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Sucrose: A Potent Stabilizer for Carbonic Anhydrase

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The effect of sucrose as a very potent osmolyte and stabilizer on the structure and activity of bovine carbonic anhydrase has been studied using different techniques. Sucrose has been shown to stabilize the enzyme against inactivation alongside of time. Besides, it seems that the enzyme got greater thermal stability in the existence of sucrose because both the transition temperature (T_m) and the standard Gibbs free energy (ΔG°_{25}) in protein thermal denaturation were increased. It seems that sucrose can have these effects through inducing some alterations in the structure of carbonic anhydrase.

Key Words: Carbonic anhydrase, Sucrose, Protein stability, Circular dichroism.

INTRODUCTION

Carbonic anhydrase (CA, EC 4.2.1.1) is a ubiquitous zinc enzyme which catalyzes the reversible hydration of carbon dioxide, and is involved in the transfer and accumulation of H^+ and HCO_3^- in a large number of tissues of vertebrates and in nearly all the major groups of invertebrates[1]. The zinc ion in the structure of the enzyme is situated at the bottom of a 15 Å deep active site cleft and is coordinated to three histidines (His 94, 96 and 119) and a water molecule/ hydroxide ion[2, 3].

Carbonic anhydrase has some other catalytic activities, such as hydrolysis of aromatic and aliphatic esters[4, 5], the hydration of cyanate to urea[6], or some other less investigated hydrolytic processes[7]. Carbonic anhydrase exhibits outstanding dynamic properties; its most active isozymes reach turnover numbers up to 10^6 s^{-1}.[8] In erythrocytes, carbonic anhydrase is the most abundant protein after hemoglobin and allows the transport of CO_2 across the cell membrane at very high rates required for accomplishing the respiratory process[9]. These unique physiologic features have stimulated great trechnological interest in the enzyme for using in extra-corporeal devices for blood oxygenation[10, 11].

Polyhydric compounds such as sugars have been used for a long time to stabilize proteins or to preserve the biological activity of organelles[12, 13]. Sucrose is a naturally occurring polyhydric osmolyte and its effect on stabilizing the proteins has been widely investigated[14–16]. The addition of sucrose to the aqueous phase of a protein solution can decrease the thermodynamic affinity of protein

for that aqueous environment[17], stabilize globular whey proteins against thermal unfolding[18] and increase denaturation temperature of some proteins[19].

Recently, we have reported structural and functional changes of carbonic anhydrase as a consequence of temperature[20] and in the presence of three different amino acids as osmolytes[21]. To find more information about the protein stability and to improve carbonic anhydrase stability, it would appear helpful to study the effect of different stabilizers on the stability and activity of the enzyme and possibly find some effective stabilizers. Hence, in this study, the effect of sucrose as a strong stabilizer on the structure and function of carbonic anhydrase has been studied.

EXPERIMENTAL

Erythrocyte carbonic anhydrase and *p*-nitrophenylacetate were obtained from Sigma. The buffer used in all experiments was 50 mM tris, pH 7.5, which was obtained from Merck. Sucrose was obtained from Sigma.

Enzyme assay: Enzyme activity was assayed using a spectrophotometer with jacketed cell holders by following the increase in absorbance at 400 nm due to the production of *p*-nitrophenol.[5] Its temperature was regulated by an external thermostated water circulator within ±0.05°C. Enzyme was incubated with 0.5 and 1 M of sucrose and the initial rate of *p*-nitrophenylacetate hydrolysis catalyzed by carbonic anhydrase was determined in different concentrations of sucrose at definite periods of time. Enzyme and substrate concentrations were 0.3 μM and 6 mM, respectively. Nonenzymatic hydrolysis rate was always subtracted from the observed rate[22, 23].

Temperature-Scanning Spectroscopy: Absorbance profiles, which describe the thermal denaturation of carbonic anhydrase, were obtained from a UV-Vis spectrophotometer CARY-100-Bio model fitted with a temperature programmer, which controls the speed of temperature change in denaturation experiments. The cuvette holder can accommodate two samples: one as a reference buffer solution and the other one for sample experimental determination. All sample cells had identical concentrations of carbonic anhydrase (0.3 mg/mL) and 0.3 mg/mL of sucrose. The recording chart reads the temperature reference line (from the reference cuvette) and the absorbance change at 280 nm for each of the samples.

Heat stability studies: The stability of enzyme against heat was studied by incubating definite concentration of enzyme in different concentrations of sucrose, at 67°C, for 10–90 min, followed by cooling at 4°C for about 1 h. Then the esterase activity of each sample was assayed as usual. Care was taken to hold the stoichiometry of carbonic anhydrase and sucrose according to other experiments.

Circular dichroism experiments: Circular dichroism (CD) spectra were recorded on an Aviv-215 spectropolarimeter (USA). The results were expressed as ellipticity [θ(deg cm^2 dmol^{-1})] considering a mean amino acid residues weight (MRW) of 112 and weight of 29 kDa for carbonic anhydrase[24]. The molar ellipticity was determined as $[\theta]_\lambda = (100 \times MRW \times \theta_{obs}/cl)$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength, c is the protein concentration in mg/mL and l is the length of the light path in cm. The instrument was calibrated with (+)-10- camphorsulfonic acid, assuming $\theta_{291} = 7820$ deg cm^2 dmol^{-1} and

JASCO standard nonhygroscopic ammonium, (+)-10-camphorsulfonate assuming $[\theta]_{290.5}$ = 7910 deg cm^2 $dmol^{-1}$.[25, 26] The noise in the data was smoothed by using the software included in the spectropolarimeter apparatus. This software uses the fast Fourier-transform noise reduction routine that allows decrease of most noisy spectra without distorting their peak shapes. The software was used to predict the secondary structure of the protein according to the statistical method[27, 28].

Far-UV CD was carried out in the presence of 0.22 mg/ml of carbonic anhydrase. In Near-UV region higher concentrations of protein should be used due to the low extinction coefficients of the chromophores. Thus 1.12 mg/mL of carbonic anhydrase was applied in Near-UV CD experiments. Consequently, the sucrose concentrations were selected so that the stoichiometry of the solution held constant.

RESULTS AND DISCUSSION

Fig. 1 shows the inactivation alongside of time scheme for carbonic anhydrase in different concentrations of sucrose. Although carbonic anhydrase is very resistant against inactivation alongside of time, increasing the concentration of sucrose can lead to a decrease in spontaneous inactivation of the enzyme. In other words, it seems that sucrose can stabilize the protein against the inactivation alongside of time.

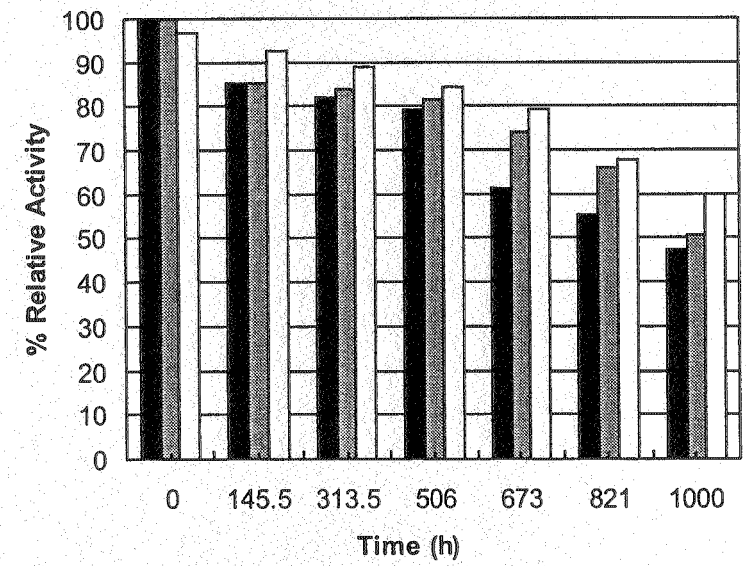

Fig. 1. The relative activity of carbonic Anhydrase alongside of time in the presence of different concentrations of sucrose: (■) 0, (▩) 0.5 and (□) 1 M.

To obtain further information on the stability of the enzyme, the thermal denaturation curves for carbonic anhydrase were obtained in different concentrations of sucrose (Fig. 2a). The transition temperature (T_m) of the enzyme increases with increasing the concentration of sucrose. The values of T_m can be obtained from the midpoint change of the curves. These values are 63.2, 65.0 and 68.1 ± 0.2 °C in the absence and presence of 0.5 and 1 M of sucrose, respectively. Therefore, the existence of sucrose led to more thermal stability. The amount of ΔG°_{25} (the standard Gibbs free energy of protein denaturation at 25°C) for different

concentrations of sucrose can be obtained from Pace analysis[28] of thermal denaturation curves (Fig 2b). Determination of the standard Gibbs free energy of denaturation ($\Delta G°$), as a criterion of conformational stability of a globular protein, is based on two state theory as follows:

$$\text{Native (N)} \rightleftharpoons \text{Denatured (D)} \quad (1)$$

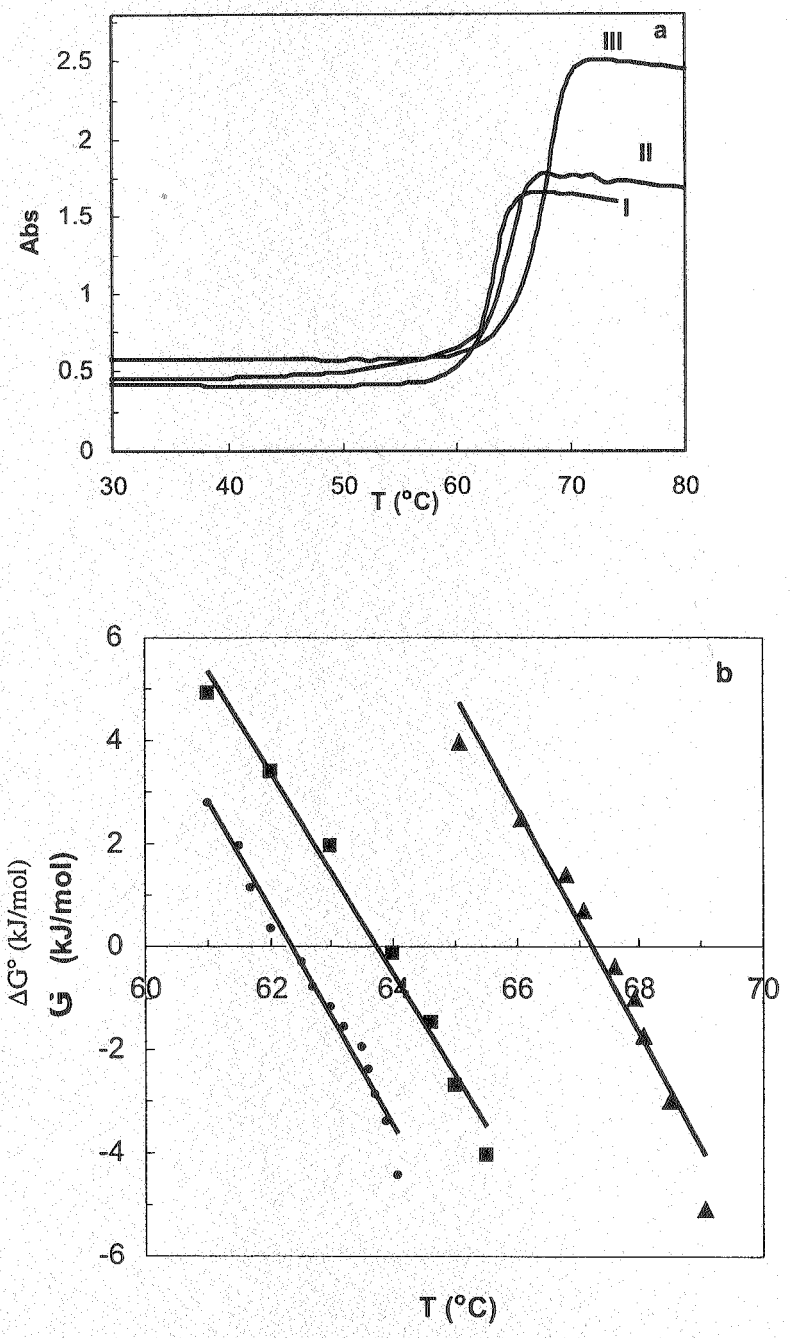

Fig. 2. (a) Thermal denaturation profiles of carbonic anhydrase in the presence of different concentrations of sucrose: (I) 0, (II) 0.5, and (III) 1 M; (b) The standard Gibbs energy of unfolding in the presence of different concentrations of sucrose: (●) 0, (■) 0.5, and (▲) 1 M.

This process was described as a single denaturant-dependent step according to the two-step theory[29]. By assuming two-state mechanism for protein denaturation by temperature, one can determine the process by monitoring the changes in the absorbance[27, 30, 31] and hence calculate the denatured fraction of protein (F_d) as well as determination of the equilibrium constant (K).

$$F_d = \frac{(Y_N - Y_{obs})}{(Y_N - Y_D)} \tag{2}$$

$$K = \frac{F_d}{(1 - F_d)} = \frac{(Y_N - Y_{obs})}{(Y_{obs} - Y_D)} \tag{3}$$

where Y_{obs} is the observed variable parameter (*e.g.*, absorbance) and Y_N and Y_D are the values of Y characteristic of a fully native and denatured conformation, respectively. The standard Gibbs free energy change ($\Delta G°$) for protein denaturation is given by the following equation:

$$\Delta G° = -RT \ln K \tag{4}$$

where R is the universal gas constant and T is the absolute temperature. $\Delta G°$ varies linearly with T over a limited region. The simplest method of estimating the conformational stability in the room temperature, $\Delta G°_{25}$, is to assume that linear dependence continues to 25°C and to use a least-square analysis. $\Delta G°_{25}$ was estimated to be 75.1, 76.2 and 93.3 ± 0.1 kJ/mol in the absence and presence of 0.5 and 1 M of sucrose, respectively. The values indicate higher structural stability of the enzyme with the existence of sucrose.

Fig. 3 depicts the profile of heat stability of the enzyme, in the presence of different concentrations of sucrose. The enzyme itself seems to be strong against

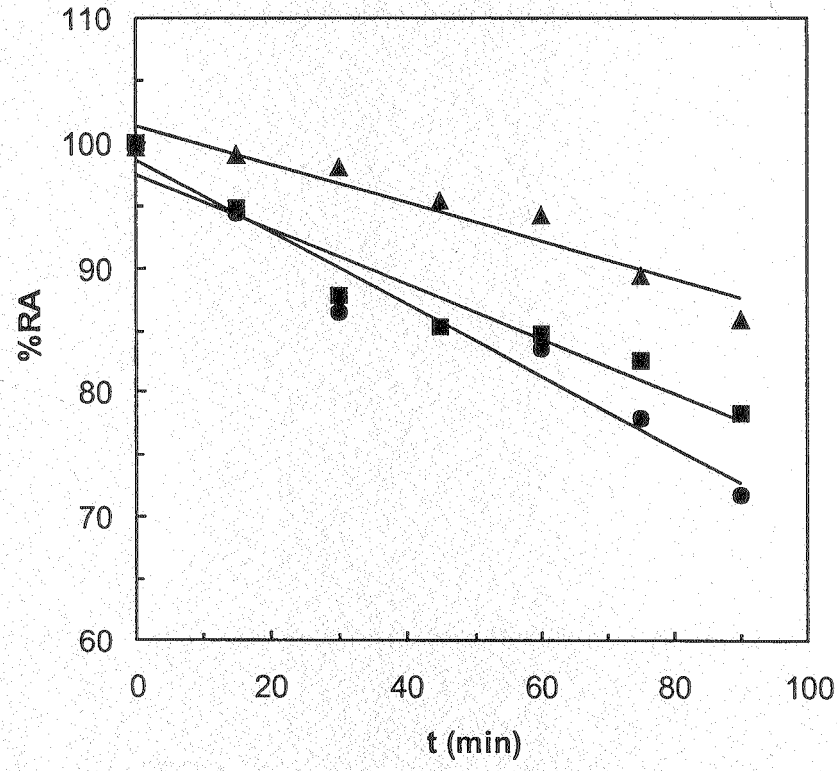

Fig. 3. The residual activity of carbonic anhydrase in the presence of (●) 0, (■) 0.5 and (▲) 1 M of sucrose

heat denaturation. As can be seen, the enzyme loses about 30% of its initial activity after being incubated for 90 min at 67°C. The results show that the existence of sucrose in the buffer protects the enzyme against heat denaturation. So it can be concluded that carbonic anhydrase in the presence of sucrose has more heat stability and is also more stable against thermal denaturation as well as inactivation alongside of time.

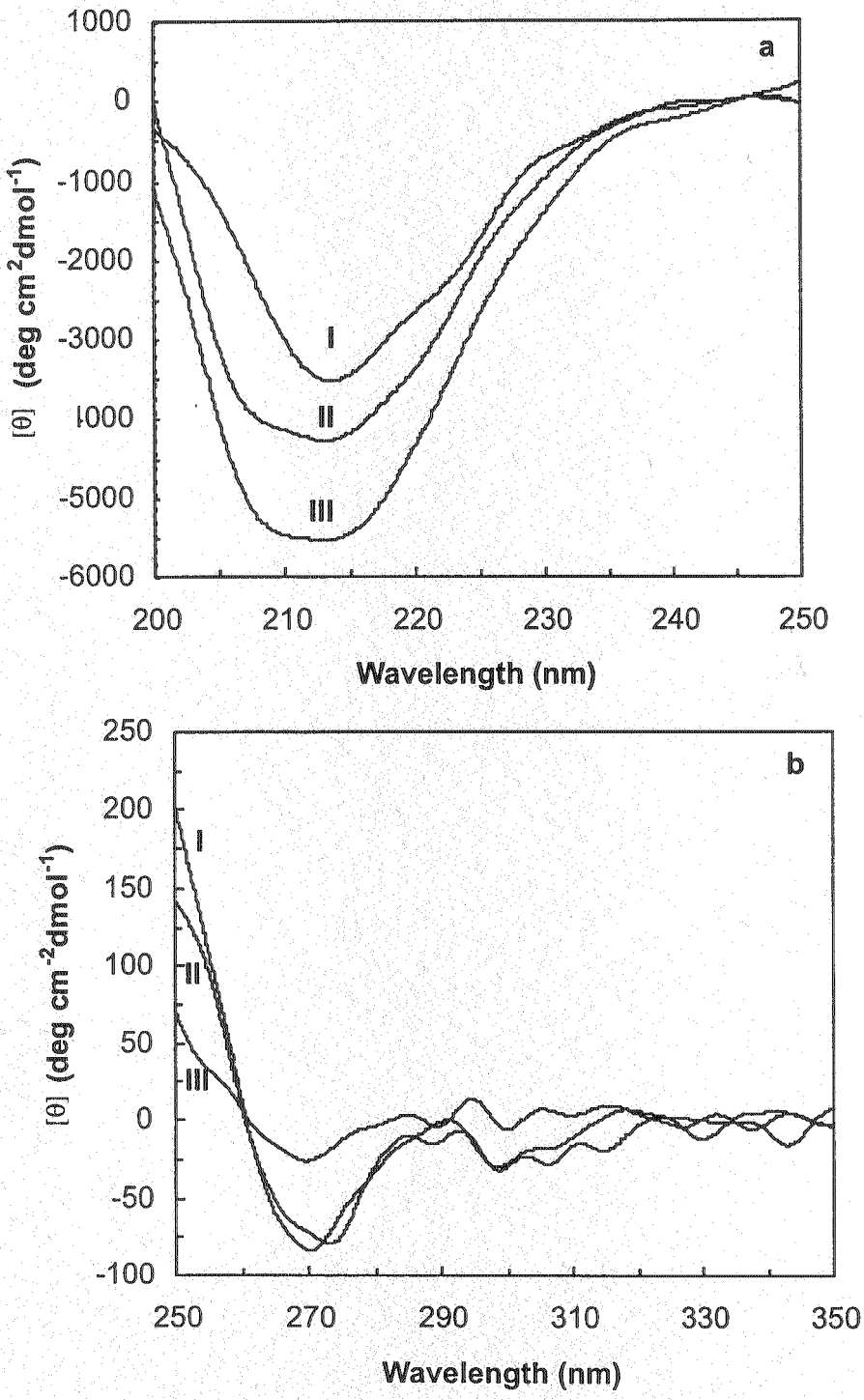

Fig. 4. Far-UV CD spectra (a) and near-UV CD spectra (b) for carbonic anhydrase in the presence of (I) 0, (II) 0.5 and (III) 1 M of sucrose.

To have a deeper look into the structural changes of the enzyme, the CD spectra of both far and near-UV regions were recorded in different concentrations of sucrose. Detailed structural differences can be calculated[32], which is reported in Table-1.

TABLE-1
THE AMOUNTS OF SECONDARY STRUCTURES OF CARBONIC ANHYDRASE IN THE PRESENCE OF DIFFERENT CONCENTRATIONS OF SUCROSE

[Sucrose] (M)	Helix (%)	β-structures (%)	Random coil (%)
0.0	16.0 ± 0.2	25.6 ± 0.2	58.5 ± 0.2
0.5	16.2 ± 0.2	33.4 ± 0.2	50.5 ± 0.2
1.0	17.7 ± 0.2	36.7 ± 0.2	45.7 ± 0.2

As can be seen, the enzyme gets more secondary structure in the presence of sucrose. The amounts of helix and β-structures increase by increasing the concentration of sucrose. This may be the reason of more stability of carbonic anhydrase against both heat denaturation and inactivation alongside of time. Besides, these changes can lead to greater ΔG°_{25} which indicates greater structural stability.

Also, there are some alterations in the near-UV CD spectra of carbonic anhydrase in different concentrations of sucrose. The most obvious among them is the amount of θ around 270 nm, which is related to the Phe residues of carbonic anhydrase. The amount of θ in this region is decreased by increasing the concentration of sucrose. So it may be concluded that the existence of sucrose can induce some structural changes in the enzyme so that the Phe residues may become farther from each other.

In an overall view, it can be concluded that sucrose can be considered as a very potent osmolyte for carbonic anhydrase, since it can increase its structural as well as its heat stability. Besides, sucrose not only preserves the enzymatic activity, but also protects it against inactivation alongside of time. It seems that sucrose can have these effects through inducing some alterations in the structure of carbonic anhydrase. All these alterations can lead to a parallel increase in both the thermodynamic stability and the stability of the enzyme against inactivation. So, sucrose can be referred to a potent stabilizer of carbonic anhydrase.

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