Acid Difference Spectra of Human Carbonic Anhydrases*

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Human carbonic anhydrase B loses enzymatic activity as it undergoes an irreversible conformational change near pH 4 (1). Riddiford (2) further found that seven of the eleven histidyl groups, which are unreactive in the native protein, become titratable during this change. Also, five or six of the eight tyrosyl groups do not ionize freely and reversibly in the native protein (2, 3). Human carbonic anhydrase C has a much greater specific activity (4) and a higher isoelectric point than enzyme B (1), but its titration behavior is very similar to that of enzyme B (5). Seven of the twelve imidazole groups are normalized near pH 4.5 (at a slightly higher pH than the value of 4.2 found for enzyme B), with a loss in enzymatic activity. Also, as in carbonic anhydrase B, only two of the eight tyrosyl groups titrate normally. However, enzyme C is more labile above pH 12.25 than is B.

Difference spectra between native and acidified carbonic anhydrase B were first studied by Ghazanfar in this laboratory (1). He observed three characteristic peaks in the difference spectra, at 280, 285, and 291.5 nm. The first of these is the most intense. He showed that there is a very abrupt transition when ΔEM is plotted against pH. The resulting curve is qualitatively similar to an ordinary titration curve, but is much steeper. At an ionic strength of 0.05 the midpoint of the curve (pH_mid) lies near pH 3.5, or slightly above. Here we report studies of the pH dependence of the acid difference spectra at Γ/2 = 0.05 and at Γ/2 = 0.15 of carbonic anhydrases B and C. The difference spectra are time dependent, as Ghazanfar had already found, and we have studied the changes with time in further detail.

Experimental Procedure

The carbonic anhydrases were prepared from packed human red blood cells by the chloroform-ethanol method, as previously described (1). Carbonic anhydrase B was obtained from the crude extract by the hydroxylapatite column method and crystallized as described by Riddiford (2). Carbonic anhydrase C was prepared from the crude chloroform-ethanol extract by DEAE-Sephadex chromatography (1), and was then concentrated with SE-Sephadex (6). Crystallization occurred upon dialysis against 2.5 M (NH₄)₂SO₄ in 0.05 M Tris buffer, pH 8.5. A second recrystallization was carried out on the preparations used for the study of difference spectra. Both enzymes B and C were stored in the crystalline form in 3.8 M (NH₄)₂SO₄ containing the respective buffers. Neither was lyophilized at any stage in the preparation.

To prepare the stock solutions, the (NH₄)₂SO₄ was filtered off and the crystals were dissolved either in water or 0.001 M phosphate buffer, pH 7, to give approximately a 1% solution. The protein solution was then dialyzed against 100 volumes of the same solvent for 3 to 4 days, with change of the outer solution every 12 hours. From this stock solution, 100 to 200 μl were pipetted into 3 ml of the appropriate buffer for measurements. Concentration of the final solution was measured at neutral pH from ultraviolet absorption at 280 μm (ΔA280 was taken as 16.3 for enzyme B, and as 17.8 for enzyme C). The molecular weight of both enzymes was taken as 30,000 (1, 7).

The buffers used (ionic strength, 0.05) were as follows: 0.01 N HCl + 0.04 N KCl (pH 2), glycine (pH 2.3 to 3), citrate (pH 2.5 to 3.8), acetate (pH 3.6 to 5.5), and phosphate (pH 5.5 to 8.5). For experiments at an ionic strength of 0.15, 0.1 M KCl was added to the buffer solutions. Either 0.05 N HCl (for Γ/2 = 0.05, pH 1.5) or 0.1 N HCl (plus 0.05 M KCl for Γ/2 = 0.15, pH 1.2) was used for the reference solutions. The protein was added to the reference solution 2 to 3 hours before the measurements to allow for complete denaturation. It was found with a pH 7 solution as a stable standard that the blue shift increased significantly during the first 1½ to 2 hours after addition of the protein to the acid. No further change was detected, even after 12 hours at room temperature.

All difference spectra were obtained on the Perkin-Elmer model 350 recording spectrophotometer at room temperature (23–25°C). Usually, the time required to scan from 400 μm to 250 μm was about 2½ minutes. Stopped matched cuvettes were used, and the instrument was compensated as often as necessary to give a flat base line with empty cell compartments. We studied changes with time as follows. One starts a stopwatch. The protein is added to the buffer solution in the cuvette. The solution is rapidly mixed, and scanning begins within 30 to 40 seconds. The time is noted at each peak. Scan...

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‡ L. M. Riddiford, unpublished results.

§ It should be emphasized here that the crystals of carbonic anhydrase B are distinctly inferior in quality to those of enzyme C, and are commonly found mixed with material that appears more or less amorphous. This is true even for preparations that yield only a single band on starch gel electrophoresis in Tris buffer at pH 8.6. However, crystallization by the method described was of considerable practical value for the removal of minor impurities.

D. V. Myers, unpublished results. The separation of the carbonic anhydrases on DEAE-Sephadex has been developed by Dr. Dirck V. Myers in this laboratory and will be reported in a later communication.
ning is repeated at 5, 10, 15, 20, and 30 minutes, if any change is detected at the early times. The solution is then removed from the cuvette and the pH taken. Additional scannings are made at, 1, 3, 24, and sometimes 48 hours, and the pH checked each time. The data for the two peaks for the first 20 to 30 minutes are plotted as a function of time and extrapolated back to zero time.

After each set of runs, the buffers used are scanned against the reference solution (without protein), and any deviations from the base line corrected in the experimental spectra. In general, we have found no deviation above 250 μm.

The Radiometer pH meter 4 equipped with the GK2021 B probe electrode was used for all pH measurements.

RESULTS

The difference spectra of both enzymes B and C (reference pH between 1.2 and 1.5) have maxima at 234 to 235, 285, and 291.5 μm, and there is some fine structure detail between 250 and 280 μm (Fig. 1). Studies of pH dependence were for the most part confined to the two maxima above 250 μm, because the time effects made the use of buffers imperative. The acetate, citrate, and glycine buffers used all showed some absorption below 250 μm. Subtraction of a blank curve from an experimental curve proved an unsatisfactory correction. We found the solutions to obey Beer’s law at 285 and 291.5 μm over a concentration range from 0.02 to 0.15%. At 234 to 235 μm, the slit width was changing rapidly with decreasing wave length and became greater than 1 mm; thus in Fig. 1 values below 240 μm should be regarded only as qualitative estimates.

At an ionic strength of 0.05 the two enzymes show identical relative heights of the maxima at 285 and 291.5 μm (pH 7 against pH 1.5). Also the total change in ε at pH 2 and pH 7 is very similar (Fig. 2). For enzyme B the value of Δε at pH 7 is 7700 and that of Δε at pH 1.5 is 8300 μm⁻¹ cm⁻¹, as compared to 8500 and 9300 μm⁻¹ cm⁻¹, respectively, for enzyme C. However, at pH 2 = 0.15 there is a significant difference between the two. For enzyme B Δε at pH 2 is 4200 and in Δε at pH 1.5 5300, whereas for enzyme C the respective values are 7100 and 7400 (Fig. 3).

The pH values at the midpoints of these curves (Figs. 2 and 3) at zero time are shown in Table I. As can be seen, the value of ΔpH at pH 2 = 0.15 is the same for both enzymes. However, at pH 7 there is a significant difference between the two. For enzyme B Δε at pH 7 is 4200 and in Δε at pH 1.5 5300, whereas for enzyme C the respective values are 7100 and 7400 (Fig. 3).

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ENZYME B: O,A.O
ENZYME C: \( l \),A,=,V

Fig. 3. \( \Delta \varepsilon \) as a function of pH at 291.5 \( \mu \) and at 285 \( \mu \) for carbonic anhydrases B (solid line) and C (dashed line) at room temperature and ionic strength of 0.15. Reference, pH 1.20 (0.1 N HCl, 0.05 M KCl). The different symbols indicate different enzyme preparations. For enzyme B (open symbols) the concentration range was 0.15 mg per ml to 0.53 mg per ml and for enzyme C (closed symbols) 0.18 mg per ml to 0.31 mg per ml.

TABLE I

<table>
<thead>
<tr>
<th>Wave length</th>
<th>pHmid ( \Gamma/2 = 0.05 )</th>
<th>pHmid ( \Gamma/2 = 0.15 )</th>
<th>( \Delta \text{pHmid} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>m( \mu )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>291.5</td>
<td>3.60</td>
<td>4.93</td>
<td>0.56</td>
</tr>
<tr>
<td>285</td>
<td>3.57</td>
<td>4.07</td>
<td>0.50</td>
</tr>
<tr>
<td>Enzyme C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>291.5</td>
<td>3.47</td>
<td>4.25</td>
<td>0.77</td>
</tr>
<tr>
<td>285</td>
<td>3.75</td>
<td>4.23</td>
<td>0.48</td>
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</tbody>
</table>

* \( \Delta \text{pHmid} = \text{pHmid} (\Gamma/2 = 0.15) - \text{pHmid} (\Gamma/2 = 0.05) \).

both ionic strengths there is a significant time dependence of the spectrum of enzyme B in the pH region 2.4 to 5. From pH 3.2 to 5, a blue shift (relative to pH 7) occurs at zero time and increases up to 24 hours, although the major increase is in the first hour. Below pH 3 in both glycine and citrate buffers (but not in HCl-KCl solution), an initial blue shift greater than that observed at pH 1.2 occurs. With time this shift decreases (Fig. 4). The changes with time in the spectrum of enzyme C occur between pH 3.7 and 5.5 at \( \Gamma/2 = 0.15 \) and between pH 3 and 5 at \( \Gamma/2 = 0.05 \). At both ionic strengths there is again a small blue shift at zero time followed by a progressive increase. Also, in glycine and citrate buffers below pH 2.7 the results are similar to those on enzyme B although the effect is not as pronounced. Also, the relative heights of the peaks are reversed in these pH ranges, that at 291.5 being slightly smaller than that at 285 \( \mu \).

For both enzymes the same type of denaturation blue shift occurs upon decreasing the ionic strength (from 0.15 to 0.05 at pH 1.5).

**DISCUSSION**

Difference spectra in the region between 270 and 300 \( \mu \) are due to changes in the environment of the aromatic chromophores of the protein molecule. The maximum between 230 and 236 \( \mu \) has been attributed partially to changes in the conformation of the peptide backbone (8), but Eisenberg and Edsall (9) have concluded that in the case of serum albumin it can be explained by change in the environment of the tyrosyl residues. Some fine structure which can be detected (see Fig. 1) between 250 and 300 \( \mu \) in the carbonic anhydrase difference spectra is presumably due to phenylalanine. Maxima at 278 and 285 \( \mu \) have been found in the difference spectrum of tyrosine and O-methyltyrosine (10, 11). The acid difference spectrum of tryptophan shows a large maximum near 295 \( \mu \) and a much smaller one near 283 \( \mu \) (11, 12). Hamaguchi and Kurono (13) have studied the difference spectrum of tryptophan in guanidine hydrochloride solutions, with tryptophan in dilute aqueous EDTA as a reference solution. The peaks in these difference spectra occur at 284 and 292 \( \mu \). The height of the peak at 292 \( \mu \) is unaffected by the addition of tyrosine to the tryptophan solution, but that of the peak at 284 \( \mu \) is greatly increased by the addition of tyrosine, which indeed on a molar basis makes a greater contribution than tryptophan to this peak (13).

The molecule of carbonic anhydrase B contains six tryptophan and eight tyrosine residues; carbonic anhydrase C contains seven tryptophans and eight (or nine) tyrosines (1, 7, 14). It appears that the molecule of human carbonic anhydrase B can be resolved into two components, one of which contains six tryptophan and nine tyrosine residues and the other seven tryptophan and eight (or nine) tyrosines. This is consistent with the results of Hamaguchi and Kurono (13), who found that the addition of tyrosine to a tryptophan solution increases the height of the peak at 284 \( \mu \) by a factor of 1.4.

4 S. Bradbury and G. Guidotti, unpublished results on enzyme C prepared in this laboratory.
safe to conclude, by analogy with the studies on the amino acids in guanidine hydrochloride solutions, and in the light of all other experience with difference spectra (15), that the peak in the acid difference spectrum of the carbonic anhydrases at 291.5 mp may be assigned entirely to changes in the environment of the tryptophanyl groups. The tyrosyl groups certainly contribute significantly to the peak at 285 mp.

On this assumption, $\Delta_{AEM}$ per tryptophanyl group at 291.5 mp is 1420 for enzyme B and 1330 for C at $\Gamma/2 = 0.05$. At $\Gamma/2 = 0.15$, $\Delta_{AEM}$ per group is 880 for enzyme B and 1060 for C. Since the effect of intramolecular electrostatic repulsions at low pH (high positive charge) decreases with increasing ionic strength, we may infer that the enzyme molecules remain partially folded at $\Gamma/2 = 0.15$, and unfold more completely as the ionic strength is lowered. The tryptophanyl and tyrosyl groups are, therefore, more exposed to the solvent at the lower ionic strength, hence the higher values of $\Delta_{AEM}$ at $\Gamma/2 = 0.05$. This shielding effect of increasing ionic strength is pronounced for enzyme B and is relatively slight for C as Figs. 2 and 3 show. This would appear to imply some structural difference between the two enzymes, but at present there is no basis for inferring the nature of this difference.

The observed values of $\Delta_{M}$ at 291.5 arc higher than any that Yanari and Bovey (11) found for the tryptophan peak in a series of proteins with varying tryptophan-tyrosine ratios. Their highest value was 750 per tryptophan residue in ovalbumin. It is of interest to compare the carbonic anhydrases with lysozyme, which contains six tryptophanyl and three tyrosyl residues in a total of 129 amino acid residues (16). Yanari and Bovey (11) reported a total $\Delta_{AEM}$ for lysozyme, at 295 mp, of 640, between pH 7 and pH 1. Donovan, Laekowski, and Scherra (12) reported a value of 400 between pH 5 and 1.5. The value per tryptophanyl residue, from the data of Yanari and Bovey, is near to 105, which is only about 10% of the value for the carbonic anhydrases. Hanauchi and Kurono (13), from studies of difference spectra in guanidine hydrochloride solutions, have proposed that three or four (probably four) of the tryptophanyl chromophores in lysozyme lie at the periphery of the native molecule, and that the other two (or three) become exposed to the solvent when the guanidine hydrochloride concentration is increased to about 4 M. In contrast one may tentatively conclude from the acid difference spectra that the tryptophanyl residues in the native carbonic anhydrase molecules are in the molecular interior, well shielded from the surrounding solvent. Presumably, they become fully exposed to the solvent during the transition that occurs in solution near pH 4.

For each of the two carbonic anhydrases the plot of $\Delta_{AEM}$ against pH (Figs. 2 and 3) shows the same pH$_{acid}$ at 285 mp and 291.5 mp, depending only on ionic strength (Table I). Apparently, the molecular changes on acidification affect both the tryptophan and the tryptophanyl groups in parallel fashion. There is clear evidence from titration studies in guanidine hydrochloride solutions, have proposed that three or four (probably four) of the tryptophanyl chromophores in lysozyme lie at the periphery of the native molecule, and that the other two (or three) become exposed to the solvent when the guanidine hydrochloride concentration is increased to about 4 M. In contrast one may tentatively conclude from the acid difference spectra that the tryptophanyl residues in the native carbonic anhydrase molecules are in the molecular interior, well shielded from the surrounding solvent. Presumably, they become fully exposed to the solvent during the transition that occurs in solution near pH 4.

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During titration with acid the uptake of protons is markedly time-dependent only from pH 3.9 to 4.3 for enzyme B (2) and from pH 4.2 to 4.7 for enzyme C (5), irrespective of ionic strength. In these pH regions the buried imidazole groups become titratable. Observations of pH in these titrations (2, 5) were continued for no more than 1 hour, and usually the pH reached a steady value within 2 minutes. The time course of the difference spectra, which are considerably more sensitive than pH titrations, reveals further changes which require several hours for completion.

The blue shift relative to pH 1 seen in glycine and citrate buffers (Fig. 4) in the pH region below 3 may result from removal of zinc by chelation. The zinc atom is essential for enzymatic activity and its removal appears to make the enzymes more unstable (17, 18). The addition of any chelating agent could therefore enhance the sensitivity of the enzyme to acid denaturation. Thus, the aromatic chromophores would be more exposed between pH 2.3 and pH 3 in either a glycine or a citrate buffer than in a HCl-KCl buffer at the same ionic strength.

**Summary**

The acid difference spectra (reference pH 1.2 to 1.5) of both human carbonic anhydrases B and C have peaks at 234 to 235 mp, 285 mp, and 291.5 mp. Since the carbonic anhydrases contain both tryptophan and tyrosine, both of these residues contribute to the 285-mp peak, but only tryptophan contributes to the 291.5-mp peak. The pH dependence of the peaks at 285 and 291.5 mp for both enzymes was studied as a function of time at ionic strength of 0.05 and 0.15.

At an ionic strength of 0.05 the total $\Delta_{AEM}$ (pH 7 against pH 1.5) at both wave lengths lies between 7500 and 9500 cm$^{-1}$ for both enzymes, although the tryptophan to tyrosine ratios are different (6:8 for enzyme B, and 7:8 or 7:9, for enzyme C). At an ionic strength of 0.15 the values of $\Delta_{AEM}$ for enzyme B are lowered to 4200 at 285 mp and 5300 at 291.5 mp, whereas the values for enzyme C, 7100 and 7400, respectively, are only a little lower than at ionic strength of 0.05. Comparison with available data on lysozyme, a protein molecule containing six tryptophan and three tyrosine residues, shows that the peaks in the acid difference spectra are greater by a factor of approximately 10 for the carbonic anhydrases than for lysozyme.

The midpoints of the pH-dependence curves of the difference spectra, at a given ionic strength, lie between pH 3.6 and 4.3. They are higher by approximately 0.18 pH unit for enzyme C than for enzyme B. Change of ionic strength from 0.05 to 0.15 displaces the curves to higher pH. For each enzyme, at fixed ionic strength, the pH dependence of the peaks at 285 and 291.5 mp is the same. The transition occurs in the pH range where other conformational changes are known to occur.

The difference spectral changes occur over a period of several hours, although the unreactive imidazole groups become titratable in much less than 1 hour.

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