Thermochimica Acta, 5 (1973) 339–350

THERMAL ANALYSIS OF PROTEIN BEHAVIOR*

R. P. McKNIGHT and F. E. KARASZ
Polymer Science and Engineering, University of Massachusetts, Amherst, Mass. 01002 (U. S. A.)
(Received 24 April 1972)

ABSTRACT

The measurement and significance of heats associated with conformational transitions in solvated proteins and their synthetic analogues are discussed. An outline is given of the principles underlying several calorimetric and non-calorimetric techniques. Some new quantitative results relating to the denaturation of the protein lysozyme, obtained with a modified commercial differential scanning calorimeter, are presented.

INTRODUCTION

The advent of sophisticated quantitative thermoanalytical instrumentation in the last decade has had great impact on the study of synthetic polymers. Parameters such as glass transition temperatures and heats of fusion of bulk systems, which hitherto had only been available after considerable labor, are now readily obtained using commercially-available equipment. Thermal analysis indeed is the method of choice for many such measurements, in particular those having to do with transitional phenomena. In biological polymers, on the other hand, such methods have been taken up more slowly. The main reason for this is that in biological systems thermal properties—especially those having to do with transitional behavior—are concerned mostly with such behavior in solution, usually in dilute solution in an effort to adhere as closely as possible to *in vivo* conditions. (The solid state transitional behavior of biological polymers is an almost completely unexplored field.) The transitions occurring in solution are uniformly more subtle and their study requires one or two orders of magnitude greater sensitivity than those in the solid state. Presently available commercial instrumentation may thus be barely capable of making such measurements quantitatively without modification.

In the present paper we wish both to review relatively briefly the different types of thermal measurements that have been undertaken in an important class of biological polymers, proteins, and in the synthetic analogues, homopolypeptides, and to discuss some recent advances in the application of commercially-available thermoanalytical equipment to such studies.

*Presented at the Twenty-Third Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Cleveland, Ohio, March, 1972.

Proteins and polypeptides are polymers of general structure, $-(\underset{\displaystyle R}{\underset{|}{CH}}-\overset{\displaystyle O}{\overset{\|}{C}}-\underset{\displaystyle H}{\underset{|}{N}})_n-$, and can also be regarded as polymers of α-amino acids. Proteins are distinguished by their wide range of molecular weights and in the variability in chemical structure and sequence in the side group R. Poly-α-amino acids with identical side groups do not occur naturally, but have been synthesized in considerable variety. Such polymers are usually termed homopolypeptides or simply polypeptides. The nature of the side groups will, of course, determine many of the properties of the polymer, and differences in behavior and the mechanism of action and specificity in the case of enzymes, for example—also proteins—are ultimately a reflection of this infinitely variable chemical structure. One arbitrary demarcation may be made in terms of solubility: naturally-occurring proteins and many synthetic polypeptides are aqueous soluble, while polypeptides in which the side groups are largely hydrophobic—consisting of aromatic or extensive aliphatic moieties, for example—may be only soluble in organic media.

A common feature and one that is central to the present discussion, however, is that most proteins and polypeptides of interest are able to assume *ordered* conformations in dilute solution. In this respect they differ greatly from the majority of synthetic polymers; the solution conformation of the latter is known to be an essentially random one produced by the free or restricted rotation of sequential skeletal bonds. The resulting conformation is known as a random coil.

The protein or polypeptide in solution can exist in an ordered conformation only under favorable conditions with respect to the external parameters—temperature, pressure, pH, nature of solvent, etc. This stabilization of ordered conformations is largely a result of the extensive hydrogen bond formation that is possible between the carbonyl oxygen and amino hydrogen of peptide residues. Certain regular arrangements of residues are particularly favored, leading, for example, to the α-helix first described by Pauling, Corey, and Branson[1]. Outside this range of ordered conformation stability, the macromolecule will assume a random-coil conformation, indistinguishable in essential features from those formed by other polymers. The transition in conformation between the two states is a co-operative one, thus thermodynamically it is useful to treat the thermally-induced transformation as a smeared first-order transition. It is, of course, this aspect which makes conformational transitions of great interest in both the theoretical and experimental frames of reference. The order–disorder transitions in proteins have been recognized for decades as the denaturation phenomenon; in synthetic polypeptides it is more commonly referred to as the helix–coil transition. It should also be observed that the ordered ("native") state in proteins is not necessarily completely regularly structured; the fraction of α-helix in natural proteins lies between 5–50%. In synthetic poly-

peptides, however, it is frequently found that a completely ordered tertiary structure can be obtained[2]. These differences are also reflected in the fact that the transition in the naturally occurring polymer may be only partially reversible, whereas in the polypeptides total reversibility is more typical.

The course of the transition in either the hetero- or homopolymer may be followed calorimetrically, as will be shown below, but it is generally more convenient to make use of differences in optical properties between the ordered and disordered conformations. In many cases a simple measurement of optical rotation in the visible region may suffice to monitor the transition. A thermally-induced conformational transition thus is characterized by a curve of typically sigmoidal appearance (Fig. 1) if, for example, $[\alpha]_D$ versus temperature is plotted.

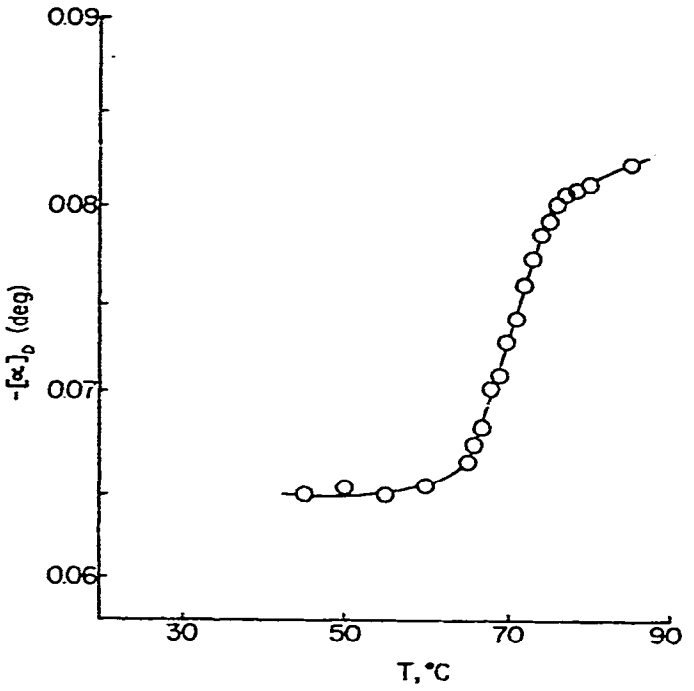

Fig. 1. Specific optical rotation at 589 nm as a function of temperature, for lysozyme, 1% aqueous solution, pH 3.

The transition mechanisms from the ordered to the disordered conformation therefore involve a co-operative disruption of the intramolecular hydrogen bonding which in the simplest case, is the prime factor in stabilizing the conformation. This disruption may be thermally induced or may be a manifestation of competitive intermolecular bonding between the peptide group and the solvent or a second solute. In aqueous systems, ionization of side groups is capable of breaking down the ordered conformation because of the resultant coulombic repulsive interaction. At any given degree of ionization, such forces may also be modified by the presence of further ionic species—thus the addition of salts can induce a conformational transi-

tion. Conformational transitions in general are therefore best regarded as a multi-dimensional phenomenon, with several degrees of freedom.

THERMODYNAMIC ASPECTS

It is also generally assumed that the extent of order in the polymer is linearly proportional to the change in whatever optical property is being used to follow the transition; thus the mid-point of a curve such as is depicted in Fig. 1 defines the transition temperature, T_c, at which $f_H = 0.5$ (f_H is fractional order); in α-helical forming synthetic polypeptides this corresponds therefore to a 50% helical content. Similar sigmoidal curves are obtained if the transition is induced by changes in parameters other than temperature, for example by an appropriate change in pH in an aqueous soluble system or, in a polypeptide soluble in organic solvent, by a solvent-titration experiment in which the solvent composition is changed from one favoring the ordered conformation to one favoring the coil. In the latter case it is appropriate, therefore, to regard the mid-point $f_H = 0.5$ as corresponding to a "transition composition," x_c, etc., implicitly at a temperature T_c.

The locus of points at which $f_H = 0.5$ in the temperature–solvent composition or temperature–pH planes then defines a phase boundary which divides regions in which the polymer is either in an ordered or disordered conformation (Fig. 2). It is to be emphasized that the transition width, however, is finite (compare Fig. 1) in contradistinction to the more common solid–solid or solid–liquid phase boundary.

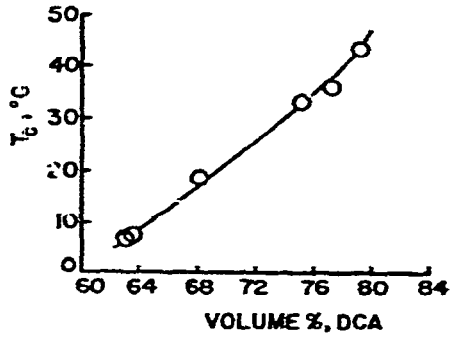

Fig. 2. Transition temperature as a function of solvent composition for poly-γ-benzyl-L-glutamate (PBG) in dichloroacetic acid (DCA)–1,2-dichloroethane (DCE) mixtures. Region *above* phase boundary corresponds to ordered conformation.

The course of the thermally induced transition, such as is shown in Fig. 1, may also be used to calculate transition enthalpies, as the ordinate f_H is a function of an equilibrium constant K for the coil-to-helix "reaction," such that

$$f_H = \frac{K}{1+K}$$

Then the van't Hoff enthalpy, ΔH_{vH}, is given by[3]

$$\Delta H_{vH} = 4RT_c^2 \left(\frac{\partial f_H}{\partial T}\right)_{f_H=\frac{1}{2}}$$

Since the transitions in proteins and peptides are invariable quite sharp (half-widths are typically between 3° and 20°C), ΔH_{vH} will lie in the range, approximately, of 20 to 150 kcal/mole. Such high values are a reflection, of course, of the co-operative nature of the transition.

The transition enthalpy may also be determined calorimetrically; we shall denote such values ΔH_{cal}. These may be evaluated on a residue mole basis or per mole of macromolecule. The latter practice is commonly used in the thermodynamic discussion of proteins. Since synthetic polypeptides of almost any molecular weight may be synthesized and studied, calorimetric transition enthalpies for this class of polymer are normally evaluated on a residue mole basis.

In proteins the equivalence or otherwise of ΔH_{vH} and ΔH_{cal} is of prime interest in regard to the question of the existence of stable intermediate conformations in the transition. The equivalence of the enthalpies is strong evidence for the intermediate-less "two-state" model[4].

In synthetic polypeptides the usual analysis is from a somewhat different point of view. The transition has been discussed as an example of a one-dimensional phase transition with less than perfect co-operativity. The dimensionality leads to simplifications which permit results to be obtained in essentially closed form; a considerable number of treatments are available[5]. There is considerable interest in differences in degree of co-operativity amongst species and the structural origin of such differences. In one such theoretical treatment, that due to Zimm and Bragg[6], this is expressed in terms of a co-operativity parameter σ defined by

$$\sigma = \left(\frac{\Delta H_{cal}}{\Delta H_{vH}}\right)^2$$

It can be seen that $\sigma = 0$ corresponds to a classical phase transition, while $\sigma = 1$ indicates a complete lack of co-operativity—the case of the typical chemical reaction. If ΔH_{cal} is evaluated on a residue mole basis, the latter situation therefore implies that each residue undergoes a transition from the ordered to the disordered state (or vice versa) completely independently of the state of adjacent residues. Further useful insight is given by the relation[6]

$$\sigma^{\frac{1}{2}} = \bar{n}^{-1}$$

where $\bar{n}$ is the average number of residues in a helical sequence at the mid-point of the transition or, stated another way, $\bar{n}$ is the number of residues which constitute a "mole" in the order–disorder reaction scheme. It is on the latter basis that the significance of the equivalence of ΔH_{cal} and ΔH_{vH} in proteins (where the former quantity is evaluated on a whole polymer basis) may be seen.

CALORIMETRIC MEASUREMENTS OF TRANSITION ENTHALPIES

As already stated, conformational transitions in proteins or polypeptides may be induced by changes in one or more of a relatively large number of parameters, but it is sufficient to consider here only two of these: temperature and solvent composition. pH changes in aqueous solutions may be considered as belonging to the latter category. The treatment is further simplified, both theoretically and experimentally, if we consider situations in which there is only one degree of freedom.

Thus ΔH_{cal} may be measured in situations which the transition is induced by varying the temperature at constant solvent composition (or pH). Conversely transitions may be studied isothermally in solvent-titration experiments. The first class is analogous to heat of fusion determinations, consequently the measurement may be essentially one of heat capacity versus temperature. Solvent induced transitions are seen to involve heat of mixing measurements, in which it is arranged that the bringing together of a solution of the polymer with an appropriate second solvent or solvent mixture results in the desired conformational transition. Clearly in such measurements corrections for the heat of mixing of the solvents or solvent mixtures themselves are required. A variation in which heats of solution constitute the experimental measurements may also be cited. If this parameter is determined, in separate experiments, for the dissolution of a protein or polypeptide in solvents favoring, respectively, the ordered and disordered conformations, ΔH_{cal} may be obtained from a First Law consideration of the enthalpies.

Heat capacity measurements

Direct thermal measurements of conformational transitions in polypeptides and proteins by the heat capacity method were first reported in 1965 by three groups: Privalov *et al.*[7], Ackermann and Ruterjans[8], and Karasz *et al.*[9]. In each case laboratory-constructed instrumentation was employed. These groups used a differential thermal analytical apparatus, a twin adiabatic calorimeter designed for

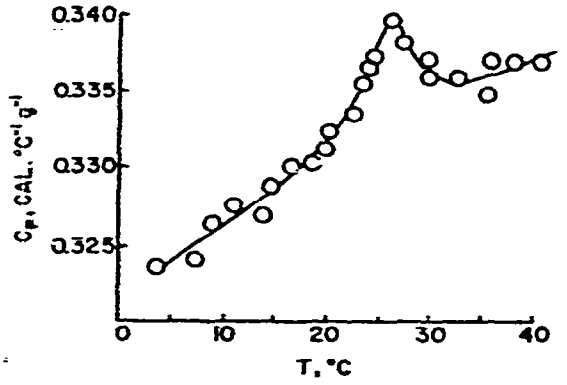

Fig. 3. Specific heat of PBG in 2 wt. % DCA–DCE (74%/26% by volume) mixed solvent. Data obtained using adiabatic calorimeter[9].

electrolyte solution heat capacity measurements, and an adiabatic calorimeter of essentially classical low-temperature type, respectively. A typical C_p versus T plot obtained with the latter is shown in Fig. 3. It will be observed that the transition is manifested as a "melting" peak superimposed on a relatively sharply sloped baseline. After the latter is subtracted from the data, the endotherm is found to be symmetric with respect to the maximum at T_c.

The unusual expansion of the ordinate scale in this and similar plots is an indication of the sensitivity required in such determinations.

A considerable amount of data obtained by such techniques has been accumulated in the last eight years[10]. Several calorimetric apparatuses specifically designed for this purpose—mostly based on the "twin" principle—have been described, but the measurement is still far from routine[11]. Nevertheless, the "fine structure" of the conformational transition phenomenon has been increasingly revealed: for example, the controversy regarding ΔH_{cal} and ΔH_{vH} in proteins, while not resolved, is at least under quite intensive examination, and there are now a number of systems, both synthetic and naturally occurring polymers, whose thermodynamic behavior has been investigated by a number of different calorimetric techniques and research groups. It may be noted that agreement amongst such sets of data is still the exception rather than the rule[10].

An impetus to heat capacity studies of conformational transitions will be provided when commercial instrumentation of sufficient sensitivity becomes routinely available. Some such work has already been reported[12]. A quantitative specification of the sensitivity required depends, of course, on many parameters: the particular polymer–solvent system, the proposed solute concentration and the instrumental signal-to-noise ratio amongst others. However, perhaps the most sensitive commercial differential scanning calorimeter currently available, the Perkin–Elmer DSC-1B, has been found to require some modification in work reported by the present author and associates to provide satisfactory results. This modification has involved one or both

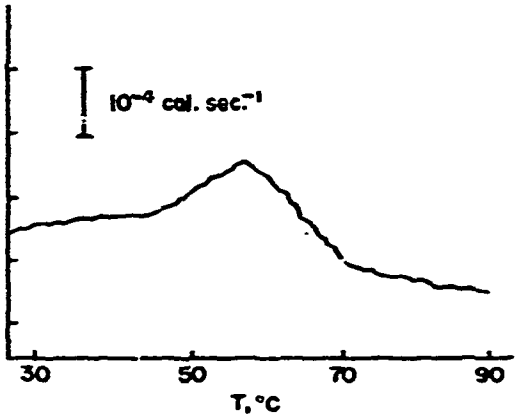

Fig. 4. Differential scanning calorimeter trace of 3% lysozyme solution (pH 2). Modified DSC used (see text).

of the following changes: (a) an enlarged sealable sample cell (increasing the capacity from about 20 μl, as supplied, to 60 μl) and (b) a further mild stage of amplification interposed between the instrument output and a strip-chart recorder. This amplification, typically by a factor of 3, can be easily provided by a Keithley 150 A microvolt meter or similar instrumentation[12]. A typical result obtained in a study of the order–disorder transition in the protein lysozyme is shown in Fig. 4. In a com-

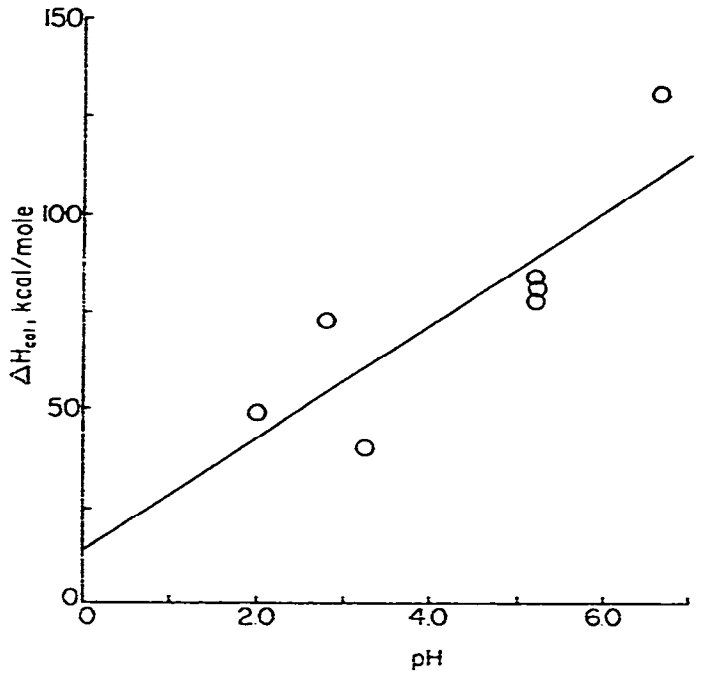

Fig. 5. Transition enthalpy for lysozyme as function of solvent pH. Data obtained with modified DSC.

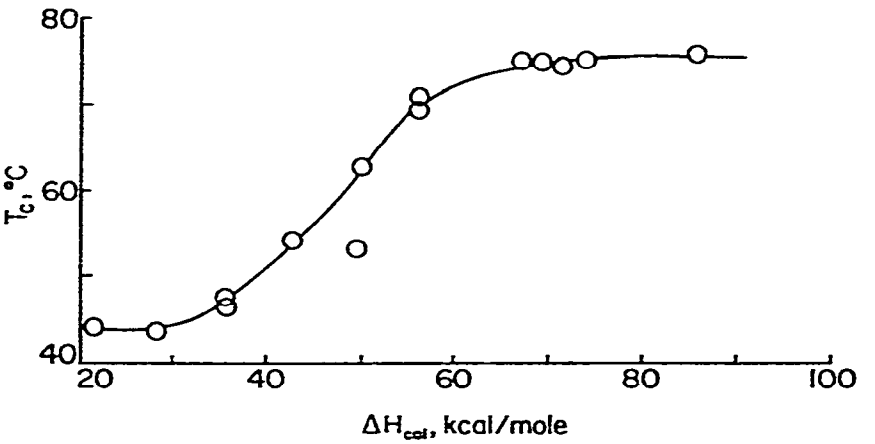

Fig. 6. Relation between transition enthalpy and transition temperature for lysozyme.

prehensive study of the thermodynamic aspects of this transition, ΔH_{cal} as a function of T_c, *i.e.* as a function of pH (see Fig. 5), was determined (Fig. 6). The present situation is still not entirely satisfactory, however; it would be desirable to use yet more dilute solutions, and higher precision will undoubtedly be required. Newly announced commercial instrumentation of enhanced sensitivity will undoubtedly be of interest in this regard[13].

Isothermal measurements of ΔH_{cal}

Isothermal calorimetric studies of denaturation in proteins antecede heat capacity investigations. Several such systems were investigated some years ago in a heat of mixing calorimeter constructed by Sturtevant and associates[14]. The first study of this type applied to synthetic polypeptides in organic solvents seems to have been by Block and Jackson[15] but only qualitative results were reported. This work was followed very closely by the first heat capacity studies, discussed above[7–9], and isothermal measurements, at least for synthetic systems, were largely ignored for some years thereafter.

The application of a commercially available calorimeter to this problem was reported by Hermans and Rialdi[16]. They adapted the well-known Tian–Calvet conduction micro-calorimeter to operate in a heat of mixing mode, and thereby studied the conformational transition of myoglobin over a wide pH range. These authors later used the same technique to determine the thermodynamic parameters of the helix–coil transition in poly-α-glutamic acid[17]. An extensive study of the transition in poly-γ-benzyl glutamate (PBG) using the heat of mixing technique has been carried out by Kagemoto and Fujishiro[18], and by Choquette[19].

Within the last few years several sensitive calorimeters which could be used in solution mixing studies have become commercially available and one may expect a proliferation of such investigations in the near future[20].

A variation of the solvent titration concept was introduced by Giacometti and collaborators[21] in studies, again, of the PBG system. In this technique, the heat of

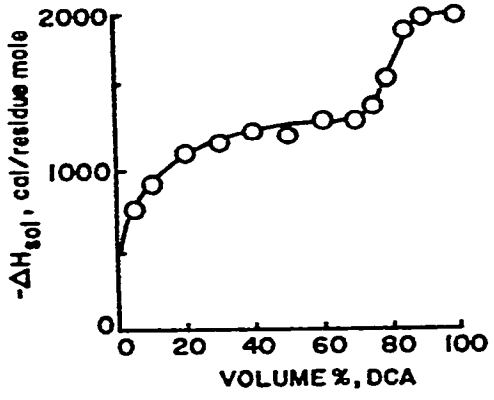

Fig. 7. Heat of solution at 25 °C of dry PBG film in DCA–DCE solvent mixtures. Conformational transition centered at about 80 vol. % DCA. The change in ΔH_{sol} at low DCA compositions believed due to preferential solvation of side-chains; data taken from ref. 21.

solution of polypeptide film in a series of DCA–DCE compositions, at 25°C, was measured. It will be seen from Fig. 7 that for compositions containing less than 76% DCA the polypeptide in the resulting solution will be in an ordered conformation; in compositions containing a higher proportion of DCA the PBG will assume a random-coil conformation. Thus, the required heat of transition is essentially the difference of these two determinations. This technique has also been used with other synthetic polypeptides[22].

DISCUSSION

It may be observed that a fairly wide range of calorimetric methods has now been described for the study of transition phenomena in biological macromolecules and their synthetic model analogues. However, it appears probable that really widespread adoption of such fundamental measurements in the biological area will only come with the availability of suitable commercial instrumentation. These experiments demand high sensitivity and careful technique and the difficulties involved in constructing such equipment in the laboratory are considerable deterrents.

Indeed, because of such difficulties, several non-calorimetric techniques of determining heats of transition have been developed. For example, in aqueous soluble pH-sensitive polymers such as poly-α-glutamic acid, this information can be elucidated from careful titration studies[23]. A second type of calculation depends essentially on the variation of the van't Hoff heat on the molecular weight of the polypeptide[24]. Molecular weight effects are observable for chains containing less than 100 or so residues. Such determinations thus require only readily obtainable f_H versus T data for a series of such samples, but it should be pointed out that the result depends on the availability of carefully characterized monodisperse samples and also is quite sensitive to the precise details of the theoretical model for the transition.

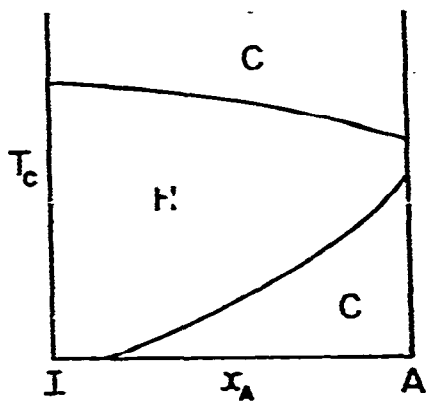

Fig. 8. Schematic of phase boundaries between ordered (H) and disordered (C) conformations of a polypeptide in a binary solvent system consisting of an "active", hydrogen-bonding solvent A, and an "inert", non-bonding solvent, I. The low temperature random-coil phase is strongly bonded to A; the high temperature "C" phase interacts[26] only weakly with A.

An analysis of phase boundaries, that is of T_c versus x_c data, provide a third non-calorimetric method for determination of enthalpies suitable in the case of polypeptides in binary organic solvent systems[25]. This determination depends essentially on the recognition that the change in T_c with solvent composition is analogous to the depression of melting points with added diluents in a solid–liquid transition. Thus measurement of the slope of such phase boundaries can yield ΔH_{cal}. Again, it should be pointed out that this calculation is predicated upon a number of assumptions concerning the details of the supposed transition mechanism (Fig. 8)[26].

In conclusion, we may comment on the agreement between the various calorimetric and non-calorimetric methods. At present there are a few systems for which comparisons can be made; in general, agreement does not appear to be good, even when the same calorimetric techniques have been employed. The comparative novelty of any results in the field have up to now prevented much attention being paid to these discrepancies. It may be anticipated that this situation will not long persist.

Thermal studies of transitional behavior in protein, polypeptides and indeed every other classification of biopolymer yields such important results in numerous ways that increasing attention to this problem is inevitable. As the parallel case of thermal analysis of bulk synthetic polymers has demonstrated the availability of suitable instrumentation and, equally important, the recognition of this availability by the interested researchers will result in greatly expanded activity.

Some of the immediate problems that will be investigated are as follows. For the proteins, further tests of the two-state model by a comparison of the ΔH_{cal} and ΔH_{vH} is required for additional systems; another important issue concerns the physical significance of the very large apparent temperature dependency of ΔH_{cal}—values for ΔC_p as large as 8000 cal/deg mole have been reported[27]. In polypeptides we may expect, for example, a more systematic assessment of the effect of side-group structure of the transition parameters, and upon solvent–polymer interaction. The relative role of intramolecular hydrogen bonding and of side-group interactions in stabilizing the ordered conformation is a matter of continuing debate. Further, it will be recalled that the principal measured parameter ΔH_{cal} represents the net effect of two processes: the formation—or dissolution, according to the transition direction—of the intramolecular bonds, and their partial replacement by intermolecular peptide–solvent interactions. Experimental and theoretical developments to separate these effects are desirable to study, again, the effect of structure upon these fundamental processes. The role of thermal measurements in these and many other problems will be crucial.

ACKNOWLEDGMENT

The work reported here was supported by the National Science Foundation NSF GB 8080 (F.E.K.).

REFERENCES

1 L. Pauling, R. B. Corey and H. R. Branson, *Proc. Nat. Acad. Sci. U. S. A.*, 37 (1951) 205.
2 G. D. Fasman, in G. D. Fasman (Ed.), *Poly-α-Amino Acids*, Volume I, M. Dekker, New York, 1967.
3 J. Applequist, *J. Chem. Phys.*, 38 (1963) 934.
4 J. F. Brandts, *J. Amer. Chem. Soc.*, 86 (1964) 4302.
5 D. C. Poland and H. A. Scheraga, *Theory of the Helix–coil Transition in Biopolymers*, Academic Press, New York, 1970.
6 B. H. Zimm and J. K. Bragg, *J. Chem. Phys.*, 31 (1959) 526.
7 P. L. Privalov, K. A. Kafiani and D. R. Monaselidze, *Dokl. Akad. Nauk SSSR*, 156 (1964) 951.
8 T. Ackermann and M. Ruterjans, *Z. Phys. Chem. (Frankfurt am Main)*, 41 (1964) 116.
9 F. E. Karasz, J. M. O'Reilly and H. E. Bair, *Nature*, 202 (1964) 693.
10 T. Ackermann, in H. D. Brown (Ed.), *Biochemical Microcalorimetry*, Academic Press, New York, 1969.
11a J. M. Sturtevant and P. A. Lyons, *J. Chem. Thermo.*, 1 (1969) 201;
b W. M. Jackson and J. F. Brandts, *Biochemistry*, 9 (1970) 2294;
c P. R. Stoesser and S. J. Gill, *Rev. Sci. Instrum.*, 38 (1967) 422.
12a J. M. Steim, *Arch. Biochem. Biophys.*, 112 (1965) 599; *Perkin–Elmer Instrum. News*, 19 (1968) 12;
b A. Kagemoto and F. E. Karasz, in R. S. Porter (Ed.), *Analytical Calorimetry*, Vol. 2, Plenum Press, New York, 1970, p. 147;
c F. Delben and V. Crescenzi, *Biochim. Biophys. Acta*, 194 (1969) 615.
13 M. J. O'Neill, S. D. Norem, H. I. Hill and R. S. Richmond, Paper No. 6, *Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy*, Cleveland, Ohio, March, 1972.
14 A. Buzzell and J. M. Sturtevant, *J. Amer. Chem. Soc.*, 74 (1952) 1983, *et seq.*
15 H. Block and J. B. Jackson, *Proc. Chem. Soc., London*, (1963) 381.
16 J. Hermans Jr. and G. Rialdi, *Biochemistry*, 4 (1965) 1277.
17 G. Rialdi and J. Hermans, Jr., *J. Amer. Chem. Soc.*, 88 (1966) 5719.
18 A. Kagemoto AND R. Fujishiro, *Makromol. Chem.*, 114 (1968) 139.
19 A. Choquette, *Ph. D. Thesis*, Université de Montréal, 1970.
20a Imass, S. Hingham, Mass.;
b LKB Instruments, Rockville, Md.;
c Tronac, Orem, Utah;
d Nissei Sangyo Co., Ltd., Tokyo.
21 G. Giacometti and A. Turolla, *Z. Phys. Chem.*, 51 (1966) 108.
22 G. Giacometti *et al.*, *Biopolymers*, 6 (1968) 441.
23 M. Nagasawa and A. Holtzer, *J. Amer. Chem. Soc.*, 86 (1964) 538.
24a B. H. Zimm, P. Doty and K. Iso, *Proc. Nat. Acad. Sci. U. S. A.*, 45 (1959) 160;
b A. Teramoto and H. Fujita, *Polymer J. (Japan)*, 1 (1970) 55;
c M. Bixon and S. Lifson, *Biopolymers*, 4 (1966) 815.
25 F. E. Karasz and J. M. O'Reilly, *Biopolymers*, 5 (1967) 27.
26 F. E. Karasz and G. E. Gajnos, in press.
27 C. Tanford, *Advan. Protein Chem.*, 22 (1968) 121; 24 (1970) 1.