Nickel Carbonic Anhydrase: A Re-examination of the Electronic Spectra with the Help of CD Spectra

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The electronic and CD spectra of nickel(II)-substituted bovine carbonic anhydrase, and of its adduct with p-toluenesulfonamide, have been re-recorded in the ranges 8–30 × 10^3 cm^{-1} and 10–30 × 10^3 cm^{-1}, respectively. Although the positions of the main absorptions are consistent with six-coordination of the metal ion in the enzyme active site, their relative intensity and the detection of other transitions suggest the operativity of strong low-symmetry components. Therefore five-coordinated rather than octahedral chromophores have been considered in order to account for the observed spectroscopic properties.

Introduction

Nickel(II) has been used in several systems as a probe for zinc enzymes. In liver alcohol dehydrogenase [1] and aspartate transcarbamylase [2], nickel(II) undertakes the same coordination number as the native zinc, which is pseudotetrahedral. Nickel(II) carboxypeptidase [3] is octahedral, while the native zinc is described as five coordinated [4]. Nickel(II) carbonic anhydrase was first reported by Coleman [5] and then re-investigated by us [6]. The electronic spectra showed two main transitions in the visible region at 15.6 and 25.6 × 10^3 cm^{-1}, whose position is slightly pH dependent. Such band positions have been interpreted as indicative of six coordination [6], since commonly six-coordinated chromophores containing nitrogens and oxygens absorb in the energy ranges 14–18 and 25–28 × 10^3 cm^{-1} [3]. The intensity of the band at 15.6 × 10^3 cm^{-1} is relatively small ($\epsilon = 20\ M^{-1}\ cm^{-1}$ at pH = 6.1), whereas the intensity of the high energy band is difficult to determine since it is located on the tail of the intense protein absorption. However the intensity of the former band for the nickel protein and for the investigated inhibitor derivatives is larger than that usually found in six-coordinated nickel(II) complexes. Although the latter statement has no theoretical background, since low symmetry components may justify an increase in intensity as sometimes proposed in the literature [7], we felt that further investigations of the electronic spectra could be worthwhile through extension to the near infrared region in concentrated D_2O solutions and through circular dichroism measurements.

Experimental

Bovine carbonic anhydrase was obtained as a lyophilized material from Sigma. The isoenzyme B was isolated through chromatography on DEAE cellulose [8], de-metallized according to the usual procedure [9] and exhaustively dialyzed against freshly double-distilled water. Nickel(II) was added as sulfate salt in slightly less than the stoichiometric amount on apoenzyme solutions at pH 5–6. The nickel(II) carbonic anhydrase samples were lyophilized and re-dissolved twice in D_2O; higher pH values (uncorrected meter readings) were obtained by addition of NaOD. No buffers were employed. Enzyme concentrations were calculated from the absorbance at 280 nm ($\epsilon = 57000\ M^{-1}\ cm^{-1}$) [10].

The electronic spectra in D_2O solutions were recorded on a Cary 17D spectrophotometer in the absorbance range 0–0.1, using microcells of 1 cm optical path and 0.5 ml volume. The reference cell contained D_2O solutions of apoenzyme at the same concentration.

The room temperature CD spectra of 4×10^{-3} *M* enzyme solutions were recorded on a Jasco spectrophotometer with cells of 1 cm optical path, using a 2 m°/cm scale and a 50 nm/min scan speed; ellipticity is expressed as $\Delta\epsilon = \epsilon_L - \epsilon_R$, or as molecular ellipticity $[\theta] = 2.303(4500/\pi)\Delta\epsilon$, with units of def. cm^2 per dmol.

Results and Discussion

The electronic spectra recorded from 8 to 30 × 10^3 cm^{-1} at several pH values in D_2O solutions show

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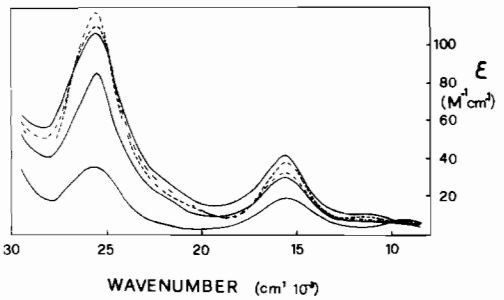

Fig. 1. Electronic spectra of Nickel(II) bovine carbonic anhydrase; spectra at pH 5.7, 7.5 and 8.2 are represented by solid lines in order of increasing absorbance, whereas spectra at pH 9.4 and 10.3 are represented by dashed lines in order of decreasing absorbance at 15.6 X 10^3 cm^{-1} and of increasing absorbance at 25.6 X 10^3 cm^{-1}.

three main absorption regions, around 10, 15.6 and 25.6 X 10^3 cm^{-1}, as already reported [6] and as shown in Fig. 1.

The difference spectroscopy technique has allowed fair estimates of the intensities of the transitions and has shown that the intensity of the absorptions increases with increasing frequency. In particular the intensity of the absorption around 10 X 10^3 cm^{-1} is about one order of magnitude smaller than that at 15.6 X 10^3 cm^{-1}. Six-coordinated complexes generally show an intensity of the band around 10 X 10^3 cm^{-1} (assigned as ν_1 in O_h symmetry) larger than that at 16 X 10^3 cm^{-1} (ν_2). Furthermore in the present case the former band is quite sensitive to pH, (Fig. 1) while in octahedral complexes it should be determined by the mean Dq value which is only sensitive to major changes in the donor set. At this point the possibility of an equilibrium between octahedral species detected through the weak band at 10 X 10^3 cm^{-1}, together with other more absorbing species (*e.g.* five coordinated), has been ruled out through variable temperature (0–40 °C) measurements which

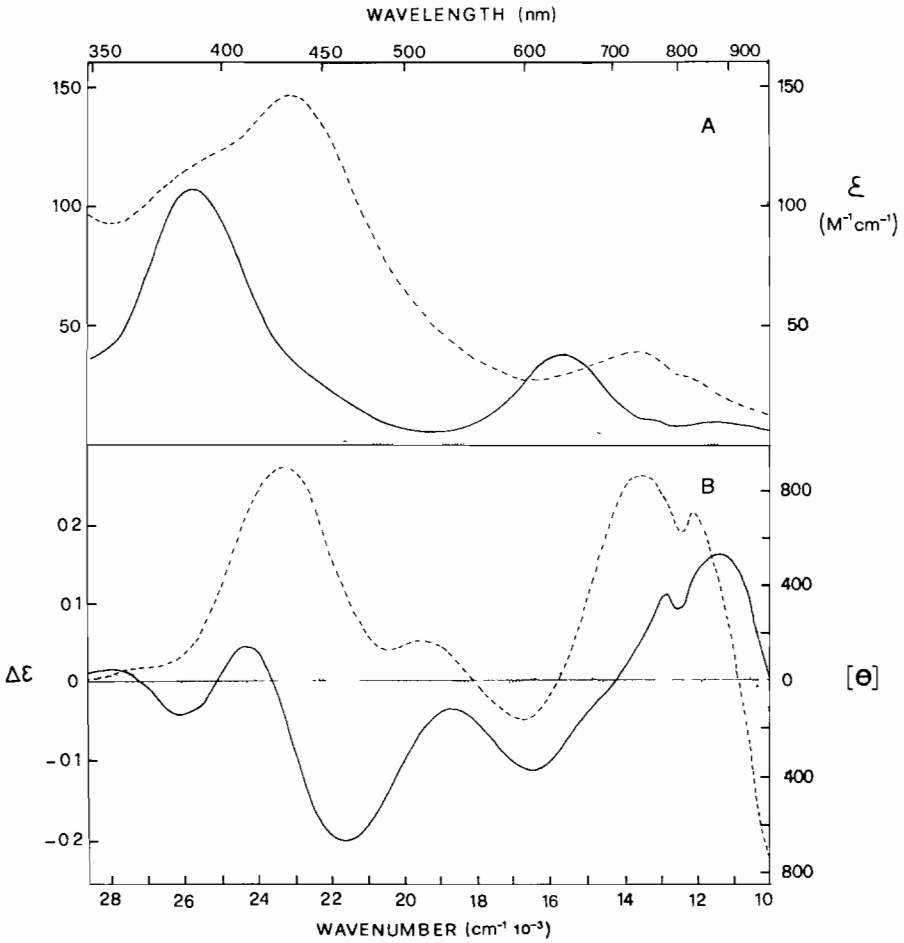

Fig. 2. Absorption (A) and CD spectra (B) of Nickel(II) bovine carbonic anhydrase at pH 10.3 in the absence (——) and presence (------) of *p*-toluenesulfonamide. The spectra of Nickel tartrate (·····) as taken from ref. 11 are also reported for comparison purposes.

showed that the relative intensity of the three absorptions is constant.

The electronic spectrum of the *p*-toluenesulfonamide derivative at pH 10.3 is reported in Fig. 2A, together with that of the pure nickel enzyme at the same pH. Transitions are observed at 13.7 × 10^3 and 23.2 × 10^3 cm^{-1} with a shoulder at 26 × 10^3 cm^{-1}. No low frequency transition is observed, down to 8 × 10^3 cm^{-1}. Sulfonamide probably binds through nitrogen, either as NH_2 or NH^-.

The CD spectra have also been recorded in D_2O solutions between 10 and 29 × 10^3 cm^{-1}. They display a strong ellipticity in the entire frequency band. The pure nickel derivative at pH 10.3 displays a positive peak at 11.4 × 10^3 cm^{-1}, a negative band in the region corresponding to the second transition, and a negative feature at 21.7 × 10^3 cm^{-1} which probably corresponds to an unresolved shoulder in the absorption spectrum, besides at least two more weak bands at 24.3 and 26.0 × 10^3 cm^{-1}. The presence of the new absorption evidenced around 22 × 10^3 cm^{-1} is indicative of strong low-symmetry components in the chromophore, since it should be assigned to a transition to a level arising from the ^3P free ion term. Never has a pseudo-octahedral complex been found to absorb so low in energy.

The CD spectrum of the *p*-toluenesulfonamide derivative indicates a positive absorption corresponding to the band at 13.7 × 10^3 cm^{-1}, and another positive absorption corresponding to the band at 23.2 × 10^3 cm^{-1}. The spectrum shows also evidence of other absorptions between 16 and 21 × 10^3 cm^{-1}. A negative absorption starts to appear in the low energy end of the spectrum. All of these features, and the absorption at 26 × 10^3 cm^{-1} present as a shoulder in the absorption spectrum, are again consistent with strong low symmetry components in the chromophore. For comparison purposes the spectra of a solution containing Ni^{2+} and excess *D*-tartrate are also reported [11]. The chromophore presumably is six-coordinated as shown by the low absorption and low ellipticity of the bands. The CD spectra show splitting of the F → P transitions in the range 24–28 × 10^3 cm^{-1}. However, the low symmetry components do not cause any absorption between 16 and 23 × 10^3 cm^{-1}.

The wealth of absorptions shown by the present spectral investigation in the nickel carbonic anhydrase system is hardly related to a simple model of coordination geometry. A pseudooctahedral coordination, which in this case would be due to three histidine nitrogens and three solvent oxygens (pure nickel protein) or four nitrogens and two oxygens (sulfonamide derivative) would hardly give rise to absorptions in every region between 9 and 30 × 10^3 cm^{-1}. In a lower symmetry, *e.g.* as in a five-coordinated chromophore with a geometry intermediate between square pyramidal and trigonal bipyramidal, the observed transitions might be grouped as F–F transitions below 17 × 10^3 cm^{-1} and as F–P transitions above 19 × 10^3 cm^{-1} [12]. Such low symmetry easily accounts for both a large number of absorptions and the relatively high intensity of the bands. Furthermore, if without loss of generality, intensity considerations are introduced on the basis of D_{3h} or C_{4v} symmetries, several low energy transitions are expected to be symmetry-prohibited and indeed are found with low intensity in model chromophores [12]. Therefore the low intensity transitions which have been evidenced with the aid of the CD spectroscopy may also be accounted for. Finally, recent evidences on the native and metal substituted enzyme seem to suggest the presence of only two coordination sites within the active cavity [13].

In the light of the above results, the water proton relaxation data on nickel carbonic anhydrase solutions [6] deserve a further comment. It was shown that the ^1H T_1^{-1} values of water solutions provide evidence for exchangeable protons attached to donor atoms of the metal ion. In the presence of ligands like *p*-toluenesulfonamide and azide, the ^1H T_1^{-1} values were markedly lower than those of the pure enzyme, and very small in the presence of oxalate. A recent investigation of the $Ni(OH_2)_6$ system [14] has shown that the correlation time for the nuclear spin–electron spin interaction is extremely field dependent, especially in the 80 MHz region. Therefore quantitative conclusions on the metal hydration cannot be drawn from the comparison of the data on the pure metalloprotein and its inhibitor derivatives. With this limitation in mind it might be noted that the results are not inconsistent with a chromophore based on three histidine nitrogens and two water oxygens in which a monodentate ligand substitutes a water molecule and the bidentate oxalate substitutes both of them.

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