Effect of Some Amino Acids on the Structure and Activity of Carbonic Anhdrase

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The effect of three amino acids, histidine, phenylalanine and aspartic acid, as three different osmolytes, on the structure and activity of bovine carbonic anhdrase has been studied using different techniques. All the three amino acids were found to stabilize the enzyme against inactivation alongside of time. While the thermal transition temperature was the same as control in all cases, ΔG°_{25}, a quantitative marker for thermal stability was found to be affected differently by the three amino acids. Besides, it seems that in the presence of phenylalanine, as a hydrophobic osmolyte, the structure of the enzyme is somehow exposed while aspartic acid, as a negatively charged amino acid, may induce the contraction of the protein. Histidine can make carbonic anhdrase get some extra secondary structure while the tertiary structure is not considerably changed. The *in-vitro* studies may make some points of view for future *ex-vivo* study on the effect of these osmolytes on the stability and activity of carbonic anhdrase.

Key Words: Carbonic anhdrase, Osmolyte, Stability, Activity, Amino acid.

INTRODUCTION

The carbonic anhdrases (CA, EC 4.2.1.1) are ubiquitous zinc enzymes, present in aechaeo and eubacteria, algae, green plants and animals[1, 2]. 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, His 96, His 119) and a water molecule/hydroxide ion[1, 3].

In addition to catalyzing the reversible hydration of CO_2 to bicarbonate, CA is involved in a variety of other reactions, such as hydrolysis of aromatic and aliphatic esters[4, 5] and the hydration of carbamic acid or of cyanate to urea[6], or some other less investigated hydrolytic processes[1]. CA exhibits outstanding dynamic properties; its most active isozymes reach turnover numbers[7] up to $10^6 \, s^{-1}$.

Since this enzyme is involved in crucial physiological or pathological processes, and its deficiency may cause problems as well as its over-activity[4, 8], the study of its activation and/or stabilization is as important as its inhibition.

The array of naturally occurring organic osmolytes falls into three general chemical categories[9, 10]: the polyols, free amino acids and their derivatives and methylamines. There are some reports on the effect of some of these substances on the structure and function of different enzymes and proteins[10–12]. Osmolytes can increase the stability of proteins while protecting them from thermal denaturation, meanwhile the enzymatic activity is not reduced[13].

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Besides, the effect of some amino acids on carbonic anhydrase activity has been studied. Most of them have been investigated to be activators of the enzyme[14, 15]. Recently, it has been reported that phenylalanine and imidazole, as CA activators, may have pharmacological applications in conditions in which learning and memory are impaired, as for example, Alzheimer's disease or aging[16]. It was previously mentioned that the levels of several CA isozymes are significantly diminished in the brain of patients affected by Alzheimer's disease[17]. So, this may constitute a new approach for the treatment of Alzheimer and other conditions in need of achieving memory therapy[15–18]. Hence, in this study, the effect of Phe, His and Asp as three osmolytes has been investigated on the stability and activity of CA.

EXPERIMENTAL

Erythrocyte bovine carbonic anhydrase and *p*-nitrophenylacetate were obtained from Sigma. The buffer used in all experiments was 50 mM Tris, pH 7.5 and amino acids, *viz.*, Histidine (His), aspartic acid (Asp) and phenylalanine (Phe) were all obtained from Merck.

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. The enzyme was incubated with 5 mM of each of the three amino acids (His, Phe and Asp) and then the initial rates of *p*-nitrophenyl acetate hydrolysis catalyzed by CA was determined in the absence and presence of each amino acid at definite periods of time. Enzyme and substrate concentrations were 0.3 µM and 6 mM, respectively. Non-enzymatic hydrolysis rate was always subtracted from the observed rate[19]. Care was taken to use adequate experimental conditions to keep the enzyme reaction linear during the first minute of reaction.

Temperature-scanning spectroscopy

Absorbance profiles, which describe the thermal denaturation of CA, were obtained from a UV-Visible 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 2 samples: one as a reference buffer solution and the other one for sample experimental determination. All sample cells had identical concentrations of CA (0.3 mg/mL) and definite concentrations of each amino acid (His, Phe and Asp). 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 the absence and presence of Phe, His and Asp, in 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.

Fluorescent experiments

Fluorescent intensity measurements were carried out on a Hitachi spectrofluorimeter, MPF-4 model, equipped with a thermostatically controlled cuvette compartment. The intrinsic emission of protein solution, 0.3 mg/mL, was recorded at the excitation wavelength of 280 nm. The experiment was repeated in the presence of definite concentrations of His, Phe and Asp, as well.

Circular dichroism experiments

Circular dichroism (CD) spectra were recorded on a JASCO J-715 spectrophotometer (Japan). The results were expressed as ellipticity [θ (deg cm^2 dmol^{-1})] considering a mean amino acid residue weight (MRW) of 112 and weight of 29 kDa for CA[20]. The molar ellipticity was determined as $[\theta]_\lambda = (100 \times$ MRWX θ_{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-camphor sulfonic acid, assuming $[\theta]_{291} = 7820$ deg cm^2 dmol^{-i}, [21] and JASCO Standard non-hygroscopic ammonium, (+)-10-camphor sulfonate[22, 23]. assuming $[\theta]_{290.5} = 7910$ deg cm^2 dmol^{-1}. The noise in the data was smoothed by using the JASCO J-715 software. This software uses the fast Fourier-transform noise reduction routine that allows decrease of most noisy spectra without distorting their peak shapes. The JASCO J-715 software was used to predict the secondary structure of the protein according to the statistical method[24, 25].

Far-UV CD was carried out in the presence of 0.22 mg/mL of CA. 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 CA was applied in near-UV CD experiments. Consequently, the amino acid concentrations were selected so that the stoichiometry of the solution is held constant.

RESULTS AND DISCUSSION

Fig. 1 shows the inactivation alongside of time scheme for CA in the presence of three amino acids, His, Phe and Asp. The enzyme itself is very resistant to time inactivation, so that after 44 days (1056 h) in lab, temperature (25°C), the activity reaches to 29 per cent of the initial activity. Besides, all three amino acids stabilize the protein against time inactivation. Histidine not only increases the enzyme activity but also stabilizes it. Although Asp decreases the initial rate of enzymatic activity, it can also stabilize the enzyme against time inactivation.

The activators of CA can generally take place in the rate-limiting step of the enzyme activity, which is proton transfer. They can structurally help in the transfer process of proton. So His can help this step and play its role as activator; nevertheless, Asp cannot do the same and may somehow decrease the activity of CA. The structure of Asp, which is not aromatic and is considered as a negatively charged amino acid, enables it to get involved in the proton transfer process successfully.

Obtaining further information on the stability of enzyme, it is possible to take a look at the thermal stability scheme of the enzyme in the absence and presence
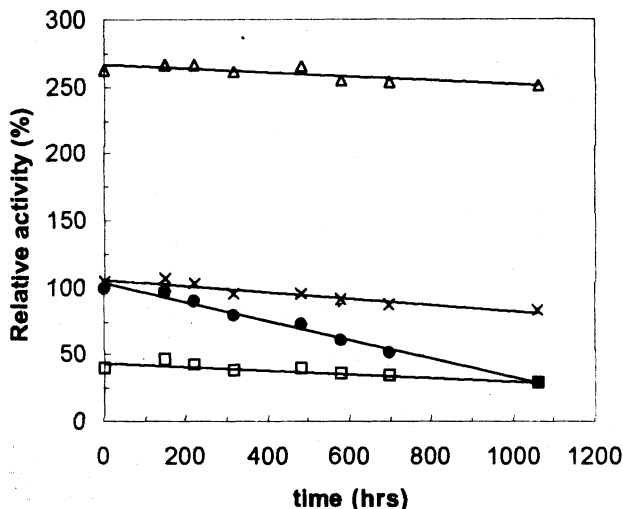
Fig. 1. The plot of inactivation of carbonic anhydrase alongside of time in the absence of osmolytes (●) and in the presence of Phe (×), His (Δ) and Asp (⊔). The buffer solution was Tris 50 mM, pH = 7.5.

of His, Phe and Asp (Fig. 2). It seems that there is no significant change in the thermal transition temperature (T_m) of the enzyme in the absence and presence of three amino acids. The amount of ΔG°_{25} (the standard Gibbs free energy of protein denaturation at 25°C) for each amino acid can be obtained from Pace analysis[26] of thermal denaturation curves (Fig. 3). The determination of standard Gibbs free energy of denaturation (ΔG°), as a criterion of conformational stability of a globular protein, is based on two state theory as follows:

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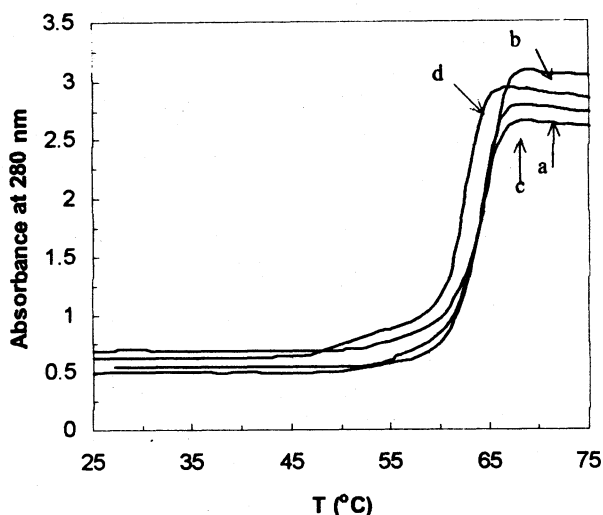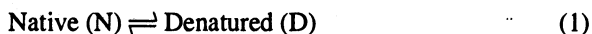

Fig. 2. Thermal denaturation curves of carbonic anhydrase in Tris 50 mM, pH = 7.5, in the absence of osmolytes (a) and in the presence of Phe (b), His (c) and Asp (d).
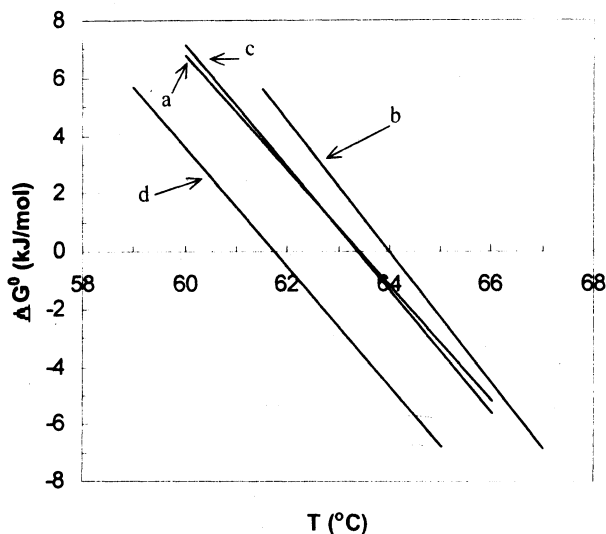
Fig. 3. Free standard Gibbs energy plots of the sole enzyme (a), and in the presence of Phe (b), His (c) and Asp (d).

This process was described as a single denaturant-dependent step according to the two step theory[26]. By assuming two-state mechanism for protein denaturation by temperature, one can determine the process by monitoring the changes in the absorbance[24, 27, 28], and hence calculate the denatured fraction of protein (F_d) as well as determination of the equilibrium constant (K).

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

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

where Y_{obs} is the observed variable parameter (e.g., absorbance) and YN and YD 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:

$$\Delta G° = -RT \ln K \qquad (4)$$

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 the highest for Phe (88.1 kJ/mol), may be due to the hydrophobic interactions which make the protein get a more stable structure. For Asp, which is a negative charged amino acid, it was the lowest (75.6 kJ/mol), and for His as a polar amino acid with a positive charge it was higher than the sole enzyme (81.5

and 76.6 kJ/mol, respectively). His and Phe increase the protein stability without any change in T_m of the protein, which indicate Phe and His may have some destabilizing effect on the denatured state of the enzyme[29].

Fig. 4 depicts the profile of heat stability of the enzyme. It can be seen that the sole enzyme loses its activity only partially even after being for 90 min at 67°C. Meanwhile, Phe and His can somehow protect the enzyme from heat denaturation while in the presence of Asp, the enzyme denatures completely. It seems that the amino acids Phe and His have some effects on the native form of
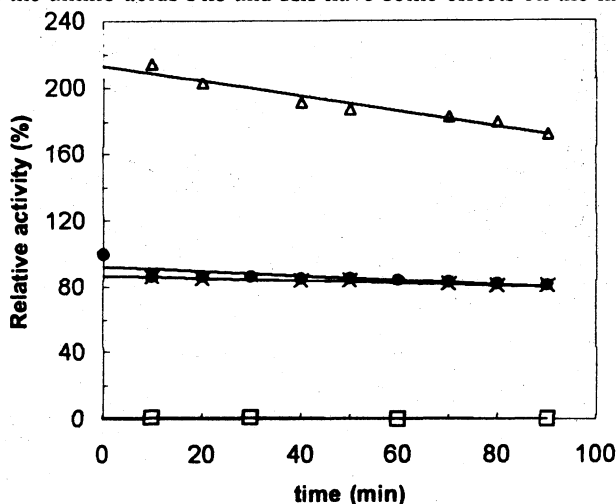

Fig. 4. Thermal stability of carbonic anhydrase in the absence (●) and in the presence of Phe (×), His (Δ) and Asp (⊔). The buffer solution was Tris 50 mM, pH = 7.5. The remaining activity of enzyme was assayed after the time of being incubated at 67°C followed by cooling for 1 h.

the enzyme while Asp cannot stabilize this folded state. So, the thermal transition temperature does not change but the ΔG^o_{25} is altered in such a way, which is almost in coincidence with the heat stability data. The experiment was repeated at 65°C, which concluded the same results. Besides, at 70°C the enzyme in the absence and presence of each of these three amino acids was irreversibly denatured (data not shown).

To understand the structural differences of the enzyme in the absence and presence of the three amino acids, CD spectra of CA in far-UV region were recorded (Fig. 5a). Detailed structural differences can be calculated[30], which is reported in Table-1. As it is seen, there are many significant structural changes in the ratio of secondary structures. It seems that Phe has decreased the per cent of helix and β-structures, while His has increased the per cent of helix and Asp has increased the per cent of both helix and β-structures. Besides, Fig. 5b shows the near-UV CD spectra of CA in the absence and presence of Phe, His and Asp. In all three cases, the amount of θ in the region of 250–270 nm is increased, which can mean that the Phe residues of the protein are somehow closer to each other. On the other hand, the amount of θ in the region of 285 nm shows different schemes. For His and Phe, θ is increased, while decreased in the presence of Asp.
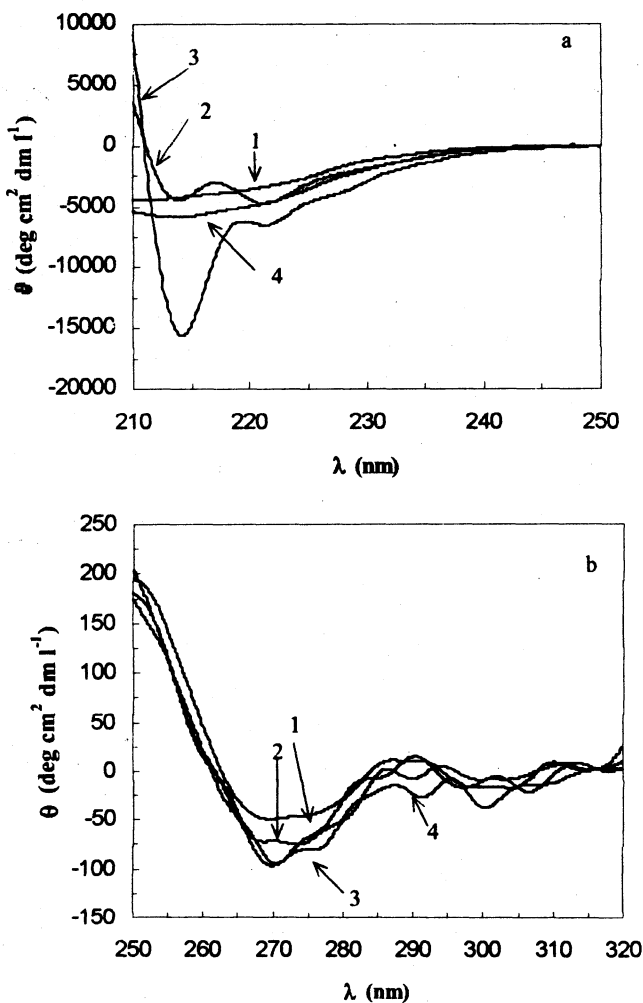
Fig. 5. Far-UV CD (a) and near-UV CD (b) spectra of carbonic anhydrase in the absence of osmolytes (1) and presence of Phe (2), His (3) and Asp (4).

TABLE-1

THE PER CENT OF SECONDARY STRUCTURES OF THE ENZYME IN THE ABSENCE AND PRESENCE OF THREE DIFFERENT AMINO ACIDS

	% Helix	% β-structures	% Random coil
CA	18.8	24.6	56.5
CA + Phe	17.0	18.2	64.8
CA + His	25.4	21.7	52.9
CA + Asp	21.0	30.3	48.7

It may be the result of some change in the conformation of the enzyme, which can make the Tyr residues closer to each other in the presence of His and Phe, and make them farther from each other, in the presence of Asp.

The amount of θ in the region of 290–320 nm, which is related to Trp residues of CA is comparable with fluorescence curves (Fig. 6). For Asp and Phe, θ in this region is somehow constant and less than the sole enzyme, respectively. These data can mean furthering the Trp residues of the enzyme in the presence of Phe,
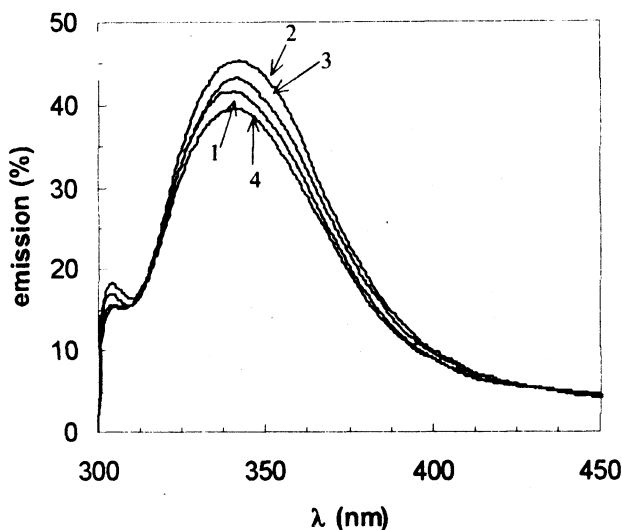

Fig. 6.   The intrinsic fluorescence plot for carbonic anhydrase in Tris 50 mM, pH = 7.5, in the absence of osmolytes (1) and in the presence of Phe (2), His (3) and Asp (4).The excitation wavelength was 280 nm.

which is in agreement with enhancement of Trp residues emission in the same case. On the other hand, in the presence of Asp, the emission of Trp residues of CA is decreased, which can be the result of some contractions in the structure of the enzyme. When CA is in the presence of His, neither the ellipticity in the region of 290–320 nm, nor the intrinsic fluorescence shows considerable change.

In an overall view, these three amino acids Phe, His and Asp as three different osmolytes, Phe with hydrophobic characteristics, His as a positive charged amino acid and Asp as a polar amino acid with negative charge, may have some differences and some similarities in their effect on CA. When CA is in the presence of Phe, it can be considered in a more hydrophobic environment, so the conformation of the enzyme is somehow exposed. However, it doesn't mean that the thermal stability of the protein is decreased. Besides, the enzyme is somehow more stable against inactivation alongside of time.

His, as a polar amino acid with positive charge, makes the enzyme get some extra secondary structure, while the tertiary structure is not significantly changed. These features in addition to the helping role of histidines in the environmental solution to the histidine residues of active site, have made His a potent activator and stabilizer.

On the other hand, it seems that Asp, as a negatively charged amino acid, may induce some contraction to the enzyme, so that although the enzymatic activity may be decreased in the presence of Asp, but that contraction may help it remain active for longer periods of time. But Asp may not act as a good stabilizer against temperature.

Since there is some evidence that Phe and imidazole may have some pharmacological effect in some conditions with decreased amount of CA, it is suggested to do some experiments *ex-vivo* with these amino acids or similar ones and have some conclusions for choosing a potent drug which increases the CA activity and stabilizes the existent molecules of CA. In such cases, *in-vitro* information about the effect of such substances on the protein can be valuable.

ACKNOWLEDGEMENTS

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