Nuclear Magnetic Resonance Studies of Human Carbonic Anhydrase B. Histidine Residues*

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ABSTRACT: Nuclear magnetic resonance spectroscopy has been used to investigate the environment and titration behavior of histidine residues in human carbonic anhydrase isoenzyme B. In the native enzyme four histidine titration curves could be distinguished, two of which had abnormal values of pK and chemical shift. On removing the zinc atom from the enzyme a further titratable peak appeared in the nuclear magnetic resonance spectrum downfield of 800 Hz, while the titration of the other peaks was almost unaffected. Addition of specific inhibitors (*p*-carboxybenzenesulfonamide, or iodide ion) caused changes in some of the titration curves while leaving others unaffected, and caused further peaks to appear.

These observations are compatible with inhibitor-induced conformational changes in the enzyme, the nature of which depends on the type of inhibitor used.

Human carbonic anhydrase, isoenzyme B, is a metalloenzyme, mol wt 28,733 (Armstrong *et al.*, 1966), containing one atom of zinc per molecule (Nyman, 1961; Laurent *et al.*, 1962; Rickli *et al.*, 1964). It catalyzes the hydration of CO_2 (Nyman, 1961; Gibbons and Edsall, 1963, 1964) and certain aldehydes (Pocker and Meany, 1965a; Pocker and Dickerson, 1968), and the hydrolysis of esters (Tashian *et al.*, 1964; Armstrong *et al.*, 1966; Duff and Coleman, 1966). It is strongly inhibited by a variety of aromatic sulfonamides (Maren, 1967; Taylor *et al.*, 1970a) and monodentate anions (Coleman, 1967a; P. W. Taylor and A. S. V. Burgen, 1970).

X-Ray crystallography of the human C isoenzyme (Fridborg *et al.*, 1967) has shown that the inhibitor acetoxymercurisulfanilamide is bound in a crevice in the enzyme close to the zinc atom which is essential for activity and for strong sulfonamide binding (Coleman, 1967b).

The CO_2 hydration, esterase, and aldehyde hydration activities of various mammalian carbonic anhydrases exhibit a sigmoidal curve of activity against pH, with an inflection point near neutrality (see, for example, Kernohan, 1964, Verpoorte *et al.*, 1967, and Pocker and Meany, 1965b). This effect has been variously ascribed to the ionization of a zinc-bound water molecule (Coleman, 1967a; Bradbury, 1969), to facilitated removal of a proton from zinc-bound water through an imidazole group in a concerted mechanism (Pocker and Meany, 1965b; Pocker and Dickerson, 1968), and to facilitated proton transfer, through an imidazole group, from a zinc-bound hydroxyl group to the substrate (Wang, 1968).

Human carbonic anhydrase B further exhibits a bell-shaped curve of affinity constant for sulfonamides, or rate of forward reaction with sulfonamides, as a function of pH (Taylor *et al.*, 1970b). This curve can be explained on the basis of the titration of two groups, one on the enzyme with a pK near neutrality and the other the sulfonamido group of the inhibitor.

Nuclear magnetic resonance spectroscopy offers an unequivocal method for determination of the microscopic dissociation constants of individual side-chain groups in intact enzymes (Roberts and Jardetzky, 1970). Without the use of selective isotopic substitution techniques the method is at present restricted to the study of residues, notably histidine, whose resonances are clearly distinguishable from the spectral envelope. Since a histidine side chain may possibly be the protein group responsible for the pH dependence of the activity and inhibition of human carbonic anhydrase B, one object of this study was to determine whether any of the observable histidine resonances could be responsible for the pH-dependent effects. In addition it was hoped to obtain more information about the conformational changes which appear to accompany sulfonamide or anion binding (King and Burgen, 1970).

Materials and Methods

Human carbonic anhydrase, isoenzyme B, was prepared from erythrocytes obtained from the blood bank, using the ion-exchange method described by Armstrong *et al.* (1966). The purified material was stored as a slurry in ammonium sulfate solution (60 g/100 ml). Except where detailed below the following method was used to prepare samples for nuclear magnetic resonance experiments.

A sample of the slurry was sedimented in a bench centrifuge. The sediment was placed in dialysis bags and dialyzed against 67 mM ($\Gamma/2 = 0.2$) K_2SO_4 solution in H_2O. Several changes of dialysate over a period of 3 days served to remove the ammonium sulfate and dissolve the enzyme. The solution was then clarified by centrifugation and concentrated using a membrane ultrafiltration cell. The concentrated solution was dialyzed against 67 mM K_2SO_4 in D_2O. A further three changes over 3 days served to remove almost all of the H_2O, as judged from the nuclear magnetic resonance signal, and to exchange the readily exchangeable protons of the enzyme. The solution was finally concentrated further by pressure dialysis in a bench centrifuge. Using this method it was possible to obtain 2 ml of a 6 mM (*ca.* 18% w/v) enzyme solution from 6 l. of blood.

Enzyme concentration was determined spectrophotometrically, taking an E^{M}_{280} value of 46.8×10^3 (Armstrong *et al.*, 1966; Nyman and Lindskog, 1964).

The esterase activity of the enzyme was monitored using the

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p-nitrophenyl acetate assay method described by Armstrong *et al.* (1966).

Apocarbonic anhydrase was prepared by dialyzing the enzyme against 40 mM sodium acetate buffer solution (pH 5.5), containing 5 mM 1,10-phenanthroline, for 3 weeks. After this period the esterase activity was 6% of that of the native enzyme, and was restored to 97% on addition of Zn^{2+} ions. The solution for nuclear magnetic resonance examination was treated as above except that in this case the deionized water used was further purified by passing through Chelex-Na resin (Bio-Rad Laboratories, Richmond, Calif.) to remove interfering metal ions. At the termination of this experiment the esterase activity of the enzyme had risen to 11% of the native value.

For the sulfonamide inhibition experiments twice recrystallized *p*-carboxybenzenesulfonamide (Aldrich Chemical Co.) was dialyzed into the enzyme solution after the H_2O dialysis. Excess sulfonamide was removed by the D_2O dialysis. Calculations using a value of 10^6 M^{-1} for the affinity constant (Taylor *et al.*, 1970a) showed that 99.5% of the enzyme was complexed with the sulfonamide after this dialysis treatment.

Potassium iodide (0.2 M) was used as the supporting electrolyte instead of K_2SO_4 for the iodide inhibition experiment. For the iodide binding curve, known volumes of concentrated KI solution in D_2O were added from a micrometer syringe to the enzyme solution in K_2SO_4–D_2O.

pH measurements are given as uncorrected glass-electrode meter readings (Roberts *et al.*, 1968) referred to as pH*, and were made with a Radiometer pHM 26 pH meter and Beckman combination electrode type 39030. The pH* of the solutions was adjusted with 0.1 N NaOD or D_2SO_4 delivered from a micrometer syringe. If the difference in the pH* readings of a sample before and after recording a spectrum was greater than 0.04 pH* unit the results were not accepted. In general the agreement was better than 0.03 pH* unit. Measurements were made at the temperature of the nuclear magnetic resonance probe, 27.5 ± 0.5°.

Spectra were recorded at 100 MHz using a Varian Associates HA-100D nuclear magnetic resonance spectrometer, equipped with a Biomac 1000 computer of average transients for spectrum accumulation. A few spectra were obtained using a Varian HA-100D-15 instrument, obtaining the sweep frequency from a Wavetek voltage-to-frequency converter driven by a linear voltage ramp from the computer. A sweep rate of 1 Hz/sec was used over a 250-Hz sweep range. Frequency calibration was obtained by counting the difference between the manual oscillator and sweep oscillator frequencies using the V-4315 counter. The samples were contained in Wilmad (Buena, N. J.) 5-mm precision-bore nuclear magnetic resonance cells with coaxial capillary inserts containing the external standard, Me_4Si (Ciba ARL Ltd., Duxford, England). All chemical shifts are given as hertz downfield from this external Me_4Si (TMS) reference.

The experimental points were fitted to theoretical titration curves using a program written in the FOCAL language for a PDP-8/L computer (Digital Equipment Corp.). The simple Henderson–Hasselbalch equation was used for the titration curve since inclusion of the electrostatic interaction factor (Linderström-Lang, 1924; Cannan *et al.*, 1942) does not improve the fit to the experimental points for histidine titration curves in proteins determined by nuclear magnetic resonance (Meadows, 1968).

A linearized version of the titration curve was derived as follows:

$$K_d = [H^+][A]/[AH]$$

where K_d is the dissociation constant and [A] and [AH] are the concentrations of unprotonated and protonated imidazole, respectively.

Since the deuterium isotope effect on the glass-electrode pH readings and on the dissociation constant of histidine are equal and opposite (Roberts *et al.*, 1968; Meadows, 1968), we shall refer throughout to pK rather than pK*.

If the exchange between protonated and unprotonated imidazole is rapid (in this case, if the rate of exchange, $k > 630$ sec^{-1}; this condition is undoubtedly fulfilled, since temperature-jump experiments (Eigen *et al.*, 1960) have shown that for imidazole in water the lowest value of k observed is 2×10^3 sec^{-1}), then

$$\delta = \frac{[A]}{[A_{TOT}]}\delta_A + \frac{[AH]}{[A_{TOT}]}\delta_{HA}$$

where δ is the observed chemical shift, δ_A and δ_{HA} are the chemical shifts of the species A and AH, respectively, and $[A_{TOT}] = [A] + [AH]$. Thus we have

$$\frac{[A]}{[A_{TOT}]} = \frac{(\delta_{HA} - \delta)}{(\delta_{HA} - \delta_A)}$$

and

$$\frac{[AH]}{[A_{TOT}]} = \frac{(\delta - \delta_A)}{(\delta_{HA} - \delta_A)}$$

so

$$K_d = [H^+]\frac{(\delta_{HA} - \delta)}{(\delta - \delta_A)} \quad (1)$$

and

$$\frac{1}{(\delta - \delta_A)} = \frac{1}{[H^+]}\frac{K_d}{(\delta_{HA} - \delta_A)} + \frac{1}{(\delta_{HA} - \delta_A)} \quad (2)$$

Figure 3, for example, shows that, due to precipitation of the enzyme at low pH*, it was often impossible to obtain a complete titration curve for each histidine C_2–H peak. When this was the case, a least-squares fit of the data to eq 2 was performed (iterating upon the value of δ_A to obtain the optimum fit), and estimates of δ_{HA} and K_d were obtained. However, since eq 2 involves the reciprocal of the hydrogen-ion concentration, while the experimental points are spaced on a pH scale, analysis of the data in this way weights the points in a way unrelated to the actual experimental error. For this reason, final estimates of δ_{HA}, δ_A, and K_d were obtained by fitting the simple Henderson–Hasselbalch titration curve. The theoretical titration curve was calculated using the values of δ_A, δ_{HA}, and K_d obtained from the linear equation, and this was compared point by point to the experimental data. The standard error of δ was calculated from the sum of the squares of the differences between calculated and experimental chemical shifts at each pH*. The value of K_d was calculated at each point using eq 1, and the standard error of K_d calculated from the squares of the residuals of these values from the mean, $\bar{K}$. Then, $SE(pK) = 0.4343SE(K_d)/\bar{K}$ (Wilkinson, 1961).

These standard errors were minimized by iterating the values of δ_A, δ_{HA}, and K_d. Relatively small changes from the

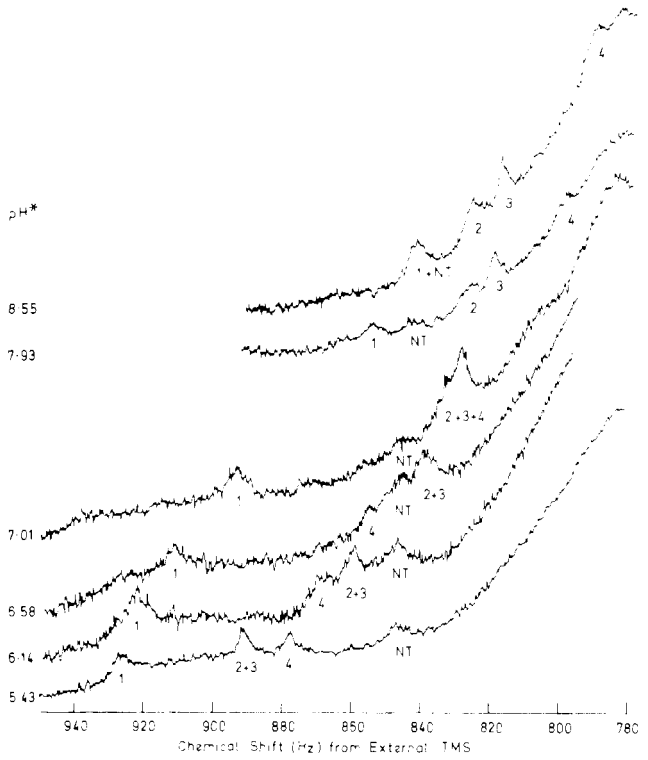

FIGURE 1: Histidine region of the nuclear magnetic resonance spectrum of human carbonic anhydrase B at various pH* values. The peaks are assigned as described in the text.

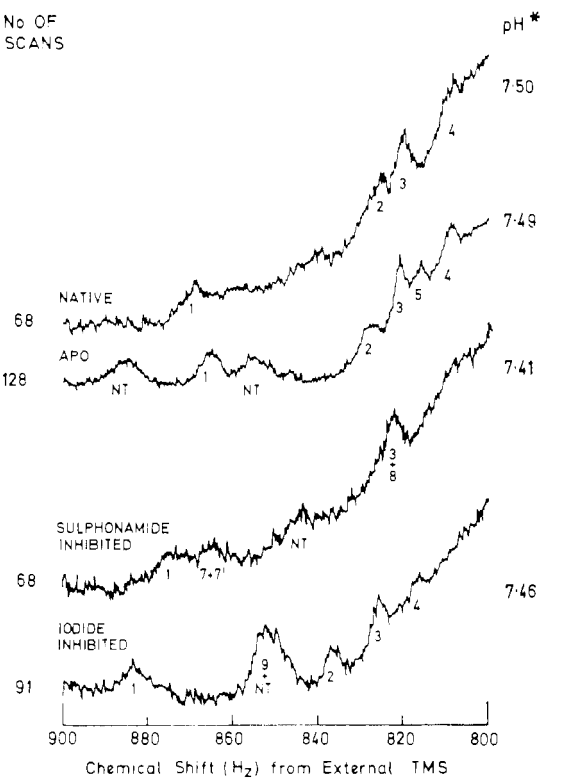

FIGURE 2: Histidine region of the nuclear magnetic resonance spectrum of human carbonic anhydrase B near pH* 7.5 in the native, apo, sulfonamide-inhibited, and iodide-inhibited forms.

values of these parameters obtained from eq 2 were required, but there was an appreciable reduction in the standard errors. When the whole titration curve could be observed, reasonable estimates of δ_{HA}, δ_A, and K_d could be obtained by inspection, and then only the second procedure was used.

Results and Discussion

Figures 1 and 2 show that the region 800–950 Hz of the nuclear magnetic resonance spectrum of human carbonic anhydrase B contains a number of peaks whose position depends upon pH, and in some cases also on the presence of inhibitors. These can be assigned to the C_2–H of the imidazole ring of histidine residues. In addition there are peaks, notably at 845 Hz, which do not change position appreciably in the pH* range 5–9. These are most probably due to peptide NH protons which have not exchanged with the solvent, though the possibility that they are the C_2–H peaks of histidine residues which are fairly free to move (see below), but which do not titrate in the region pH* 5.5–9, cannot be definitely excluded. Finally the region 800–830 Hz also contains the resonances of the C_5 and C_6 ring protons of some or all of the tryptophan residues.

The presence of these nontitrating peaks, the rather poor signal-to-noise ratio of the spectra, and the fact that the imidazole C_2–H peaks themselves overlap at some pH* values combine to make accurate determination of the position of the histidine peaks difficult. However, by examination of a particular region of the spectrum at a convenient pH* value where it contains no pH-dependent peaks a "base line" can be obtained; the positions of the pH-dependent peaks present in this region at other pH* values can then be estimated to within 2 Hz (or 5 Hz when overlapping of peaks is serious). Having estimated the positions of the peaks at various pH* values, the next problem is to join the points to give titration curves. Due to the extensive overlapping of peaks, it is not possible to do this unambiguously. The curves shown in Figures 3–6 were chosen on the basis of (a) area measurements and (b) the somewhat different line widths of the peaks. In these figures, the positions of the nontitrating peaks have been omitted for clarity; they are given in Table II. As will be seen from the figures, the majority of these curves can be fitted very well by the Henderson–Hasselbalch equation. We have also tried drawing the titration curves through the points in a number of other ways; these uniformly gave a much worse fit to the theoretical curves, and we are confident that the curves shown in Figures 3–6 represent true titration curves. The values of δ_{HA}, δ_A, and pK which define these curves are shown in Table I. It is worth emphasizing that, since essentially all the points on the curve contribute to the determination of these parameters, their accuracy is substantially greater than that of individual points on the curve.

Native Enzyme. The histidine titration curves observed for native human carbonic anhydrase B are shown in Figure 3. It is notable that, while the enzyme contains eleven histidine residues, only four titration curves can be seen. The line widths of proton resonances in low-viscosity solvents are effectively determined by the rotational correlation time of the protein. An imidazole group buried within a compact globular protein can rotate only when the whole molecule rotates, and its correlation time is determined by the rotational diffusion of the macromolecule. Interpolation of results obtained with other proteins suggests that the line width for such a buried group in human carbonic anhydrase B would be about 20 Hz. Under our present experimental conditions, a peak as broad as this would not be distinguishable from the

TABLE I

Curve No.		pK	± SE	δ_A (Hz)	δ_{HA} (Hz)	SE (δ)
1	Native	7.23	0.06	836	928	1.1
	Apo	7.19	0.06	836.5	926	1.2
	Sulfonamide inhibited	7.23	0.08	836	930	1.1
	Iodide inhibited	7.38	0.06	839	930	1.2
2	Native	5.91	0.13	823	915	1.3
	Apo	5.84	0.12	825	915	1.5
	Iodide inhibited			827.5		
3	Native	6.04	0.06	817	910	1.0
	Apo	6.10	0.09	816	907	1.6
	Sulfonamide inhibited	6.06	0.09	816	910	1.4
	Iodide inhibited	6.18	0.08	818	909	1.6
4	Native	7.00	0.09	786	880	1.1
	Apo	7.00	0.03	786	879	1.0
	Iodide inhibited	7.02	0.05	790	882	2.0
5	Apo	6.02	0.08	812	892	1.2
7	Sulfonamide inhibited	7.30	0.10	847	884	1.6
7′	Sulfonamide inhibited	7.00	0.13	848	898.5	2.8
8	Sulfonamide inhibited	6.77	0.17	809	883	1.5
9	Iodide inhibited	6.80	0.04	830	939	1.8

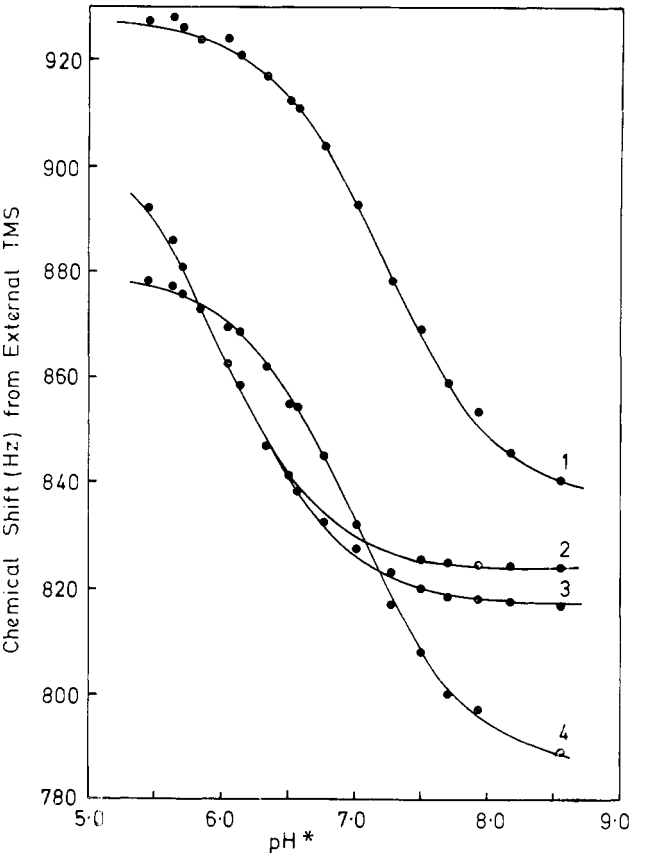

FIGURE 3: Titration of the histidine residues of native human carbonic anhydrase B. Curves labeled 1–4 correspond to peaks 1–4 in Figure 1. Solid lines are the curves calculated from the experimental points (dots).

base line. On the other hand, the imidazole groups of histidine residues in which rotation about the C_α–C_β and/or C_β–C_δ bond is relatively rapid would show appreciably narrower resonances. Therefore, we conclude that in human carbonic anhydrase B there are four histidine residues in which rotation about the side-chain bonds is relatively unrestricted, and which are therefore probably at or near the surface of the enzyme, and seven histidine residues which are not free to move rapidly with respect to the rest of the protein molecule. This agrees with the results of Riddiford (1964) and Riddiford *et al.* (1965) who showed from the hydrogen-ion titration curves of human carbonic anhydrase B that only four histidine residues were titratable in the native enzyme.

It is clear from Figure 3 and Table I that even the four histidines near the surface of the enzyme are in quite different environments, as indicated by their different values of δ_{HA}, δ_A, and pK. In discussing the environments of these histidines it is useful to take some, necessarily rather arbitrary, values of these parameters for an imidazole group in a solvent environment. We shall use the values of δ_{HA} = 910 Hz, δ_A = 811 Hz, and pK = 6.65 observed for the C_2–H peak of His-105 of pancreatic ribonuclease (Meadows *et al.*, 1968). This imidazole group has similar values of pK, and ΔH and ΔS of ionization (Roberts *et al.*, 1968), to those observed for imidazole and for histidine derivatives, including small histidine peptides (Kirby and Neuberger, 1938; Cohn and Edsall, 1943; Nozaki *et al.*, 1957; Koltun *et al.*, 1959; Schneider, 1963), and the values of δ_{HA} and δ_A are close to those observed for the C_2–H peak of imidazole (Cohen, 1968). X-Ray crystallographic studies of ribonuclease (Kartha *et al.*, 1967; Wyckoff *et al.*, 1967, 1970) show that His-105 is on the surface of the molecule, freely accessible to the solvent. Since very slight changes in the environment will change the observed values of δ_{HA}, δ_A, and pK from those given above, one would not necessarily expect any other histidine C_2–H peak to have exactly these values of chemical shift and pK; however, large deviations from these values can be taken to indicate an environment substantially different from the solvent.

Thus curve 1 in Figure 3 yields values of δ_{HA} = 930 Hz, δ_A = 836 Hz, and pK = 7.23 (Table I); the histidine residue corresponding to this curve, which we shall refer to as H_1, is clearly not in a solvent-like environment. Histidine H_4 also has a slightly high pK, but its chemical shift in both the protonated and unprotonated forms is upfield of the normal position. The increased pK of these two residues could be due, for example, to the proximity of a carboxylate group. If this is the case, the orientation of the imidazole ring with respect to the carboxylate group is most probably different in the two cases, since the chemical shifts of the two C_2–H resonances differ from the normal values in opposite directions. It is never possible to define unambiguously the environment of a histidine residue from its chemical shift and pK alone, though many possible environments would be incompatible with these values. Histidines H_2 and H_3 have chemical shifts in the normal range, though their pK values

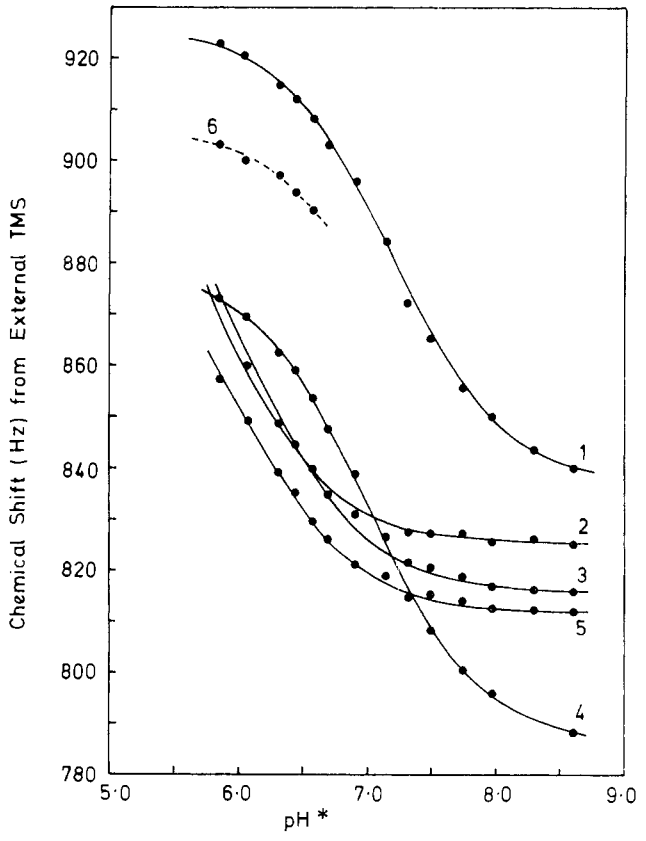

FIGURE 4: Titration of the histidine residues of human apocarbonic anhydrase B.

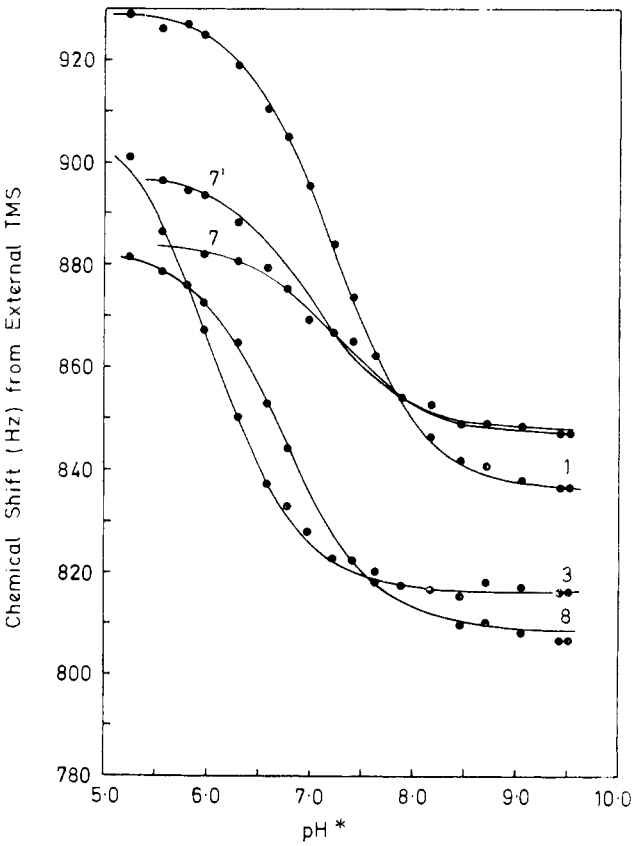

FIGURE 5: Titration of the histidine residues of human carbonic anhydrase B in the presence of a saturating concentration of *p*-carboxybenzenesulfonamide.

are somewhat low, suggesting that their environments are not wholly solventlike.

Apoenzyme. When the zinc atom is removed from carbonic anhydrase there is very little effect on histidines H_1–H_4 (Figure 4 and Table I). It is clear therefore that any conformational change which occurs on removal of the metal does not alter the environment of these residues. Since at least H_1 and H_4 appear to be in nonsolvent environments, the conformational change does not extend to the region of the molecule containing these residues. Curve 5 in Figure 4 represents a histidine residue (H_5) which was immobilized and hence not observable in the native enzyme, but which can be clearly seen in the apoenzyme (see Figure 2). It is tempting to suggest that this may be one of the histidine ligands to the metal atom (A. Liljas, personal communication), though of course it may equally well be a residue affected by a relatively localized conformational change on removal of the metal atom.

As indicated by the fact that the titration curves in Figure 4 extend down only to pH* 5.8, while those for the native enzyme (Figure 3) extend to pH* 5.4, the apoenzyme precipitates at a somewhat higher pH than the native enzyme. Curve 6 in Figure 4, which is observed only below pH* 6.6 (area measurements show that it disappears above this pH, rather than joining curve 1), probably represents a histidine residue which is buried in the native enzyme and in the apoenzyme at neutral pH and above, but which becomes accessible to the solvent as the acid unfolding begins. The guanidine- and urea-induced unfolding of carbonic anhydrase is known to occur in stages (Edsall *et al.*, 1966) and this seems to be true also of the acid unfolding, since residue H_6 seems to become substantially more mobile at pH* values at which there is no discernible change in the environments of H_1 and H_4, and at which none of the other five buried histidines shows an observable peak. An additional nontitrating peak at 883–887 Hz is also observed in the apoenzyme, and the nontitrating peak which appeared at 840–846 Hz in the native enzyme is at 854–855 Hz in the apoenzyme (Table II).

Sulfonamide-Inhibited Enzyme. The titration curves observed for human carbonic anhydrase B in the presence of stoichiometric amounts of *p*-carboxybenzenesulfonamide are shown in Figure 5. Curves 1 and 3 are virtually identical with the corresponding curves in the native enzyme (compare the values for the chemical shifts and p*K*'s in Table I). This shows that the environment of residues H_1 and H_3 is unaffected by sulfonamide binding; these residues are therefore probably remote from the sulfonamide binding site, and in addition are not affected by the conformational change produced by sulfonamide binding (King and Burgen, 1970).

The remaining titration curves observed in the presence of sulfonamide (curves 7, 7′, and 8) bear no obvious resemblance to any of the titration curves seen in the native enzyme. Since sulfonamides bind very tightly to carbonic anhydrase (for *p*-carboxybenzenesulfonamide, $K_a = 10^6\ M^{-1}$), their exchange between bound and free states is slow on the nuclear magnetic resonance time scale, and it is not possible to observe progressive shifts of the histidine C_2–H peaks on addition of increasing amounts of sulfonamide. We cannot, therefore, say with certainty which of curves 7, 7′, and 8 corresponds to residues H_2 or H_4 identified from Figure 3. It is clear, however, that an additional titration curve has appeared, imply-

TABLE II: Position of Nontitrating Peaks in the 800–950-Hz Region of the Nuclear Magnetic Resonance Spectrum of Human Carbonic Anhydrase B.

	Chemical Shift (Hz) Downfield from External Me_4Si	
	pH* 6	pH* 8
Native enzyme	846	840
Apo enzyme	855	854
	887	883
Iodide inhibited	846, 856	850
	925	926
Sulfonamide inhibited	842	842

ing that a histidine residue which was buried in the native enzyme is able to rotate much more freely when sulfonamide is bound to the enzyme. Thus the conformational change produced by sulfonamide binding involves imparting additional motional freedom to one imidazole side chain. Postulating only the minimum necessary changes in the titration curves on sulfonamide binding, we can suggest tentatively that curve 8 corresponds to residue H_4, and that either curve 7 or curve 7′ corresponds to residue H_2, the other one of this pair being the new titration curve. Thus sulfonamide binding clearly affects at least three of the eleven histidine residues of human carbonic anhydrase B.

The titration curves of Figure 5 also provide evidence for a pH-induced conformational change in the sulfonamide–enzyme complex. The difference in chemical shift between the protonated and unprotonated forms of the residues giving rise to curves 7 and 7′ is only 50.5 and 37 Hz, respectively, compared to a normal value of *ca.* 100 Hz. This suggests that these two residues are in different environments in the protonated compared to the unprotonated forms, and that these environments differ in such a way as to reduce the apparent difference in chemical shift between the two forms. Since the chemical shifts at the low pH end of curves 7 and 7′ are more nearly normal than those at the high pH end, it appears that in the unprotonated form these residues are in a deshielding environment, while in the protonated form their environment more closely resembles that of the solvent. Similarly, although the chemical shift span ($\delta_{HA} - \delta_A$) of curve 8 is almost normal, there is a downfield shift of 23 Hz in the unprotonated form if this curve is due to residue H_4. Since these curves can be fitted tolerably well by a Henderson–Hasselbalch curve, the pK of the perturbing group must be very close to that of the histidines themselves. Although it is possible that the peculiarities in these three titration curves reflect three separate local changes, it is perhaps more likely that a single change is affecting all three residues. It is not possible to say from the available evidence whether the group whose titration produces these effects is one of the observable histidine residues or another group (histidine or other) of similar pK. It is notable that there is no evidence for a pH-dependent change of this sort in the native enzme.

Iodide-Inhibited Enyzme. The titration curves of human carbonic anhydrase in the presence of a saturating concentration of iodide ion (Figure 6) are fairly similar to those of the

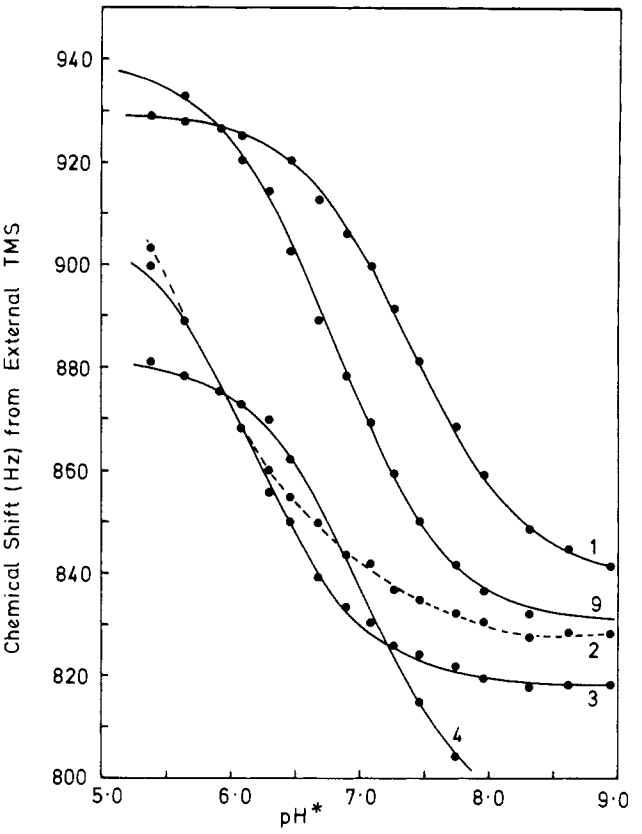

FIGURE 6: Titration of the histidine residues of human carbonic anhydrase B in the presence of a saturating concentration of iodide ion.

native enzyme. Curves 1, 3, and 4 of Figure 6 are very similar to the corresponding curves for the native enzyme, and curve 2 of Figure 6 also resembles curve 2 of Figure 3. As with sulfonamide binding, however, a fifth titration curve has appeared (curve 9).

Since iodide binds relatively weakly to human carbonic anhydrase B (K_a = 5.0 × 10^2 M^{-1} at pH 6.5), the exchange is fast on the nuclear magnetic resonance time scale and the progressive changes on addition of increasing concentrations of iodide can be followed. The results of such an experiment at pH* 6.3 are shown in Figure 7. The value for the affinity constant of 2 × 10^2 M^{-1} obtained from these curves, which is likely to be rather unreliable because of the small chemical shift changes being measured, agrees satisfactorily with that obtained by Taylor from kinetic studies (P. W. Taylor, personal communication) and quoted above. The data shown in Figure 7 demonstrate unequivocally that the curves 1–4 in Figure 6 do correspond to the curves with the same numbers in Figure 3, and hence to histidine residues H_1–H_4. Unfortunately, the peak giving curve 9 is rather broad (line width 12 Hz), and its appearance as the iodide concentration was increased could not be followed with any accuracy. Measurement of the areas shows, however, that this peak could not have come from any of the four curves observed for the native enzyme; as in the case of sulfonamide binding, the binding of iodide confers a certain amount of motional freedom on an imidazole group which is buried in the native enzyme.

Of the other four histidine residues which can be observed, only H_2 is substantially affected by iodide binding (though the pK's of H_1 and H_3 are increased somewhat, the difference is

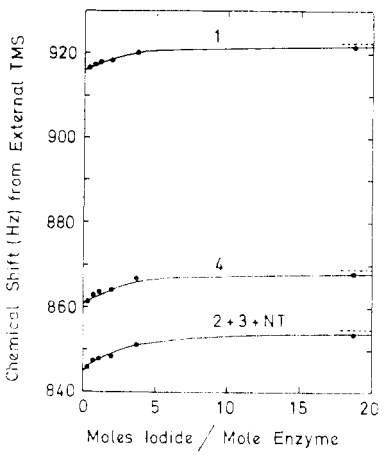

FIGURE 7: Chemical shifts of the histidine residues of human carbonic anhydrase B as a function of added iodide ion at pH* 6.3. Dashed lines give the chemical shifts at saturation with iodide.

not statistically significant). In the presence of iodide the titration curve of H_2 cannot be fitted by the Henderson–Hasselbalch equation. The slope of the dotted line drawn through the points for curve 2 in Figure 6 is less than that expected, suggesting a pH-dependent effect on the chemical shift other than that due to simple titration.

The nontitrating peaks in the iodide-inhibited enzyme are quite different from those in the native or sulfonamide-inhibited enzyme. An additional nontitrating peak appears at 925 Hz, and the peak which is at 840 Hz above pH* 6.7 splits into two below this pH*. One of these two peaks stays at 846–850 Hz, and the other moves downfield as the pH* decreases below pH* 6.7, reaching 862 Hz at pH* 5.35.

It is possible that one or both of these peaks are histidine C_2–H peaks, in which case the pK of the corresponding residues would have to be 5 or less. In the present state of our knowledge of the nuclear magnetic resonance spectrum of this enzyme, no useful conclusions can be drawn from the behavior of these nontitrating peaks. They do, however, indicate a difference between iodide- and sulfonamide-inhibited human carbonic anhydrase which is worthy of further investigation.

Conclusions

Only four of the eleven histidine residues of native human carbonic anhydrase B have sufficiently rapid side-chain motion to give observable C_2–H resonances. Riddiford (1964) and Riddiford *et al.* (1965) calculated from hydrogen-ion titration curves that only four histidine residues are titratable in the native enzyme, suggesting that we can observe peaks corresponding to all the titratable histidine residues. If this is so, then we can conclude that native human carbonic anhydrase B contains only two imidazole groups with pK values near neutrality (H_1 and H_4).

Histidine residue H_1 has a pK of 7.23, which could conceivably explain the kinetic results referred to earlier. However, this residue is wholly unaffected by iodide or sulfonamide binding, and *cannot* therefore be the group controlling the pH dependence of inhibitor binding.

It has previously been suggested that sulfonamides may donate a proton to an imidazole on binding to human carbonic anhydrase B (King and Burgen, 1970); this would lead to the imidazole group being protonated at all pH values at which the sulfonamide is bound—in this case up to high pH* values (sulfonamides bind strongly until the pH of the solution exceeds the pK of the sulfonamido group (Taylor *et al.*, 1970b); we have measured the pK of the sulfonamido group of *p*-carboxybenzenesulfonamide by nuclear magnetic resonance methods and obtained a value of 10.2 ± 0.05). Residues H_2 (pK = 5.91) and H_4 (pK = 7.00) are affected by sulfonamide binding and residue H_2 by iodide binding. However, neither of these residues is affected by inhibitor binding in such a way as to indicate that the inhibitor binds only to the unprotonated form. Provided that, as argued above, we can indeed observe all the titratable histidine residues in the native enzyme, these considerations lend weight to the suggestion that the titratable group controlling inhibitor binding is a zinc-coordinated water molecule. Nevertheless, it must still be emphasized that several histidine residues do not give observable resonances.

An additional histidine C_2–H peak is observed in the apoenzyme and in the enzyme inhibited with iodide or sulfonamide. The behavior of this peak is quite different in the three situations, and there is no evidence for or against the possibility that the same residue is involved in all three cases. The appearance of a mobilized histidine residue may be due either to the displacement of one of the histidine ligands of the metal ion or to a conformational change affecting a more remote residue. Recent crystallographic results (A. Liljas, personal communication) suggest the latter is the correct interpretation for the case of inhibitor binding.

Again, recent studies of inhibitor-induced ultraviolet difference spectra (King and Burgen, 1970) indicate that sulfonamides apparently cause greater conformational change than anions. This may be reflected in the observation that histidine residue H_4 is affected by sulfonamide binding but not by iodide binding, though this difference could also be due to a direct effect of, for example, the aromatic ring of the sulfonamide. At present, we have no indication of which of the residues we observe (if any) are in the active site, and therefore likely to be affected directly by inhibitor binding. In this connection it is interesting that two histidine residues near the active site of human carbonic anhydrase B can be chemically modified, one by iodoacetate (Bradbury, 1969) and one by chloroacetyl chlorothiazide (Whitney *et al.*, 1967). The study of the nuclear magnetic resonance spectrum of these derivatives (currently in progress) should help to clarify some of these points.

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Reversible, pH-Dependent Formation of a Conformer of Rabbit Liver Fructose 1,6-Diphosphatase with Low Catalytic Activity*

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ABSTRACT: The catalytic activity of rabbit liver fructose 1,6-diphosphatase at neutral pH was found to markedly decrease with increasing hydrogen ion concentration. More than 90% of the activity was lost at pH values below 6.7. The presence of certain carboxylic acids such as EDTA, oxaloacetate, and malonate both prevented and reversed the inactivation. Also, recovery of full neutral activity was found upon incubation at pH 7.3. In both instances, the presence of low concentrations of substrate prevented the restoration of activity.

No evidence could be found for any change in molecular size in the two forms of fructose 1,6-diphosphatase. It was therefore concluded that the low-activity form represented a new conformer of the enzyme. A significant consequence of this conformational change was reflected in the increased sensitivity of the enzyme toward its allosteric inhibitor, AMP, and the loss of cooperative interactions among the inhibitor sites.

Our early experiments on rat and rabbit liver fructose 1,6-diphosphatases (D-fructose 1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) indicated the existence of a different form of the enzyme with low catalytic activity at neutral pH (Taketa and Pogell, 1963, 1965). Using a continuous spectrophotometric assay for the enzyme at pH 7.3, reproducible maximal activities were not observed unless the enzyme was

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