Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 7715–7718, September 1991
Biochemistry

Cold denaturation and ^2H_2O stabilization of a staphylococcal nuclease mutant

(protein stability/mutation)

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Communicated by Julian Sturtevant, May 8, 1991 (received for review September 21, 1990)

ABSTRACT **Cold denaturation is now recognized as a general property of proteins but has been observed only under destabilizing conditions, such as moderate denaturant concentration or low pH. By destabilizing the protein using site-directed mutagenesis, we have observed cold denaturation at pH 7.0 in the absence of denaturants in a mutant of staphylococcal nuclease, which we call NCA S28G for a hybrid protein between staphylococcal nuclease and concanavalin A in which there is the point mutation Ser-28 → Gly. The temperature of maximum stability (t_{max}) as determined by circular dichroism (CD) was 18.1°C, and the midpoints of the thermal unfolding transitions (t_m) were 0.6°C and 30.0°C. These values may be compared with the t_m of 52.5°C for wild-type staphylococcal nuclease, for which no cold denaturation was observed under these conditions. When the stability of the mutant was examined in ^2H_2O by NMR, CD, or fluorescence, a substantial increase in the amount of folded protein at the t_{max} was noted as well as a decrease in t_{max}, reflecting increased stability.**

Cold denaturation is an inherent property of proteins based on the thermodynamics of denaturation (1–8). It is likely to be observed in cases where the heat-capacity difference between native and denatured states is large and the difference in enthalpy is small; thus, the temperature at which the denaturation enthalpy becomes zero is high (8). Because these conditions are usually met at temperatures below 0°C, cold denaturation has only been observed under denaturing conditions, such as in the presence of denaturant (3, 5–7) or at low pH (1, 4). Recently, cold denaturation of lactate dehydrogenase was reported in a water-in-oil emulsion at subzero temperatures (9). Cold denaturation has previously been reported for wild-type staphylococcal nuclease (SNase) in the presence of 2 M urea or low pH (6). Here we report that cold denaturation can be observed when the intrinsic stability of the protein is decreased by a change in the amino acid sequence, rather than by manipulating the solution conditions. The SNase mutant used in these studies is a simple amino acid variant of a nuclease–concanavalin A hybrid protein (NCA) (10) in which residues 27–30 of SNase (Tyr-Lys-Gly-Gln), found in a type I′ β-turn conformation, were replaced with a type I + G_1 β-bulge structure from concanavalin A (residues 160–164, Ser-Ser-Asn-Gly-Ser). Here the stability of a point mutant of that hybrid protein (Ser-28 → Gly, which we refer to as S28G) is characterized. Although both the original hybrid protein, NCA, and its mutant, NCA S28G, show cold denaturation, the mutant showed higher enthalpy changes (more cooperative transitions) and was thus more suited for an in-depth study.

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MATERIALS AND METHODS

Thermal Denaturation Curves. The thermal denaturation of SNase and its mutated hybrid protein, NCA S28G, was determined at pH 7.0 and pH 5.5 in aqueous buffer and in buffer containing 10 mM $CaCl_2$ and 0.1 mM 3′,5′-deoxythymidine bisphosphate (pdTp) (pH 7.0) by using far-UV circular dichroism (CD) and fluorescence. Protein concentrations were in the range of 4–10 μM and were determined by absorbance with $A_{280}^{1\%} = 9.2$ (11). CD at 222 nm was measured with an Aviv Associates (Lakewood, NJ) model 60DS CD spectrometer. Fluorescence measurements were made with a Perkin–Elmer model MPF-4 fluorescence spectrophotometer equipped with a computer interface (excitation wavelength, 295 nm; emission wavelength, 330 nm). A Neslab Instruments (Portsmouth, NH) Endocal refrigerated circulating bath with a temperature controller was used to change the temperature of the cell block at a rate of 0.25°C or 0.3°C per min. The NMR thermal-unfolding data were measured from the ratio of areas of histidine proton $H^{\varepsilon 1}$ resonances assigned to folded and unfolded protein (10). Samples were 3 mg of ^2H-exchanged protein per ml in 200 mM acetate-buffered ^2H_2O (pH* 5.3 uncorrected pH-meter reading). NMR measurements were made with a Bruker (Karlsruhe, F.R.G.) AM-500 instrument. Temperatures were measured with a digital electronic thermometer inserted into the probe before and after each protein spectrum. Fluorescence and CD measurements in ^2H_2O at pH* 5.3 were also made to compare the results directly with the NMR data. For both CD and fluorescence data, the native protein fraction, F_N, was obtained by using the signal for wild type SNase at 20°C as the 100% native protein value and the signal for the guanidine hydrochloride (Gdn·HCl)-denatured (5 M, pH 2) protein as the denatured protein value ($[\theta]_{222} = -350$ deg·cm^2·dmol^{-1}) and subtracting the latter from the former. The midpoint of the thermal transition curve (t_m) was defined as the temperature corresponding to 50% of the maximum change in signal. The fluorescence measurements were corrected for the effect of temperature on the signal for the native and denatured protein by linear extrapolation.

Guanidine Thiocyanate (GdnSCN) and Gdn·HCl Denaturation Curves. The stability of SNase and NCA S28G was determined as a function of GdnSCN and Gdn·HCl concentrations by using tryptophan fluorescence. Approximately 800 μl of buffer with a protein concentration of 5–10 μM was cooled to 0°C in a quartz fluorometer cell. Aliquots of concentrated denaturant were added, and the fluorescence at equilibrium was recorded as a function of the denaturant

Abbreviations: SNase, staphylococcal nuclease; NCA, nuclease–concanavalin A hybrid protein; t_{max}, temperature of maximum stability; t_m, midpoint of thermal unfolding transitions (melting temperature); GdnSCN, guanidine thiocyanate; Gdn·HCl, guanidine hydrochloride; pH*, uncorrected pH meter reading.

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concentration. The following buffers were used: 10 mM cacodylate (pH 7.0 and pH 5.5) and 10 mM cacodylate/10 mM $CaCl_2$/0.1 mM pdTp, pH 7.0. The data were converted to correspond to the native protein fraction F_N, and the free energy of denaturation in the transition region was calculated from the expression $\Delta G = -RT\ln K$, where $K = (1 - F_N)/F_N$. A linear extrapolation of the data was used to determine $\Delta G(H_2O)$ in the absence of denaturant. The C_m is defined as the denaturant concentration corresponding to $F_N = 0.5$.

CD Spectra. Spectra of the CD between 200 and 260 nm of the wild-type and mutant proteins were made by using a 0.2-mm path-length circular cell and a protein concentration of 40–55 μM. The protein spectra were determined in 10 mM cacodylate in H_2O or ^2H_2O (pH 7.0), 5 M Gdn·HCl (pH 2.0), and HCl (pH 2).

RESULTS AND DISCUSSION

NCA S28G Exhibits Cold Denaturation. The thermal unfolding transitions of wild-type SNase and mutant NCA S28G as monitored by CD are shown in Fig. 1*A*. For wild-type SNase, a single unfolding transition is observed as the temperature is raised, with the t_m in agreement with that reported previously (12). On the other hand, the NCA S28G mutant has a high-temperature t_m that is 20°C lower than that of the wild type at pH 7.0 and shows a maximum stability in the 14–18°C region, where it is 96% folded compared to the wild type. At pH 5.5 in aqueous buffer, the behavior is similar except that only 71% of the mutant protein is folded at the t_{max} of 16.7°C. The transitions were fully reversible. The addition to the buffered (pH 7) mutant protein of Ca^{2+} and the inhibitor 3′,5′-deoxythymidine bisphosphate, which is known to stabilize wild-type SNase, stabilized the mutant protein completely against cold denaturation and increased the t_m to 53.8°C as measured by CD.

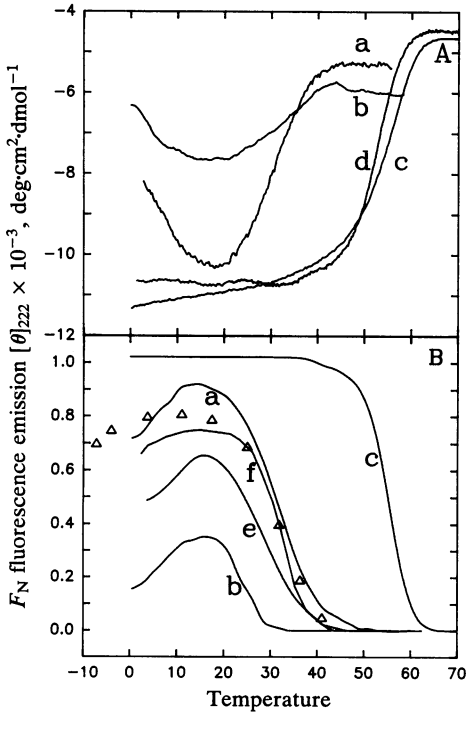

FIG. 1. Thermal unfolding transitions for wild-type SNase and the NCA S28G mutant. The data shown by solid lines in *A* were measured by CD and those in *B*, by tryptophan fluorescence emission (excitation at 295 nm, emission at 330 nm). In *B*, only data for the mutant NCA S28G are shown, and the triangles represent ^1H NMR data for NCA S28G. Proteins were in 10 mM sodium cacodylate buffer with the additions and pH as indicated. Curves: a, NCA S28G mutant at pH 7.0; b, mutant at pH 5.5; c, mutant + 10 mM Ca^{2+} + 0.1 mM pdTp at pH 7.0; d, wild-type SNase at pH 7.0; e, mutant in ^2H_2O at pH* 5.3; f, mutant in ^2H_2O at pH* 5.5.

The cold denaturation was confirmed by fluorescence measurements. Comparison of the tryptophan fluorescence emission in water (Fig. 1*B*) with the CD measurements (Fig. 1*A*) shows similar thermal denaturation behavior, although t_{max} measured by fluorescence was 14.2°C at pH 7.0 and 16.1°C at pH 5.5 compared with 18.1°C and 16.7°C, respectively, by CD. These discrepancies between the transitions detected by fluorescence and CD may reflect the existence of an intermediate state or structure in the unfolded state (13). The latter possibility is supported by the observation that thermally unfolded SNase shows residual structure by CD but not by fluorescence (Fig. 1).

Because CD and fluorescence experiments were carried out both at pH 7 to compare with previous work and at pH around 5.5 to compare with NMR data, a complete pH titration was performed to determine the effect of pH on the stability of NCA S28G. The mutant was maximally stable at pH 8.5–10, with a cooperative unfolding transition observed around pH 6. Wild-type SNase is fully native at pH 5.5 and has a pH-induced unfolding transition in the vicinity of pH 3.8 (14). Thus, in addition to decreased thermal stability, the mutant is more sensitive to acid denaturation.

Cold denaturation was also observed with NMR. The fraction folded was measured from the relative intensities of histidine $H^{\varepsilon 1}$ resonances assigned to folded and unfolded protein (12). To resolve the histidine $H^{\varepsilon 1}$ resonances, NMR experiments were carried out in ^2H_2O at pH* 5.3. The thermal denaturation curve for NCA S28G (Fig. 1*B*, triangles) clearly shows cold denaturation, although it shows t_{max} 5°C lower as well as a higher fraction folded at t_{max} than in the fluorescence studies in H_2O.

The discrepancy between the NMR studies in ^2H_2O and the fluorescence studies in H_2O is due in large part to strong isotope effects in the deuterated solvent. Whereas the protein used in the NMR experiments was fully ^2H-exchanged, this was not the case for the protein studied by fluorescence in ^2H_2O. When fluorescence measurements were repeated in ^2H_2O, the fraction folded at t_{max} increased from 36% in H_2O (pH 5.5) to 54% in ^2H_2O (p^{2}H 5.5). Fluorescence measurements between 5.1 and 7 show substantial increases in the amount of folded protein at t_{max} in ^2H_2O compared with that in H_2O. In pH titration, the unfolding transition observed at pH 6 in H_2O was shifted to lower pH in ^2H_2O, further reflecting the increased stability in ^2H_2O relative to H_2O. Stabilization was also observed by CD at pH 7 (see Fig. 3*B*).

A small stabilizing effect of deuterium oxide on thermal inactivation of oligomeric proteins has been observed previously and attributed to an increased strength of hydrogen bonds containing deuterons (15–17). The observed increase in protein stability reported here could result from a change in the free energy of the folded structure due to the increased strength of intramolecular amide H bonds. Alternatively, or additionally, an effect of the solvent interaction with exposed hydrophobic groups (i.e., the "hydrophobic interaction") could influence the relative free energies of the unfolded and folded states. The observed isotope effect may be more dramatic for this mutant protein than for proteins in general, as the relative contribution of H bonds to hydrophobic interactions is greatest at low temperatures where this protein is only marginally stable. The low temperature arm of the denaturation profile is shifted to lower temperature in ^2H_2O due to this stabilization.

The stability of NCA S28G was confirmed to be concentration independent in the range used in these studies, although NMR studies on the effects of protein concentration (data not shown) did show an effect at higher concentrations: a 10% decrease in F_N at t_{max} and a decrease in the high-

temperature t_m from 33.2°C to 26.2°C was observed as the protein concentration was increased from 3 to 30 mg/ml. t_{max} and the fraction folded were independent of concentration below 3 mg/ml. The observed concentration dependence is probably due to favorable interactions between unfolded molecules, as aggregation or dimerization have been observed with SNase (J. Flanagan, personal communication).

NCA S28G Is Less Stable Than Wild-Type SNase. Use of tryptophan fluorescence to monitor the stability of SNase and NCA S28G as a function of GdnSCN concentration at pH 7.0 and 0°C is shown in Fig. 2. The dramatic decrease in stability of NCA S28G relative to wild-type SNase is quite evident. The ΔG values (0°C) in the absence of denaturant were calculated as 4.6 kcal·mol^{-1} for wild-type SNase, 0.96 kcal·mol^{-1} for the NCA S28G mutant, and 3.5 kcal·mol^{-1} for the mutant in the presence of Ca^{2+} and the inhibitor pdTp. Binding of these ligands stabilizes the native state sufficiently so as to overcome the cold denaturation and to increase the stability toward guanidine denaturation. At pH 5.5 and 0°C, addition of a small amount of GdnSCN stabilized the mutant (Fig. 2*A*). This was shown to be due to a salt effect, since the addition of NaCl brought about the same effect. There was little difference between the effects of GdnSCN and Gdn·HCl on the derived free energy of unfolding at pH 7.0 and 0°C; however, the slopes of the free-energy plots were significantly different (Fig. 2*B*).

The free energy of unfolding as a function of temperature for wild-type SNase and the NCA S28G mutant was determined from the thermal unfolding curves as monitored by CD, and the enthalpy change for unfolding at t_m (ΔH_m) was determined by a least-squares analysis of the plots of ΔG as a function of temperature ($\Delta H_m = T_m \times$ slope at t_m). The heat capacity difference between the native and denatured protein (ΔC_P) was determined by the method described by Pace and Laurents (18) using the $\Delta G(H_2O)$ determined from the GdnSCN stability curves and the Gibbs–Helmholtz equation:

$$\Delta G(T) = \Delta H_m(1 - T/T_m) - \Delta C_P[(T_m - T) + T\ln(T/T_m)]. \quad [1]$$

The thermodynamic parameters for SNase and the NCA S28G mutant at pH 7.0 and 0°C are summarized in Table 1. The enthalpy change for unfolding, ΔH_m, for the NCA S28G mutant was 32.1 kcal·mol^{-1} less than that of wild type, and the ΔC_P was about 400 cal·deg^{-1}·mol^{-1} greater than that of the wild type. The ΔC_P value for the wild type determined by this method is in the same range as that reported by Shortle *et al.* (13) but is somewhat lower than the values reported for differential scanning calorimetry measurements (6).

Thermally Unfolded and pH-Unfolded SNases Possess Some Secondary Structure. The observation of a significant residual ellipticity at 222 nm after unfolding prompted further investigation into the presence of secondary structure in the denatured state. Fig. 3 shows the far-UV CD spectra of wild-type SNase and mutant NCA S28G under various denaturing conditions. In comparing these spectra with those of the proteins in the fully native and fully denatured states, it is evident that there is significant secondary structure in both acid-denatured and heat-denatured protein [even when the effect of temperature on the spectrum of denatured protein is taken into consideration (19)]. In contrast, the fluorescence spectra of the thermally denatured and GdnSCN-denatured proteins are indistinguishable from each other. These observations indicate that the acid- and heat-denatured proteins more closely resemble compact intermediate states such as the molten globule state in which there is some secondary structure but little or no tertiary structure (20). It is of interest to note that the tryptophan fluorescence signal shows complete unfolding at [Gdn·HCl] > 0.25 M (Fig. 2), whereas there is still secondary structure as judged by θ_{222} at [Gdn·HCl] to 5 M (pH 7) (Fig. 3).

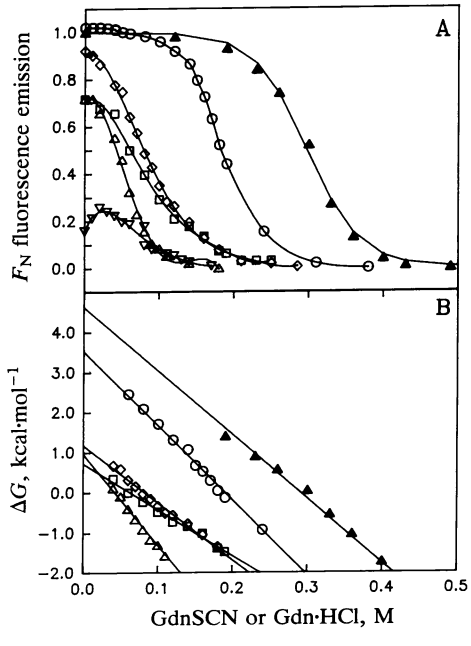

FIG. 2. Denaturant-induced unfolding of wild-type SNase and mutant NCA S28G as monitored by tryptophan fluorescence. (*A*) Proteins were with GdnSCN at the concentrations shown in 10 mM cacodylate buffer unless otherwise noted. △, Mutant at pH 7.0 and 0°C; ○, mutant + 10 mM Ca^{2+} + 0.1 mM pdTp at pH 7 and 0°C; ▽, mutant at pH 5.5 and 0°C; ◇, mutant at pH 7.0 and 18°C; ▲, wild-type SNase at pH 7.0 and 0°C; □, mutant at pH 7.0 and 0°C with Gdn·HCl. The fraction native for the mutant was calculated relative to the wild type. (*B*) Corresponding free-energy plots extrapolated to 0 denaturant.

CONCLUSIONS

The pronounced decrease in stability of NCA S28G relative to the wild-type SNase probably arises from a decrease in ΔS from the ordering of water molecules due to exposure of hydrophobic residues in and adjacent to the altered loop region, which are normally buried in a hydrophobic pocket as is seen in the unmutated NCA hybrid protein (10). This is supported by the observation that unmutated NCA is also less stable than wild-type SNase and shows evidence of cold denaturation (data not shown).

SNase is known to have a large ΔC_P (21) and thus to be more likely to show cold denaturation (8). The destabilization induced by the mutation results in an increase in ΔC_P and a decrease in ΔH, which is sufficient to raise the t_{max} to temperatures well above 0°C in the absence of denaturants

Table 1. Thermodynamic parameters for GdnSCN and thermal unfolding of SNase and its NCA S28G mutant in 10 mM cacodylate buffer at pH 7.0

Parameter	SNase	NCA S28G
$\Delta G(H_2O)$,* kcal·mol^{-1}	4.6 ± 0.4	0.96 ± 0.15
C_m,† M	0.30 ± 0.01	0.04 ± 0.01
t_m, °C	52.5 ± 0.5	30.0 ± 0.5
ΔH_m,‡ kcal·mol^{-1}	73.9 ± 6.5	41.5 ± 3.1
ΔC_P,§ cal·deg^{-1}·mol^{-1}	1634 ± 296	2045 ± 485

*ΔG at 0°C and no GdnSCN.
†Midpoint of the GdnSCN unfolding transition.
‡Determined from the slope of the plot of ΔG vs. T at t_m.
§Calculated from Eq. 1 with t_m, ΔH_m, and $\Delta G(H_2O)$ at 0°C and T = 273 K.

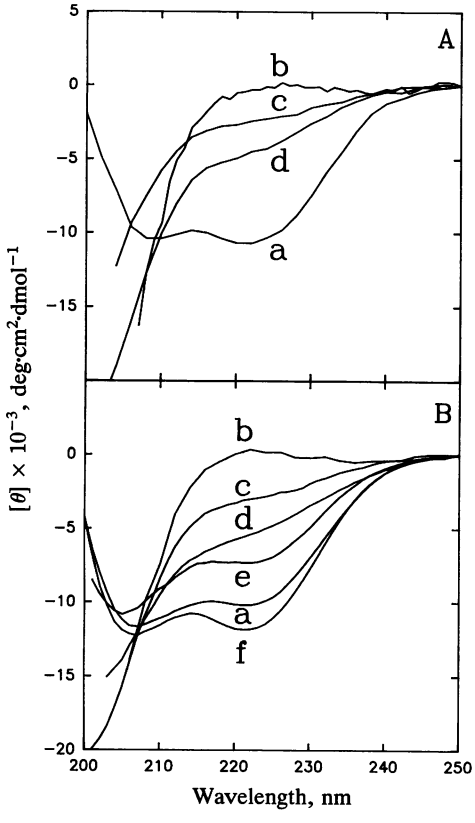

FIG. 3. CD spectra of wild-type SNase (*A*) and mutant NCA S28G (*B*). (*A*) Curves with wild type: a, native protein at pH 7.0 and 20°C; b, with 5 M Gdn·HCl at 0°C and pH 2; c, at 70°C and pH 7.0; d, at pH 2 and 0°C. (*B*) Curves with mutant: a, at 18°C and pH 7.0; b, with 5 M Gdn·HCl at 0°C and pH 2; c, at 56°C and pH 7.0; d, at 0°C and pH 2.0; e, at 0°C and pH 7.0; f, at 20°C and pH* 7.0 in ^2H_2O.

and makes the cold-denaturation transition observable at experimentally accessible temperatures at neutral pH. Thus, disruptions in the structure of a protein by mutagenesis can produce a protein that has a combination of ΔC_P and ΔH changes that result in a cold denaturation temperature well above 0°C. Such a system provides an excellent model in which to investigate further the effects of amino acid substitutions on the intrinsic cold-denaturation behavior of proteins.

We thank Chuck Wilson and John Gill for early contributions to this work. This research was supported by grants from the National Science Foundation (to A.L.F.) and the National Institutes of Health (to A.L.F. and R.O.F.).

1. Brandts, J. F. (1964) *J. Am. Chem. Soc.* **86**, 4291–4301.
2. Brandts, J. F. (1964) *J. Am. Chem. Soc.* **86**, 4302–4314.
3. Pace, N. C. & Tanford, C. (1968) *Biochemistry* **7**, 198–208.
4. Privalov, P. L., Griko, Y. V., Venyaminov, S. Y. & Kutyshenko, V. P. (1986) *J. Mol. Biol.* **190**, 487–498.
5. Chen, B.-L. & Schellman, J. A. (1989) *Biochemistry* **28**, 685–691.
6. Griko, Y. V., Privalov, P. L., Sturtevant, J. M. & Venyaminov, S. Y. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3343–3347.
7. Franks, F., Hatley, R. H. M. & Friedman, H. L. (1988) *Biophys. Chem.* **31**, 307–315.
8. Privalov, P. L. (1990) *Crit. Rev. Biochem. Mol. Biol.* **25**, 281–305.
9. Hatley, R. H. M. & Franks, F. (1989) *FEBS Lett.* **257**, 171–173.
10. Hynes, T. R., Kautz, R. A., Goodman, M. A., Gill, J. F. & Fox, R. O. (1989) *Nature (London)* **339**, 73–76.
11. Fuchs, S., Cuatrecasas, P. & Anfinsen, C. B. (1967) *J. Biol. Chem.* **242**, 4768–4770.
12. Evans, P. A., Kautz, R. A., Fox, R. O. & Dobson, C. M. (1989) *Biochemistry* **28**, 362–370.
13. Shortle, D., Meeker, A. K. & Freire, E. (1988) *Biochemistry* **27**, 4761–4768.
14. Epstein, H. F., Schecter, A. N., Chen, R. F. & Anfinsen, C. B. (1971) *J. Mol. Biol.* **60**, 499–508.
15. Masson, P. & Laurentie, M. (1988) *Biochim. Biophys. Acta* **957**, 111–121.
16. Henderson, R. F., Henderson, T. R. & Woodfin, B. M. (1970) *J. Biol. Chem.* **245**, 3733–3737.
17. Gomez-Puyou, M. T., Gomez-Puyou, A. & Cerbon, J. (1978) *Arch. Biochem. Biophys.* **187**, 72–77.
18. Pace, C. N. & Laurents, D. V. (1989) *Biochemistry* **28**, 2520–2525.
19. Privalov, P. L., Tiktopulo, E. I., Venyaminov, S. Y., Griko, Y. V., Makhatadze, G. I. & Khechinashvili, N. N. (1989) *J. Mol. Biol.* **205**, 737–750.
20. Kuwajima, K. (1990) *Proteins* **6**, 87–103.
21. Calderon, R. O., Stolowich, N. J., Gerlt, J. A. & Sturtevant, J. M. (1985) *Biochemistry* **24**, 6044–6049.