Biochimica et Biophysica Acta, 452 (1976) 239—244

BBA 67945

INTERACTION OF COBALT-BOVINE CARBONIC ANHYDRASE WITH THE ACETATE ION

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(Received March 2nd, 1976)

Summary

The visible spectra of solutions of cobalt(II) bovine carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) with increasing amounts of acetate have allowed the determination of the apparent inhibition constants and of the extrapolated limit spectra of the completely bound enzyme. The electronic spectra have been interpreted on the basis of the presence of five coordinate adduct species.

^{13}C and ^{1}H NMR spectra have also been recorded and discussed on the basis of the metal enzyme-acetate type of interactions. Titrations by means of NMR spectroscopy of the acetate-cobalt(II) bovine carbonic anhydrase with *p*-toluenesulfonamide and azide indicate the existence of two binding sites for the acetate group.

Introduction

The acetate ion is known to be a noncompetitive inhibitory agent of Zn(II), Mn(II) and Co(II) carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) [1—5]. Owing to the complexity of the system under investigation, just a little is known on the structural properties of the metal enzyme-acetate adduct. From the electronic spectra of the cobalt(II) bovine carbonic anhydrase with various amounts of acetate, both the apparent equilibrium constants at various pH values and the limit spectra of the fully bound enzyme have been determined through a computer programme. This procedure is particularly useful for obtaining the limit spectra under those conditions in which the inhibitor has little affinity towards the enzyme.

^{13}C and ^{1}H NMR spectra have added some experimental data which may lead to a deeper insight on the structural properties of the system cobalt(II)-enzyme-acetate ion.

Materials and Methods

Bovine carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) was obtained as lyophilized material from Sigma Chemical Comp., and used without further purification. Acetate, azide, *p*-toluenesulfonamide and all other materials were of analytical grade.

Protein concentrations were determined from the absorbance at 280 nm, using a molar extinction coefficient of $5.7 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ [6].

Enzymatic activity was assayed using *p*-nitrophenylacetate as substrate, at 22°C [7].

Apocarbonic anhydrase was prepared from the commercial enzyme by dialysis against 1,10-phenanthroline, 10^{-2} M in 10^{-1} M acetate buffer, pH 5.2, to a residual activity of less than 5% [8]. The cobalt enzyme was obtained by dialysis of apocarbonic anhydrase against cobalt sulphate, pH = 6.1, 7.5 or 8.7. Esterase activity assayed on cobalt(II)-carbonic anhydrase showed no significant change with respect to native enzyme.

Cobalt(II)-bovine carbonic anhydrase concentrations were determined from the absorbance at both 280 and 550 nm (ϵ_{550} = 270, 360 and 390 $M^{-1} \cdot cm^{-1}$ at pH 6.1, 7.5 and 8.7 respectively). Concentration values obtained from the measurements at the two wavelengths were the same within 10% error, indicating that the protein was almost entirely in the cobalt enzyme form. The spectra of the cobalt enzyme were strictly similar to those already reported [9].

Samples for ^1H NMR measurements were prepared by lyophilizing buffered solutions of cobalt(II)-bovine carbonic anhydrase in deuterium oxide. The deuterated enzyme was then dissolved in ^2H_2O solutions of acetic acid/acetate at the appropriate pH. Titrations were performed by adding small volumes of a solution of *p*-toluenesulfonamide $1.37 \cdot 10^{-1}$ M in acetone (d_6 for ^1H measurements), and of NaN_3, 1 M in ^2H_2O.

NMR measurements. ^1H NMR spectra were recorded on a Perkin-Elmer R32 spectrometer (90 MHz) at 35°C; ^{13}C NMR spectra were recorded on a Varian CFT-20 at 15°C.

Spectrophotometric measurements. Optical spectra were recorded on a Cary 17 spectrophotometer, in the absorbance range 0—0.1, using 10^{-3}—10^{-4} M solutions of the enzyme at 22°C.

The experimental limit spectrum at pH 6.1 was recorded down to 8000 cm^{-1} using a $2.8 \cdot 10^{-3}$ M solution of the enzyme in ^2H_2O, the acetate concentration being 0.5 M.

Calculations. Spectrophotometric data were treated with a least squares programme described in ref. 10. The experimental spectra are always reproduced by the computer within an accuracy of $\pm 1 \cdot 10^{-3}$ absorbance units assuming the equilibrium is

$$\text{Enzyme} + \text{Inhibitor} \rightleftharpoons \text{Enzyme} \cdot \text{Inhibitor}$$

No fitting is obtained if a 1 : 2 enzyme-to-inhibitor stoichiometric ratio is assumed for the above equilibrium.

Results

The features of the electronic spectra of the system cobalt(II)-bovine carbonic anhydrase with increasing amounts of acetate ion show a dramatic dependence on the acetate concentration, with an isosbestic point at approx. 480 nm which is only slightly affected by the pH. The apparent stability constants of the adducts at pH 6.1, 7.5 and 8.7 are respectively 389 (standard deviation ±5), 135 (±3) and 13.8 (±0.3) M^{-1}. The value obtained at pH 7.5 is close to the value of 130 M^{-1} at pH 7.6 previously obtained through studies of fluorescence quenching [4,11]. The calculated limit spectra are practically coincident at the three pH values and correspond to the experimental spectrum obtained at pH 6.1 (see Materials and Methods). Such a spectrum is reported in Fig. 1. Although the gross features of this spectrum are those common to cobalt(II)-enzyme with most inhibitory agents [12—14], the molar absorbance is unusually low, i.e. about one third of that of the pure cobalt-enzyme system. The benzoate-cobalt-enzyme spectrum is also reported (Fig. 1) for comparison purposes (pH = 7.5, K = 67 (±2) M^{-1}). It appears that the latter spectrum is intermediate between that of the pure cobalt enzyme and that of the cobalt-enzyme-acetate adduct.

^{13}C NMR spectra of a solution 10^{-3} M in enzyme and $3.3 \cdot 10^{-1}$ M in acetate, at pH 7.5, gives two signals shifted downfield with respect to the positions of pure acetate of about 0.5 ppm for both COO^- and CH_3. The line halfwidth is 20 Hz for both signals. By titrating the solution with *p*-toluenesulfonamide,

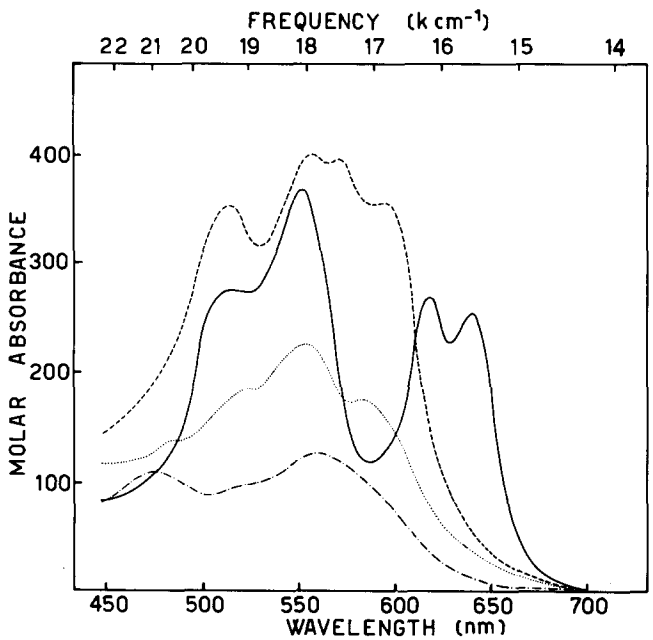

Fig. 1. Limit electronic spectra of cobalt(II)-bovine carbonic anhydrase with acetate (— · — ·, pH = 6.1) and benzoate (. . . . . ., pH = 7.5). Electronic spectra of the pure cobalt(II)-carbonic anhydrase (———, pH = 7.5) and of its 1 : 1 adduct with *p*-toluenesulfonamide (- - - - -).

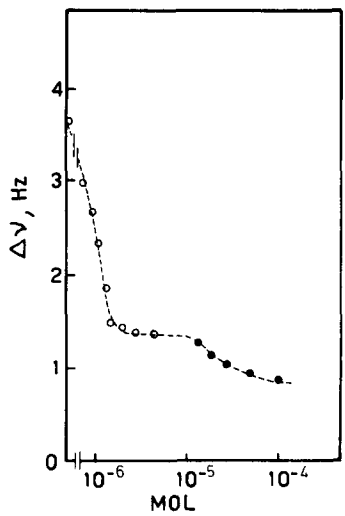

Fig. 2. Titration of $5.6 \cdot 10^{-5}$ mol of acetate in presence of $2.2 \cdot 10^{-6}$ mol of cobalt(II)-bovine carbonic anhydrase (pH = 7.5) with *p*-toluenesulfonamide (○) and N_3^- (●). Semilogarithmic diagram of ^1H acetate line halfwidth vs. mol of the appropriate inhibitor added.

which is a strong inhibitor, both the linewidths and the shifts decrease, following the same qualitative pattern, in agreement with a competition of acetate and *p*-toluenesulfonamide at the same inhibitory site [15—17]. After an addition of twice as much *p*-toluenesulfonamide as there is the enzyme, the signals do not show any isotropic shifts and are only slightly broader than the peaks of the pure acetate. Further additions of *p*-toluenesulfonamide do not further affect the spectra. On the contrary, addition of N_3^- eliminates the residual broadening (2 Hz) of the signals.

The same experiment has been repeated with ^1H NMR spectroscopy. The ^1H NMR spectra of a solution containing $2.2 \cdot 10^{-6}$ mol of enzyme and $5.6 \cdot 10^{-5}$ mol of acetate at pH 7.5 show a small shift of the CH_3 signal (—3.2 Hz) from the diamagnetic position, and a remarkable broadening (line halfwidth 3.6 Hz). Titration with *p*-toluenesulfonamide first and then with N_3^- follows a similar pattern as found for the ^{13}C spectra. The dependence of the ^1H line halfwidth on the amount of titrating inhibitor is shown in Fig. 2.

Discussion

Both electronic and NMR spectra confirm that the acetate ion binds to the metal of cobalt enzyme, as previously proposed [1—5]. The broadening of the ^{13}C NMR signals confirms [4,15] that the linewidth is governed by $\Delta\omega^2 \tau_m$, where $\Delta\omega$ is the isotropic shift and τ_m is the residence time of the ligand in the coordination sphere. Therefore the linewidths cannot give any structural information. The observed downfield ^{13}C isotropic shifts can be due either to a contact mechanism or to a dipolar electron spin-nuclear spin coupling or to both mechanisms. Cobalt(II) complexes, independently of the coordination geometry, often give rise to a large dipolar contribution to the observed isotropic

shifts [18]. However, the fact that the shifts as well as the linewidths are almost the same for both the resonances of the acetate rules out the possibility that the dominating contribution is dipolar in origin, as in this case an r^{-3} dependence of the shifts and therefore an r^{-6} dependence of the linewidths would be expected [19]. Contact shifts in the acetate group are the final evidence of direct metal-oxygen bonds. The inhibitor *p*-toluenesulfonamide titrates this binding site abolishing the contact shifts. The titration with azide indicates the existence of a second binding site which does not involve the metal ion and therefore only slightly affects the ^{13}C and ^{1}H signal widths as the relaxing dipolar mechanism is scarcely operative.

If these data are considered on the light of the work of Lanir and Navon [16, 17] on zinc(II) and manganese(II) bovine carbonic anhydrase, it may be concluded that the acetate ion binds at two different sites, only one of which is directly bound to the metal and therefore titrated by *p*-toluenesulfonamide. However, the small effect on the linewidth of the second site combined with the large effect due to the bulky susceptibility of the sample prevented us from investigating this second site more deeply.

Some information on the structure of the chromophore, which the acetate of the first site belongs to, can be obtained by analyzing the electronic spectra of the system cobalt-enzyme-acetate. A molar absorbance of about 100 $M^{-1} \cdot cm^{-1}$ in the visible region is completely unusual for cobalt(II)-bovine carbonic anhydrase with any other investigated inhibitors and is hardly accounted for by assuming a tetrahedral environment around the metal ion as has been proposed for most cobalt(II)-carbonic anhydrase-inhibitor systems [1,2,13,14,20]. (See for example the spectrum of cobalt(II)-bovine carbonic anhydrase with *p*-toluenesulfonamide in Fig. 1). In fact pseudotetrahedral cobalt(II) complexes have larger molar absorbances irrespectively of the nature of the donor atoms [21].

Moreover the spectrum extended down to 8000 cm^{-1} and showed weak absorptions at 13 800 cm^{-1} ($\epsilon = 10$) and 10 000 cm^{-1} ($\epsilon = 5$). The band at 13 800 cm^{-1} which has the shape and the intensity of an F-F transition can reasonably be taken as diagnostic of the presence of five coordinate species, as tetrahedral and octahedral species do not have F-F transitions that high in energy [21]. Five-coordination can be reached through three donor atoms of the proteic part of the enzyme, the acetate group and a water molecule, as has been already suggested by Dennard and Williams [22] and Morpurgo et al. [23] in order to account for the low intensity of some adducts of cobalt(II)-bovine carbonic anhydrase with anionic inhibitors.

Addition of *p*-toluenesulfonamide to the sample causes the disappearance of the band at 13 800 cm^{-1} and the formation of a new broad band around 8000 cm^{-1} characteristic of tetrahedral species. The low intensity of the spectrum might suggest the possibility of the existence to some extent of six-coordinated species, whose absorptions would be so low not to be detected. Such species could have the acetate group acting as bidentate if the calculated metal-to-acetate ratio of 1 : 1 is followed; since bidentate ligands have exchange kinetics lower than monodentate ligands [24] the latter suggestion is consistent with the observation that the ^{13}C linewidth of the acetate ion in the presence of cobalt(II)-bovine carbonic anhydrase is much larger than that of the benzoate and anthranilate adducts, whose electronic spectra are much more intense [25].

Acknowledgements

Thanks are expressed to Professor L. Sacconi for encouragement and discussions and to Professor A. Vacca for the computer programme.

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