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Conformational Stability of Mixed Disulfide Derivatives of β-Lactoglobulin B†

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ABSTRACT: To probe the relationship between chemical structure and conformational stability, the urea denaturation of bovine β-lactoglobulin B and four mixed disulfide derivatives of this protein was investigated. The following groups were attached to the single sulfhydryl group of β-lactoglobulin B through a disulfide bond: propyl, aminoethyl, carboxyethyl, and hydroxyethyl. The optical rotatory properties in the near- and far-UV wavelength range suggest that β-lactoglobulin B and the propyl, carboxyethyl, and hydroxyethyl derivatives have similar conformations but that the conformation of the aminoethyl derivative differs substantially from that of the unmodified protein. In all cases, denaturation was shown to be reversible, and the derivatives were less stable than unmodified β-lactoglobulin B. The midpoints of the isothermal urea denaturation curves at pH 2.83 and 25 °C occur at 4.97 M urea for β-lactoglobulin B and at 4.46, 4.23, 4.19, and 1.68 M urea for the carboxyethyl, propyl, hydroxyethyl, and aminoethyl derivatives, respectively. An analysis of these data shows that the corresponding decreases in the conformational stability are approximately 1.1, 1.6, 1.7, and 7.3 kcal/mol for the modified proteins. The conformational stability of the aminoethyl derivative is decreased to such an extent that the protein is partially unfolded even in the absence of a denaturant. The urea denaturation curves were less steep for the derivatives than for the unmodified protein. The dependence of the free energy of denaturation on urea concentration, $d(\Delta G_D)/[d(\text{urea})]$, was 2.23 kcal/mol per M urea for β-lactoglobulin B and 1.46, 1.26, 0.92, and 0.43 kcal/mol per M urea for the carboxyethyl, hydroxyethyl, propyl, and aminoethyl derivatives, respectively. This suggests a greater deviation from a two-state mechanism for the unfolding of the derivatives.

Estimates of the conformational stability of about 25 proteins are now available (Pace, 1975; Privalov, 1979; Pfeil, 1981). In general, the globular conformation is from 2 to 15 kcal/mol more stable than unfolded conformations. There is considerable interest in determining how small changes in the chemical structure of a protein can alter the conformational stability. One approach is to compare genetic variants of a protein which differ only slightly in amino acid sequence (Knapp & Pace, 1974; Yutani et al., 1980; Schellman et al., 1981; Matthews et al., 1980). Another approach is to compare proteins whose structure has been changed slightly by chemical modification (Imoto & Rupley, 1973; Jacobson & Braun, 1977; Stoesz & Lumry, 1979; Hollecker & Creighton, 1982). These experimental studies of the relationship between structure and conformational stability are essential to test predictions based on theory and model compound data and the increasingly well-defined and well-understood three-dimensional structures of globular proteins determined by using X-ray diffraction (Richardson, 1981). The successful development of a useful approach for analyzing electrostatic interactions in globular proteins by Gurd's laboratory provides a good example of the progress which can be made when reliable experimental results are available for refining a theoretical approach (Friend & Gurd, 1979; Matthew & Richards, 1982).

We report studies of the effect of chemical modification of β-lactoglobulin B on the conformational stability. β-Lactoglobulin is well suited for this purpose since large amounts of the pure protein are readily available and the denaturation of the protein has been investigated in detail (Pace & Tanford, 1968; Alexander & Pace, 1971; Creighton, 1980). In addition, β-lactoglobulin contains a single sulfhydryl group (cysteine residue 121) which can be chemically modified (Townend et al., 1969; Ralston, 1972). We have prepared the propyl (P),[1]

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hydroxyethyl (HE), carboxyethyl (CE), and aminoethyl (AE) mixed disulfide derivatives of β-lactoglobulin. An analysis of urea denaturation has allowed us to estimate the effect of chemical modification on the conformational stability.

Experimental Procedures

Materials. Bovine β-lactoglobulin B was purified from the milk of a typed homozygous cow by the method of Armstrong et al. (1967). Ultrapure ammonium sulfate purchased from Schwarz/Mann was used in the purification. Ultrapure urea was also purchased from Schwarz/Mann. The mercaptans and other reagents used in the chemical modification of the protein had a degree of purity of 98% or better. Iodine, potassium iodide, *p*-(chloromercuri)benzoate, mercaptoethanol, mercaptopropionic acid, and mercaptoethylamine were purchased from Sigma. Propanethiol was purchased from Aldrich. The water used was glass redistilled.

Methods. The protein concentration was determined spectrophotometrically by using the extinction coefficient $E^{1\%}_{278nm} = 9.1$ (Prakash et al., 1981) and corrections for light scattering by the method of Leach & Scheraga (1960). For the calculation of the specific rotations in the denaturation curves, the protein concentration was determined in 8.0 M urea by using $[\alpha']_D = -330.2°$ (Alexander & Pace, 1971; Alexander, 1970).

Urea stock solutions were prepared by weight and their molar concentrations calculated by using densities given by Kawahara & Tanford (1966). The urea concentration was checked before use with an Abbe Model 10450 refractometer (Warren & Gordon, 1966).

All pH measurements were made at room temperature with a Radiometer Model 26 pH meter. The pH values reported are those at the midpoint of the urea denaturation curve.

Chemical modification of the sulfhydryl groups was performed according to the procedure of Cunningham & Nuenke (1959, 1960). Protein at concentrations of 0.4–0.5 g/100 mL was reacted with iodine at pH 6.5 and 0 °C. Iodine uptake was monitored spectrophotometrically at 355 nm. The extent of sulfhydryl group modification was determined by a *p*-(chloromercuri)benzoate (PCMB) titration (Boyer, 1954). The entire modification procedure was carried out twice, and PCMB titration showed 90–94% modification of the sulfhydryl group. Protein purity was assessed by polyacrylamide gel electrophoresis (Davis, 1964; Chrambach et al., 1967). A single protein band was observed for all of the modified proteins.

Optical rotations were measured on a Cary Model 60 spectropolarimeter by using quartz cells with a 1-cm path length. For the denaturation curves, a wavelength of 365.4 nm and a slit width of 0.25 mm were used. All solutions contained 0.15 M KCl–HCl and were filtered through a 0.45-μm Millipore filter before use. The temperature of the solutions was maintained at 25.1 ± 0.05 °C. The optical rotation of individual protein solutions at varying urea concentrations was measured after a minimum of 20 min was allowed for equilibrium to be reached. The results are reported as the reduced specific rotation, $[\alpha'] = 3[\alpha]/(n^2 + 2)$, where $[\alpha]$ is the specific rotation and n is the refractive index of the solvent (Adler et al., 1973).

Results

The urea denaturation curves for β-lactoglobulin B and the four derivatives are shown in Figures 1 and 2. The reduced specific rotations of the unmodified protein and the P, HE, and CE derivatives are identical in the absence of urea (=−90 ± 1°). In contrast, the rotation of the AE derivative is −119°. In terms of the a_0 and b_0 parameters of the Moffitt equation (Van Holde, 1972), this difference is entirely due to a more negative value of a_0 ($a_0 = -208°$) for the AE derivative. Similar results are observed in the near- and far-UV regions of the spectra. The aromatic Cotton effects in the 280–300-nm region are superimposable for β-lactoglobulin and the P, HE, and CE derivatives, but the Cotton effect for the AE derivative is shifted to a more negative rotation. Similarly, the low-UV optical rotatory dispersion (ORD) spectrum of the AE derivative differs substantially from those of the other derivatives and the unmodified protein. Thus, the conformations of the P, HE, and CE derivatives appear to be similar to that of unmodified β-lactoglobulin, but the conformation of the AE derivative differs significantly. The AE derivative appears to be partially unfolded even in the absence of a denaturant.

The optical rotations shown in Figures 1 and 2 were measured after the unfolding reaction had reached equilibrium.

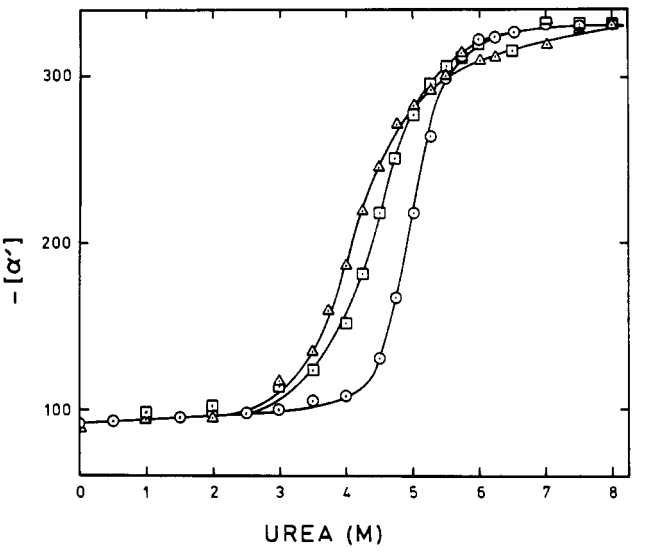

FIGURE 1: Urea denaturation curves at 25.1 °C, 0.15 M KCl–HCl for β-lactoglobulin B (○) (pH 2.83), the carboxyethyl mixed disulfide derivative (□) (pH 2.82), and the propyl mixed disulfide derivative (△) (pH 2.75).

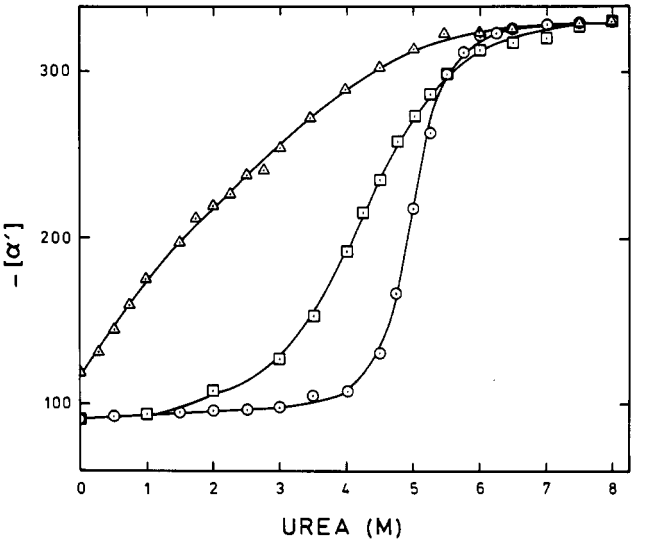

FIGURE 2: Urea denaturation curves at 25.1 °C, 0.15 M KCl–HCl for β-lactoglobulin B (○) (pH 2.83), the hydroxyethyl mixed disulfide derivative (□) (pH 2.83), and the aminoethyl mixed disulfide derivative (△) (pH 2.76).

[1] Abbreviations: P, propyl; AE, aminoethyl; CE, carboxyethyl; HE, hydroxyethyl.

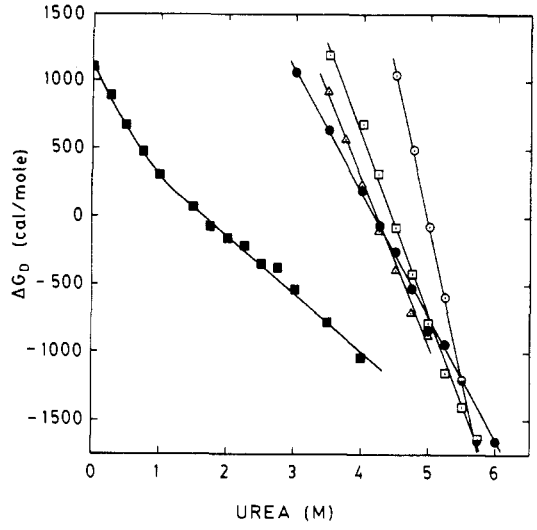

FIGURE 3: ΔG_D as a function of urea concentration for β-lactoglobulin B (○) and the carboxyethyl (□), propyl (●), hydroxyethyl (△), and aminoethyl (■) mixed disulfide derivatives. ΔG_D was calculated by using eq 1 from the data in Figures 1 and 2. The conditions are the same as those given in Figures 1 and 2.

Under the conditions used here, urea denaturation is completely reversible for β-lactoglobulin and each of the derivatives. When solutions equilibrated at higher urea concentrations are diluted, the optical rotations are identical with those measured on solutions prepared directly at the lower urea concentrations.

The urea denaturation of β-lactoglobulins A and B closely approaches a two-state mechanism under the condition used here, and dissociation to monomers occurs at lower urea concentrations than those required for unfolding (Pace & Tanford, 1968; Creighton, 1979, 1980). In contrast, as discussed below, the results presented here suggest that unfolding of all of the derivatives departs significantly from a two-state mechanism. Nevertheless, it will prove useful to assume a two-state mechanism to analyze the urea denaturation curves of the derivatives.

For a two-state mechanism, the free energy of unfolding, ΔG_D, can be calculated from the data in Figures 1 and 2 by using

$$\Delta G_D = -RT \ln \frac{[\alpha']_N - [\alpha']}{[\alpha'] - [\alpha']_D} \quad (1)$$

where $[\alpha']$ is the observed rotation and $[\alpha']_N$ and $[\alpha']_D$ are the rotations the native and denatured states, respectively, would have under the same conditions (Pace, 1975). Values of $[\alpha']_N$ and $[\alpha']_D$ are obtained by extrapolating the pre- and posttransition rotations into the transition region. In Figure 3, ΔG_D is plotted as a function of urea concentration for β-lactoglobulin and each of the derivatives. The dependence of ΔG_D on urea concentration is linear for β-lactoglobulin and the P, CE, and HE derivatives. A least-squares analysis was used to fit these data to the following equation (Pace, 1975):

$$\Delta G_D = \Delta G_D^{H_2O} - m(\text{urea}) \quad (2)$$

Values of ΔG_D and m are given in Table I along with the urea concentration at the midpoint of the denaturation curve, $(\text{urea})_{1/2}$. Small corrections were made to adjust all of the data in Table I to a common pH of 2.83. The results for unmodified β-lactoglobulin B are in excellent agreement with earlier studies of Alexander & Pace (1971). For the AE derivative, the dependence of ΔG_D on urea concentration is roughly linear above 1 M urea but increases at lower urea concentrations. The parameters given for the AE derivative in Table I are based on data between 1 and 4 M urea.

Table I: Parameters Characterizing the Dependence of ΔG_D on Urea Concentration[a]

protein	m[b] (kcal mol^{-1} M^{-1})	$\Delta G_D^{H_2O}$[b] (kcal/mol)	$(\text{urea})_{1/2}$[c] (M)
β-lactoglobulin-SH	−2.23	+11.08	4.97
β-lactoglobulin-S-S-CH_2CH_2COOH	−1.46	+6.54	4.47
β-lactoglobulin-S-S-$CH_2CH_2CH_3$	−0.92	+3.89	4.23
β-lactoglobulin-S-S-CH_2CH_2OH	−1.26	+5.30	4.19
β-lactoglobulin-S-S-$CH_2CH_2NH_2$	−0.43	+0.71	1.68

[a] The data in Figure 3 were fit to eq 2 by a least-squares analysis. [b] Equation 2. [c] The midpoint of the urea denaturation curve, where $\Delta G_D = 0$.

Discussion

The sulfhydryl group of β-lactoglobulin reacts sluggishly, at least below pH 7 (Leslie et al., 1962; Dunnill & Green, 1965). We could not obtain good yields of the mixed disulfide derivatives by adding an excess of a disulfide such as oxidized glutathione or oxidized mercaptoethylamine directly to the protein. We also had difficulty getting yields above 25% with the fluorescent probe *N*-[[(iodacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid (Hudson & Weber, 1973). The sulfenyl iodide procedure had been used previously by Cunningham & Nuenke (1960), Townend et al. (1969), and Ralston (1972) to prepare mixed disulfide derivatives of β-lactoglobulin. Only by repeating the procedure twice could we obtain 90–95% yields of the mixed disulfide derivatives. This may indicate that the sulfhydryl group is sterically hindered. The observation that all of the derivatives are less stable than native β-lactoglobulin is consistent with this possibility. Despite the difficulty encountered in modifying the sulfhydryl group, the optical rotatory properties of the P, CE, and HE derivatives are almost identical with those of the unmodified protein, suggesting that they have similar conformations. In addition, only a single band is observed on polyacrylamide disc gel electrophoresis for the four mixed disulfide derivatives.

The denaturation curves for the derivatives are less steep than those for native β-lactoglobulin (Figures 1 and 2). This is quantitatively reflected in the m values given in Table I which measure the dependence of ΔG_D on urea concentration, $m = d(\Delta G_D)/[d(\text{urea})]$. This was not expected. For a two-state mechanism, the m value will depend mainly on the size and composition of the part of the polypeptide chain which is freshly exposed to denaturant on unfolding (Greene & Pace, 1974). Thus, for the P, CE, and HE derivatives, we expected the m value to be at least as large as it is for native β-lactoglobulin, because the conformations are similar. The most reasonable interpretation of the decreases in the m values is that unfolding deviates markedly from a two-state unfolding mechanism for all three of the derivatives. This would suggest that one or more unfolding intermediates is specifically stabilized by the groups introduced so that larger concentrations are present in the transition region than for unmodified β-lactoglobulin. Perhaps this should not be too surprising. Single amino acid substitutions can significantly change the unfolding mechanism of the α-subunit of tryptophan synthetase (C. R. Matthews, personal communication).

The analysis of the denaturation curve for a mixture of two proteins differing in conformational stability would generally lead to an m value lower than that observed for either of the proteins investigated individually (Pace, 1975). As noted above, there could be as much as 10% unmodified β-lacto-

Table II: Difference in Conformational Stability between β-Lactoglobulin B and Mixed Disulfide Derivatives

protein	change in ΔG_D (kcal/mol)
β-lactoglobulin-SH	
β-lactoglobulin-S-S-CH_2CH_2COOH	1.1
β-lactoglobulin-S-S-$CH_2CH_2CH_3$	1.6
β-lactoglobulin-S-S-CH_2CH_2OH	1.7
β-lactoglobulin-S-S-$CH_2CH_2NH_2$	7.3

globulin present in the derivatives. It is important, therefore, to show that this could not account for observed decreases in the *m* value for the derivatives. As shown below, the CE, P, and HE derivatives are approximately 1.1, 1.6, and 1.7 kcal/mol less stable than native β-lactoglobulin. Calculations show that the *m* value would be decreased from 2200 to 2060, 1880, and 1840 cal/mol per M urea for each of these derivatives, respectively, if they contained 10% unmodified β-lactoglobulin. Consequently, the presence of some unmodified β-lactoglobulin may contribute to the decrease observed in the *m* values, but some other factor, probably stabilization of an intermediate, makes a much larger contribution.

The problem of using data such as those shown in Figure 3 for estimating the free energy of unfolding in the absence of denaturant, $\Delta G_D^{H_2O}$, has been discussed elsewhere (Pace, 1975; Schellman, 1978; Vanderburg & Pace, 1979; Ahmad & Bigelow, 1982). Here our interest is in estimating how much the conformational stability of a protein is changed as a result of chemical modification. Even when intermediate states are present, the observed equilibrium constant for unfolding approaches the value expected for a two-state mechanism near the midpoint of the transition (Pace, 1975). Consequently, taking the difference between the $(urea)_{1/2}$ values for β-lactoglobulin and each of the derivatives and multiplying these by the *m* value for the unmodified protein (2230 cal/mol per M urea) should lead to reasonable estimates of the decrease in conformational stability. The values obtained in this way are shown in Table II.

This approach is dubious for the AE derivative where $(urea)_{1/2}$ is shifted from 4.97 to 1.68 M, and there is an indication from the denaturation curve that unfolding may occur in two stages. In this case, the ΔG_D measurements extend to 0 M urea and, as shown in Figure 2, lead to an estimate of 1.1 kcal/mol for $\Delta G_D^{H_2O}$. Using this and the value of $\Delta G_D^{H_2O}$ = 11.1 kcal/mol for unmodified β-lactoglobulin B given in Table I leads to an estimate of the difference in stability of 10.0 kcal/mol. This differs substantialy from the estimate of 7.3 kcal/mol given in Table II. Thus, it is clear that the AE derivative is much less stable than the unmodified protein, but it is difficult to estimate the difference in stability with certainty.

Ralston (1972) modified the sulfhydryl group of β-lactoglobulin A with *N*-ethylmaleimide and mercaptoacetic acid and determined urea denaturation curves for these derivatives at pH 5.2. As observed here, the urea denaturation curves were shifted to lower urea concentrations and were less steep for the derivatives than for the unmodified protein. From his data, we estimate $(urea)_{1/2}$ values of 6.6, 6.1, and 5.0 M for β-lactoglobulin, the carboxyethyl derivative, and the *N*-ethylmaleimide derivative, respectively. The decrease in $(urea)_{1/2}$ for the carboxyethyl derivative is identical with that which we observe at pH 2.83.

All of the derivatives of β-lactoglobulin in which the sulfhydryl group has been modified are less stable than the native protein. This, plus the difficulty encountered in modifying the sulfhydryl group, suggests that there may be unfavorable steric interactions between the groups introduced and adjacent groups on the protein for all the derivatives. The significantly larger decrease in stability for the AE derivative suggests an unfavorable electrostatic interaction between the positive charge on the amino group and neighboring charges on the protein. If so, it might be expected that the negative charge of the CE group would stabilize the protein through favorable electrostatic interactions. In line with this, the CE derivative is more stable than the P and HE derivatives even though the carboxyl group should be only partially ionized at pH 2.83. Model compound data would suggest a p*K* of 3.0 for this group in the absence of any influence of the protein (Kortum et al., 1961). However, as noted above, Ralston (1972) observed the same decrease in $(urea)_{1/2}$ at pH 5.2 as we observed at pH 2.83 for the CE derivative. The 6-Å resolution structure of β-lactoglobulin has been determined (Green et al., 1979). It will be interesting to reconsider the results reported here when a more detailed structure is available.

It is clear from our results that a small change in the chemical structure of protein can dramatically affect both the conformational stability and the mechanism of unfolding of the protein. This should be kept in mind when considering the physiological significance of the many different types of chemical modification which are observed in vivo after the biosynthesis of a protein is completed (Uy & Wold, 1977). Our results are of particular interest in connection with suggestions that formation of a mixed disulfide with glutathione or other compounds may influence the turnover (Bond & Offermann, 1981) or regulate the activity (Gilbert, 1982) of enzymes in vivo.

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Nitrogen-15 Nuclear Magnetic Resonance Study of Benzenesulfonamide and Cyanate Binding to Carbonic Anhydrase†

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ABSTRACT: The binding of inhibitors, cyanate and benzenesulfonamide, to the active-site zinc of human carbonic anhydrase B was studied by ^{15}N nuclear magnetic resonance spectroscopy. The cyanate nitrogen resonance moved 34 ppm upfield on binding to the enzyme. The shielding is comparable to that reported for a zinc–isocyanate complex and strongly suggests complexation of cyanate to zinc through nitrogen. The proton-coupled ^{15}N resonance of the enzyme-bound benzenesulfonamide was a doublet. Hence, benzenesulfonamide is bound as $C_6H_5SO_2NH^-$. The proton-decoupled ^{15}N resonance of the bound benzenesulfonamide was observed 17 ppm upfield of that of free benzenesulfonamide anion. A model ligand, 2-aminobenzenesulfonamide anion, undergoes binding of zinc through the sulfonamide nitrogen which results in an 11.8 ppm shielding of the ^{15}N resonance. In contrast, *N*-(2-aminophenyl)benzenesulfonamide, which is reported to bind zinc through an oxygen, has its sulfonamide nitrogen deshielded by 4.3 and 1.2 ppm on complexation of zinc to the neutral and anionic ligands, respectively. Thus, coordination to the nitrogen causes shielding and to the oxygen deshielding of the sulfonamide resonance. The observed shielding of the enzyme-bound sulfonamide resonance strongly suggests that benzenesulfonamide binds primarily to zinc through the sulfonamide nitrogen. The implications of these results for the high affinity of association of the inhibitor are discussed.

Carbonic anhydrase, a zinc metalloenzyme widespread in nature, is a highly efficient catalyst for reversible hydration of CO_2:

$$CO_2 + H_2O \rightleftarrows HCO_3^- + H^+$$

The essential zinc ion at the active site has four tightly coordinated ligands with zinc–ligand distances of ~2 Å: three imidazolyl nitrogens of histidyl residues and a water molecule. There is also a more distant (2.9 Å) fifth ligand site. The possible sixth ligand site is sterically hindered by the protein (Nostrand et al., 1975; Kannan et al., 1977). The activity of human carbonic anhydrase B is governed by the ionization of a group with a pK_a of 7.3–7.6 or higher (Coleman, 1967b; Khalifah, 1971; Bauer et al., 1976). In one proposed model for the catalytic mechanism, this ionizable group is thought to be the zinc-bound water molecule, which on deprotonation to –ZnOH is postulated to act as a nucleophile to carbon dioxide (Pocker & Sarkanen, 1978; Pocker & Deits, 1982). A possible sequence for catalysis of the hydration of carbon dioxide by carbonic anhydrase is shown in Figure 1.

The activity of carbonic anhydrase is strongly inhibited by aromatic sulfonamides. A typical inhibitor, benzenesulfon-

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