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Mechanism of denaturation of cytochrome-c by lithium salts†

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Guanidine hydrochloride, LiCl and LiBr denaturations of cytochrome-c have been followed by observing changes in difference spectral intensities in the visible and UV regions and by circular dichroism. It has been observed that the former induces a highly cooperative and reversible transition between the native and denatured conformations. On the other hand, lithium salts induce a three-state transition in the protein. Characterization of the native state, X (intermediate) state and the end products by measurements of all optical properties and intrinsic viscosity suggests (i) that in each denaturant the end product is a random coil, and (ii) that the X state corresponds to a highly folded conformation in which heme seems to be completely exposed to the solvent. It has been observed that the second transition induced by the action of lithium salts corresponds to the unfolding of the polypeptide backbone. Measurements of the free energy change associated with all transitions suggest that protein polypeptide chain cannot exist in the folded conformation without the interaction between heme and globin.

If protein stability ($\Delta G_D^{H_2O}$) is defined as the gain in Gibbs energy during the transition between the structureless denatured (D) conformation and the native (N) conformation in water (or dilute buffer), it is then obvious that the evaluation of $\Delta G_D^{H_2O}$ is connected to the study of unfolding by a denaturant that induces a cooperative reversible transition between N and D conformations, for equilibrium between N and D states cannot be measured in water. This is one of the reasons why proteins were treated with different kinds of denaturants. A large number of data suggest that different denaturants may give rise to different denatured states of a protein[1-6]. A question arises: Why do proteins behave like that? We thought that this could be due to the presence of two or more independent stable substructures that a folded protein may contain. If this is so then treatments of cytochrome-c (cyt-c) which contains only one domain[7,8] with different denaturants should give only one denatured state. This is the first such study in which we report results of denaturation of cyt-c by inorganic salt denaturants. From these equilibrium results a possible mechanism of denaturation by lithium salts has been suggested.

Materials and Methods

Horse heart cyt-c, type VI, was purchased from Sigma Chemical Company. Its purity was checked by gel chromatography. Ultrapure GdnHCl was obtained from Schwarz/Mann, Biotech. All other chemicals purchased from Aldrich Chemical Company were analytical grade reagents.

Cyt-c was oxidized with 0.1% potassium ferricyanide, and dialysed extensively against several changes of 0.1 M KCl solution (pH 7.0) in the cold. Concentration of the stock solution of cyt-c was estimated spectrophotometrically using a value of $106 \times 10^3\ M^{-1}\ cm^{-1}$ for the molar extinction coefficient at 410 nm[9].

Stock solutions of denaturants were prepared in 0.03 M cacodylate buffer containing 0.1 M KCl. Concentrations of the stock solutions of GdnHCl[10] and LiCl[11] were determined by measuring the difference refractive index of each solution and 0.03 M cacodylate buffer (pH 7.0) containing 0.1 M KCl. Concentration of LiBr was determined by weighing dry powder.

Cyt-c solutions containing denaturants were prepared as follows: For unfolding experiments, known amounts of stock protein solution, buffer and denaturant solutions were mixed and incubated overnight which was sufficient for completion of the reaction. A similar procedure as used in unfolding experiments was employed in preparing the solutions for refolding experiments with the only exception that cyt-c was first denatured in concentrated denaturant solution and then diluted with the buffer. pH of each solution was measured with the help of Consort P-907 pH-meter.

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Difference spectroscopic measurements were performed in Shimadzu 2100 UV/VIS spectrophotometer. The temperature of both cells were maintained at 25 ± 0.05°C by circulating water from an external thermostated water-bath (Shimadzu TB-85). Viscosity measurements were made on an Ostwald type viscometer with a flow rate of 60 seconds for one ml of distilled water. Viscometer was kept in a thermostated water-bath. All measurements were performed at 25 ± 0.05°C. The timing was done manually with a stop watch which can be read to 0.1 sec. η_{red}, the reduced viscosity of the protein solution was calculated using Eq. (1)[12].

$$\eta_{red} = \frac{t - t_0}{t_0 \times c} + \frac{1 - \bar{v}_2 \rho_0}{\rho_0} \quad \ldots (1)$$

where t_0 and t are the times of fall of one ml solvent and one ml protein solution, respectively, c is the protein concentration in g/ml, $\bar{v}_2$ the partial specific volume of cyt-c and ρ_0 the density of the solvent. A value of 0.724 ml/g for $\bar{v}_2$ was used[13].

Circular dichroism measurements were made in Jasco 500-A spectrophotometer at 25 ± 0.01°C. Mean residue ellipticity at 222 nm ($[\theta]_{222}$) was calculated from the measured ellipticity θ at the same wavelength using Eq. (2):

$$[\theta]_{222} = \frac{\theta_{222} \cdot M_0}{10\, lc} \quad \ldots (2)$$

where M_0 is the mean residue weight of the protein, l the path length in cm, and c the protein concentration in mg/ml.

Analysis of the transition curves

The denaturation transition curves of cyt-c fall into two categories, and these were analyzed as follows: The transition curve induced by GdnHCl is assumed to follow a two-state mechanism, $\mathbf{N} \Leftrightarrow \mathbf{D}$. The state of equilibrium can be represented in terms of the optical property by the relation shown in Eq. (4)

$$K_{app} = \frac{y - y_N}{y_D - y} \quad \ldots (3)$$

where y_N and y_D represent values of y obtained, respectively, for the pure native and denatured states under identical conditions in which y has been measured. From K_{app} values ΔG_{app}, the Gibbs energies for the reaction can be calculated using Eq. (4).

$$\Delta G_{app} = -RT \ln K_{app} \quad \ldots (4)$$

where R is gas constant and T is the temperature in Kelvin.

The denaturation of cyt-c by lithium salts, followed by observing changes in the visible region involves two separable stages, namely, $\mathbf{N} \Leftrightarrow \mathbf{X}$ and $\mathbf{X} \Leftrightarrow \mathbf{D}$, where $\mathbf{X}$ is the intermediate state. It has been assumed that each stage follows a two-state mechanism. The equilibrium constants and free energy changes for the process $\mathbf{N} \Leftrightarrow \mathbf{X}$ are given by Eq. (5),

$$K_I = \frac{y - y_N}{y_X - y}$$

$$\Delta G_I = -RT \ln K_I, \quad \ldots (5)$$

and for the process $\mathbf{X} \Leftrightarrow \mathbf{D}$ by Eq. (6)

$$K_{II} = \frac{y - y_X}{y_D - y}$$

$$\Delta G_{II} = -RT \ln K_{II} \quad \ldots (6)$$

For each process, be it $\mathbf{N} \Leftrightarrow \mathbf{D}$, $\mathbf{N} \Leftrightarrow \mathbf{X}$ or $\mathbf{X} \Leftrightarrow \mathbf{D}$, ΔG values in the range $-1.36 \leq \Delta G$ (kcal mol^{-1}) ≤ 1.36 were plotted against the molar concentration of the denaturant. A least-squares analysis was used to fit the data using the relation shown by Eq. (7),

$$\Delta G = \Delta G^{H_2O} - m\,[\text{denaturant}] \quad \ldots (7)$$

where ΔG^{H_2O} is the value of ΔG at zero molar denaturant concentration, [denaturant], and m is the slope of the line, i.e., $(\partial \Delta G / \partial\, [\text{denaturant}])_{T,P}$.

Results and Discussion

Different chemical denaturants are known to give different denatured states of a protein at room temperature[1-6,14-17]. Only urea, GdnHCl and GdnHCNS give the maximally denatured state in which protein is devoid of all the elements of native conformation, whereas inorganic salts give rise to partial denatured states whose conformational properties lie between those of the native and maximally unfolded states. At present we are interested in understanding the question why do different denaturants give different denatured states of a protein? One of the proteins which we have chosen is cyt-c. During the course of this study we have observed some very interesting results on the denaturational behaviour of this protein which are presented and discussed below.

Fig. 1A shows the GdnHCl induced denaturation of cyt-c followed by observing changes in the

molar extinction coefficient at 403 nm, which is the maximum wavelength observed for the hemopeptides[18]. This spectral property measures the interaction of heme with the globin. It has been observed (i) that the denaturation is reversible, (ii) that the spectral property increases linearly with the denaturant concentration in the range 0-1.6 *M* (pretransition region), (iii) that sigmoidal change occurs in the range 1.6-3.2 *M* (transition region), and (iv) that $\Delta\varepsilon_{403}$ increases linearly in the range 3.2-6 *M* (posttransition region). A linear extrapolation of the results obtained in the posttransition region to zero [GdnHCl] using a least-squares analysis gave a value of $24130 \pm 256\ M^{-1}\ cm^{-1}$ for the denatured state in the buffer, which is in excellent agreement with those reported earlier[19,20].

GdnHCl induced denaturation of cyt-c was also followed by observing changes in $\Delta\varepsilon_{290}$, which measures local change in the environment of tryptophan and heme (see Fig. 1B). It has been observed that the transition is completely reversible and all the three regions coincided with those given in Fig. 1A. The linear extrapolation of the posttransition region data gave a value of $-13000\ M^{-1}\ cm^{-1}$ for $\Delta\varepsilon_{290}$. A value of $-1600\ M^{-1}\ cm^{-1}$ is, however, expected if one mole of tryptophan is transferred from protein interior to water[21]. As observed earlier[22], it appears that the major contribution to the property of the denatured protein comes from the heme moiety.

Fig. 1C shows a plot of the change in relative mean residue ellipticity ($[\theta]/[\theta]_N$) at 222 nm as a function of [GdnHCl]: This property measures the change in conformation, be it α-helix, β-structure, β-turn or "unordered" structure[23]. It has been observed that the transition shown in Fig. 1C is completely reversible. Pretransition, transition and posttransition regions are identical to those shown in Figs 1A and B. A linear extrapolation of the posttransition results to 0 *M* GdnHCl gave a value of -2900 deg cm^2 dmol^{-1} for the denatured protein in the absence of the denaturant; for the native protein a value of -12200 deg cm^2 dmol^{-1} for $[\theta]_{222}$ was observed which is in excellent agreement with that reported by Yang *et al.*[24]. It has been observed that the entire far-UV spectrum of the denatured cyt-c is well within the range of spectra of the randomly coiled polypeptides in concentrated solutions of GdnHCl[25].

The product of GdnHCl denaturation was also characterized by $[\eta]$ and intrinsic viscosity measurements. Values of η_{red} measured at different protein concentrations were analyzed according to Eq 1. We have observed that the value of $[\eta]$

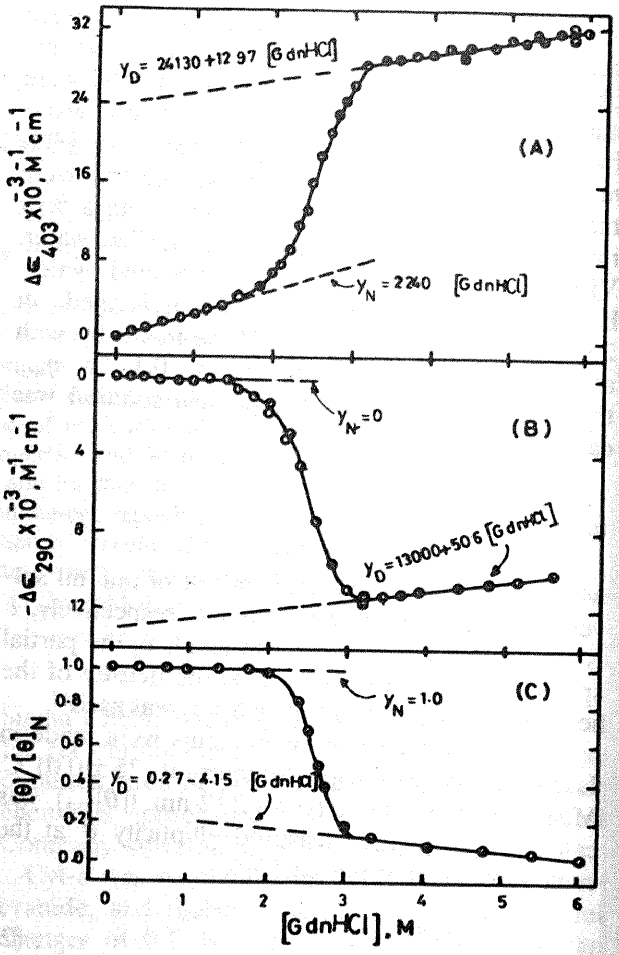

Fig. 1—GdnHCl denaturation of cyt-c at 25 ± 0.05°C and *p*H 7.0, followed by observing changes in $\Delta\varepsilon_{403}$ (A), $\Delta\varepsilon_{290}$ (B) and $[\theta]/[\theta]_N$ (C) as a function of denaturant concentration.

increased from 2.67 ml/g, obtained for the native protein, to a value of 14.61 ± 0.19 ml/g for the protein in 3.5 *M* GdnHCl. This is in excellent agreement with the values reported in concentrated solutions of GdnHCl and urea[19,13,26]. Furthermore, this value of $[\eta]$ is also in excellent agreement with the values calculated from the empirical equations given earlier; Eq. 18 in Tanford[14] gave a value of 14.3 ml/g and Eq. 2 in Ahmad and McPhie[27] yielded a value of 14.51 ml/g. Privalov *et al.*[28] have recently shown that apocytochrome-c in its linearly random coiled state gives a value of 15 ml/g for $[\eta]$. Thus, $[\eta]$ measurements provide strong evidence that the denatured cyt-c having covalently linked heme behaves as a linear random coil.

Isothermal denaturation of cyt-c by LiCl was followed by observing changes in difference spectral intensity in both visible and UV regions and by measuring changes in relative mean residue ellipticity (see Fig. 2). Difference molar extinction

coefficient values at 399 nm at which peak occurs were plotted as a function of LiCl concentration. It can be seen in Fig. 2A that the difference molar extinction coefficient increases linearly with an increase in LiCl concentration from 0 to 4.0 *M*. This linear increase is described by the relation $y_N = 1322\ (\pm 61)$ [LiCl]. As shown below (Figs 2B and 2C), no change in the conformation of the protein occurs in the range 0-4.0 *M*. This region is taken as pretransition region. Above 4.0 *M* a sigmoidal increase occurs upto 6 *M* where the value of the optical property is 24000 M^{-1} cm^{-1}, a value observed for GdnHCl denatured protein in the buffer (see Fig. 1A). It is interesting to note that $\Delta\varepsilon$ decreases with an increase in the denaturant concentration above 6 *M*. This change in the range between 6-7.84 *M* is sigmoidal. It seems that this represents another change in the conformation of the protein. $\Delta\varepsilon$ above 7.84 *M* was found to be linear and can be described by a straight line $y_D = 24311\ (\pm 682) - 1076\ (\pm 76)$ [LiCl]. Here again the value of y_D is 24311 M^{-1} cm^{-1} in the absence of denaturant (see Fig. 2A). Results shown in Fig. 2A clearly suggest that LiCl induced denaturation of cyt-c involves at least two stages namely **N** ⇔ **X**, where **X** is the intermediate state and **X** ⇔ **D**.

Fig. 2B shows the denaturation transition curve followed by observing changes in $\Delta\varepsilon_{290}$ as a function of LiCl concentration. In the region 0-4.0 *M* LiCl concentration there is no change in the difference spectral intensity. This suggests that there is no change in the environment of heme and tryptophan. Change in the environment of heme and tryptophan occurs above 4.0 *M* and is complete only above 7.84 *M* LiCl concentration. The linear extrapolation of all the results above 7.84 *M* gave a value of $-13000\ M^{-1}\ cm^{-1}$ for y_D which is identical to what has been found for GdnHCl denatured cyt-c. Contrary to the results shown in Fig. 2A no separable intermediate occurred.

When the denaturation was followed by observing changes in the relative mean residue ellipticity, it was observed that the CD property does not change in the range 0-6 *M* [LiCl]. Melting of secondary structure can be seen when LiCl was added at concentrations above 6 *M*. This change in peptide conformation is, however, complete at 7.84 *M*. CD measurements strongly suggest that no change in the secondary structure of cyt-c occurs during **N** ⇔ **X** transition, and **X** ⇔ **D** transition represents the unfolding of the polypeptide backbone.

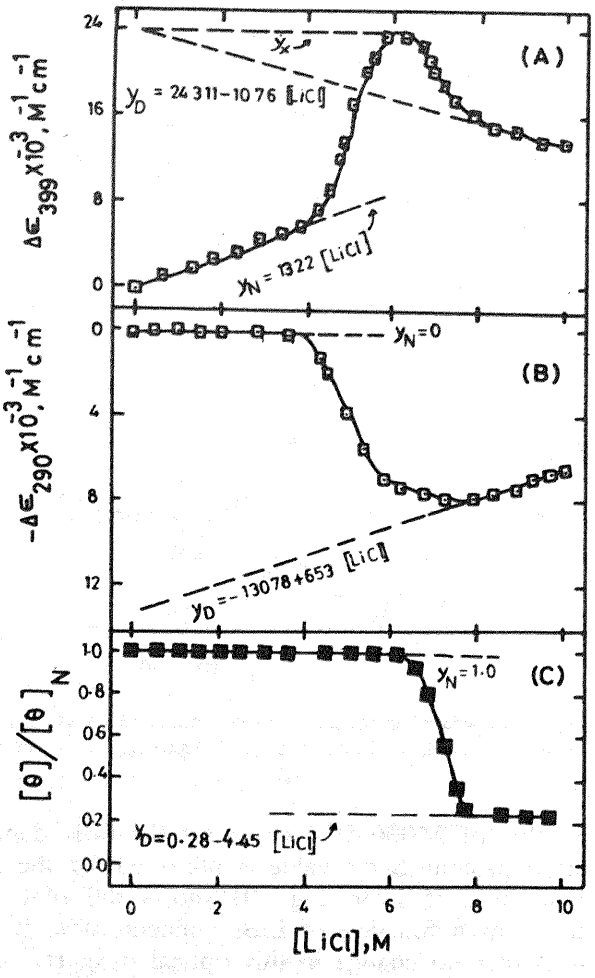

Fig. 2—Plots of changes in $\Delta\varepsilon_{399}$ (A), $\Delta\varepsilon_{290}$ (B) and relative mean residue ellipticity (C) as a function of LiCl concentration at *p*H 7.0 (0.03 *M* cacodylate buffer containing 0.1 *M* KCl) and 25 ± 0.05°C.

We have measured η_{red} of cyt-c in 6 *M* LiCl at which cyt-c is in the **X** state and 8 *M* LiCl at which protein is in the **D** state, as a function of protein concentration. Analysis of both sets of data using Eq. 1, gave values of 3.49 and 14.53 ml/g for the value of $[\eta]$ of the **X** and **D** states, respectively.

We decided to see whether this denaturational behaviour of cyt-c in LiCl is shared by another lithium salt namely, LiBr. It has been observed that the denaturational behaviour of cyt-c in both LiCl and LiBr is same. Fig. 3 shows denaturation transition curve followed by observing changes in $\Delta\varepsilon_{399}$ as a function of [LiBr]. As can be seen in this figure, the pretransition occurs in the range 0-2.5 *M*. The first transition **N** ⇔ **X** occurs in the range 2.5-4 *M*. The second transition occurs in the concentration range 4-6.5 *M* above which the optical property decreases linearly. The linear extrapolation of all the results above 6.5 *M* gave

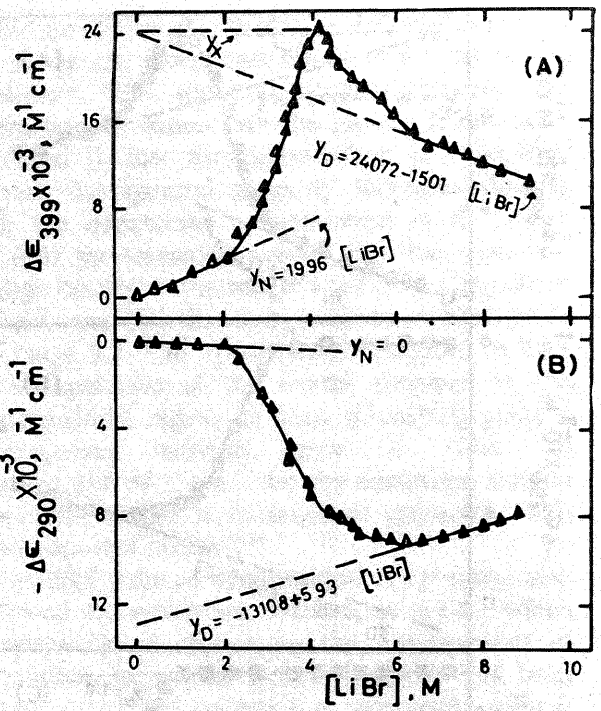

Fig 3 —Denaturation transition curves induced by LiBr at *p*H 7.0 and 25 ± 0.05°C: Plots of $\Delta\varepsilon_{399}$ (A) and $\Delta\varepsilon_{290}$ (B) versus [LiBr].

a value of 24000 M^{-1} cm^{-1} for the LiBr denatured protein. Same value is observed for the **X** state in 4 *M* LiBr. Fig. 3B shows the plot of $\Delta\varepsilon_{290}$ as a function of LiBr concentration. It is seen that no change in this optical property occurs in the pretransition region (0-2.5 *M*). The optical property decreases in a sigmoidal fashion when concentration is increased from 2.5 to 6.5 *M* above which a linear increase occurs. The linear extrapolation of all the results above 6.5 *M* LiBr gave a value of -13000 M^{-1} cm^{-1} for the LiBr denatured protein.

Intrinsic viscosity measurements were carried out for the protein in 4 *M* at which **X** state exists and in 6.5 *M* at which **D** state exists. It has been observed that the intrinsic viscosity of **X** state was 3.96 ml/g which is the same as observed for the **X** state obtained in LiCl. Intrinsic viscosity of the LiBr denatured protein was 14.84 ml/g which is same as observed for LiCl and GdnHCl.

Both LiCl and LiBr induce a biphasic transition in cyt-c. Characterization of intermediates and end products by difference spectral, far-UV CD and intrinsic viscosity measurements suggests that (i) heme is exposed to the solvent in the intermediate state **X** which retains all the secondary structure of polypeptide backbone, (ii) viscosity of the **X** state is in the range 3.49-3.96 ml/g which is very close to the value of $[\eta]$ observed for the native protein, and (iii) that the final product **D** of denaturation by these salts is as unfolded as that observed in concentrated solution of GdnHCl. On the basis of difference spectral and CD measurements it may be concluded that the unfolding of cyt-c in lithium salts occurs in two distinct reversible steps. During the first step heme is exposed to the solvent completely without any change in polypeptide backbone conformation, and the second step, $\mathbf{X} \Leftrightarrow \mathbf{D}$ represents the actual unfolding of the polypeptide backbone.

Mayer *et al.*[29] have described the folding of cyt-c at neutral *p*H by a mechanism involving two intermediates $\mathbf{X}_1$ and $\mathbf{X}_2$ on the path between **N** and **D** states. They have presented convincing data to support their argument that transitions, $\mathbf{X}_1 \Leftrightarrow \mathbf{X}_2$ do not involve any change in the polypeptide conformation of the protein and the lone tryptophan at position-59 is exposed to solvent but the state of heme-tryptophan, i.e., the deepest part of the crevice is maintained during the transition. The presence of intermediates during the denaturation of cyt-c has been shown by Ikai *et al.*[19] and Drew and Dickerson[30]. Ikai *et al.*[19] have postulated that one of the intermediates found during the kinetics of denaturation of cyt-c by GdnHCl is highly folded and its spectral property is very close to the one obtained for the unfolded protein polypeptide in concentrated GdnHCl solution. Although we do not see more than one separable intermediate in cyt-c denaturation, our equilibrium results are consistent with the overall denaturation scheme derived from thermodynamic and kinetic studies of GdnHCl, urea and alcohol[19,29,30].

Ohgushi and Wada[31] found an ordered structure in cyt-c which situates in between the native and denatured forms and is as compact as the native protein. The molecular characteristics of this state, inferred from the intrinsic viscosity, radius of the protein molecule, and ^{1}H NMR spectra are that the main change in the intermediate state folds as compactly as in the native state and intramolecular fluctuations of atoms is distinctly large compared to that in the native state. The "**A**" state (intermediate state obtained by lowering *p*H at room temperature) of cyt-c has comparable amount of ordered secondary structure to the native state[32]. It seems that the properties of the "**A**" state observed here are similar to that of the **X** state observed here.

All the measurements on the characterization of the final product of denaturation of cyt-c by all denaturants seem to support the idea that the number of denatured states that a protein can

give depends upon the folding pattern of the native protein. The reason for saying this is that since cyt-c contains only one domain as revealed by X-ray diffraction studies[7,8] all denaturants gave the same denatured state.

For each mode of denaturation, normalization of transition curves suggested that all data points obtained from the transition curves, followed by observing changes in different optical properties fell on the same curve. The coincidence among the results obtained by two or more different conformational properties suggest that the denaturation of cyt-c by GdnHCl and lithium salts is highly cooperative. It should be noted that this coincidence neither proves nor disproves the existence of intermediate states on the path between N and D[16]. We have however, assumed that all transitions $\mathbf{N} \Leftrightarrow \mathbf{X}$, $\mathbf{X} \Leftrightarrow \mathbf{D}$ and $\mathbf{N} \Leftrightarrow \mathbf{D}$ follow a two-state mechanism.

In order to assess the contribution of heme to the folded structure of cyt-c, denaturation transitions, shown in Figs 1-3, were analyzed for thermodynamic parameters using appropriate equations given under the section of Materials and Methods. Analysis of the GdnHCl induced transition curve followed by all three optical properties gave a value of 6.9 ± 0.1 kcal mol^{-1} which is in excellent agreement with those reported earlier[33,34]. Analysis of the lithium salts induced transition curves for the process $\mathbf{N} \Leftrightarrow \mathbf{X}$ gave a value of 8.2 ± 0.5 kcal mol^{-1} for $\Delta G_{\mathrm{I}}^{\mathrm{H_2O}}$ which measures the strength of interaction between folded polypeptide chain and the heme. The GdnHCl and lithium salts results seem to suggest that the entire stability of the holoprotein comes from the heme and globin interaction. Removal of heme should therefore lead to the unfolding of protein. This conclusion is concomitant with the finding of Fisher *et al.*[35] and Stellwagen *et al.*[36] who reported that the apocyt-c exists in an unfolded conformation in water. Analysis of the transition curve for the reaction $\mathbf{X} \Leftrightarrow \mathbf{D}$ gave a value of 1.6 ± 0.5 kcal mol^{-1}. This again suggests that the apocytochrome-c alone cannot exist in the folded confirmation.

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References

1 Bigelow C C, *J molec Biol*, 8 (1964) 696.
2 Kugimiya M & Bigelow C C, *Can J Biochem*, 51 (1973) 581.
3 Sharma R N & Bigelow C C, *J molec Biol*, 88 (1974) 247.
4 Sarfare P S & Bigelow C C, *Can J Biochem*, 45 (1967) 651.
5 Ahmad F & Bigelow C C, *J molec Biol*, 131 (1979) 607.
6 Ahmad F & Khan L A, *Indian J Biochem Biophys*, 26 (1989) 301.
7 Dickerson R E, Takano T, Eisenberg D, Kallai O B, Samson L, Cooper A & Margoliash E, *J biol Chem*, 246 (1971) 1511.
8 Takano T, Kallai O B, Swanson R & Dickerson R E, *J biol Chem*, 248 (1973) 5234.
9 Margoliash E & Frohwirt N, *Biochem J*, 71 (1959) 570.
10 Nozaki Y, *Methods Enzymol*, 26 (1972) 43.
11 *CRC handbook of chemistry and physics*, edited by R C Weast (CRC Press, Inc. Cleveland) 1972.
12 Ahmad F & Salahuddin A, *Biochemistry*, 13 (1974) 245.
13 Stellwagen E, *Biochemistry*, 7 (1968) 2893.
14 Tanford C, *Advan Protein Chem*, 23 (1968) 121.
15 Lapanje S, *Physicochemical aspects of protein denaturation* (John Wiley, New York) 1978, 651.
16 Dill K A & Shortle D, *Annu Rev Biochem*, 60 (1991) 795.
17 Ahmad F, *J biol Chem*, 259 (1984) 4183.
18 Harbury H A & Loach P A, *Proc Natl Acad Sci, USA*, 45 (1959) 1344.
19 Ikai A, Fish W W & Tanford C, *J molec Biol*, 73 (1973) 165.
20 Henkens R W & Turner S R, *Biochemistry*, 12 (1973) 1618.
21 Ghelis C & Yon J, in *Protein folding* (Academic Press, New York) 1982.
22 Nail B T & Landers T A, *Biochemistry*, 20 (1981) 5403.
23 Ahmad F, *Indian J Biochem Biophys*, 28 (1991) 168.
24 Yang J T, Wu C S C & Martinez H M, *Methods Enzymol*, 130 (1986) 208.
25 Cortijo M, Panijpan B & Gratzer W B, *Intern J Pept Pro Res*, 5 (1973) 179.
26 Tsong T Y, *Biochemistry*, 14 (1975) 1542.
27 Ahmad F & McPhie P, *Intern J Biochem*, 9 (1978) 647.
28 Privalov P L, Tikatopula E I, Vinyamino S Yu, Girko Yu V, Makhatudze G I & Khechinashvilli N N, *J molec Biol*, 205 (1989) 737.
29 Mayer Y P, MacDonald L H, Verma B C & Pande A, *Biochemistry*, 19 (1980) 199.
30 Drew H R & Dickerson R E, *J biol Chem*, 253 (1978) 8420.
31 Ohgushi M & Wada A, *FEBS Lett*, 164 (1983) 21.
32 Goto Y, Calciano L J & Fink A L, *Proc Natl Acad Sci, USA*, 87 (1990) 573.
33 Knapp J A & Pace C N, *Biochemistry*, 13 (1974) 1289.
34 McLendon G & Smith M, *J biol Chem*, 253 (1978) 4004.
35 Fisher W R, Taniuchi H & Anfinsen C B, *J biol Chem*, 248 (1973) 3188.
36 Stellwagen E, Rysavy R & Babul G, *J biol Chem*, 247 (1972) 8074.