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Effects of salts of alkali earth metals and calcium chloride on the stability of cytochrome *c* and myoglobin

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Abstract

This study suggests a procedure by which binding of denaturants could be detected without any additional information other than that provided in the denaturation profiles of proteins. Two predominantly α-proteins, namely ferricytochrome *c* and metmyoglobin were denatured by guanidine hydrochloride (GdnHCl) in the presence of low fixed concentrations of salts at 25°C and transition between native and denatured states was followed by absorbance measurements in the visible region (500–350 nm). The raw data were converted into transition curves from which C_m, the midpoint of GdnHCl-induced transition, and ΔG_{app}, the free energy changes on denaturation, were calculated assuming a two-state mechanism, and values of ΔG_{app} at zero concentration of the denaturant were estimated. It has been observed (1) that chlorides of Na, K, Cs, and Rb do not affect the native conformation of proteins, (2) that GdnHCl-induced denaturations of proteins in presence and absence of sodium bromide, sodium perchlorate and salts of lithium and calcium are reversible, (3) that optical properties of the GdnHCl-denatured state of proteins remain unchanged in presence of the second denaturant, (4) C_m decreases with an increase in the denaturant concentration, and (5) that except for GdnHCl there exist one or more binding sites on the native proteins for the denaturants.

Keywords: Myoglobin; Cytochrome *c*; Denaturation; Protein stability; Ionic denaturant

1. Introduction

In order to express their biological functions in water (or dilute buffer) many proteins exist in folded conformation. If we define stability of this conformation as the gain in Gibbs energy ($\Delta G_D^{H_2O}$) associated with the transition between N, the native conformation, and D, the denatured conformation, it is then evident that both N and D states are equally important in determining the protein stability. However, the D state of a protein can only be studied and measured in the presence of one or more denaturants. It is well known that different denaturants may give rise to different denatured states, i.e., the states with different amounts of secondary and tertiary structures [1–6]. Indeed, it has been shown that many partially denatured proteins undergo extraoptical transition upon the addition of a stronger denaturant [7–9].

Many attempts have been made to make a quantitative comparison of the effectiveness of various chemical denaturants. In the earlier attempt thermal denaturation of proteins were studied in their presence, and the concentration of the denaturant in question required to bring about fifty percent change in the optical transition is used as a measure of its effectiveness in unfolding the protein [10]. The problem with this approach is that the nature of the N ⇆ D transition is not known, for no attempt has been made to characterize the product of heat denaturation in the presence of high concentrations of the ionic denaturants. Pace and Marshall [11] performed another procedure in which protein was first exposed to a urea concentration just enough to start urea denaturation followed by measuring the conformational transition curve induced by the other denaturant. The value of C_m, the molar concentration of the denaturant in question at which 50% change in the optical transition between N and D states has occurred, is taken as a measure of the effectiveness of the denaturant. The conclusion arrived from this procedure may be questionable, for the dependence of the optical property of the

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native protein on the urea–salt mixture which is needed for the accurate estimation of C_m, is not known. In order to overcome the problems associated with these two approaches, it has been suggested to study guanidine hydrochloride (GdnHCl) denaturation of proteins in presence of low concentrations of other ionic denaturants [12]. This approach has been successfully used by Thomson and Bigelow [13], to rank the order of effectiveness of various salts in denaturing ribonuclease A. The main conclusion of their studies is that, be it C_m or $\Delta G_D^{H_2O}$ the observed value of each of these parameters is the algebraic sum of the values of the constituent ions of the salt.

All earlier studies [10–13] have used $\alpha + \beta$ proteins. In order to see whether the effect of various ionic denaturants

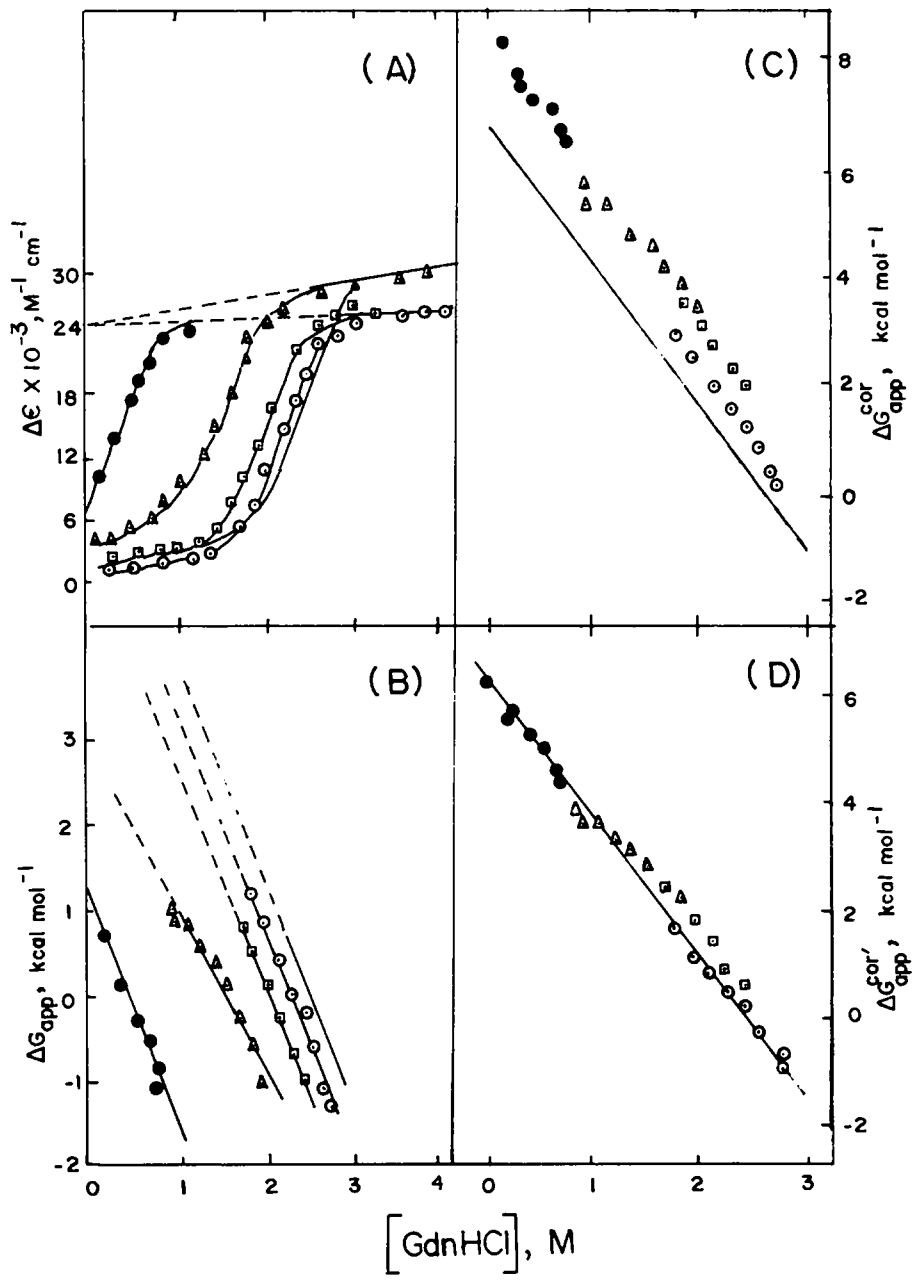

Fig. 1. GdnHCl-induced denaturation of cyt-*c* in presence of different concentrations of LiCl at pH 7.0 and 25°C. (A) —, 0 M; ○, 0.9 M; □, 1.75 M; △, 2.58 M; and ●, 4.35 M. In order to maintain clarity experimental points for the denaturation of protein in absence of LiCl (i.e. the control experiment) are not shown. (B) Values of ΔG_{app} were calculated from the results given in (A) and plotted as function of [g]. Symbols have the same meaning as in (A). Solid lines were drawn according to Eq. (2) with the fitting parameters given in Table 2. (C) The solid line represents the control experiment, and it was drawn according to Eq. (2) using values of 6.85 kcal mol^{-1} for $\Delta G_{app}^{H_2O}$ and 2.70 kcal mol^{-1} M^{-1} for m_g (see Table 2). Symbols represent results shown in (B) after their correction according to Eq. (4) using m value given in Table 4. (D) Assuming that there exists one binding site on the native protein ΔG_{app}^{cor} values, shown in (C), were further corrected, using the value of k_s given in Table 4. These $\Delta G_{app}^{cor'}$ values were plotted as a function of [g]. The solid line is the same as in (C).

on proteins has some secondary structure specificity, we have been studying the effect of various denaturants on different classes of proteins. In this communication we report the effect of salts of alkali earth metals and calcium chloride on the GdnHCl-induced denaturation of predominantly all α-proteins, namely cytochrome c and myoglobin. The thermodynamic analysis of equilibrium data suggests that proteins have strong binding site(s) for the denaturing agents, LiCl, LiBr, $LiClO_4$, NaBr, $NaClO_4$ and $CaCl_2$.

2. Materials and methods

Cytochrome c and myoglobin from horse heart, obtained from Sigma were used throughout this work. Purity of each protein was checked on Sephadex G-75 and on polyacrylamide gel electrophoresis. Ultrapure GdnHCl was obtained from Schwarz/Mann Biotech. All other chemicals purchased from Aldrich Chemical Company were analytical grade reagents.

Both proteins were oxidized with 0.1% potassium ferricyanide, as described earlier [6,14]. These preparations of ferricytochrome c and metmyoglobin are abbreviated as cyt-c and Mb respectively. The concentration of the dialysed solution of cyt-c was determined using a value of $106.1 \cdot 10^3$ M^{-1} cm^{-1} for ϵ_{410}, the molar absorbance coefficient at 410 nm [15]. The concentration of stock solution of Mb was determined using a value of $171 \cdot 10^3$ M^{-1} cm^{-1} for ϵ_{410} [16]. Each stock denaturant solution was prepared in 0.03 M cacodylic acid buffer which always contained 0.1 M KCl except in cases of stock solutions of salts of perchlorate which contained 0.1 M NaCl instead. The pH values of cacodylate buffer were 6.0 and 7.0 during denaturation studies on Mb and cyt-c, respectively. Concentrations of stock solutions of LiBr, $LiClO_4$, $NaClO_4$ and RbCl for which refractive indices were not available, represent the amount of dry samples that were carefully weighed. Concentrations of the stock solutions of GdnHCl [17] and other salts [18] were determined by measuring the difference between refractive index of each solution and that of 0.03 M cacodylate buffer containing 0.1 M NaCl/KCl. Spectral measurements were made in Shimadzu 2100 UV/Vis spectrophotometer using well matched 1 ml quartz cuvettes of 1 cm path length. The temperature of the solutions were maintained at 25 ± 0.05°C by circulating water through jacketed cell holders from an external thermostated waterbath (Shimadzu TB-85). A difference spectrum was obtained by subtracting the spectrum of the native protein from that of the protein exposed to denaturants.

The values of Gibbs energy change (ΔG_{app}) were estimated from the optical transition curves assuming a two-state process. It has been observed that all the denaturation transition curves of cyt-c and Mb were sigmoidal that can be divided into three regions, i.e., GdnHCl concentration ranges, (i) pretransition region in which protein exists in the N state, (ii) the transition region in which there exists an equilibrium between N and D states, and (iii) the post-transition region in which protein exists in the D state. It has also been observed that denaturation of both proteins was reversible, i.e., spectrum of the native protein and that of the renatured protein are indistinguishable. Eq. (1) describes the relation between ΔG_{app} and the optical property, y,

$$\Delta G_{app} = -RT \ln \frac{y - y_N}{y_D - y} \quad (1)$$

where y_N and y_D are the optical properties of the native and denatured states of the protein under identical conditions in which y has been measured.

ΔG_{app} values obtained from each transition curve were plotted against [g], the molar concentration of GdnHCl. All ΔG_{app} versus [g] plots were found to be linear and analysed using a least-squares method according to Eq. (2)

$$\Delta G_{app} = \Delta G_{app}^{H_2O} - m_g [g] \quad (2)$$

where $\Delta G_{app}^{H_2O}$ is the value of ΔG_{app} at [g] = 0 M, and m_g is the slope of the straight line. The midpoint of transition curve, C_m was estimated from $C_m = \Delta G_{app}^{H_2O}/m_g$.

Table 1
Fitting parameters for y_N, the optical property in the pretransition region of the GdnHCl-induced denaturation of cyt-c at pH 7.0 and 25°C

Salt	b_N (M^{-1} cm^{-1} $[g]^{-1}$)	c_N (M^{-1} cm^{-1} $[s]^{-1}$)	Salt	b_N (M^{-1} cm^{-1} $[g]^{-1}$)
LiCl	1380 ± 170	1320 ± 40	control [b]	2240 ± 90
LiBr	2240 ± 90	2000 ± 160	NaCl	1720 ± 110
$LiClO_4$	2240 ± 91	8573 ± 1500	KCl	1720 ± 110
$NaClO_4$	3930 ± 210	3470 ± 120	RbCl	1990 ± 130
NaBr	2280 ± 110	2000 ± 1100	CsCl	1990 ± 130
$CaCl_2$	1860 ± 150	1570 ± 90		

[a] Results in the presence of all salts given in the extreme left column were described by the equation: $y_N = b_N[g] + c_N[s]$, whereas results of the control experiment as well as in presence of NaCl, KCl, RbCl and CsCl were described by the relation $y_N = b_N[g]$.
[b] 0.03 M cacodylic acid/0.1 M KCl.

3. Results

In order to rank the ability of different salts to stabilize/destabilize the native conformation of predominantly α-proteins we have studied the GdnHCl-induced denaturation in presence of various concentrations of second salts near neutral pH at 25°C. Results of such studies on cyt-*c* and Mb are presented below.

The GdnHCl-induced denaturation of cyt-*c* in presence of different concentrations of the second salt was followed by observing changes in the $\Delta\epsilon$ at 25°C; the maximum change in $\Delta\epsilon$ showed dependence on GdnHCl–salt mixture and was observed in the range 410 to 399 nm. The following observations were made: (i) In the cases of LiCl (see Fig. 1A), LiBr, $LiClO_4$, $NaClO_4$, NaBr and $CaCl_2$, y_N showed dependence on both [g] and [s], the molar

Table 2
Optical and thermodynamic parameters of cyt-*c* denaturation by GdnHCl in presence of various concentrations of salts at pH 7.0 and 25°C

[s]	a_D ($M^{-1}\,cm^{-1}$)	b_D ($M^{-1}\,cm^{-1}\,[g]^{-1}$)	$\Delta G^{H_2O}_{app}$ (cal mol^{-1})	$m_g^{[s]}$ (cal $mol^{-1}\,M^{-1}$)	$C_m^{[s]}$ (M)
Control					
None	24 130 ± 260	1297 ± 57	6850 ± 120	2700 ± 50	2.54
LiCl					
0.90	24 052 ± 192	290 ± 51	6140 ± 230	2670 ± 100	2.30
1.75	23 996 ± 435	343 ± 125	5540 ± 100	2710 ± 50	2.04
2.58	23 607 ± 247	1361 ± 76	3020 ± 170	1970 ± 120	1.53
4.35	24 153 ± 159	146 ± 135	1100 ± 90	2720 ± 170	0.40
LiBr					
1.00	23 309 ± 504	462 ± 171	6640 ± 340	3630 ± 180	1.63
1.50	24 540 ± 333	54 ± 100	5840 ± 280	3900 ± 190	1.50
2.00	23 484 ± 313	387 ± 125	4250 ± 120	3580 ± 100	1.19
2.80	23 754 ± 52	356 ± 28	1720 ± 120	3150 ± 180	0.55
$LiClO_4$					
0.20	24 114 ± 185	284 ± 53	5430 ± 120	2500 ± 50	2.17
0.40	24 226 ± 357	907 ± 109	2730 ± 140	1780 ± 90	1.53
0.80	23 783 ± 90	1072 ± 32	1160 ± 60	1630 ± 60	0.71
$NaClO_4$					
0.48	24 393 ± 276	156 ± 89	2960 ± 130	1730 ± 80	1.71
0.76	24 516 ± 231	88 ± 85	2360 ± 90	1670 ± 70	1.26
1.90	23 962 ± 224	500 ± 156	920 ± 30	3140 ± 90	0.29
NaBr					
1.40	23 809 ± 197	906 ± 63	6100 ± 490	3210 ± 260	1.90
2.10	24 072 ± 457	12 ± 145	3540 ± 440	2120 ± 240	1.67
3.15	23 809 ± 197	906 ± 63	2930 ± 230	2280 ± 150	1.29
$CaCl_2$ [a]					
0.50	23 642 ± 230	673 ± 70	6360 ± 130	3100 ± 60	2.05
1.00	23 965 ± 71	17 ± 24	5570 ± 150	3400 ± 90	1.64
1.40	23 921 ± 133	47 ± 52	3230 ± 250	2620 ± 210	1.23
2.25	24 418 ± 176	227 ± 154	680 ± 230	4110 ± 880	0.17
NaCl					
0.49	23 428 ± 420	358 ± 111	7650 ± 310	3000 ± 120	2.55
0.97	24 641 ± 539	560 ± 143	7760 ± 210	3060 ± 80	2.54
KCl					
0.49	23 729 ± 251	462 ± 68	6800 ± 320	2680 ± 120	2.54
0.86	23 139 ± 836	904 ± 234	7570 ± 460	2940 ± 190	2.58
RbCl					
0.60	24 461 ± 1157	411 ± 317	6990 ± 660	2710 ± 270	2.58
1.20	23 511 ± 2787	693 ± 792	7210 ± 310	2760 ± 120	2.61
CsCl					
0.70	23 427 ± 524	483 ± 141	7330 ± 490	2920 ± 200	2.51
1.40	23 519 ± 452	446 ± 122	6490 ± 380	2550 ± 150	2.55

[a] Taken from Ref. [6].

concentrations of GdnHCl and the second salt respectively. A least-squares analysis of results in the pretransition region suggested that the dependence of y_N on the composition variables can be described by the function $y_N = b_N[g] + c_N[s]$, where b_N and c_N are fitting parameters. The values of these parameters are given in Table 1. We have used them to determine y_N in the transition region. (ii) The results in the pretransition region of the denaturation of the protein by GdnHCl in the presence of different concentrations of NaCl, KCl, RbCl and CsCl suggested that y_N depends only on the GdnHCl concentration and that the function $y_N = b_N[g]$ describes this dependence where b_N is the fitting parameter whose values are given in Table 1. (iii) The optical property measured in the posttransition region, showed dependence on GdnHCl concentration. A least-squares analysis of these results yielded the function $y_D = a_D + b_D[g]$, where a_D and b_D are fitting parameters. Values of these parameters at different concentrations of each salt are given in Table 2, and they were used to estimate the value of y_D in the transition region. It is interesting to note that in all cases (e.g., see Fig. 1A) the value of a_D is, within the error of experiment, the same, and is in excellent agreement with those reported earlier [6,19–21]. It is seen in Table 2 that in some cases b_D showed dependence on the second salt concentration (e.g., see Fig. 1A). (iv) All denaturation curves were sigmoidal and reversible. We have therefore assumed a two-state mechanism for the denaturation in the estimation of ΔG_{app} from the equilibrium curves. (v) The ΔG_{app} versus [g] plots in the presence of different concentrations of all salts were found to be linear (e.g., see Fig. 1B), and they were therefore analyzed using Eq. (2). Table 2 shows the values of $\Delta G_{app}^{H_2O}$ and $m_g^{[s]}$, where the superscript '[s]' represents the fact that measurements were made in the presence of a second salt at a concentration [s].

The GdnHCl-induced denaturation of Mb in presence of various concentrations of the second salt was followed by observing changes in ϵ_{409}, and the following observations

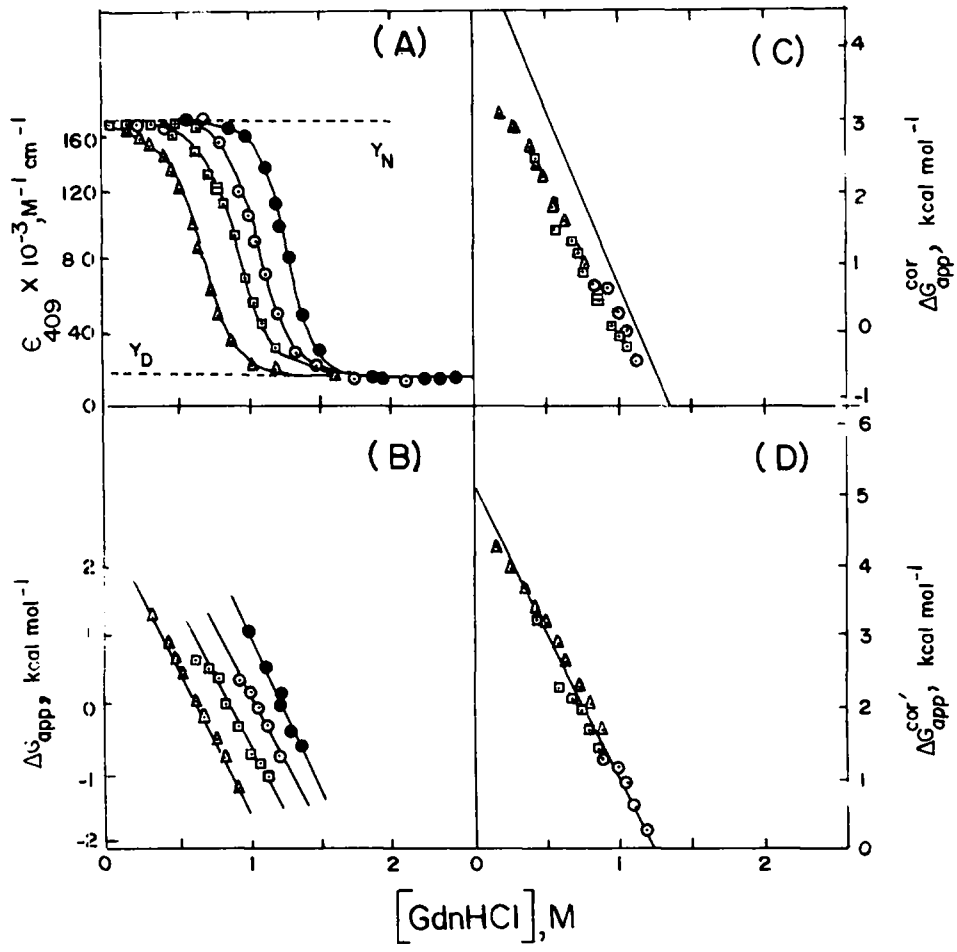

Fig. 2. Isothermal denaturation of Mb by GdnHCl at pH 6.0 and 25°C. (A) Denaturation profiles in presence of 0 M (●), 0.20 M (○), 0.40 M (□), and 0.80 M (△) LiBr. (B): Dependence of ΔG_{app}, calculated from the results shown in (A) on GdnHCl concentration. Lines were drawn using values of $\Delta G_{app}^{H_2O}$ and $m_g^{[s]}$ given in Table 3. (C) The solid line represents the results of the control experiment and drawn using $\Delta G_{app}^{H_2O}$ = 5.23 kcal mol^{-1} and m_g = 5.00 kcal mol^{-1} M^{-1} (see Table 3). ΔG_{app} values given in (B) were corrected according to Eq. (4) using values of m given in Table 4. (D) The solid line is the same as in (C). Each symbol represents results shown in (C) after the correction using Eq. (5) that assumes that there exists one binding site on the native protein. The value of k_s for LiBr is given in Table 4.

were made: (i) It was observed that y_N is independent of both [g] and [s]. A value of 171 000 M^{-1} cm^{-1} for y_N was used in all analysis. (ii) y_D, the optical property in the posttransition region was also found to be independent of the [g] and [s]; a value of 16 576 ± 408 M^{-1} cm^{-1} for y_D has been obtained. (iii) All denaturation curves were found to be sigmoidal and reversible. A two-state mechanism was assumed for the denaturation processes, and accordingly ΔG_{app} values were estimated from the transition curve using Eq. (1). (iv) All plots of ΔG_{app} versus [g] plots in presence of the second salt were found to be linear (see Fig. 1B) and were analyzed according to Eq. (2). All the thermodynamic parameters thus obtained are given in Table 3.

Table 3
Fitting parameters of Eq. (2) used to analyse the GdnHCl-induced denaturation of Mb in presence of various concentrations of salts at pH 6.0 and 25°C

[s]	$\Delta G_{app}^{H_2O,[s]}$ (cal mol^{-1})	$m_g^{[s]}$ (cal mol^{-1} M^{-1})	$C_m^{[s]}$ (M)
Control [a]			
None	5230 ± 110	5000 ± 120	1.04
LiCl			
0.58	2980 ± 60	3020 ± 60	0.98
1.16	2610 ± 70	3010 ± 70	0.86
LiBr			
0.20	3700 ± 270	3630 ± 260	1.01
0.40	3210 ± 50	3630 ± 60	0.88
0.80	2260 ± 40	3690 ± 80	0.61
$LiClO_4$			
0.17	2970 ± 180	3430 ± 210	0.86
0.35	1810 ± 50	3420 ± 110	0.53
$NaClO_4$			
0.20	3670 ± 100	4090 ± 130	0.89
0.40	2490 ± 80	3880 ± 120	0.64
0.60	1610 ± 80	3560 ± 160	0.45
NaBr			
0.34	3440 ± 120	3720 ± 120	0.92
0.68	3190 ± 130	3890 ± 160	0.82
1.36	2370 ± 60	3590 ± 120	0.66
$CaCl_2$			
0.18	4690 ± 200	5430 ± 250	0.86
0.36	3420 ± 70	4940 ± 100	0.69
0.72	2110 ± 40	4730 ± 70	0.45
NaCl			
0.45	5230 ± 110	5000 ± 120	1.04
0.90	5230 ± 110	5000 ± 120	1.04
KCl			
0.30	5230 ± 110	5000 ± 120	1.04
0.90	5230 ± 110	5000 ± 120	1.04
RbCl			
0.20	5230 ± 110	5000 ± 120	1.04
0.40	5230 ± 110	5000 ± 120	1.04
CsCl			
0.25	5230 ± 110	5000 ± 120	1.04
0.50	5230 ± 110	5000 ± 120	1.04

[a] 0.03 M Cacodylic acid buffer containing 0.1 M KCl.

Fig. 2A shows the representative curve of Mb in presence of different concentrations of LiBr. A plot of ΔG_{app} values in the range of −1.302 to +1.302 kcal mol^{-1} versus [g] is shown in Fig. 2B. It can be seen in Fig. 2B that ΔG_{app} is linear in the denaturant concentration. A least-squares analysis according to Eq. (2) gave the thermodynamic parameters that are given in Table 3. It can seen in this table that $\Delta G_{app}^{H_2O}$ shows a strong dependence on the [LiBr].

4. Discussion

Many salts are known to induce conformational transition between the native and denatured states of proteins at room temperature ([22] and references therein, [23]). In some cases this transition is complete in their presence at high denaturant concentrations. On the other hand, some salts induce only partial transition due to their limited solubility. A comparison of the denaturing effects of these salts is not possible for two reasons: (i) In cases in which the transitions between N and D states are complete, the extent of unfolding in the denatured state may differ in different denaturants. (ii) In those cases in which salt-induced transition is incomplete due to their limited solubility, the D state can not be measured and characterized. One may, however, examine the effect of both classes of salts by studying the temperature- or urea- or GdnHCl-induced denaturation of a protein in their presence [10–12].

Von Hippel and Wong [24,25] were the first ones who quantitatively compared the effects of neutral salts on the stability of proteins. Their procedure involved the study of thermal denaturation of the protein in presence of different concentrations of various salts. The concentration of the salt denaturant required to bring 50% change in the optical transition is used to rank salts ability to destabilize the protein conformation. In their studies they did not characterize the product of denaturation by the mixed denaturants, namely heat and the salt. It is therefore not known whether this product corresponds to heat-denatured state, or to a state obtained on isothermal denaturation by the salt denaturant, or to a state which is different from the heat- and salt-denatured ones owing to the mixed-denaturant system. These possibilities make the comparison of denaturation results problematic [12,13].

Pace and Marshall [11] Performed another procedure in which the concentration of the denaturant under investigation was varied while the second denaturant namely urea was held constant at a concentration just high enough to initiate the unfolding transition in the protein. The concentration of the denaturant required to bring 50% change in

the optical transition, C_m was used as a measure of the effective ability to destabilize the protein; on the molar scale, the lowest the C_m value, the greatest the destabilizing ability. Although their method allowed for direct quantitative comparison of effectiveness of various denaturants, unlike the method proposed by von Hippel and Wong [24,25], it did not lend itself to the analysis of the effectiveness of individual ions and their additivity [13]. It should also be noted that in the analysis of the conformational transition curve, it has been assumed that the optical property of the native protein in presence of urea is independent of the denaturant concentration used to induce denaturation.

A procedure very similar to the one used by von Hippel and Wong [24,25] has been suggested by Ahmad [12] who has used GdnHCl instead of heat to carry out denaturation. This method involves the isothermal study of GdnHCl-induced denaturation in presence of various fixed low concentrations of the second denaturant. The advantage of this procedure is that the end product of the mixed-denaturant system is the same in every case [5]. Furthermore, properties of the native (y_N) and denatured (y_D) protein molecules are well characterized in presence of the mixed-denaturant system. Thus a comparison of the ability of ionic denaturant to destabilize the protein is more realistic. Thomson and Bigelow [13] have successfully used this procedure and reported GdnHCl-induced denaturation of ribonuclease A in presence of various salts. The main conclusion of their studies is that the effectiveness of each salt to shift the midpoint of GdnHCl-denaturation and to alter the Gibbs energy of stabilization can be quantitatively predicted from the effects of individual constituent ions. We have used this procedure [12] to rank the denaturing effect of various ionic denaturants on two predominantly α-proteins, Mb and cyt-c. These results are discussed below.

Mechanism by which denaturing solutes promote the denaturation reaction is not certain. Results from studies with model compounds have been used to argue for the binding of urea, GdnHCl and Li^+ with the peptide backbone, leading to denaturation of proteins [10,26]. However, some of these solutes also promote solubilization of hydrophobic solutes lacking groups with which denaturant could bind, raising the possibility that denaturant may act indirectly with proteins via their effects on the solvent properties of water [27]. Recently Schellman has proposed a solvent denaturation model which is based on thermodynamic grounds [28]. Later this model has been justified theoretically [29] and experimentally [30–32]. According to Schellman's model, for a very dilute protein solution containing two non-interacting denaturants namely GdnHCl and a second salt denaturant in our case, the Gibbs energy change on denaturation may be written as

$$\Delta G_{app} = \Delta G_D^{H_2O} - m_g[g] - m_s[s] \quad (3)$$

where $m_s[s]$ is the salt contribution to measured ΔG_{app} at the salt concentration [s] and $m_g[g]$ is the GdnHCl contribution to the measured ΔG_{app} at the denaturant concentration of [g]. $\Delta G_D^{H_2O}$ is the value of ΔG_{app} in absence of denaturants. There are two inherent assumptions in Eq. (3). First, the contributions of GdnHCl and the second ionic denaturant to ΔG_{app} are mutually independent. This may be justified in the light of the findings of Thomson and Bigelow [13] who showed that the contributions to Gibbs energy is additive in the mixed-denaturant system. Second, their exists a linear dependence of ΔG_{app} on the molar concentration of each denaturant. Recently it has been shown that the plot of ΔG_{app} versus [g] for each protein studied here is linear in the entire GdnHCl concentration range and there exists no binding site for GdnHCl on the proteins [32,33]. To the best of our knowledge no such studies were carried out in cases of other denaturants. However, a large body of data from isothermal denaturation of proteins by these denaturants suggests that the ΔG_{app} dependence on the denaturant concentration is linear in the transition region.

It is evident from Eq. (3) that ΔG_{app} measured in presence of the second denaturant can be corrected for its contribution to the Gibbs energy change associated with the GdnHCl-induced transition. Thus, all the data from the measurements of GdnHCl denaturation in presence of the second denaturant can be corrected for its presence using m_s values obtained from the isothermal denaturation of proteins in absence of GdnHCl (Table 4).

$$\Delta G_{app}^{cor} = \Delta G_{app} + m_s[s] = \Delta G_{app}^{H_2O} - m_g[g] \quad (4)$$

When GdnHCl denaturation results in presence of $CaCl_2$, salts of lithium, NaBr and $NaClO_4$ were treated according to Eq. (4), it was observed that all the normalized data unexpectedly fall above or below the line describing the

Table 4
Thermodynamic parameters for Mb (pH 6.0) and cyt-c (pH 7.0) at 25°C

Denaturant	Mb		cyt-c	
	m_s [a] (cal mol^{-1} M^{-1})	k_s [b] (M^{-1})	m_s [a] (cal mol^{-1} M^{-1})	k_s [b] (M^{-1})
LiCl	2590 ± 160	5 [c]	1730 ± 60	5 [c]
NaBr	1220 ± 80	7	nd.	nd.
LiBr	2220 ± 80	7	1120 ± 120	7
$NaClO_4$	2390 ± 70	25	nd.	nd.
$LiClO_4$	3430 ± 150	25	nd.	nd.
$CaCl_2$	2920 ± 110	6	3550 ± 80	5 [c]

nd: Parameters for cyt-c could not be determined due to either limited solubility of the denaturant in the case of NaBr or monotonous change in the optical property on denaturation by the salts of perchlorate.

[a] Values of $m_s = (\partial \Delta G_{app} / \partial [s])_{P,T}$ are obtained from the isothermal study of denaturation of each protein by various salts. These values for cyt-c were taken from Refs. [6,14] and are unpublished results for Mb.

[b] k_s is the binding constant obtained from the analysis of the GdnHCl-induced denaturation of protein according to Eq. (5).

[c] Binding of the salts causes stabilization of the protein.

dependence of ΔG_{app} on [g] in absence of the second ionic denaturant (e.g., see Fig. 1C and 2C). This discrepancy could be due to inappropriateness of Eq. (3) in describing the denaturation process in presence of the mixed-denaturant system. One possibility for this behaviour may be the existence of strong binding site(s) for the denaturant molecule on the native protein, i.e., our assumption that the dependence of Gibbs energy change on [s] may be incorrect. In such a case Eq. (3) can be generalized to the form [27],

$$\Delta G_{app} = \Delta G_{app}^{H_2O} - m_g[g] - m_s[s] \pm RT \ln(1 + k_s[s]) \quad (5)$$

where the last term on the right-hand side of Eq. (3) (the free energy contribution due to the presence of the salt at a concentration [s]) has been partitioned into two factors. The first takes into account the nonstoichiometeric aspect of solvent interaction. The second deals with the binding of the salt with the native protein having one specific binding site. k_s is the specific binding constant and '+' and '−' represent respectively the stabilization and destabilization of the native protein due to salt binding. Analysis of the denaturation results according to Eq. (5) with k_s values given in Table 4, suggested that in each case all the values of $\Delta G_{app}^{cor'} (= \Delta G_{app}^{cor} + RT \ln(1 + k_s[s]))$ fall on the plot of ΔG_{app} versus [g] obtained from the control experiment (see Fig. 1D and 2D). Thus this study reveals a means by which binding of salts could be detected without any additional information other than that provided in the denaturation profiles.

It has been observed that C_m of cyt-*c* decreases in presence of all concentrations of LiCl and $CaCl_2$ (see Fig. 1A and Table 2), suggesting that these denaturants destabilize the native protein. On the other hand, analysis of the GdnHCl-induced denaturation profiles of the protein in presence of different concentrations of LiCl (Fig. 1A) and $CaCl_2$ [6] according to Eq. (5) suggested that there exists binding site(s) for the denaturants on the native protein, and that the binding of Li^+ and Ca^{2+} ions stabilizes the protein.

In the context of Eq. (5), it could then be argued that the solvation effect leading to denaturation is sufficiently strong to override stabilization effect due to binding. In the cases of ionic denaturants which destabilize the protein due to their binding to the native protein, the results shown in Table 2 suggest that the solvation effect and the denaturant binding cooperate in denaturing the protein. These arguments may be used to explain the results of Mb denaturation by GdnHCl in presence of various ionic denaturants (Table 3).

We have arrived at several important conclusions form the analysis of GdnHCl-induced transition profiles in presence of other denaturants. (i) NaCl, KCl, RbCl and CsCl have no effect on the protein stability (see Tables 2 and 3. (ii) LiCl binds to native proteins with an association constant of about 5 M^{-1} leading to stabilization of both proteins. Assuming that Na^+ and Cl^- have no effect on the protein stability, the observed stabilization of proteins in presence of LiCl may be attributed to Li^+ binding. (iii) The k_s values for $LiClO_4$ and $NaClO_4$ is not only the same but it is about five times larger than that for Li^+, it seems the destabilization of native Mb is due the binding of ClO_4^-. (iv) Since the value of k_s for the salts LiBr and NaBr is about 7 M^{-1}, it may be argued that Br^- binds to proteins and its binding destabilizes proteins. (v) The binding of Ca^{2+} to the native cyt-*c* stabilizes the conformation, whereas it destabilizes the Mb. (vi) Finally, qualitatively the rank ordering of the effects of salts on the stabilities of Mb and cyt-*c* follows the classical Hofmeister series [34].

Finally, in order to understand the question whether the conclusions drawn from the study of the effect of ionic denaturants on cyt-*c* and Mb have some structural specificity, one must relate this work to the earlier such works on proteins belonging to other structural classes. We are aware of reports of the effect of ionic denaturants on a few proteins containing both α and β structures [10–13]. It is interesting to note that the results of those studies led to the same conclusions (i) and (vi) given in the preceding paragraph. Thus, as far as these findings are concerned, it seems that ionic denaturants have no structural preferences. Furthermore, our work on the predominantly α-proteins also suggests that protein stability is affected by nonspecific interaction as well as the specific binding of the ionic denaturants with the proteins (see Eq. (5)). However, we cannot relate this finding with other proteins, for the possibility of the binding of ionic denaturants to α-plus β-proteins was not considered in the analysis of the earlier results [10–13].

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