Electron Spin Resonance Studies of Carbonic Anhydrase: Transition Metal Ions and Spin-Labeled Sulfonamides

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Communicated by Joseph S. Fruton, August 17, 1970

Abstract. Electron spin resonance (esr) spectra of Cu(II) and Co(II) carbonic anhydrase, and a spin-labeled sulfonamide complex of the Zn(II) enzyme, are reported. The coordination geometry of Cu(II) bound in the enzyme appears to have approximately axial symmetry. Esr spectra of enzyme complexes with metal-binding anions also show axial symmetry and greater covalency, in the order ethoxzolamide < SH< N3 < CN. Well-resolved superhyperfine structure in the spectrum of the cyanide complex suggests the presence of two, and probably three, equivalent nitrogen ligands from the protein. Esr spectra of the Co(II) enzyme and its complexes show two types of Co(II) environment, one typical of the native enzyme and the 1:1 CN complex, and one typical of a 2:1 CN complex. Co(II) in the 2:1 complex appears to be low-spin and probably has a coordination number of 5. Binding of a spin-labeled sulfonamide to the active center immobilizes the free radical. The similarity of the esr spectra of spin-labeled Zn(II) and Co(II) carbonic anhydrases suggests that the conformation at the active center is similar in the two metal derivatives.

The Zn(II) ion of native carbonic anhydrase can be replaced by the transition metal ions Cu(II) or Co(II) to provide a paramagnetic probe at the active site. The divalent first transition metal ions from Mn(II) to Cu(II) occupy the same binding site as Zn(II), although only the Zn(II) and Co(II) derivatives are enzymatically active. Esr studies of Cu(II) and Co(II) human carbonic anhydrases, and their complexes with monovalent anions, were undertaken to elucidate both the nature of the ligands to the metal ion and the coordination geometry. A second type of paramagnetic probe used was a spin-labeled sulfonamide, which is coordinated to the metal ion.

Experimental. Esr spectra were measured with a Varian E-4 spectrometer using a 100 kHz modulation frequency. Temperature was controlled to ±0.5°C with a Varian E-257 variable temperature controller. All spectra are presented as the first derivative of the absorption curve. The field was calibrated with crystalline DPPH, taking g = 2.0036 for the radical. The synthesis of the spin-labeled sulfonamide inhibitor 2,2,6,6-tetramethyl-4-piperidone-1-oxy1-p-sulfamylphenyldrazone (I) will
be described in detail elsewhere. Human and bovine carbonic anhydrases (HCAB, HCAC, and BCAB) and their metal derivatives were prepared as previously described.

Results and Discussion. Cu(II) carbonic anhydrase anion complexes: While Cu(II) carbonic anhydrase does not catalyze the hydration of CO\textsubscript{2} or the hydrolysis of esters, we have shown previously that it does react with the anionic inhibitors of the enzyme. The Cu(II) enzyme was chosen for esr studies because the esr signal for Cu(II) (S = 1/2) is one of the most readily interpreted in terms of the ligand environment of the metal ion.

Esr spectra of Cu(II) HCAB and its complexes with ethoxzolamide, SH\textsuperscript{−}, N\textsubscript{3}\textsuperscript{−}, and CN\textsuperscript{−} at 77\textdegree K are shown in Fig. 1. The spectrum of the enzyme in 0.5 M bicarbonate, not shown, is identical with that of the enzyme itself. Copper nuclear-hyperfine structure is clearly resolved in the g\textsubscript{∥} region. In addition, nitrogen super-hyperfine (shf) splitting is observed in the g\textsubscript{⊥} region of all complexes except the bicarbonate complex. The nitrogen splitting becomes increasingly well resolved as the ligand field strength of the anion increases. The esr spectra of the enzyme and its complexes have the general shape expected for Cu(II) in an axially symmetric environment. Table 1 gives approximate g values and hyperfine splittings.

These data suggest that the copper in carbonic anhydrase is in an axially symmetric environment, probably rather close to square coplanar, and that the addition of the anion ligands does not significantly distort this geometry. The visible absorption spectra for Cu(II) HCAB and Cu(II) monkey carbonic anhydrase B (MCAB) are compatible with such a geometry and show three absorption maxima in the region 550–900 nm. The major band is located at 750 nm, ε = 100. It is significant that the intensities of the d-d transitions of the Cu(II) enzyme and its anion complexes, ε = 50–100, are those observed for more normal square-planar Cu(II) complexes rather than the high intensities observed for those complexes believed to have significant distortions from a square-planar arrangement.

Calculation of the "covalency" of the in-plane σ bonding for these complexes, by the methods of Kivelson and Nieman, allows some interesting conclusions.
The covalency, or the degree to which the unpaired d electron is delocalized onto the ligand nuclei, can be calculated from the copper nuclear-hyperfine structure by the formula $\alpha^2 = -(A_{||}/P) + (g_{||} - 2) + (3/7) (g_{\perp} - 2) + 0.04$, derived from molecular orbital approximations, where $A_{||}$ is the copper hyperfine splitting constant and $P = 2y_\beta y_\delta < r^{-3}>_0$ (ref. 9). $P$ is equal to 0.035 cm$^{-1}$. One cannot strictly separate the "ionic" and "covalent" character of a bond, but under such an assumption if $\alpha^2 = 1$, the $\sigma$ bonding would be completely ionic, while if $\alpha^2 = 0.5$, the bond would be completely covalent. The $\alpha^2$ values for the five Cu(II) carboxylic anhydrase complexes are given in Table 1 and show the increasingly covalent character of the complexes in the order CuCA $<$ CuCA-ethoxzolamide $<$ CuCA-SH $<$ CuCA-N$_3$ $<$ CuCA-CN$^-$. The azide and cyanide complexes, $\alpha^2$ = 0.67 and 0.69, respectively, have a greater covalency than any model Cu(II) complex reported. The only smaller $\alpha^2$ values reported are for Cu(II) at the active centers of laccase and ceruloplasmin. $^9$ The Cu(II) enzyme, $\alpha^2$ = 0.84, is quite comparable to the more ionic complexes of Cu(II) with oxalate and EDTA. The latter both have $\alpha^2$ values of 0.84.$^9$

In the $g_{\perp}$ region of the spectrum of Cu(II) HCAB-CN$^-$, the first signal shows 7 shf lines spaced 17 G apart. On the higher-field--or "foldover"--peak, at least 5 shf lines are also visible. While the shf lines at high field may be unambiguously assigned to nitrogen shf structure, $^{10}$ present resolution does not permit us to distinguish between 2 and 3 nitrogens. Two arguments suggest that the nitrogen shf lines observed in the cyanide complex may all be attributed to ligand nuclei contributed by the protein. In mononuclear mixed complexes, cyanide coordination is likely to be to the carbon. Seven shf lines also appear in the sulfide complex where a nitrogen donor from the anion is not present.

The electron density map of the Zn(II) human enzyme C determined by x-ray diffraction at 2 Å resolution shows three protein ligands to the zinc ion. Although the amino acid sequence of the protein is not known in the region contributing the ligand side chains, the electron densities are best fitted by histidyl side chains (Liljas et al., in preparation). The esr findings are compatible with the assignment of three equivalent nitrogen nuclei as ligands to the metal ion.$^{11}$

**Co(II) carboxylic anhydrase anion complexes:** In contrast to the Cu(II) enzyme, Co(II) carboxylic anhydrase is a functional enzyme; hence, the charac-
teristics of its coordination geometry are more pertinent to the mechanism of action. Cobalt esr signals, however, may be quite complex and in many ligand environments are not observed at all because of rapid electron spin relaxation. Most signals reported have been observed for low-spin Co(II) in a planar, square-pyramidal, or octahedral environment.\textsuperscript{12–17} High-spin Co(II) spectra have not been reported except at very low temperatures for Co(II) inserted in various crystal lattices.\textsuperscript{18} No cobalt-protein spectra have been reported.

Co(II) carbonic anhydrase and its anion complexes demonstrate rather complex esr and we report some representative examples here. Susceptibility measurements show that the Co(II) enzyme, and its complexes with sulfanilamide, NCO\textsuperscript{-}, Cl\textsuperscript{-}, and NO\textsubscript{3}\textsuperscript{-}, are high-spin at room temperature.\textsuperscript{19} At liquid nitrogen temperatures, Co(II) carbonic anhydrase and its common anion complexes, such as the 1:1 complex with CN\textsuperscript{-}, show weak but detectable esr signals with g values near 2 (Fig. 2A). It is unclear at present whether these signals represent the total complex or a minor species. The width of the signals prevents accurate estimation of the total spin. The signal is modified by the combination of one equivalent of cyanide with the active center, and thus is arising from the active-center cobalt.

When a second equivalent of CN\textsuperscript{-} is added, an intense narrow signal appears, characterized by g values of 2.30 and 2.00 (Fig. 2B). On the high-field g\textsubscript{||} signal, the hyperfine lines attributable to the cobalt nucleus of spin 7/2 are clearly re-
solved. Each of the high-field hyperfine lines is split into a triplet, implying that at least one of the ligands is a nitrogen atom. $A_{\text{Co}||}$ is 95 G, $A_{\text{Co}\perp}$ is $\sim 20$ G, and $A_{N\parallel}$ is 14 G.

Esr and absorption-spectral titrations show that the intense signal is abolished by the addition of one equivalent of ethoxzolamide. Thus, the intense esr signal represents a second cyanide complex involving 2 cyanide ions bound at the active center. Transformation of the 1:1 CN$^-$ complex to the 2:1 complex is accompanied by minimal changes in the visible absorption spectrum, accounting for the failure to detect the 2:1 complex by spectral titrations. The esr signal of the 2:1 complex is characteristic of low-spin Co(II) in an axially-symmetric environment in which the unpaired electron is in the $d_{z^2}$ orbital of cobalt. The data are consistent with a shift from a coordination number of 4 to 5 upon the addition of the second cyanide. The observed shf structure is probably due to an axial cyanide nitrogen, although this does not necessarily require that the CN$^-$ be $N$-coordinated. Alternatively, a protein nitrogen could be the axial ligand in a five-coordinate complex. It is common in low-spin, square-pyramidal or octahedral Co(II) complexes to observe only the shf interactions of the vertical ligands. The Co(II) esr spectra suggest that the anion interaction may be more complex than heretofore believed.

It is interesting, in view of these results, to speculate on the reasons for the activity of the Zn and Co enzymes, and the lack of activity of the derivatives formed from the other divalent metal ions of the first transition and IIB series. It may be that the protein conformation allows some variation in the geometry assumed by the metal complex, perhaps varying between a square-planar and distorted tetrahedral geometry. The distorted geometry may be required for carbonic anhydrase activity to facilitate rapid exchange of monodentate ligands. Thus, we speculate that the transition state involves a tetrahedral distortion of the coordination geometry. This type of geometry would be accommodated relatively easily by Zn(II) and Co(II), but would be energetically quite unfavorable for Cu(II). The formation of the 2:1 CN$^-$ complex of the Co(II) enzyme implies some flexibility of the coordination site and suggests that it is possible to add an apical ligand to the Co(II) complex forcing axial symmetry and a low-spin state.

**Spin-labeled sulfonamide binding**: The esr spectrum of the sulfonamide-nitroxide (I) in solution is shown in Fig. 3A. The spectra for the 1:1 complexes of this sulfonamide with Zn(II) BCAB, Zn(II) HCAB, and Co(II) HCAB are shown in Figs. 3B, C, and D. The esr signal of the free label consists of three narrow lines, due to the hyperfine interaction of the radical electron with the nitrogen nucleus. The enzyme-bound sulfonamide gives a typical powder-type esr spectrum for an immobilized nitroxide. The origin and interpretation of the esr spectra of immobilized nitroxides has been discussed extensively by McCon nell and McFarland.

The esr signals of the enzyme complexes given in Fig. 3 may be compared with the computed spectra for immobilized nitroxides presented by Itzkowitz. For the bovine enzyme, the rotational correlation time so obtained is $2-5 \times 10^{-8}$ sec. It is of interest that the rotational relaxation time of bovine carboxic
anhydrase calculated by Chen and Kernohan, from fluorescence data on the complex with a fluorescent sulfonamide, is $2.89 \times 10^{-8}$ sec. The latter is very close to the rotational relaxation time of $2.43 \times 10^{-8}$ sec calculated from Stokes' law, assuming that carbonic anhydrase is an anhydrous sphere of molecular weight 30,000. Thus, in the case of the bovine enzyme, the esr spectrum indicates that the sulfonamide-nitroxide is almost completely immobilized and has almost no mobility apart from the rotational motion of the protein. In the HCAB complexes, the esr spectra indicate that the sulfonamide-nitroxide is slightly less immobilized. Apparently, the compound is not quite so rigidly fixed in the binding cavity and is slightly flexible in relation to the protein surface, in agreement with the independent observation that the bovine enzyme appears to bind the sulfonamide more tightly than the human enzyme. We have found that the precise signal profiles from the bound sulfonamide-nitroxides are characteristic of each species and isozyme variant of carbonic anhydrase, another confirmation that the immediate environment of the binding cavity varies between the different carbonic anhydrase isozymes.

The identical signals observed for the nitroxide bound to native Zn(II) HCAB, and its Co(II) derivative, (Fig. 3C and D) suggest that the conformation of the active center cavity does not differ appreciably in the two derivatives. Thus, it would appear that data on the active Co(II) derivative can be interpreted as arising from a similar conformation of the active center.

Another significant feature of the signal from the nitroxide incorporated into the enzyme is that there appears to be no spin-spin interaction between the paramagnetic Co(II) and the nitroxide. This is perhaps not surprising, since according to present models of the mode of binding of the sulfonamide, 15–20 Å must separate the sulfonamide coordinated to Co(II) from the free radical. If the spin-labeled sulfonamide is added to Cd(II) or Hg(II) carbonic anhydrase, a signal typical of the free label is observed (Fig. 3A.) This confirms the finding that these metal ions at the active site do not induce the binding of the sulfon-
amide. Likewise if $10^{-2}$ M cyanide is added to the spin-labeled Zn(II) enzyme, the signal reverts to that of the free label. Thus, the interaction of the spin-labeled sulfonamide is clearly one mediated by the nature of the metal ion, and the site overlaps the anion-binding site. This overlap appears to involve coordination to the metal ion, as further documented by the shifts in the Cu(II) esr spectra observed upon binding of both ethoxzolamide and the anions (Fig. 1).

We thank Dr. W. E. Blumberg for valuable discussions.

* Supported by grant AM009070-06 from the National Institutes of Health and grant GB-13344 from the National Science Foundation.

† Abbreviations: HCAB, human carbonic anhydrase B; HCAC, human carbonic anhydrase C; MCAB, monkey carbonic anhydrase B; BCAB, bovine carbonic anhydrase B; shf, superhyperfine; DPPH, 2,2-diphenyl-1-picrylhydrazyl.

6 Coleman, J. E., Biochemistry, 4, 2644 (1965).
11 The esr spectrum of Cu(II) HCAC is not identical to that of Cu(II) HCAB, apparently reflecting some isozyme differences in the environment of the metal ion. While the changes in geometry accompanying anion complexation of Cu(II) HCAC show the same trend as in the case of Cu(II) HCAB, the cyanide complex does not reveal the highly resolved nitrogen hyperfine structure.
18 Low, W., Solid State Physics, Suppl., 2, 76 (1960).