Electron-Paramagnetic-Resonance Studies on Cobalt(II) Carbonic Anhydrase

LOW-SPIN CYANIDE COMPLEXES

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The low-spin cyanide complexes of three Co(II) carbonic anhydrases were investigated by electron paramagnetic resonance (e.p.r.) at 9 and 35 GHz. Well-defined and closely axial spectra were obtained only in the absence of oxygen. Several mole equivalents of cyanide were required for complete formation of the complexes in frozen solution, although large excesses caused abstraction of the cobalt. Experiments with [^{13}C]cyanide showed that the low-spin complexes contained two CN^- groups in an environment similar to that of the in-plane ligands in $[\text{Co}(\text{CN})_5]^{3-}$. A combined e.p.r. and spectrophotometric titration confirmed the presence of two CN^- ligands. A 5-co-ordinate square pyramidal structure involving three protein ligands was proposed. The dicyanide complex could be oxygenated reversibly, producing a characteristic new e.p.r. spectrum. The O_2 molecule was thought to occupy the remaining octahedral metal site in a formally Co(III) species. The optical spectrum of the dicyanide lacked the prominent d–d bands of the high-spin monocyanide. Both e.p.r. and optical data indicated that the low-spin complex was formed much more fully in frozen solution than at room temperature. Differences in behaviour between the high- and low-activity enzymes suggested some variation in conformational flexibility at the metal binding site.

The zinc ion in native carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) may be replaced by cobalt(II) with retention of enzymic activity, thus enabling a number of spectroscopic methods to be used for probing the catalytic centre (see Lindskog, 1970). Co(II) in the active enzyme and in 1:1 complexes with sulphanilamide and several anions has been shown to exist in the high-spin $S = \frac{3}{2}$ state at room temperature (Lindskog & Ehrenberg, 1967). Owing to rapid electron spin relaxation, intelligible e.p.r. (electron paramagnetic resonance) spectra have been observed only at temperatures below about 30°K (Grell & Bray, 1971). However, treatment of the Co(II) enzyme with excess of cyanide gives rise to a quite different species whose e.p.r. spectrum, detectable even at ambient temperature, is characteristic of the low-spin $S = \frac{1}{2}$ state (Grell & Bray, 1971; Taylor & Coleman, 1971). This has been regarded as a 1:2 enzyme–cyanide complex in which Co(II) is probably five-co-ordinate. The present paper reports some detailed e.p.r. investigations on the structure and stoichiometry of the higher cyanide complex, by using ^{13}C -enriched KCN and both 9 and 35 GHz instruments. The evident sensitivity of the system to

O_2 has also led to the discovery of an oxygen adduct, which thus exhibits a high co-ordination number and an unusual oxidation state for cobalt bound at the active site of carbonic anhydrase. A preliminary account of some of this work has been published (Cockle, 1973).

Materials and Methods

Reagents

Spectrographically pure cobalt sulphate ($\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$) was obtained from Johnson Matthey Chemicals Ltd., London EC1P 1AE, U.K. K^{13}CN enriched to 61% and to 87.43% were from, respectively, Bio-Rad Laboratories, St. Albans, Herts., U.K., and Merck, Sharpe and Dohme (Canada) Ltd., Pointe Claire, P.Q., Canada. All solutions of KCN were freshly prepared before each experiment. Ethoxzolamide (6-ethoxybenzothiazole-2-sulphonamide) was purchased from the Upjohn Co., Kalamazoo, Mich., U.S.A. A sample of aquocobalamin (vitamin B_{12a}) was kindly provided by Dr. H. A. O. Hill, Inorganic Chemistry Laboratory, University of Oxford. Other chemicals were of analytical or reagent grade, as appropriate.

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Cobalt carbonic anhydrase

Native carbonic anhydrase was isolated from human and bovine erythrocytes by established procedures (Henderson & Henriksson, 1973; Lindskog, 1960; Nilsson & Lindskog, 1967). The zinc was extracted by dialysis at 5°C against 0.01 M-1,10-phenanthroline in 0.1 M-sodium acetate buffer, pH 5.5 (25°C), for the human isoenzymes B and C, and pH 5.2 for the bovine enzyme. Dialysis was continued until the residual esterase activity was no greater than 0.5% with respect to reconstituted apoenzyme. Cobalt was introduced by treating the apoenzyme with a 5% molar excess of CoSO₄ in unbuffered solution at pH 8–9, followed by extensive dialysis against distilled deionized water.

Enzyme concentration

Protein concentrations were determined from measurements of optical absorbance at 280 nm. The following molar extinction coefficients were used, determined from the molecular weights given in parentheses (see Lindskog *et al.*, 1971): human isoenzyme B (29000) 47300 litre·mol⁻¹·cm⁻¹, human isoenzyme C (30000) 56100 litre·mol⁻¹·cm⁻¹, bovine enzyme (30000) 57000 litre·mol⁻¹·cm⁻¹. The concentration of active-site Co(II) could be readily evaluated from the integrated intensity of the e.p.r. spectrum of the fully-formed low-spin cyanide complex (see the Results section).

Enzyme activity

Preparations were assayed for esterase activity in the following medium: enzyme (0.5–1.5 μM), *p*-nitrophenyl acetate (0.40 mM), Tris-sulphate buffer (0.08 M), acetone (1%, v/v), pH 7.60 at 25°C (Whitney *et al.*, 1967). Specific activities were: Co(II) human isoenzyme B 0.31, Co(II) human isoenzyme C 0.90, Co(II) bovine enzyme 0.43 mol of ester hydrolysed/s per mol of enzyme.

The proportion of active enzyme in these samples was determined by titrating the esterase activity against Ethoxzolamide. Owing to the high binding constant of this inhibitor, zero activity is reached at a 1:1 molar equivalence of sulphonamide to active enzyme (Fabry *et al.*, 1970). Purities were: Co(II) human isoenzyme B 92%, Co(II) human isoenzyme C 86%, Co(II) bovine enzyme 100% with respect to protein concentration derived from optical absorbance at 280 nm.

Anaerobic technique

Anaerobic e.p.r. samples were prepared in Thunberg tubes to which standard e.p.r. tubes were joined via graded seals. Solutions could thus be deoxygenated either before or after mixing enzyme with

cyanide. The normal practice was to purge the system by alternate evacuation and flushing with O₂-free Ar or N₂, while the reagent solutions were kept frozen in liquid N₂. When the solutions had been allowed to thaw and equilibrate with the new atmosphere, the complete cycle was repeated three or four times. In this way, volatilization of HCN was prevented, and concentration changes were minimal. A similar technique was adopted for oxygenation of enzyme-cyanide samples. The repeated freezing and thawing did not appear to affect the e.p.r. characteristics of the higher cyanide complex.

Electron paramagnetic resonance

Spectra were obtained with a Varian E-3 spectrometer operating at 9 GHz, or with a Varian E-9 instrument at both 9 and 35 GHz. Except for measurements at room temperature, spectra were recorded at 77°K on the E-3, or at 120°K on the E-9 instrument, with a modulation amplitude of 0.5 mT (5 G). Double integration was carried out by the method of Wyard (1965), with CuSO₄ in 2 M-NaClO₄ or Cu(II)-EDTA as concentration standards. A correction for the variation of transition probability with *g* value was applied as described by Aasa & Vänngård (1970).

Optical spectra

Visible spectra of the Co(II) enzymes were run on a Cary 15 or Unicam SP.1800 recording spectrophotometer, over 0–0.1 or 0–0.2 absorbance ranges.

Results

Anaerobic formation of the low-spin Co(II) enzyme complex

Addition of more than 1 mole equiv. of KCN to Co(II) carbonic anhydrase in Tris-sulphate buffer, pH 9 (25°C), gave rise, under anaerobic conditions, to well-defined e.p.r. spectra in the frozen solutions (Figs. 1 and 2). Comparison of the 9 and 35 GHz traces confirmed that these spectra represent single chemical species, and that the *g*₁ and *A*₁ parameters could be derived quite accurately by inspection (Table 1). Only slight differences were noted among several enzyme variants (see also Taylor & Coleman, 1971). A prominent feature of the spectra is the triplet splitting of the eight ⁵⁹Co hyperfine lines, attributed to a nitrogen ligand from the protein (Grell & Bray, 1971). However, spectra reported by previous authors have frequently been distorted by an overlying narrow resonance at *g* = 2.0 arising from the presence of oxygen.

Unlike Taylor & Coleman (1971), I did not find that the low-spin enzyme complex could be fully formed by addition of 2 mole equiv. of KCN. With

Table 1. *E.p.r. parameters of low-spin cyanide complexes*

The stoichiometry of the Co(II) carbonic anhydrase complexes is deduced elsewhere in this paper (see the Discussion section). Cyanocobalt(II) cobalamin was obtained by reduction of aquo-cobalt(III)cobalamin with ascorbate, followed by treatment with excess of K¹³CN. Other samples were prepared as described in the Figure legends. Parameters were measured directly from experimental spectra, except for the $A_{\parallel}(^{13}\text{C})$ values (indicated by *) derived by simulation, for which the consequent error limits are given. Abbreviations: HCAB and HCAC, human carbonic anhydrase B and C; BCA, bovine carbonic anhydrase.

Complex	$g_{\parallel}$	$g_{\perp}$	$10^4 \times A_{\parallel}(^{59}\text{Co}) $ (cm ⁻¹)	$10^4 \times A_{\perp}(^{59}\text{Co}) $ (cm ⁻¹)	$10^4 \times A_{\parallel}(^{14}\text{N}) $ (cm ⁻¹)	$10^4 \times A_{\parallel}(^{13}\text{C}) $ (cm ⁻¹)
CoBCA(CN) ₂	2.005	2.27	88	—	13.5	
CoHCAC(CN) ₂	2.004	2.27	87	—	13.6	
CoHCAB(CN) ₂	2.004	2.27	89	—	13.2	
CoHCAB(¹³ CN) ₂	2.004	2.27	89	—	—	10.3 ± 0.5*
Co(¹³ CN)cobalamin	2.005	—	80	—	—	60.0
[Co(¹³ CN) ₅] ³⁻	2.004	—	82	—	—	39.5 ± 1.0*
						9.0 ± 0.5*
CoHCAB(CN) ₂ (O ₂) [†]	2.070	2.006	14.6	13		
[Co(CN) ₅ (O ₂)] ^{3-‡}	2.06	2.007	—	12		

[†] Peak-to-peak width 9.6 mT (96 G).

[‡] Tentative formulation; peak-to-peak width 6.5 mT (65 G).

Fig. 1. *E.p.r. spectra of low-spin cyanide complexes of Co(II) carbonic anhydrase*

All samples were prepared anaerobically in 0.04M-Tris-sulphate, pH9.0 at 25°C. (a), Co(II) human isoenzyme C 0.42mM, KCN 5.0mM; (b), Co(II) bovine enzyme 0.41 mM, KCN 4.8mM; (c), Co(II) human isoenzyme B 0.40mM, KCN 2.8mM; (d), Co(II) human isoenzyme B 0.40mM, K¹³CN (87.43% ¹³C) 3.2mM. Spectra were recorded at 9.15 GHz, with arbitrary instrument gain; cavity temperature was 120°K (a) or 77°K (b-d).

Fig. 2. *E.p.r. spectrum at 35GHz of the low-spin cyanide complex of Co(II) human carbonic anhydrase B*

The sample was prepared under Ar in 0.04M-Tris-sulphate, pH9.0 at 25°C; the composition was Co(II) enzyme 0.41mM, KCN 3.3mM. The spectrum was recorded at 34.78 GHz, with cavity temperature 120°K.

0.4mM-enzyme, the degree of formation was 40–45% for the high-activity variants Co(II) human C and Co(II) bovine carbonic anhydrases, and only 15% for the low-activity Co(II) human isoenzyme B. This result led to some unusual observations when a mixture of Co(II) human isoenzyme B and KCN (mole

ratio 1:2) was titrated with Ethoxzolamide, which is expected to displace enzyme-bound CN^- almost completely (Taylor & Coleman, 1971). Although the spectrum detectable at 77°K was indeed virtually abolished at a 1:1 molar equivalence of sulphonamide to enzyme, the intensity rose threefold during addition of the first 0.4 mole equiv. of Ethoxzolamide. Thus the yield of the low-spin complex was initially enhanced by the effective decrease in the enzyme: cyanide mole ratio.

Saturation of the Co(II) enzymes by CN^- was complete at 4 mole equiv. of KCN with Co(II) human C and Co(II) bovine carbonic anhydrases, and at 6 mole equiv. with the human isoenzyme B; no further alterations in signal shape or amplitude occurred up to 10 or more mole equiv. Under these conditions, the Co(II) concentration determined by spectral integration accounted for 98–100% of the catalytically active enzyme. However, no firm conclusion could be reached on the stoichiometry of the higher cyanide complex.

E.p.r. spectra of complexes with ^{13}C cyanide

Fig. 1(d) shows a 9GHz spectrum obtained anaerobically from Co(II) human isoenzyme B and K^{13}CN (87.43% enrichment). Additional nuclear hyperfine structure is evident, although the g values and the coupling constant $A_1(^{59}\text{Co})$ remain unchanged. Since ^{13}C has nuclear spin $I = \frac{1}{2}$, the observed quintet patterns in the g_1 region could not arise through the interaction of a single CN^- ligand. A Varian C-1024 Time-Averaging Computer was used to simulate the $M_I = +\frac{1}{2}$ ^{59}Co hyperfine line on the basis of two CN^- groups with equal coupling constant $A_1(^{13}\text{C})$. Five partial spectra of the Co(II) human isoenzyme B/ $^{12}\text{CN}^-$ system with appropriate gains and field offset were added in to represent the contributions from species with two, one or no ^{13}C nuclei (cf. Malkin *et al.*, 1968). The experimental spectrum was closely matched at an input value $|A_1(^{13}\text{C})| = 1.1 \text{ mT}$ (11 G; $10.3 \times 10^{-4} \text{ cm}^{-1}$) (Fig. 3a). The partial spectrum from Co(II) isoenzyme B and K^{13}CN (61% enrichment) was also simulated, again with good agreement (Fig. 3b). An attempt to synthesize the whole spectrum with a single hyperfine constant $|A(^{13}\text{C})| = 1.1 \text{ mT}$ gave fair agreement in the poorly resolved g_1 region, suggesting that the ^{13}C -hyperfine interaction includes a dominant isotropic term. No detailed spectral analyses were carried out for Co(II) human isoenzyme C and Co(II) bovine carbonic anhydrase, but the spectra obtained with K^{13}CN closely resembled those described and illustrated for the human B isoenzyme. The results therefore indicate that the low-spin complex contains two equivalent CN^- ligands.

To provide a comparison for the enzyme system, e.p.r. measurements have also been carried out on

Fig. 3. Experimental and simulated ^{13}C hyperfine splitting in Co(II) human carbonic anhydrase B [^{13}C]dicyanide

All samples were prepared under N_2 in 0.04M-Tris-sulphate, pH 9.0 at 25°C. The experimental samples comprised: E(a), Co(II) enzyme 0.41 mM, K^{13}CN (87.43% ^{13}C) 3.2 mM; E(b), enzyme 0.40 mM, K^{13}CN (61% ^{13}C) 3.2 mM. The simulations (S) were carried out as described in the text, by using a sample of composition: Co(II) enzyme 0.39 mM, K^{12}CN 3.2 mM. The spectra show structure on the $M_I = +\frac{1}{2}$ ^{59}Co hyperfine line in the g_1 region and were recorded at 9.18 GHz, with cavity temperature 77°K. A value $|A(^{13}\text{C})| = 1.1 \text{ mT}$ (11 G; $10.3 \times 10^{-4} \text{ cm}^{-1}$) was used in the simulations.

some model Co(II) complexes in which the disposition of CN^- ligands is known (Cockle, 1973). Solutions of cyanocobalt(II) cobalamin and pentacyanocobaltate(II) were prepared from the highly enriched K^{13}CN . The vitamin B_{12} derivative has a single CN^- group co-ordinated perpendicular to the Co(II)-corrin plane, giving a prominent ^{13}C hyperfine splitting (Table 1). Pentacyanocobaltate(II) has CN^- ligands in two distinct environments; four occupy an equatorial square plane, with the fifth in an axial position (Tsay *et al.*, 1971). The e.p.r. spectra of both $[\text{Co}(^{12}\text{CN})_5]^{3-}$ and $[\text{Co}(^{13}\text{CN})_5]^{3-}$ are shown in Fig. 4. Since the unpaired d electron occupies the $3d_{z^2}$ orbital, which has its main density along the 4-fold axis, the large observed ^{13}C -splitting is assigned to the axial CN^- ligand, whereas the equatorial group is responsible only for the line-broadening effect. The $M_I = -\frac{1}{2}$ ^{59}Co hyperfine line in the g_1 region was simulated as before with the aid of a computer program (27 individual spectra of $[\text{Co}(^{12}\text{CN})_5]^{3-}$ were needed), and close-fit values are given in Table 1. The results clearly favour an equatorial rather than an axial disposition of CN^- ligands in Co(II) carbonic anhydrase.

Fig. 4. E.p.r. spectra of pentacyanocobaltate(II) with $^{12}\text{CN}^-$ and $^{13}\text{CN}^-$ ligands

The samples were prepared under vacuum in methanol, and comprised: CoCl_2 (anhydrous) 1mM, K^{12}CN or K^{13}CN (87.43% ^{13}C) 6mM. Spectra were recorded at 9.10GHz, with cavity temperature 120°K.

Effect of excess of cyanide on Co(II) carbonic anhydrase

When the Co(II) enzyme was treated with an approx. 100-fold excess of cyanide, still under anaerobic conditions, grossly distorted, e.p.r. spectra were observed. In the extreme case, the original spectrum was completely replaced by a new signal virtually identical with that of $[\text{Co}(\text{CN})_5]^{3-}$ in Fig. 4. This change was accompanied by an appreciable fall in integrated intensity, consistent with the tendency of $[\text{Co}(\text{CN})_5]^{3-}$ in aqueous solution to dimerize on freezing (Maher, 1968). The CN^- ion is therefore capable of abstracting active-site cobalt with some ease, though individual enzyme variants showed marked differences in behaviour. Co(II) human isoenzyme B was slightly decomposed by only 10mole equiv. of KCN in Tris-sulphate buffer at pH9 (25°) whereas the bovine enzyme was hardly affected by 40mole equiv.

On brief exposure to air, the $[\text{Co}(\text{CN})_5]^{3-}$ spectrum rapidly disappeared, leaving a sharp but less intense residual signal at $g = 2.0$ (see Fig. 6b). This spectrum is typical of many Co(II) -oxygen adducts studied in recent years (see, e.g. Bayston *et al.*, 1969; Cockle *et al.*, 1970; Hoffman *et al.*, 1970), and undoubtedly represents an intermediate such as $[\text{Co}(\text{CN})_5\text{O}_2]^{3-}$ in the oxidation of $[\text{Co}(\text{CN})_5]^{3-}$ (Bayston *et al.*, 1963). The same signal was of course generated in enzyme-cyanide solutions prepared aerobically, but it should be emphasized that the process giving rise to it was quite irreversible. During an extended incubation, a

progressive loss of total e.p.r. intensity took place, as Co(II) was removed from the enzyme and subsequently oxidized to Co(III) .

Oxygenation of Co(II) carbonic anhydrase-cyanide solutions

Enzyme samples treated in air with insufficient cyanide to cause detectable metal abstraction still exhibited a narrow resonance at $g = 2.0$ overlying the principal spectrum. With the high-activity enzymes Co(II) human C and Co(II) bovine carbonic anhydrases, this signal accounted for only 2–3% of the total integrated intensity, but with Co(II) human isoenzyme B the proportion rose to 16%. These values were independent of the enzyme-cyanide ratio, even when the Co(II) ion was only partially saturated. Similarly, both components of the e.p.r. spectrum were removed simultaneously when a 1:4 Co(II) human isoenzyme B-KCN solution was titrated with Ethoxzolamide (see also Taylor & Coleman, 1971). Although the sharp signal at $g = 2.0$ is clearly characteristic of a $\text{Co(II)}-\text{O}_2$ adduct, close inspection reveals some appreciable differences from that of the inorganic artifact above, for example the peak-to-peak width (see Fig. 6 and Table 1).

The new oxygen adduct was thus concluded to be a complex of active-site cobalt, existing in equilibrium with the higher cyanide. This was further demonstrated by varying the atmosphere in contact with the solution before freezing. Rigorous deoxygenation was found to abolish completely the narrow signal, restoring the original spectrum to its full intensity, as indeed was observed when enzyme and cyanide were mixed anaerobically. On fresh exposure to air or O_2 , the signal reappeared at a corresponding intensity. Provided that sufficient KCN was present to saturate the enzyme and no appreciable HCN was lost, several cycles of oxygenation and deoxygenation could be carried out with little or no effect on the total integrated e.p.r. intensity. Fig. 5 illustrates some spectra obtained from the Co(II) human isoenzyme B system. The proportion of oxygen adduct at 0.1 MPa (1 atm) of O_2 was around 40%, but up to 70% formation was achieved under pressure. Assuming that the frozen solution reflected the composition existing in the liquid at 0°C, a dissociation constant K_d of about 3mM was estimated for the equilibrium:

where E/CN is the parent low-spin complex. For Co(II) bovine carbonic anhydrase, $K_d \approx 18\text{mM}$. The 9GHz spectrum of the adduct from Co(II) human isoenzyme B in Fig. 6(a) exhibits ^{59}Co hyperfine structure on both $g_{\parallel}$ and $g_{\perp}$ branches; the parameters given in Table 1 were deduced with the additional aid of 35GHz measurements.

Fig. 5. *E.p.r. spectra during oxygenation and deoxygenation of Co(II) human carbonic anhydrase B dicyanide*

Sample composition was Co(II) enzyme (0.40mM), KCN (2.8mM), in 0.04M-Tris-sulphate, pH9.0 at 25°C. Spectra were recorded at 9.20GHz with the same instrument gain; cavity temperature was 77°K. (a), Sample prepared in air; proportion of O₂ adduct 16%; (b), after oxygenation at 0.1MPa (1atm); proportion of O₂ adduct 40%; (c) after rigorous flushing with N₂.

Spectrophotometric and e.p.r. titration of Co(II) carbonic anhydrase with cyanide

Although Co(II) enzyme-cyanide solutions at room temperature were usually mauve in colour, like the high-spin 1:1 anion complexes, a distinct fading, which was quite reversible, was observed on cooling and freezing. In cases where the low-spin complex was fully formed, the frozen solution retained only a pale-yellow colour. This behaviour was unrelated to the bleaching that also accompanied Co(II)-abstraction by excess of CN⁻. In view of the conflicting spectrophotometric data of other workers (Grell & Bray, 1971; Taylor & Coleman, 1971), it seemed appropriate to investigate the optical properties of the higher cyanide, and to discover whether a genuine composition difference existed between liquid and frozen media.

E.p.r. measurements on three enzyme variants verified that formation of the low-spin complex was always much less complete at ambient temperature than in frozen solution. With Co(II) human isoenzyme B, a room-temperature signal was only detected when the KCN concentration was already high enough to cause some decomposition. At suitable enzyme:cyanide ratios, it was therefore possible to record an optical spectrum of the fully formed 1:1 cyanide complex at room temperature, and a fully intense e.p.r. spectrum of the higher cyanide at 77°K.

Fig. 6. *E.p.r. spectra of cobalt(II)-oxygen adducts*

Spectra were recorded at 9.20GHz, with arbitrary instrument gain; cavity temperature was 77°K. (a), O₂ adduct from Co(II) human carbonic anhydrase B dicyanide. This is the central portion of Fig. 5(b); the stick diagram at the top locates the hyperfine components of the underlying spectrum of the dicyanide complex. (b), O₂ adduct from [Co(CN)₅]³⁻. Co(II) human isoenzyme B (0.40mM) in 0.04M-Tris-sulphate, pH9.0 at 25°C, was treated in air with a 100-fold excess of KCN, then quickly frozen after agitation. The signal intensity corresponded to 56% of the original enzyme concn.; the residual enzyme-dicyanide complex was approx. 1%.

Fortunately, the high-activity enzymes were more suitable for room-temperature experiments, since they bind CN⁻ more strongly and are more resistant to Co(II) abstraction and O₂ binding.

Fig. 7 illustrates the results of a titration of Co(II) human isoenzyme C and KCN in which spectrophotometry and e.p.r. were used to follow disappearance of the monocyanide and appearance of the higher cyanide respectively. E.p.r. measurements were later carried out on the frozen samples. Over the composition range investigated, the enzyme was fully saturated with CN⁻ in the frozen state, with about 3% decomposition evident at the highest KCN concentrations. In the liquid phase, progressive formation of the higher cyanide was mirrored by loss of the d-d optical bands of the monocyanide and emergence of

Fig. 7. Combined spectrophotometric and e.p.r. titration of Co(II) human carbonic anhydrase C with KCN

Samples contained Co(II) enzyme (0.31 mM) and various amounts of KCN in 0.04M-Tris-sulphate, pH 9.0 at 27°C; no attempt was made to exclude air. Consecutive optical and e.p.r. spectra were recorded at about 27°C for each sample, which was then frozen for e.p.r. measurement at 120°K. ■, Concn. of monocyanoide complex at about 27°C from optical spectra. The molar extinction coefficient of the Co(II) d-d band at $\lambda_{\max}$, 589 nm was taken as 760 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹; this is the mean of values previously found by Lindskog and co-workers for the bovine enzyme complex (Lindskog, 1963; Lindskog & Ehrenberg, 1967). ▲, Concn. of dicyanoide complex at about 27°C from e.p.r. spectra; ●, sum of ■ and ▲; ○, concn. of dicyanoide complex in frozen solution from e.p.r. spectra at 120°K. Slight decomposition (approx. 3%) occurred at the highest cyanide concn.

Fig. 8. Spectrophotometric titration of Co(II) human carbonic anhydrase C with KCN

The experiment is described in the legend to Fig. 7. Spectra 1 to 6 denote mixtures of Co(II) enzyme (0.31 mM) with increasing amounts of KCN as follows: (1), 1.0 mM; (2), 1.3 mM; (3), 1.7 mM; (4), 2.6 mM; (5), 3.4 mM; (6), 5.2 mM.

Fig. 9. Stoichiometry of the low-spin cyanide complex of Co(II) human carbonic anhydrase C

The experiment is described in the legend to Fig. 7. Concentration data are plotted in the form:

$$\log R = n \log C - \log K'_d$$

where $R = [E(CN)_2]/[E(CN)]$ and $C = [CN^-] + [HCN]$. The straight line is the least-squares fit to the experimental points ●, and gives $n = 1.06$ and $K'_d = 1.55$ mM. Thus the low-spin complex is formed by addition of one CN⁻ ligand to the monocyanoide.

a new band around 390 nm (Fig. 8). Hence the bleaching effect observed on freezing was clearly associated with generation of the low-spin complex.

The concentration data also confirmed that the higher cyanide has the stoichiometry E(CN)₂, with apparent dissociation constant $K'_d = 1.55$ mM at pH 9 and 27°C (Fig. 9). Hence $K_d = 0.61$ mM for the equilibrium:

allowing for cyanide hydrolysis according to the relationship:

$$K_d = K'_d \left/ \left\{ \left(\frac{[H^+]}{K_a} \right) + 1 \right\} \right.$$

with $pK_a(\text{HCN}) = 9.17$ at 27°C (see Izatt *et al.*, 1962). A comparable result has been derived by Grell & Bray (1971) for the bovine enzyme from spectrophotometric work ($K'_d = 3.5$ mM at pH 8.7 and 12°C, giving $K_d = 0.42$ mM).

Discussion

Structure of the higher cyanide complex and oxygen adduct

Two independent pieces of evidence have now verified that the low-spin Co(II) enzyme contains two CN⁻ ligands. Since the protein contributes three

histidine ligands according to the crystal structure of native human carbonic anhydrase C (Liljas *et al.*, 1972), the cobalt is expected to be 5-co-ordinate, although the second CN^- ligand might conceivably displace one histidine ligand. The e.p.r. spectrum is very closely axial, with no detectable splitting in the $g_{\perp}$ region at 35 GHz, and the g values themselves ($g_{\parallel} \approx g_e$, $g_{\perp} > g_e$) indicate tetragonal rather than trigonal symmetry. Previous authors (Grell & Bray, 1971; Taylor & Coleman, 1971) have pointed out the strong resemblance between the enzyme spectra and those of Co(II) cobalamin (vitamin B_{12r}) and other low-spin chelates of formal C_{4v} or D_{4h} symmetry. The single unpaired electron in these systems is largely localized in the cobalt $3d_{z^2}$ orbital, so well-resolved nuclear hyperfine structure from axial nitrogen ligands is observed (e.g. Bayston *et al.*, 1970; Walker, 1970). In Co(II) carbonic anhydrase, this interaction is attributed to a protein group, since substitution of C^{15}N^- has no effect on the spectrum (Grell & Bray, 1971). The complex therefore contains no axial CN^- bound through nitrogen, and the present experiments with $^{13}\text{CN}^-$ rule out the possibility of an axial CN^- bonded through carbon. The spectra are compatible only with two equivalent CN^- groups, and the coupling constant is much smaller than in model complexes with axial CN^- ligands. On the other hand, the hyperfine interaction is very similar to that of the four equatorial CN^- groups in $[\text{Co}(\text{CN})_5]^{3-}$. By this analogy, it may be concluded that the low-spin Co(II) enzyme has two in-plane CN^- ligands bound via carbon directly to the metal ion. It is noteworthy that Feeney *et al.* (1973) have shown that the monocyanide complexes of native human B and bovine carbonic anhydrases also contain the CN^- ion linked to Zn(II) via carbon.

The data thus imply a square-pyramidal geometry for the Co(II) enzyme derivative. The apparent axial symmetry is unlikely to be maintained if one of the in-plane positions is occupied by an H_2O molecule from the active-site crevice, so there is no reason to suspect displacement of a histidine ligand. Since the metal ion is located at the base of the crevice (Liljas *et al.*, 1972), the complex probably has a *cis* rather than a *trans* configuration, as shown schematically in formula (I). Assignment of the three histidines is not possible at the moment, but some deviation from

(I)

ideal geometry is anticipated. Nevertheless, the structure would seem to be more regular than in vitamin B_{12r} , which has a rhombic e.p.r. spectrum at 35 GHz (Cockle *et al.*, 1972) and a site symmetry as low as C_2 (Pilbrow & Winfield, 1973). Here bond-angle distortions may be $5\text{--}10^\circ$ by inference from crystallographic data on vitamin B_{12} itself, cyanocobalt(III) cobalamin (Hodgkin *et al.*, 1962).

The ability of the dicyanide complex to bind oxygen reversibly is typical of low-spin Co(II) chelates such as vitamin B_{12r} (Bayston *et al.*, 1969). In the enzyme adduct, the O_2 molecule undoubtedly takes up the remaining octahedral site around the cobalt. Displacement of other groups is unlikely, since a strong tetragonal ligand field and a *trans*-axial heterocyclic base are important stabilizing features in such complexes. A space-filling model of the active-site crevice in native human carbonic anhydrase C shows that there is indeed sufficient room to accommodate three additional ligands about the metal ion. The e.p.r. spectrum of the O_2 adduct is strikingly different from that of the parent complex, owing to a smaller g -tensor anisotropy and cobalt hyperfine interaction, and a reversal in g -value inequality ($g_{\parallel} > g_{\perp}$). These and other considerations (Hoffman *et al.*, 1970) indicate that the unpaired spin now resides mainly on the O_2 ligand, with cobalt formally oxidized to Co(III). The fact that irreversible oxidation does not occur, as for example with $[\text{Co}(\text{CN})_5]^{3-}$, is due in part to the impossibility of obtaining a peroxo-bridged dicobalt species, which is usually the next intermediate (Bayston *et al.*, 1963).

Optical properties of the low-spin Co(II) enzyme

The difference in spin state and geometry between mono- and di-cyanide complexes of Co(II) carbonic anhydrase must inevitably lead to a difference in optical spectrum. It is clear that the prominent d-d bands in the region 500 to 600 nm are absent from the dicyanide. The origin of the new band near 390 nm remains uncertain; the titration could not be completed, as e.p.r. measurements at ambient temperature detected the onset of cobalt abstraction, although optical absorbance at 390 nm continued to rise as before. The authenticity of the absorption band near 400 nm reported by Grell & Bray (1971) must therefore be doubtful, particularly in view of the very large excess of cyanide in their sample.

The apparent temperature-dependence of formation of the dicyanide requires further investigation. It cannot be due to any variation in the degree of dissociation of HCN , since this is very small in the present work; ΔpK_a between 27°C and 0°C is almost the same for Tris buffer as for HCN (Good *et al.*, 1966; Izatt *et al.*, 1962). An abrupt co-ordination of CN^- during the freezing process cannot be excluded.

Differences between carbonic anhydrase variants

In view of the close uniformity in e.p.r. spectra, there can be little variation in metal-ion environment among different Co(II) carbonic anhydrases. Studies on vitamin B_{12} derivatives (Cockle *et al.*, 1969, 1972) suggest that changes in bond strength to the axial base ligand should be reflected by an inverse relationship between the hyperfine coupling constants, $A_{\parallel}(\text{Co})$ increasing and $A_{\parallel}(\text{N})$ decreasing as the bond weakens. For the human isoenzymes, there are small but significant variations of this sort (Table 1) which indicate a slightly weaker, perhaps longer, bond to the axial histidine in the B form. Other differences in behaviour between the high- and low-activity variants are related to this conclusion. Although both Co(II) human C and bovine enzymes accept a second cyanide ligand more readily than does Co(II) human isoenzyme B, they are also less susceptible to removal of cobalt by excess of cyanide. The latter point shows that Co(II) is bound more firmly in the high-activity enzymes, either because the mutual arrangement of ligand histidine residues is more favourable, or because there is greater flexibility in conformation. In contrast, Zn(II) binds equally well to all three apoenzymes, though anyway more strongly than Co(II) (Lindskog & Nyman, 1964). Other conformational adjustments are undoubtedly required to create the 5-co-ordinate dicyanide complex, and again these are accommodated more easily by the high-activity enzymes. A degree of conformational flexibility at the metal site, allowing changes in co-ordination number and geometry, may have an important bearing on catalytic activity.

The variations in ability to bind oxygen probably relate to other features of the active-site region. The O_2 ligand in the Co(II) human isoenzyme B complex may owe its comparative stabilization to a favourable hydrogen bond, since crystallographic work on human isoenzyme C (Liljas *et al.*, 1972) has shown that a number of polar side chains are available for interaction with anionic inhibitors. A potential hydrogen-bond donor in the B form is histidine-67, which is modified by an active-site-directed reagent, and is absent in the isoenzyme C sequence (Anderson *et al.*, 1972; Henderson *et al.*, 1973). A possible analogy may be drawn with the proposed role of the 'distal' histidine in oxyhaemoglobin and oxymyoglobin (see, e.g., Stynes *et al.*, 1973).

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