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THERMODYNAMIC INVESTIGATIONS OF PROTEINS.
II. Calorimetric study of lysozyme denaturation by guanidine hydrochloride

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The thermodynamic parameters of the denaturation of lysozyme are determined at various temperatures (25–60°C) by isothermal calorimetric titrations with guanidine hydrochloride (GuHCl) and by scanning calorimetry in the presence of GuHCl. An approach for the determination of the enthalpy of preferential binding of GuHCl is proposed. It has been shown from GuHCl denaturation experiments that the net enthalpies of denaturation and the denaturational change in the heat capacity of protein can be obtained if preferential binding is taken into consideration. These results are nearly the same as in the case of thermal denaturation in the absence of denaturants. It is concluded that the states of both heat- and GuHCl-denatured lysozyme are thermodynamically indistinguishable.

1. Introduction

It is generally accepted that proteins on heat denaturation undergo incomplete unfolding in contrast with denaturation in concentrated guanidine hydrochloride (GuHCl) solutions [1, 2]. The evidence for this suggestion is provided not only by a smaller viscosity [3] of the heat-denatured protein, but also by the additional changes in the optical properties of heat-denatured proteins with the addition of GuHCl [4]. From rather complicated equilibrium curves it was concluded that in the case of lysozyme the state of heat-denatured protein (state X) corresponds to 70% of the fully unfolded state (state D) which is achieved in concentrated GuHCl [2]. The major evidence for this conclusion was the apparent heat-capacity change Δc_p which appeared as $\Delta c_p = 0.950$ kcal mol^{-1} K^{-1} for heat denaturation of lysozyme and $\Delta c_p = 1.375$ kcal mol^{-1} K^{-1} for complete unfolding in GuHCl.

The importance of this parameter (Δc_p) in discussing the nature of the denaturation is obvious. Since it is commonly assumed according to Kauzmann [5, 6] that the heat capacity is strictly dependent on the ordering of water molecules around exposed hydrophobic groups, it is evident that the change of the heat capacity at denaturation should be connected with the disruption of apolar contacts in protein. Thus, it was assumed that the remaining structural elements must be maintained by the hydrophobic contacts in heat-denatured proteins. This conception seemed quite probable, and therefore the experimental result that the real heat-capacity changes on heat denaturation measured by direct calorimetric experiments [7, 8] were greater ($\Delta c_p = 1.6$ kcal mol^{-1} K^{-1}) than the apparent heat-capacity changes even on complete unfolding of lysozyme in GuHCl obtained by equilibrium treatment was very confusing.

It becomes evident that the understanding of the denatured states and of the process of denaturation itself is far from clear. Speculation on the nature of protein denaturation can be eliminated only by using direct experimental approaches to the problem, i.e., by a direct calorimetric determination of the thermodynamical parameters. Some preliminary investigations of calorimetrically technical possibilities have shown

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that modern precise instruments provide good prospects for helping to solve this problem *.

2. Materials and methods

The reagents and the instrumentation used in these studies were the same as described in the preceding paper [9]. GuHCl (Reachim, USSR) was purified and checked according to ref. [10], but was twice recrystallized. Protein solutions were prepared by weighing lyophilized lysozyme and adding predetermined amounts of water or GuHCl solutions. Concentration determinations were performed optically (see ref. [9]) only in the absence of GuHCl. The protein concentrations before mixing were in the range of 5×10^{-4} to 5×10^{-3} moles l^{-1} and the flow rate was about 1.4×10^{-3} ml sec^{-1}. The mixing ratios of protein solutions with GuHCl solutions were 1:1 and 1:2.5. It was shown that the heat effect does not depend on the initial concentration.

Optimal pH and temperature conditions were chosen in the previous experiments to exclude limiting effects (e.g., slow kinetics). Completeness of the reaction in the calorimetric system was checked using different flow rates (1.4×10^{-3} to 7×10^{-3} ml sec^{-1}) as well as by the stepwise attainment of given final concentrations. pH changes due to the influence of GuHCl on the hydrogen ion activity were found to be ± 0.1 but in some cases (pH 4.5) up to ± 0.2.

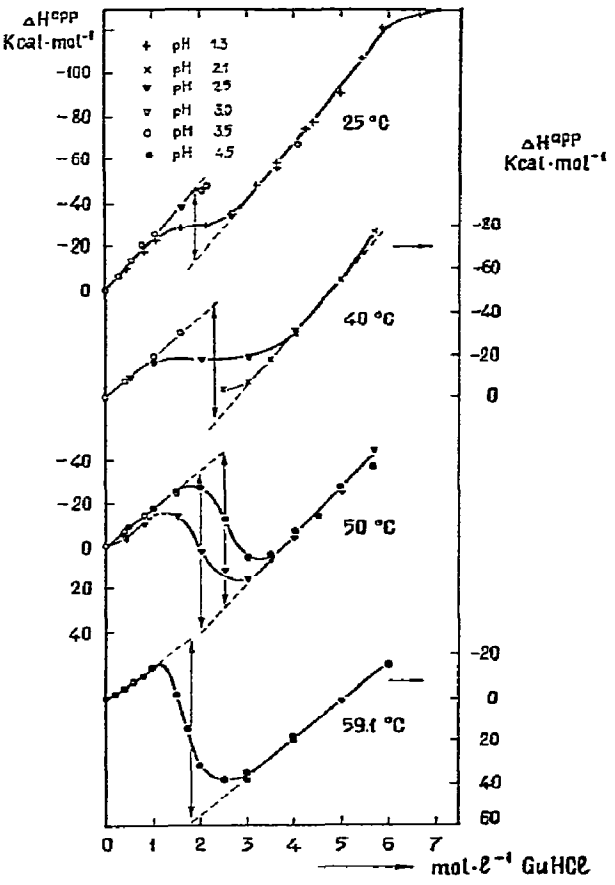

Fig. 1. Results of calorimetric titrations of lysozyme at given temperature. For results see also table 1.

3. Results

3.1. Calorimetric titrations

Results of calorimetric titrations of lysozyme with GuHCl at various temperatures and pH values are represented in fig. 1. The experimental curves can be divided into three parts. At relatively low GuHCl concentrations exothermal effects with a nearly linear concentration dependence are observed. At a higher concentration of GuHCl a sigmoidal curvature is seen, and in cases of high temperatures dominating endothermic characteristics are displayed. At a following increase of concentration, the curves again become linear up to saturation. The slope in both linear parts is pH-independent but after the sigmoidal part it is, as a rule, steeper.

Comparing the calorimetric titration curves with the changes in the optical properties of lysozyme (see fig. 2 and also curves in ref. [11]), it becomes evident that only the sigmoidal part of these curves corresponds to the denaturation of proteins. Thus, the slope before and after the sigmoidal part must be a manifestation of the heat of progressive solvation of native and denatured protein by GuHCl with an increase of its concentration. This conclusion is in agreement with the results of ref. [12] showing that native protein is also able to interact with GuHCl.

The apparent heats (ΔH^{app}) given in table 1 were

* See, for example, the review papers, refs. [34–36].

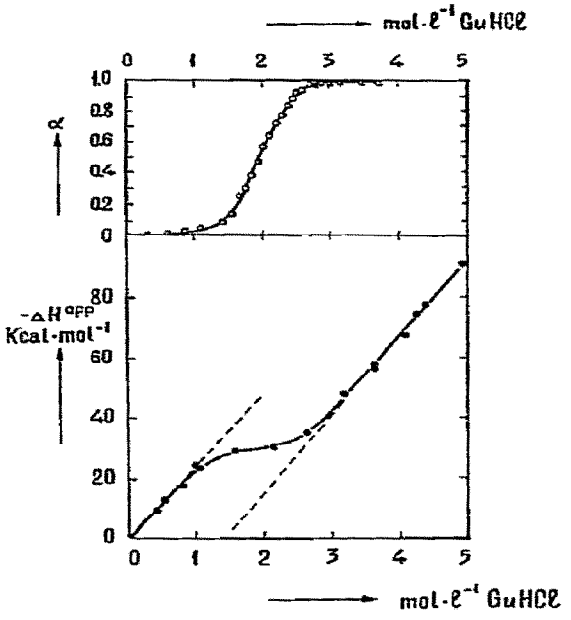

Fig. 2. Comparison of the normalized conversion curve of lysozyme in GuHCl (top) with the heat evolved in calorimetric measurements at 25°C and pH 1.3. The conversion curve is taken from the difference spectral intensity at 301 nm.

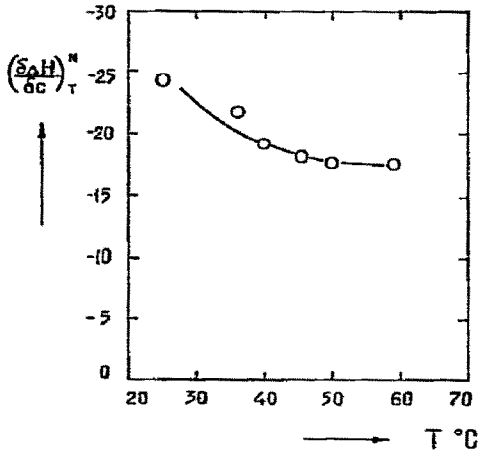

Fig. 3. Pre-denaturational slope of the calorimetric titration curves.

obtained by using a linear extrapolation to the midpoint of the transition. They are in agreement with the data reported in the literature and obtained under comparable conditions but using other experimental techniques: at 25°C in 3.0 M GuHCl the apparent heat was 29 ± 2 kcal mol^{-1} [13] and 36.1 kcal mol^{-1} [14]; at 58°C in 2.0 M GuHCl it was 85 and 89 kcal mol^{-1} [15]; at 60°C in 1.6 M GuHCl ΔH^{app} = 90 kcal mol^{-1} [16].

It should be mentioned that the predenaturational slope (s_N) in the calorimetric titration curves is temperature dependent (fig. 3) indicating the temperature dependence of the solvation phenomena.

Using high temperatures and low pH values we can also perform calorimetric titrations of thermally denatured lysozyme with GuHCl. The results, represented in fig. 4, show a strong interaction of GuHCl with thermally unfolded lysozyme reaching about −120 kcal mol^{-1} in 6.0 M GuHCl at 59.1°C. Thus, it is evident that the solvation enthalpies in thermodynamic considerations can no longer be neglected. The shape of the curve in fig. 4 corresponds to the usual mono-

Table 1
Results of isothermal enthalpimetric titrations of lysozyme with GuHCl

T (°C)	$c_{0.5}$ (mol l^{-1} GuHCl)	ΔH^{app} (kcal mol^{-1})	$-\Delta H_{pref}$ (kcal mol^{-1})	$\Delta H^{d} = \Delta H^{app} - \Delta H_{pref}$ (kcal mol^{-1})
25	1.9 (pH 1.3)	32 ± 2	$21._8$ ± 2	54 ± 4
40	2.2 (pH 2.5)	56 ± 2	$19._8$ ± 2	76 ± 4
50	2.0 (pH 3.0)	75 ± 2	$16._5$ ± 1.5	92 ± 4
50	2.5 (pH 4.5)	73 ± 2	$20._7$ ± 2	94 ± 4
59.1	1.8 (pH 4.5)	87 ± 5	$14._9$ ± 1.5	102 ± 6
59.1	1.8 (pH 4.5)	87 ± 5	$15._5$ ± 1 *	103 ± 6
59.1				106 ± 5 **

* Using the initial slope s_D from the experimental corve in fig. 4 instead of from eq. (3b).
** Taken from the extrapolation represented in fig. 6.

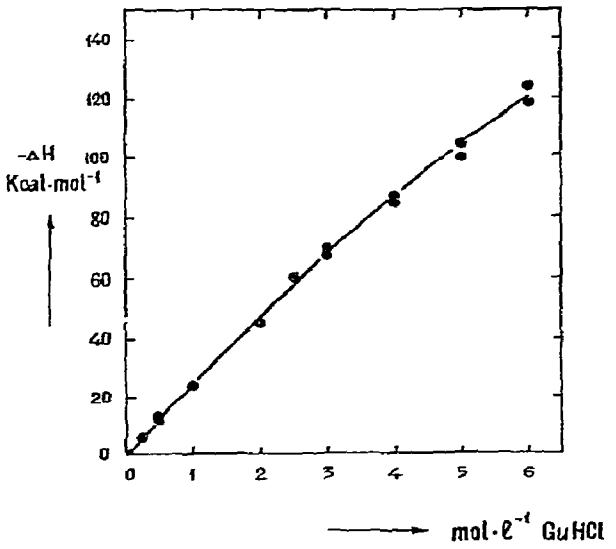

Fig. 4. Calorimetric titration of heat-denatured lysozyme at pH 1.5 and 59.1°C.

tonic saturation function. An additional exothermal transition in the range of 1 to 4 mol l^{-1} GuHCl which was determined from optical measurements [4] can neither be confirmed nor excluded due to the curvature in this region, but, even if it exists, its enthalpy must be less than −4 kcal mol^{-1}.

From the calorimetric titration data an average value of the interaction enthalpy of one mole of GuHCl with lysozyme can be estimated, knowing that in a 6 molar GuHCl solution at 25°C lysozyme binds 67 moles of GuHCl [12]. The corresponding ΔH can be taken from fig. 1 when the postdenaturational slope is extrapolated to zero GuHCl concentration to exclude the denaturation enthalpy. This gives an average ΔH of

$$\Delta H = (-160 \pm 5)/67 = -(2.4 \pm 0.1) \text{ kcal/mole}$$

of GuHCl bound to lysozyme at 25°C.

Before further conclusions are drawn it has to be shown that the transition observed in our denaturation experiments really corresponds to thermodynamically defined states. This can be verified if it is shown that the enthalpy of state is not dependent on the pathway. An example for reaching the new state by different pathways is given in fig. 5. Here step 1 corresponds to denaturation in the absence of GuHCl.

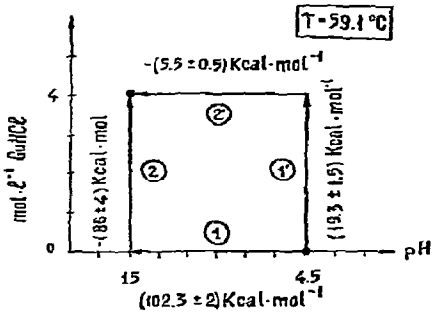

Fig. 5. Apparent enthalpy changes of lysozyme in different pathways of state changes.

Step 2 is the subsequent reaction of the denatured lysozyme with GuHCl. Step 1' represents the process of denaturation and solvation under the influence of GuHCl. Step 2' is the subsequent pH change of denatured lysozyme in GuHCl. It can be easily seen that the enthalpies on pathways 1 + 2 are equal to the enthalpies on pathways 1' + 2'. Thus in both cases identical enthalpy changes were observed within the experimental error.

3.2. Heat effects of preferential binding

When a protein undergoes denaturation, additional binding sites become available for the interaction with denaturant molecules due to disruption of the structure. This change in the preferential binding of denaturants is usually expressed by the parameter $\Delta\nu$ representing the amount of denaturant bond per mole protein at given conditions, whereas Δn is the difference between two states in the number of binding sites per molecule. The term preferential binding is used here in a broad sense (see also refs. [1, 17, 18]), i.e. including changes in hydration, binding of counter ions, etc.

Having in mind the heat effects shown in fig. 4, it is obvious that the change in preferential binding must also be accompanied by large negative heat effects. Therefore this binding effect is not negligible at all. But the question is, how can we introduce a correction to this preferential binding heat to obtain just the net enthalpy of conformational transition from the apparent enthalpy of denaturation?

We can exclude the preferential binding effect by extrapolation of the enthalpies of the native and de-

natured state to zero concentration using the functional dependence obtained for the denatured protein (see fig. 4) assuming that we do not have any transition here, and that this curve does not change with pH. In this way we obtain ΔH_d = 106 kcal mol^{-1} for the enthalpy of conformational transition at zero concentration of GuHCl in contrast to the apparent heat ΔH^{app} = 87 kcal mol^{-1} (see fig. 6). Generally the use of such an extrapolation procedure is limited because a titration of both the native and the denatured protein at the same temperature is not possible in every case. Therefore a different procedure must be found for calculating the enthalpy of preferential binding.

Having in mind that the number of binding sites for a denaturant molecule in denatured proteins (a_D) is greater in comparison with the native ones (a_N), then it is obvious from experimental observation that the slope of the titration curves of these forms (s_N, s_D) is also different:

$$s_N \equiv \left(\frac{\delta \Delta H}{\delta c}\right)_T^N < s_D \equiv \left(\frac{\delta \Delta H}{\delta c}\right)_T^D . \qquad (1)$$

Assuming that the binding sites have identical binding constants and enthalpies, it then follows that for $c_{GuHCl} \gg c_{protein}$:

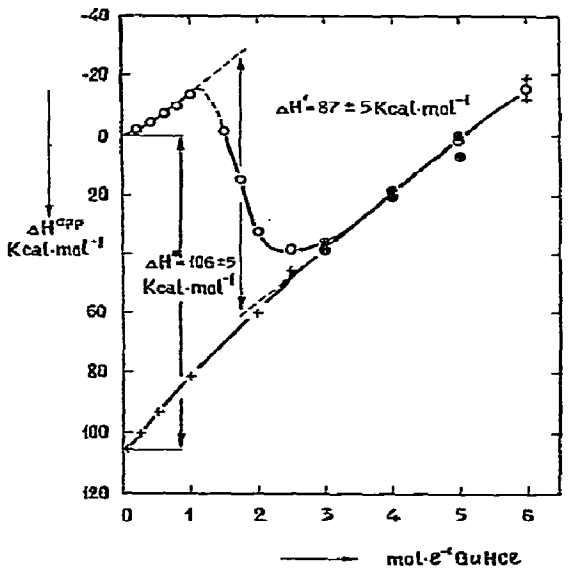

Fig. 6. Estimation of the heat of denaturation at 59.1°C by various extrapolation procedures (see the text).

$$s_D = \left(\frac{\delta \Delta H}{\delta c}\right)_T^D = \left(\frac{\delta \Delta H}{\delta c}\right)_T^N \frac{a_D}{a_N} = s_N \frac{a_D}{a_D - \Delta n} . \qquad (2)$$

At the midpoint of transition (denaturant concentration $c_{0.5}$) the correction value, ΔH_{pref}, is given by

$$\Delta H_{pref} = s_D c_{0.5} - s_N c_{0.5} \qquad (3a)$$

$$= \left(\frac{\delta \Delta H}{\delta c}\right)_T^N \frac{c_{0.5} \Delta n}{a_D - \Delta n} . \qquad (3b)$$

Here the ΔH of preferential binding is expressed in experimentally observable parameters. In addition to the terms explained above, Δn can be obtained from equilibrium measurements [1, 17] and a_D from isopiestic methods or density measurements [12].

It should be mentioned here that the actual mechanism of GuHCl binding is still under discussion. From studies on model compounds it was assumed that one molecule of GuHCl binds with two peptide bonds [19]. Besides this, contacts with aromatic residues [1, 20] and ionic groups are possible. This model is consistent with recently obtained results from equilibrium measurements and has been characterized as the most likely form of GuHCl binding [2, 12, 20, 21]. In this paper, Δn = 21.5 [17] and a_D = 67 [12] was used for lysozyme denaturation in GuHCl.

If peptide bonds are the main binding sites for GuHCl then the parameter Δn should be correlated with the amount of slowly exchanging peptide bonds in hydrogen exchange experiments. If one GuHCl molecule binds indeed to two peptide bonds [19] then we should have

$$\Delta n \simeq \tfrac{1}{2} \times (\text{amount of slowly exchanging peptide bonds}) . \qquad (4)$$

From hydrogen exchange experiments it was concluded that lysozyme possesses 44 slowly exchanging peptide groups [22]. In this case Δn = 22 which is in excellent agreement with the experimental value Δn = 21.5 for lysozyme [17].

The experimental results of the isothermal calorimetric titrations and corrections for preferential binding obtained by eq. (3b) are listed in table 1. From the temperature dependence of ΔH_d the corresponding $\Delta[c_p]_d$ = 1.5 ± 0.2 kcal mol^{-1} K^{-1} is obtained (fig. 7).

3.3. Determination of thermodynamic parameters from scanning experiments

As mentioned above, changes in heat capacities are correlated with disruption (and solvation) of apolar contacts. These Δc_p values correspond to the temperature dependence of the reaction enthalpies (d ΔH/dT) according to Kirchhoff's law. But here we must have in mind that all variable parameters except temperature are assumed to be constant. In GuHCl denaturation experiments, where not only the temperature but also the denaturant concentration is variable, the use of Kirchhoff's law leads to an erroneous Δc_p^{app} since the ΔH^{app} already represents a sum. Denoting the observed reaction enthalpies by ΔH^{app}, the true denaturation enthalpies by ΔH_d and the heat of additional reaction by ΔH^*, then the corresponding terms in Δc_p are given by

$$\Delta c_p^{app} = \frac{(\Delta H_d + \Delta H^*)_{T_1} - (\Delta H_d + \Delta H^*)_{T_2}}{T_1 - T_2}$$

$$= \Delta[c_p]_d + \Delta c_p^* . \qquad (5)$$

Substituting here eq. (3b) which describes the relationships in the GuHCl denaturation experiments, it follows that

$$\Delta c_p^{app} = \Delta[c_p]_d + \frac{1}{T_1 - T_2}\frac{\Delta n}{a_D - \Delta n} \times \left\{ \left[\left(\frac{\delta \Delta H}{\delta c} \right)^N c_{0.5} \right]_{T_1} - \left[\left(\frac{\delta \Delta H}{\delta c} \right)^N c_{0.5} \right]_{T_2} \right\} . \qquad (6)$$

The difference arising in Δc_p^{app} and $\Delta[c_p]_d$ is therefore dependent on the second term containing the denaturant concentrations used in each experiment, the temperature dependent differential quotient and the individual values of a_D and Δn for the given protein.

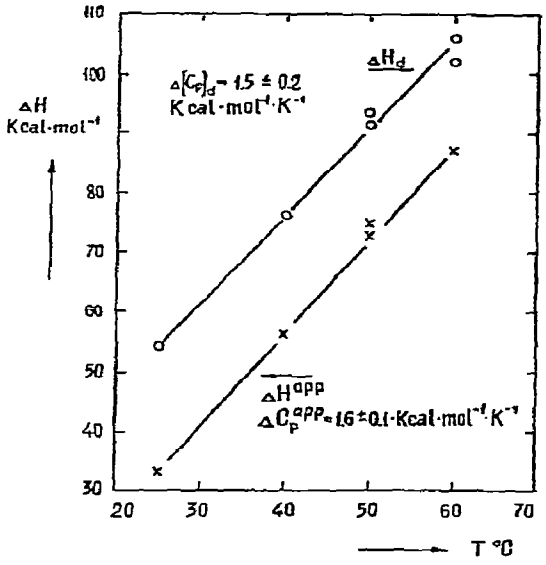

Fig. 7. Graphical determination of $\Delta[c_p]_d$.

The contribution of these parameters can be demonstrated using the results of scanning calorimetric measurements which is a method allowing direct observation of ΔH^{app} and $\Delta[c_p]_d$ at given GuHCl concentrations. The data listed in table 2 for several concentrations of GuHCl obtained by scanning experiments demonstrate the difference which can arise in Δc_p^{app} and $\Delta[c_p]_d$. After correcting ΔH^{app} for pref-

Table 2
Results of scanning calorimetric investigations of lysozyme in the presence of GuHCl at pH 2.

c_{GuHCl} (mol l^{-1})	T_d (°C)	ΔH^{app} (kcal mol^{-1})	$-\Delta H_{pref}$ (kcal mol^{-1})	ΔH_d (kcal mol^{-1})	$\Delta[c_p]_d$ * (kcal mol^{-1} K^{-1})
0	55	$102._5 \pm 3.0$	0	$102._5$	1.6 ± 0.15
0.25	52	$92._5 \pm 3.0$	$2._1$	$94._5$	1.4 ± 0.15
1.0	45.5	$80._5 \pm 4.0$	$8._7$	89	1.6 ± 0.15
2.0	36	49 ± 5.0	$20._6$	71	1.3 ± 0.3
		$\Delta c_p^{app} = 2.5 \pm 0.3$ **		$\Delta[c_p]_d = 1.6 \pm 0.2$ ***	

* Values obtained from direct scanning calorimetric recordings.
** Calculated from the temperature dependence of apparent enthalpies without corrections for preferential binding.
*** Calculated from the temperature dependence of ΔH_d, i.e. after the corrections for preferential binding have been made.

erential binding, its temperature dependence ($\Delta[c_p]_d = 1.6 \pm 0.2$ kcal mol^{-1} K^{-1}) corresponds to average $\Delta[c_p]_d$ values from each calorimetric curve, indicating that the use of eq. (3b) is correct.

4. Discussion

The interaction of denaturant molecules with proteins is one of the limiting problems in the evaluation of thermodynamic data in denaturation studies. As shown in this work the heat of solvation of lysozyme in concentrated GuHCl is over −100 kcal mol^{-1} and the partial enthalpy changes due to preferential binding are considerable in comparison with the denaturational enthalpy changes.

For the calculation of ΔH_{pref} an equation is proposed making use of the nearly linear initial slope of a saturation function in which different numbers of identical binding sites for the native and the denatured protein are included. The assumed mechanism for the action of GuHCl is discussed above, but further evidence for the correctness of the number of GuHCl molecules included in the unfolding process is given above in comparison with hydrogen exchange data.

True ΔH_d values can be obtained from isothermal calorimetric titrations with GuHCl if corrections are made with the help of eq. (3b). They are of the same order of magnitude as the denaturation enthalpies obtained in scanning calorimetric measurements performed in the absence of GuHCl [7–9] and emphasize the universal character of the temperature dependent function of ΔH_d [7,8] (fig. 8). Denaturational changes in molar heat capacities obtained in GuHCl denaturation studies were found to be $\Delta[c_p] = 1.5 \pm 0.2$ kcal mol^{-1} K^{-1} in agreement with $\Delta[c_p] = 1.6 \pm 0.1$ kcal mol^{-1} K^{-1} from scanning calorimetry without denaturants [7,8]. This result is not consistent with assumptions considering an additional structure breaking mechanism in GuHCl. Thus, the degree of unfolding in thermal denaturation must be much greater than commonly expected.

Comparing the enthalpy and heat-capacity changes for the different ways of denaturation, we cannot find evidence for the residual structural elements in heat denaturation and we conclude that the thermodynamic states of both heat- and GuHCl-denatured lysozyme are identical as regards ΔH_d and $\Delta[c_p]_d$. This result is

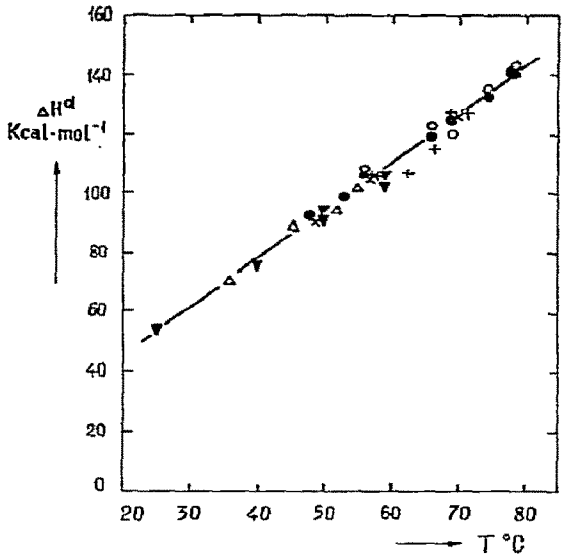

Fig. 8. Temperature function of the denaturation enthalpy of lysozyme using experimental results of various methods. Without GuHCl: • scanning calorimetry [7], ○ scanning calorimetry (Van 't Hoff) [7], × optical methods [7], + titrimetric method [9]. In the presence of GuHCl (this paper): ▼ isothermal titrations, △ scanning calorimetry.

coincident with conclusions drawn from NMR, hydrogen exchange and Raman studies showing that in thermally denatured lysozyme all groups are exposed [23,24,31]. On the other hand, divergences in hydrodynamic and optical parameters remain [3,4].

The most striking argument for incomplete unfolding in heat denaturation of lysozyme is the moderate increase in viscosity from about 3 cm^3 g^{-1} for the native state at 25°C [1,3,27,28,30] to about 4.7 cm^3 g^{-1} (reduced viscosity η_r) for thermally denatured lysozyme at 60–75°C [3,28]. For comparison, the intrinsic viscosity (η_i) equals 6.5 cm^3 g^{-1} at 25°C in 6 molar GuHCl [1]. The end-to-end distance of the polypeptide chain corresponding to the random coil state is only reached if the disulfide bonds in lysozyme are broken (η_i about 17 cm^3 g^{-1} [1,26,30]). Having in mind the position of the disulfide bridges in lysozyme, an explanation of the decrease of η_i to about 6.5 cm^3 g^{-1} was found using a semi-quantitative calculation; i.e., this value represents the maximum possible one at 25°C for cross-linked lysozyme if all other structural elements are broken [1].

An often done, direct comparison of $\eta_r^{70°}$ =

4.7 $cm^3\ g^{-1}$ for heat-denatured lysozyme with $\eta_r^{25°} = 6.5\ cm^3\ g^{-1}$ after GuHCl-denaturation is physically not correct without taking into account the temperature dependence of the viscosity of the polypeptide chains. It was determined that the intrinsic viscosity decreases with increasing temperatures [1]. On heating lysozyme in GuHCl from 25°C to 55°C η_r drops from about 6.5 $cm^3\ g^{-1}$ to about 4.8 $cm^3 g^{-1}$ at 55°C [26] and coincides with the results of heat-denatured lysozyme in the absence of GuHCl. There is no doubt that the structure breaking of proteins in 6 molar GuHCl is complete, even at elevated temperatures [1,4]. The identity of η_r for cross-linked denatured lysozyme after thermal transition with the results for GuHCl denaturation at comparable temperatures supports the suggested relationship of both thermodynamical states.

It follows that indirect conclusions on structure breaking in heat-denatured lysozyme by GuHCl as suggested earlier [4] must be seen in a new light. We should have in mind that the optical effects observed here have the sign opposite to that expected for rupture of structure [4] and this might be due to the preferential binding which was not taken into account. Anomalous optical effects due to the interaction of proteins with denaturants were found in studies with urea, GuHCl [32,33] and dodecylsulfate [25].

We conclude that the states of both heat- (state X) and GuHCl-denatured lysozyme (state D) are thermodynamically indistinguishable as far as their denaturational enthalpy and molar heat capacity change are concerned. A further manifestation of the thermodynamical identity of the considered denatured states is the coincidence of the values of the Gibbs energy of stabilization of the native structure obtained by quite different approaches, as will be shown in the following paper [29].

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