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Acid-Base Properties of Cobalt(II)-Substituted Carbonic Anhydrases

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The acid-base properties of two cobalt(II)-substituted carbonic anhydrases have been analyzed through electronic absorption spectroscopy in terms of deprotonation of two interacting acidic groups. The pattern of pK_a values observed in water and in water-organic solvent mixtures is consistent with the two groups being a coordinated water and a histidinium residue. The pH dependence of the apparent affinity constants of NO_3^- for the cobalt(II) derivative of bovine carbonic anhydrase II has also been accurately measured through electronic spectroscopy. The analysis of the data provides a basis for the understanding of the pH dependence of the enzymatic properties.

Introduction

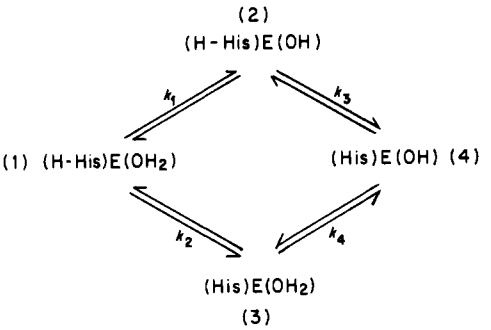
There is today agreement that the enzymatic properties of carbonic anhydrase (CA) depend on more than one acidic group,[1-4] as do the spectroscopic properties of the cobalt(II)-substituted enzyme (CoCA hereafter).[5-7] One acidic group is the metal-coordinated water molecule. Recently, Simonsson and Lindskog proposed[8] that in carbonic anhydrase a histidine hanging in the active site cavity is a second acidic group capable of affecting the catalytic properties of the enzyme. Such a group would be His 64 for bovine CA II and His-64 or His-200 for human CA I.[4] As already pointed out,[8] the general treatment for a species experiencing two proton dissociations requires four microconstants of which only three are independent[9] (Scheme I). Simonsson and Lindskog[8] have given a set of four microconstant values that are consistent with the overall behavior of the enzyme. The four constants cannot be determined unless some assumptions are made. In the absence of assumptions, only two macroscopic or apparent acidic constants can be obtained. Since this is a crucial point in the understanding of carbonic anhydrase, we have undertaken a thorough investigation of the pH dependence of the electronic spectra of bovine CoCA II and human CoCA I. For each experiment the two macroscopic constants have been obtained through nonlinear least-squares treatment of the spectral variations with pH. Then, it is assumed that (H-His)E(OH) and (His)E(OH) have the same electronic spectra, as well as (H-His)E(OH_2) and (His)E(OH_2), since the donor groups are pairwise the same. Such assumption has allowed us to calculate the values of the four microconstants. Furthermore, other independent sets of data could be obtained on water-Me_2SO and water-dioxane mixtures. Finally, the affinity constants of NO_3^- have been obtained as a function of pH in order to reveal their sensitivity to both acidic groups.

Experimental Section

Bovine CA II was purchased from Sigma Chemical Co. and purified through chromatography on DEAE cellulose;[10] human CA I was a gift of S. Lindskog and was used without further purification. Cobalt substitution was achieved through (i) zinc removal by dialysis against 10^{-2} M 2,6-dipicolinic acid solutions at pH 7.0;[11] (ii) extensive (10 changes in 3 days) dialysis of the apoenzyme against twice-distilled water to remove traces of chelator; (iii) dialysis (three to four changes in 2 days) against unbuffered 10^{-3} M cobalt(II) sulfate solutions; (iv) exhaustive (12 changes in 4 days) dialysis against twice-distilled water. The final pH of the solutions was close to the isoelectric values of each isoenzyme (5.6 and 5.8 for bovine and human I enzymes, respectively), if the dialysis of point iv were carried out under nitrogen. CoCA concentrations were determined by measuring the absorbance at both 280 and 550 nm (ϵ_{280} = 5.6×10^4 and 4.7×10^4 M^{-1} cm^{-1} and ϵ_{550} = 290 and 200 M^{-1} cm^{-1} (pH 6.0),[5,12] for bovine II and human I isoenzymes, respectively). Concentration data obtained at the two wavelengths were the same within 10% error.

Dimethyl sulfoxide (ERBA RPE) was kept over sodium hydroxide overnight and then distilled at reduced pressure. Dioxane (ERBA RPE) was distilled over sodium. Room-temperature electronic absorption spectra were recorded on a Cary 17D spectrophotometer in the range 13 000-25 000 cm^{-1}. The pH of the samples was changed by adding increasing amounts of 1 M sodium hydroxide. The pH values were always measured in situ with a glass electrode after recording the spectra. The spectra have also been recorded in 50 mM HEPES buffer. The affinity constants of nitrate were measured from the spectral variations on samples obtained by titration of either unbuffered or 50 mM HEPES buffered solutions of enzyme with nitrate solutions at the same pH (HEPES = *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid).

Scheme I



Results and Discussion

The spectra of human CoCA I and bovine CoCA II at different pH values have been measured in water and found to be very similar to those previously reported.[5] Figure 1 shows the pH dependence of the absorbance at 640 nm, i.e. where the variation in absorbance with pH is largest, for bovine CoCA II in water and in water-Me_2SO mixtures. The spectral data are absolutely reproducible even with samples obtained from different preparations. Any attempt of analyzing the observed data with a single pK_a is unsatisfactory (Figure 1); it is shown here that the analysis with two macroscopic pK_a's is quite adequate. Two macroscopic acidic constants can be related to the absorption data A through eq 1, where A_1 and A_3 are the absorption of biprotonated and fully

$$A = \frac{A_1[H^+]^2 + A_2K_1[H^+] + A_3K_1K_2}{[H^+]^2 + K_1[H^+] + K_1K_2} \quad (1)$$

deprotonated species and A_2 is a fictitious absorbance of the monodeprotonated species. The pK_a values obtained through a

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Table I. Experimental pK_1 and pK_2 and Calculated Microconstant Values Associated with Proton-Transfer Equilibria of Cobalt(II)-Substituted Carbonic Anhydrase in Water and in Mixed Solvents at 20 °C[a]

	medium[b]	pK_1	pK_2	pk_1	pk_2	pk_3	pk_4	pK_W[c]
CoHCA I	water	6.87 (6)	8.71 (8)	7.14 (4)	7.21 (7)	8.45 (9)	8.38 (6)	14.1
CoBCA II	water	5.89 (5)	7.97 (8)	6.12 (4)	6.28 (7)	7.75 (9)	7.59 (6)	14.1
	water–Me_2SO 5%	6.05 (4)	8.35 (6)	6.25 (3)	6.49 (5)	8.16 (6)	7.92 (4)	
	water–Me_2SO 10%	6.21 (4)	8.28 (7)	6.41 (3)	6.61 (6)	8.07 (8)	7.87 (5)	14.4
	water–Me_2SO 20%	6.32 (9)	8.27 (10)	6.60 (6)	6.65 (11)	8.00 (12)	7.95 (7)	14.7
	water–dioxane 10%	6.48 (5)	8.79 (6)	6.73 (4)	6.85 (7)	8.55 (8)	8.43 (5)	14.4

[a] Standard deviations in parentheses. [b] Percentages are v/v. [c] Determined by potentiometric titration.

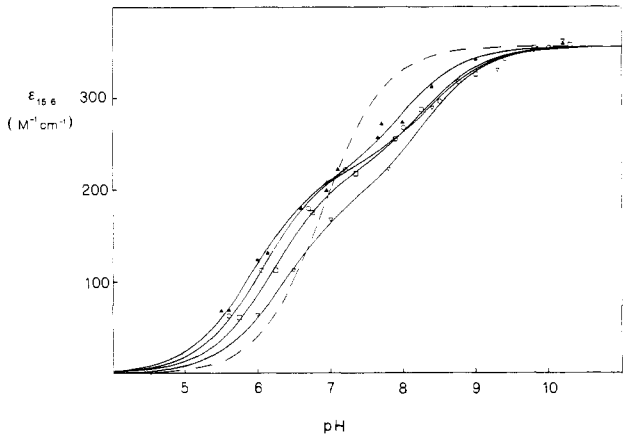

Figure 1. pH dependence of the molar absorption coefficient at 15.6 × 10^3 cm^{-1} of unbuffered cobalt(II)-substituted bovine carbonic anhydrase II solutions in water (▲), water–Me_2SO 5% (○), water–Me_2SO 10% (□), and water–Me_2SO 20% (▽) at 20 °C. The dashed line is a fit of CoCA II in water (▲) with a single pK_a = 6.9; the solid lines are best fits according to eq 1 or 2 (see text).

five-parameter fitting are reported in Table I, and the best fit curves are reported as solid lines in Figure 1. It appears that the two isoenzymes sensibly differ in their pH-dependent properties: the two apparent pK_a's in water are separated by about 2 units in both cases, but those of the human enzyme are approximately 1 unit higher than those of the bovine enzyme.

We have noticed that if the final dialysis of the cobalt enzymes is carried out in the air, as it is generally done, the final pH ranges between 5.6 and 5.2, depending on the time of dialysis against water. Presumably this is due to CO_2 titrating basic groups of the enzyme. The electronic spectra of solutions that came out at pH values lower than the isoelectric points are slightly different from the other and provide sets of macroscopic constants somewhat lower than those reported in Table I. In particular, pK_2 decreases with the decrease of the pH of the starting solution up to 0.3 unit and pK_1 up to 0.5 unit. These discrepancies in the obtained data have puzzled us for a considerable time, since we first reported evidence of more than one ionizing group from the electronic spectra of CoCA.[5] In the exclusion of air, the spectra in HEPES of human CA I and bovine CA II are equal to those in unbuffered solutions.

In the case of bovine isoenzyme, the spectra have been measured also in water–Me_2SO and water–dioxane mixtures as a function of pH. Figure 1 shows that the overall pH-dependent profiles shift to higher pH with increasing percent of organic solvent. The best fit pK_1 and pK_2 values are reported in Table I. In order to check whether the organic solvents did not directly interact with the catalytic metal ion, their ^1H NMR line widths and T_1 values were measured in solutions containing bovine CoCA II. No significant changes in the above nuclear relaxation parameters were observed, suggesting the lack of any direct interaction between the paramagnetic metal ion and the organic molecule.[13]

An attempt of further understanding the chemical meaning of the calculated macroscopic acidity constants can be made by analyzing the data in terms of Scheme I. The experimental absorbance at every frequency is related to the microconstants through the equation (2), where a_i is the molar absorbance of the

$$A = \frac{a_1[H^+]^2 + a_2k_1[H^+] + a_3k_2[H^+] + a_4k_1k_3}{[H^+]^2 + k_1[H^+] + k_2[H^+] + k_1k_3} \quad (2)$$

*i*th species of Scheme I. In this case it is assumed that $a_2 = a_4$ and $a_1 = a_3$, i.e. the H_2O-containing species and the OH-containing species are pairwise assumed to give rise to the same electronic spectra. If these assumptions were strictly valid, the spectra at various pH values would show isosbestic points; indeed, the deviations from spectra with isosbestic points are rather small.[5] Equation 2 can then be used to best fit the data of Figure 1 to obtain the values of the four microconstants (pk_4 being equal to $pk_1 + pk_3 - pk_2$). Such values are also reported in Table I. It should be noted that the best fit curves coincide with those of Figure 1, since eq 2 can be mathematically transformed into eq 1; also, in the above assumption about the pairwise identity of the chromophores, the number of unknowns is the same (five) in the two cases.

Inspection of Table I can now be more instructive from the chemical point of view. The value of K_4 is associated with dissociation of water coordinated to cobalt(II) when the histidine residue is uncharged. The pk_4 values of 7.6 for bovine CA and 8.4 for the human isoenzyme are absolutely consistent with the pK_a values found for water dissociation in model complexes.[14-20] The fact that the enzyme works also at pH values below 6 is then due to the presence of the histidinium, which causes about 40% of the hydroxo-containing species to be present at pH values as low as the value of pk_1. The interaction between the two acidic groups (coordinated water and histidinium residue in the cavity) causes the lowering of pK_a of the cobalt(II)-coordinated water molecule down to pk_1 and, therefore, the lowering of the apparent pK_a for enzymatic activity. Therefore, the rationalization of the pH dependence of the enzymatic activity does not require any entatic state representation nor any peculiar thermodynamic behavior of the cavity, but it simply requires regularly coordinated water interacting with a histidine residue. The higher pk_4 values of human CoCA I with respect to the bovine CoCA II is consistent with a larger share of five-coordination in the low-pH species of the former derivative.[21]

Unfortunately, the data obtained in water–organic solvent mixtures do not sensibly improve the resolution of the two apparent pK values, which remain separated by approximately 2 units. Both the apparent pK's and microconstant values for the various mixed solvents are reported in Table I. It appears that while pK_1 steadily

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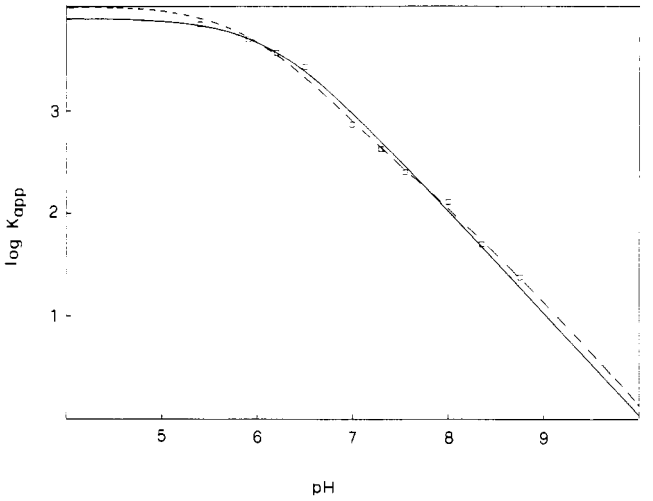

Figure 2. pH dependence of the apparent affinity constant of nitrate ion for cobalt(II)-substituted bovine carbonic anhydrase II in HEPES buffered water solutions at 20 °C. The data are fitted to a single pK_a (full line) assuming a negligible affinity for the high-pH species (best fitting parameters: $pK_a = 6.14$, $\log K = 3.09$) and microconstant values reported in Table I (best fit parameters: $\log K_1 = 4.01$, $\log K_2 = 2.40$) (dashed line).

increases with percent of Me_2SO, pK_2 increases from pure water to 5% Me_2SO and then essentially levels off. Such behavior, that is of course reflected in the pk_1 and pk_2 pair with respect to the pk_3 and pk_4 pair, seem scarcely related to the increase of pK_w in the various solvent mixtures. Possibly these patterns bear a further chemical meaning with respect to the thermodynamic parameters of the various dissociation processes; however, a further discussion of the data may become unsound, also because of the relatively large errors.

The presence of the two acidic groups in the active site of CoCA should also be reflected in the pH dependence of the apparent affinity constant of anions. Again this has been suggested by Lindskog through activity measurements on the zinc enzyme inhibited by iodide.[8] We have measured through spectrophotometric titrations the apparent affinity constants of NO_3^- against bovine CoCA II both in the absence of buffering species and anions and in the presence of the noncoordinating HEPES buffer.[5] The k_{app} values were found virtually the same in the two cases.

The pH dependence of such affinity constants is shown in Figure 2. A qualitative inspection of the data indicates that (i) there is very little evidence of two inflections in the pH dependence of the NO_3^- affinity and (ii) the main pK_a value can be located around pH 6.0. Indeed, the data can be satisfactorily fitted to a single pK_a of 6.14 ± 0.06 (full line in Figure 2); such a value should be compared with the apparent pK_a of 5.9 in Table I. A deeper insight can be obtained by fitting the data using the microconstant values of Table I to estimate the affinity of NO_3^- for the two water-containing species, assuming the affinity for the hydroxo species to be negligible. Such fitting (dashed line in Figure 2) provides values of $\log k = 4.01 \pm 0.02$ and 2.40 ± 0.04 for the affinity constant of NO_3^- for the (H-His)E(OH_2) and (His)E(OH_2) species, respectively. However, there is no significant improvement in the goodness of the fitting with respect to the single pK_a case. This means that, if it were not for the independent characterization of the ionization process of the system, the pH dependence of NO_3^- binding would have given no evidence of more than one acid–base equilibrium. Indeed, the second acid–base process has always escaped detection from inhibitor binding measurements, even in the absence of competing anions or buffering species.[6] In any case, it appears that the affinity of NO_3^- for the diprotonated species is much higher than that for the monoprotonated species, providing a rationalization of all the anion-binding data that in the past have always indicated a lower pK_a value than that obtained from the midpoint of variation of the spectral and catalytic properties of the enzyme.

The above findings bear a chemical significance well beyond the understanding of the acid–base properties of carbonic anhydrase: The present enzyme is usually reported to have much higher affinity for anions than any other zinc- or cobalt(II)-substituted enzyme with related function, and much higher than expected from the coordination chemistry of small model complexes. It appears now that such enhancement of anion affinity is largely brought about by protonation of a nearby histidine side chain, which increases by 1 unit the positive charge in the active site cavity. Interestingly, a similar behavior is shown by carboxypeptidase A, which also undergoes two active site ionizations and shows a dramatic increase in anion-binding affinity on passing from the monoprotonated to the diprotonated species.[22]

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Lewis Acid Induced Intramolecular Redox Reactions of Difluoramino Compounds

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It is shown that strong Lewis acids, such as AsF_5 or SbF_5, which are good fluoride ion acceptors, strongly catalyze an intramolecular redox reaction of difluoramino compounds, such as CF_3NF_2, SF_5NF_2, $ClNF_2$, CF_3ONF_2, and SF_5ONF_2. In the $ClNF_2$–AsF_5 system a thermally unstable intermediate is formed at −78 °C, which on the basis of its Raman spectra is the fluorine-bridged donor–acceptor adduct $ClNF_2 \cdot AsF_5$. The nature of the final decomposition products can be rationalized in terms of their stability. In connection with the low-temperature Raman studies, an unidentified, unstable, blue-green species was observed that gives rise to a resonance Raman spectrum with $\nu = 177$ cm^{-1} and that is also formed from $Cl_3^+AsF_6^-$ and excess Cl_2. For NF_2Cl, ^{14}N–^{19}F spin–spin coupling was observed in its ^{19}F NMR spectrum.

Introduction

During experiments aimed at the oxidative fluorination of CF_3NF_2 to $CF_3NF_3^+AsF_6^-$ by $KrF^+AsF_6^-$, an unusual observation was made. Besides the NF_3 and CF_4 products expected for an oxidative fission of the C–N bond, significant amounts of gaseous *trans*-N_2F_2 and solid $N_2F^+AsF_6^-$ were obtained. Since KrF^+ is the strongest oxidative fluorinating agent presently known[1] and N_2F_2 is a reduction product of CF_3NF_2, KrF^+ was unlikely to cause the observed N_2F_2 formation. Since $KrF^+AsF_6^-$ is thermally unstable and decomposes to Kr, F_2, and AsF_5,[2] we have considered

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