Effects of pH and inhibitors on the absorption spectrum of cobalt(II)-substituted carbonic anhydrase III from bovine skeletal muscle

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Received 27 February 1984; revised version received 19 March 1984

Bovine apocarbonic anhydrase III has been prepared by incubation with 2-carboxy-1,10-phenanthroline at pH 5.5. The Co(II)-substituted enzyme has been prepared and its absorption spectrum has been studied. The spectrum is nearly pH-independent above pH 6. It is very similar to the high pH spectral forms of Co(II)-carbonic anhydrases I and II. The spectra of complexes with the sulfonamide inhibitor, acetazolamide, and with CN^- and NCO^- are virtually identical to the spectra of the corresponding complexes with Co(II)-isoenzymes I and II. The spectrum of the N_3^- complex indicates that this anion is bound somewhat differently in Co(II) isoenzyme III than in the other Co(II)-substituted isoenzymes.

Carbonic anhydrase *Isoenzyme* *Muscle* *Cobalt(II)* *Absorption spectrum* *Inhibitor*

1. INTRODUCTION

Carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) is a zinc-containing enzyme catalyzing the reversible reaction: $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$. In mammals, birds, and reptiles three genetically and immunologically distinct isoenzyme forms have been shown to occur [1]. These isoenzymes have homologous structures but they have different kinetic and inhibitor-binding properties. Isoenzymes I (or B) and II (or C), which are present in the red cells of most mammals, have been studied in great detail. Isoenzyme III was discovered in red skeletal muscle only a few years ago [2–4], and its specific properties are just beginning to be understood.

Carbonic anhydrase III has a much lower turnover number for CO_2 hydration than isoenzymes I and II [2,3,5]. Furthermore, while isoenzymes I and II are effective catalysts of 4-nitrophenyl acetate hydrolysis, isoenzyme III has a very low esterase activity [2,3]. In addition, most of the aromatic and heterocyclic sulfonamides that are potent and specific inhibitors of isoenzymes I and II are rather poor inhibitors of the muscle isoenzyme [3,5]. Recently, it was shown [6] that rat muscle isoenzyme III is identical with the 'sulfonamide-resistant' carbonic anhydrase discovered in liver of male rats [7–9].

The amino acid sequence of bovine carbonic anhydrase III has been reported [10]. Extensive homologies with isoenzymes I and II are apparent. For example, the metal ion ligands are conserved as well as several amino acid residues participating in hydrogen-bond networks connected with the metal ion center [1,10]. A feature that is peculiar to the muscle isoenzyme is the presence of a number of basic amino acid residues, Lys 64, Arg 67, and Arg 91, in the active site cavity.

One important aim of our work on carbonic anhydrase is to elucidate the structural basis of the functional differences between the isoenzymes. The present paper concerns the coordination geometry of the metal ion in bovine isoenzyme III as reflected in the absorption spectrum of the Co(II)-substituted derivative.

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2. EXPERIMENTAL

Carbonic anhydrase III was prepared from bovine muscle tissue by a modification of the procedure in [5]. Details of the purification method will be presented elsewhere. Enzyme concentrations were estimated spectrophotometrically at 280 nm taking $\epsilon_{280} = 64\ mM^{-1} \cdot cm^{-1}$ as determined from amino acid analysis of a sample with known absorbance. Carbon dioxide hydration activity was estimated at 2°C by the Veronal-bromothymol blue assay described in [11]. Esterase activity was estimated spectrophotometrically at 348 nm, room temperature (20–22°C) in 50 mM Tris–H_2SO_4 (pH 7.2) with 0.4 mM 4-nitrophenyl acetate as substrate [12]. Cobalt analyses were performed with a Perkin Elmer 3030 atomic absorption spectrometer equipped with an HGA graphite furnace. Zinc analyses were performed with a Varian AA-875 atomic absorption spectrometer. Absorption spectra were recorded at room temperature with a Perkin Elmer 320 or a Beckman Acta CIII spectrophotometer.

Acetazolamide (2-acetylamido-1,3,4-thiadiazole-5-sulfonamide) was obtained from American Cyanamide Co. Our initial supply of 2-carboxy-1,10-phenanthroline was a generous gift from Dr Ralph G. Wilkins (New Mexico State University, Las Cruces, NM) while additional quantities were synthesized as in [13]. To remove metal ion impurities from buffer solutions, these were stored over Chelex 100 (200–400 mesh, BioRad), and the resin was filtered off before the buffer was used. Glassware was cleared by soaking in 6 M HNO_3 for 3 days.

3. RESULTS

3.1. *Preparation of apoenzyme*

Initial attempts to remove the metal ion from bovine isoenzyme III using the chelating agents 1,10-phenanthroline [14] or pyridine-2,6-dicarboxylate [15] were not successful (cf. also [3]). However, following the observation [16] that 2-carboxy-1,10-phenanthroline rapidly removes the metal ion from bovine Co(II)-isoenzyme II, we found that this chelating agent can also remove Zn(II) from the bovine muscle enzyme. The following procedure is now routinely used. A concentrated solution of bovine isoenzyme III (15–20 mg/ml) in 25 mM sodium phosphate buffer (pH 6.5) is diluted with 2 mM chelating agent in 50 mM 4-morpholineethanesulfonic acid (Mes)–NaOH buffer (pH 5.5) to a final protein concentration of 1 mg/ml at room temperature. Aliquots are taken at suitable intervals and the CO_2 hydration activity is measured. Fig.1A shows the decrease in activity as a function of time. When the activity has decreased to less than 3% of the initial value, the chelating agent is removed by dialysis against several changes of 50 mM Mes–NaOH (pH 6.0) containing Chelex resin. Thus prepared, the apoenzyme contains 0.1 mol Zn(II) or less per mol protein. Fig.1B shows that the apoenzyme is reactivated by one molar equivalent of Zn(II). The recovery of activity was 90%.

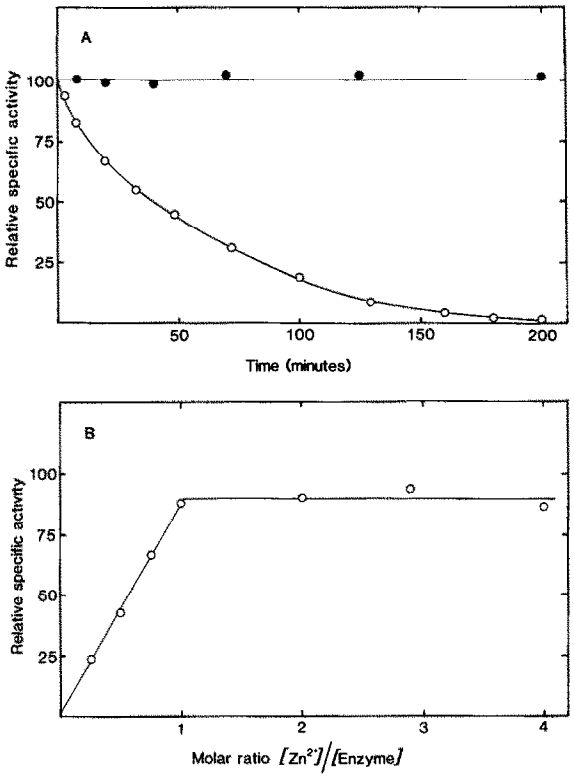

Fig.1. (A) Effect of 2-carboxy-1,10-phenanthroline on the CO_2 hydration activity of bovine carbonic anhydrase III. The enzyme (1 mg/ml) was incubated at 22°C in 50 mM Mes–NaOH buffer (pH 5.5): (○) with 2 mM chelating agent; (●) without chelating agent. (B) Titration of apocarbonic anhydrase III with Zn(II). Aliquots of 1 mM $ZnCl_2$ were added to a solution of 30 μM apoenzyme in 50 mM Mes–NaOH (pH 6.0).

3.2. *Preparation of Co(II) enzyme*

Apoenzyme (1 mg/ml), still containing chelating agent, was dialyzed overnight at 4°C against 10 mM $CoCl_2$ in 50 mM Mes–NaOH buffer (pH 5.5). Excess Co(II) was then removed by dialysis for two days against several changes of 50 mM Hepes–NaOH buffer (pH 8.0). Cobalt analysis showed that the enzyme now contained 0.95 mol Co/mol protein. The CO_2 hydration activity of the Co(II) enzyme was about 45% of that of the native enzyme under the assay conditions used. The 4-nitrophenyl acetate hydrolase activity was about the same as that of the native enzyme. This activity is very low, only about 0.2% of that of isoenzyme II under the same conditions (pH 7.2).

3.3. *Absorption spectra of Co(II) enzyme*

The absorption spectrum of bovine Co(II) isoenzyme III at pH 8.0 is shown in fig.2. There are 4 distinct maxima at 640, 617, 550, and 508 nm. The spectrum is quite similar to the high pH forms of the spectra of Co(II)-isoenzymes I and II [1,17–20]. To investigate a possible pH dependence of the spectrum, the Co(II) enzyme

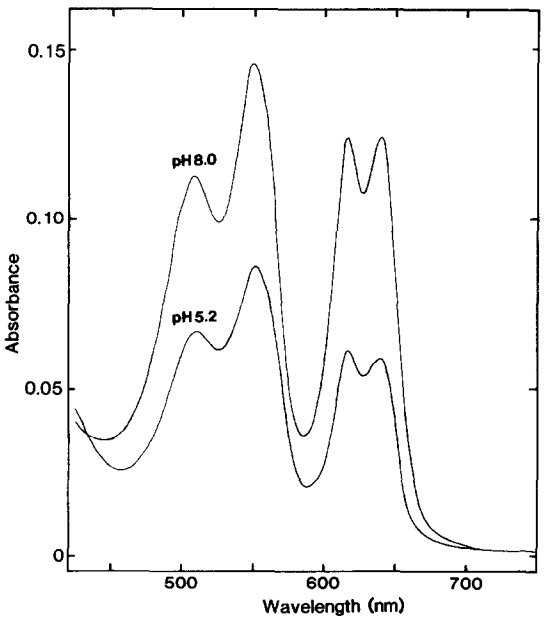

Fig.2. Visible absorption spectrum of bovine Co(II)-carbonic anhydrase III at pH 8.0 and pH 5.2. Enzyme concentration, 0.44 mM; room temperature; 50 mM Mes–NaOH (pH 5.2) or 50 mM 4-morpholinepropanesulfonic acid–NaOH (pH 8.0).

Table 1

Molar absorption coefficient at 640 nm (ϵ_{640}) of bovine Co(II)-carbonic anhydrase III at different pH values

pH	ϵ_{640} ($M^{-1} \cdot cm^{-1}$)
9.9	230
9.0	250
8.0	280
7.0	270
6.0	210
5.2	130

Buffers: pH 5.2 and 6.0, 50 mM Mes–NaOH; pH 7.0 and 8.0, 50 mM 4-morpholinepropanesulfonic acid–NaOH; pH 9.0, 50 mM 2-hydroxy-1,1-bis(hydroxymethyl)ethylamino-1-propanesulfonic acid–NaOH; pH 9.9, 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid–NaOH

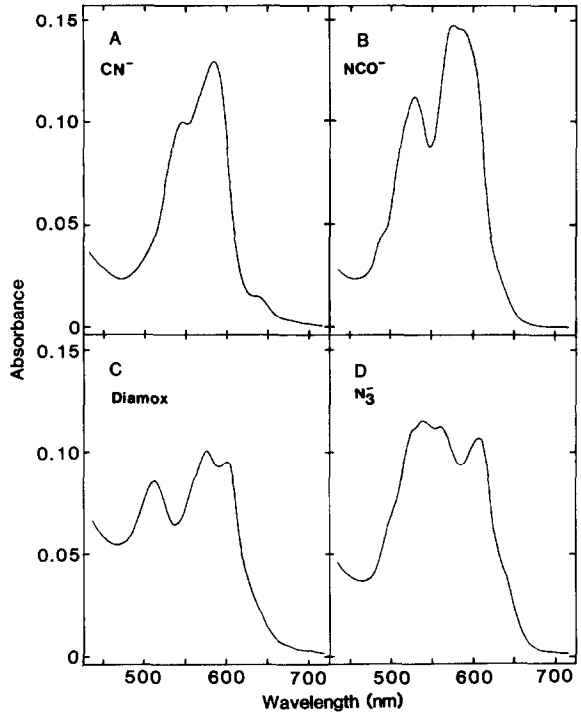

Fig.3. Visible absorption spectrum of inhibitor complexes of bovine Co(II)-carbonic anhydrase III. Room temperature; 50 mM 4-morpholinepropanesulfonic acid–NaOH (pH 8.0). (A) 0.44 mM CN^- + 0.25 mM Co(II) enzyme. (B) 8.7 mM NCO^- + 0.31 mM Co(II) enzyme. (C) 5.4 mM acetazolamide + 0.27 mM Co(II) enzyme. (D) 7.5 mM N_3^- + 0.30 mM Co(II) enzyme.

was dialyzed against various buffers in the pH range 5.2–9.9. The results showed that the shape of the spectrum is virtually independent of pH in the whole investigated range (see fig.2). However, there is significant bleaching of the spectrum at low pH and possibly also a small bleaching at high pH (table 1). By dialysis of the pH 5.2 solution against pH 8.0 buffer, it was shown that the bleaching at low pH is essentially reversible indicating that it is not due to loss of Co(II). Sulfate has been shown to interact with Co(II)-isoenzyme II at low pH yielding a spectrum similar to that of the low pH form [21]. However, the addition of 50 mM Na_2SO_4 to bovine Co(II)-isoenzyme III at pH 6.5 had no significant effect on the absorption spectrum.

The effects of some inhibitors on the spectrum of Co(II)-isoenzyme III were investigated. Spectra obtained with acetazolamide, N_3^-, NCO^- and CN^- are shown in fig.3.

4. DISCUSSION

The absorption spectra of Co(II) derivatives of carbonic anhydrases I and II show a characteristic pH dependence in the neutral pH range that can be described as an equilibrium between a low pH spectral form and a high pH spectral form [1,18]. The pH dependence is complex in the absence of salts or in the presence of Mes–NaOH or Hepes–NaOH buffers [18,22]. However, in the presence of sulfate ions (say, 50 mM) a simple titration curve with pK_a near 7 is obtained [21]. It is thought that these spectral changes reflect changes in coordination geometry associated with a dissociation of H^+ from metal-bound H_2O to form metal-bound OH^- [1,18,23].

The spectrum of bovine Co(II)-isoenzyme III does not show a corresponding pH dependence. A comparison with the spectra of Co(II)-isoenzymes I and II yields two major conclusions. Firstly, above pH 8 the coordination geometry appears to be very similar in all three isoenzymes. Secondly, it appears that a Co(II)-OH^- species prevails in Co(II)-isoenzyme III in the whole investigated pH range and that the pK_a for the ionization of metal-bound H_2O is well below 6. Such a low pK_a fits with the finding in [24] that the CO_2 hydration activity (k_{cat}/K_m) of cat isoenzyme III is pH-independent between pH 5 and 8.5. A low pK_a might result from electrostatic interactions with positive charges from the isoenzyme III-specific active-site residues Lys 64, Arg 67, and Arg 91.

The nature of the observed bleaching of the spectrum of bovine Co(II)-isoenzyme III is not clear, but a partial conversion to a form of low spectral intensity, perhaps a 6-coordinated Co(II) species, seems to take place.

The spectra of the adducts of Co(II)-isoenzyme III with acetazolamide, CN^- and NCO^- are virtually identical to the corresponding spectra obtained with Co(II)-isoenzymes I and II [17,19, 20,25], again implying similar structures of the metal ion centers in the three isoenzymes. This is particularly interesting in the case of acetazolamide since this sulfonamide inhibitor binds much more weakly to isoenzyme III than to isoenzymes I and II. The spectrum of the N_3^- complex of Co(II)-isoenzyme III has an additional maximum at 609 nm and is more intense than that of the N_3^- complex of bovine Co(II)-isoenzyme II. This might be rationalized by assuming that the N_3^- complex of Co(II)-isoenzyme III is essentially 4-coordinated while the complex with Co(II)-isoenzyme II has been assumed to be a mixture of 4- and 5-coordinated species [18,20].

ACKNOWLEDGEMENTS

This investigation has been financially supported by the Swedish Natural Science Research Council (K 2911). We thank Dr Ralph G. Wilkins for a generous gift of 2-carboxy-1,10-phenanthroline. We are grateful to Ms Katarina Wallgren for excellent technical assistance.

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