Research Paper

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Effects of Sarcosine on the Stability of Cytochrome C

Chemistry

KEYWORDS : Sarcosine; Cytochrome c; unfolded state; stability; GuHCl and GuSCN

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ABSTRACT

The effect of sarcosine on the stability of Cytochrome c (Cyt c) was investigated in the presence of GuHCl, GuSCN and sarcosine by chemical and heat-induced unfolding studies. The chemical unfolding data in the presence of GuHCl and GuSCN demonstrated that GuSCN destabilizes the protein more effectively than GuHCl. The thermodynamic parameters obtained clearly indicate that sarcosine stabilizes the protein by forcing the protein to acquire a native like structure. This study supports the earlier reports and clearly shows that sarcosine can effectively offset the destabilizing effects of GuHCl and GuSCN to protect the protein by acquiring a native like conformation.

Introduction

The stability of proteins is important for their biological activity and to be used in various biotechnological applications. However, most biologically active proteins under different environmental stress conditions lost their activity and a very few recovered to regain the stability [1,2]. Several approaches such as protein engineering, chemical or enzymatic modifications and use of co-solutes/co-solvents have been employed to overcome such problems and regain the protein stability [3-5]. Among the co-solutes used, osmolytes have gained much attention because of their competence effects and availability in the cellular systems [6]. Sarcosine is a naturally occurring non-compatible osmolyte and is frequently used for protein stabilization [7–11]. Earlier reports showing the different mechanisms such as preferential hydration, preferential exclusion from the surface of denatured/unfolding protein has been attributed to understand the stabilizing effect of sarcosine [12–16]. However, all the mechanism in the molecular basis is still under debate and need to be address more precisely.

For this study we have chosen Cytochrome c, which is a highly conserved ~12 kDa protein consisting of a single 104 amino acid peptide with a single heme group, covalently attached to Cys14 and Cys17. Because of its ubiquitous nature and sequence homology, Cyt c has been used as a model protein for molecular evolution studies [17]. We have examined the competence of sarcosine to offset and stabilize Cyt c under chemical and heat-induced stress conditions.

Material and methods

Material

Cytochrome c (horse heart, lyophilized powder), guanidine hydrochloride (GuHCl), guanidine thiocyanate (GuSCN), sarcosine and sodium acetate buffer were obtained from Sigma Chemical Company, USA. The water used for solution preparation was double distilled-deionized using Cole-Parmer research cartridge resin followed by degassing. All the masses were determined on a Sartorius BP 211D balance, which had a readability of ± 0.01 mg. The pH of the buffer solution was measured on a Control Dynamics pH meter at room temperature.

Fluorescence measurements

The equilibrium unfolding profiles of Cyt c at pH 4.0 in the presence of increasing concentrations of GuHCl and GuSCN were measured on a Perkin Elmer LS-55 spectrofluori meter at 394 nm and 25 °C. All the spectra are average of three accumulations and blank corrected. A protein concentration of 5 μM was used for all the fluorescence measurements. The unfolding profile of Cyt c at pH 4.0 was highly cooperative and fitted to the linear extrapolation model with the assumption of two-state mechanism, N ⇌ U. Then the values of ΔG_u were calculated from the fluorescence data points using the relation,

$$\Delta G_u = \Delta RT \ln \left[\frac{(y - y_n)}{(y_u - y_n)} \right] \quad (1)$$

where, y is the observed intensity, y_n and y_u are the intensities in the native and unfolded states respectively. From equation 2, the calculated values of ΔG_u were plotted against [GuSCN] and a linear least-square analysis was used to fit the data to the relation:

$$\Delta G_u = \Delta G_u^{H_2O} - m[GuSCN] \quad (2)$$

where, $\Delta G_u^{H_2O}$ is the value of ΔG_u at zero molar GuSCN concentration, and *m*-value (cooperatives of the unfolded reaction) gives the linear dependence of ΔG_u on [GuSCN] were used to calculate C_m (midpoint of denaturant concentration).

UV-visible measurements

The heat-induced unfolding of Cyt c was monitored using a Shimadzu-260 UV-visible spectrophotometer equipped with a temperature controller (ETC-505T). The protein solutions were loaded in a 3 ml masked Teflon-stopper quartz cuvette and heated from 10 to 80 °C with a heating rate of 1 °Cmin^{-1}. For this scan rate adequate time was provided for equilibration and the change in absorbance at 295 nm with the increasing temperature were measured. Reheating the sample after cooling to the room temperature checked the reversibility of the each thermal scan. All scans were blank corrected for respective reference solutions. Each heat-induced transition curve was analyzed using a linear extrapolation method (LEM) as described by Pace et. al., [18]. The temperature dependence free energy change at temperature T, $\Delta G_u(T)$was calculated using Gibbs-Helmholtz equation,

$$\Delta G_u(T) = \Delta H_m \left(\frac{T_m - T}{T_m} \right) - \Delta C_p \left[(T_m - T) + T \ln \left(\frac{T}{T_m} \right) \right] \quad (3)$$

where, T_m is the melting temperature at the midpoint of unfolding curves; ΔH_m is the unfolding enthalpy change at T_m and ΔC_p is the molar heat capacity change accompanying the thermal unfolding process.

Results

Chemical unfolding of Cyt c

The GuHCl and GuSCN-induced unfolding profile of Cyt c at pH 4.0 was monitored by observing the change in fluorescence intensity at 394 nm and 25 °C (Fig.1).

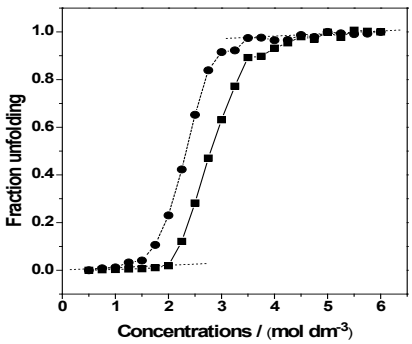

Figure 1. Fraction unfolding of Cyt c at pH 4.0 and 25 °C with the increasing GuHCl (■) and GuSCN (●) concentrations.

The thermodynamic parameters obtained using equation 1 & 2 are listed in Table 1.

Table1. Thermodynamic parameters of the chemical unfolding of Cyt c at pH 4.0

Parameters	GuHCl	GuSCN
$\Delta G_u^{H_2O}$ (kJmol^{-1})	18.2 (0.5)	14.1(0.1)
m (mol^{-2} dm^3)	6.7(1.2)	5.7 (0.8)
C_m (mol dm^3)	2.7	2.3

Heat-induced unfolding of Cyt c

Heat-induced unfolding of Cyt c at pH 4.0 was monitored by observing the change in absorbance at 295 nm in the absence and presence of GuHCl and in the mixture of sarcosine and GuHCl. All the UV-visible melting profiles of Cyt c at pH 4.0 were found to be reversible (>90%) and the unfolding profile of Cyt c follows a simple two state processes, native (N) ⇌ unfolded (U) and allow the application of equilibrium thermodynamic to evaluate the unfolding thermodynamic parameters.

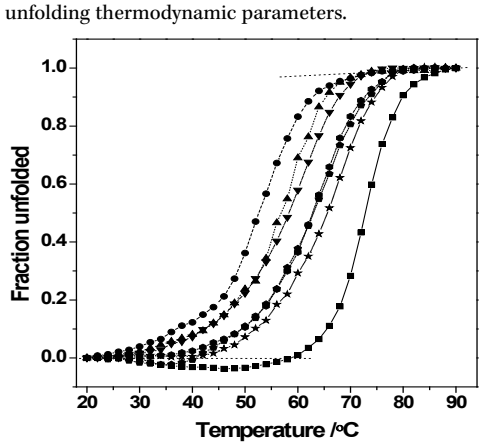

Figure 2. Fraction unfolding of Cyt c (■), and in 1.0 M GdmCl(●), and in the presence of + 0.5 M sarcosine (▲), + 1.0 M sarcosine(▼), + 1.5 M sarcosine (●), + 2.0 M sarcosine (⬟), + 2.5 M sarcosine (✱).

Here, T_m (the mid-point of melting curve) and ΔH_m (enthalpy at T_m) are used to calculate ΔS_m at T_m and free energy of unfolding at 298.15 K (ΔG_U^o). A linear least-square analysis of T_m and ΔH_m gave the value of molar heat capacity $\{\Delta C_p = \partial \Delta H_m / \partial T_m)\}$ = 1.63 ± 0.47 kJ mol^{-1} K^{-1}. The ΔC_p value obtained was used to calculate the standard Gibbs free energy of unfolding ΔG_U^o using equation 3 and are listed in Table 2.

Table 2. Thermodynamic parameters of heat-induced unfolding of Cyt c at pH 4.0 in GuHCl and in sarcosine and GuHCl mixture.

Conditions	T_m °C	ΔH_m kJmol^{-1}	ΔG_U^o kJmol^{-1}
Cyt C at pH 4.0	72	332 (2)	31.95 (0.64)
1.0 M GuHCl	53	202 (4)	15.32 (0.59)
+0.5M sarcosine	57	206 (2)	17.35 (0.99)
+1.0 M sarcosine	59	211 (2)	18.89 (1.16)
+1.5 M sarcosine +2.0 M	63	216 (4)	20.82 (1.84)
sarcosine	64	218 (3)	21.44 (1.56)
+2.5 M sarcosine	67	224 (4)	23.53 (1.47)

The value of standard free energy change of unfolding (ΔG_U^o) has been used as criteria to define the conformational stability of globular proteins following a simple two state theory of native (N) ⇌ unfolding (U) [19,20].

For comparison studyof Cyt c, the heat-induced unfolding in the absence and presence of GuSCN, sarcosine and their mixtures were carried out (Fig. 3).

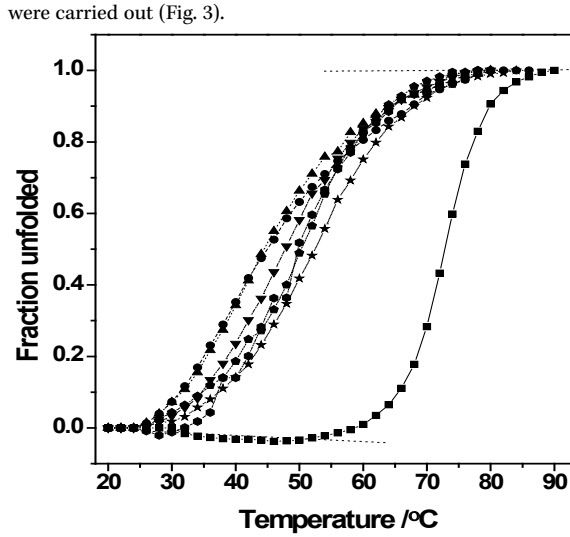

Figure 3. Fraction unfolding of Cyt c (■), in 1.0 M GuSCN (●) and the presence of +0.5 M sarcosine (▲), +1.0 M sarcosine (▼), + 1.5 M sarcosine (●), + 2.0 M sarcosine (⬟), + 2.5 M sarcosine (✱).

All heat-induced experiments were found to be complete reversible and following a simple two-sate model of protein folding and that allows the application of equilibrium thermodynamics to derive the parameters; T_m, ΔH_m ΔS_m at T_m, ΔC_p and ΔG_U^o are listed in Table 3.

Table 3. Thermodynamic parameters of the heat-induced unfolding of Cyt c in GuSCN and sarcosine at pH 4.0

Conditions	T_m °C	ΔH_m kJmol^{-1}	ΔG_U^o kJmol^{-1}
Cyt C at pH 4.0	72	332 (2)	31.95 (0.64)
1.0 M GuSCN	42	122 (5)	5.36 (0.06)
+0.5 M sarcosine	43	132 (3)	6.14 (0.37)
+1.0 M sarcosine	46	139 (2)	7.38 (0.54)
+1.5 M sarcosine +2.0	47	146 (3)	8.04 (1.02)
M sarcosine	50	150 (2)	9.00 (0.36)
+2.5 M sarcosine	52	156 (5)	9.97 (0.97)

Further, the difference in the standard free energy change of unfolding, ΔG_U^o and change in melting temperature, ΔT_m for both the mixture conditions have been compared (Fig. 4).

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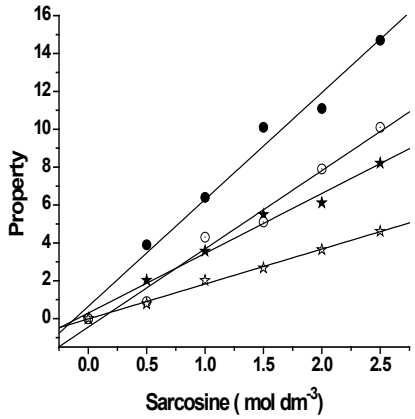

Figure 4. Plots of $\Delta\Delta G_U^o$ [$(\Delta G^o_{U(GuHCl+Sarcosine)})$ - $(\Delta G^o_{U(GuHCl)})$] (●) and ΔT_m ($T_{m\,(GuHCl+Sarcosine)}$ - $T_{m\,(GuHCl)}$) (○) and $\Delta\Delta G_U^o$ [$(\Delta G^o_{U(GuSCN+Sarcosine)})$ - $(\Delta G^o_{U(GuSCN)})$] (★) and ΔT_m ($T_{m\,(GuSCN+Sarcosine)}$ - $T_{m\,(GuSCN)}$) (✲) and of Cyt c at pH 4.0.

Figure 5, represents the overall energetics of osmolyte-induced folding of Cyt c at pH 4.0 under various experimental conditions. The upper bars represent the thermal stabilization of Cyt c in the presence of sarcosine, sarcosine and GuHCl, sarcosine and GuSCN compare to control, GuHCl and GuSCN. The lower bars represent the thermal destabilization of Cyt c in the presence of GuHCl and GuSCN. The shaded portion of the lower bars represents the counteraction of sarcosine compare to the unfolded Cyt c at pH 4.0.

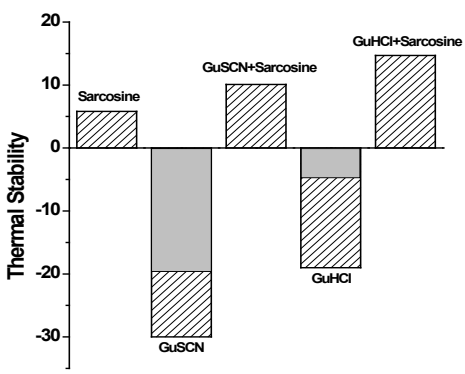

Figure 5. The comparison of the energetics of heat-induced unfolding of Cyt c at pH 4.0 under various solution conditions.

Discussion

It has been observed that sarcosine stabilizes Cyt c at pH 4.0 by enhancing conformational stability in the presence of GuHCl and GuSCN in all studied conditions. The results obtained from the equilibrium unfolding experiments of the protein in the presence of GuHCl and GuSCN clearly indicates that GuSCN is a stronger denaturant of Cyt c at pH 4.0 than GuHCl as the $C_{m,GuSCN} < C_{m,GuHCl}$.

For Cyt c heat-induced unfolding, there is effective increase in T_m values and significant changes in enthalpy and comparative entropy values in the mixture of sarcosine and GuHCl, which indicates the fine balance between the preferential binding of GuHCl to protein backbone and preferential exclusion of sarcosine by solvophobic effect to generate water structure around the protein molecule. Further in Fig. 3, it was observed that in the presence of 1.0 M GuSCN, the melting curve dramatically shifted towards lower temperature and signifying the destabilizing effects of GuSCN. However in the presence of increasing concentrations of sarcosine in sarcosnie and GuSCN mixture, the melting curves shifted towards the control protein, a similar effect of stability was observed for sarcosine and GuHCl mixture as well. In the counteraction ratio of 1:2.5 M for GuHCl or GuSCN to sarcosine is dominated by the denaturants. The extent of counteraction or stabilization by sarcosine in the mixture is more effective for sarcosine and GuHCl than sarcosine and GuSCN. The reason is clear that GuSCN is a stronger denaturant than GuHCl. From this study we observed that sarcosine is able to counteract the denaturing effects of GuHCl and GuSCN to regain native like conformation but to different extents.

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