Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 3343–3347, May 1988
Biochemistry

Cold denaturation of staphylococcal nuclease

(protein stability/calorimetry)

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Contributed by Julian M. Sturtevant, January 15, 1988

ABSTRACT **Denaturation of staphylococcal nuclease was studied in a temperature range from −7 to 70°C by scanning microcalorimetry and spectropolarimetry. It was found that the native protein is maximally stable at about 20°C and is denatured upon heating and cooling from this temperature. The heat and cold denaturation processes are approximated rather well by a two-state transition showing that the molecule is composed of a single cooperative system. The main difference between these two processes is in the sign of the enthalpy and entropy of denaturation: whereas the heat denaturation proceeds with increases in the enthalpy and entropy, the cold denaturation proceeds with decreases in both quantities. The inversion of the enthalpy sign occurs at about 15°C in an acetate buffer, but this temperature can be raised by addition of urea to the solvent.**

It is known that the denaturation of a protein is always accompanied by a significant increase in its enthalpy (for reviews, see refs. 1 and 2). The usually observed positive denaturational increment of heat capacity means that the enthalpy of protein denaturation is a temperature-dependent function. Thus, one can expect that the enthalpy of denaturation can, in principle, become zero and then even invert its sign at some low enough temperature, T_{inv}^{H}, changing from the factor stabilizing the native protein structure into a factor destabilizing this structure. Therefore, one can imagine that protein denaturation can occur not only upon heating but also upon cooling. In contrast with heat denaturation, which proceeds with heat absorption—i.e., an increase of enthalpy and entropy—cold denaturation should proceed with a release of heat—i.e., a decrease of enthalpy and entropy (3).

The decrease of entropy upon the breakdown of the ordered native structure of protein molecules sounded like a paradox when it was first predicted more than 20 years ago by Brandts (4, 5). As this prediction of cold denaturation was based on a long extrapolation of indirect data, there have been many attempts since then to confirm it by direct experiment. The first successful demonstration of cold denaturation was accomplished with myoglobin and apomyoglobin (3, 6).

However, to show that cold denaturation is a general phenomenon specific for all globular proteins and not only for globins, it is highly desirable to demonstrate it on some other proteins. One of them was found to be lactate dehydrogenase on which cold denaturation was recently observed by Hatley and Franks (7). The other is staphylococcal nuclease (Nase), the denaturation of which is considered in this paper.

Heat denaturation of Nase was studied calorimetrically by Calderon *et al.* (8) and it was shown that it is accompanied by a rather large increase of heat capacity, which indicated that cold denaturation of this protein might be observable under experimentally realizable conditions.

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MATERIALS AND METHODS

The Nase was prepared as described in detail by Calderon *et al.* (8) and was kindly supplied by John Gerlt (University of Maryland). This preparation contains seven extra amino acid residues at the N terminus, raising the molecular mass from 16,807 Da (9, 10) to 17,601 Da.

Protein solutions were prepared by dissolving the necessary amount of the lyophilized preparation in an appropriate solvent. After thorough dialysis against the same solvent, it was centrifuged for 40 min at 24,000 × *g*. The protein concentration in the solution was determined spectrophotometrically with consideration for the light scattering effect, according to Winder and Gent (11), which is rather significant in the case of Nase. For solutions with pH from 3.0 to 5.0 without urea we used the absorbance values $A_{277}^{1\%} = 9.84$ and $A_{280}^{1\%} = 9.39$, which we determined by measuring the nitrogen content in the stock solution according to the method of Jaenicke (12). These values are close to those reported earlier for the neutral solutions: $A_{277}^{1\%} = 9.7$ and $A_{280}^{1\%} = 9.3$ (13, 10, respectively). The extinction coefficients in acidic solutions in the presence of 2 M urea were found to be almost the same as in the absence of urea.

All measurements were done in 10 mM acetate buffer solutions containing 0.1 M NaCl. In some cases 2 M urea was added to the solvent.

The calorimetric measurements were done with the scanning microcalorimeter DASM-4 as described by Privalov and Potekhin (14).

The circular dichroism (CD) spectra in the range of 183–320 nm were measured with a Jasco-41A spectropolarimeter (Japan), and the molar ellipticity, $[\Theta]_\lambda$, was calculated by taking the mean molecular mass of an amino acid residue, $\overline{M}_{ar}$, to be 116.4.

RESULTS

Fig. 1 represents the temperature dependence of the partial specific heat capacity of Nase as measured during continuous heating of the precooled acidic solutions at various pH values. The extensive heat absorption peaks observed at temperatures above 15°C are due to the usual thermal denaturation. With an increase of pH the heat absorption peak increases in size and shifts to a higher temperature, as it usually does (2), showing that the protein structure becomes more stable against heating.

The denaturation process is accompanied also by a significant increase in the heat capacity of the protein. The heat capacity of the denatured protein is likely to be pH-independent at any temperature above 65°C, at which temperature under the considered solvent conditions Nase is in a completely thermally denatured state. One can suppose that at temperatures below 65°C the heat capacity function of the denatured state at all values of the pH is represented by the same function as found for a solution with pH 3.23, where

Abbreviation: Nase, staphylococcal nuclease.

the protein is certainly denatured over the temperature range from −10 to 70°C, according to the CD test (see below). At 20°C the apparent specific heat capacity of the denatured Nase is 1.75 ± 0.05 $J \cdot K^{-1} \cdot g^{-1}$. Since this quantity is essentially independent of the protein concentration, it may be identified as the partial specific heat capacity, $C_{p,pr}$.

The heat capacity of the native Nase can be determined only in a solution with pH ≥ 4.5, as at lower pH values its acid denaturation takes place even at room temperature. At 20°C the partial specific heat capacity of the native Nase equals 1.30 ± 0.05 $J \cdot K^{-1} \cdot g^{-1}$, which is very close to the values found for many other globular proteins (1.25–1.40 $J \cdot K^{-1} \cdot g^{-1}$; see, e.g., ref. 2). Therefore, at 20°C the denaturational increment of heat capacity of Nase, $\Delta_N^D C_p$, amounts to 0.45 $J \cdot K^{-1} \cdot g^{-1}$ or 7.60 $kJ \cdot K^{-1} \cdot mol^{-1}$. By extrapolating the heat capacity function for the native and denatured states to the mid-denaturation temperature, T_t [which is close to the temperature of maximal denaturational heat absorption (14)], as indicated by the dashed line in Fig. 1, we can determine the heat absorption peak area corresponding to the true (calorimetric) enthalpy of the protein denaturation at the transition temperature, $\Delta_N^D H(T_t)^{cal}$.

From the sharpness of the observed heat absorption peak we can determine the effective (van't Hoff) enthalpy of the process under study, assuming that this process is a two-state transition

$$\Delta_N^D H(T_t)^{vH} = \frac{4RT_t^2 \delta C_p^{max}}{\Delta_N^D H(T_t)^{cal}}, \quad [1]$$

where

$$\delta C_p^{max} = C_{p,pr}^{max} - \frac{1}{2}(C_{p,pr}^N + C_{p,pr}^D)T_t$$

is the maximal value of the excess heat capacity measured from the mean heat capacity of the native and denatured states at the mid-transition temperature, T_t (see ref. 14 for details).

Table 1. Calorimetric and van't Hoff enthalpies for the denaturation of Nase in 10 mM acetate buffer solutions containing 100 mM NaCl

pH	T_t, 0°C	$\Delta_N^D H^{cal}$, $kJ \cdot mol^{-1}$	$\Delta_N^D H^{vH}$, $kJ \cdot mol^{-1}$	$\Delta H^{cal}/\Delta H^{vH}$
7.0	51.7	353	323	1.09
6.8	50.0	332	312	1.06
5.0	48.3	290	272	1.07
4.5	43.3	268	238	1.13
3.98	35.0	186	159	1.17

The values obtained for the calorimetric and van't Hoff enthalpies of denaturation of Nase in solutions where the native state is clearly defined (pH ≥ 4.5) are given in Table 1. As seen, they are in rather good agreement: the calorimetric enthalpy exceeds only slightly the van't Hoff enthalpy, as has been found to be the case for many other small globular proteins (2). Discrepancies with previously published data (8) are due at least in part to light scattering effects that were not taken into account in determinations of protein concentrations and are especially significant in the case of Nase.

The slope of the plot of the denaturation enthalpy vs. denaturation temperature,

$$\frac{d\Delta_N^D H(T_t)}{dT_t},$$

in Fig. 2 equals 8.23 $J \cdot K^{-1} \cdot mol^{-1}$—i.e., it is close to the value of the denaturational heat capacity increment,

$$\Delta_N^D C_p = \left(\frac{\partial \Delta_N^D H(T)}{\partial T}\right)_{pH}$$

This shows that ionization effects do not influence significantly the slope of the enthalpy function and that the dependence presented in Fig. 2 can be regarded in the first approximation as a temperature dependence of the denaturational

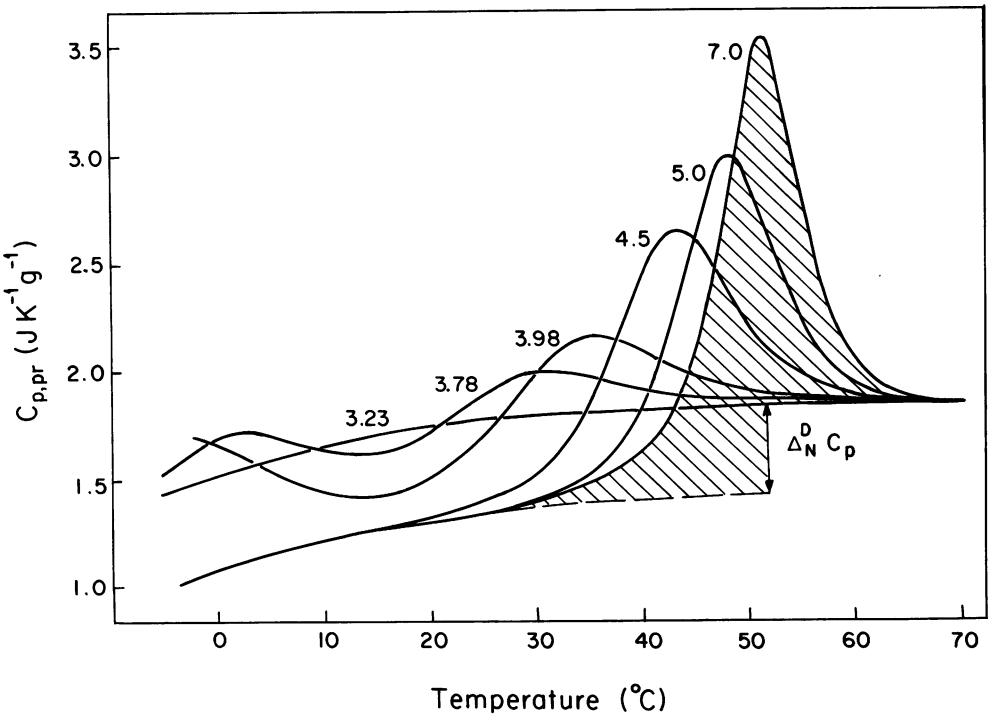

FIG. 1. Temperature dependence of the partial specific heat capacity of Nase in solutions of various pH values. The dashed line shows the extrapolation of the experimentally determined heat capacity function for the native state. The hatched area corresponds to the enthalpy of protein denaturation (*inset*).

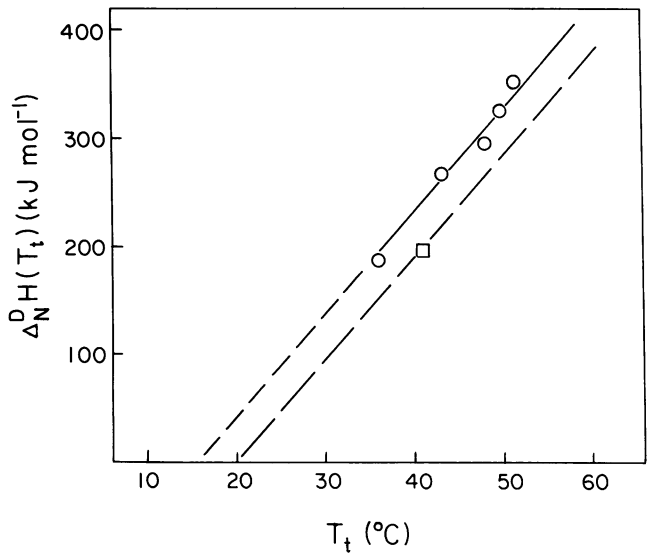

FIG. 2. Calorimetric enthalpy of Nase denaturation plotted vs. denaturation temperature T_t. The circles represent the results obtained in this study in acetate solutions without urea, and the square represents the result obtained in acetate solution containing 2 M urea. The dashed lines show the $\Delta_N^D H$ extrapolation with the slope determined calorimetrically,

$$\left(\frac{\partial \Delta_N^D H}{\partial T} = \Delta_N^D C_p\right).$$

enthalpy. Its extrapolation to lower temperatures shows that the enthalpy of the protein denaturation should be zero at about 15°C and below this temperature its sign should be reversed—i.e., this temperature should be considered as T_{inv}^H for Nase in acetate buffer solution. At the same time, according to Fig. 1, just at this temperature (15°C) the heat capacity minimum is observed for Nase in solutions with pH $\leq$ 4.5. Since the denaturational heat effect is zero at this temperature, it is clear that a somewhat higher heat capacity of protein observed at these pH values is caused by the presence of partially denatured protein in the solution. The amount of the denatured protein increases on heating or cooling the solution from this temperature.

Unfortunately, the low temperature part of the heat capacity function that is connected with cold denaturation takes place at too low temperatures to be traced to its completion even in a supercooled solution. To observe the end of cold denaturation we cannot go down to temperatures lower than −7°C because of the danger of damaging the calorimetric cell upon spontaneous freezing of the solution. We also cannot decrease the lower limit of supercooling of an aqueous solution by the addition of various organic compounds, since, as we pointed out in a previous communication (3), all of the compounds that depress the freezing temperature of water change the thermodynamic properties not only of water but of the protein as well—that is, they lower the temperature of cold denaturation making its observation impossible. Thus, the only possibility to observe the completion of the cold denaturation of Nase consists in raising its T_{inv}^H. This can be done by adding denaturants to the protein solution, such as urea or guanidine hydrochloride, which have a positive solvation enthalpy, and by solvating the protein groups on denaturation, they decrease the overall enthalpy of denaturation (see ref. 15). The efficiency of urea in raising the temperature of cold denaturation was demonstrated recently in the case of metmyoglobin (6).

Fig. 3 presents the results of the calorimetric study of the heat capacity of Nase in solutions containing 2 M urea. The heat absorption peaks are considerably shifted toward higher temperatures in the presence of urea and the minimum between the two peaks is located now at about 20°C. The denaturational increment of heat capacity is also somewhat increased due to an addition of the heat effect of a gradual dissolution of urea upon continuous heating of the protein (see ref. 15). As for the enthalpy of denaturation, it is significantly lower than it was at similar temperatures in the absence of urea (Fig. 2). By using the previously found value of $\Delta_N^D C_p$ as the slope of the enthalpy function, 8.0 kJ·K^{-1}·mol^{-1}, we can find that $T_{inv}^H \approx$ 20°C, which directly corresponds to the temperature of the heat absorption minimum of Nase in the presence of urea (Fig. 3).

In Fig. 4 we present an original microcalorimetric recording of the heat effect observed upon scanning a Nase solution containing 2 M urea in both directions over the temperature range at ± 0.50 K·min^{-1}. As seen, the recorded effects are quite symmetrical as if one of the curves is the mirror

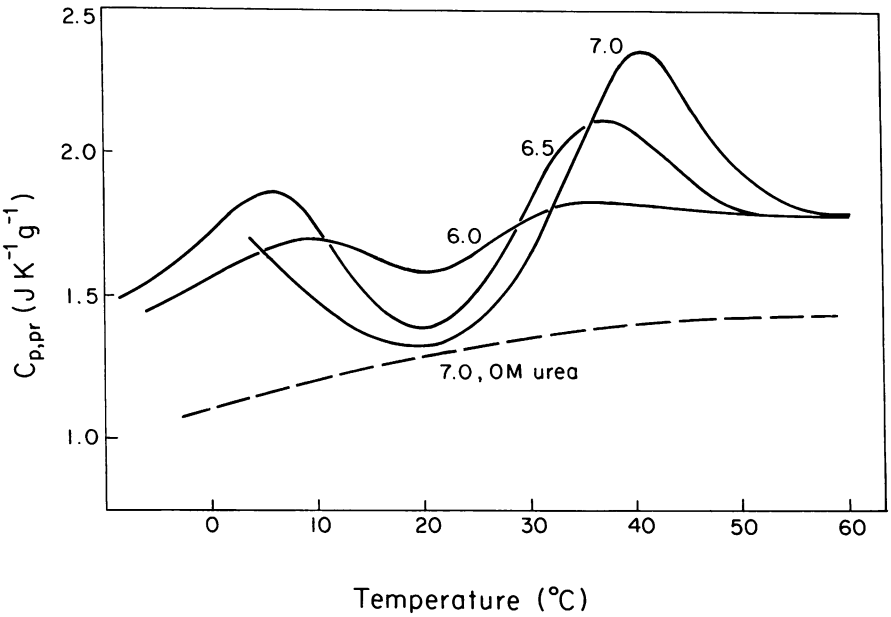

FIG. 3. Temperature dependence of the partial specific heat capacity of Nase in acetate buffer containing 2 M urea at various pH values. The dashed line corresponds to the native protein in a solution without urea at pH 7.0.

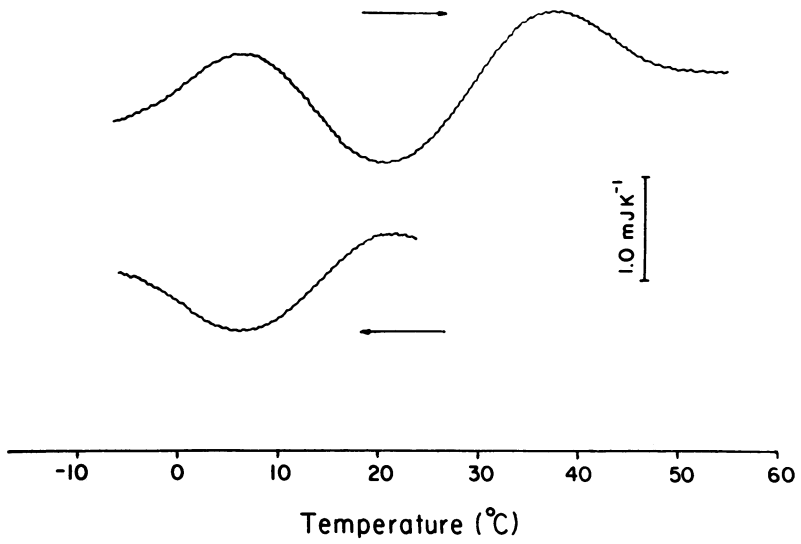

FIG. 4. Microcalorimetric recording of cooling and subsequent heating of a Nase solution containing 2 M urea (pH 6.5) at scan rate 0.5 $K \cdot min^{-1}$. The protein concentration in the solution was 3.9 mg/ml.

image of the other. This shows that the temperature-induced processes in Nase, its heat and cold denaturation and renaturation, are perfectly reversible and close to equilibrium at the scan rate used—i.e., these processes are rather fast in contrast to those in myoglobin, where cold denaturation and renaturation are slow processes judging by their shift over the temperature scale (3).

Fig. 5 shows the changes observed in the ultraviolet CD spectra of Nase in the considered temperature range. Judging by the ellipticity at 222 nm, which, according to Chen *et al.* (16), reflects the content of α-helical structure in a protein, the heat and cold denaturation of Nase lead to an almost complete loss of helicity in this protein.

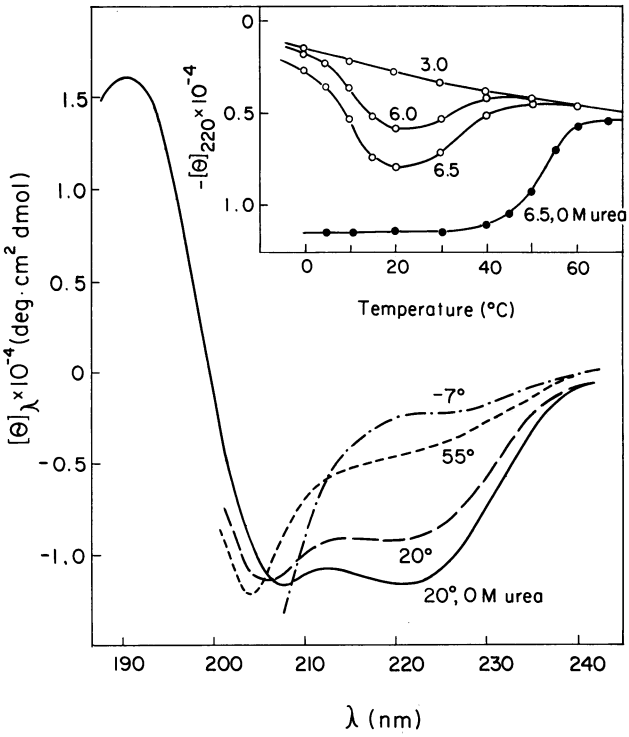

FIG. 5. CD spectra of Nase in the ultraviolet in solutions containing 2 M urea (pH 6.5) at different temperatures and the temperature dependence of Nase ellipticity at 220 nm in solutions with various pH values (*Inset*, open circles). The solid line with filled circles shows the results for a solution without urea.

DISCUSSION

Among the questions that are usually raised when considering the mechanism of stabilization of a native protein structure, one of the first is whether this structure represents a single cooperative system that integrates the contributions of all of the forces maintaining it. In the case of Nase it looks as if we have just this situation according to Taniuchi and Anfinsen (9) and Anfinsen *et al.* (17), who studied the stability of various fragments of a Nase molecule and showed that almost all its residues, with the exception of the few residues at the terminal parts of the chain, play crucial roles in the stabilization of its native structure. The same conclusion was made when studying the acid denaturation of Nase, which showed that it is a highly cooperative process as judged by simultaneous changes of its various characteristics and, especially, of the NMR resonances corresponding to different histidines (18, 19).

However, the most rigorous thermodynamic test for the cooperativity of any molecular structure consists in a comparison of the calorimetric and van't Hoff enthalpies of a

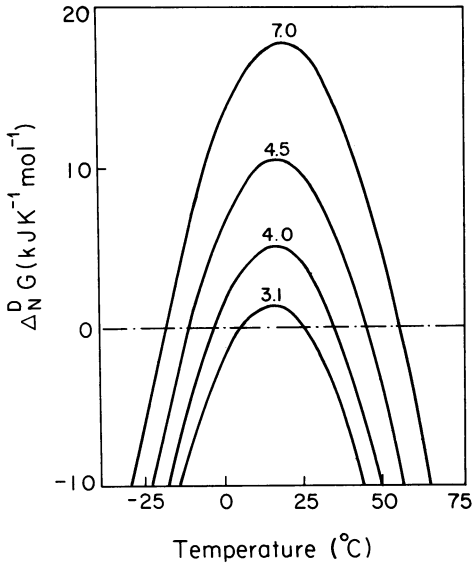

FIG. 6. Temperature dependence of the Gibbs energy difference of the native and denatured states of Nase at various pH values in the absence of urea.

temperature-induced disruption of the structure (2, 14).

As was shown above, the calorimetric enthalpy, $\Delta_N^D H^{cal}$, of the thermal denaturation of the Nase is close to the van't Hoff enthalpy, $\Delta_N^D H^{vH}$, at all conditions where the native state is clearly defined, as it is at pH $\geq$ 4.5. The deviation between these two quantities does not exceed a few percent (see Table 1) and in all cases the calorimetric enthalpy is larger than the van't Hoff enthalpy. This means that the Nase heat denaturation is not an absolutely two-state transition, but is close to such a transition—i.e., the population of all intermediate states between the native and denatured macroscopic states is low and can be neglected in the first approximation.

However, if the Nase denaturation can be regarded as a transition between two macroscopic states that differ significantly in their heat capacities and this difference does not depend noticeably on temperature, then we have for the enthalpy and entropy of this transition:

$$\Delta_N^D H(T) = \Delta_N^D H(T_t) - (T_t - T)\Delta_N^D C_p = (T - T_{inv}^H)\Delta_N^D C_p \quad [2]$$

$$\Delta_N^D S(T) = \frac{\Delta_N^D H(T_t)}{T_t} - \Delta_N C_p \ln(T_t/T)$$

$$= \left[\frac{T_t - T_{inv}^H}{T_t} - \ln(T_t/T)\right]\Delta_N C_p, \quad [3]$$

where T_t is the transition temperature at which both states, N and D, are equally populated and thus

$$\Delta_N^D S(T_t) = \Delta_N^D H(T_t)/T_t;$$

with T_{inv}^H the temperature at which the denaturation enthalpy is zero:

$$\Delta_N^D H(T_{inv}^H) = \Delta_N^D H(T_t) - (T_t - T_{inv}^H)\Delta C_p = 0.$$

The stability of the native state is determined by the work required for its disruption—i.e., by the Gibbs energy difference between the native and denatured states:

$$\Delta_N^D G(T) = \Delta_N^D H(T) - T\Delta_N^D S(T)$$

$$= \left[\frac{T \cdot T_{inv}}{T_t} - T_{inv}^H + T\ln(T_t/T)\right]\Delta_N^D C_p. \quad [4]$$

By using the experimentally found values of $\Delta_N^D C_p$ and T_{inv}^H we can determine by Eq. 4 the temperature dependence of the Gibbs energy difference between the native and denatured states for any chosen T_t. As seen from Fig. 6, it is a function with an extremum and crosses the zero level at two different temperatures, T_t and T_t'. As the stability of the native structure of the protein is zero at these temperatures, it is clear that the first of these temperatures is the temperature of the heat denaturation of the protein molecule and the second one is that of its cold denaturation.

The demonstration of the cold denaturation of Nase makes it clear that this phenomenon is characteristic not only for the globins with which it was first studied but that it is a general property of all globular proteins.

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