STABILITY OF PROTEINS

Small Globular Proteins

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I. Introduction

One of the most important recent achievements of protein science is the advance on structural analysis of macromolecules. With the accumulation of crystallographic information on proteins, we become increasingly convinced that protein structures are extremely sophisticated and precise. This was not even unexpected since, *a priori*, it was believed that only an exclusively ordered system could perform such delicate functions as those performed by proteins. But, in considering the fascinating models of pro-

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teins resulting from crystallographic analyses, the question arises: How stable are real protein structures? It is evident that without an answer to this question we cannot hope to solve the problem of the intramolecular interactions responsible for this structure, i.e., the problem of assembling all the elements of a polypeptide into one system, which is called the native protein macromolecule. Without a quantitative definition of stability of structure, the discussions on the mechanism of structure organization from random polypeptide chains and on the mechanism of changes in this structure in relation to protein functioning are groundless.

The problem of stability of proteins is not as simple as it seems. Moreover, this is one of the most complicated and obscure problems of present protein physics. Indeed, what does the stability of a very precisely defined structure mean? Should any deviation from a structure presented by crystallographers be considered as a different structure and as a different state of protein? In this case the stability of protein at all temperatures above 0°K will evidently be zero; or we have to assume that not all the changes in protein structure are significant. However, the definition of a boundary between significant and insignificant structural changes does not seem to be probable in structural terms, especially if we have in mind that real protein structures cannot be as fixed as their models and that fluctuations at the molecular level are not negligible at all (see Cooper, 1976). At the same time, we feel that there is a boundary which separates the "native" protein from the "nonnative," or the "denatured." It is supposed that not all the actions, i.e., not all the changes in external variables, cause denaturation or transition from the native to the denatured state. It is also believed that "denaturational" changes in protein are connected with changes in structure or conformation (Anson, 1945; Putnam, 1953; Kauzmann, 1959; Tanford, 1968).

Before anything about protein structure was known, it was easy to assume that the native and the denatured are the only states of a protein macromolecule and to explain all the observed (at a sufficiently low resolution) changes in protein properties by a shift of the equilibrium between these states. This gave a great advantage since only in this case was it possible to present all the observed effects through some abstract effective parameter—the "equilibrium constant":

$$K^{eff} = \frac{\theta_x - \theta_N}{\theta_D = \theta_N} \tag{1}$$

where θ_N and θ_D are values of any observed indices characterizing the pure native and the pure denatured state, respectively, and θ_x represents the value of this index under given conditions. Studying the dependence of

this effective equilibrium constant on external variables such as temperature, pressure, and ion activity, we could derive effective parameters characterizing the denaturation process. If these characteristics were derived using equations of equilibrium thermodynamics, they would have dimensions of thermodynamic parameters and should be interpreted physically as changes of:

Gibbs energy $$-RT \ln K^{\text{eff}} = \Delta G^{\text{eff}} \tag{2}$$

enthalpy $$RT^2 \frac{d \ln K^{\text{eff}}}{dT} = \Delta H^{\text{eff}} \tag{3}$$

volume $$-RT \frac{d \ln K^{\text{eff}}}{dp} = \Delta V^{\text{eff}} \tag{4}$$

amount of bound ligands $$\frac{d \ln K^{\text{eff}}}{d \ln a_i} = \Delta \nu^{\text{eff}} \tag{5}$$

These possibilities of a qualitative treatment of denaturation and its description by physical terminology seemed very attractive, and it is not surprising that it became popular after the pioneering work of Anson and Mirsky (1934) followed by Eisenberg and Schwert (1951).

The thermodynamic studies of protein denaturation were greatly encouraged when it was shown that renaturation of even completely unfolded proteins is a reversible, thermodynamically driven process (Anfinsen, 1956); thus, equilibrium thermodynamics is in principle applicable to the study of denaturation. These studies of equilibrium led to the conclusion that protein denaturation is accompanied by an enormous increase in enthalpy. This was regarded as an indication that protein denaturation is a highly cooperative process involving the whole macromolecule (Anson, 1945).

The "all-or-none" character of denaturation has been generally accepted, and—as can be seen—only by accepting it could we define the meaning of "stability of protein." Indeed, in this case under "stability of proteins" we could assume the work required for the cooperative disruption of the entire protein structure. For a macroscopic system in equilibrium with its surroundings, such as a protein in solution, this work will correspond to the Gibbs energy difference between the native and the denatured state and could be found from equilibrium studies by Eq. (2), even outside the transition range, by using a simple extrapolation procedure (see Brandts, 1964; Aune and Tanford, 1969a; Ptitsyn and Birstein, 1969). Thus, for the case of the two-state transition the problem of protein structure stability seemed to be quite solvable thermodynamically. But with further studies

of this problem, and with advances in the precision of measurements, many doubts arose as to the correctness of the two-state concept and of the entire thermodynamic approach to the problem of protein stability.

First, it was found that changes in protein properties were observed over a much broader range of conditions than had been previously supposed and that an unambiguous definition of the pure native and the pure denatured states is not as simple as it had appeared. This produced doubt about the correctness of the equilibrium constant defined by Eq. (1) and about the correctness of a conclusion on the extreme cooperativity of protein denaturation.

In fact, the conclusion concerning the extreme cooperativity of denaturation was achieved only because it was assumed *a priori* that protein is a cooperative system and behaves in an all-or-none fashion. Thus, all thermodynamic treatment seemed to be nothing other than a *circulus vitiosus.* Indeed, sharp changes in the properties of a protein do not mean anything in themselves, since sequential multistep transitions exhibit, as was shown by Tsong *et al.* (1972), the same sharp sigmoidal changes in the observed parameters. For such a large and complicated molecule as protein, multistep transitions seemed to be much more probable (Anfinsen, 1973), the more so in that all attempts to explain the extreme cooperativity of proteins in terms of the concepts of existing physics were unsuccessful. But if the native structure of a protein is assembled sequentially, moving through the kinetically closest intermediate states, does the final state correspond to the global or local minimum of the thermodynamic potential; i.e., will the native state be the macroscopic equilibrium state, or should it be considered as a metastable state trapped kinetically (Levinthal, 1968; Wetlaufer and Ristow, 1973; Anfinsen and Scheraga, 1975; Ptitsyn and Rashin, 1975)? In spite of the scholastic character of all discussions on the behavior of thermodynamic potentials of proteins, they cast doubt on the capacity of equilibrium thermodynamics to describe the native protein.

There is only one way to escape from this *circulus vitiosus* and to prove that protein can be studied thermodynamically, and that is to determine all thermodynamic functions describing a protein by direct experimental methods, i.e., by such methods as calorimetry, dilatometry, and titrimetry. Only by comparing experimental values with values derived from equilibrium studies is it possible to decide whether a protein can be treated thermodynamically as a macroscopic system.

Although the necessity of investigation of proteins by direct thermodynamic methods was realized long ago (see Lumry *et al.,* 1966), a practical realization of this program was delayed because of great experimental difficulties. These difficulties proceeded mainly from the principal require-

ment that protens be studied in dilute solutions, since only in dilute solutions can the effects of intermolecular interactions be neglected and the properties of individual macromolecules be investigated. But in dilute solutions the partial quantities to be measured are also very small, and their investigation needs an extremely sensitive and precise technique. These requirements for sensitivity and precision are even at present far from satisfactory, particularly in volumetry (dilatometry) at various pressures. The technical difficulties of studying volume effects at denaturational pressures (i.e., at several thousand atmospheres) are so grave that no studies of protein partial volume dependence on pressure have yet been done. The situation with titrimetry is somewhat better, but there are still no detailed thermodynamic investigations of protein denaturation. Only in the case of calorimetry are all the sensitivity and precision requirements fulfilled as a result of a recently developed scanning microcalorimetry technique, which was specially designed to study the problem of stability (Privalov, 1974). By means of this technique it is possible to obtain a direct relation between two of the most fundamental conjugate intensive and extensive variables—temperature and enthalpy (heat capacity), which is principally important for the development of thermodynamics. In this chapter the results obtained by scanning microcalorimetry are treated extensively, the more so because these studies present the main new achievements of protein thermodynamics after the thorough review of Tanford (1968).

The problem of stability of native proteins is closely connected with the problem of protein denaturation, since we can judge stability only by breaking the native structure, i.e., denaturing protein by various treatments. But it should be emphasized that a review of all publications on denaturation is not the purpose of this chapter. We have included here only the results which directly pertain to the problem under consideration. In this chapter we will still use the old word "denaturation," in the sense defined by Kauzmann (1959), in spite of many objections as to its ambiguity. As will be seen, this word is not misleading in the case of small compact globular proteins, but for the large and nonglobular proteins it indeed becomes unclear because of the inadequacy of the two-state model for these systems. This qualitative difference in proteins leads us to separate our discussion into two parts. This chapter includes the results obtained on small compact globular proteins which represent one single cooperative system. A chapter which will appear in a later volume will include the results obtained on proteins which cannot be considered as a single cooperative system. These are large proteins consisting of subunits and also fibrillar proteins.

II. Temperature-Induced Changes in Protein

A. Temperature Dependence of Protein Characteristics

It was known from the earliest days of biochemistry that, on heating of protein solutions, the protein solubility drastically decreases over a narrow temperature range, resulting in intensive aggregation. Although the decrease in solubility is one of the most characteristic features of protein denaturation, it is the least studied quantitatively. Moreover, just this decrease of solubility is the major complication in studying the state of denatured protein, since aggregation causes the greatest problem for all physical methods. Because of this tendency to aggregate, denatured protein and the process of denaturation could be studied only under conditions preventing aggregation, i.e., far from the isoelectric point and in highly dilute solutions. Just this requirement was the cause of the great popularity of optical methods in studying denaturation. Usually in thermodynamic studies the observed changes in optical parameters are considered only as the abstract index of change in the protein state. Figure 1 presents a typical result of a spectrophotometric study of the influence of temperature on a protein solution and the temperature dependence of the difference spectra. The protein, pancreatic ribonuclease A, a typical small compact globular protein, was widely used to study denaturation because of its perfect reversibility after eliminating the denaturing condition. Ultraviolet (UV) ab-

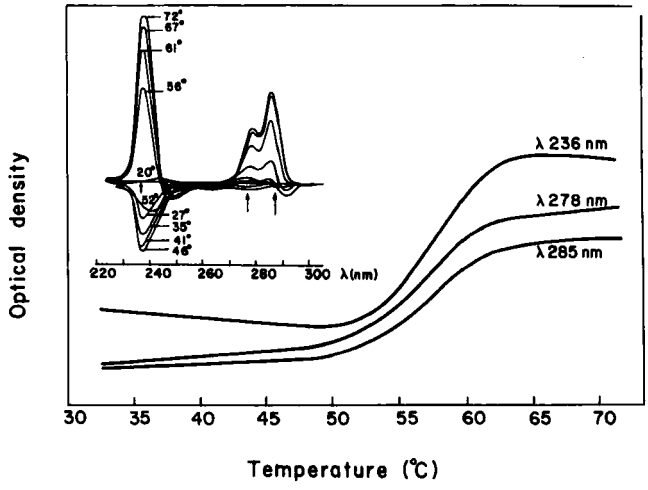

Fig. 1. Change of difference spectra of ribonuclease A in solution with pH 4.0 at heating. From Tiktopulo and Privalov (1974).

sorbance measurements were used in most studies on denaturation of ribonuclease (see, for example, Hermans and Scheraga, 1961; Scott and Scheraga, 1963; Ginsburg and Carroll, 1965; Brandts and Hunt, 1967; Tiktopulo and Privalov, 1974). We can summarize the main results of these studies in the following statements: (1) The change in protein absorption starts from the lowest temperatures and proceeds up to the highest temperature studied; (2) the character of the changes observed at different wavelengths is different; (3) in all cases we can distinguish the temperature region where the changes in absorption are most pronounced. The minor changes below and above this region are much less pronounced and might even pass unnoticed at a low precision of measurements.

Quite the same situation is observed on studying the other properties of ribonuclease such as circular dichroism (Simons *et al.*, 1969; Tiktopulo and Privalov, 1974), optical rotation (Klee, 1967), viscosity (Holcomb and van Holde, 1962), stability against proteolysis (Klee, 1967), and rate of hydrogen exchange (Tiktopulo and Privalov, 1975). In all cases it appears that the dependence on temperature of the various observables can be generally represented by a sum of simpler functions—one sigmoidal function and two very smooth functions below and above the sigmoidal one. The sigmoidal changes, which in all cases are more pronounced, are usually called the denaturational changes, and the corresponding temperature range is denoted as the denaturational temperature range. Consequently, the minor changes below and above this region can be called the pre- and postdenaturational changes in protein.

For a quantitative analysis of denaturation we must separate these functions. This can be done by the extrapolation of the low-temperature and high-temperature functions into the denaturational temperature range. It is evident that the less pronounced the pre- and postdenaturational changes in protein, the easier is the extrapolation procedure and the more reliable is the function which is assumed to describe only the process of denaturation. But in all cases, when the pre- and postdenaturational changes are observed, we can never be sure that the extrapolation and decomposition procedure is correct and that the obtained constitutent function of the complex curve represents a unique set. Indeed, in extrapolating the pre- and postdenaturational changes, we know nothing about the functions which should be used for this extrapolation and therefore nothing about the shape of the function which should be evaluated. The only conclusions which can be drawn with some degree of certainty from the experimental transition curves are that the pre- and posttransitional functions are not linear and that the sigmoidal curve which characterizes the transition range is not symmetric. Thus, the constitutent functions cannot be simple functions. This severely complicates all the procedures of decomposition and

renders their results less unambiguous. It follows that a comparison of the constituent functions of the complex transition curves obtained by observing various parameters characterizing the state of the protein is not very informative. Indeed, the coincidence, and noncoincidence, of these functions is mainly the result of the particular decomposition procedure chosen and cannot be considered as evidence of an all-or-none character of transition. As was shown, the observed asymmetry of the sigmoidal function corresponding to denaturation can be explained by the existence of several sequential stages (Scott and Scheraga, 1963), or equally well by a large temperature dependence of the enthalpy of denaturation (Brandts, 1964).

The situation is the same with regard to several other reversibly denaturing globular proteins studied at present: lysozyme (Hamaguchi and Sakai, 1965; Khechinashvili *et al.*, 1973), chymotrypsinogen (Brandts and Lumry, 1963), myoglobin (Acampora and Hermans, 1967; Atanasov and Mitova, 1971; Kinderlehrer *et al.*, 1973), and *Aplysia* myoglobin (Brunori *et al.*, 1968, 1972). In all cases the influence of temperature is apparent from the very beginning of the heating of the solution, and the temperature dependence of any observable parameter specifying the state of the protein is characterized by a complex curve. In all cases we have the problem of decomposition of the experimental curve into its constituent functions as well as the problem of postulating a reaction mechanism which is consistent with the functions obtained by the decomposition procedure.

Usually in discussions of the mechanism of denaturation of proteins, the simultaneous changes in all observables are considered to provide a valid criterion for the applicability of the two-state model. But evidently this is only a necessary but not a sufficient criterion (see Lumry *et al.*, 1966). Indeed, it can be shown that multistep transitions exhibit identical overall transition patterns, provided all the steps are identical. At the same time, as was shown earlier the question of coincidence or noncoincidence of functions, which are evaluated from a complex curve, is in itself open for discussion. It is evident also that the coincidence of all the obtained functions is a rare occasion, since a deviation in results is a natural tendency in all experiments. Thus, the reports of simultaneous changes of all characteristic integral observables do not really prove that the process is a two-state transition. However, contrary reports favoring multistate transitions should also be considered with caution, since in principle they suffer from the same limitations.

Among the special attempts to study the nature of the denaturation reaction, the method sensitive to a change in specific sites of protein should be noted. One of them is the method of studying the kinetics of proteolysis by specific proteolytic enzymes.

According to Klee (1967), different proteolytic enzymes digest pancreatic

ribonuclease A at different, and not even overlapping, temperature regions. This was regarded as an indication that the conformation of ribonuclease changes in a gradual manner, and that a central region becomes disordered at a lower temperature than either end of the molecule. But Klee's results can also be interpreted as an indication that the resistance of ribonuclease against some proteolytic enzymes decreases before denaturation at a temperature where other techniques detect minor changes in the properties of the protein. As for the denaturational temperature range, it is highly questionable whether it is possible to distinguish slight shifts (1°-2°C) between the temperature profiles of digestion by different enzymes because of the many weak points in the application of the method of proteolysis at elevated temperatures. The results obtained by Burgess *et al.* (1975) on digestion of pancreatic ribonuclease A by immobilized carboxypeptidase could be interpreted also as an indication that the unfolding of the C-terminal residue takes place only at the temperature region of the proper denaturation, although some increase of accessibility of the C-terminal amino acid to the exopeptidase occurs below this temperature region. Thus, proteolytic studies of ribonuclease neither disprove nor substantiate the two-state mechanism of its denaturation.

Much more definite results were obtained in proteolytic studies of lysozyme. Matthyssens *et al.* (1972) studied the digestion of lysozyme and found complete agreement of the thermodynamic values characterizing denaturation obtained from both the change in proteolytic stability and in optical rotation. Imoto *et al.* (1974) studied digestion of lysozyme by four different proteolytic enzymes and analyzed the products chromatographically. These authors showed that in no case could intermediate-sized products be found and concluded that protease digestion proceeds only by the all-or-none type mechanism and that protease digests only the unfolded molecule.

In considering the mechanism of a change in protein state with temperature, NMR studies attract the most attention because of the possibility of simultaneously observing the state of several definite sites of the macromolecule by chemical shifts of the corresponding resonance lines. Using this method, it should be possible in principle to determine whether or not the different parts of the macromolecule undergo changes simultaneously, i.e., to provide unambiguous evidence for the cooperativity of the observed process. But unfortunately in practice this potential of NMR technique cannot be realized as yet. The main difficulties arise from the insufficient sensitivity of NMR at present and from the high degree of complexity of NMR spectra. Because of low sensitivity, denaturation studies are usually done at concentrations so high (above 1%) that aggregation effects are unavoidable. Although the resolution of the spectra can be improved by using selectively deuterated analogs of the protein, this method is so ex-

pensive that until recently it has not been effectively used to study the problem of cooperativity of the denaturation process.

Detailed studies of the influence of temperature on the state of the protein by NMR have so far been performed only on two globular proteins: ribonuclease A (Zaborsky and Millman, 1972; Westmoreland and Matthews, 1973) and lysozyme (McDonald *et al.*, 1971). These studies clearly showed a qualitative difference between the changes occuring in the protein below the transition range and those occurring at denaturation. While only gradual changes in chemical shifts of resonance lines without a change in the shape of these lines observed at all temperatures in the predenaturational region, the area of the lines decreases in the denaturational region and a new line, corresponding to the resonance of free amino acids in solution, appears simultaneously. In the course of denaturation no other new resonances are observed. Thus, no stable intermediates in the course of denaturation have been seen. The curves describing the state of different parts of the macromolecule coincide within the error of the measurements (see Fig. 2). Unfortunately, these errors are too large to allow an ultimate conclusion about the nature of the transition. The situation is the same with ribonuclease, although Westmoreland and Matthews (1973) drew the opposite conclusion. Thus, the problem of applicability of the two-state model cannot be considered solved. The doubt about the reality of the

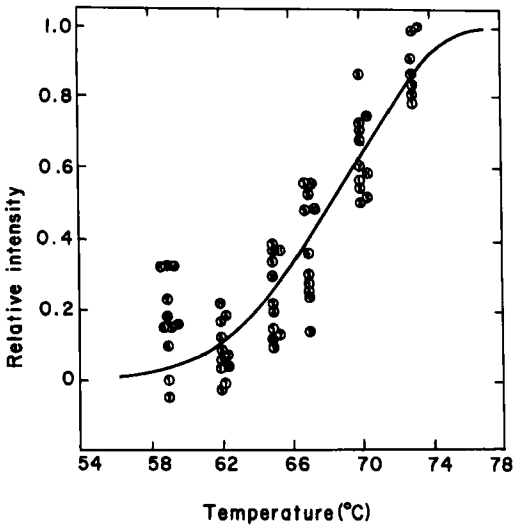

FIG. 2. Relative changes in intensity of 11 different proton resonances in H_2O, pH 3.3, at thermal denaturation of lysozyme. Reprinted with permission from McDonald *et al.*(1971), *J. Am. Chem. Soc.* **93**, 235–246. Copyright by the American Chemical Society.

NMR results is further aggravated by a comparison of the van't Hoff enthalpies derived from other studies. According to McDonald *et al.* (1971), the van't Hoff enthalpy of lysozyme denaturation is 73 kcal mol^{-1} in the 65°–75°C temperature region, while the calorimetrically measured enthalpy of this protein at these temperatures is 120 kcal mol^{-1} (Khechinashvili *et al.*, 1973). Due to the great uncertainty in the decomposition of the observed complex transition curve of protein into its constituent parts, and in view of the ambiguity of the models used to treat the obtained functions thermodynamically, it is not surprising that the equilibrium data available at present deserve a critical review. This situation is well illustrated in Table I, where the thermodynamic data on the denaturation of ribonuclease A published by various authors and even by the same authors in different years are collected. We can conclude by citing Poland and Scheraga (1965): "The thermodynamic information which authors are extracting from the experimental data is surely the kind one wishes to have. It is based on a set of assumptions that seems to lack any solid ground for support."

B. Calorimetric Studies on the Influence of Temperature on the State of Protein

The calorimetric studies of the influence of temperature on the state of the protein require the determination of the partial heat capacity of the protein in solution as a function of temperature. At present only a few calorimetric measurements of heat capacities of protein solutions exist. The first calorimetric studies (Privalov, 1963; Beck *et al.*, 1965; Tsong *et al.*, 1970; Jackson and Brandts, 1970; Privalov *et al.*, 1971) were performed using instruments of low resolution and insufficient stability of the base line. Therefore, it was impossible to determine the partial heat capacity of the protein from these measurements. The only information which could be obtained from these measurements was the enthalpy change associated with a sharpe change in the state of the protein on heating. But this information was quite insufficient for a detailed quantitative analysis of the influence of temperature on the protein. The determination of partial heat capacity of the protein in dilute solution become possible only after the appearance of a precise scanning microcalorimeter (Privalov, 1974; Privalov *et al.*, 1975).

The calorimetric recording resulting from heating a dilute solution of lysozyme is presented in Fig. 3. As can be seen, the heat capacity of this solution is significantly lower than the heat capacity of the same volume of pure solvent, which is presented as the base line. From the distance ΔC_p^{app} between the two recordings for solvent and solution, it is possible to determine the partial specific heat capacity of the protein, $C_{p,pr}(T)$, at any

TABLE I

Thermodynamic Parameters for Thermal Denaturation of Ribonuclease Derived from Equilibrium Studies

Reference	pH	T_d (°C)		$\Delta_d H$ (kcal mol^{-1})		$\Delta_d C_p$ (cal K^{-1} mol^{-1})	Method
Hermans and Scheraga (1961)	2.0	35		51		0	Optical density
	3.0	44		51		0	
	4.0	56–60		51		0	
		I	II	I	II		
Scott and Scheraga (1963); two steps in denaturation assumed	2.0	23.2	32.9	48.5	74.7	—	Optical density
	2.5	27.0	37.0	32.8	78.7	—	
	3.3	29.5	48.0	21.1	106.2	—	
Brandts and Hunt (1967)	2.1	28		58.3		1980	Optical density
	2.5	38		73.0		1985	
	3.15	44		60.7		1987	
Ginsburg and Carroll (1965)	2.1	28.8		46		—	Optical density
Holcomb and van Holde (1962)	2.8	44.6		68		—	Sedimentation
		42.9		67		—	Viscosity
Tiktopulo and Privalov (1974)	2.5	35		75		1200	Optical density
	3.0	42		80		1200	Circular dichroism
	4.0	57		105		1200	Optical rotation

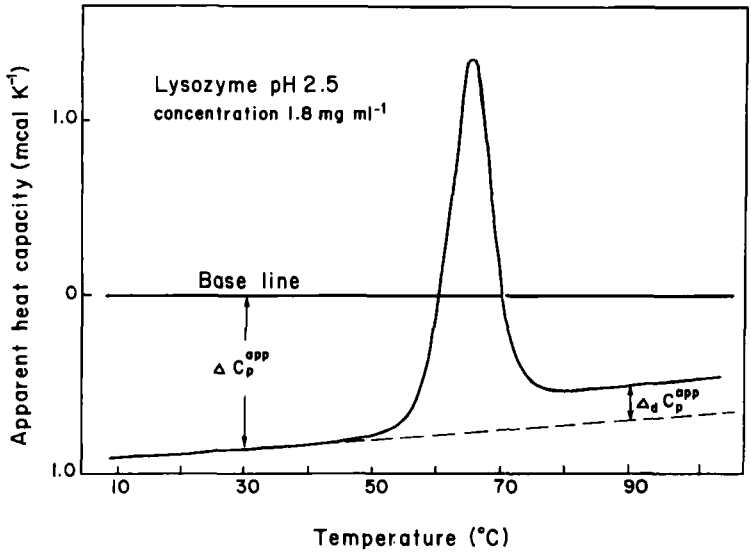

FIG. 3. An example of scanning microcalorimetric recording of apparent heat capacity of dilute protein solution on heating over a broad temperature range.

temperature if the specific partial volume of the protein, $V_{pr}(T)$, is known:

$$C_{p,pr}(T) = C_{p,sol}(T)\frac{V_{pr}(T)}{V_{sol}(T)} - \frac{\Delta C_p(T)^{app}}{m_{pr}} \tag{6}$$

where m_{pr} is the amount of protein in the calorimetric cell (for details, see Privalov and Khechinashvili, 1974a).

The temperature dependence of the partial heat capacity of lysozyme in solution at different pH values is given in Fig. 4. Figure 5 summarizes the results obtained with several globular proteins. In these figures we present the specific values of heat capacity instead of the more commonly used molar values, since the specific partial heat capacity, i.e., the heat capacity per unit of protein mass, *is very much the same for all compact globular proteins*: At 25°C it is (0.32 ± 0.02) cal K^{-1} g^{-1}. As seen from the figures, it changes with temperature and this correlates with a change in the other characteristics considered previously. Here we can also distinguish the temperature regions corresponding to the main change in the state of the protein, the denaturation, which appears on the heat capacity curve as a peak of intensive heat absorption. Below and above this denaturational peak, only slight changes in heat capacity are observed, the pre- and postdenaturational changes in protein heat capacity.

In comparing heat capacity curves with any other curves describing the

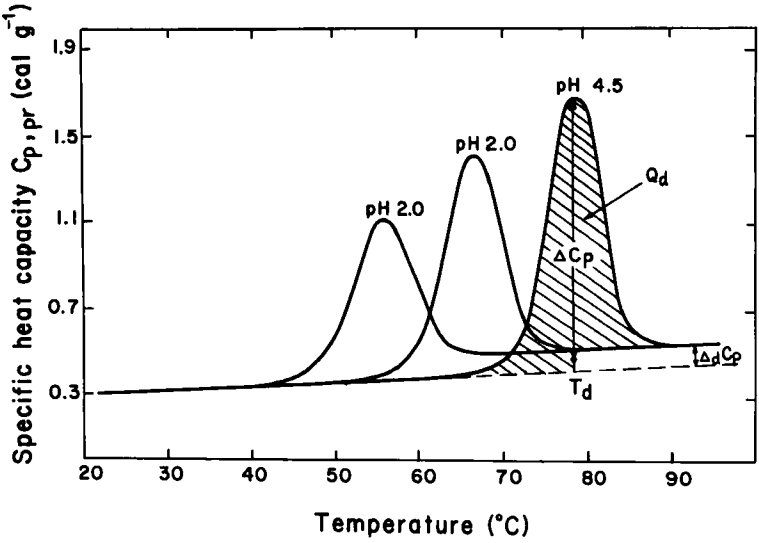

FIG. 4. Temperature dependence of partial specific heat capacity of lysozyme in solution at different pH values. From unpublished results in the author's laboratory.

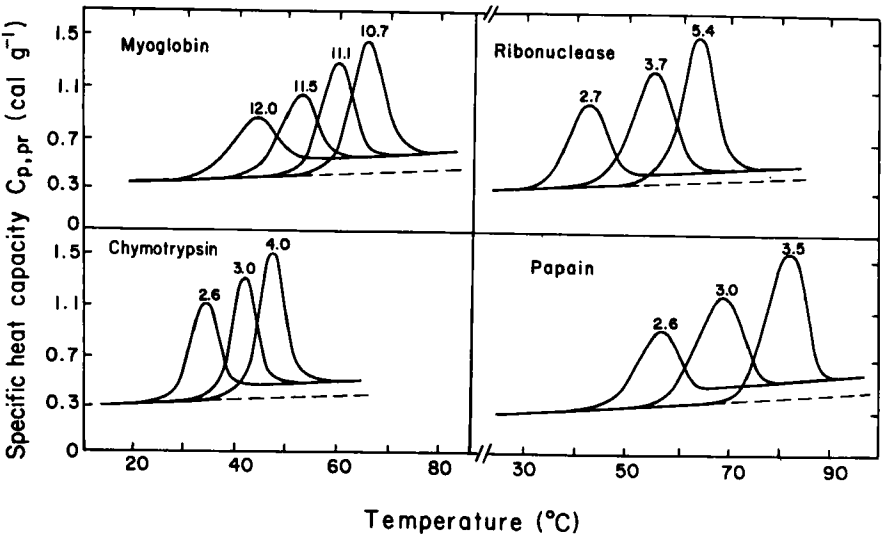

FIG. 5. Temperature dependence of partial specific heat capacity of four proteins at different pH values. Data for papain from Tiktopulo and Privalov (1978); for the other proteins from Privalov and Khechinashvili (1974a).

change in the protein state with temperature, we must bear in mind that heat capacity is a temperature derivative of an integral characteristic, i.e., of enthalpy. As a derivative by the variable parameter, this function more precisely describes all the changes in a state of protein with temperature and has a simpler shape. At the same time, since heat capacity is the derivative of enthalpy, no temperature-induced change in state can occur without being reflected in the heat capacity curve. This is in contrast to the other characteristics of protein which are not always sensitive to some changes in its state, e.g., the optical characteristics at some wavelengths (see Fig. 1).

In analyzing the heat capacity curve, let us assume as a first approximation that the pre- and postdenaturational heat capacity changes in the protein can be described by linear functions of temperature. In this case we can easily extrapolate the heat capacity of the pure native and the pure denatured protein into the transition range and evaluate the heat of the denaturational process Q_d. As seen from Fig. 3, Q_d corresponds to the peak area above the heat capacity functions extrapolated to a midpoint of transition. The difference between the extrapolated heat capacity functions at the temperature of the midpoint of the denaturation corresponds to the difference between the heat capacity of the native and denatured state of the protein, $\Delta_d C_p(T_d) = C_p^D(T_d) - C_p^N(T_d)$. It is evident that the change in heat capacity must determine the temperature dependence of the enthalpy of denaturation, since, according to Kirchhoff's relation, $(d\Delta_d H)/(dT) = \Delta_d C_p$.

The temperature dependence of the enthalpy of denaturation can also be determined from the change in enthalpy of denaturation at changing stabilities, e.g., from the change in both the denaturational peak area δQ_d and temperature δT_d, induced by changing pH if ionization effects are excluded (see Section II,D). By comparing $(\delta Q_d/\delta T_d)$ with $\Delta_d C_p$ it is possible to decide whether the procedure used of separating the heat of denaturation from the intrinsic effects of the heat capacities of the native and the denatured protein is correct. Such a comparison for several globular proteins is presented in Fig. 6. The coincidence of both values leads to the conclusion that this procedure for evaluating the enthalpy of denaturation is correct, and that we can extrapolate linearly the heat capacities of proteins before and after the denaturational heat uptake, and can consider these extrapolated functions as heat capacities of the pure native and the pure denatured state. This conclusion is supported also by the following: (a) In studying the varying stability of a protein with pH, its heat capacities below and above the heat absorption zone in all cases are presented by the same functions (see Fig. 5). (b) The slopes of the heat capacity functions below and above the heat absorption zone are almost the same.

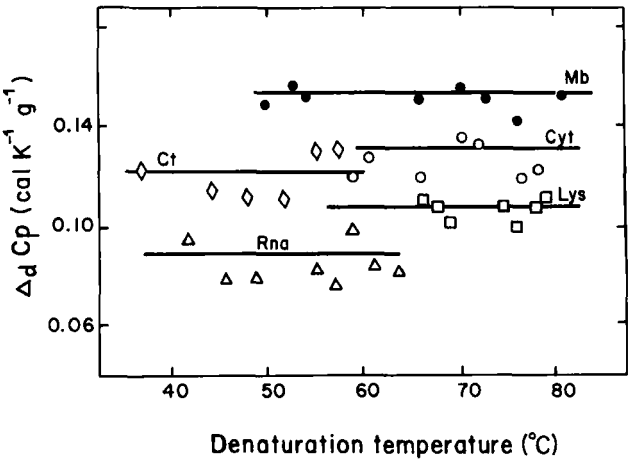

FIG. 6. Denaturational change in partial specific heat capacity $\Delta_d C_p$ for metmyoglobin (Mb), α-chymotrypsin (Ct), ribonuclease A (Rna), lysozyme (Lys), and cytochrome c (Cyt). The lines correspond to values of the observed dependence of specific enthalpy of denaturation on the temperature of denaturation $(\delta\Delta_d h)/(\delta T_d)$. Reprinted with permission from Privalov and Khechinashvili (1974a), *J. Mol. Biol.* **86,** 665–684. Copyright by Academic Press, Inc. (London) Ltd.

The conclusion that both below and above the heat absorption zone we have the heat capacities of pure native and pure denatured protein, respectively, and that these heat capacities are linear functions of temperature (within the considered limited region of temperature), is substantiated only by the whole body of evidence presented in this chapter. The justification of this assumption requires simultaneous consideration of the problem of the two-state model of protein denaturation and also the proof of the existence of thermodynamic potentials describing these states.

C. Validity of the Two-State Model for Denaturation

One of the greatest advantages of scanning calorimetric recording is that it is possible to obtain simultaneously not only the real (calorimetric) enthalpy of any process induced by temperature, but also the effective (van't Hoff) enthalpy of this process. Thus, it becomes possible to compare these two quantities immediately, excluding any doubts as to the similarity of objects and the identity of treatment of the observed effects (Privalov, 1963; Jackson and Brandts, 1970).

Calorimetric enthalpy of the process is determined from the heat capacity versus temperature curve by the area of the heat absorption peak. For the molar denaturational enthalpy we have:

$$\Delta_d H^{cal} = MQ_d \tag{7}$$

At the same time, the relative amount of the heat absorbed to a given temperature $\vartheta(T) = Q(T)/Q_d$ is a direct measure of progress of the temperature-induced reaction. Assuming that this reaction is a two-state transition, we will have for the equilibrium constant $K^{eff} = \vartheta/(1 - \vartheta)$, which can be used to determine the effective enthalpy by Eq. (3).

$$\Delta H^{eff} = \frac{RT^2}{\vartheta(1 - \vartheta)} \frac{d\vartheta}{dT} \tag{8}$$

For the calorimetric curve,

$$\frac{d\vartheta}{dT} = \frac{1}{Q_d} \frac{dQ}{dT} = \frac{\Delta C_p}{Q_d} \tag{9}$$

i.e., it is nothing other than the normalized intensity of heat absorption or excess heat capacity at a given temperature, divided by the total heat of denaturation. Thus, for the effective enthalpy at a given temperature we have:

$$\Delta_d H^{eff}(T) = \frac{RT^2}{\vartheta(1 - \vartheta)} \frac{\Delta C_p(T)}{Q_d} \tag{10}$$

from the middle of transition where $\vartheta \equiv \frac{1}{2}$, and $T \equiv T_d$,

$$\Delta_d H^{eff} = \frac{4\,RT_d^2}{Q_d} \Delta C_p(T_d) \tag{11}$$

Here we must only have in mind that for the asymmetric peak T_d is close to the temperature of the maximum of heat absorption T_{max} but does not equal it (see Privalov and Khechinashvili, 1974a).

If the process considered is indeed of a two-state transition type, the effective enthalpy should be equal to the real one; i.e., the relation $\Delta H^{cal}/\Delta H^{eff}$ should be close to unity. In practice the evaluation of this relation amounts to the analysis of the shape of the calorimetrically obtained melting curve and can be done with high accuracy.

This kind of analysis had been done by Privalov (1963) on ovalbumin, by Jackson and Brandts (1970) on chymotrypsinogen, and with greater accuracy by Privalov and Khechinashvili (1974a) on several small compact globular proteins: ribonuclease A, lysozyme, α-chymotrypsin, cytochrome c, and metmyoglobin at different pH values of solutions. The obtained ratio $\Delta H^{cal}/\Delta H^{eff}$ is presented in Fig. 7 as a function of transition temperature. As seen in the figure , it is very close to, but not exactly, unity. The average value for these five proteins is $\Delta H^{cal}/\Delta H^{eff} = 1.05 \pm 0.03$. The same results were obtained later for the heat of denaturation of calcium-free parvalbumin (Filimonov *et al.*, 1978), lactalbumin (W. Pfeil, personal

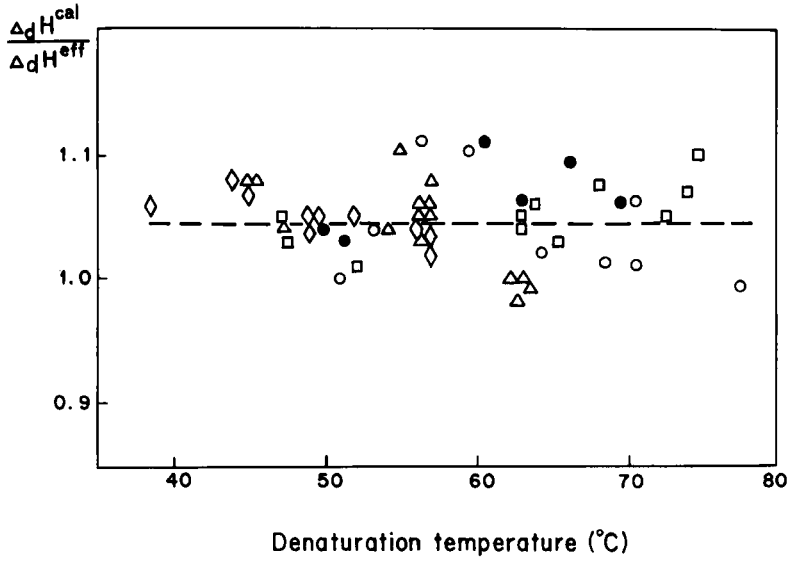

FIG. 7. Ratio of calorimetric and effective denaturation enthalpy $(\Delta_d H^{cal})/(\Delta_d H^{eff})$ of five globular proteins under different conditions plotted against the corresponding temperature of denaturation. Metmyoglobin (●), ribonuclease (△), cytochrome c (○), α-chymotrypsin (◇), lysozyme (□). Reprinted with permission from Privalov and Khechinashvili (1974a), *J. Mol. Biol.* **86**, 665–684. Copyright by Academic Press, Inc. (London) Ltd.

communication, 1978), carbonic anhydrase, trypsin, and soybean trypsin inhibitor (unpublished results from our laboratory).

The observed deviation of $\Delta H^{cal}/\Delta H^{eff}$ from unity cannot be explained by artifacts (see Privalov and Khechinashvili, 1974a), and it was concluded that it is due to the inadequacy of the two-state model in describing the real process of denaturation. In other words, it can be considered as an indication that there are some intermediates between the native and the denatured macroscopic states, but that their concentration is small. Recently, Freire and Biltonen (1978) carried out a detailed analysis of the temperature dependence of the heat capacity function of ribonuclease and showed that the sum of distributions for the native and the denatured states which can be determined from the heat capacity curve is indeed very close to unity (see Fig. 8). The deviation from unity, which is equal to the concentration of the intermediate states, does not exceed 5%.

The low concentration of the intermediates in the denaturation of compact globular proteins means that they are highly unstable thermodynamically. Thus we can neglect them in a first approximation if the mechanism of transition is not considered, and we can regard denaturation as

a cooperative transition between only two macroscopic states, the native and the denatured, i.e., as an all-or-none process.

However, it should be pointed out that we cannot generalize from this conclusion, which was obtained by studying the denaturation of small and compact globular proteins, and apply it to all globular proteins and treat their equilibrium data by the two-state approximation. Indeed, it was shown that the relation $\Delta H^{cal}/\Delta H^{eff}$ for papain, which is also a typical average-sized globular protein (MW = 23,000) is 1.80 ± 0.01 (Tiktopulo and Privalov, 1978), while for the Bence-Jones protein (MW 48,000) it is 1.90 ± 0.01 (Zavyalov *et al.*, 1977). Thus, it becomes evident that the denaturation of these proteins is far from being a two-state transition type. The most probable explanation for this deflection from the two-state model is that these proteins consist of two quite independent and equal cooperative regions. This is supported by the known three-dimensional structure of these proteins. Indeed, papain has a very deep cleft which bisects this macromolecule into two nearly equal domains (Drenth *et al.*, 1970). The Bence-Jones protein consists of four domains (Edelman, 1970), and we have to assume that here the pairs of the domains are connected into one cooperative region. Thus, a knowledge of the number of domains constituting the protein is in itself insufficient to predict the number of the cooperative regions in a macromolecule. Indeed, parvalbumin, which has two calcium-binding domains, behaves as one cooperative unit in the absence of calcium (Filimonov *et al.*, 1978). Quite the opposite is demonstrated by pancreatic trypsin inhibitor, for which the calorimetric enthalpy

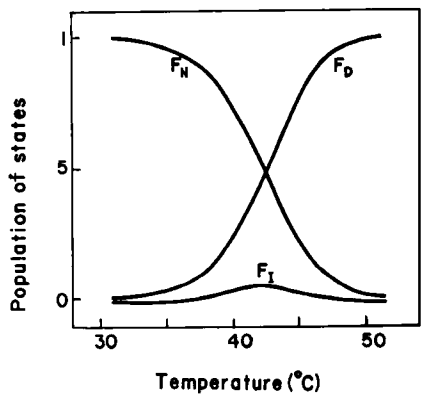

FIG. 8. Relative population of states associated with the thermal denaturation of ribonuclease A. F_N and F_D are the fraction of molecules populating the initial and final states, respectively. F_I is the summed population of all intermediates; at no temperature is F_I greater than 5% of the total population. Reprinted with permission from Freire and Biltonen (1978).

calculated per 6500-dalton monomer unit is twice as small as the effective enthalpy (Tischenko and Gorodkov, 1978). This means that the cooperatuve unit of this protein is a dimer (see also Kraut *et al.*, 1960; Anderer and Hörnle, 1965).

From the examples presented it becomes evident how dangerous it is to treat equilibrium without special studies on the mechanism of transition, and how uncertain are the thermodynamic data obtained in this way. Indeed, in considering only the denaturational curves of papain (see Fig. 5), it is impossible to guess that the process of its denaturation is not represented by the two-state transition model, and that its effective enthalpy is only half as great as the true value.

At present there is nonthermodynamic evidence which also supports the all-or-none mechanism of denaturation and renaturation of small compact globular proteins. The failure of all attempts to trace the intermediates in the thermal transition process by the kinetic method can be considered as a first indication (see Baldwin, 1975). After several years of intensive studies and clarification of numerous contradictory results, it became evident that the conformational transition of globular proteins into the denatured state, as well as the back-transition into the native state, is a fast process. The complications observed in kinetics, with the appearance of slow phases, are most probably the result of secondary phenomena which are not of primary significance in studying the mechanism of cooperative conformational transitions of globular proteins (Brandts *et al.*, 1975, 1977; Garel *et al.*, 1976; Nall *et al.*, 1978).

Other evidence was obtained by studying the intermediate disulfide linkages in the process of refolding of a native structure of globular proteins (Creighton, 1977a–d; Creighton *et al.*, 1978) and from the studies of the interchange of the fragments of nuclease T′ (Taniuchi and Anfinsen, 1969; Taniuchi, 1970). These studies led to the conclusion that all the intermediate states of the macromolecule are unstable relative to the unfolded and fully folded conformations. It appears that essentially all elements of the native conformation are attained simultaneously at the final stage of folding, and the native conformation requires all its stabilizing interactions for stability.

D. Enthalpy of Denaturation

It is evident that the calorimetrically measured heat of denaturation is a complex effect which includes not only the heat of the conformational transition of a compact macromolecule, but also the heat of ionization of protein accompanying the transition and the heat of concomitant ionization of the buffer compound if denaturation is studied in a buffered solution.

Denoting the observed overall denaturational change of enthalpy by $\Delta_d H^{app}$, we can present it as the following sum:

$$\Delta_d H^{app}(T_d) = \Delta_d H_{pr}^{conf}(T_d) + \Delta_d H_{pr}^{ion}(T_d) + \Delta_d \nu \Delta H_{buf}^{ion}(T_d) \qquad (12)$$

To exclude the effect of buffer ionization, we must know the change in protonation of protein $\Delta_d \nu$ at denaturation and the enthalpy of buffer ionization ΔH_{buf}^{ion}. Knowing $\Delta_d \nu$, it is possible to exclude also the heat effect of ionization of protein groups, since enthalpies of ionization of the individual groups of protein are known. Where denaturation can be considered a two-state transition, $\Delta_d \nu$ can be obtained from the pH dependence of the transition temperature and the transition enthalpy by the following equation (see Ptitsyn and Birstein, 1969):

$$\Delta_d \nu = -\frac{\Delta_d H(T_d)}{2.3\, RT_d^2} \frac{dT_d}{d\mathrm{pH}} \qquad (13)$$

It is evident that in the pH region where T_d does not depend on pH (see Fig. 9), $\Delta_d \nu$ is zero; but, in the acidic and the alkaline pH regions, it can

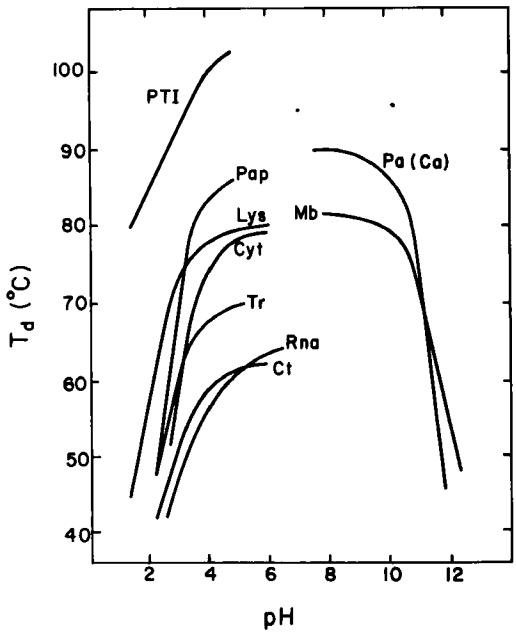

FIG. 9. pH dependence of temperature of denaturation (T_d) for various globular proteins: metmyoglobin (Mb), ribonuclease A (Rna), cytochrome c (Cyt), α-chymotrypsin (Ct), parvalbumin with calcium [Pa(Ca)], papain (Pap), pancreatic trypsin inhibitor (PTI), lysozyme (Lys). For references, see Fig. 10.

be rather significant. The enthalpies of ionization of buffers and protein groups are small in acidic pH (<1 kcal mol^{-1}) and are quite substantial in the alkaline pH region (>10 kcal mol^{-1}) (Izatt and Christensen, 1968). Therefore, the correction for ionization effects is significant only when studying denaturation in alkaline solutions (and neutral solutions if the protein has several histidine residues), but at acidic pH this correction in most cases does not exceed the error of calorimetric measurements.

It can be seen that we can easily exclude all the ionization heat effects by the appropriate choice of buffers. Indeed, if the enthalpy of ionization of the buffer is equal to the enthalpy of ionization of the protein group, they will automatically compensate each other, and the apparent enthalpy of denaturation will just correspond to the enthalpy of conformational transition of the protein. From this point of view, one of the most convenient buffers for studying protein denaturation in the acidic and alkaline pH regions is glycine. This method of automatic compensation of ionization effects essentially facilitates all treatment and increases the accuracy of determination of the enthalpy of conformational transition of the protein. The molar enthalpies of denaturation, corrected for ionization effects, i.e., enthalpies of conformational transitions of proteins, for denaturation in solutions with different pH, are presented for several compact globular proteins in Fig. 10. Corresponding pH values can be found Fig. 9.

The remarkable feature of the functions presented in Fig. 10 is that they are all linear. With the increase of temperature, the enthalpy of conformational transition increases. Since the temperature of denaturation is not a linear function of pH and these functions are not identical for all the considered proteins, the observed regularity in behavior of enthalpy leads us to the assumption that the enthalpy of transition does not depend directly on pH, but is a direct function of temperature. The other remarkable feature of the enthalpy function is that the slope of these functions, i.e., $(\delta\Delta_d h^{conf})/(\delta T_d)$, is equal to the observed denaturational heat capacity change of protein $\Delta_d c_p$ as has been already shown in Fig. 6. In fact, we used this equality as an *a priori* condition for defining the procedure for separating the denaturational heat effect from the effects of the heat capacities of the native and the denatured protein. Therefore, we draw our conclusion from the independence of conformational enthalpy on pH on the simultaneous basis of both the mentioned features of the enthalpy function, i.e., on its linear dependence on temperature and on the equality of the slope of this function to the heat capacity change. The fact that we do not encounter any contradictions in interpreting the effects observed for the denaturation of very different proteins at different pH conditions convinced us of the correctness of the presented treatment.

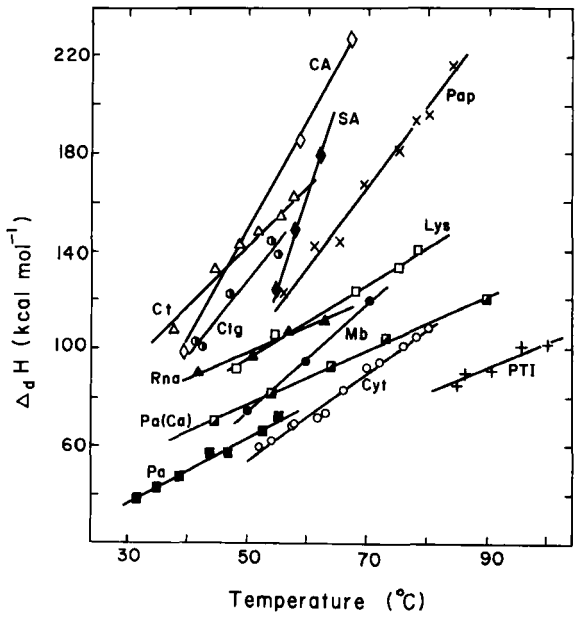

FIG. 10. Plot of molar enthalpy of denaturation measured at different pH values ($\Delta_d H_{pH}$) versus corresponding temperature of denaturation (T_d). The proteins presented are: ribonuclease A (Rna) (Tsong *et al.*, 1970; Tiktopulo *et al.*, 1974), lysozyme (Lys) (Khechinashvili *et al.*, 1973), chymotrypsinogen (Ctg) (Jackson and Brandts, 1970), α-chymotrypsin (Ct) (Tischenko *et al.*, 1974), metmyoglobin (Mb) (Privalov and Khechinashvili, 1974a), cytochrome c (Cyt) (Privalov and Khechinashvili, 1974b), papain (Pap) (Tiktopulo and Privalov, 1978), parvalbumin (Pa) (Filimonov *et al.*, 1978), pancreatic trypsin inhibitor (PTI) (Tischenko and Gorodkov, 1978), carbonic anhydrase B (CA) (unpublished results of our laboratory), and serum albumin (SA) (Leibman *et al.*, 1975).

E. Predenaturational Changes in Proteins

Although predenaturational changes in protein are much less profound than the denaturational ones, they attract no less attention, since they are observed with the native protein and thus may have some biological significance. This interest was exaggerated by numerous reports on the observation of a very sharp change in protein properties just at physiological temperatures. It was indeed tempting to assume that at this temperature we have two distinct native conformations and the transition between them is just the mechanism of the enzymatic action of protein.

A sharp break of the Arrhenius plot for a change in the fluorescence intensity in time was observed for α-chymotrypsin in the vicinity of 25°C. The van't Hoff enthalpy evaluated from the sharpness of the observed effect was reported to be 48.4 kcal mol^{-1} (Kim and Lumry, 1971).

A change of the spin–lattice relaxation rate in myoglobin in the temperature region of 35°–40°C was observed (Atanasov *et al.*, 1968) as was a change in the paramagnetic label (Likhtenstein *et al.*, 1970). The reported van't Hoff enthalpy of this transition was 50 kcal mol^{-1}. A break of the Arrhenius plot of data obtained by ESR studies of ribonuclease A at 40°C was reported by Matheson *et al.* (1977).

The temperature-dependent structural transition was assumed for lysozyme at 25°C, since it was found that below and above this temperature different crystalline forms of this protein grow (Jollès and Berthou, 1972). This assumption was supported by the observation of a sharp break in the Arrhenius plot for the kinetics of enzymatic reaction (Saint-Blancard *et al.*, 1977) and by the change in NMR spectra (Cozzone *et al.*, 1975).

We have cited here only a few of the publications on this subject, selecting reports concerning only small compact globular proteins. Calorimetric studies were regarded as crucial tests in evaluating predenaturational transitions in proteins, since temperature-induced transitions are impossible without heat absorption and this expected heat was even calculated. As has been already shown (see Figs. 4 and 5), calorimetrically no heat absorption is observed on heating monomolecular solutions of small compact globular proteins. A slight deflection from linearity was found only when aggregates preexist in solution. Thus, it follows from calorimetric studies that the observed changes in protein properties in the temperature region before denaturation cannot be interpreted as a cooperative structural transition induced by temperature.

Critically reconsidering reports of the sharp break in the van't Hoff or the Arrhenius plots, or in other temperature-dependent protein characteristics, it can be said that they might be due to an erroneous approximation of the slightly bent curves by two intersecting lines. As is evident, these lines will always intersect in the middle of the temperature interval studies. In most reported cases this middle temperature interval is just in the range of 25°–30°C. NMR studies often present an overly straightforward interpretation of the observed denaturational changes in proteins. Indeed, from the fact that the very complicated NMR spectra of protein at two temperatures are different, it does not follow that we have a cooperative structural transition. This conclusion can be made only on the basis of a detailed analysis of the total course of a change in individual resonances over a broad range of temperature, to exclude the possibility that changes in protein in this region are not gradual changes in its structure.

The fact that has been firmly established in studying temperature-induced changes in protein by the NMR technique is that the changes in resonances in the denaturational and in the predenaturational regions of temperature are qualitatively different (McDonald *et al.*, 1971). Denatur-

ation leads to the disappearance of resonances specific for the native state and to the appearance of resonances specific for free amino acids in solution, while in the predenaturational temperature region there are only monotonic changes in chemical shifts of specific resonances. This fact is, perhaps, the clearest indication that the nature of the predenaturational and the denaturational changes in protein are qualitatively different. It follows that these changes are connected with the process proceeding with absolutely different rates. If denaturational changes are connected with a relatively slow transition between two clearly distinguished macroscopic states of protein, the predenaturational changes are connected with the processes which are faster by several orders of magnitude. It is most probable that these fast processes are the local fluctuations of the native structure. In some cases, as was shown by Nakanishi *et al.* (1972, 1973) for lysozyme and Tiktopulo and Privalov (1975) for ribonuclease, the equilibrium constant of these local or micro-unfoldings of protein compact structure could be obtained as a function of temperature from hydrogen exchange studies. Figure 11 shows that the concentration of micro-unfoldings slowly increases with the increase of temperature until the denaturation region is reached, but at these temperatures this functional dependence changes drastically. From the slope of a curve in the coordinates used in Fig. 11, one can get the enthalpy of a process responsible for the unfolding of a compact structure. In the case of lysozyme, Nakanishi *et al.* found

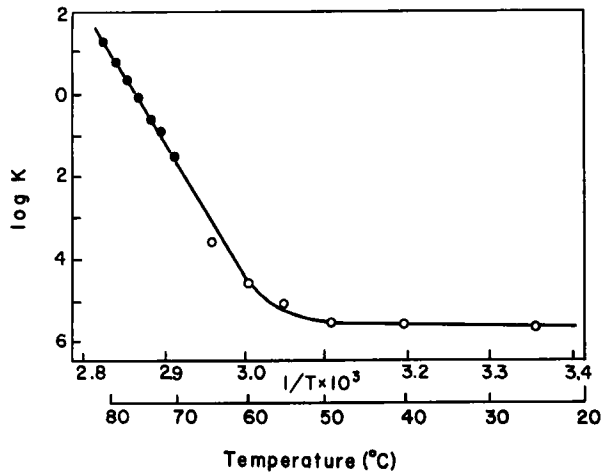

FIG. 11. Temperature dependence of equilibrium constant K for unfolded forms of lysozyme determined by hydrogen exchange experiment (O) and optical studies (●). Reprinted with permission from Nakanishi *et al.* (1973), *J. Mol. Biol.* **75**, 673–682. Copyright by Academic Press, Inc. (London) Ltd.

(1973) that the enthalpy of unfolding at the predenaturational temperature region is 2 kcal mol^{-1}, whereas at the denaturational region it is 127 kcal mol^{-1}, which just equals the value found calorimetrically for lysozyme denaturation under corresponding conditions (Khechinashvili *et al.*, 1973). From the estimated enthalpy values, if follows that the unfoldings of the compact structure of globular proteins which lead to the exchange of hydrogens are of an absolutely different scale in the predenaturational and denaturational temperature regions. If denaturational unfolding is a cooperative process which involves all the internal hydrogen bonds, then unfolding in the predenaturational region is connected with the rupture of a single bond and, what is important, the amount of simultaneously disrupting bonds does not increase with the increase of temperature; only the frequency of this noncooperative disruption increases. From the results obtained from hydrogen exchange studies it is also evident that the calorimetrically observed heat capacity increase cannot be explained by the disruption of hydrogen bonds with the increase of temperature. At room temperatures the equilibrium constant for micro-unfolded forms does not exceed 10^{-5} (Nakanishi *et al.*, 1973; Tiktopulo and Privalov, 1975). This value is too small to explain the observed heat capacity effect. Unfortunately, thermodynamic analysis of hydrogen exchange data has been done only on two globular proteins, lysozyme and ribonuclease, since the kinetics of hydrogen exchange in other proteins is more complex and quantitative interpretation becomes impossible in the frame of a simple model (Hvidt and Nielsen, 1966; Willumsen, 1971).

III. Thermodynamic Presentation of Protein

A. The Influence of pH on the State of Protein

The pH of the solution is one of the most important factors determining the state of a protein, and it is natural that during the last several decades there have been numerous publications on studies of pH-induced changes in proteins. It was shown that the observed overall change in protein is a complex process. Here also, as in the case of temperature effects, intensive changes in all parameters, characterizing protein conformation in a narrow region of pH, are clearly distinguished (see Fig. 12) above a smooth background of gradual changes. Potentiometric titration of protein revealed that smooth changes are connected with the titration of groups with a pK not very different from that of free amino acids, while the gross conformational changes associated with pH denaturation are accompanied by the unmasking of buried groups (see Edsall and Wyman, 1958). But most thermodynamic studies on the influence of pH on the state of protein

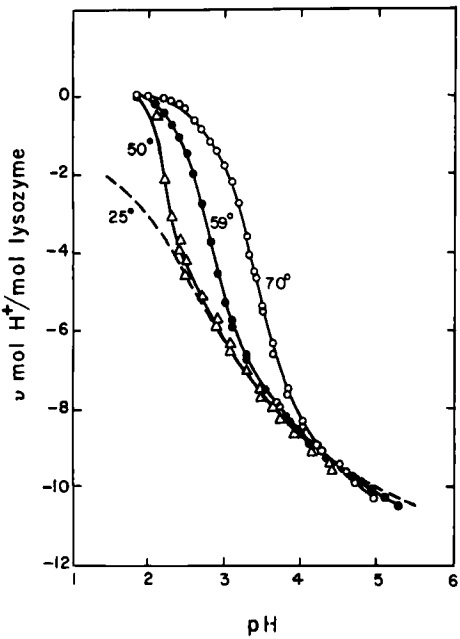

FIG. 12. pH dependence of lysozyme protonation at different temperatures. At 25°C lysozyme does not denature at any pH. From Pfeil and Privalov (1976a).

were devoted to the studies of temperature-induced denaturation at different pH values, because it made possible a description of this influence in energetic terms.

Since the temperature-induced denaturational transition of protein seemed to be a two-state type, it was assumed that the transition induced by the variation of pH at the corresponding fixed temperature is also of a two-state type, so that the initial and final characteristics of the protein seemed independent of the sequence of pH or temperature variation. This independence of sequences follows from the interconvertibility of the curves characterizing protein obtained by varying pH and temperature (Fig. 13).

Assuming that pH-induced denaturation is indeed a two-state transition, it is possible to calculate the amount of groups unmasked at denaturation, $\Delta_d \nu$, by Eq. (5). The comparison of these calculated values with those measured by potentiometric titration supported the assumption of the validity of the two-state approximation for pH denaturation (Hermans and Acampora, 1967; Pfeil and Privalov, 1976a). But it should be noted that this comparison cannot be done with the same accuracy as in the case of calculated and measured enthalpies in temperature-induced denaturation,

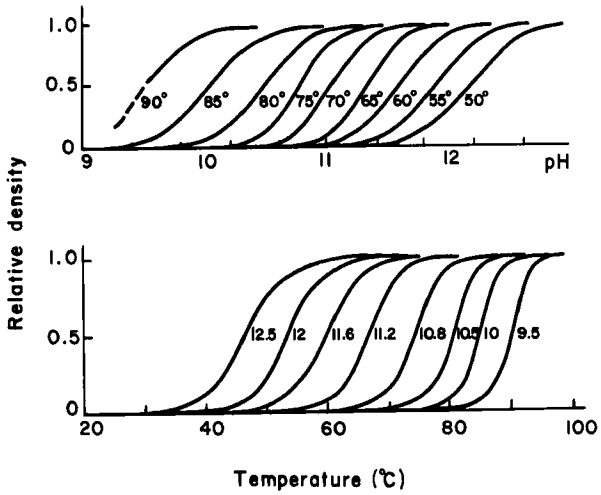

FIG. 13. Relative changes in optical density of myoglobin (MbCN) in the Soret region at temperature denaturation (fixed pH values) and at pH denaturation (fixed temperatures). Reprinted with permission from Atanasov and Mitova (1971).

since the resolution of titrimetry is much lower than that of scanning microcalorimetry. Thus, to confirm the conclusion on the two-state character of transition, nonthermodynamic evidence is important. Here NMR studies attract the most attention. But up to the present, only one detailed NMR study of pH-induced denaturation of globular protein was carried out with all the requisite precautions for such an investigation. This is the investigation of the pH denaturation of staphylococcal nuclease, done on a 220 MHz spectrometer by Epstein *et al.* (1971). The alkali-induced denaturation of the same protein was studied by Jardetzky *et al.* (1971), but this result will not be considered here, due to lack of evidence that the observed effects correspond to real equilibrium and are not artifacts.

The resonance corresponding to the C2 protons of imidazoles is clearly distinguished on the NMR spectra of staphylococcal nuclease obtained by Epstein *et al.* (1971) (Fig. 14). As pH varies, all resonances shift gradually, but the redistribution of the area of individual resonances takes place in the lower pH region, which is quite narrow. At the same pH range a simultaneous change is observed in the intensity of tryptophanyl fluorescence and in the ellipticity and viscosity of this molecule, demonstrating that it is just in this region that its compact structure unfolds. Thus, this study can be considered as supporting the two-state transition model for pH denaturation of globular protein.

B. Calorimetric Studies of pH-Induced Changes in Protein

Until recently only a few calorimetric investigations of pH changes in protein have been published. This was because of the great experimental difficulties encountered in calorimetric titration of dilute protein solutions in a broad pH region. The detailed calorimetric titration curve which is obtained point by point using the isothermal reaction microcalorimetric technique is not only time-consuming, but also requires a large amount of highest purity preparation. Thus it is not surprising that the first experiments in this field, using myoglobin (Hermans and Rialdi, 1965), ribonuclease (Kresheck and Scheraga, 1966), and chymotrypsinogen (Biltonen *et al.*, 1971), were qualitative and revealed only the general possibilities of calorimetric technique in studying pH denaturation. Detailed calorimetric titration at several temperatures has been done only recently on lysozyme (Pfeil and Privalov, 1976a) and parvalbumin (Filimonov *et al.*, 1978). These studies revealed the qualitative difference between heat effects on change in proteins before and at cooperative denaturational transition. The heat effect before the transition presents only the heat of ionization of titrable groups, which at acidic pH is at least an order less than the observed heat effect of conformational transition (Fig. 15).

The other important fact revealed from the curves in Fig. 15 is the great temperature dependence of the observed heat effect. It is remarkable that this heat effect, corrected for ionization heat, just corresponds to the enthalpy changes for heat denaturation of the same protein, if this denaturation takes place at the corresponding temperature (Pfeil and Privalov,

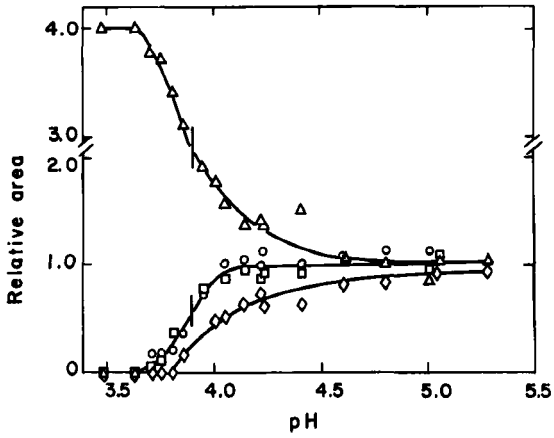

FIG. 14. The relative areas of the imidazole C2 proton resonances of the staphylococcal nuclease, as a function of pH. Reprinted with permission from Epstein *et al.* (1971).

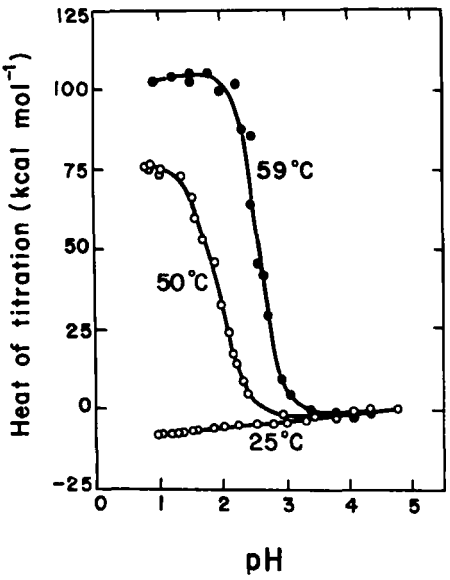

FIG. 15. Calorimetric titration of lysozyme at different temperatures. From Pfeil and Privalov (1976a).

1976a; Filimonov *et al.*, 1978). This correspondence between the enthalpies of pH and heat denaturation supports the idea that both these processes are nothing but two views of the same phenomenon—the cooperative conformational transition of protein from the native to the denatured state—and that this denatured state is universal for pH and heat denaturation. Thus, it is now attractive to assume that the denatured, as well as the native, states are the macroscopic states of protein dependent on external variables such as temperature, pH, and ionic strength. If that is really the case, these states should be described by thermodynamic functions and these functions should have the property of a potential—they should not depend on the pathways needed to reach the definite point in a phase space. Until recently, no one had proved the correctness of this hypothesis, although it was often used, even unconsciously, in discussing protein properties. But in reality it is far from self-evident, and before using it for protein analysis, its correctness should be shown experimentally.

C. Standard Functions of Temperature and pH for Protein

Infinitesimal changes in any thermodynamic function describing a macroscopic state of a system can be expanded in different terms which represent variable parameters influencing the state. Where the variables are

temperature and pH, we have for a function F:

$$dF = \left(\frac{\partial F}{\partial T}\right)_{\mathrm{pH}} dT + \left(\frac{\partial F}{\partial \mathrm{pH}}\right)_{T} d\mathrm{pH} \tag{14}$$

Taking the state of the native protein at $\mathrm{pH}^0 = 7.0$, $T^0 = 25°\mathrm{C}$, and the ionic strength $I^0 = 0.1$ as standard from Eq. (14), we have for the partial enthalpy and entropy functions of the native protein:

$$H^{\mathrm{N}}(T,\mathrm{pH}) = \int_{T^0}^{T} \left(\frac{\partial H}{\partial T}\right)_{\mathrm{pH}} dT + \int_{\mathrm{pH}^0}^{\mathrm{pH}} \left(\frac{\partial H}{\partial \mathrm{pH}}\right)_{T} d\mathrm{pH}$$

$$= \int_{T^0}^{T} C^{\mathrm{N}}_{\mathrm{p,pH}}\, dT + (H^{\mathrm{N}}_{\mathrm{pH}} - H^{\mathrm{N}}_{\mathrm{pH}^0})_{T^0}$$

$$= \int_{T^0}^{T} C^{\mathrm{N}}_{\mathrm{p,pH}}\, dT + H^{\mathrm{N}}(\mathrm{pH})_{T^0} \tag{15}$$

$$S^{\mathrm{N}}(T,\mathrm{pH}) = \int_{T^0}^{T} \left(\frac{\partial S}{\partial T}\right)_{\mathrm{pH}} dT + \int_{\mathrm{pH}^0}^{\mathrm{pH}} \left(\frac{\partial S}{\partial \mathrm{pH}}\right)_{T} d\mathrm{pH}$$

$$= \int_{T^0}^{T} \frac{C^{\mathrm{n}}_{\mathrm{p,pH}}}{T}\, dT + S^{\mathrm{N}}(\mathrm{pH})_{T^0} \tag{16}$$

The temperature-dependent terms contain the partial heat capacity of native protein in solution at given pH, i.e., $C^{\mathrm{N}}_{\mathrm{p}}(T)_{\mathrm{pH}}$, which is determinable by scanning microcalorimetry. The pH-dependent term $H^{\mathrm{N}}(\mathrm{pH})_T$, which describes the molar heat of titration of native protein with the initial pH 7.0 at T^0, can be determined by isothermal microcalorimetry. The pH-dependent term of standard entropy $S^{\mathrm{N}}(\mathrm{pH})$ cannot be obtained directly, but can be calculated if the standard enthalpy $H^{\mathrm{N}}(\mathrm{pH})$ and the Gibbs function $G^{\mathrm{N}}(\mathrm{pH})$ are known for the given standard temperature.

Determination of $G^{\mathrm{N}}(\mathrm{pH})$ is possible by the direct use of titration curves. From the general equation

$$G(x) = G(x_0) + \int_{x_0}^{x} \left(\frac{\partial G}{\partial x}\right) dx \tag{17}$$

follows the equation for the multiple equilibrium of protein ionization:

$$G(\alpha) = \mathrm{const} + \int_{\alpha_0}^{\alpha} \mu(\alpha)\, d\alpha$$

$$= G(\alpha_0) - 2.303\, RT \int_{\alpha_0}^{\alpha} \mathrm{pH}(\alpha)\, d\alpha \tag{18}$$

Introducing instead of the degree of ionization (α) the relative number of protons (ν), measured potentiometrically for a standard condition where the integration constant $G^0(\nu_0)$ is equal to zero, we have for the Gibbs function

$$G^N(\nu) = -2.303\,RT \int_{\nu_0}^{\nu} \mathrm{pH}(\nu)\, d\nu \tag{19}$$

which can be easily transformed into $G^N(\mathrm{pH})$ on the basis of the experimental titration curve (see Pfeil and Privalov, 1976a). Having $G^N(\mathrm{pH})$, we will have for the pH-dependent entropy term:

$$S^N_{\mathrm{pH}} = \frac{H^N_{\mathrm{pH}} - G^N_{\mathrm{pH}}}{T^0} \tag{20}$$

For the Gibbs energy function of native protein we have:

$$G^N(T,\mathrm{pH}) = H^N(T,\mathrm{pH}) - TS^N(T,\mathrm{pH}) \tag{21}$$

Corresponding functions for the denatured state can be obtained from standard functions of the native state and denaturational changes in enthalpy $\Delta_d H$, entropy $\Delta_d S$, and Gibbs energy $\Delta_d G$. Since Gibbs energies of native and denatured states are equal at the transition temperature T_d [i.e., $\Delta_d G(T_d) = 0$], we have for the entropy of denaturational transition:

$$\Delta_d S(T_d) = \frac{\Delta_d H(T_d)}{T_d} \tag{22}$$

Thus, for the denatured state we obtain:

$$\begin{aligned} H^D(T,\mathrm{pH}) &= \int_{T^0}^{T_d} C^N_{p,\mathrm{pH}}\, dT \\ &\quad + \Delta_d H_{T_d} + \int_{T_d}^{T} C^D_{p,\mathrm{pH}}\, dT + H^N(\mathrm{pH}) \\ &= H^N(T,\mathrm{pH}) + \Delta_d H_{T_d} + \int_{T}^{T_d} \Delta_d C_{p,\mathrm{pH}}\, dT \end{aligned} \tag{23}$$

$$S^D(T,\mathrm{pH}) = S^N(T,\mathrm{pH}) + \frac{\Delta_d H}{T_d} - \int_{T}^{T_d} \Delta_d C_{p,\mathrm{pH}}\, d\ln T \tag{24}$$

$$G^D(T,\mathrm{pH}) = H^D(T,\mathrm{pH}) - TS^D(T,\mathrm{pH}) \tag{25}$$

As can be seen, all the data necessary for establishing the complete thermodynamic standard functions of protein are determinable experimentally using scanning microcalorimetry, isothermal microcalorimetry, and poten-

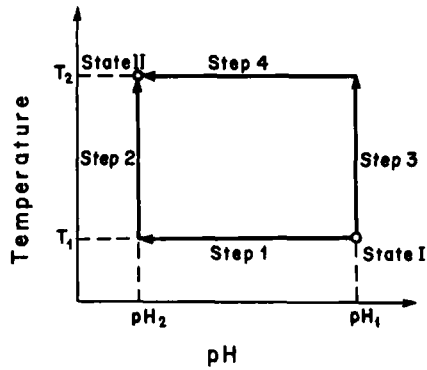

FIG. 16. Scheme of different pathways from state I (native) to state II (denatured).

tiometric titration; and their establishment does not need any *a priori* assumptions. The validity of these functions in a nonalternative description of a state can be tested using a cyclic pathway. The defined function will be potential if its value does not depend on the pathway, and, for any closed cycle, the total change will be zero; i.e., if the total change at passing from state I to state II in Fig. 16 is the same for any pathway. An example based on lysozyme is presented in Table II taken from Pfeil and Privalov (1976a).

The most important conclusion which follows from the validity of defined thermodynamic functions for protein is that the states of protein which are described by these functions can be considered as real macroscopic states.

The example of standard functions of protein is given in Figs. 17 and

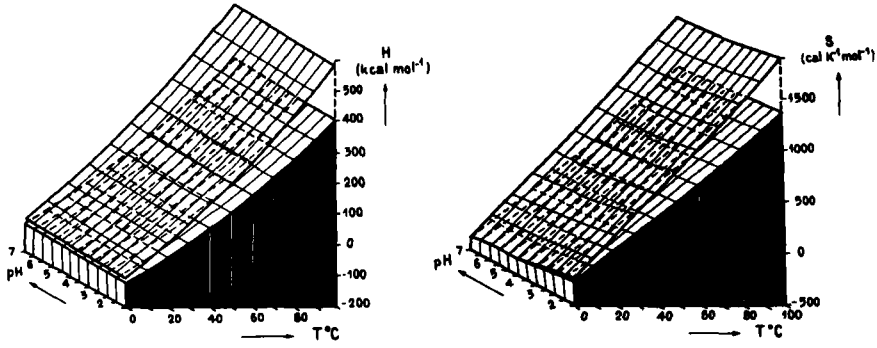

FIG. 17. Standard enthalpy $H(T,\text{pH})$ and standard entropy $S(T,\text{pH})$ functions for the native and denatured states of lysozyme. In both cases the denatured state is represented by the upper surfaces. From Pfeil and Privalov (1976c).

TABLE II

Comparison of Thermodynamic Values for Lysozyme Denaturation Obtained Using Independent Pathways[a,b]

Step	Thermodynamic expression	Methods used	Results for T_2 = 50°C (kcal mol^{-1})	Results for T_2 = 59.1°C (kcal mol^{-1})
ΔH				
1	$H^N_{T_1,pH_2} - H^N_{T_1,pH_1}$	Isothermal calorimetry at 25°C	−6.7	−6.7
2	$H^D_{T_2,pH_2} - H^N_{T_1,pH_2}$	Scanning calorimetry	218.9	289.2
Sum (1 + 2)	$H^D_{T_2,pH_2} - H^N_{T_1,pH_1}$		212.2	282.5
3	$H^N_{T_2,pH_1} - H^N_{T_1,pH_1}$	Scanning calorimetry	123.8	178.8
4	$H^D_{T_2,pH_2} - H^N_{T_2,pH_1}$	Isothermal calorimetry at T_2	81.5	103.7
Sum (3 + 4)	$H^D_{T_2,pH_2} - H^N_{T_1,pH_1}$		205.3	282.5
Deviation			3.3%	—
ΔS				
1	$S^N_{T_1,pH_2} - S^N_{T_1,pH_1}$	Isothermal calorimetry and potentiometric titrations at 25°C	78.2	78.2
2	$S^D_{T_2,pH_2} - S^N_{T_1,pH_2}$	Scanning calorimetry	694.2	908.2
Sum (1 + 2)	$S^D_{T_2,pH_2} - S^N_{T_1,pH_1}$		772.4	986.4
3	$S^N_{T_2,pH1} - S^N_{T_1,pH_1}$	Scanning calorimetry	397.9	563.8
4	$S^D_{T_2,pH_2} - S^N_{T_2,pH_1}$	Isothermal calorimetry and potentiometric titration at T_2	379.2	454.0
Sum (3 + 4)	$S^D_{T_2,pH_2} - S^N_{T_1,pH_1}$		777.1	1017.8
Deviation			0.6%	3.1%

[a] From Pfeil and Privalov (1976a).

[b] T_1 = 25.00 ± 0.05°C, pH_1 = 4.80 ± 0.02, pH_2 = 1.50 ± 0.02.

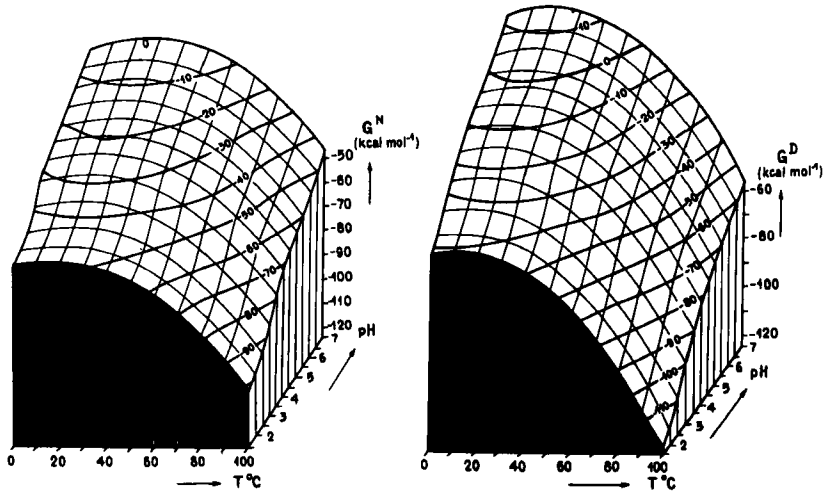

FIG. 18. Standard Gibbs energy functions for the native $G^N(T,\mathrm{pH})$ and denatured $G^D(T,\mathrm{pH})$ states of lysozyme. From Pfeil and Privalov (1976c).

18. Each of these functions is presented by two continuous surfaces in the corresponding phase spaces separated by a considerable gap. Each gap is equal to the enthalpy, entropy, and Gibbs energy change at the transition from the native to the denatured state:

$$\Delta^{D}_{N}H(T,\mathrm{pH}) = H^{D}(T,\mathrm{pH}) - H^{N}(T,\mathrm{pH}) \tag{26a}$$

$$\Delta^{D}_{N}S(T,\mathrm{pH}) = S^{D}(T,\mathrm{pH}) - S^{N}(T,\mathrm{pH}) \tag{26b}$$

$$\Delta^{D}_{N}G(T,\mathrm{pH}) = G^{D}(T,\mathrm{pH}) - G^{N}(T,\mathrm{pH}) \tag{26c}$$

It is evident that in the regions where denaturation is observed experimentally, these difference functions will be equal to measured changes of the enthalpy, the entropy, or the Gibbs energy. For example at the middle of the denaturational transition we will have:

$$\Delta^{D}_{N}H(T = T_d;\ \mathrm{pH} = \mathrm{pH}_d) = \Delta_d H(T_d;\ \mathrm{pH}_d) \tag{27a}$$

$$\Delta^{D}_{N}S(T = T_d;\ \mathrm{pH} = \mathrm{pH}_d) = \Delta_d S(T_d;\ \mathrm{pH}_d) \tag{27b}$$

$$\Delta^{D}_{N}G(T = T_d;\ \mathrm{pH} = \mathrm{pH}_d) = \Delta_d G(T_d;\ \mathrm{pH}_d) = 0 \tag{27c}$$

But the important advantage of presentation of data by standard thermodynamic functions is that it becomes possible to describe the state of the protein over a much broader range of variables, even for conditions where pure states are not observed experimentally. Unfortunately, the functional description of the thermodynamic properties of protein is still rarely used

although it is evident that the publication of particular thermodynamic values obtained under varied conditions in most cases is meaningless.

D. Proteins with Several Native States

The case in which the protein has only one native macroscopic state is not the only possibility even among small compact globular proteins. The alternative situation can be illustrated by the example of the calcium-binding protein parvalbumin. On binding of two calcium ions, the stability of parvalbumin increases by 10 kcal mol^{-1} (Filimonov *et al.*, 1978). This specific binding with a very high binding constant ($K_a \simeq 10^7\ M^{-1}$) cannot be regarded as simple chelation (as is Ca^{2+} binding by EDTA), since in contrast to chelation it is accompanied by a significant enthalpy decrease of 20 kcal mol^{-1}. This enthalpy effect alone plays the major role in the strong binding of calcium ions by parvalbumin, but it can be explained only by assuming that there is a significant rearrangement, with formation of a more compact conformation, of the entire native structure of parvalbumin. Thus, the change in the state of parvalbumin on binding the calcium ions has to be regarded as a cooperative transition into another more stable macroscopic state. This state must be presented by an additional surface in the phase space separated by the corresponding values of the enthalpy or entropy or Gibbs energy of calcium binding from the surface presenting calcium-free native parvalbumin. The same situation exists for other proteins that specifically bind ligands, since a strong binding always means that the binding process is cooperative and involves a great part of the macromolecule.

The other example of cooperative rearrangement of protein native structure is a pH-induced isomerization reaction, e.g., N⇌F or N⇌B transition of a serum albumin which takes place in a definite pH region (see Sogami and Foster, 1968; Harmsen *et al.*, 1971). In all these cases, different forms of the macromolecule cannot be presented by the same continuous surface in a phase space, i.e., they cannot be regarded as gradual changes of the same compact structure. Unfortunately, the thermodynamics of the cooperative isomerization of proteins is not yet well studied.

Finally, it should be noted that in all cases when several native forms are present simultaneously, the denaturation process appears to be complicated [for example, see denaturation of parvalbumin in the presence of calcium, studied by Filimonov *et al.* (1978)]. But the observed complexity does not in itself mean that transition of the individual forms is not of a two-state type. With this possibility in mind, we can avoid misinterpretation of some experimental results when discussing the all-or-none character of denaturation.

E. The Influence of Pressure on Proteins

Pressure is one of the fundamental physical parameters determining any system, and it is natural that studies of its effect on protein were begun a long time ago, apparently in 1914 when the first experiment was done by Bridgman. But quantitative investigations of the effect of pressure on protein were begun much later, in the middle 1960s, when it became clear that this was the only practical way to acquire information on volume effects associated with conformational transitions of proteins (Gill and Glogowsky, 1965; Tanford, 1968).

Even early studies of the influence of pressure on proteins revealed that proteins are not very sensitive to pressure, and only at extremely large values of pressure do they exhibit the changes which are very similar to those observed in temperature and pH denaturation. This pressure-induced denaturation of proteins takes place in a relatively narrow pressure interval which depends strongly on the temperature and pH of the solution (Fig. 19). At the same time, the temperature and the pH of denaturation are themselves dependent on pressure. Thus, these three parameters are interdependent, and a variation of any of them at fixed values of the

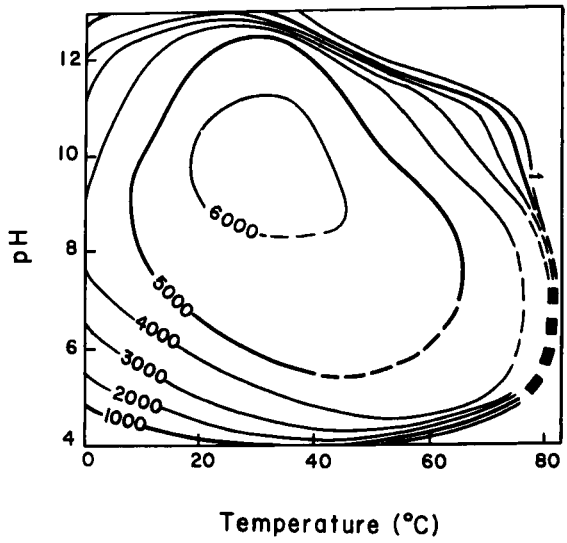

FIG. 19. Isobar of metmyoglobin half-conversion to the denatured state in the pH–temperature plane according to Zipp and Kauzmann. The native state is more stable than the denatured state inside each contour [contour lines are pressures (kg/cm^2)]. Reprinted with permission from Zipp and Kauzmann (1973), *Biochemistry* **12**, 4217–4228. Copyright by the American Chemical Society.

others leads to a denaturational change in protein. Since denaturation by temperature and pH is a two-state transition, we can conclude that pressure-induced denaturation must be also a transition between two states, the native and the denatured, and that these states are macroscopic states of protein; i.e., they are described by thermodynamic functions of all three variables. This statement is a very serious one and undoubtedly needs special substantiation, as has been done previously for the variables of temperature and pH. But up to the present this has not been shown experimentally, and we can regard this statement as only a very probable hypothesis, since it is supported by the fact that intermediate states are absent at pressure-induced denaturation. This was convincingly demonstrated by Hawley and Mitchell (1975) on chymotrypsinogen, using an electrophoretic technique at high pressure. The practical significance of this hypothesis is evident, since at high pressure, where experimental difficulties are enormous, indirect equilibrium studies are the only sources of thermodynamic information. Moreover, equilibrium studies on the dependence of protein denaturation on pressure and treatment of results by Eq. (4) are the only possible methods of obtaining any information on the volume effect accompanying denaturation because the existing volumetric technique is incapable of measuring it with the necessary accuracy, even at atmospheric pressure.

It follows from the inefficiency of pressure in causing protein denaturation that the volume effect of denaturation, $\Delta_d V$, is very small. According to Gill and Glogowsky (1965), this volume decrease at denaturation of ribonuclease is (-30 ± 10) ml mol^{-1} at an average pressure of about 1000 atm; Brandts *et al.* (1970) found that it drops from -4 ml mol^{-1} to -5 ml mol^{-1} when the temperature increases from 25° to 50°C and depends strongly on pressure. A strong dependence of $\Delta_d V$ on temperature and pressure has been found for chymotrypsinogen by Hawley (1971). Here the effect recalculated for standard temperature (0°C) and pressure (1 atm) is -14.3 ml mol^{-1}. For metmyoglobin $\Delta_d V$ was found to be about -100 ml mol^{-1} in a broad pressure interval from 600 to 6000 atm (Zipp and Kauzmann, 1973). Unfortunately, we cannot check these values of $\Delta_d V$ by direct measurement and thus cannot prove the applicability of the two-state model for pressure-induced denaturation as we did for temperature-induced denaturation (see Section II). The only published value of $\Delta_d V$ (-240 ml mol^{-1}) measured directly for ribonuclease by Holcomb and van Holde (1962), is very doubtful, being six times larger than that found from pressure studies. But values obtained from studying equilibrium at high pressures should also be regarded with caution, since the error in determining the equilibrium constant at high pressures cannot be smaller than

it is at atmospheric pressure and, as has been shown in Section II, this error is not small.

IV. Thermodynamics of the Denaturant Action on Protein

A. Protein Treatment by a Denaturant

Under the action of a denaturant such as guanidine hydrochloride (GuHCl) and urea, protein exhibits a sharp change in state in the narrow region of the denaturant concentration, depending on the pH and temperature at which titration of the protein solution by a denaturant is carried out. This change in protein state could be recorded by any indices sensitive to a conformational change (see Fig. 20), and they are all evidence that denaturation of protein by a denaturant can be interpreted as an unfolding of its compact structure. It was shown by viscosimetric investigation that the unfolded state achieved at a high concentration of GuHCl and urea can be regarded as the random coil (Tanford, 1968). This was confirmed later by NMR studies (McDonald and Phillips, 1969; Bradbury *et al.*, 1972; Bradbury and Norton, 1973). At the same time it was concluded from the sharpness of the observed changes that this transition is highly cooperative and can be considered as an all-or-none type transition of protein from a

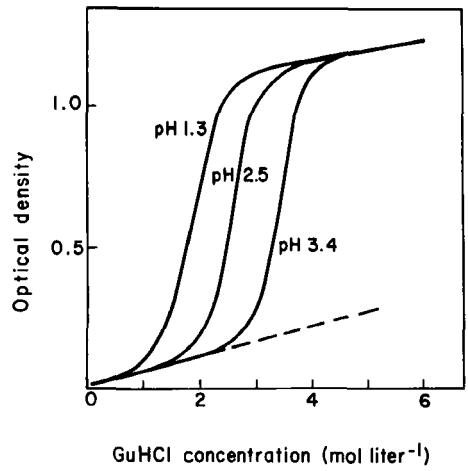

FIG. 20. Change of optical density at 300 nm for lysozyme solution at different pH values with the increase in GuHCl concentration. From unpublished results in the author's laboratory.

compact native state to a completely unfolded state (Tanford *et al.*, 1966). This was also supported by kinetic studies of the denaturation process by a denaturant (Ikai and Tanford, 1973; Tanford *et al.*, 1973) and by NMR spectroscopy studies McDonald *et al.*, 1971), although in some cases the change in NMR spectra reveals that during titration by the denaturant, the change in the outer parts of the molecule proceeds before the unfolding of the hydrophobic core (Bradbury and King, 1969; Benz and Roberts, 1973).

The studies of protein denaturation by a denaturant attracted great attention of those working in the field of thermodynamics for several reasons. Of prime importance was the generally accepted opinion that only denaturants could unfold the compact globular protein completely, up to the state of the random coil and, moreover, could do so reversibly, The high reversibility of the action of denaturants on proteins is provided by their ability to prevent aggregation. As for the possibility of studying the process of complete unfolding, it was indeed very important, since only the random coil state could be taken as a universal basic state in thermodynamic considerations of the stability of any structure. It was essential also that a simple extrapolation of the equilibrium titration data to the zero concentration of denaturant be suggested to obtain all the thermodynamic characteristics of protein unfolding in the absence of the denaturant. The fact that no special equipment for thermodynamic studies of authentic unfolding of proteins was needed naturally produced great enthusiasm for this approach to the problem of protein stability, notwithstanding some *a priori* doubts concerning its real significance.

The starting assumption for this approach is that denaturation by a denaturant is a two-state transition from the native to the completely unfolded state. In this case, according to Aune and Tanford (1969a,b), the slope of a conversion curve at any given value of activity of denaturant (a_{GuHCl}) can be rigorously represented in terms of the "preferential binding" of the denaturant to the protein, i.e.,

$$\left[\frac{\partial \ln K_d}{\partial \ln a_{\mathrm{GuHCl}}}\right] = \left[\frac{\partial \ln f(a_{\mathrm{GuHCl}})}{\partial \ln a_{\mathrm{GuHCl}}}\right] = \Delta\nu^{\mathrm{pref}}_{\mathrm{GuHCl}} \tag{28}$$

where

$$\Delta\nu^{\mathrm{pref}}_{\mathrm{GuHCl}} = \Delta\bar{\nu}_{\mathrm{GuHCl}} - \frac{m_{\mathrm{GuHCl}}}{55.5}\Delta\bar{\nu}_{\mathrm{w}} \tag{29}$$

Here m_{GuHCl} is the molar concentration of GuHCl in the solution; $\Delta\bar{\nu}_{\mathrm{GuHCl}}$ is the difference between the number of moles of GuHCl bound to 1 mole of denatured and native protein; $\Delta\bar{\nu}_{\mathrm{w}}$ is the similar expression for the

"bound" water molecule; and K_d is the observed equilibrium constant which is believed to be the product of two independent functions at constant temperature, one of the functions depending only on the pH and the other on the activity of GuHCl, i.e.,

$$K_d = K_d^0 F(a_H) f(a_{GuHCl}) \tag{30}$$

where K_d^0 is a constant that formally represents the value of K_d under conditions where $F(a_H)$ and $f(a_{GuHCl})$ are both equal to unity. The form used for $F(a_H)$ is such that it becomes unity at a very low pH:

$$F(a_H) = \prod^{t} (1 + K_{t,D}/a_H) / \prod^{t} (1 + K_{t,N}/a_H) \tag{31}$$

The function $f(a_{GuHCl})$ cannot be formulated without models that will account for the observed values of $\Delta\bar{\nu}_{GuHCl}$. It was assumed that the difference between the native and the denatured states lies in differences between the number of binding sites $\Delta n = n_D - n_N$, and several possibilities for binding have been considered: (a) The protein exhibits noninteracting binding sites, each capable of binding one GuHCl molecule; (b) binding of GuHCl includes the competitive release of two water molecules; (c) guanidine cation and anion are bound independently; (d) the same as (c), including the competitive release of water at binding of the denaturant.

These models lead to the different equations which can be solved by least-square fits:

$$K_d/F(a_H) = K_d^0(1 + K a_{GuHCl})^{\Delta n} \tag{32a}$$

$$K_d/F(a_H) = K_d^0(1 + K^I a_{GuHCl})^{\Delta n} \tag{32b}$$

$$K_d/F(a_H) = K_d^0(1 + K^{II} a_{\pm})^{\Delta n} \tag{32c}$$

(assuming $a_{\pm} = a_{GuH^+} = a_{Cl^-} = a_{GuHCl}^{1/2}$)

$$k_d/F(a_H) = K_d^0(1 + K^{II} a_{\pm})_w^{\Delta n/q} \tag{32d}$$

(q is the number of sites on the surface of the native protein molecule which accommodates water molecules but excludes ions in general).

The results obtained by Eq. (32c) have been believed to be the most plausible (Tanford and Aune, 1970). The assumed model of GuHCl action is indeed most consistent with the results of Robinson and Jencks (1965) and with the more recent studies of Lee and Timasheff (1974) and Roseman and Jencks (1975).

It has been assumed that the functions in Eq. (30) are temperature-independent; i.e., the enthalpy of interaction of a denaturant with the protein is zero. Under this assumption the enthalpy of unfolding and its

dependence on temperature, i.e., the heat capacity change, were found from the temperature dependence of the apparent equilibrium constant (Tanford and Aune, 1970). The data compiled for lysozyme have been interpreted in terms of different states for thermal (X) and guanidine hydrochloride (RC) denaturation which has already been suggested by Aune *et al.* (1967). Both states are distinguishable by the preferential interaction parameters Δn and by different heat capacity changes ΔC_p (see Table III).

The importance of the observed heat capacity change in discussing the nature of denaturation is obvious, since it is commonly accepted since the work of Kauzmann (1959) that the heat capacity of a system with nonpolar groups is larger, when these groups are in contact with water. The difference in the heat capacities of state X and state RC were interpreted as proof that state X is less unfolded than state RC which presents a random coil, i.e., structural elements maintained by hydrophobic contacts in thermally denatured protein remain (Tanford and Aune, 1970). This concept seemed quite probable until the heat capacity changes associated with heat denaturation were measured directly by scanning microcalorimetry (see Section II). It was found that in heat denaturation, the heat capacity of lysozyme changes by 1.6 kcal K^{-1} mol^{-1} (Khechinashvili *et al.*, 1973; Privalov and Khechinashvili, 1974a). This value is much larger than the heat capacity change expected from equilibrium treatment even for the complete unfolding of lysozyme in concentrated GuHCl solution. The enormous discrepancy between the expected and measured values was confusing since it meant that one had to reconsider the concept of the denatured state of protein which seemed to be already settled, or to reconsider the treatment of equilibrium data in the presence of denaturants, or even to revise them both. These doubts were aggravated by the failure of all attempts to disclose experimentally the heat effect associated with the additional unfolding of heat-denatured protein by guanidine hydrochlo-

TABLE III

Thermodynamic Parameters for Lysozyme Transitions in Different States at 25°C, pH 7[a]

Transition	Log K	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal K^{-1} mol^{-1})	ΔC_p (cal K^{-1} mol^{-1})
$N \rightleftharpoons RC$[b]	−5.8	7.9	22.4	49	1375
$N \rightleftharpoons X$[c]	−5.7	7.8	41.2	112	950
$X \rightleftharpoons RC$	−0.1	0.1	−18.8	−63	425

[a] Reprinted with permission from Tanford and Aune (1970), *Biochemistry* **9**, 206–211. Copyright by the American Chemical Society.

[b] RC = the random coil state obtained by guanidine hydrochloride denaturation.

[c] X = the state obtained by thermal denaturation.

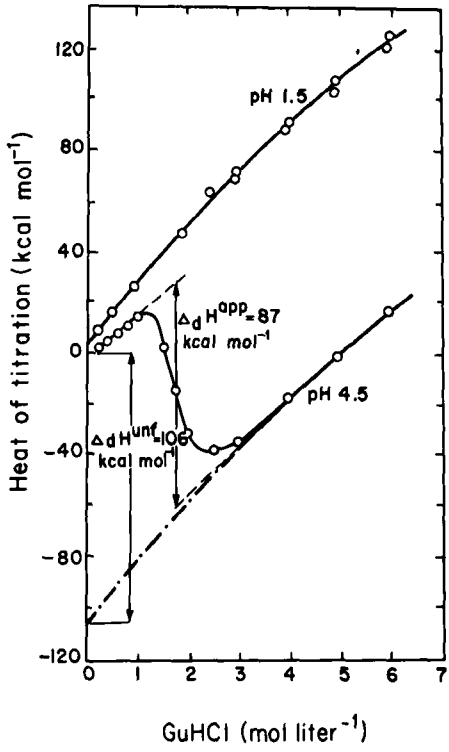

FIG. 21. Calorimetric titration of lysozyme by GuHCl at 59.1°C in solution at pH 1.5 and 4.5. The lysozyme at pH 1.5 is already heat-denatured at this temperature. From Pfeil and Privalov (1976b).

ride. Calorimetric titration of lysozyme in solution at pH 1.5 and 59.1°C, where this protein is already heat denatured (Fig. 21), revealed only a monotonically increasing curve with a slight curvature. This guaranteed, up to 6 *M* GuHCl, the absence of any transition with a negative enthalpy of more than 4 kcal mol^{-1}. At the same time, the large endothermic effect, clearly observed in solutions of lysozyme at pH 4.5 and 59.1°C, essentially exceeded the enthalpy value expected from the equilibrium treatment (Pfeil and Privalov, 1976b).

Calorimetric studies revealed a very strong solvation effect of a denaturant (see also Delben and Crescenzi, 1969; Atha and Ackers, 1971; Lapanje and Wadsö, 1971; Paz Andrade *et al.*, 1976). The heat of solvation of heat-denatured lysozyme in 6 *M* GuHCl at 59.1°C is about 120 kcal mol^{-1}. Assuming that there are about 67 binding sites at this concentration of GuHCl (Lee and Timasheff, 1974), we obtained 2 kcal mol^{-1} for the average interaction enthalpy of GuHCl, in agreement with the expectation

of Robinson and Jencks (1965). Thus, the enthalpy of denaturant interaction is not negligible at all, and its disregard in the treatment of the temperature dependence of apparent equilibrium constants is unjustified. But this means that we can consider as reliable only the data obtained at a fixed temperature, i.e., only the apparent equilibrium constant (if this transition is indeed of a two-state type) and corresponding effective Gibbs energy of unfolding, but not its temperature derivatives—the enthalpy and, more so, the heat capacity. These estimates of effective Gibbs energies of unfolding of various compact globular proteins are collected in Table IV. For comparison, the Gibbs energies of protein denaturation at 25°C obtained by calorimetric studies at heat denaturation are also included in Table IV. The coincidence of the corresponding values is rather surprising and seems inexplicable from the point of view that different treatments unfold proteins to different extents.

B. Calorimetric Studies of Protein Unfolding by a Denaturant

The remarkable feature of calorimetric titration curves of proteins by a denaturant is an essential increase in enthalpy before and after denaturation due to the intensive heat effect of solvation (see the case of lysozyme at pH 4.5 in Fig. 21). To exclude the solvation effect and to obtain the net enthalpy of unfolding, it is necessary to extrapolate the enthalpy function for denatured protein to zero concentration of a denaturant. For this extrapolation the function can be taken for the protein, which is already heat-denatured, at pH 1.5. In the absence of GuHCl, this procedure gives the value of $\Delta_d H^{unf} = (106 \pm 5)$ kcal mol^{-1} for the enthalpy of lysozyme unfolding. As seen in Fig. 21, this value is much larger than the value obtained by the interpolation of solvation functions for the native and denatured states in the middle of the transition zone $\Delta_d H^{app} = (87 \pm 5)$ kcal mol^{-1}. This difference is caused by the preferential binding of GuHCl on unfolding of protein since the number of binding sites in the unfolded protein is greater than in the compact one. For the same reason the observed slopes of enthalpy functions for native and denatured proteins s_N and s_D are also different. Denoting the number of binding sites in native and denatured protein at given activities of denaturant by a_N and a_D, we have for the slopes:

$$s_D = \left(\frac{\partial \Delta H}{\partial c}\right)_T^D = \left(\frac{\partial \Delta H}{\partial c}\right)_T^N \frac{a_D}{a_N} = s_N \frac{a_D}{a_D - \Delta n} \tag{33}$$

where $\Delta n = a_D - a_N$ is the increment of the binding sites at unfolding. The Δn value can be estimated from equilibrium measurements (Tanford, 1968, 1970), and a_D can be determined from density measurements and

TABLE IV

Gibbs Energy Change for Protein Unfolding at 25°C Obtained by Different Approaches

Protein	pH	ΔG (kcal mol^{-1})	Approach	Reference
Bovine pancreatic ribonuclease A	6.6	9.7 ± 1.7	Denaturant	Greene and Pace (1974)
	6.0	13.0	Denaturant	Salahuddin and Tanford (1970)
	5.5	10.6 ± 0.6	Temperature (calorimetry)	Privalov and Khechinashvili (1974a)
Hen egg white lysozyme	7.0	14.2	Denaturant	Aune and Tanford (1969b)
	5.7	14.5 ± 0.8	Temperature (calorimetry)	Privalov and Khechinashvili (1974a)
Bovine pancreatic α-chymotrypsin	4.3	8.3 ± 0.4	Denaturant	Greene and Pace (1974)
	—	12.3	Denaturant	Knapp and Pace (1974)
	4.0	11.6 ± 0.5	Temperature (calorimetry)	Privalov and Khechinashvili (1974a)
Bovine heart ferricytochrome c	6.5	15.4	Denaturant	Knapp and Pace (1974)
	4.8	9.0 ± 0.6	Temperature (calorimetry)	Privalov and Khechinashvili (1974a)
Sperm whale ferrimyoglobin	7.0	13.2	Denaturant	Puett (1973)
	10.0	12.0 ± 0.8	Temperature (calorimetry)	Privalov and Khechinashvili (1974a)
	9.0	13.6	pH (titration)	Hermans and Acampora (1967)
α-Lactalbumin	6.7	6.5	Denaturant	Kuwajima *et al.* (1976)
	5.0	5.4	Temperature (calorimetry)	W. Pfeil (personal communication, 1978)

from isosbestic determination (Lee and Timasheff, 1974). Knowing Δn and a_D, it is possible to determine the enthalpy of the preferential binding from the observed slopes of titration curves, since at the midpoint of transition c_t

$$\Delta_d H^{pref} = s_D c_t - s_N c_t = \left(\frac{\partial \Delta H}{\partial c}\right)_T^N \frac{c_t \Delta n}{a_D - \Delta n} \tag{34}$$

Thus, it is possible to obtain the net unfolding enthalpy without extrapolation procedures to zero concentration of the denaturant since

$$\Delta_d H^{unf} = \Delta_d H^{app} - \Delta_d H^{pref} \tag{35}$$

(for details, see Pfeil and Privalov, 1976b).

It is evident that the same correction for preferential binding of a denaturant should also be introduced in studying heat or pH denaturation of protein in the presence of a denaturant and the omission of this factor was the greatest fault of many publications on this subject. Indeed, in studying heat denaturation of protein in the presence of various amounts of a denaturant by scanning calorimetry, it can be seen (Table V) that with the increase of the denaturant concentration, the observed apparent enthalpy decreases much faster than the enthalpy corrected for the preferential binding $\Delta_d H^{corr}$. At the same time, the apparent heat capacity change calculated from the temperature dependence of the apparent enthalpies is in drastic conflict with calorimetrically measured heat capacity change, while the latter is in good agreement with the heat capacity calculated from enthalpies corrected for preferential binding.

It was striking that the proper change of the enthalpy and heat capacity on unfolding of protein does not depend on the presence of the denatur-

TABLE V

Scanning Calorimetric Studies of Lysozyme in the Presence of GuHCl at pH 2[a]

C_{GuHCl} (M)	T_d (°C)	$\Delta_d H^{app}$ (kcal mol^{-1})	$\Delta_d C_p^{app}$ (kcal K^{-1} mol^{-1})	$\Delta_d H^{corr}$ (kcal mol^{-1})
0	55	102 ± 3	1.6 ± 0.15	103 ± 3
0.25	52	92 ± 3	1.4 ± 0.15	94 ± 3
1.0	45.5	80 ± 4	1.6 ± 0.15	89 ± 5
2.0	36	49 ± 5	1.3 ± 0.3	71 ± 7

$$\frac{\partial \Delta_d H^{sss}}{\partial T_d} = (2.5 \pm 0.3) \text{ kcal K}^{-1} \text{ mol}^{-1}$$

$$\frac{\partial \Delta_d H^{corr}}{\partial T_d} = (1.6 \pm 0.2) \text{ kcal K}^{-1} \text{ mol}^{-1}$$

[a] According to Pfeil and Privalov (1976b).

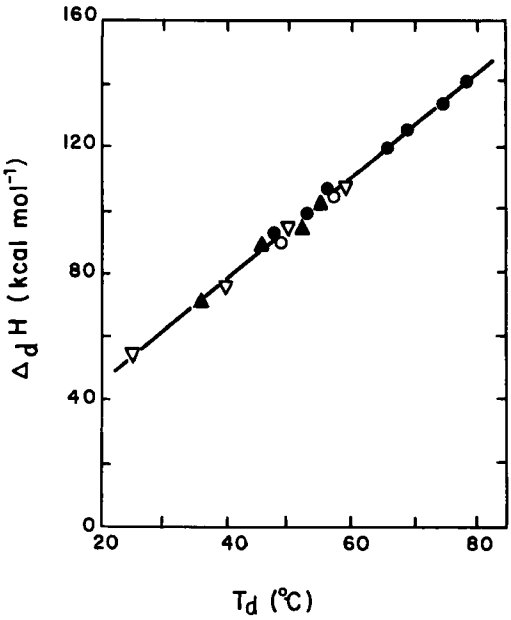

FIG. 22. Enthalpies of lysozyme denaturation obtained by various methods and at different conditions plotted against temperature of denaturation. *In solutions without GuHCl*: scanning calorimetry (denaturation by temperature at fixed pH) (●), isothermal calorimetry (denaturation by pH at fixed temperatures) (○). *In solutions with GuHCl*: scanning calorimetry (denaturation by temperature at fixed concentration of GuHCl) (▲), isothermal calorimetry (denaturation by GuHCl at fixed temperature and pH) (▽). From Pfeil and Privalov (1976b).

ant. But even more striking was the fact that the enthalpy of unfolding does not depend on the manner of unfolding—whether it was achieved by increasing the amount of GuHCl at constant temperature or by elevating temperature in the presence or absence of a denaturant. If the corrections for preferential binding are made correctly, the same values for unfolding enthalpies are obtained either by titration or by scanning calorimetry, and these values are found to be functions only of the temperature at which unfolding occurs (Fig. 22). The temperature dependence of unfolding enthalpy or heat capacity change at unfolding was found to be (1.6 ± 0.1) kcal K^{-1} mol^{-1} for lysozyme (Pfeil and Privalov, 1976b). The same excellent correspondence of unfolding enthalpies and heat capacity changes has been found in studying the denaturation of α-lactalbumin induced by temperature or by GuHCl (W. Pfeil, personal communication, 1978). It is evident that these results are not consistent with the concept that denaturants, particularly GuHCl, are more efficient in breaking the compact struc-

tures than change in pH and increase in temperature and could break residual structures in heat-denatured protein. This conclusion is supported also by the observed linear temperature dependence of apparent enthalpies of heat denaturation for various proteins in the presence of different amounts of denaturant (Khechinashvili, 1977), and by the fact that their extrapolation to zero denaturant concentration gives precisely the point which corresponds to the enthalpy of heat denaturation in the absence of the denaturant $\Delta_d H$ (Fig. 23a). It is remarkable that the difference between the functions $\Delta_d H$ and $\Delta_d H^{app}$, which is exactly the enthalpy of preferential binding at a given concentration of the denaturant is a linear function of denaturant concentration (Fig. 23b). The slope of this function will be proportional to the increase in the number of binding sites of the denaturant on protein unfolding and can be used in structural studies of proteins. From the observed linear dependence of enthalpy of preferential binding on the denaturant concentration, we can conclude that the cooperative penetration of the denaturant into the protein interior does not take place in the case of compact globular proteins. But in all cases this needs special consideration. For example, $\delta\Delta_d H$ is not a linear function of guanidine hydrochloride concentration for α-lactalbumin (W. Pfeil, personal communication, 1978). At the same time, according to Kuwajima *et al.* (1976) and Kuwajima (1977), this protein exhibits a predenaturational

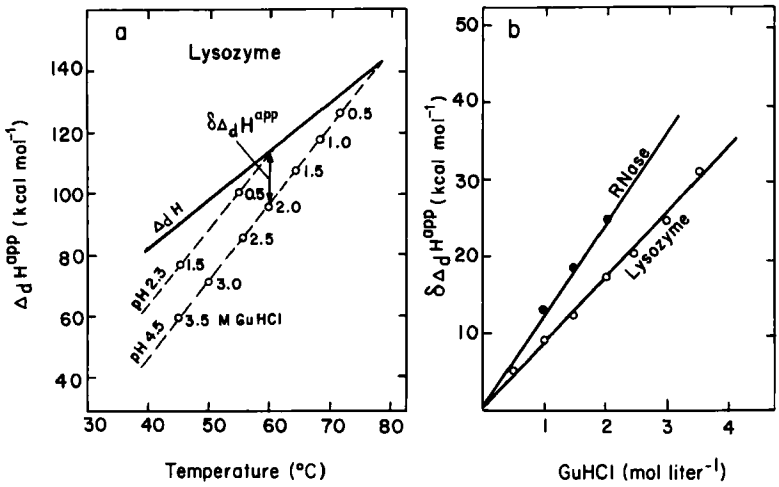

FIG. 23. (a) Enthalpy of heat denaturation of lysozyme in solutions at fixed pH in the presence of different concentrations of GuHCl ($\Delta_d H^{app}$) versus temperature of denaturation. The line $\Delta_d H$ corresponds to the enthalpy function in the absence of GuHCl. (b) Plot of enthalpy decrement ($\delta\Delta_d H^{app}$) versus GuHCl concentration for lysozyme and ribonuclease. Reproduced from Khechinashvili (1977).

transition on titration by GuHCl. It is not yet clear whether this transition of α-lactalbumin is of a two-state type into a less compact state or whether it should be regarded as a gradual swelling of its not very compact native structure (see also Section V,B).

V. Thermodynamics of Protein Unfolding

A. Unfolded State of Protein

It has been generally accepted that on denaturation the compact protein structure unfolds, but that the extent of its unfolding is different for different denaturing agents. It was believed that complete unfolding is achieved only in a concentrated GuHCl solution, whereas unfolding due to temperature changes is only partial. This viewpoint was based on the following facts: (a) on treating heat-denatured protein with GuHCl, the optical parameters exhibit changes which were interpreted as an additional unfolding of the residual structure (Aune *et al.*, 1967). (b) The intrinsic viscosity of protein increases to a lesser extent when temperature is increased than when the concentration of denaturant is increased. For instance, the intrinsic viscosity of lysozyme, which is 3.0 $cm^3\ g^{-1}$ at 25°C for native protein, increases to only 4.7 $cm^3\ g^{-1}$ upon heating to 75°C (at which temperature the protein is heat-denatured) (Hamaguchi and Sakai, 1965; Kugimiya and Bigelow, 1973), while in 6 *M* GuHCl at 25°C, it is 6.5 $cm^3\ g^{-1}$. This value corresponds to the maximum possible for the cross-linked polypeptide chain of lysozyme (Tanford, 1968). (c) According to equilibrium studies, the increase of the partial heat capacity of protein is less for heat denaturation than for denaturation by GuHCl (Tanford and Aune, 1970).

But a careful investigation of these facts revealed that: (a) The observed changes of the optical parameters on the addition of GuHCl to heat-denatured protein cannot be interpreted within the framework of additional unfolding of the structure, since the observed changes are in the opposite direction and seem to be only a manifestation of the solvation phenomena (Pfeil and Privalov, 1978). (b) The comparison of the intrinsic viscosities corresponding to different temperatures is physically incorrect since there is a strong dependence of intrinsic viscosities on temperature. The intrinsic viscosity of lysozyme in 6 *M* GuHCl at 55°C is already 4.8 $cm^3\ g^{-1}$ (Ahmad and Salahuddin, 1974), which is identical to the values obtained on heat denaturation; i.e., in reality there is no difference in the viscosities of lysozyme with the intact disulfide bridges denatured either by temperature or denaturant. (c) The heat capacity increase on heat denaturation is just the same as on denaturation by GuHCl (see Section IV.). Moreover, the enthalpies associated with unfolding due to temperature,

pH and GuHCl are the same, and the Gibbs energies of unfolding are also very similar, if possible errors of determination are taken into account.

The careful analysis of all existing publications shows that at present we have no quantitatively interpretable experimental fact which could be considered as unequivocal evidence of the existence of a difference in the extent of unfolding of protein resulting from different denaturational treatments, but we do have thermodynamic evidence that the extent of unfolding in all cases is the same. The question of whether this extent is 100%, i.e., whether unfolding is complete up to the ideal random coil, remains to be answered. It is evident that this question is a principal one in developing the thermodynamics of protein. But in considering it we must bear in mind that the denatured state is the only random state of protein experimentally available, and the future of thermodynamics and statistical physics of proteins depends greatly on our desire for its effective use. It is clear in advance that the protein polypeptide cannot be an ideal random coil in any solution, but it is far from clear how essential this fact is for thermodynamic studies, even if it is essential in some other relation.

Judging from viscosity studies, the denatured state of protein is not far from the random coil. The intrinsic viscosity of denatured lysozyme with broken disulfide bridges amounts to 17 $cm^3\ g^{-1}$, which is in good agreement with the viscosity expected for a random coil polypeptide of this size (Tanford, 1968). Bearing in mind the position of the disulfide bridges in lysozyme, the decrease in viscosity to 6.5 $cm^3\ g^{-1}$ on restoration of the bridges can be explained.

Infrared and Raman spectroscopy provide evidence that there are no elements of the secondary structure in denatured protein if aggregation is prevented (Chen *et al.*, 1974; Fedorov and Khechinashvili, 1976).

According to hydrogen exchange studies, all protons in denatured proteins are exchanged very fast (Hvidt and Nielsen, 1966).

NMR spectroscopy indicates that all groups in GuHCl- and urea-denatured proteins are in a homogeneous environment and their spectra are much the same as the spectra of solutions of free amino acids and short peptides (McDonald and Phillips, 1969). The same situation seems to exist with protein denatured by heat and pH when aggregation is excluded (Epstein *et al.*, 1971; McDonald *et al.*, 1971; Bradbury *et al.*, 1972; Bradbury and Norton, 1973; Matthews and Westmoreland, 1975).

From all these facts it follows indisputably that the denatured protein has no fixed residual structure, but it is impossible to estimate quantitatively how far its conformation is from the ideal random coil. Because of this we have to assume initially that the conformation of denatured protein is that of a random coil, and thus the process of denaturation is nothing but a transition to this unfolded state. How efficient and realistic this assumption

may be, and how essential might be the correction for nonideality of the unfolded state, will be clear only after carrying out a thermodynamic analysis of unfolding of numerous proteins.

B. Enthalpy of Protein Unfolding

In considering the denatured state of protein as an unfolded conformation, we can characterize the process of unfolding of compact native conformation by the difference of the standard functions for the denatured and the native states at given values of external variables defined by Eq. (26). As previously noted, these difference functions are determined over a broader range of variables than those for which denaturation is observed experimentally. Thus, they should be regarded as abstract functions characterizing the potential possibility of conformation at transition, irrespective of whether or not transition into another state could take place in reality.

The molar enthalpy of protein denaturation has already been presented in Fig. 10. The important conclusion which has been made in considering Fig. 10 is that the enthalpy of conformational transition of protein is a linear function of temperature. But the values of these enthalpies and their temperature dependences are so different for different proteins that no other regularities could be noticed from the presented picture. Quite a different situation appears if we consider the specific, and not the molar, quantities, i.e., the enthalpy values calculated per unit of protein mass.

The most remarkable feature of the specific enthalpy of unfolding of globular protein is that the differences between enthalpy values of different proteins decrease with the increase of temperature and even disappear at 100°–110°C (see Fig. 24). At this temperature all specific enthalpies reach values of about 13 cal g^{-1}. But this regularity is not general for all proteins which are regarded as globular. Indeed, it is not observed for serum albumin (Leibman *et al.*, 1975), α-lactalbumin (W. Pfeil, personal communication, 1978), or ribosomal protein L7 (Khechinashvili *et al.*, 1978). Also, it is not fulfilled for histone (see Fig. 24b). It is intriguing that for all the proteins in group A the three-dimensional structure is known, while it is not known for the proteins in group B (except for pancreatic trypsin inhibitor). The question arises: What is the nature of these qualitative differences between these two groups of proteins?

In discussing this problem, α-lactalbumin is one of the best examples because its chemical structure is very similar to that of lysozyme (Brew *et al.*, 1970; Vanaman *et al.*, 1970), and it was thought that the three-dimensional structures of both of these proteins are also similar (Browne *et al.*, 1969). But in contrast to lysozyme, the stability of α-lactalbumin is low (Takase *et al.*, 1976), it exchanges all the internal hydrogens much faster (Takesada *et al.*, 1973; Bradbury and Norton, 1975), and its partial specific

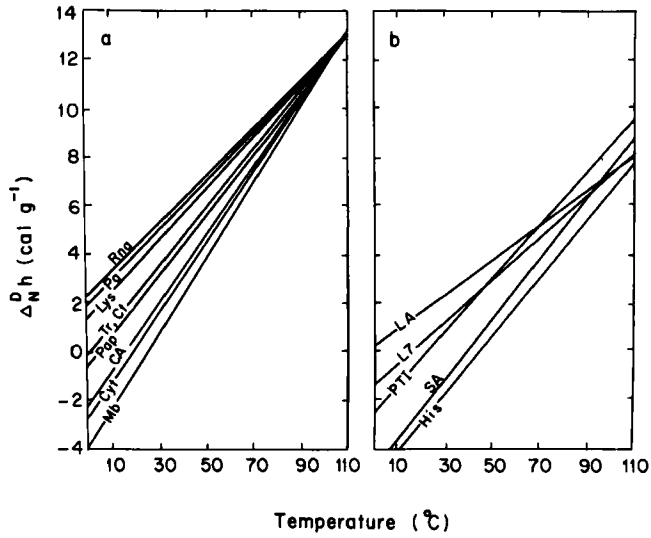

FIG. 24. Temperature dependence of specific enthalpies of unfolding $\Delta^{Ph}_{N}h(T)$ for proteins. (a) Ribonuclease A (Rna), parvalbumin (Pa), lysozyme (Lys), α-chymotrypsin (Ct), β-trypsin (Tr), cytochrome c (Cyt), carbonic anhydrase B (CA), metmyoglobin (Mb), and papain (Pa). (b) Serum albumin (SA), histone H1 (His), ribosomal protein L7, pancreatic trypsin inhibitor (PTI). For references, see legend to Fig. 10.

heat capacity is much larger than that found for compact proteins, being 0.38 cal K^{-1} g^{-1} at 25°C instead of 0.32 cal K^{-1} g^{-1} (W. Pfeil, personal communication, 1978). Therefore, although we do not yet know the three-dimensional structure of α-lactalbumin, there is some doubt that it is as compact as the structure of lysozyme (Imoto *et al.*, 1972). The same situation seems to hold for serum albumin. At present we do not know anything definite about its three-dimensional structure, but it may be suspected that this protein is not quite compact (Foster, 1960; Bloomfield, 1966; King and Spencer, 1970). In the case of α-lactalbumin and serum albumin it is not easy to decide what some lack of compactness could mean structurally. Should we interpret it as a structure loosened throughout or as a structure compact in part? For these two proteins the first interpretation seems more probable. An opposite example is presented by ribosomal protein L7 from *Escherichia coli*. According to much indirect evidence, a part of this molecule, namely residues 1-54 of the total 120 residues (i.e., 45%), is in a noncompact conformation (Gudkov *et al.*, 1977; Gudkov and Behlke, 1978). This unstructured tail of the macromolecule seems to be used for its dimerization which can be prevented by oxidation of Met_{25} (Gudkov *et al.*, 1978). At the same time the specific enthalpy of

L7, extrapolated to 110°C, is 8 cal g^{-1}, i.e., it is just 45% lower than 13 kcal g^{-1}, which is characteristic for the proteins presented in Fig. 24a. It is very interesting that when fragmenting L7 by removing a different number of residues from the N-terminal, the specific enthalpy of the remaining fragment increases and, for the fragment 45–120, approaches 11 cal g^{-1} (Khechinashvili *et al.*, 1978). The same situation is observed with histone H1. According to many lines of evidence, in solution this protein has a compact body and unstructured wings (Hartman *et al.*, 1977). The wings constitute about 40% of the entire weight of the molecule. At the same time, the specific enthalpy of unfolding of the histone at 110°C is about 7 cal g^{-1}.

Thus, we can conclude that 13 cal g^{-1} at 110°C is some characteristic value for compact structures and that the regularity which is presented in Fig. 24a is specific only for compact globular proteins. Since this regularity appears only when considering the specific and not the molar enthalpies, we have to conclude that it reflects some internal properties of proteins which do not depend on the protein size. We can assume that compact globular proteins should have some common features in their structural organization responsible for their thermodynamic behavior. The correlation analysis of structural and thermodynamic characteristics has been done on several proteins with well-known structures (Privalov and Khechinashvili, 1974a; Khechinashvili *et al.*, 1978). The results are collected in Table VI. Two main conclusions were drawn from this study: (1) Native structures of all compact proteins studied are equally saturated by the hydrogen bonds between the groups of the macromolecule; i.e., the number of hydrogen bonds per unit mass of this protein, n_H, is very much the same. The spread of values does not exceed 8% (on this aspect, also see Chothia, 1975). The only exception is pancreatic trypsin inhibitor. Its native structure is much less saturated by hydrogen bonds, but the specific enthalpy of its unfolding at 110°C is also significantly lower (see Fig. 24b). (2) Saturation of the structures of these proteins by nonpolar contacts, i.e., the number of pairs of nonpolar groups n_φ located at a distance up to 4.0 Å calculated per unit mass of protein, is different for different proteins. This specific value correlates with the observed change in specific heat capacity of protein on unfolding or with a temperature dependence of specific enthalpy of unfolding (see Fig. 25).

The last finding was not unexpected. After Kauzmann (1959), it was generally believed that unfolding of a compact protein structure has to be accompanied by a heat capacity increase as a result of interaction of nonpolar groups with water. But this effect is not the only possible source of a heat capacity increase in a system where the degrees of freedom largely increase as well. The direct correlation between the observed heat capacity

TABLE VI

Hydrogen Bonds and Contacts between Nonpolar Groups in Proteins

Protein	Molecular weight MW	Number of internal hydrogen bonds N_H	$N_H/MW = n_H$ ($\times 10^3$)	Number of nonpolar contacts N_φ	$N_\varphi/MW = n_\varphi$ ($\times 10^3$)	Observed change of heat capacity at denaturation $\Delta_d c_p$ (cal K^{-1} g^{-1})
Ribonuclease	13,600	81	6.0	90	6.6	0.090
Parvalbumin	11,500	71	6.2	71	6.2	0.095
Lysozyme egg white	14,300	89	6.2	108	7.5	0.100
Lysozyme T4	18,600	98	5.3	138	7.4	0.110
β-Trypsin	23,800	165	6.9	196	8.2	0.120
α-Chymotrypsin	25,200	173	6.9	238	9.4	0.120
Papain	23,400	139	5.9	204	8.7	0.125
Cytochrome c	12,400	70	5.6	136	11.0	0.140
Myoglobin	17,900	133	7.4	213	11.9	0.155
Pancreatic trypsin inhibitor	6,500	28	4.3	49	7.5	0.110

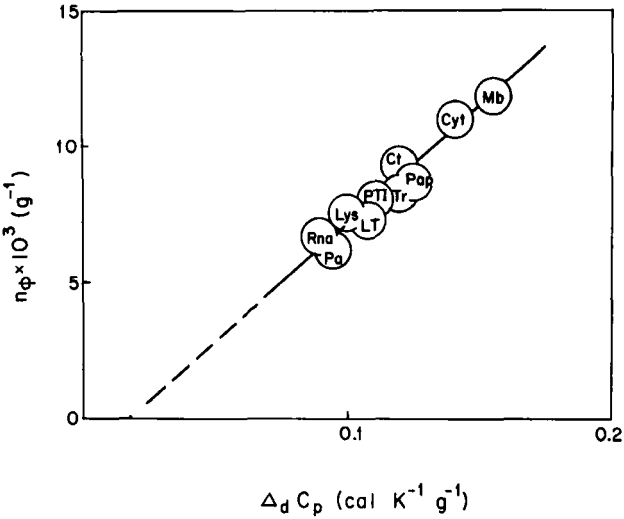

FIG. 25. Plot of concentration of nonpolar contacts in proteins n_φ against the observed denaturational specific heat capacity change $\Delta_d C_p$. See Privalov and Khechinashvili (1974a) and Privalov and Pfeil (1978).

effect and the specific amount of nonpolar contacts in a compact structure shows that the interaction of nonpolar groups with water is indeed a most important contributor to heat capacity change on unfolding. Thus, at least 80% of the temperature dependence of enthalpy of unfolding can be explained by the negative heat of disruption of hydrophobic contacts, i.e., by the negative heat of ordering water around the exposed nonpolar groups. If we assume that this ordering influence of nonpolar groups on water drops to zero at 110°C, we can explain the physical meaning of the point where the specific enthalpies of globular proteins coincide. The enthalpy of unfolding of a compact structure at 100°–110°C should correspond to the enthalpy of disruption of all the other, except the hydrophobic, bonds involved in maintaining the compact structure. The temperature dependence of this enthalpy as is seen from Fig. 25 does not exceed 0.02 cal K^{-1} g^{-1}. The bonds responsible for this enthalpy might be the hydrogen bonds and the van der Waals interaction between groups packed in the compact structure. The number of hydrogen bonds, as has been shown, is the same per unit of mass of considered protein. The concentration of van der Waals contacts also seems to be the same, judging by the same density of globular proteins. If we assume that the van der Waals contacts are much less important contributors to the enthalpy of unfolding and attribute all the enthalpy to the disruption of intermolecular hydrogen bonds, we will get 1.7 kcal mol^{-1} of hydrogen bonds at 110°C

and 1.5 kcal mol^{-1} of hydrogen bonds at 25°C. One could regard the curious coincidence of this value with the expected value for the intrapeptide hydrogen bond disruption in water media (see Schellman, 1955a; Kauzmann, 1959a,b) as confirmation of the correctness of the assumption. But the real situation does not seem to be so simple. Studies of fibrillar protein unfolding (Potekhin and Privalov, 1978a,b; to be considered also in Part B of this review) reveal that the enthalpy of disruption of the intrapeptide hydrogen bond in the fibrillar structure is about 1.2 kcal mol^{-1} at 110°C; i.e., it is 30% less than that found for globular protein. Thus, either of the following may obtain: (a) The enthalpy of the hydrogen bond in fibrillar structure is smaller; this might be due to the great influence of the polar water surroundings in the elongated system, while in globular proteins the hydrogen bonds are mainly screened from water by material with a low dielectric constant. (b) The globular structure has some additional energy resource; this could be the van der Waals interactions which are usually neglected in considering protein stabilization, since it was assumed that they are somewhat the same between protein groups and water molecules.

The results of recent analyses of group packing in globular proteins provide a forcible argument favoring the rehabilitation of the van der Waals contribution to the stabilization of compact structure. According to Klapper (1971) and Richards (1977), the compactness of the protein interior is very close to the highest found in amino acid crystals, i.e., it is quite possible that the packing of protein groups in the globule is higher than the packing of water molecules around these groups when they are exposed to water. Thus the contribution of the van der Waals interaction in maintaining the native structure of proteins cannot be negligible, but it is far from clear how realistic are the old estimates of Némethy and Scheraga (1962), who used values of 0.15 kcal mol^{-1} for a pair of aliphatic groups and 0.50 kcal mol^{-1} for a pair of aromatic groups.

It should be said that, although the assignment of the enthalpies and heat capacity values obtained for model compounds to protein is very attractive, the correctness and efficiency of this procedure is at present doubtful. These doubts are caused by the understanding that the compact native state of protein cannot be adequately approximated by a droplet of nonpolar liquids, nor can the unfolded state be regarded as a dilute solution of the corresponding groups. The concentration of the groups, even in an ideal random coil peptide, is too large for use in the thermodynamic description of data obtained for dilute solutions (see also Boje and Hvidt, 1972). That is why in this chapter we prefer to avoid a direct comparison of data obtained on proteins with the data known from studying model compounds.

C. *Entropy of Protein Unfolding*

Since the enthalpy of protein unfolding is independent of pH, it follows from the observed pH dependence of the stability of the native conformation that the entropy of unfolding is a pH-dependent function. In reality the pH-dependent function is the apparent entropy of denaturation which also includes, besides the entropy of conformational transition, the entropy of concomitant ionization of protein and buffer:

$$\Delta_d S^{app} = \Delta_d S_{pr}^{conf} + \Delta_d S_{pr}^{ion} + \Delta S_{buff}^{ion} \qquad (36)$$

In the case of enthalpy, the apparent value (in the absence of the denaturant) is close to the net enthalpy of protein unfolding, since ionization effects are small, and they can be easily compensated for by the appropriate choice of buffer. However, in the case of entropy, complete compensation is impossible, since entropy effects of ionization of protein and buffer compounds are essential and different. Thus, in considering the stability of protein in a given buffer solution, we are in reality considering a broader system which includes not only the protein, but also the buffer. This is practical, since unfolding is not only an intramolecular process and the stability of protein depends on environment. But if we want to consider the entropy change associated only with the conformational transition of protein, we have to exclude the buffer system and all entropy effects of protein ionization.

It has been shown by Pfeil and Privalov (1976c) that the conformational entropy of protein unfolding $\Delta_N^R S^{conf}$ in the first approximation is pH independent but depends strongly on temperature (see Fig. 26). Thus, there is a great analogy between the entropy and the enthalpy of protein unfolding. The entropy function is also a monotonically increasing function of temperature; but, in contrast to enthalpy, it is not linear. The difference between the specific entropies of unfolding of different proteins is diminished at higher temperatures, but the spread of values in the vicinity of 110°C is larger than in the case of enthalpy. To some extent the observed spread of entropy values at 110°C is associated with the different cross-linking of polypeptides of the studied proteins. According to the statistical theory of the random coil, a single cross-linking between two segments separated by N segments reduces conformational entropy by $\frac{3}{2}R \ln N$ (Kuhn and Majer, 1956). As is seen in Fig. 26 from the proteins studied, pancreatic trypsin inhibitor exhibits the lowest unfolding entropy at 110°C. At the same time this molecule has the shortest polypeptide chain consisting of only 59 residues cross-linked by 3 disulfides (Kassell and Laskowski, 1966). The number of residues in the 7 interdisulfide segments of its chain does not exceed 15. The other example which illustrates the role of disulfide links in regulating the entropy value of unfolding of protein is trypsin.

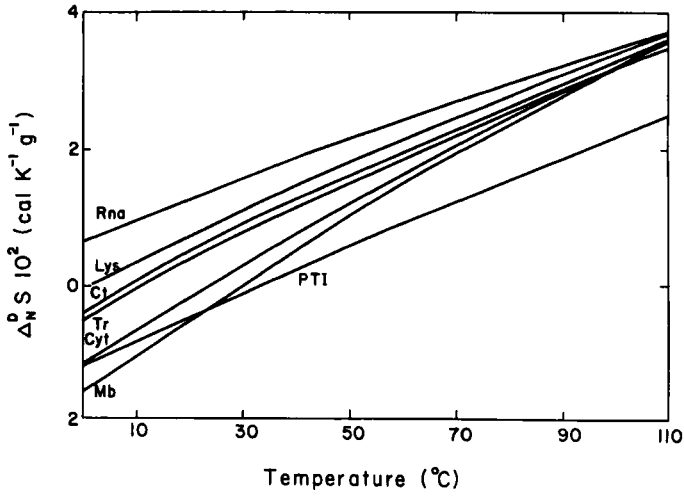

FIG. 26. Temperature dependence of specific entropies of protein unfolding $\Delta^P_N s(T)_{pH}$ at a pH where the extent of macromolecule ionization does not change. Notation the same as in Fig. 24. For references, see Fig. 10.

The structure of trypsin is somewhat similar to the structure of chymotrypsin, but with a lower molecular weight it has one extra disulfide bond. As a result, the entropy of its unfolding is lower than that of chymotrypsin, although the enthalpy is the same (see Figs. 24 and 26). A detailed analysis of the mechanism of entropy depression by specific cross-linking of the chain does not seem reliable at present because of the errors in experimental entropy values and the rough theoretical estimates of the entropy effects (see also Kauzmann, 1959a,b).

In accordance with the interpretation of the enthalpy function we might assume that at 110°C, the contribution of water in the entropy of unfolding is small, and all the observed effects can be attributed to the increase in configurational freedom of polypeptides on unfolding. The average entropy value for this temperature reaches 0.032 cal K^{-1} g^{-1}, i.e., 4.2 cal K^{-1} mol^{-1} per residue, which corresponds to about an 8-fold increase in possible configurations. This value is close to that expected for the transition of the polypeptide from the helical to the random coil conformation (Schellman, 1955b). This agreement can be considered to support the assumption that the denatured state is close to a random coil, in any case at elevated temperatures.

D. Stability of the Native State

Since the difference in Gibbs energy functions of the denatured and the native state, $\Delta^P_N G$, is the work required to transfer protein from the native

to the denatured macroscopic state, i.e., to unfold its compact structure, it can be regarded as a direct measure of stability of the native macroscopic state at the given conditions, or in short, of the macrostability of protein. But as is evident, the $\Delta_N^R G$ function will have this meaning of macrostability only if it is calculated for an entire cooperative system. As has been shown in Section II,C, in the case of small compact globular proteins the macromolecule usually, but not always, presents a single cooperative system, and, *a priori*, without calorimetric studies this cannot be decided definitely. That is why the investigation of protein stability became possible in practice only after the appearance of the microcalorimetric technique, although attempts to estimate it from equilibrium studies were begun much earlier (Brandts, 1964).

The $\Delta_N^R G(T,\mathrm{pH})$ function for several proteins is presented in Fig. 27. All of these functions are characterized by the different locations of the maxima and the different shapes of contour corresponding to the condition $\Delta_N^R G(T,\mathrm{pH}) = 0$, i.e., of half-conversion of protein to the unfolded state. But it is noteworthy that in all cases the stability increases with decreasing temperature and its maximal value is achieved at temperatures close to physiological or lower. This can be seen clearly in Fig. 28, where the cross-sections of difference Gibbs functions are collected for various proteins at pH corresponding to maximal stability, i.e., $\Delta_N^R G(T)_{\mathrm{pH}}$. The existence of the maximum of the $\Delta_N^R G(T)_{\mathrm{pH}}$ function is stipulated by the heat capacity change at unfolding, since it follows from Eqs. (25)–(32) that:

$$\Delta_N^R G(T)_{\mathrm{pH}} = \Delta_d H(T_d) \frac{T_d - T}{T_d} - \int_T^{T_d} \Delta_d C_p dT + T \int_T^{T_d} \Delta_d C_p d \ln T \quad (37)$$

It is also evident that at the temperature of maximal stability the entropy of protein unfolding is zero and stabilization of the native structure is achieved only by the enthalpy factor (Fig. 29). At a lower temperature the entropy of unfolding changes sign and becomes a stabilizing factor for the native state. The enthalpy of unfolding also changes sign, but at still lower temperatures, and becomes a destabilizing factor. Thus, *the stability of the native state is achieved only as a result of a small shift of the enthalpy and the entropy functions on the temperature scale.*

From the decrease in stability at lower temperatures we might expect, as was first pointed out by Brandts (1964), the phenomenon of "cold denaturation." Although this has not yet been observed experimentally for globular proteins, the decrease in stability at low temperature has been shown by studies of denaturation at high pressures (Hawley, 1971; Zipp and Kauzmann, 1973). This is illustrated in Fig. 30, where a projection of the Gibbs energy function on the pressure–temperature plane is given for

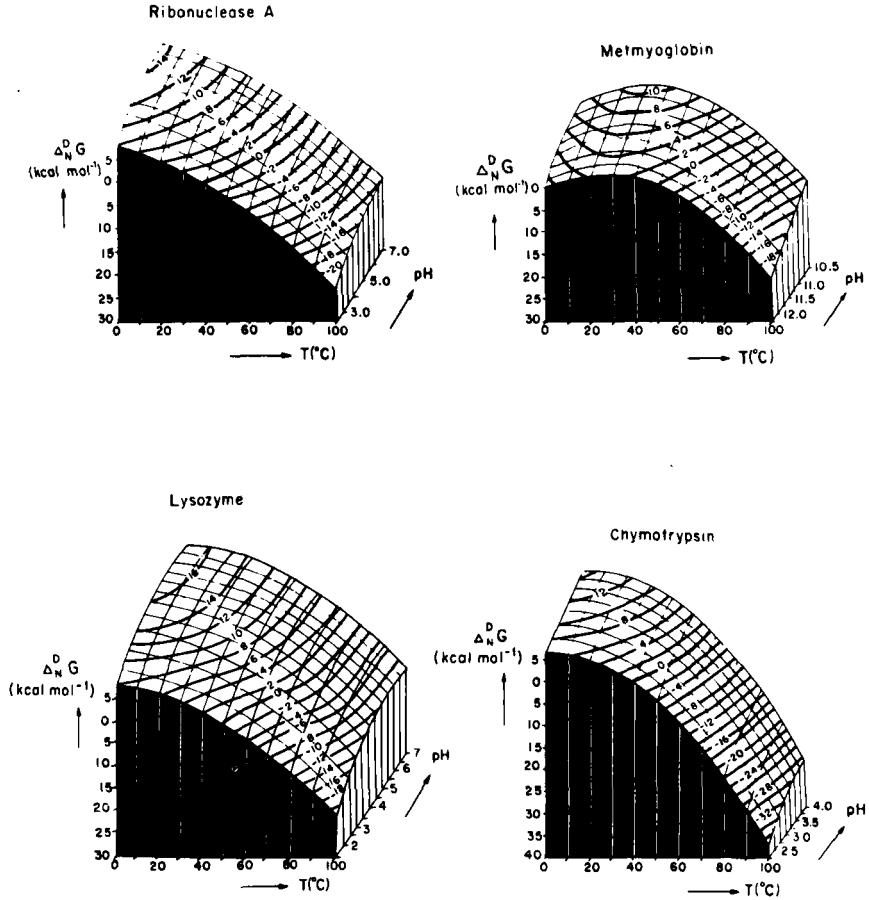

FIG. 27. $\Delta_N^D G(T, \mathrm{pH})$ functions of four different globular proteins. Reproduced from Privalov and Pfeil (1978).

chymotrypsinogen at pH 2.07, derived from the combination of calorimetric data with equilibrium data obtained by Hawley (1971) at high pressures. Unfortunately, at present the possibilities of presenting such findings for globular protein are very limited because of the absence of experimental data. But it should be emphasized that the protein phase space can be expanded in the direction of any needed variable if sufficiently complete experimental data become available.

Figures 27 and 28 reveal three remarkable properties of globular proteins: (a) Stability of very different proteins does not differ greatly. (b) Stability does not correlate with molecular weight. (c) Stability of proteins

at near-physiological temperatures is not at all large. For all the proteins considered it is about (12 ± 5) kcal mol^{-1}.

The value for the energy of stabilization of protein structure may be regarded as essential only as far as a protein can be regarded as one indivisible entity, i.e., a single cooperative unit. Dividing this total energy by the number of residues constituting the protein, we obtain the stabilization energy value per residue, which is an order less than the thermal energy RT. Thus, it becomes evident that cooperativity is a most important property of this system, since it integrates in some manner the contribution of elements constituting the protein. But what is remarkable, *proteins do not integrate more constituents than is necessary to achieve some distinct level of stability of the total system.*

It is also noteworthy that when the size of the protein molecule is too small to reach the necessary level of stability, this level is raised by specific cross-linking of the polypeptide chain. These cross-linkings influence the stability of the entire macromolecule by decreasing the entropy of total unfolding. Pancreatic trypsin inhibitor presents a good illustration of this point.

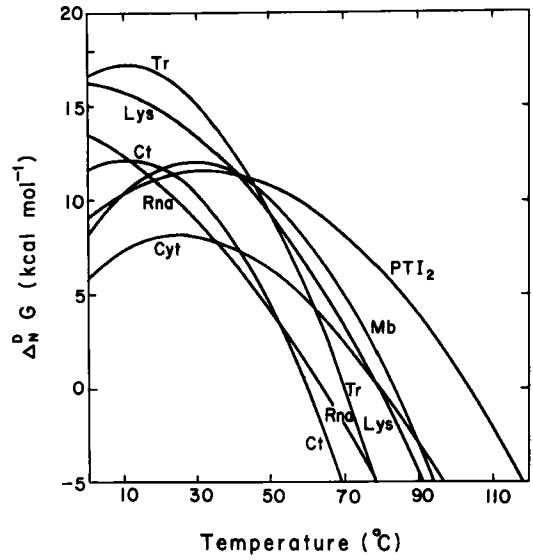

FIG. 28. Cross-sections of the $\Delta_R^D G$ functions at pH values corresponding to optimal stability of protein for lysozyme (Lys), ribonuclease A (Rna), α-chymotrypsin (Ct), cytochrome c (Cyt), metmyoglobin (Mb), dimer of pancreatic trypsin inhibitor (PTI_2). See Privalov and Khechinashvili (1974a,b) and Khechinashvili *et al.* (1978).

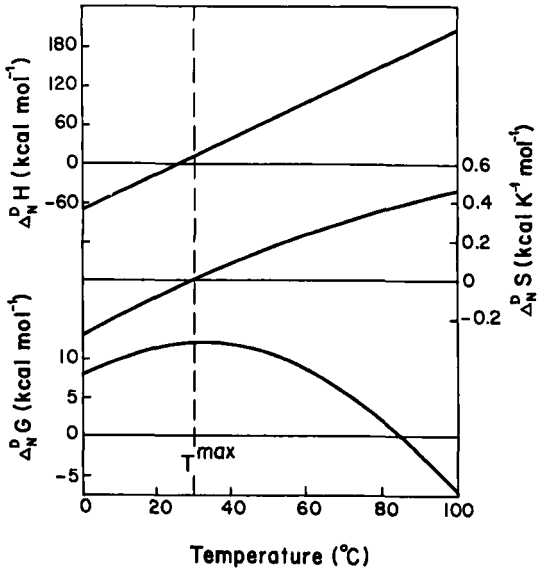

FIG. 29. The enthalpy, entropy, and Gibbs energy of protein unfolding on the example of myoglobin according to Privalov and Khechinashvili. Reprinted from Privalov and Khechinashvili (1974a), *J. Mol. Biol.* **86**, 665–684. Copyright by Academic Press, Inc. (London) Ltd.

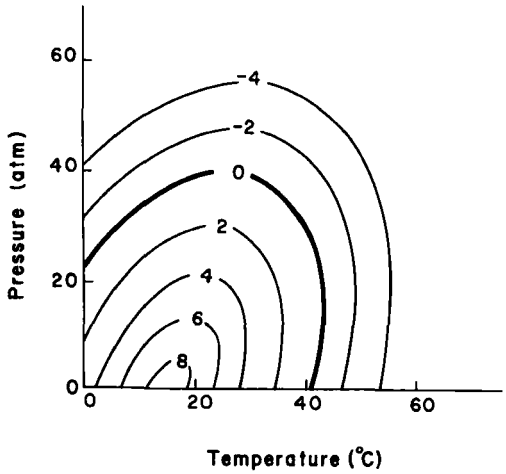

FIG. 30. Projection of isoenergic contours of the $\Delta_N^R G(T_{p,pH})$ function of protein on the temperature–pressure plane at fixed pH. (Chymotrypsinogen, pH 2.0.) Values on the curves are in kcal mol^{-1}. Calculated from calorimetric data of Jackson and Brandts (1970) and equilibrium studies at high pressure by Hawley (1971).

*E. Motility of Protein Structure**

The motility of protein structure is usually understood as the ability of the protein structure to fluctuate or to "breathe." The extent of breathing of a protein structure seems to be the property which is somewhat inverse to stability, but only to the stability of the microscopic state and not to that of the macroscopic state. In contrast to macrostability, microstability is defined by the energy required for microdisruption of protein structure, i.e., its micro or local unfolding which brings the internal groups of the protein into rapid contact with the solvent. This energy can be determined experimentally by studying the exchange of peptide hydrogens.

Assuming that peptide hydrogens can be exchanged only if they are in contact with water, the rate constant of exchange in a compact structure can be expressed through the rate constant of exchange k_0 in a noncompact structure by the equation

$$k = Kk_0 = \exp \frac{-\Delta G^{mic}}{RT} k_0 \tag{38}$$

where K is the equilibrium constant for micro-unfolding and ΔG^{mic} is the Gibbs energy of micro-unfolding (for details, see Linderstrøm-Lang and Schellman, 1959; Hvidt and Nielsen, 1966). Experimental studies on hydrogen exchange of globular protein revealed that only in exceptional cases such as lysozyme (Nakanishi *et al.*, 1973) and ribonuclease (Tiktopulo and Privalov, 1975) are exchange kinetics more or less simple. In most cases, different peptide hydrogens of globular protein are exchanged at different rates. This was interpreted as a manifestation of the difference in unfolding energies for the different parts of the macromolecule, i.e., as differences in microstabilities within the protein structure. This difference in microstabilities is best demonstrated by the "relaxation spectra" of protein proposed by Willumsen (1966); it presents a plot of the percentage of unexchanged peptide hydrogens at a given time versus $\log k_0 t$ (see Fig. 31). On this plot the distance between the curves for native protein and unfolded polypeptide in the random coil conformation corresponds to the energy of unfolding, ΔG^{mic}, which is required for the exchange of the given fraction of peptide hydrogens. This value varies between the limits of zero and 20 kcal mol^{-1} for the different peptide hydrogens of globular protein. The average energy of unfolding required for the exchange of one peptide hydrogen $\langle \Delta G^{mic} \rangle$ corresponds to the area between the curves for protein and the random-coiled polypeptide. This average energy can be considered as a measure of the microstability of a protein structure. The values of $\langle \Delta G^{mic} \rangle$ for several compact globular proteins, taken from

* See also Gurd and Rothgeb, this volume.

TABLE VII

Microstability of Protein $\langle \Delta G^{mic} \rangle$[a]

Proteins	$\langle \Delta G^{mic} \rangle$ (kcal mol^{-1})	$\Delta_N^R g$ (cal g^{-1})	Δ_N^{Dh} (cal g^{-1})	$\Delta_N^R s$ (cal K^{-1} g^{-1}) ($\times 10^3$)	$\Delta_d C_p$ (cal K^{-1} g^{-1})
Ribonuclease A	2.5[b]	0.8	5.0	1.42	0.090
Lysozyme	2.7	1.0	4.4	1.14	0.100
β-Trypsin	3.1	0.5	2.6	0.64	0.120
α-Chymotrypsin	3.3	0.5	2.7	0.72	0.120
Cytochrome c (ferri)	3.4	0.7	1.7	0.34	0.135
Chymotrypsinogen	4.7	—	2.0	—	0.130[c]
Carbonic anhydrase B	6.6	0.4	0.1	−0.10	0.140
Myoglobin	7.1	0.7	0.0	−0.22	0.155

[a] According to Willumsen (1971).

[b] Estimated from the data of Tiktopulo and Privalov (1975).

[c] According to Jackson and Brandts (1970).

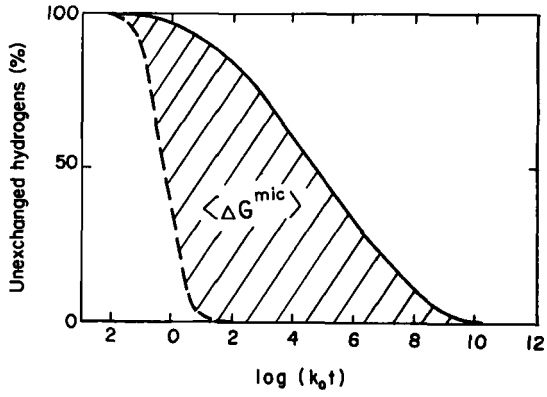

FIG. 31. Relaxation spectra of an abstract protein.

Willumsen's (1971) review, are presented in Table VII. We have selected here proteins with a known three-dimensional structure, since only in that case is there a chance of finding any structural grounds for the observed property. It would also be interesting to know whether there is any correspondence between micro- and macrostabilities of protein. But in searching for this correspondence we must bear in mind that microstability is an intensive characteristic of proteins (although it is measured in kilocalories per mole, but in peptide hydrogens and not protein), and that it can be compared only with other intensive characteristics of proteins. A comparison with the specific thermodynamic characteristics of protein unfolding (see Table VII) reveals that $\langle \Delta G^{mic} \rangle$ does not correlate with $\Delta^{D}_{N}g$, but correlates with $\Delta^{D}_{N}h$ and $\Delta^{D}_{N}s$: The greater the microstability, the lower is the specific enthalpy and specific entropy of macro-unfolding. The absence of any correlation between $\langle \Delta G^{mic} \rangle$ and $\Delta^{D}_{N}g$ when there is a correlation of the former with $\Delta^{D}_{N}h$ and $\Delta^{D}_{N}s$ might seem surprising if we do not bear in mind that the Gibbs energy is determined by the difference between the enthalpy and the entropy factors, and not by the factors themselves. In this case the similarity in enthalpy and entropy correlations is caused by $\Delta_d C_p$ which also correlates with $\langle \Delta G^{mic} \rangle$. At the same time, as was already shown in Section V,B., $\Delta_d C_p$ is directly connected to the concentration of contacts between nonpolar groups in the native structure n_φ. Thus, it is not surprising that $\langle \Delta G^{mic} \rangle$ is connected to n_φ. The connection between ΔG^{mic} and $\Delta_d C_p$, i.e., with n_φ, is not a simple one (see Fig. 32), but it is remarkable that the quantity, inverse to microstability, $\langle \Delta G^{mic} \rangle^{-1}$, which can be considered as a measure of motility of protein structure, increases almost linearly with the decrease of $\Delta_d C_p$.

In considering the structural grounds of motility we must bear in mind that n_φ reflects the nonuniformity of distribution of nonpolar groups in

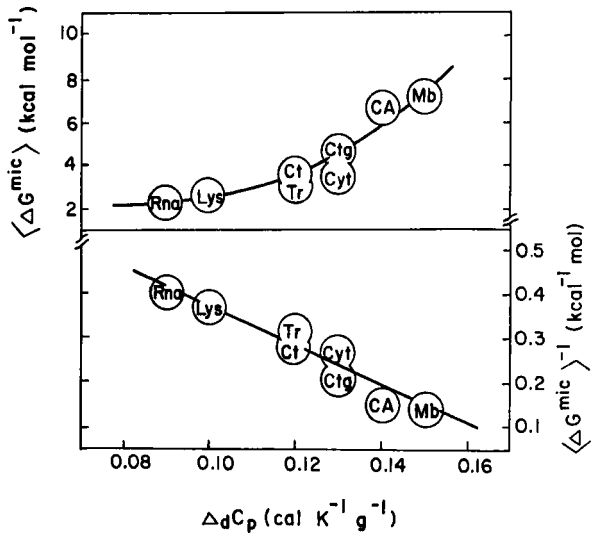

FIG. 32. Plot of experimental values of $\langle \Delta G^{mic} \rangle$ and $\langle \Delta G^{mic} \rangle^{-1}$ of six globular proteins versus the corresponding denaturational change of specific heat capacity ($\Delta_d c_p$).

the protein interior, i.e., the clustering of nonpolar groups, but not the overall concentration of these groups, which is almost the same for all mid-sized globular proteins. According to Nakanishi *et al.* (1973), the major contributor to the $\langle \Delta G^{mic} \rangle$ value is the entropy factor, since the enthalpy of micro-unfolding is small, judging by the small dependence of the equilibrium constant of micro-unfolding on temperature (see Section II, D). The large, negative value of entropy of micro-unfolding which follows from the $\langle \Delta G^{mic} \rangle$ value cannot be explained by the interaction of nonpolar groups with water at unfolding. It was explained by Nakanishi *et al.* (1973) by assuming that the micro-unfolding of protein structure requires a localization of several disruptions of the hydrogen bond. If that is the case, we might assume that clusters of nonpolar groups in the protein structure are serious obstacles along the pathway of migrating disruption in the protein structure.

VI. Thermodynamic Properties of Protein

A. Physical Models

After the formulation of the concept of structural hierarchy in globular proteins by Linderström-Lang and the successful explanation of coopera-

tivity of linear helical structures by Gibbs and Di Marzio (1959) and Zimm and Bragg (1959), there were many attempts to consider denaturation of globular proteins as the melting of secondary structures (see, for example, Scheraga, 1960). But even the first calorimetric studies on heat denaturation of globular proteins revealed that this process could not be regarded as the melting of separate blocks of secondary structure, and the observed cooperativity could be explained only by assuming that strong interactions integrate all the blocks into one system (Privalov, 1963).

Ptitsyn and Eisner (1965) tried to explain the cooperativity of globular proteins by introducing three-dimensionality; they treated the coil-to-globule transition as a condensation of gas into a liquid drop, ignoring the connections of residues in the polymer chain.

In a general way the problem of interacting links of the flexible chain was considered by Lifshits (1968), and it was shown that under some conditions this system can collapse into a compact structure, and that the process of collapse is a first-order phase transition. It was then concluded that the number of sequences of a heterogeneous polymer capable of collapse in the ordered structure is extremely limited (Lifshits and Grosberg, 1973). The last conclusion was clear even without the elaborated theory. Indeed, as had been already pointed out by Edsall (1968), from the specificity of folding it follows that the formation of the native conformation of a protein is a rare event and not a common property of any polypeptide chain. That is why consideration of the chain-folding problem in general does not seem to be promising, and the main interest at present is concentrated on studying the transition of an *a priori* folded chain which approximates a globular protein.

According to Go (1975, 1976), two types of conformational fluctuations can be considered in proteins: small and large amplitude fluctuations. At small amplitude fluctuations the relative distances between various parts of a protein fluctuate with small amplitudes maintaining the topological connectivity between them. At large amplitude fluctuations the native conformation of protein suffers occasional partial destruction. The large amplitude fluctations are assumed to be independent while they do not affect the basic conformational architecture of the molecule. But this is possible only so long as a small number of sites are fluctuating. With the increase in the number of fluctuating sites, the interaction between them becomes nonnegligible and it leads at the end to the collapse of a system. Ueda and Go (1976) derived the functional dependence between the entropy gain and the corresponding enthalpy loss accompanying disruption of secondary bonds and showed that the character of structural transitions in proteins is determined by the form of this function; the concave function leads to

the development of a cooperative process which could be considered as a first-order phase transition, while the convex function leads to a second-order phase transition.

In the theory developed by Ikegami (1977), protein is considered as a complex unique system which is maintained by specific bonds between definite links in a chain. It was assumed that cooperation between fluctuating secondary bonds exists since the bonded sites are connected to each other by the polypeptide backbone which leads to the loss of energy when the neighboring bonds are in different states. This theory also yielded two types of probability density function variations: one with two maxima in the temperature range of transition separated by zero probabilities, and another with a single maximum which is shifted with the temperature (Fig. 33a,b). In Ikegami's terminology the first type corresponds to a "structural transition," which is like the first-order phase transition. The second type corresponds to "gradual structural changes," which in some cases can be regarded as a second-order phase transition. The "structural transition" satisfactorily describes the experimentally observed heat capacity change of globular protein during denaturation, if the assumption concerning the contribution of hydrophobic bonding is introduced into the model (Kanehisa and Ikegami, 1977). "Gradual structural changes" have not yet been discovered experimentally, but investigations in this field have great promise.

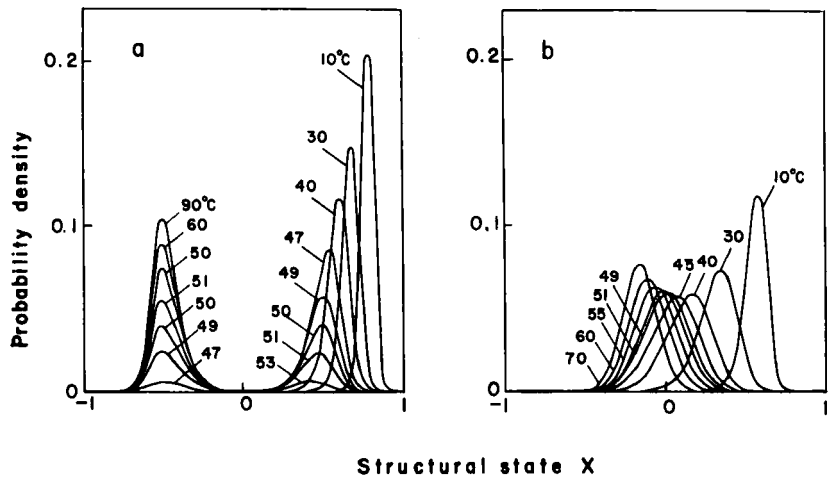

FIG. 33. The probability density ρ at various temperatures versus the parameter X representing the structural state calculated by Ikegami (1977) for the "structural transition" (a), and the "gradual change" (b) of hypothetical protein. Reproduced with permission from Ikegami (1977).

It should be emphasized that all the recent theories focus attention on long-range interactions in protein, i.e., interactions between neighbors in space and not in a chain (see also Lim, 1974; Filippi *et al.*, 1976; Warshel and Levitt, 1976). Still, it seems that the role of these interactions in the cooperation of all elements of protein structure into a single system is underestimated (Creighton *et al.*, 1978; Go and Taketomi, 1978).

The exceptional role of specific long-range interactions in protein follows directly from the concept of dense packing in the protein interior which has now been well substantiated (see Richards, 1977). Bearing in mind the unique organization of residues in this compact structure, the native protein can be regarded as an "aperiodic crystal," but of a critical size, i.e., as a nucleus which appears or disappears in the all-or-none fashion (Schellmann, 1955b). Here the problem of cooperativity reduces to the problem of compact packing of different residues connected into one chain, which has, if any, only one solution—the native state. It is evident that this packing is not only a physical problem but also a technological one, and without special assumptions concerning the technology of protein folding a solution seems improbable.

The technology of protein folding is being hotly discussed at present (see reviews by Anfinsen and Scheraga, 1975; Schultz, 1977). According to Tsong *et al.* (1972), the cooperativity of protein is connected with difficulties at the first step of folding, i.e., nucleation, while successive steps of growth are rapid and favorable. In contrast, Karplus and Weaver (1976) assume that microdomains of protein fold rapidly, but are intrinsically unstable and are stabilized by collision. Ptitsyn (1975) considers the multicentral folding pathway as the main cause of extremal cooperativity of large proteins. According to Ptitsyn, the enthalpy gain on merging of the large structural blocks which are comparable in size with the entire protein essentially exceeds the entropy loss. Because of this, only the last step locks the folded structure. If this mechanism is really important in stabilizing the native protein structure, we must expect that the organization into domains might be a general feature of the protein interior. This indeed seems to be the case (see Kretsinger, 1972; Wetlaufer, 1973; Rossman and Liljas, 1974; Schultz, 1977). Thus, although denaturation cannot be regarded as the melting of elements of the secondary structure, the structural blocks in protein might be important for stabilizing its compact structure.

At present, the existence of some short pathways of protein folding is well substantiated (Baldwin, 1978). But the native structure which is reached by a definite pathway need not correspond to the absolute energy minimum in the entire conformational space. This point led to confusing debate on the efficacy of thermodynamics in studying proteins. The paramount point missed was that the minima, which are for any reason

inaccessible for a system, are irrelevant even if they include an absolute minimum. As has been noted by Schultz (1977), "it is fundamentally impossible to distinguish between a local and the global minimum because scrutinization of the entire conformational space would be required. Neither the chain itself nor any kind of computer simulation can do that."

B. Concluding Remarks on Globular Proteins

We have considered proteins with one common property: extremal cooperativity which integrates all the elements of these macromolecules into a single unit—the globule. As will be seen in the chapter to appear in a later volume, this is not the only case for proteins in general, but it is a most important one since it is the simplest and can serve as a clue to understanding more complex cases.

In spite of great progress in our recent understanding of protein, we do not yet clearly understand how its exceptional cooperativity is achieved. Accordingly, we cannot create a model polymer which can fold as a protein into a compact structure with distinct stability and motility. But we hope that the general requirements which were realized by Nature in constructing these molecular systems are understood:

1. For the effective and reliable functioning of protein, its structure must be well defined over a wide range of conditions. A system which may be damaged even by a slight action cannot be reliable and cannot fulfill a specific function. The reserve of protein stability ensuring constant relative disposition of all its elements must considerably exceed RT, i.e., it must be not less than a few kilocalories per mole.

2. The system must not be too rigid: It must permit a relative displacement of the structure elements in the process of protein functioning and must allow easy dismantling of protein without considerable energy expenditure.

3. The system must not be too large; moreover, it must be as small as possible to facilitate synthesis, folding, and transportation.

In conclusion, we have to return to the general question raised in the Introduction on the applicability of thermodynamics to proteins. It seems that from the material presented we can already conclude that thermodynamics, if its direct methods are used, is quite efficient and reliable in studying proteins and might be helpful for the further understanding of these systems.

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