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RELAXATION OF SOLVENT PROTONS BY COBALT BOVINE CARBONIC ANHYDRASE

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In a recent report, Bertini et al. (*Biochem. Biophys. Res. Comm.* **78**, 158-160 (1977)) argued that the low-pH form of Co^{2+}-substituted bovine carbonic anhydrase contains a rapidly exchanging water molecule at the cobalt site. The basis for this was the observation of a pH-independent contribution to the solvent water proton relaxation rate; it was suggested that the result was unobserved by previous workers because of the presence of sulfate in the sample buffer. We have repeated the experiments of Bertini et al. and find that the results can be attributed to an ionic strength-induced shift of the pK of the group responsible for the relaxation enhancement. The amount of high-pH form of the enzyme present (determined spectrophotometrically) at every pH correlates with the relaxation rate, whereas the fraction of high-pH form present at a given pH depends on ionic strength. These results are in agreement with earlier data indicating that the low-pH form of the enzyme does not contribute to solvent water proton relaxation.

Fabry et al. (1) reported the pH dependence of the spin-lattice relaxation rate ($1/T_1$) of solvent water protons in solutions of Co^{2+}-substituted bovine carbonic anhydrase. They found that the contribution to $1/T_1$ attributable to the paramagnetism of the Co^{2+} ions, for samples in 0.1 M Tris-sulfate buffer, varied with pH as did the activity for enzymatic hydration of CO_2, increasing from a value of essentially zero at low pH to a saturation value at high pH, with a pK near 7. These authors also measured the optical spectra of buffered samples, and of similar samples prepared in distilled water with the pH adjusted by addition of NaOH. The high- and low-pH forms of the enzyme have different characteristic spectra in the visible region (2), and the observation was that the presence of sulfate ions did not affect the spectra. Implicit in the report of Fabry et al. (1) was that $1/T_1$ was also unaltered by the presence of sulfate.

Recently, Bertini et al. (3) reported data on $1/T_1$ of solvent water protons in solutions of bovine Co^{2+}-carbonic anhydrase in distilled water, in 0.1 M Na_2SO_4, and in 0.1 M Tris-sulfate buffer. They found no pH-dependence for $1/T_1$ for the distilled-water samples in the range 5.8-9, and a pH-dependence in agreement with Fabry et al. (1) for samples in

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sulfate-containing buffer. They concluded from their data that the low-pH form of the enzyme has an exchangeable water molecule coordinated to the metal, and that previous researchers failed to observe this because of interactions of sulfate with the cobalt ions.

As discussed by Koenig and Brown (4), the pH-dependence of $1/T_1$ and the question of an exchangeable water-ligand of the metal at low pH has important ramifications for the mechanism of enzymatic action of carbonic anhydrase. Accordingly, these authors not only relied on the results of Fabry et al. (1) for their arguments but, since the possible involvement of buffer in transient kinetic measurements of enzymatic activity was becoming appreciated at that time (5), they remeasured $1/T_1$ in both buffered and unbuffered solutions of bovine Co^{2+}-carbonic anhydrase; they found essentially no difference (unpublished). Because of apparent inconsistencies between the data of Bertini et al. (3) and Fabry et al. (1), we have repeated these measurements.

MATERIALS AND METHODS

Native bovine carbonic anhydrase, purchased from Sigma, and purified by chromatography on DEAE-cellulose according to Kandel et al. (6) was demetallized in the usual way (7), and lyophilized after extensive dialysis against distilled water. Co^{2+}-substituted enzyme in deionized water was prepared in two ways: following Fabry et al. (1), and Bertini et al. (3), respectively 1) **Direct Addition**: Lyophilized apo-carbonic anhydrase was dissolved in deionized water, a stoichiometric amount of $CoCl_2$ was added, and the solution was subjected to rapid dialysis for 90 minutes using a Crowe-Englander Micro-Dialyzer; and 2) **Exhaustive Dialysis**: Enzyme in deionized water was dialyzed against 1 mM $CoCl_2$ (rather than $CoSO_4$ as used by Bertini et al.) for one day, and then against deionized water, with frequent changes, for three days. Sample pH was measured with a radiometer PHM 64 meter and combination electrode. The electrode was immersed for less than one minute to minimize chloride uptake by the sample.

Proton relaxation rate measurements, using an automated apparatus previously described (8,9), were taken over a wide range of values of magnetic field, corresponding to proton Larmor precession frequencies in the range 0.01-30 MHz. The uncertainty in $1/T_1$ is less than $\pm$ 1%.

RESULTS

Figure 1 shows the optical spectra of two samples of Co^{2+}-carbonic anhydrase in deionized water made by the two procedures described above. The sample made by extensive dialysis had a pH of 5.7, the one made by direct addition a pH of 5.6. A substantial difference in the ratio of the concentration of high-pH form (which gives rise to the peaks at 620 and 640 nm) to low-pH form for the two samples is apparent. The sample made by extensive

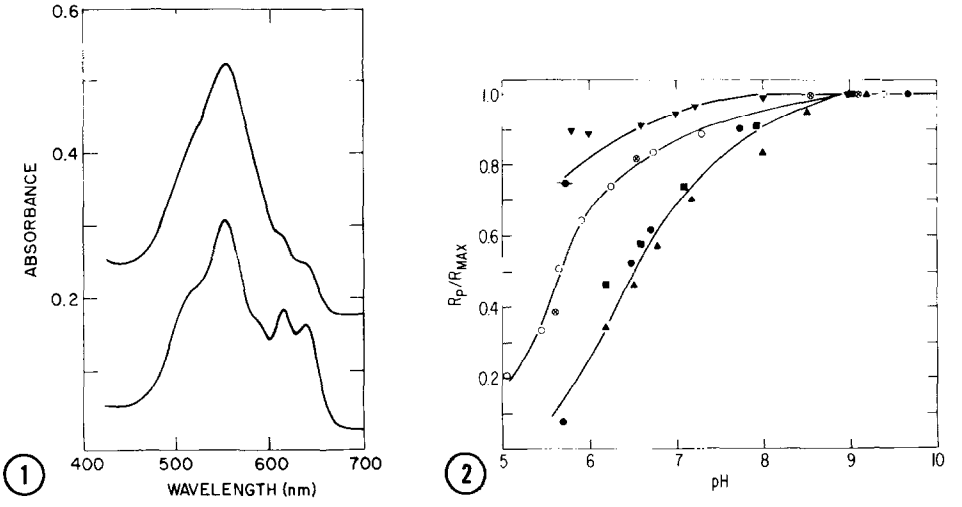

Fig. 1. Optical spectra of cobalt bovine carbonic anhydrase in deionized water. Samples were made according to procedures described in Materials and Methods. *Upper curve* - 1.4 mM enzyme, pH 5.6, made by direct addition. (optical path length = 0.8 cm). *Lower curve* - 0.8 mM enzyme, pH 5.7, made by exhaustive dialysis. (optical path length = 1 cm).

Fig. 2. Plot of normalized paramagnetic relaxivity at 20 MHz (4.7 kOe) versus pH for Co^{2+}-bovine carbonic anhydrase. Solid lines are hand-drawn to emphasize the shift of the titration curve with ionic strength. Data of Bertini et al. (2) are represented by filled squares, triangles, and inverted triangles: (▼,-●-) exhaustively dialyzed enzyme solution; (⊗) enzyme solution made by direct addition method; (○) back-titration study of dialyzed enzyme solution; (●, ▲) enzyme in 0.1 M sodium sulfate; (■) enzyme in 0.1 M Tris-sulfate.

dialysis was also found to have a larger $1/T_1$ than an equimolar sample of enzyme produced by the other method, in accordance with the presence in the first sample of more of the high-pH form, the form proposed to be responsible for the relaxation enhancement (1). Since neither sample had prior contact with sulfate, the phenomenon responsible for the different results for supposedly equivalent samples *cannot* be due to the effect of sulfate. The

Co^{2+}-substituted enzyme prepared by direct addition gave results similar to Fabry et al. (1), with the exception that the pK of the group responsible for the contribution to $1/T_1$ was 5.8, which is unusually low. An explanation for this low value is given below.

The Co^{2+}-enzyme prepared by extensive dialysis was further studied in the following ways: 1) The pH was lowered to 5 by the addition of H_2SO_4, and then back-titrated with NaOH. Relaxation rates and optical spectra were monitored. 2) Samples of the enzyme in 1 mM and 0.1 M Na_2SO_4 were made and studied as a function of pH.

Results at 20 MHz are compared with those of Bertini et al. (3) in Figure 2, where the $1/T_1$ data are expressed as molar paramagnetic relaxivity R_P given by:

$$R_P = ((T_{obs}^{-1} - T_{1w}^{-1})/[BCA]) - R_D \quad (1)$$

where T_{obs}^{-1} is the total observed spin-lattice relaxation rate of the Co^{2+}-enzyme sample, T_{1w}^{-1} is the buffer contribution, and R_D is the (diamagnetic) relaxivity of the apoprotein. The data have been normalized by plotting R_P/R_{MAX} on the ordinate axis where R_{MAX} is the value of the paramagnetic relaxivity attained at the high pH limit. Though only data obtained at 20 MHz are shown, because Bertini et al. (3) used only this value, similar results were obtained at all fields.

The extensively dialyzed Co^{2+}-enzyme, in deionized water at pH 5.7, has a relaxivity equal to 75% of R_{MAX}. This value decreased substantially when the pH was lowered to 5; the fraction of the high-pH enzyme form also decreased in a corresponding manner, as indicated by the optical spectra. Back-titration with NaOH produced an increase in R_P as well as an increase in the fraction of high-pH form; the back-titration curve has a calculated pK of 5.8 (open circles). This curve, however, does not intersect the initial value for the exhaustively dialyzed Co^{2+}-enzyme; rather, that value is not attained until the pH is raised to 6.3. Thus, addition of acid, resulting in a concentration of approximately 1 mM sulfate, shifts the pK of the activity-linked group one-half pH unit, from a calculated pK of 5.2 for the exhaustively dialyzed sample to the observed value of 5.8. Indicated in Figure 2 is the titration curve of Co^{2+}-enzyme in deionized water made by the direct method (not exposed to any sulfate),

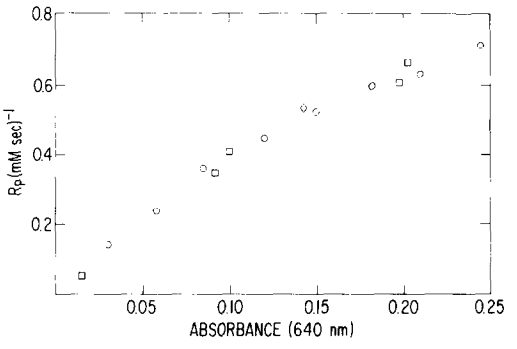

Fig. 3. Plot of paramagnetic relaxivity as a function of absorbance at 640 nm: (diamond) exhaustively dialyzed cobalt carbonic anhydrase; (circles) back-titration study of exhaustively dialyzed enzyme sample; (squares) enzyme in 0.1 M sodium sulfate.

which is seen to be indistinguishable from the one just indicated. Thus, the presence of only 1 mM salt would explain the rate and spectral differences between the enzymes in deionized water made by the two methods.

Also shown in Figure 2 are data from this study and from Bertini et al. (3) on samples in 0.1 M Na_2SO_4. The results are equivalent for the two studies, both giving a calculated pK of 6.4.

Figure 3 shows a plot of the paramagnetic relaxivity, R_p, against optical absorbance, $A^{640\ nm}_{1\ cm}$, for a number of samples with different pH and ionic strengths. The data clearly indicate that the paramagnetic relaxivity is proportional to the amount of the high-pH form of Co^{2+}-bovine carbonic anhydrase present, as it is this form of the enzyme that is responsible for the absorbance at 640 nm.

DISCUSSION

The results of these experiments indicate that the pK of the titratable group responsible for enzymatic activity is highly dependent on ionic strength: the pK can vary from 5.2, for an exhaustively dialyzed sample, to a value of 6.4 for enzyme in 0.1 M Na_2SO_4. Bertini et al.

(3) did not observe the titration curve for the exhaustively dialyzed sample (cf. Figure 2) because it was already at the high pH end of the curve at their lowest value of pH. Their report that addition of sulfate altered the optical spectra of the samples ("the bands at 16,000 cm^{-1} typical of the basic form, which are detectable down to pH 5.8, disappear by adding sodium sulfate to the solution") is, we assert, an ionic strength-induced shift of pK to higher pH, and is not an indication of sulfate metal ion interaction.

Our major finding is that the amount of high-pH form of the enzyme present at every pH correlates with the relaxation rate data, whereas the fraction of high-pH form at a given pH depends on ionic strength. It is not necessary, and indeed inconsistent, to invoke the concept that the low-pH form of bovine Co^{2+}-carbonic anhydrase contains a rapidly exchanging water molecule at the metal site.

A word is in order about the importance of these relaxation results. As emphasized previously (1,4), the observed value of $1/T_1$ at the higher pH values sets a lower limit to the off-rate of protons liganded to the Co^{2+} ions in the active site. This limit is faster, by one to two orders of magnitude, than can be explained by assuming acid-base catalyzed exchange of protons, assuming the high-pH form of the enzyme contains an OH^- liganded on the Co^{2+}; the previous conclusion was that the exchanging protons must be carried by exchanging water molecules. Thus, unless this exchanging water is a fifth ligand of the Co^{2+} acting as a reporter group to indicate events near the active site, the conclusion is reconfirmed that the ionization responsible for the pH-dependence of the enzymatic activity of carbonic anhydrase cannot be that of a water molecule ligand of the metal.

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