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On the Optical Spectrum of Cobalt(III) in the Active Sites of Enzymes

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The optical absorption spectra of cobalt(III) carbonic anhydrase and several of its derivatives was recorded. The rule of average environment was tested for the first spin-allowed d–d band in the spectra of cobalt(III) inorganic complexes and was found to be useful in predicting the positions of the absorption peaks in various complexes. It is shown how, using this rule, the optical spectra can assist in the identification of the ligands bound to cobalt(III) in the active sites of enzymes.

Introduction

Cobalt(III) has been used recently to probe the active sites of enzymes [1–8] mainly because of its substitution inert properties [see *e.g.* ref. 9]. The cobalt(III) in the active sites of bovine carbonic anhydrase B and bovine pancreatic carboxypeptidase A was found to be diamagnetic [8]. This result indicated that the cobalt(III) ion in these enzymes is octahedrally coordinated since both tetrahedral and five coordinate geometries such as square pyramidal or trigonal bipyramidal are expected to be paramagnetic. The optical absorption spectra of cobalt(III) ions bound to enzymes have been given in several instances [1, 3, 4, 7]. It was noted [7, 8] that they are in the range of analogous inorganic octahedral complexes. However no quantitative treatment was given. In the present work we would like to show that the first spin-allowed transition of cobalt(III) obeys the rule of average environment and to use this rule in order to obtain information about the ligands to which the cobalt(III) ion is coordinated in the enzymes.

Materials and Methods

Cobalt(II) bovine carbonic anhydrase was prepared as previously described [8], except for the apoenzyme which was prepared using pyridine-1,6-dicarboxylic acid according to the method of Hunt *et al.* [10]. Co(III) carbonic anhydrase was prepared by the gradual addition of H_2O_2 into a 0.3 m*M* solution of the cobalt(II) enzyme until about 80% oxidation was observed spectrophotometrically (about a five-fold excess). The enzyme was then dialyzed against 1 m*M* $ZnSO_4$ solution in order to convert the remaining unoxidized cobalt(II) enzyme to zinc enzyme. The reactions of cobalt(II) carbonic anhydrase with azide and cyanate ions were performed by the addition of 0.01–0.1 *M* NaN_3 or KOCN, and incubating at room temperature for a few hours. The products of these reactions were stable; their spectra (see Fig. 1) remained unchanged after dialysis for more than two days. They could be reduced by dithionite to give Co(II) carbonic anhydrase–dithionite complex as shown previously for the Co(III) carbonic anhydrase [8].

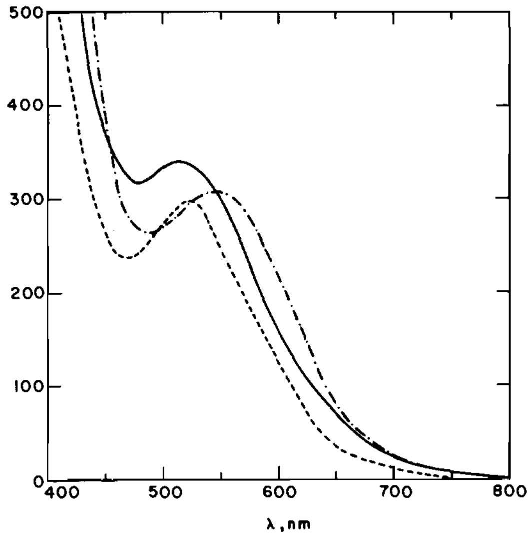

Fig. 1. Visible absorption spectra of cobalt(III) carbonic anhydrase (——) and its N_3^- (–.–.) and NCO^- (-----) derivatives.

Results and Discussion

The rule of average environment [11] has been used in the early development of ligand field theory by Tsuchida [12] in order to derive the spectrochemical series. It can be stated in the following form:

TABLE I. A Comparison between Observed and Predicted Transition Energies of Cobalt(III) Complexes.

	Ref.	ν_{max} (observed)	$\nu_{cal.}$
$Co(H_2O)_6^{3+}$	13	16.5	–
$Co(NH_3)_6^{3+}$	14, 15, 16	21.05	–
$Co(CN)_6^{3-}$	16	32.17	–
$Co(NH_3)_5(H_2O)^{3+}$	16	20.4	20.3
cis-$Co(NH_3)_4(H_2O)_2^{3+}$	17	20.0	19.5
trans-$Co(NH_3)_4(H_2O)_2^{3+}$	18	19.5	19.5
fac-$(Co(NH_3)_3(H_2O)_3^{3+}$	16	18.95	18.8
mer-$Co(NH_3)_3(H_2O)_3^{3+}$	16	18.3	18.8
$Co(NH_3)_5CN^{2+}$	15, 19	22.7	22.9
cis-$Co(NH_3)_4(CN)_2^+$	19	25.3	24.8
trans-$Co(NH_3)_4(CN)_2^+$	19	~24	24.8
fac-$Co(NH_3)_3(CN)_3$	16	26.35	26.7
mer-$Co(NH_3)_3(CN)_3$	16	26.7	26.7
$Co(NH_3)_5N_3^{2+}$	16, 20	19.3	19.5
cis-$Co(NH_3)_4(N_3)_2^+$	16, 20, 21	18.6	18.0
trans-$Co(NH_3)_4(N_3)_2^+$	16, 20, 21	17.6	18.0
mer-$Co(NH_3)_3(N_3)_3$	16	16.9	16.5
$Co(CN)_5N_3^{3-}$	21	26.3	28.7
$Co(NH_3)_5(CH_3CO_2)^{2+}$	16, 22, 23	19.9	19.9
cis-$Co(NH_3)_4(CH_3CO_2)_2^+$	22	19.1	18.7
trans-$Co(NH_3)_4(CH_3CO_2)_2^+$	22	18.1	18.7
mer-$Co(NH_3)_3(CH_3CO_2)_3$	24	16.8	17.5
$Co(NH_3)_5(C_2H_5CO_2)^{2+}$	22	19.9	19.9
trans-$Co(NH_3)_4(C_2H_5CO_2)_2^+$	22	18.1	18.7

TABLE II. Predicted Shifts and Splittings of the First Spin-allowed d–d Band of CoA_nB_{6-n} Complexes Relative to CoA_6 Complex According to Yamatera [13].

Complex Symmetry	CoA_5B C_{4v}		*trans*-CoA_4B_2 D_{4h}		*cis*-CoA_4B_2 C_{2v}		*mer*-CoA_3B_3 C_{2v}		*fac*-CoA_3B_3 C_{3v}	
	$(^1A_2)$	0	$(^1A_{2g})$	0	$(^1B_1)$	$\frac{1}{2}\delta$	$(^1B_2)$	$\frac{1}{4}\delta$		
	(^1E)	$\frac{1}{4}\delta$	(^1E_g)	$\frac{1}{2}\delta$	$(^1A_2, {}^1B_2)$	$\frac{1}{4}\delta$	$(^1A_2)$	$\frac{1}{2}\delta$	$(^1A_2, {}^1E)$	$\frac{\delta}{2}$
							$(^1B_1)$	$\frac{3}{4}\delta$		
Average[a]		$\frac{1}{6}\delta$		$\frac{2}{6}\delta$		$\frac{2}{6}\delta$		$\frac{3}{6}\delta$		$\frac{3}{6}\delta$

[a] Average shift relative to the transition energy of CoA_6 calculated using Eq. 2.

$$\nu = \frac{1}{6} \sum_{i=1}^{6} \nu_i^0 \qquad (1)$$

where ν is the absorption maximum of a cobalt(III) complex and ν_i^0 are the absorption maxima of the same band for the hexacoordinated complexes of the kind $Co(L_i)_6$. Although eqn. 1 is expressed for monodentate ligands it can be used for multidentate ligands as well, after appropriate changes in the subscripts.

In order to test the validity of eqn. 1 for the first spin-allowed d–d of cobalt(III) complexes we compare in Table I, experimental and calculated transition energies of various cobalt(III) complexes. In the calculations, the contribution of the ligands H_2O, NH_3 and CN^- were calculated from the transition energies of the hexakis complexes $Co(L_i)_6$, given at the top of the table.

The contributions of the azide ion and that of acetate and propionate ions were calculated as $\nu^0(N_3^-)$

= 12.0 kK and $\nu^0(\mathrm{Ac}^-) = 14.0$ kK respectively; values which most closely agreed with the experimental values are listed in Table I. As seen from the table, the agreement between experimental and calculated values is very good except for the case of $Co(CN)_5N_3^{2-}$ which will subsequently be discussed.

The theoretical justification for use of the rule of average environment for the first spin-allowed d–d transition of low spin d^6 complexes will now be presented. This transition is ${}^1T_{1g} \leftarrow {}^1A_{1g}$ in octahedral (O_h) complexes and is split at lower symmetries. Since this splitting is too small to be resolved in many complexes it will be assumed that the frequency of the absorption maximum is a weighted average of the various components. Using this assumption, eqn. 1 can be derived from the molecular orbital results of Yamatera [26] for the special case of only two different kinds of ligands. His results for all possible combinations of complexes of the structure CoA_nB_{6-n} are given in Table II. Similar results can be obtained from the more intuitive description of Ballhausen [27]. In this table δ is the difference in the effect on the orbital energies due to ligands A and B, where both σ and π bonding and antibonding contributions were taken into account. The value of δ can be approximated by the difference of these contributions in the complexes CoA_6 and CoB_6 [26]. The average values given in the table were calculated by taking the double degeneracy of the E states into account:

$$\bar{\nu} = \frac{1}{3}\,[\nu(A_2) + 2\nu(E)] \qquad (2)$$

Eqn. 1 can be also derived from the electrostatic crystal field theory of Wentworth and Piper [28] for the complexes of the type CoA_5B and trans CoA_4B_2 provided that eqn. 2 is assumed. They compared their theory with experimental results using the assumption that when the tetragonal splitting was not resolved the peak maximum corresponded to a simple average of the E and A_2 levels. In fact, recalculation of their data using eqn. 2 provides better consistency with experimental data.

The rule of average environment can be used only in cases where the peak occurs very close to the weighted average position of the transition. In those cases where splitting is large, and the peak of the absorption band occurs at one of the split components of the transition, eqn. 1 cannot be applied. For example in the case of $Co(CN)_5N_3^{3-}$, the splitting is expected to be exceptionally large since CN^- and N_3^- are listed in the two extreme ends of the spectrochemical series. It is likely in this case that the observed maximum is that of the ${}^1E \leftarrow {}^1A_1$ transition while the ${}^1A_2 \leftarrow {}^1A_1$ transition lies at a shorter wavelength and is masked by the next d–d or charge transfer transition. Thus the rule of average environment is more likely to apply to complexes where the difference in the ligand field strengths of the various ligands is small. This rule has been previously used [19, 29] for the prediction of the transition energies of the second spin allowed d–d band in cobalt(III) complexes where the splittings due to lower fields are generally smaller than those of the first band.

Table III gives a comparison between peak maxima found for Co(III) enzymes and those calculated for analogous model compounds. The observed positions of the absorption maxima had to be corrected for the residual uv absorption. This was done by assuming symmetrical Gaussian absorption bands in the λ scale. The results, with the estimated errors, are given in Table III. For the two complexes Co(III) CA–CN^- and Co(III) CPA, where not enough data is available

TABLE III. A Comparison of Transition Energies for Cobalt(III) Bound to Enzymes and Those Predicted for Model Compounds.

Enzyme	λ_{max}	ν_{max}	ν_{max} (corrected)	Model Compound	ν_{max} (Predicted)[a]
Co(III)CA	515[b]	19.4	18.5 ± 0.3	$Co(Im)_3(H_2O)_3^{3+}$	18.3
Co(III)CA–N_3^-	550[b]	18.2	17.9 ± 0.2	$Co(Im)_3(H_2O)_2N_3^{2+}$	17.6
Co(III)CA–CN^-	482[c]	20.7	–	$Co(Im)_3(H_2O)_2CN^{2+}$	20.9
Co(III)CA–NCO^-	525[b]	19.0	18.9 ± 0.1	$Co(Im)_3(H_2O)_2NCO^{2+}$	18.0
				$Co(Im)_3(H_2O)_2OCONH_2^{2+}$	17.8
Co(III)CPA	565[d]	17.7	–	$Co(Im)_2(H_2O)_3Ac^{2+}$	17.3
				$Co(Im)_2(H_2O)_2(Ac)Cl^+$	15.7

[a] The following ligand contributions ν_i^0 were used in the calculation: 16.5(H_2O), 12(N_3^-), 32.2(CN^-) and 14 (Ac = CH_3COO^- or $C_2H_5COO^-$) taken from Table I; 14.8(NCO^-), 13.6(NH_2COO^-) and 7.1(Cl^-) based on the absorption peaks of the corresponding $Co(NH_3)_5X^{n+}$ complexes at 20.0 kK (X = NCO^-) [30]; 19.8 kK (X = NH_2COO^-) [31] and 18.72 kK (X = Cl^-) [14]. The contribution of imidazole (Im) $\nu^0 = 20.1$ kK, is based on the absorption maximum of $Co(Im)_6^{3+}$ at 497 nm (G. Navon and R. Panigel, unpublished results). It gives for $Co(NH_3)_5Im^{3+}$ $\nu_{cal} = 20.9$ kK as compared with ν_{max} (observed) = 21.2 kK [25]. [b] Present work. [c] Ref. 8. [d] Ref. 7.

for the substraction of the residual uv absorption, the visible absorption peaks seem to be well separated so that the correction is expected to give a shift to lower energies of less than 0.2 kK.

X-ray data indicate that the zinc atom in the native carbonic anhydrase is bound to three histidine residues and presumably one water molecule [32]. However, since the Co(III) ion was found to be diamagnetic [8] we concluded that it is six-coordinated with water molecules probably occupying the remaining two sites. The good agreement of its spectrum with that calculated for three imidazoles and three water molecules seems to confirm this conclusion. The agreement is also good for the azide and the cyanide derivatives of cobalt(III) carbonic anhydrase. The position of the spectrum of the product of the reaction between Co(III) carbonic anhydrase with NCO^- agrees fairly well with the spectrum calculated for the model compound $Co(Im)_3(H_2O)_2NCO^{2+}$. However, since the product of the reaction of $Co(NH_3)_5(H_2O)^{3+}$ with NCO^- is the carbamato complex $Co(NH_3)_5OCONH_2^{2+}$ [31], it is possible that a carbamato complex might also be a product of the reaction between Co(III) carbonic anhydrase and NCO^-. Unfortunately it is impossible to distinguish between these two possibilities on the basis of the optical spectrum alone since the cyanato and carbamato complexes are expected to have almost the same transition energies (see Table III).

For carboxypeptidase A, X-ray crystallography indicates two histidines, one glutamic acid and one water molecule as ligands of the zinc atom [33]. Again since the cobalt(III) in carboxypeptidase A was also found to be diamagnetic [8] three water molecules were assumed to be in its coordination sphere. Acetate ion is a good model for the γ carboxylic acid of glutamic acid as can be inferred from the negligible change in spectra of Co(III) complexes when acetate is replaced by propionate (Table II). On the basis of its absorption peak, Van Wart and Vallee [7] have concluded that the cobalt(III) in Co(III)–carboxypeptidase A is bound, in addition to the known ligands of the zinc in the native enzyme (*i.e.*, two histidines, glutamic acid and water) to either another two water molecules or one water molecule and one chloride ion. According to our analysis the predicted absorption wavelength for $Co(Im)_2(CH_3CO_2)(H_2O)_2Cl^+$ is 637 nm (15.7 kK) which does not agree with the experimental value, while the agreement with $Co(Im)_2(CH_3CO_2)(H_2O)_3^{2+}$ seems good, as seen in Table III. It is interesting to note that the spectrum given by Kang and Storm [1] for their preparation of Co(III) carboxypeptidase A has a peak at about 505 nm (19.8 kK); corrected for the residual UV absorbance it peaks at abut 520 nm (19.2 kK). This position agrees better with four nitrogenic ligands and therefore the cobalt atom is not likely to be bound in the active site of the enzyme.

The analysis of the optical spectrum of nickel(II) in carboxypeptidase A [34] indicates an octahedral coordination with two nitrogen-donor and four oxygen-donor ligands, similar to our finding with cobalt(III) carboxypeptidase A.

The general agreement found between the spectra of the metal ions imbedded in the active sites of enzymes and those in inorganic complexes may be interpreted as an indication for a lack of distortion imposed by the proteins. On the other hand there is the question of how sensitive is the optical transition positions to such distortions. The root mean square deviation between experimental and predicted peak positions according to Table III is 0.5 kK (1.4 kcal). A similar figure applies also for the list of inorganic complexes given in Table I. Such a figure can serve as an order of magnitude estimate for the sensitivity of the optical spectrum to possible distortions in the active sites of enzymes. It may be applied for a quantitative assessment of the entatic state hypotheses that was suggested from the mechanism of catalysis by metalloenzymes [35].

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