Inorganica Chimica Acta, 46 (1980) 211–214

Cyanometallates and Cobalt(II) Bovine Carbonic Anhydrase B. Five Coordination with Dicyanoaurate(I)

IVANO BERTINI, GIORGIO CANTI, CLAUDIO LUCHINAT and PAOLO ROMANELLI

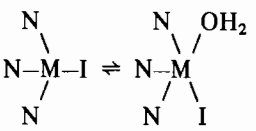
Istituto di Chimica Generale ed Inorganica della Facoltà di Farmacia, Università di Firenze, and Laboratorio per lo Studio dei Composti di Coordinazione del CNR, Florence, Italy

Received March 4, 1980

The binding affinities of various cyanometallates for cobalt(II) bovine carbonic anhydrase B have been investigated by means of optical and magnetic resonance spectroscopies. Dicyanoaurate(I) and dicyanoargentate(I) ions show a large affinity for the enzyme, giving rise to a five coordinate adduct in the former case and to equilibria between four and five coordinate species in the latter case. Five coordination is reached through three histidine nitrogens, a cyanide nitrogen and a water molecule. $Hg(CN)_2$, $Fe(CN)_6^{4-}$, $M(CN)_4^{2-}$ (M = Ni, Pd) and $Au(CN)_4^-$ do not show appreciable affinity for the enzyme either on account of the charge ($Hg(CN)_2$) or of the ionic dimensions.

Introduction

The possibility that five coordination occurs as one of the steps in the catalytic interaction of the metalloenzyme carbonic anhydrase with the various substrates has been proposed by several authors [1–3]. During the investigation of the coordination number of the cobalt(II) substituted enzyme with various inhibitors five coordination was proposed to occur, as well as equilibria between four- and five-coordinate species [4]. The HCO_3^- ion belongs to the latter class of ligands [4].



Among the various X-ray studies through a difference map it appears [5] that the dicyanoaurate(I) ion can be present in the active cavity of the enzyme without removing the water molecule which is bound to the metal ion in the non-inhibited enzyme. The authors [5] could not, however, establish whether the latter ligand was bound to the metal at all, and therefore could not establish the geometry of the resulting chromophore. In the case where the dicyanoaurate ion is bound, five coordination should occur. With this in mind the systems cobalt(II) bovine carbonic anhydrase B (CoBCAB hereafter) with dicyanoaurate(I) and dicyanoargentate(I) ions have been investigated by means of electronic and NMR spectroscopies.

Furthermore, the study of the binding capability of other cyanoderivatives like $Hg(CN)_2$, $Au(CN)_4^-$, $Ni(CN)_4^{2-}$, $Pd(CN)_4^{2-}$, and $Fe(CN)_6^{4-}$ with respect to CoBCAB may allow to appreciate the importance of the charge and geometry of inhibitors among closely related compounds.

Materials and Methods

Bovine erythrocyte carbonic anhydrase (carbonate dehydratase, EC 4.2.1.1.) was obtained as a lyophilized material from Sigma. Carbonic anhydrase B was obtained through chromatography on DEAE Cellulose [6]; apocarbonic anhydrase was prepared by dialyzing the enzyme against two changes of pyridine-2,6-dicarboxylic acid in phosphate buffer at pH 6.9 [7], then dialyzing the solution against bidistilled water. Cobalt(II) bovine carbonic anhydrase B was obtained by dialyzing apoenzyme solutions against unbuffered 10^{-3} *M* solutions of $CoSO_4$ at a pH around 7 and then removing the excess metal ions by exhaustive dialysis against the appropriate buffers.

CoBCAB concentrations were determined by measuring the absorbance at 280 nm, using a molar absorbance of 5.7×10^4 M^{-1} cm^{-1} [8]. $Hg(CN)_2$ and the potassium salts of all the cyanocomplexes investigated were prepared following the procedures described in the literature [9, 10], and checked through elemental analysis. Enriched $K^{13}CN$ for the preparation of $KAu(^{13}CN)_2$ and $KAg(^{13}CN)_2$ was purchased from Prochem B.O.C.

Spectrophotometric Measurements

Optical spectra were recorded in the 0–0.1 absorbance range using 10^{-3}–10^{-4} M solutions of CoBCAB. For the determination of the affinity constants of the cyanocomplexes with CoBCAB, solutions of the cobalt enzyme were prepared by dialysis against buffer solutions of HEPES or TAPS 10^{-2} *M* at the appropriate pH in the range 6–10. Enzyme and buffer concentrations were kept constant within each

experiment. The affinity constant values were determined by following the spectral changes in the visible region under addition of increasing amounts of the cyanocomplexes.

The spectrophotometric data were treated with a least squares program described in [11]. Although the standard deviation given by the computer treatment was very low (< 3%), larger sources of errors are the uncertainty in the enzyme concentration as well as in the constancy of pH within each experiment. The overall precision is estimated to be within ± 10%.

The electronic spectra in the near infrared region were recorded using ^2H_2O solutions of enzyme and cyanocomplexes, with enzyme concentrations in the range $1-3 \times 10^{-3}$ *M*.

^{13}C Nuclear Magnetic Resonance Measurements

The ^{13}C NMR spectra of ^{13}C enriched cyanocomplexes were recorded on a Varian CFT 20 spectrometer, operating at 15 or 25 °C, and using ^2H_2O lock. The T_1 measurements were performed using the inversion recovery method, while T_2 was calculated through the relation $T_2 = (\pi\Delta\nu)^{-1}$. The reproducibility of the reported values for several measurements is within ± 5%.

Results

The Electronic Spectra

The addition of increasing amounts of cyanocomplexes to CoBCAB, both in buffered aqueous solutions, was followed by electronic spectroscopy in the visible region. Of all the cyanoderivatives investigated only $Au(CN)_2^-$ and $Ag(CN)_2^-$ cause a marked variation in the electronic spectrum of the cobalt enzyme, while $Hg(CN)_2$, $Au(CN)_4^-$, and $Fe(CN)_6^{4-}$, added up to 2×10^{-1} *M*, 10^{-1} *M, and* 2×10^{-2} *M*, respectively, do not cause any appreciable change. In the case of $Ni(CN)_4^{2-}$ and $Pd(CN)_4^{2-}$ the addition to the CoBCAB solutions yields spectra identical to that of the CoBCAB–CN adduct [4]: the K_4 values for the above complexes are in fact expected [9] to be lower than the stability constant of the enzyme–cyanide derivative [4], thus a cyanide ion is transferred from the Ni(II) or Pd(II) complexes to the cobalt enzyme.

From the spectral data obtained with $Au(CN)_2^-$ and $Ag(CN)_2^-$ the limit spectra of the inhibited enzyme and the affinity constants were obtained through a computer program; the data could only be reproduced by assuming a 1:1 equilibrium.

The affinity constants of the dicyanoaurate(I) and dicyanoargentate(I) ions (Table I) show the usual increase with decreasing pH; this behaviour has been attributed to the presence [12] of at least [13, 14] one ionizable group in the active site of the enzyme with pK_a around 7.

TABLE I. Apparent[a] Affinity Constants of Dicyanometallates for CoBCAB.

	pH	k_{app}
$Ag(CN)_2^-$	8.0	1.6×10^3
	9.0	3.8×10^2
$Au(CN)_2^-$	8.0	6.8×10^2
	9.0	2.3×10^2

[a]The affinity of ligands for carbonic anhydrase depends on at least one ionization in the active site; the enzyme cannot be regarded as a single chemical entity.

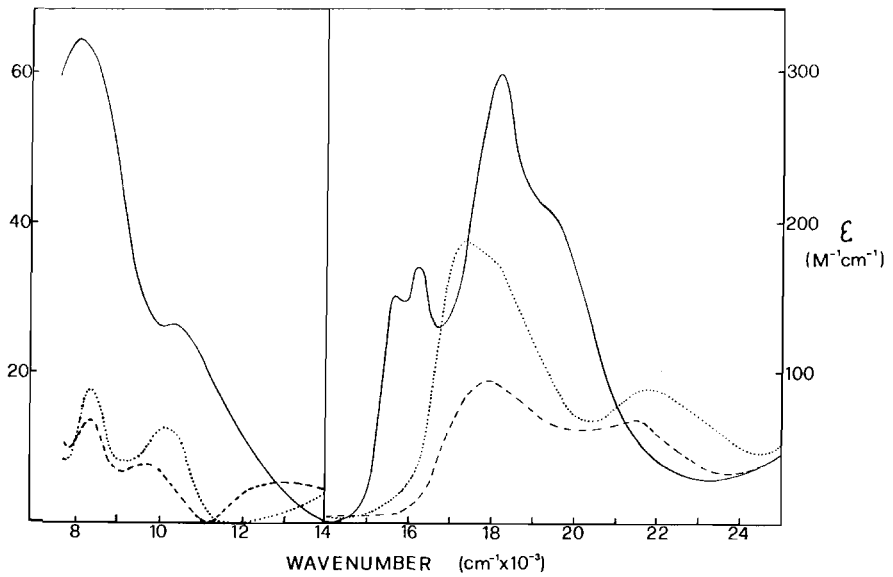


Fig. 1. Electronic spectra of cobalt(II) bovine carbonic anhydrase at pH 6.1 (—), and of its adducts with dicyanoaurate(I) (– – –) and dicyanoargentate(I) (·····) ions.

TABLE II. ^{13}C Relaxation Rates (s^{-1}) and Isotropic Shifts (ppm) at 20 MHz of Dicyanoargentate(I) and Dicyanoaurate(I) in the Presence of CoBCAB at pH 9.0 and 25 °C.

	T_1^{-1}	T_2^{-1}	$\Delta\nu$	T_{1M}^{-1}	T_{2M}^{-1}	$\Delta\nu_p$
$Ag(CN)_2^-$	0.02	2	–	–	–	–
$Ag(CN)_2^-$ + CoBCAB (460:1)	6.9	6.6×10^2	7.0	3.2×10^3	3.0×10^5	1.6×10^3
$Au(CN)_2^-$	0.04	10	–	–	–	–
$Au(CN)_2^-$ + CoBCAB (140:1)	7.0	6.9×10^2	10.5	9.7×10^2	9.5×10^4	1.5×10^3

The electronic spectra of the $Au(CN)_2^-$ and $Ag(CN)_2^-$ adducts recorded in the visible and near infrared region are reported in Fig. 1, together with the spectrum of the pure enzyme; the spectra of the adducts are pH independent.

^{13}C NMR Data

The ^{13}C NMR spectra of $Au(^{13}CN)_2^-$ and $Ag(^{13}CN)_2^-$ have been registered; they compare well with those described in the literature [15] both with respect to chemical shifts and relaxation rates. The extremely low T_1^{-1} values are particularly suitable for detecting even small paramagnetic effects, which are expected to increase the nuclear relaxation rates.

Addition of CoBCAB to cyanocomplexes solutions results in a dramatic increase of such relaxation rates as well as in sizeable isotropic shifts, as summarized in Table II. The transverse relaxation rates (T_2^{-1}) of both cyanocomplexes in the presence of CoBCAB are about two orders of magnitude larger than the respective longitudinal relaxation rates (T_1^{-1}). Measurements at different CoBCAB:$M(CN)_2^-$ ratios show a linear dependence of the paramagnetic effects on the enzyme concentration; the effect is also decreased by increasing temperature, indicating that the chemical exchange of $M(CN)_2^-$ between the paramagnetic enzyme site and the bulk solution is fast with respect to both T_1 and T_2 time scale. In Table II the relaxation rates experienced by the ^{13}C nuclei in the enzyme adduct (T_{iM}^{-1}) are also shown; they are calculated through the known molar fraction of bound ligand.

Discussion

The T_{iM}^{-1} values under fast ligand exchange conditions are related to the sixth power of the metal–nucleus distance according to the Solomon–Bloembergen–Morgan equations [16].

$$T_{iM}^{-1} = k\,1/r^6 f(\tau_c) + \text{spin delocalization contributions}$$

The first term, which is dipolar in origin, is valid in the point-dipole approximation. Although quantitative distance calculations cannot be performed since the additional contributions due to spin delocalization (both contact and pseudocontact) cannot be evaluated, it is easy to distinguish whether the $M(CN)_2^-$ ions are directly bound to the metal or not: in the second case there are no contributions from spin delocalization, and the dipolar term should give a distance larger than that expected for the first coordination sphere, *i.e.* > 350 pm. The values obtained substituting the experimental T_{1M} and a range of reasonable values for τ_c [16] are: 210 ÷ 260 pm for $Au(CN)_2^-$ and 170 ÷ 220 pm for $Ag(CN)_2^-$, and even lower using T_{2M}. The above values are actually too short even for direct coordination, definitely showing the existence of a large spin delocalization term and, hence, of metal–ligand chemical bond.

Such a conclusion is quite consistent with the large change in the spectral features of the cobalt(II) chromophore upon addition of the cyanocomplexes. Since from X-ray data [5] it appears that the metal ion maintains a coordinated water molecule in the presence of the $Au(CN)_2^-$ ion, the latter can only add to a fifth coordination position, giving rise to a five coordinate chromophore. In a thorough investigation of the spectral properties of CoBCA with numerous inhibitors three types of spectra were recognized and associated to structural properties [4]: (*i*) spectra with low intensity ($\epsilon \leqslant 150\ M^{-1}\ cm^{-1}$ in the visible region) and an absorption at 12–14 $\times 10^3$ cm^{-1}; (*ii*) spectra with intense absorptions ($\epsilon > 300\ M^{-1}\ cm^{-1}$ in the visible region) and absence of bands in the 12–14 $\times 10^3$ cm^{-1} region; (*iii*) intermediate spectra.

The first class of compounds were assigned as five-coordinate, the second as tetrahedral, and the third to equilibria between the two species. The electronic spectra of the CoBCAB–$Au(CN)_2^-$ system (Fig. 1) belong to the first class since the absorptions are weak and a band at 12.5 $\times$ 10^3 cm^{-1} is nicely evident. The spectra of the system containing $Ag(CN)_2^-$ belong to the third class and are therefore indicative of an equilibrium between tetra and pentacoordinate species.

The differences in T_{1M} values of the two adducts are also consistent with this picture, since they are related to the electronic relaxation rates τ_e of the

cobalt chromophores which in their turn determine τ_c. Pseudotetrahedral cobalt(II) complexes are expected to experience larger τ_e with respect to five or six coordinate chromophores [4, 17, 18]. A fraction of tetracoordinate cobalt(II) species in the dicyanoargentate–metalloprotein system may account for the shorter τ_e of the cobalt(II) ion with respect to the fully five coordinate dicyanoaurate adduct.

Isotropic Shifts

In Table II the isotropic shifts experienced by the ^{13}C nuclei in the two adducts are also reported: $\Delta\nu$ refer to the experimental values, while $\Delta\nu_p$ represent the isotropic shifts of the fully paramagnetic species, calculated through the ligand:metal ratios; the latter values are downfield and of the same magnitude for both dicyanometallate complexes, indicating that similar mechanisms are operative in the transmission of the paramagnetic effect. Although the origin of the isotropic shift may be controversial owing to the different mechanisms which can be operative, it may be worthy to compare the present data with literature data relative to ^{13}C and ^{14}N in paramagnetic cyano-complexes [19, 20]. In systems of the type M–C≡N the nitrogen atom always shows downfield shifts, while the carbon invariably undergoes upfield shifts about one order of magnitude larger. In the present case the paramagnetic center is bound to the nitrogen, *i.e.* M–N≡C, and, indeed, the measured shifts on ^{13}C compare well in sign and magnitude with those of ^{14}N in the above examples, suggesting that similar spin delocalization mechanisms are operative when the cyanide group binds the paramagnetic center through the nitrogen or the carbon atoms.

Conclusions

The $M(CN)_2^-$ complexes show remarkable affinity for the metal enzyme. They bind at the metal, as shown by the ^{13}C NMR data and, consistently, by the electronic spectra. The latter suggest, at least for $Au(CN)_2^-$, a five coordination of the donor atoms around the cobalt(II) ion, the donor atoms being the three histidine nitrogens from the protein, a water molecule and a cyanide nitrogen; this coordination seems consistent with the X-ray diffraction data [5]. The uncharged isosteric $Hg(CN)_2$ species does not interact with the metal ion within the sensibility of the electronic spectroscopy thus confirming the primary importance of the charge [21] in the affinity of the inhibitors. Other complexes like $Au(CN)_4^-$, $Ni(CN)_4^{2-}$, $Pd(CN)_4^{2-}$, and $Fe(CN)_6^{4-}$ do not show any detectable affinity for the metal, probably on account of their large dimensions with respect to the active site crevice.

Acknowledgments

Thanks are expressed to Prof. L. Lunazzi for providing NMR facilities.

References

1 E. T. Kaiser and K. W. Lo, *J. Am. Chem. Soc., 91,* 4912 (1969).
2 M. Caplow, *J. Am. Chem. Soc., 93,* 230 (1971).
3 J. M. Pesando, *Biochemistry, 14,* 681 (1975).
4 I. Bertini, G. Canti, C. Luchinat and A. Scozzafava, *J. Am. Chem. Soc., 100,* 4873 (1978).
5 P. C. Bergstén, I. Waara, S. Lövgren, A. Liljas, K. K. Kannan and U. Bengtsson, in 'Proceedings of the Alfred Benzon Symp. IV', (M. Rorth and P. Astrup Ed.), p. 363 (1972), Munksgaard, Copenhagen.
6 S. Lindskog, *Biochim. Biophys. Acta, 39,* 218 (1960).
7 J. B. Hunt, M. J. Rhee and C. B. Storm, *Anal. Biochem., 55,* 615 (1977).
8 P. O. Nyman and S. Lindskog *Biochim. Biophys. Acta, 85,* 141 (1964).
9 A. G. Sharpe, 'The Chemistry of the Cyano Complexes of the Transition Metals', Academic Press (1976), London, New York, San Francisco.
10 J. M. Smith, L. H. Jones, I. K. Kressin and R. A. Penneman, *Inorg. Chem., 4,* 369 (1965).
11 R. Barbucci, L. Fabbrizzi and P. Paoletti, *J. Chem. Soc. Dalton,* 1099 (1972).
12 J. E. Coleman, in 'Inorganic Biochemistry' (G. L. Eichhorn Ed.), Vol. I, p. 488 (1973), Elsevier, London.
13 D. Barzi, I. Bertini, C. Luchinat and A. Scozzafava, *Inorg. Chim. Acta, 36,* L431 (1979).
14 I. Bertini, C. Luchinat and A. Scozzafava, *Inorg. Chim. Acta, 46,* 85 (1980).
15 J. P. Pesek and W. R. Mason, *Inorg. Chem., 18,* 924 (1979).
16 I. Bertini, C. Luchinat and A. Scozzafava, *J. Chem. Soc. Dalton,* 1962 (1977).
17 M. E. Fabry (Riepe), S. H. Koenig and W. E. Shillinger, *J. Biol. Chem., 245,* 4256 (1970).
18 L. Luz and S. Meiboom, *J. Chem. Phys., 40,* 1058 (1964).
19 A. Loewenstein, M. Shporer and G. Navon, *J. Am. Chem. Soc., 85,* 2855 (1963).
20 D. G. Davis and R. J. Curland, *J. Chem. Phys., 46,* 388 (1967).
21 S. Lindskog, L. E. Enderson, K. K. Kannan, A. Liljas, P. O. Nyman and B. Strandberg, 'Enzymes', 3rd Ed., *5,* 587 (1971).