Journal of Biochemistry and Molecular Biology, Vol. 39, No. 5, September 2006, pp. 636-641

JOURNAL OF Biochemistry and Molecular Biology

Interaction of Native and Apo-carbonic Anhydrase with Hydrophobic Adsorbents: A Comparative Structure-function Study

Zahra Salemi[1], Saman Hosseinkhani[2], Bijan Ranjbar[2] and Mohsen Nemat-Gorgani[1,3,*]

[1]Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran

[2]Department of Biochemistry, Faculty of Basic Science, Tarbiat Modarres University, Tehran, Iran

[3]Stanford Genome Technology Center, Stanford University, Palo Alto, CA, USA

Received 4 April 2006, Accepted 14 June 2006

Our previous studies indicated that native carbonic anhydrase does not interact with hydrophobic adsorbents and that it acquires this ability upon denaturation. In the present study, an apo form of the enzyme was prepared by removal of zinc and a comparative study was performed on some characteristic features of the apo and native forms by far- and near-UV circular dichroism (CD), intrinsic fluorescent spectroscopy, 1-anilino naphthalene-8-sulfonate (ANS) binding, fluorescence quenching by acrylamide, and Tm measurement. Results indicate that protein flexibility is enhanced and the hydrophobic sites become more exposed upon conversion to the apo form. Accordingly, the apo structure showed a greater affinity for interaction with hydrophobic adsorbents as compared with the native structure. As observed for the native enzyme, heat denaturation of the apo form promoted interaction with alkyl residues present on the adsorbents and, by cooling followed by addition of zinc, catalytically-active immobilized preparations were obtained.

Keywords: Adsorptive immobilization, Apo-carbonic anhydrase, Hydrophobic matrices, Molten globule

Introduction

A significant proportion of all known enzymes, perhaps as high as one-third, are metalloproteins and require metal cofactors for their biological function. Zinc is one of the two most commonly bound transition metals, second only to iron (Holm *et al*., 1996).

Bovine carbonic anhydrase (EC 4.2.1.1) is a metalloenzyme containing one atom of bound zinc per molecule of 30,000 molecular weight. Carbonic anhydrase is the earliest known zinc metalloenzyme and catalyzes the reversible hydration of CO_2 in solution (Cleland and Wang, 1990). The enzyme has been extracted from animal and plant tissues and is also found in some strains of bacterial genus *Niesseria*. The main role of carbonic anhydrase in erythrocytes is the transport of hydrogen and bicarbonate ions through the cell membrane (Waygood, 1955). The metal is firmly bound, since it is not removed by extensive electrodialysis (Scott and Mendiv, 1941) and does not exchange with radioactive zinc at neutral pH (Tupper *et al.,* 1952).

It has been found, however that on dialysis against a chelating agent such as 1,10- phenanthroline, at pH 5, zinc is released with concomitant loss of catalytic activity. On addition of zinc to the zinc-depleted enzyme, the activity is completely regained (Lindskog and Malmström, 1960). Thermodynamic parameters for binding of zinc to the apoenzyme, obtained by combining the calorimetric results of Henkens *et al.* (Henkens *et al.,* 1969) with equilibrium measurements of Lindskog and Malmström (Lindskog and Malmström, 1962) show that zinc is very tightly bound and that an unfavorable enthalpy of binding is overwhelmed by a very large entropy increase. The binding of zinc is relatively rapid while its dissociation is very slow (Henkens and Sturtevant, 1968). Apo carbonic anhydrase has been widely used for preparation of zinc sensors. Apo carbonic anhydrase has also been employed for fluorescence–based biosensing of zinc in nano - picomolar amounts (Thompson and Patchan, 1995; Thompson *et al.,* 2000). The approach is based upon the energy transfer from a fluorescent label, when bound to the enzyme. Moreover, non-Redox active proteins and enzymes have been immobilized on a variety of electrode surfaces in an effort to understand and utilize their electrochemistry in applications such as biosensors. For example, The BCA-graphite rod system provides an excellent opportunity to investigate the effect of an applied potential on immobilized

*To whom correspondence should be addressed.
Tel: 1-650-812-1961; Fax: 1-650-812-1975
E-mail: mohsenn@stanford.edu

enzyme activity (McLachlan and Crumbiss, 1991).

Our previous studies indicated that carbonic anhydrase is not normally immobilized on palmityl-Sepharose, a hydrophobic support used to immobilize a number of enzymes with retention of native properties (Nemat-Gorgani and Karimian, 1982). This was taken to suggest unavailability of the hydrophobic side chains in the native protein structure for interaction with alkyl arms present on the surface of the matrix. More recent studies indicated the effectiveness of reversible denaturation for its immobilization on hydrophobic adsorbents (Azari and Nemat-Gorgani, 1999).

In the present communication, a comparative study was performed on some of the structural properties of the native and apo forms in addition to their capacity for binding to hydrophobic adsorbents. It was found that the apo form had a greater affinity for interaction with the hydrophobic ligands as compared with the native structure. Also, as for the native enzyme (Azari and Nemat-Gorgani, 1999), heat denaturation promoted immobilization of the apo structure and, by heating and subsequent cooling, followed by addition of zinc; an active immobilized preparation was obtained. Comparative studies on the structural properties of the apo and native forms explained the reasons for the observed differences in relation to binding affinities of the two forms for hydrophobic supports.

Material and Methods

Materials. Bovine carbonic anhydrase (BCA) and all other biochemicals were purchased from Sigma. All chemicals were of analytical reagent grade. Reproducibility of the data presented in this manuscript was confirmed by repeating the experiments at least twice.

Enzyme assay. A stock solution of p-nitro phenyl acetate in acetonitril (5×10^{-3} M) was used. The esterase activity of bovine carbonic anhydrase was normally measured at 25°C by addition of 10 μl of the substrate solution to 1 ml of Tris-sulfate buffer, pH 7.5. Rate of appearance of p-nitrophenolate ion at 400 nm was determined by a previously reported procedure (Azari and Nemat-Gorgani, 1999). Protein concentration was determined by the Lowry method (Lowry *et al.,* 1951).

Preparation of apo carbonic anhydrase. All buffers and solutions were prepared using deionized water and stored in plastic ware. Metal-free carbonic anhydrase was prepared using dialysis of enzyme solution against 0.1 M sodium acetate, pH 5.0, containing 2×10^{-3} M 1,10-phenanthroline at 4°C, for seven days. Finally, dialysis against few changes of metal-free buffer was carried out (Lindskog and Malmström, 1962). Atomic absorption and activity measurements indicated that removal of zinc was at least 96% complete. The apoenzyme preparations were fully reactivable with exactly one equivalent of Zn^{2+}.

Preparation of Sepharose-lipid and immobilized native and apo forms. Preparation of hexyl, octyl, dodecyl, and palmityl glycidyl ethers and their coupling to Sepharose 4B were carried out following the procedure described earlier (Nemat-Gorgani and Karimian, 1982).

An enzyme solution (native or apo) of a known concentration (150 μg/ml) in 50 mM Tris-sulfate pH 7.5 was incubated at 60°C for 30 min. 0.5 ml of pre-heated adsorbent at the corresponding temperature was added. The mixture was shaken for another 30 minutes at the same temperature followed by cooling at 4°C, for 30 min. Upon centrifugation, the pellet was washed with the Tris-sulfate buffer to remove unbound protein. Protein concentration was subsequently determined for all fractions. Zinc was added to the immobilized apo form (1 : 1) followed by activity determination using a procedure reported earlier (Azari and Nemat-Gorgani, 1999) and employing 0.1 ml of packed matrix.

Fluorescence measurements. Fluorescence emission spectra were recorded on a Perkin-Elmer luminescence spectrofluorimeter LS 50B. Experiments were carried out at 25°C. The tryptophan residues were selectively excited at 295 nm. Emission spectra were recorded between 300 and 450 nm, for the native and apo forms. Final protein concentration was 300 μg/ml.

Fluorescence quenching experiments were performed to measure solvent accessibility of Trp residues. The experiments were performed in 0.05 M Tris buffer adjusted to pH 7.5 with H_2SO_4. Fluorescence quenching was carried out by the addition of 2 M acrylamide to protein solutions (20 μg/ml) using an excitation wavelength of 280 nm and emission spectra were recorded between 300 and 400 nm. Quenching data were analyzed in terms of the Stern-Volmer constant, Ksv, which was calculated from the ratio of unquenched and quenched fluorescence intensities, F_0/F, using the relationships $F_0/F = 1 + Ksv\ [Q]$. Q is the molar concentration of the quencher (Eftink and Ghiron, 1976). The intrinsic protein fluorescence F was corrected for the acrylamide inner filter effect f, the latter being defined as $f = 10^{-\varepsilon[Q]/2}$ using an extinction coefficient of 4.3 $M^{-1}\,cm^{-1}$ for acrylamide at 280 nm.

Extrinsic fluorescence studies were carried out as outlined earlier (Azari *et al.,* 2001), using ANS (8-anilino-1-naphthalene-sulfonate) as a fluorescence probe. Protein concentration was 100 μg/ml and ANS was 50 μM, using 50 mM Tris-sulfate pH 7.5. The ANS emission was scanned between 400 and 600 nm with an excitation wavelength of 350 nm.

Circular dichroism (CD) studies. CD spectra were recorded on a JASCO J-715 spectropolarimeter equipped with a thermostatically-controlled cell holder. The instrument was calibrated with (+)-10-camphorsulfonic acid using protein concentration of about 0.2 and 1.2 mg/ml for far- and near-UV regions respectively. Results are expressed as molar ellipticity [Θ] (deg cm^2 $dmol^{-1}$), based on a mean amino acid residue weight (MRW) of 112 for BCA. The molar ellipticity [Θ] was calculated from the formula $[\Theta]_\lambda = (\theta \times 100\ MRW)/\ (cl)$, where c is the protein concentration in mg/ml, l the light path length in centimeters, and θ is the measured ellipticity in degrees at wavelength λ.

Determination of T_m. The enzyme was dissolved in 50 mM Tris-sulfate buffer pH 7.5 to a final concentration of 100 μg/ml. T_m was determined by continuous heating of the protein solution at a rate of 1°C/min using a Cary 100 spectrophotometer. Melting curve and

Tm determination were obtained based on increase in absorbance at different temperatures. Melting curves were constructed automatically at the constant wavelength of 280 nm and analysis of thermal denaturation data for this experiment was performed by the use of "Derivative method", which displays the first derivative of hyperchromacity (dH/dt) as a function of temperature.

Determination of thermal stability. To determine the time course of irreversible thermoinactivation of free and immobilized enzyme in its native and apo forms, samples were incubated at 60ºC for 10-90 min, followed by cooling at 4ºC for 1 h. The esterase activity was subsequently measured at 25ºC in the usual manner.

Aggregation measurements. The extent of aggregation was determined by measuring turbidity at 415 nm as reported earlier (Miroliaei and Nemat-Gorgani, 2001; Zhang *et al.,* 1993). The protein solution (100 μg/ml in 50 mM Tris-sulfate pH 7.5) was incubated at 65ºC. The increase in absorbance (apparent) against time was determined using a Shimadzu (UV-160) spectrophotometer.

Results and Discussion

We have recently reported that carbonic anhydrase takes up intermediate structures during renaturation of its denatured forms, and thereby acquires the capacity to interact with alkyl-substituted Sepharose (Azari and Nemat-Gorgani, 1999). In the present study, an apo form of the enzyme was prepared and its structural properties were compared with those of the native enzyme, especially in relation to its capacity to interact with hydrophobic ligands, presented to the surface of the protein molecule. Using the same strategy as for the native structure (Azari and Nemat-Gorgani, 1999), the propensity for available hydrophobic sites was enhanced.

Carbonic anhydrase provides an interesting example of a protein which takes up the so-called "molten-globular" intermediate structure in the process of going through a fully-unfolded form to a native conformation (Ikai *et al*., 1978; Hughson *et al*., 1991; Uversky *et al*., 1992). The intermediate state is characterized by high mobility of side chains (Mitaku *et al.,* 1991).

Interaction of native and apo forms with hydrophobic adsorbents. Interaction of native and apo carbonic anyhdrase and their intermediate structures with hydrophobic adsorbents was carried out (Table 1). As evident, the apo form showed a relatively higher affinity for binding. Also, heat denaturation, reported previously to promote adsorptive immobilization of the native enzyme to palmityl Sepharose (Azari and Nemat-Gorgani, 1999), was effective in improving immobilization of the apo enzyme to hydrophobic adsorbents (Table 1). In all cases, catalytically-active immobilized preparations were obtained with improved activities, as compared to the free forms (Table 1). This occurred in spite of diffusion and mass-transfer limitations brought about by immobilization, as evident by a clear increase in Km (app) (Hosseinkhani and Nemat-Gorgani, 2003). The Human carbonic anhydrase shares high similarities with the bovine enzyme (Lindskog *et al.,* 1971). Crystal structure of apo-carbonic anhydrase reveals absence of waters 381 and 389 upon removal of zinc, which may explain differences in the affinity of the two forms of the enzyme for interaction with hydrophobic adsorbents. It has been shown that metal binding specificity in carbonic anhydrase is influenced by conserved hydrophobic core residues (Hunt *et al.,* 1999).

Intrinsic fluorescence. Fluorescence emission spectra of native and apo-carbonic anhydrase carried out at the same protein concentration are depicted in Fig. 1A. As indicated, the native structure shows a higher fluorescence emission, presumably due to a greater structural rigidity. Upon addition of zinc to the apo form, the protein exhibited native fluorescence intensity (results not shown).

Fluorescence quenching by acrylamide. The results of quenching experiments allow us to assess relative solvent exposure of different types of fluorophores. The more exposed a flurophore is, the more effective collisional quencher will be in reducing the fluorescence intensity displayed by that molecule (Eftink and Ghiron, 1976; Varley and Pain, 1991; d'Amico *et al.,* 2003). The Stern-Volmer plots for quenching of intrinsic protein fluorescence by acrylamide are depicted in

Table 1. Immobilization (%) and specific activity of native and apo-carbonic anhydrase on alkyl-substituted Sepharose 4B

Matrix	Immobilization (%)		Specific activity *(U/mg)	
	Native	Apo	Native	Apo
Hexyl Sepharose	35 (2)	50 (5)	1.80 ± 0.05	1.60 ± 0.05
Octyl Sepharose	45 (4)	61 (12)	1.60 ± 0.05	1.50 ± 0.05
Dodecyl Sepharose	70 (6)	75 (15)	1.20 ± 0.05	1.00 ± 0.05
Hexadecyl Sepharose	80 (9)	85 (20)	0.60 ± 0.03	0.50 ± 0.03

To 0.25 ml of Sepharose-lipid, 0.5 ml of the enzyme solution (150 μg/ml) pre-incubated for 30 min at 60ºC was added. The mixtures were left for an additional 30 min at this temperature followed by placing on ice for 15 min. The extent of immobilization and specific activity was then determined. The number in parentheses indicate immobilization (%) at room temperature (without denaturation-renaturation). Specific activities of native and apo carbonic anhydrase in free form were 0.40 ± 0.02 and 0.35 ± 0.02 respectively.
*Specific activity determined as micromoles of p-nitrophenol produced/min/mg protein.

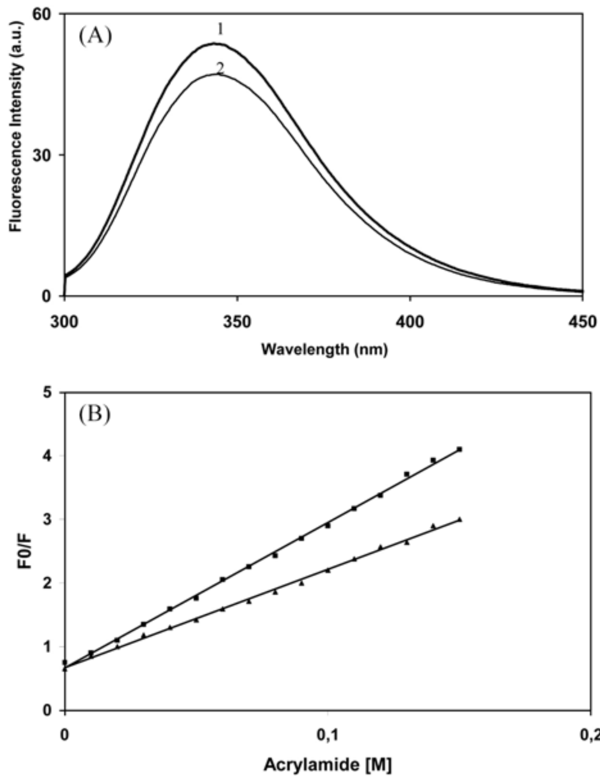

Fig. 1. (A) Fluorescence emission spectra of carbonic anhydrase in native (1) and apo (2) forms. Final protein concentration was 300 μg/ml and the experiment was performed at 25°C. (B) Stern-Volmer plots of native (▲) and apo (■) forms of carbonic anhydrase obtained by quenching with acrylamide. Please see Materials and Methods for details.

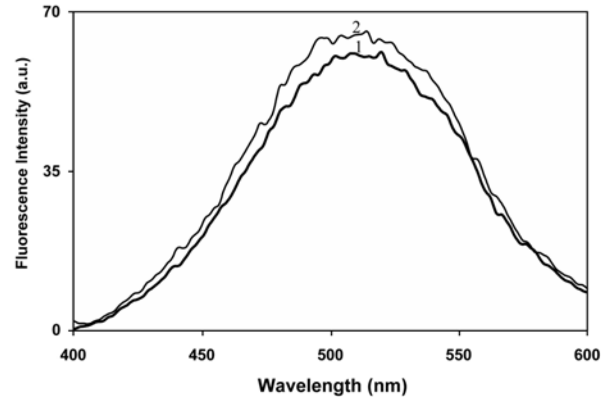

Fig. 2. Fluorescence spectra of 50 μM ANS in the presence of native (1) and apo (2) forms of carbonic anhydrase at pH 7.5, using 50 mM Tris sulfate.

Fig. 1B. Results indicate that fluorescence of the aromatic amino acids in the apo form is more quenched than it is in the case of native protein.

ANS binding. It has been established that the semi-flexible structure of the molten globular state permits exposure of hydrophobic groups in the protein structure. Accordingly, this probe has been found to provide a particularly useful test for the molten globule state (Ptitsyn *et al.,* 1990). It is bound to the equilibrium molten globule much more strongly than the native and unfolded states with a dramatic increase in its fluorescence intensity. Our own previous reports involving enhancement of surface hydrophobicity via provision of such intermediate structures, indicated that this is indeed the case (Hosseinkhani and Nemat-Gorgani, 2003). In the present study, protein adsorption on hydrophobic supports was improved (Table 1) concomitantly with ANS fluorescence enhancement (Fig. 2), upon formation of the apo structure. The relatively small increase in ANS fluorescence upon removal of zinc from native carbonic anhyhdrase (Fig. 2), may be taken to suggest that the enzyme takes up a structure similar to the intermediate forms attained upon denaturation of the native molecule (Hosseinkhani and Nemat-Gorgani, 2003).

CD spectra. The near-UV CD spectra of bovine carbonic anhydrase and its apo form are shown in Fig. 3A. The spectrum corresponding to the native enzyme contains a major positive dichroic absorption band near 250 nm, a negative band near 270 nm, and a number of small bands above 270 nm, similar to that reported previously (Coleman, 1968). As for the apo form, the spectrum is identical to the native enzyme, suggesting that loss of zinc does not extensively alter protein structure around the aromatic chromophores.

The far-UV spectra presented in Fig. 3B indicate relatively small differences in the secondary structures of the two forms. Many metal ion-binding proteins have been shown to unfold through the molten globule state. It has been suggested that removal of zinc alters the propensity of BCA to adopt an intermediate state and destabilizes the protein structure (Rajaraman *et al.,* 1996)

T_m. Tm, determined spectrophotometrically, was found to be 68°C for the native and 60°C for the apo form. This difference provides further support for the indication that the enzyme becomes more flexible upon loss of zinc.

Thermal stability. Irreversible thermoinactivation runs involving native and apo carbonic anhydrase in free and immobilized forms were carried out at 60°C (Fig. 4). As indicated, both forms were substantially stabilized upon immobilization, especially on hexyl Sepharose. A greater degree of stabilization on this matrix consisting of the shortest alkyl residues employed may be due to a higher degree of substitution (Hosseinkhani *et al.,* 2003), resulting in multipoint attachment and/or a lower possibility of denaturation due to unfavorably-extensive hydrophobic interactions involving the longer alkyl residues at the high temperature utilized. It is also noteworthy that the patterns of stabilization by immobilization on various adsorbents tested were similar for both forms of the enzyme, suggesting that the overall density-distribution of hydrophobic patches on the native protein structure was not dramatically

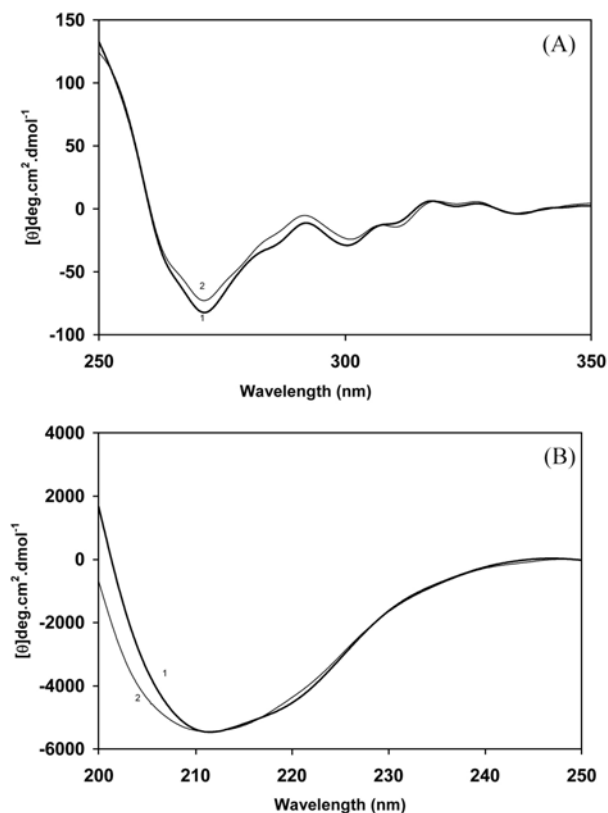

Fig. 3. Near-UV (A) and far-UV (B) CD spectra of native (1) and apo (2) forms of carbonic anhydrase. Further details are described in Materials and Methods.

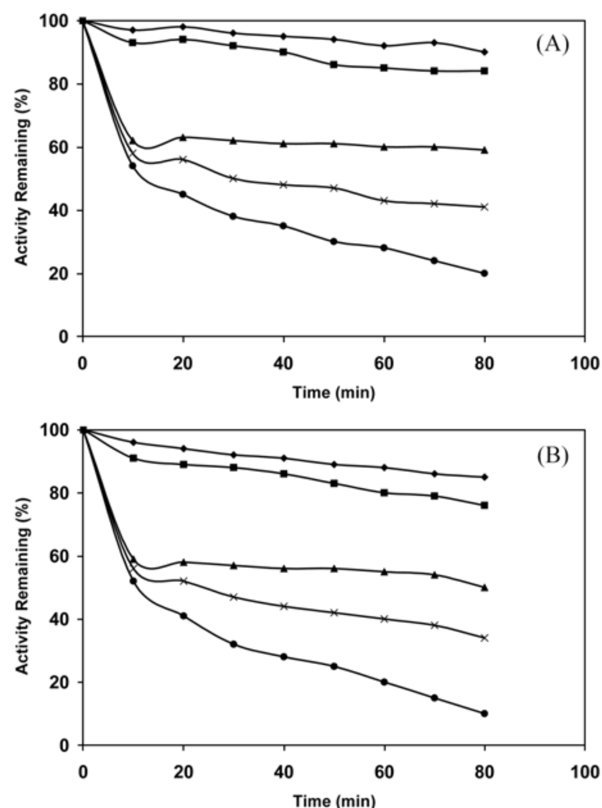

Fig. 4. Thermal stability of native (A) and apo (B) carbonic anhydrase in free and immobilized forms. Preparations of native and apo forms, either free (•) or immobilized on hexyl (◆) octyl (■), dodecyl-(▲) and palmityl-(×) Sepharose 4B, were incubated at 60°C for 10-90 min followed by cooling on ice and remaining activities were determined. Additional details are provided under Materials and Methods.

altered upon loss of zinc. This is in line with the data presented in Fig. 2, indicating a limited increase in preponderance of hydrophobic sites in the protein structure upon formation of the apo structure.

Aggregation of native and apo-carbonic anhydrase. Aggregation was found to be more extensive for the apo form than for the native structure (Fig. 5). This is in line with an increase in hydrophobicity and flexibility combined with a lower thermostability, upon loss of zinc. Proline and some other additives tested (including sugars, glycerol, acetaldehyde and ethylene glycol) afforded protection against aggregation (results not shown). Moreover, in the present study, proline was found to prevent immobilization involving the two forms (results not shown) as reported earlier for the native structure (Hosseinkhani and Nemat-Gorgani, 2003). It may therefore be concluded that aggregation, an important mechanism of irreversible thermoinactivation, is enhanced upon conversion to the apo form and is diminished by its interaction with the hydrophobic chains present on the adsorbents.

Data presented above may be of important physiological significance related to function of proteins in general and metalloproteins in particular. Accordingly, a distinct destabilization of the apo form, and not the holo structure, may contribute to important pathological consequences (Lindberg *et al.,* 2002).

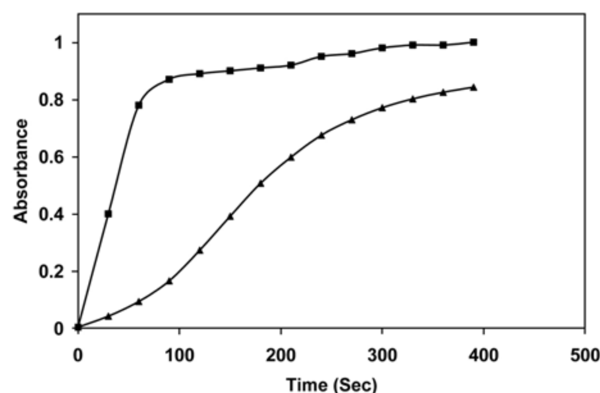

Fig. 5. Aggregation of native (▲) and apo (■) forms of carbonic anhydrase. Further details are described under Materials and Methods.

In conclusion, it is suggested that the type of studies presented here may give useful information in connection with structure-activity of native and apo forms of carbonic anhydrase as a metalloprotein. Moreover, the role of zinc in

providing the protein molecule with its native structural characteristics is delineated.

Acknowledgment Financial support for this work was provided by the Research Council, University of Tehran.

References

Azari, F., Hosseinkhani, S. and Nemat-Gorgani, M. (2001) Use of reversible denaturation for adsorptive immobilization of Urease. *Appl. Biochem. Biotechnol*. **94**, 265-277.

Azari, F. and Nemat-Gorgani, M. (1999) Reversible denaturation of carbonic anhydrase provides a method for its adsorptive immobilization. *Biotechnol. Bioeng.* **62**, 193-197.

Cleland, J. L. and Wang D. I. C. (1990) Refolding and aggregation of bovine carbonic anhydrase B: Quasi-elastic light scattering analysis. *Biochemistry* **29**, 11072-11078.

Coleman, J. E. (1968) Carbonic anhydrase-azosulfonamide complexes. *J. Biol. Chem.* **243**, 4574-4587.

d'Amico, S., Marx, J. C., Gerday, C. and Feller, G. (2003) Activity-stability relationships in extremophilic enzymes. *J. Biol. Chem.* **278**, 7891-7896.

Eftink, M. R. and Ghiron, C. A. (1976) Exposure of tryptophanyl residues and protein dynamics. *Biochemistry* **16**, 5546-5551.

Håkansson, K., Carlsson, M., Svensson, L. A. and Liljas, A. (1992) Structure of native and apo carbonic anhydrase II and structure of some of its anion-ligand complexes. *J. Mol. Biol*. **227**, 1192-1204.

Henkens, R. W. and Sturtevant, J. M. (1968) The kinetics of the binding of zinc (II) by apocarbonic anhydrase. *J. AM. Chem. Soc*. **90**, 2669-2676.

Henkens, R. W., Watt, G. D. and Sturtevant, J. M. (1969) The enthalpy of binding of various transition metal ions to bovine apocarbonic anhydrase. *Biochemistry* **8**, 1874-1878.

Holm, R. H., Kennepohl, P. and Solomon, E. I. (1996) Structural and functional aspects of metal sites in biology. *Chem.Rev.* **96**, 2239-2314.

Hosseinkhani, S. and Nemat-Gorgani, M. (2003) Partial unfolding of carbonic anhydrase provides a method for its immobilization on hydrophobic adsorbents and protects it against irreversible thermoinactivation. *Enzyme. Microb. Technol.* **33**, 179-184.

Hosseinkhani, S., Szittner, R., Nemat-Gorgani, M. and Meighen, E. (2003) Adsorptive immobilization of bacterial luciferases on alkyl-substituted Sepharose 4B. *Enzyme Microb.Technol*. **32**, 186-193.

Hughson, F. M., Barrick, D. and Baldwin, R. L. (1991) Probing the stability of a partly folded apomyoglobin intermediate by site-directed mutagenesis. *Biochemistry* **30**, 4113-4118.

Hunt, J. A., Ahmed, M. and Fierke, C. A. (1999) Metal binding specificity in carbonic anhydrase is influenced by conserved hydrophobic core residues. *Biochemistry* **38**, 9054-9062.

Ikai, A., Tanaka, S. and Noda, H. (1978) Reactivation kinetics of guanidine-denatured bovine carbonic anhydrase B. *Arch. Biochem. Biophys*. **190**, 39-45.

Lindberg, M. J., Tibell, L. and Oliveberg, M. (2002) Common denominator of Cu/Zn superoxide dismutase mutants associated with amyotrophic lateral sclerosis: Decreased stability of the apo state. *P.N.A.S.* **99**, 16607-16612.

Lindskog, S., Henderson, L. E., Kannan, K. K., Liljas, A., Nyman, P. O. and Strandberg, B. (1971) Carbonic anhydrase; in *The Enzymes*, Boyer, P. D. (ed.), pp. 587-665, Academic Press, New York, USA.

Lindskog, S. and Malmström, G. (1960) Reversible dissociation of zinc in bovine carbonic anhydrase. *Biochem. Biophys. Res. Commun*. **2**, 213-217.

Lindskog, S. and Malmström, B.G. (1962) Metal binding and catalytic activity in bovine carbonic anhydrase. *J. Biol. Chem.* **237**, 1129-1137.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

McLachlan, K. L. and Crumbliss, A. L. (1991) The effect of an applied potential activity of carbonic anhydrase immobilized on graphite rods. *Biotechnol. Bioeng.* **37**, 491-496.

Miroliaei, M. and Nemat-Gorgani, M. (2001) Sugars protect native and apo yeast alcohol dehydrogenase against irreversible thermoinactivation. *Enzyme. Microb. Technol*. **29**, 554-559.

Mitaku, S., Ishido, S., Itoh, H., Kataoka, R. and Saito, N. (1991) Hydrophobic core of molten globule state of bovine carbonic anhydrase B. *Biophy. Chem*. **40**, 217-222.

Nemat-Gorgani, M. and Karimian, K. (1982) Non-ionic adsorptive immobilization of proteins to palmityl-substituted Sepharose 4B. *Eur. J. Biochem*. **123**, 601-609.

Ptitsyn, O. B., Pain, R. H., Semisotnov, G. V., Zerovnik, E. and Razgulyaev, O. I. (1990) Evidence for a molten globule state as a general intermediate in protein folding. *FEBS Lett.* **262**, 20-24.

Rajaraman, K., Raman, B. and Rao, C. M. (1996) Molten-globule state of carbonic anhydrase binds to the chaperon-like á-crystallin. *J. Biol. Chem.* **271**, 27595-27600.

Scott, D. A. and Mendiv, J. R. (1941) Chemical observations on carbonic anhydrase. *J. Biol .Chem.* **140**, 445-451.

Thompson, R. B. and Patchan, M. W. (1995) Lifetime-based fluorescence energy transfer biosensing of zinc. *Anal. Biochem.* **227**, 123-128.

Thompson, R. B., Maliwal, B. P. and Zeng, H. H. (2000) Zinc biosensing with multiphoton excitation using carbonic anhydrase and improved fluorophores. *J. Biomed. Optics* **5**, 17-22.

Tupper, R., Watts, R. W. and Wormall, A. (1952) Some observations on the zinc in carbonic anhydrase. *Biochem. J.* **50**, 425-429.

Uversky, V. N., Semisotnov, G. V., Pain, R. H. and Ptitsyn, O. B. (1992) "All-or-none" mechanism of the molten-globule unfolding. *FEBS Lett.* **314**, 89-92.

Varley, P. G. and Pain, R. H. (1991) Relation between stability, dynamics and enzyme activity in 3-phosphoglycerate kinases from yeast and *Thermus thermophilus. J. Mol. Biol.* **220**, 531-538.

Waygood, E. R. (1955) Carbonic anhydrase (plant and animal). *Meth Enzymol.* **2**, 836-846.

Zhang, Y. L., Zhou, J. M. and Tsou, C. L. (1993) Inactivation procedes conformation change during thermal denaturation of adenylate kinase. *Biochem. Biophys. Acta* **1164**, 61-67.