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Molten Globule-Like State of Bovine Carbonic Anhydrase in the Presence of Acetonitrile

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We have evaluated the effects of acetonitrile on the structure and function of bovine carbonic anhydrase II. The potential structural and functional changes in carbonic anhydrase in the presence of different acetonitrile/buffer ratios (0%, 17.5% and 47.5% v/v) were determined using a variety of methods. These included simple spectrophotometric methods to record enzyme velocity, fluorescence measurements and calculation of accessible surface area (ASA) to identify possible alterations in tertiary structure of the protein, CD measurements to search for secondary structure conversions, and thermal scanning to determine structural stability of the protein in different media. The Far-UV CD studies indicated that carbonic anhydrase, for the most part, retains its secondary structure in the presence of acetonitrile. Fluorescence measurements using iodide ion and ANS along with ASA calculations revealed that in the presence of acetonitrile some degree of conformational change occurs in the carbonic anhydrase structure. In addition to the hydrophobic pockets, two additional tryptophanyl residues become exposed to the solvent, thereby increasing the surface hydrophobicity of the protein. These alterations dramatically reduce the catalytic activity, thermal stability, and aggregation velocity of the enzyme. Thus, our results support a molten globule-like structure of carbonic anhydrase in the presence of acetonitrile.

Key words: acetonitrile, carbonic anhydrase, molten globule, organic solvent, thermal stability.

Abbreviations: *p*-NPA, *p*-nitrophenylacetate; THF, tetrahydrofuran; Trp, tryptophan; His, histidine; ε, extinction coefficient; ANS, anilinonaphtalene sulfonic acid; KI, potassium iodide; CD, circular dichroism; ASA, accessible surface area; mAb/min, milliabsorbance per minute; a.u., arbitrary unit; v/v, volume per volume; $Å^2$, surface unit equal to one square of Angstrom.

Bovine carbonic anhydrase II [BCAII; EC 4.2.1.1] catalyzes the reversible hydration and dehydration of carbon dioxide ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$), the fundamental process in the transport of carbon dioxide in animals, plants and bacteria (*1*). The molecule consists of a single polypeptide chain with 259 amino acid residues giving a molecular weight of about 29,000. This enzyme consists of thirteen β-strands and seven α-helices surrounding the β-sheets (*1*, *2*). It contains one zinc ion in the active site coordinated to three histidine residues (His 93, His 95, His 118) and one putative water molecule in a tetrahedral geometry (*3*). The hydrolysis of some esters is also catalyzed by the enzyme, and this activity has been utilized by several investigators using *p*-nitrophenylacetate (*p*-NPA) and other phenolic esters as substrates (*4*). These materials can be handled more easily than carbon dioxide, and the reaction rates can be measured more accurately by simple spectrophotometric methods (*5*).

The enzyme behavior in anhydrous media has important applications in biotechnology. Thus far, chemical modification and solvent engineering of proteins are used to alter the catalytic power of enzymes. A typical comment of a classical enzymologist, when considering enzyme-catalyzed reactions in non-aqueous media, is that the enzyme suspended in organic solvent must have a different structure than the same enzyme in water. Indeed, activity alterations typically occur on many occasions when the enzyme is transferred into an organic solvent. However, in an organic solvent, the catalyst particle can be hydrated and remain active (*6–8*). The protein activity is sensitive to the type of the organic solvent employed. As the polarity of the organic solvent increases (*e.g.* octan → tetrahydrofuran, THF → acetonitrile, ACN) the enzymatic activity drops dramatically. The observed decrease in enzyme activity reflects the stripping of essential water molecules from around the surface of the protein, which becomes greater when the polarity of the solvent increases (*9–12*). In addition to the solvent polarity, the ability of the solvent to strip water molecules is dependent on the size of the solvent molecules. Thus, penetration of ACN molecules into the crevices of the protein structure and the stripping of the water molecules is aided by its relative small size (*12*).

The structural properties of several proteins have been determined in organic solvents such as acetonitrile, dioxane, and hexane (*13–19*). These studies showed that the overall protein structure in water and non-aqueous solutions is similar except for the reorientation of some of the

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side chains. Such reorientations near the active site may be responsible for the loss of enzymatic activity in organic media. In some reports, the decrease in enzyme activity in organic solvents was attributed to a lower conformational mobility of the protein structure (*7*). The decrease in the dielectric constant of the medium that surrounds the protein structure promotes the formation of additional hydrogen bonds among the polar residues on the protein surface. Molecular dynamic simulations (*20*, *21*) also support the establishment of more hydrogen bonds and salt bridges on the protein. Moreover, organic molecules replace some water molecules, which act as a lubricant for the protein. Thus, it seems that the protein should be more rigid in organic media as supported by crystal structures (*13*, *14*).

It has become clear that in aqueous or neat organic solvents enzymes may not only retain their catalytic activities, but also exhibit new unusual properties compared with those in water (*22–24*). For example, substrate specificity or molecular memory of the enzyme in organic solvent may be completely different from that in a water medium. Therefore, to understand the mechanistic basis of these phenomena, it is critical to elucidate protein conformations in organic media and compare them with that in aqueous solution. In the present work, this task was achieved for carbonic anhydrase by means of several methods in order to illustrate the possibility of structural alterations when acetonitrile exists as an organic solvent in the medium.

MATERIALS AND METHODS

Materials—Bovine carbonic anhydrase from erythrocytes, *p*-nitrophenylacetate (*p*-NPA), Trizma base (molecular biology grade), and anilinonaphtalene sulfonic acid (ANS) were obtained from Sigma. Acetonitrile (extra pure), potassium iodide and sodium dithionite were purchased from Merck. All other reagents were of analytical grade. All of the solutions were prepared in Tris buffer (100 mM Tris, pH 7.5) utilizing double distilled water with very low conductivity obtained by SANYO Gallencamp PLC (Fistreem Cyclon).

Methods—Enzyme assays: Carbonic anhydrase assays were performed at 20°C as described by Armstrong *et al.* (1966) and Pocker and Stone (1966) (*25*, *26*). The reaction catalyzed by the enzyme was followed at 400 nm using a Shimadzu UV-260 spectrophotometer in quartz cells with a 1 cm path length. The enzyme concentration was 1.92 μM in the presence of *p*-nitrophenylacetate (2.5 mM) as the substrate. The enzyme stock solution was prepared by dissolving 3 mg of enzyme powder in 1 ml of Tris buffer followed by dialysis at 4°C against several changes of buffer. The enzyme concentration was subsequently determined after dialysis using the extinction coefficient of $\varepsilon_{280}^{1\%} = 19.0$ previously reported for the enzyme (*27*). The stock solution of *p*-NPA was prepared by dissolving 0.05 g of the related compound in 2 ml acetonitrile to obtain a final concentration of 138 mM. In all steps of the enzyme assay, 13 μl of enzyme and 20 μl of *p*-NPA from the related stock solutions were transferred into a total volume of 500 μl for the reaction mixture. The esterase activity of the enzyme was monitored at 400 nm by the appearance of *p*-nitrophenol as the product.

Fluorescence measurements: Fluorescence emission spectra of the enzyme in three concentrations acetonitrile/buffer solution (0%, 17.5% and 47.5% v/v) were recorded using a Hitachi spectrofluorimeter model MPF-4. The selected excitation wavelength of 291 nm was almost specific for excitation of the tryptophanyl residues of the protein. Emission spectra were recorded between the wavelengths of 300 nm to 450 nm at the bandwidth of 10 nm. A filter was placed on the pathway of the emitted light to reduce the interfering effects from the scattered photons of the wavelengths under 290 nm.

The presence of accessible hydrophobic pockets in the native protein was determined using ANS under similar acetonitrile/buffer conditions. ANS is a good fluorogenic probe to demonstrate significant changes in the emission spectrum based on the hydrophobic power of the surrounding medium. The emission spectra of the enzyme-ANS complexes at 20°C were recorded between the wavelengths of 400 nm to 700 nm at a bandwidth of 10 nm and excitation wavelength of 350 nm. The enzyme and ANS concentrations in all of protein samples were 1.92 μM and 50 μM in a total volume of 500 μl, respectively. Before recording of the emission spectrum an incubation time of 2 min was allowed for ANS penetration into the accessible hydrophobic pockets in the protein structure.

The accessibility of the tryptophanyl residues was tested using iodide ion as a fluorescence quencher of the emitted photons leaving the excited tryptophanyl residues. Enzyme (1.92 μM) samples in medium containing increasing concentrations of KI (0 to 100 mM obtained from 1 M stock solution of KI containing 2 mM sodium dithionite to prevent oxidation of the iodide ions) were incubated for two minutes under the three acetonitrile/buffer conditions (0%, 17.5% and 47.5% v/v). This was done until the maximum quenching was obtained. Recording of the emission spectra was performed in the range of 300–450 nm at an excitation wavelength of 291 nm.

Calculation of the accessible surface area: Calculation of the Accessible Surface Area (ASA) was carried out at the amino acid level using the FANTOM program provided by the Sealy center of Structural Biology at the University of Texas Medical Branch. Cartesian coordinates of the atoms in carbonic anhydrase were obtained using the web service of the Protein Data Bank (PDB).

Circular dichroism studies: Far-UV CD spectra were recorded on an Aviv CD spectropolarimeter model 215 (Aviv Associates, Inc., USA) using three different protein solutions (0.5 mg/ml) prepared in 0%, 17.5% and 47.5% v/v acetonitrile/buffer. The results were expressed as molar ellipticity, [θ] (deg cm^2 $dmol^{-1}$), based on a mean amino acid residue weight (MRW). The molar ellipticity was determined as $[\theta]_\lambda = (\theta \times 100\ \mathrm{MRW})/(cl)$, where c is the protein concentration in mg/ml, l is the light path length in cm, and θ is the measured ellipticity in degrees at a given wavelength. The instrument was calibrated with (+)-10-camphorsulfonic acid, assuming $[\theta]_{291} = 7{,}820$ deg cm^2 $dmol^{-1}$, and with standard nonhygroscopic ammonium (+)-10-camphorsulfonate, assuming $[\theta]_{290.5} = 7{,}910$ deg cm^2 $dmol^{-1}$. Noise reduction was accomplished using Aviv software, including the fast Fourier-transform noise reduction routine, which allows the enhancement of most noisy spectra without a distortion of peak shapes. Spectral deconvolution was performed by the method of Bohm *et al.*

employing CDNN version 2.1 (http://bioinformatik.biochemtech.uni-halle.de/cdnn) (*28*).

Thermal scanning: The thermal stability of carbonic anhydrase was investigated by spectrophotometric methods using a Varian UV spectrophotometer (model Carry 100) equipped with a thermal accessory worked by the Peltier effect between the range of 10 to 85°C with a scanning rate of 1°C/min. Protein concentration was 1.92 μM dissolved in buffer or buffer-acetonitrile media (17.5% and 47.5% v/v).

RESULTS

Figures 1 to 3 depict the quenching effect of the iodide ion exerted on the accessible tryptophanyl residues on the carbonic anhydrase structure when the enzyme exists under three different conditions (acetonitrile/buffer = 0%, 17.5% and 47.5% v/v). In these figures, the *y* axis has been scaled using relative units (F/F_0). This ratio depicts the recorded fluorescence intensity of the protein solution in the presence of potassium iodide (F) at the emission λ_{max} of the protein (344 nm for 0% as well as 17.5% v/v and 349 nm for 47.5% v/v acetonitrile/buffer) relative to the fluorescence intensity of the protein in the related acetonitrile/buffer solution when the KI concentration is zero (F_0). The increase in iodide ion concentration was accompanied by a decrease in the recorded fluorescence intensity ratio since iodide ions existing near the accessible tryptophanyl residues in the protein are more likely at higher KI concentrations (Figs. 1–3). The point of maximum quenching can be detected by drawing a double reciprocal curve (1/[KI] against F_0/F) and extrapolating the resultant curve (relevant to the last shape of the curve) to the 1/*y* axis (dashed lines in inset a of Figs. 1, 2 and 3). With respect to the point of maximum quenching, calculation of the number of accessible tryptophanyl residues is possible. This calculation can be performed by multiplication of the F/F_0 ratio (related to the point of the maximum fluorescence quenching) that is subtracted from 1 (percent value of the protein in the absence of KI, $F/F_0 = 1$) to 7 (the total number of the existed tryptophanyl residues in the protein) (*29*). Therefore, the number of accessible tryptophanyl residues calculated under the three cited conditions are 0.92 (≈1.00), 1.75 (≈2.00) and 3.01 (≈3.00), respectively. These calculated numbers show that when the acetonitrile concentration increases from 0 to 47.5% in the medium, two additional tryptophanyl residues become exposed to the outer environment of the native structure of the protein.

Inset *b* of Fig. 1 indicates the fluorescence intensity of the protein recorded under the three described conditions at the related emission λ_{max} in the absence of KI. Thus, increasing the concentration of acetonitrile in the medium did not cause a rigorous alteration in the fluorescence

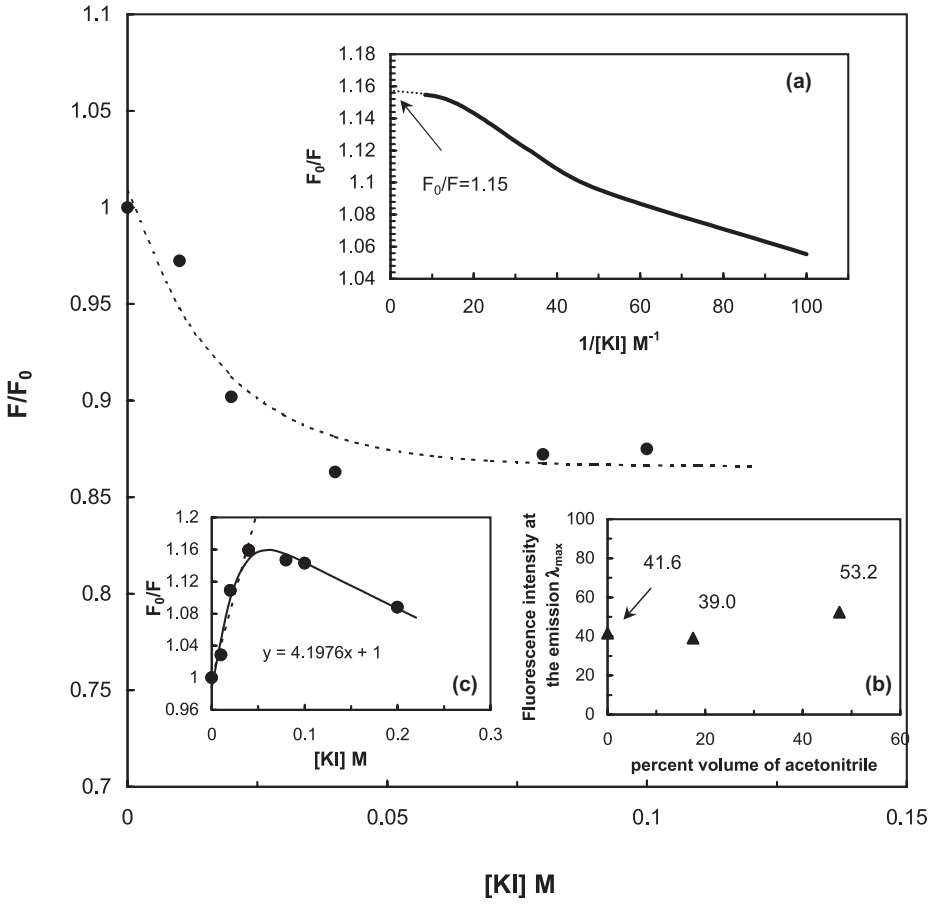

Fig. 1. **Fluorescence quenching of the tryptophanyl residues of the native structure of carbonic anhydrase.** Quenching experiments were carried out in the presence of different concentrations of KI (0–100 mM). The excitation wavelength was 291 nm and protein concentration was 1.92 μM prepared in 100 mM Tris buffer, pH 7.5. Inset a: The point of maximum quenching was determined by extrapolation of the curve (dotted curve) to the *y* axis in the double reciprocal plot. Inset b: Fluorescence alterations of three conformations of the native enzyme (prepared in 100 mM Tris buffer pH 7.5) and the protein structures in 17.5% and 47.5% v/v acetonitrile/buffer solution. The excitation wavelength was 291 nm and the protein concentration was 1.92 μM. Note that when acetonitrile is added to the buffer, there is no appreciable change in the fluorescence intensity (compare the related numbers above each point showing the emission intensity at λ_{max} = 344 nm for 0% as well as 17.5% v/v and λ_{max} = 349 nm for 47.5% v/v acetonitrile/buffer). Inset c: Stern-Volmer plot of native carbonic anhydrase. $K_{S\text{-}V}$ is calculated from the slope of the first linear phase and is equal to 4.2 M^{-1} (the mathematical equation of the dotted line is brought in the figure as a function of *y* to calculate $K_{S\text{-}V}$). *x* is a variable equivalent to [KI].

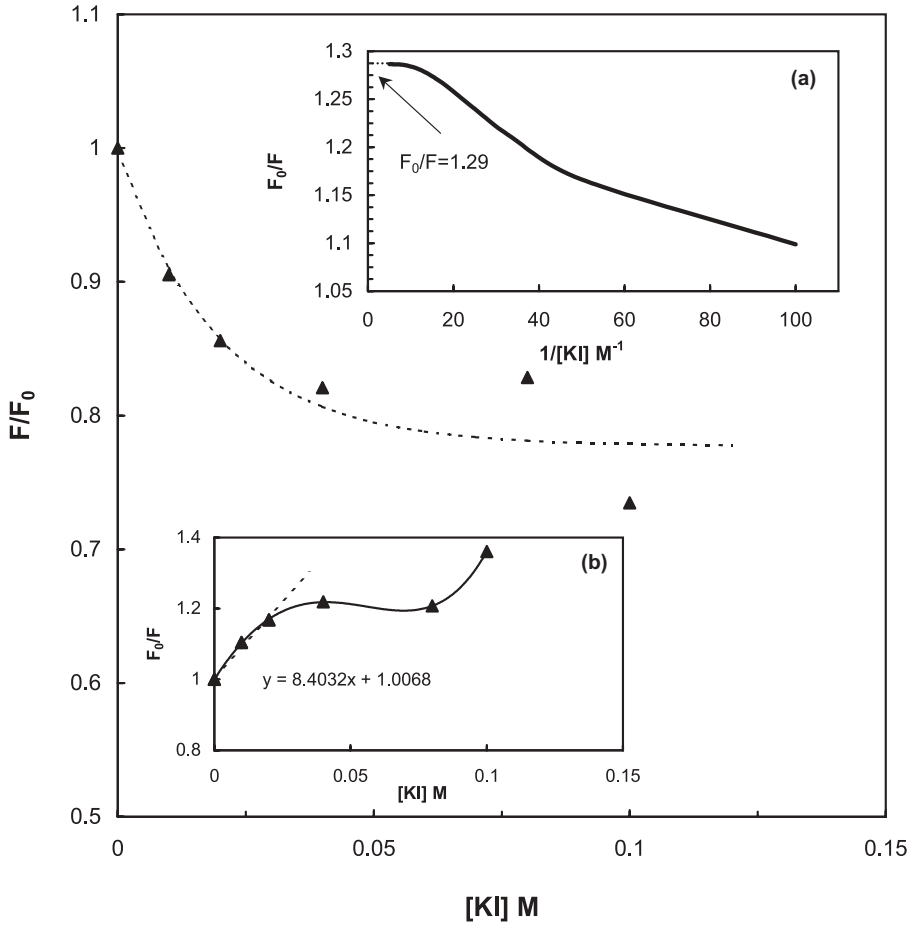

Fig. 2. **Fluorescence quenching of the tryptophanyl residues of the protein in 17.5% v/v acetonitrile/buffer.** Quenching experiments were carried out under the same conditions as described in the legend to Fig. 1. Inset a: The point of maximum quenching was determined by extrapolation of the curve (dotted curve) to the *y* axis in the double reciprocal plot. Inset b: Stern-Volmer plot for carbonic anhydrase in 17.5% v/v acetonitrile. $K_{S\text{-}V}$ is calculated from the slope of the first linear phase and is equal to 8.4 M^{-1} (the mathematical equation of the dotted line is brought in the figure as a function of *y* to calculate $K_{S\text{-}V}$). *x* is a variable equivalent to [KI].

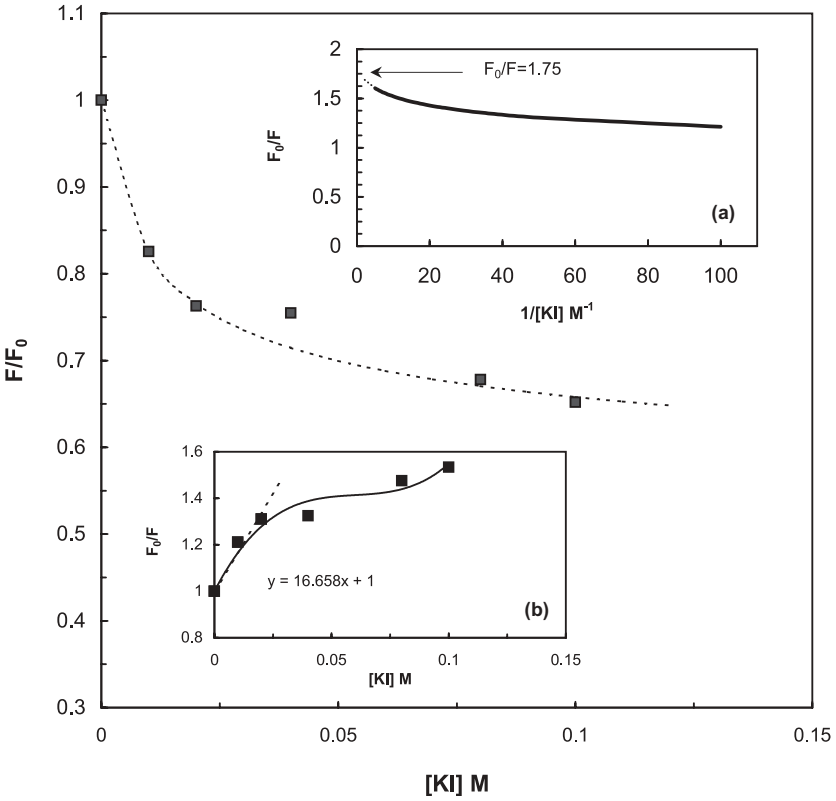

Fig. 3. **Fluorescence quenching of the tryptophanyl residues of the protein in 47.5% v/v acetonitrile/buffer.** Quenching experiments were carried out under the same conditions as described in the legend to Fig. 1. Inset a: The point of maximum quenching was determined by extrapolation of the curve (dotted curve) to the *y* axis in the double reciprocal plot. Inset b: Stern-Volmer plot for carbonic anhydrase in 47.5% v/v acetonitrile. $K_{S\text{-}V}$ is calculated from the slope of the first linear phase and is equal to 16.6 M^{-1} (the mathematical equation of the dotted line is brought in the figure as a function of *y* to calculate $K_{S\text{-}V}$). *x* is variable equivalent to [KI].

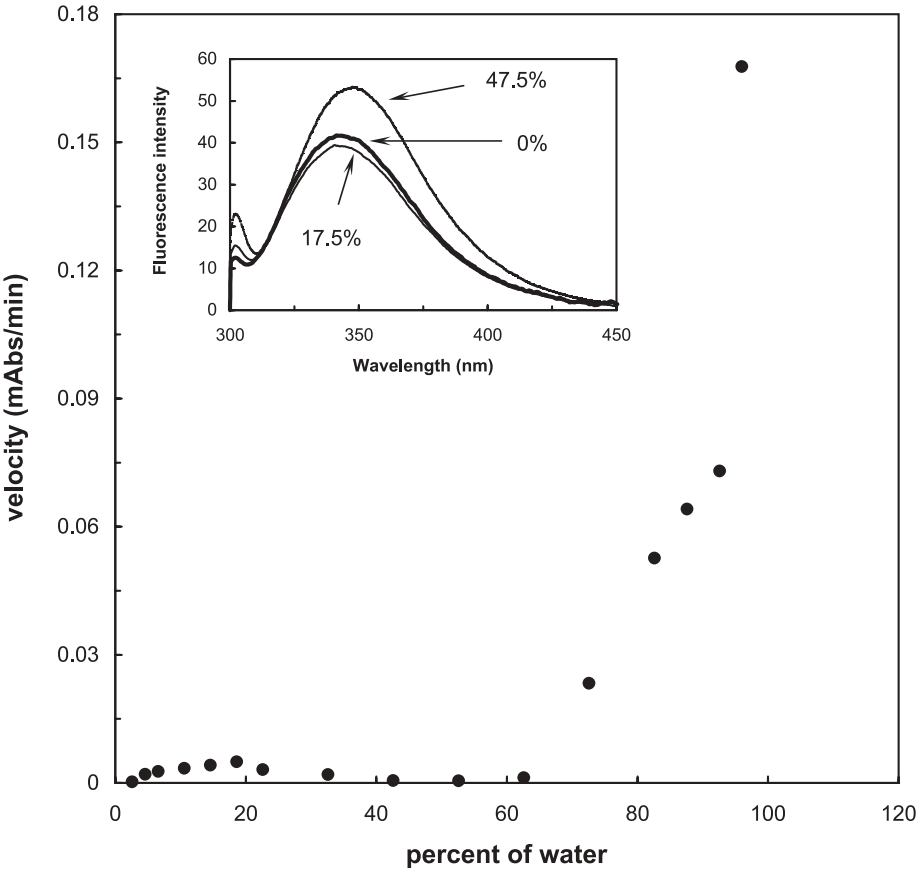

Fig. 4. **Velocity measurements of carbonic anhydrase in different percent volume acetonitrile-buffer solutions.** The enzyme and *p*-nitrophenylacetate concentrations were 1.92 μM and 2.5 mM, respectively. Inset: The fluorescence emission spectra of the protein in 0%, 17.5% and 47.5% v/v acetonitrile/buffer. The excitation wavelength was 291 nm and the protein concentration was 1.92 μM prepared in 100 mM Tris buffer, pH 7.5.

intensity, indicating that the tertiary structure of the protein was not drastically altered. However, the red shift of about 5 nm (observed when the acetonitrile concentration in the medium was equal to 47.5% v/v; see inset of Fig. 4) indicates the possibility of exposing tryptophanyl residues to the outside where polar water molecules have an adequate opportunity to accumulate around them (*29*). Moreover, the slope ($K_{S\text{-}V}$) of the first linear phase under each environmental condition was 4.2, 8.4 and 16.7 M^{-1} for 0%, 17.5% and 47.5% v/v acetonitrile, respectively, thus, showing an increase in the accessibility of tryptophanyl residues when the acetonitrile concentration in the medium is increased (inset c of Fig. 1 and insets b of Figs. 2 and 3). The slope of the Stern-Volmer plot is equal to the Stern-Volmer constant ($K_{S\text{-}V}$), indicating the degree of exposure of the tryptophanyl residues on the protein. Fully exposed Trp residues typically have a $K_{S\text{-}V}$ of 8–9 M^{-1}, whereas the $K_{S\text{-}V}$ for the buried or inaccessible tryptophans is lower and can be close to 0 M^{-1} (*30*, *31*).

The relationship between an increase in acetonitrile concentration and catalytic activity is shown in Fig. 4. The increase in acetonitrile concentration in the medium was followed by a decrease in enzyme velocity, especially at 47.5% acetonitrile. In fact, the selection of the acetonitrile/buffer ratios was first performed in our experiments relative to the selection of three critical points in the sharp declining phase of the velocity curve (Fig. 4). The possibility of the existence of structural alterations of a protein in the presence of acetonitrile can also be evaluated using ANS as a fluorogenic probe to detect exposed and accessible hydrophobic pockets in the protein (Fig. 5). The differences between each ANS-protein emission spectrum relative to the free ANS spectrum in related medium was recorded as an indication of no drastic structural alterations in the enzyme. These findings were consistent with the constancy of the intrinsic fluorescence emission of the tryptophanyl residues under the three different solvent conditions (inset b of Fig. 1), but are contradictory to the activity and thermal stability measurements that were the result of severe structural alterations in the enzyme (Figs. 4 and 7).

The CD spectra of the protein under the three different solvent conditions were recorded to determine the occurrence of the secondary structural changes (Fig. 6). Our results indicate that the presence of acetonitrile in the medium has minimal effect on the secondary structure of the protein. This was further confirmed when the molar ellipticity of the protein at 222 nm was drawn against the percent volume of acetonitrile (inset of Fig. 6). The thermal stability of the protein structure was determined relative to the native structure and is shown in Fig. 7. The stability of the protein structure in the presence of acetonitrile was drastically decreased, especially at 47.5% acetonitrile. This is consistent with the sharp decline observed in the enzyme velocity (Fig. 4).

DISCUSSION

There has been increasing interest in the determination of protein structural properties in organic solvents. Proteins may exhibit unusual characteristics under these solvent

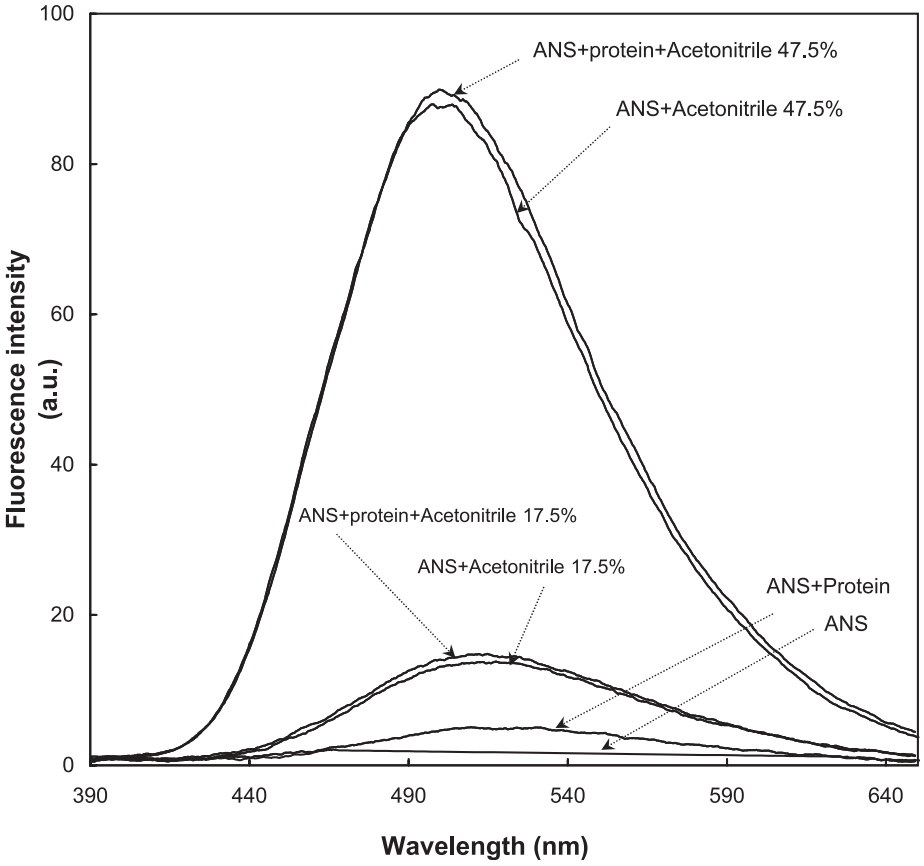

Fig. 5. **ANS checking to identify accessible hydrophobic pockets on the protein structure under three different conditions (0%, 17.5% and 47.5% v/v acetonitrile/buffer).** Each ANS-protein spectrum was drawn in relation to the spectrum of free ANS.

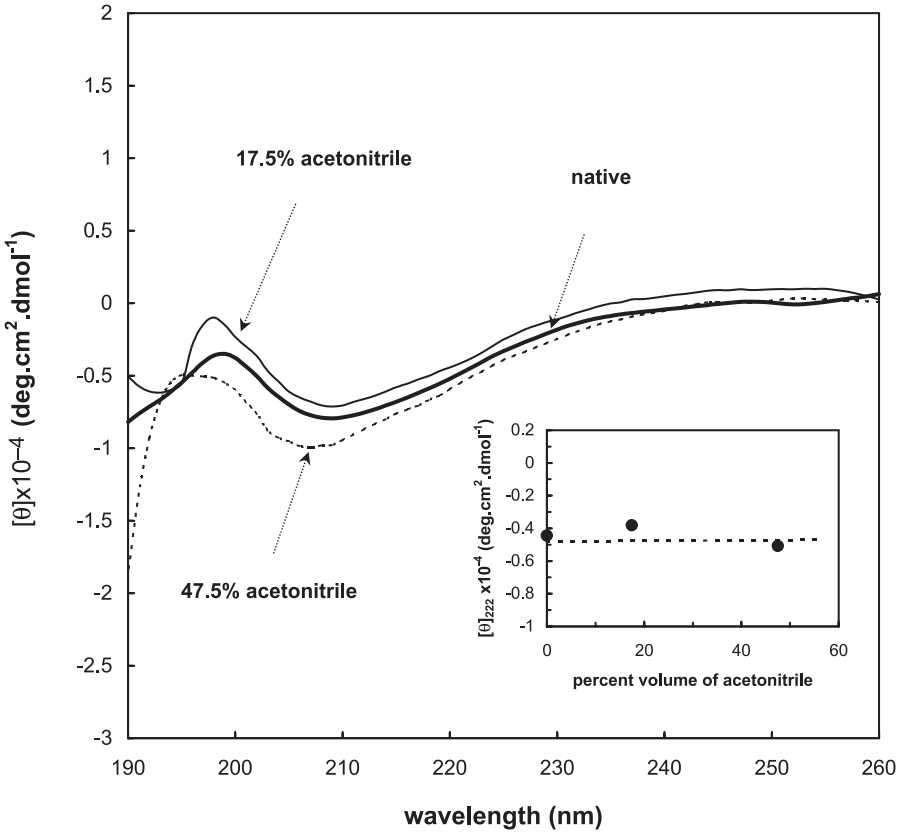

Fig. 6. **CD spectra of carbonic anhydrase solutions prepared in 0%, 17.5% and 47.5% v/v acetonitrile/buffer.** The bold, solid and dashed lines indicate the protein structures in 0%, 17.5% and 47.5% v/v acetonitrile/buffer solution, respectively. The inset indicates that relative to the native structure no considerable changes occurred in the secondary structure of the protein in 17.5% or 47.5% acetonitrile/buffer. The protein concentration was 0.5 mg/ml.

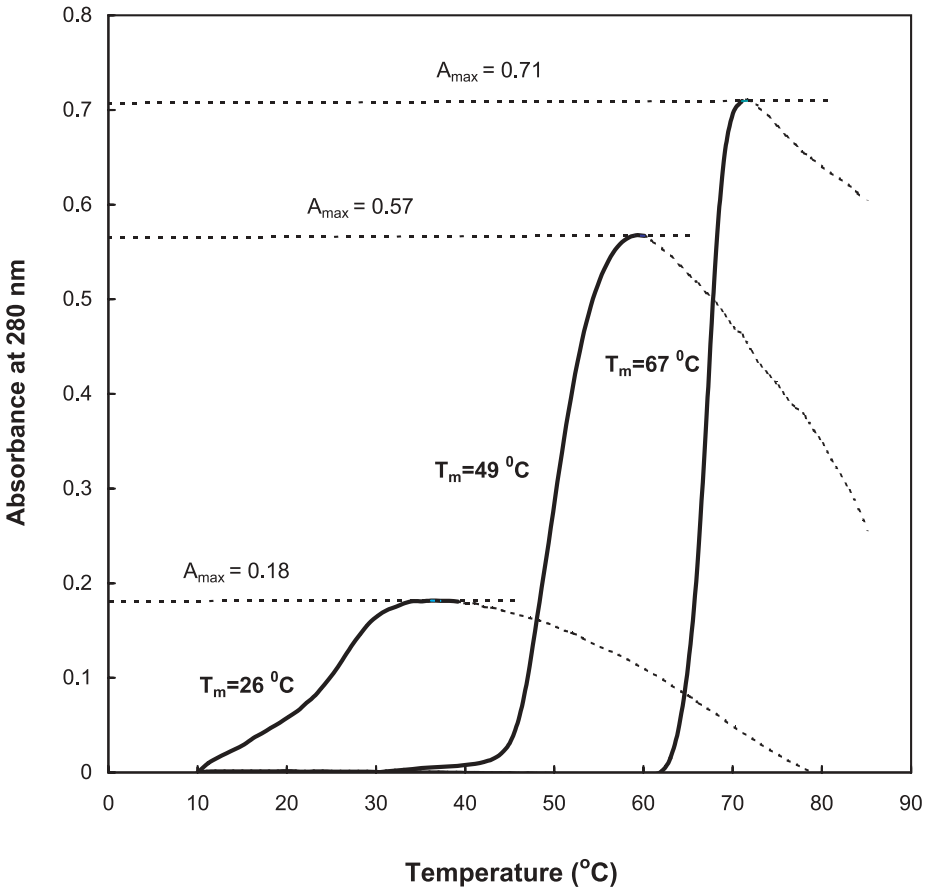

Fig. 7. **Thermal profiles of carbonic anhydrase in 0%, 17.5% and 47.5% v/v acetonitrile/buffer solutions.** The value of melting temperature (T_m values) of the protein decreases drastically with increasing acetonitrile concentration in the solution. The protein concentration was 1.92 μM. The bold lines indicate those related sigmoid curves utilized to calculate the T_m values. A_{max} is the maximum absorbance of the thermally unfolded protein solution recorded at a wavelength of 280 nm.

conditions that are important for specific applications. Acetonitrile is commonly used in many biochemical investigations as a mobile phase, especially for the separation of protein molecules from each other in biological samples. Here the effects of acetonitrile on the structure and function of bovine carbonic anhydrase were examined using several experimental techniques. Although some of the methods utilized in our studies indicate the possibility of drastic structural changes in the enzyme, other methods do not. Recording of the CD and fluorescence emission spectra of the protein under different solvent conditions did not indicate large structural changes in the enzyme (inset b of Fig. 1; Figs. 5 and 6; comparison between the calculated amounts of secondary structures tabulated in Table 1). The CD technique can distinctly determine secondary or tertiary structural alterations in proteins; however, a simple fluorescence experiment will not reveal tertiary structural changes. Inset b of Fig. 1, drawn using the emission intensity at the emission λ_{max} of the protein under three different solvent conditions, shows that the tertiary structure of the protein remains unchanged. Moreover, the fluorescence experiments using the ANS fluorogenic probe indicated that small structural alterations may occur in the protein such that the exposure of the hydrophobic pockets on the protein surface is minimal (Fig. 5). However, shifting of the fluorescence emission spectrum in parallel with the other fluorescence experiments performed using KI confirmed the existence of considerable conformational alterations induced by the acetonitrile molecules in the protein structure (Figs. 1–3 and inset of Fig. 4). These conflicting observations can be explained by the fact that when the fluorescence intensity of a protein sample is determined based on the excitation of the tryptophanyl residues, the results may fail to reveal structural alterations that are independent of significant alterations in the microenvironment around most tryptophanyl residues. It is also possible that the increase in emission of some tryptophanyl residues is compensated by quenching by others depending on their positioning near some of the existing quenching chemical groups or the polar media. Moreover, protein structural alterations may occur in such a way that ANS penetration into the exposed hydrophobic pockets is physically prevented by a distinct structural domain of the protein (*i.e.* a cap). A histidine cluster in the active site of carbonic anhydrase II, which protrudes in to the surface of the enzyme assuring a very efficient proton

Table 1. **Calculated amounts of the five types of secondary structure in bovine carbonic anhydrase II.**

Secondary structure (200–260 nm)	Native	17.5% acetonitrile	47.5% acetonitrile
Helix	20.0%	18.7%	22.5%
Antiparallel	13.0%	13.2%	12.5%
Parallel	11.6%	12.0%	10.9%
β-Turn	18.5%	18.5%	18.8%
Random coil	37.0%	37.8%	35.2%

transfer process (*32*), may fulfill such an activity. Thus, in such a situation the penetration of ANS molecules into the hydrophobic pocket(s) (*e.g.* active site) may be limited, thereby, causing the disappearance of considerable common increases in the fluorescence emission intensity (Fig. 5). Therefore, this type of structural alteration will not be distinguished in the obtained fluorescence spectra and should be ascertained using other methods. For example, in 47.5% v/v acetonitrile, the specific exposure of the hydrophobic pocket(s) of the protein was confirmed by in the intrinsic emission spectrum toward higher wavelengths (illustrating the exposure of the Trp residues to the outside that is synonymous with the exposure of the hydrophobic pockets) and the KI experiments showing the accessibility of two additional tryptophanyl residues that are naturally distributed in the hydrophobic pocket(s) of the protein structure (Fig. 3 and inset of Fig. 4). After the addition of acetonitrile to the medium and the decrease in the dielectric constant, the translocation of tryptophanyl residues (or hydrophobic pockets) from inside to outside the protein structure could be possible. This is supported by the increase in the $K_{S\text{-}V}$ values when the acetonitrile concentration in the medium is increased.

Our theoretical methods for calculating accessible surface area (ASA) and determining the two additional exposed tryptophanyl residues (described above) are based on the method of Shrake and Rupley (*33*). These calculations indicate that among the seven tryptophanyl residues of carbonic anhydrase, one residue (Trp 243) has the highest accessibility to the solvent or other small molecules (*e.g.* iodide ions). The calculated ASA for Trp 243 was greater than those of other Trp residues (ASA_{Trp243} = 35.96 Å^2; ASA_{Trp4} = 23.41 Å^2; ASA_{Trp190} = 13.63 Å^2; ASA_{Trp122} = 8.86 Å^2; ASA_{Trp207} = 2.23 Å^2; ASA_{Trp15} = 0.73 Å^2; ASA_{Trp96} = 0.00 Å^2) indicating a greater chance of colliding with small molecules in solution such as the iodide ions. The findings are also consistent with our results obtained in the fluorescence quenching experiments. In these experiments the exposure of only one tryptophanyl residue (logically Trp 243) was proposed for the native structure of the protein in pure buffer (Fig. 1). With respect to the crystallographic coordinates, Trp 243 and the other two more accessible tryptophanyl residues (Trp 4 and Trp 190), are placed near the enzyme active site. Thus, reorientation and exposure of the Trp 4 and Trp 190 may be responsible for the decreased enzyme velocity.

The decrease in catalytic activity of an enzyme in the presence of organic solvents can be attributed to less conformational flexibility of the protein structure (*7*). This possibility can be ruled out for carbonic anhydrase placed in aqueous acetonitrile medium, since the decrease in the melting temperature (Fig. 7) confirms an increase in the structural flexibility of the protein. In addition, the existence of an organic solvent in the medium can induce reorientation of some critical residues, especially residues near the active site of the protein, thus causing a decrease in enzyme activity (*13–19*). Therefore, the tertiary structural alterations of carbonic anhydrase and the reorientation of some essential residues (especially Trp4 near the active site) can explain the rigorous drop in the velocity of carbonic anhydrase in the presence of acetonitrile.

It should be noted that in 47.5% acetonitrile, the melting temperature of the enzyme is 26°C, which is near to the room temperature (27°C). Therefore, at 27°C, the protein molecules may be very sensitive to unfolding and denaturation. All of our experiments were performed at temperatures at least 7°C below room temperature. The thermal stability of carbonic anhydrase was determined under the three solvent conditions, and showed the occurrence of a rigorous decrease in the melting temperature of the enzyme (Fig. 7). This is consistent with the strict structural alterations resulting in decreased enzyme stability, and is further supported by the fluorescence and activity experiments (Figs. 1–3 and 4). In addition, the recorded absorbance differences between the thermally unfolded structures of the enzyme and their related folded structures (the related absorbance is lower than 0.1) are very high, especially in 0% and 17.5% acetonitrile (compare the absorbances of 0.18, 0.57 and 0.71 with 0.1 absorbance unit in Fig. 7). These results indicate the existence of a high aggregation velocity in the medium when the acetonitrile concentration is low. In fact, when the aggregation velocity of the unfolded enzyme molecules is lower than the applied scan rate of the instrument (1 K/min) at each exerted temperature point, higher amounts of aggregated protein molecules may form, resulting in more scattering of the radiated photons, and in more recorded absorbance of the sample protein solution (Fig. 7). When the acetonitrile concentration in the medium becomes adequate (47.5% v/v), resulting in a considerable lowering of the dielectric constant and weakening of hydrophobic interactions, the intermolecular hydrophobic interactions that are the main cause for the aggregation of unfolded enzyme molecules can be greatly weakened. This will lower the tendency of the unfolded protein molecules (with their hydrophobic cores exposed) to come into close proximity with each other and aggregate. Furthermore, the addition of acetonitrile to the medium could result in the strengthening of electrostatic interactions (*e.g.* salt bridges) and hydrogen bonds (with electrostatic nature) established between the potent polar residues on the protein. The strengthening of electrostatic interactions generally causes an increase in the structural stability of a protein. We showed that the structural stability of carbonic anhydrase decreases in the presence of acetonitrile. We believe the decrease in the dielectric constant of the medium disturbs intramolecular hydrophobic interactions of the protein resulting in decreased protein structural stability. Thus, we propose that the stability and structural continuity of carbonic anhydrase is more dependent on hydrophobic interactions (which are disturbed due to the presence of acetonitrile) than on electrostatic interactions.

In conclusion, the addition of acetonitrile to the aqueous medium lowers the dielectric constant of water allowing some concealed or semi-concealed hydrophobic residues (*e.g.* Trp 4 and Trp 190) to become partially exposed to the solvent. Under these conditions, the tertiary structure of the protein is altered without any considerable changes in the secondary structure. These finding coincide with the molten globule or compact denatured state in which the secondary structure is retained but the tertiary structure is flexible. Therefore, it is reasonable to state that

acetonitrile is a mild denaturant, at least, in the case of bovine carbonic anhydrase II.

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REFERENCES

1. Hakansson, K., Carlsson, M., Svensson, L.A., and Lilijas, 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
2. Ohta, S. Alam, M.T., Arakawa, H., and Ikai, A. (2004) Origin of mechanical strength of bovine carbonic anhydrase studied by molecular dynamics simulation. *Biophys. J.* **87**, 4007–4020
3. Saito, R. Sato, T. Ikai, A., and Tanaka, N. (2004) Structure of bovine carbonic anhydrase II at 1.95 Å resolution. *Acta Crystallogr. D. Biol. Crystallogr.* **60**, 792–795
4. Tashian, R.E., Plato, C.C., and Shows, T.B. (1963) Inherited variant of erythrocyte carbonic anhydrase in Micronesians from Guam and Saipan. *Science* **140**, 53–54
5. Thorslund, A. and Lindskog, S. (1967) Studies of the esterase activity and the anion inhibition of bovine zinc and cobalt carbonic anhydrases. *Eur. J. Biochem.* **3**, 117–123
6. Koskinen, A.M.P. and Klibanov, A.M. (1996) *Enzymatic Reactions in Organic Media*, Blackie Academic and Professional, New York
7. Klibanov, A.M. (1997) Why are enzymes less active in organic solvents than in water? *Trends Biotechnol.* **15**, 97–101
8. Gupta, M.N., Tyagi, R., Sharma, S., Karthikeyan, S., and Singh, T.P. (2000) Enhancement of catalytic efficiency of enzymes through exposure to anhydrous organic solvent at 70 degrees C. Three-dimensional structure of a treated serine proteinase at 2.2 Å resolution. *Proteins* **39**, 226–34
9. Dordick, J.S. (1991) Non-aqueous enzymology. *Curr. Opin. Biotechnol.* **2**, 401–407
10. Burke, P.A., Griffin, R.G., and Klibanov, A.M. (1992) Solid-state NMR assessment of enzyme active center structure under non-aqueous conditions. *J. Biol. Chem.* **267**, 20057–20064
11. Lee, C.S., Ru, M.T., Haake, M., Dordick, J.S., Reimer, J.A., and Clark, D.S. (1998) Multinuclear NMR study of enzyme hydration in an organic solvent. *Biotechnol. Bioeng.* **57**, 686–693
12. Yang, L., Dordick, J.S., and Garde, S. (2004) Hydration of enzyme in non-aqueous media is consistent with solvent dependence of its activity. *Biophys. J.* **87**, 812–821
13. Fitzpatrick, P.A., Steinmetz, A.C.U., Ringe, D., and Klibanov, A.M. (1993) Enzyme crystal structure in a neat organic solvent. *Proc. Natl. Acad. Sci. USA* **90**, 8653–8657
14. Yennawar, N.H., Yennawar, H.P., and Farber, G.K. (1994) X-ray crystal structure of gamma-chymotrypsin in hexane. *Biochemistry* **33**, 7326–7336
15. Allen, K.N., Bellamacina, C.R., Ding, X., Jeffery, C.J., Mattos, G.A., Petsko, D., and Ring, D. (1996) An experimental approach to mapping the binding surfaces of crystalline proteins. *J. Phys. Chem.* **100**, 2605–2611
16. Schmitke, J.L., Stern, L.J., and Klibanov, A.M. (1997) The crystal structure of subtilisin Carlsberg in anhydrous dioxane and its comparison with those in water and acetonitrile. *Proc. Natl. Acad. Sci. USA* **94**, 4250–4255
17. Schmitke, J.L., Stern, L.J., and Klibanov, A.M. (1998) Comparison of x-ray crystal structures of an acyl-enzyme intermediate of subtilisin Carlsberg formed in anhydrous acetonitrile and in water. *Proc. Natl. Acad. Sci. USA* **95**, 12918–12923
18. Schmitke, J.L., Stern, L.J., and Klibanov, A.M. (1998) Organic solvent binding to crystalline subtilisin1 in mostly aqueous media and in the neat solvents. *Biochem. Biophys. Res. Commun.* **248**, 273–277
19. Gao, X.G., Maldonado, E., Perez-Montfort, R., Garza-Ramos, G., de Gomez-Puyou, M.T., Gomez-Puyou, A., and Rodriguez-Romero, A. (1999) Crystal structure of triosephosphate isomerase from *Trypanosoma cruzi* in hexane. *Proc. Natl. Acad. Sci. USA* **96**, 10062–10067
20. Hartsough, D.S. and Merz, K.M. (1992) Protein flexibility in aqueous and non-aqueous solutions. *J. Am. Chem. Soc.* **114**, 10113–10116.
21. Hartsough, D.S. and Merz, K.M. (1993) Protein dynamics, and salvation in aqueous, and nonaqueous environments. *J. Am. Chem. Soc.* **115**, 6529–6537
22. Wescott, C.R. and Klibanov, A.M. (1993) Solvent variation inverts substrate specificity of an enzyme. *J. Am. Chem. Soc.* **115**, 1629–1631
23. Wescott, C.R. and Klibanov, A.M. (1994) The solvent dependence of enzyme specificity. *Biochim. Biophys. Acta* **1206**, 1–9
24. Carrea, G., Ottolina, G., and Riva, S. (1995) Role of solvents in the control of enzyme selectivity in organic media. *Trends Biotechnol.* **13**, 63–70
25. Armstrong, J.M., Meyrs, D.V., Verpoorte, J.A., and Edsall, J.T. (1966) Purification and properties of human erythrocyte carbonic anhydrases. *J. Biol. Chem.* **241**, 5137–5149
26. Pocker Y. and Stone J. T. (1967) The catalytic versatility of erythrocyte carbonic anhydrase. III. Kinetic studies of the enzyme-catalyzed hydrolysis of *p*-nitrophenyl acetate. *Biochemistry* **6**, 668–678
27. Nyman, P.O. and Lindskog, S. (1964) Amino acid composition of various forms of bovine and human erythrocyte carbonic anhydrase. *Biochim. Biophys. Acta* **85**, 141–151
28. Bohm, G., Muhr, R., and Jaenicke, R. (1992) Quantitative analysis of protein far UV circular dichroism spectra by neural networks. *Protein Eng.* **5**, 191–195
29. Freifilder, D. (1985) *Physical Chemistry with Application to Biological Science*, Jones and Bartlett Publishers, Inc, Bostone, MA
30. Wimley, W.C., Creamer, T.P., and White, S.H. (1996) Solvation energies of amino acid side chains and backbone in a family of host-guest pentapeptides. *Biochemistry* **35**, 5109–5124
31. Liu, L.P. and Deber, C.M. (1997) Anionic phospholipids modulate peptide insertion into membranes. *Biochemistry* **36**, 5476–5482
32. Briganti, F., Mangani, S., Orioli, P., Scozzafava, A., Vernaglione, G., and Supuran, C.T. (1997) Carbonic anhydrase activators: X-ray crystallographic and spectroscopic investigations for the interaction of isozymes I and II with histamine. *Biochemistry* **36**, 10384–10392
33. Shrake, A. and Rupley, J.A. (1973) Environment and exposure to solvent of protein atoms. Lysozyme and insulin. *J. Mol. Biol.* **79**, 351–371