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The Electronic Spectra of Cobalt(II) Bovine Carbonic Anhydrase

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It is generally assumed that the catalytic activity of the native zinc containing enzyme carbonic anhydrase depends on a single acid–base equilibrium [1–4]. The cobalt substituted enzyme is the only metal substituted enzyme which maintains a comparable activity [5, 6] and the variation of its electronic spectra with pH is believed to depend on the same dissociating group [7, 8]. However, affinity constants of iodide for the cobalt substituted human B enzyme appeared to be consistent with the presence of more than one dissociating group [9]. The recent ^1H T_1 measurements of water solutions of CoCA as a function of pH [10], definitely showing that a water molecule is bound to the metal under any circumstance, as well as the ^1H NMR shift studies of histidines as a function of their deprotonation [11, 12] induced us to register the electronic spectra of the cobalt derivative of carbonic anhydrase in order to check whether they depend on a single dissociating group.

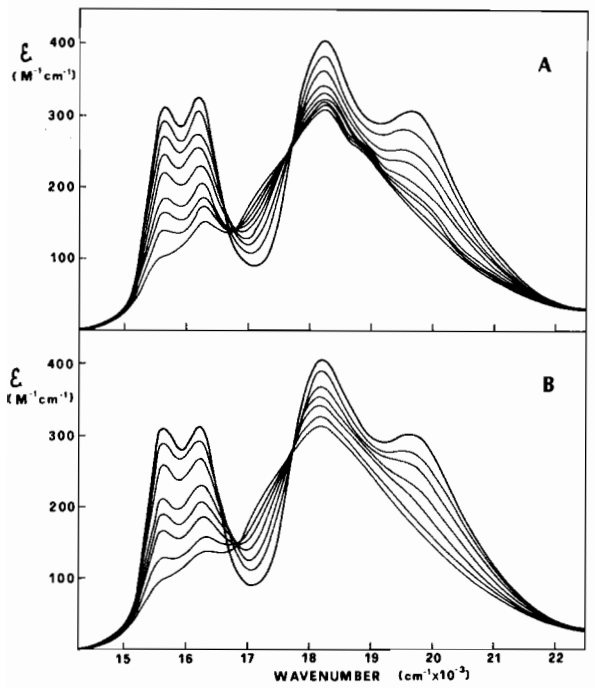

Fig. 1. Electronic spectra of cobalt bovine carbonic anhydrase B as a function of pH. A: unbuffered solutions at pH 5.8, 6.0, 6.3, 6.7, 7.3, 7.7, 7.9, 8.2, 8.8, in order of increasing ϵ_{640}; B: 0.1 *M* Hepes solutions at pH 5.9, 6.2, 6.5, 7.0, 7.3, 7.8, 8.4, 9.1, in order of increasing ϵ_{640}.

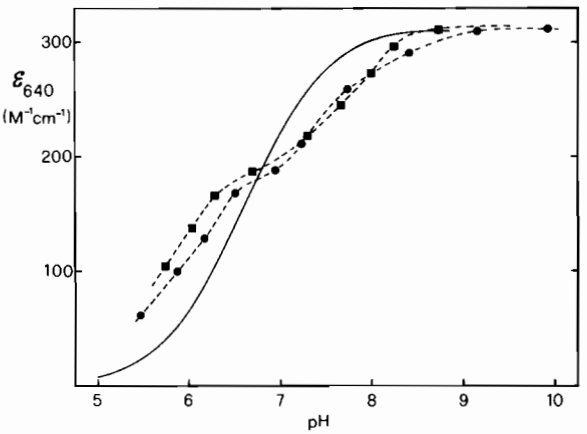

Fig. 2. Intensity of the 640 nm d–d transition of cobalt bovine carbonic anhydrase B as a function of pH in unbuffered (■) and 0.1 *M* Hepes buffered (●) solutions. The solid line is calculated for a single $pK_a = 6.6$.

The electronic spectra were registered on a Cary 17 D operating in the 0–0.1 absorbance range between 700 and 400 nm; they are reported in Fig. 1 as a function of pH for unbuffered and Hepes-buffered solutions. Unbuffered samples were obtained from enzyme solutions prepared as previously reported [10] and brought to the isoionic pH (*ca.* 5.6) through extensive dialysis against freshly bidistilled water; the pH was varied by addition of increasing amounts of NaOH. The pH of the samples was measured after recording the electronic spectra. The buffered samples were obtained by mixing equal amounts of unbuffered enzyme solutions with 0.2 *M* Hepes solutions to which different calculated amounts of NaOH had been added. The pH of each sample was again measured only after having recorded the electronic spectra, and was found to be negligibly different from the calculated value. In such a way the samples never got in touch with the electrode, avoiding the risk of chloride leakage [12] which could alter the measurements. The plot of the absorbance at 640 nm as a function of pH is reported in Fig. 2. The curves obtained for the unbuffered and Hepes-buffered solutions are very similar, indicating that: i) contrary to a previous proposal [13], no major ionic strength effect is present; ii) Hepes, unlike most of the usually employed buffers [10, 14] does not appreciably interact with the enzyme. The experimental curves, outside the experimental error, do not fit with any sigmoid calculated on the basis

of a single acid–base equilibrium. At least two dissociating groups are necessary to closely reproduce the observed pattern. The presence of buffering systems different from Hepes, which usually contain anions able to bind the metal ion, are capable to mask the inflection in the low pH region of the above curve. This is probably the reason for which the curve had been fitted with a single pK_a, and the reason for which several pK_a values were proposed [5, 15, 16], depending on the nature and concentration of the buffering systems. Preliminary data on the human carbonic anhydrase B confirm a complex pH dependence of the spectra.

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