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The Nature of the Ground States of Cobalt(II) and Nickel(II) Carboxypeptidase A

(metallocarboxypeptidase A/magnetic susceptibility/coordination geometry/spectra)

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ABSTRACT **The magnetic susceptibilities of cobalt(II) and nickel(II) derivatives of carboxypeptidase A (CPA) follow the Curie law over a wide temperature range. The observed magnetic moments of Co(II)CPA and Ni(II)CPA are 4.77 ± 0.15 and 2.53 ± 0.10 Bohr Magnetons, respectively. The magnetic and spectral properties of Ni(II)CPA are consistent only with an octahedral ground-state geometry, whereas Co(II)CPA has a probable five-coordinate structure. The results establish ordinary metal-ion ground states for two metallocarboxypeptidase A derivatives which exhibit full peptidase activity.**

We have been concerned lately with the role of the metal ion in the catalytic activity of carboxypeptidase A. The Zn(II) ligands contributed by the polypeptide chain at the active site have been identified through a combination of x-ray diffraction (1–3) and sequence (4, 5) studies as His 69, Glu 72, and His 196. The x-ray results have shown further that the probable coordination geometry of the Zn(II) site is distorted tetrahedral, with the fourth ligand presumably being a H_2O molecule in the resting enzyme. A distorted tetrahedral coordination site assignment also appears to be compatible with the electronic absorption spectrum of Co(II)-CPA (6), which is a fully active derivative (7, 8).

Our efforts have been channeled into experiments designed to elucidate the electronic ground and excited states of several metallocarboxypeptidase A derivatives, in the hope of establishing some connection between metal-ion structure and activity. We have been particularly interested in the question of whether the metal ion in its role in the multifunctional catalytic apparatus has been endowed with enhanced substrate-attacking properties by virtue of a special (9, 10) ground-state structure. The present paper deals with the ground states of cobalt(II)CPA and nickel(II)CPA, as characterized by measurements of variable-temperature magnetic susceptibility by our ultrasensitive, superconducting, quantum mechanical magnetometer.

MATERIALS AND METHODS

Preparation of Sample. Bovine carboxypeptidase A, prepared by the method of Cox (11), was obtained from Sigma Chemical Co. Cobalt(II) and nickel(II) carboxypeptidase A derivatives were prepared by the method of Coleman and Vallee (12). Extreme care was taken to eliminate metal contamination. Plastic laboratory ware was used throughout and all buffers were extracted with dithizone in CCl_4 (13). Metal solutions were prepared from minimum 99.9% pure metal powders obtained from Alfa Inorganic, Inc. Crystalline metallocarboxypeptidase A, spread on a clean slide and allowed to air dry in a scrupulously dust-free enclosure, was placed in a quartz tube for magnetic measurements. A sample of Co(II)CPA treated in this manner retains its red–purple color through the entire procedure. The principal visible absorption spectral band of a pellet of air-dried Co(II)CPA has a maximum at 558 nm, in excellent agreement with the reported (6) solution spectrum. Total metal analysis on each sample was performed by digestion of the protein in 1:1 HNO_3–H_2SO_4, dilution to a known volume, and determination of the metal concentration by means of a Varian Techtron Atomic Absorption Spectrometer.

Measurements of Magnetic Susceptibility. All measurements were made on a superconducting, quantum mechanical magnetometer of ultrahigh sensitivity. Full details of the operation of the system will be described elsewhere (Wang, R. H., Hoenig, H. E., Rossman, G. R., and Mercereau, J. E., to be published). In our experimental setup, a superconducting sensor inductively coupled to an rf tank circuit induces a current due to a change of magnetic flux in the sample, arising either from movement of the sample or change of sample temperature. This induced current is used to drive a feedback loop so that the compensation current, which depends linearly on the sample-related flux, can be accurately converted into magnetic flux.

Two types of magnetic susceptibility measurements were made. In the first type, the total magnetic susceptibility in an axial field was determined by moving the sample from one pickup coil into another wound in the opposite direction so as to compensate the effect of the sample holder, which extends through both primary coils of the dc superconducting flux transformer. In a second mode of operation, the relative change of susceptibility was measured as a function of temperature up to 120°K. This change is independent of the large diamagnetic background arising from the host protein. The relative susceptibility measurement on Ni(II)CPA was made on a 14-mg sample containing 16.8 μg of Ni^{2+} in a field of 150 G from 8 to 75°K. A total susceptibility measurement

Abbreviations: CPA, Carboxypeptidase A; B. M., Bohr Magnetons.

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TABLE 1. *Visible spectral band maxima and magnetic moments of cobalt(II) complexes*

Compound	Donor set	μ_{eff} (B.M.)	$\bar{\nu}_{max}$, cm^{-1} (ϵ_{molar})
Four coordinate			
Co(EDM)Cl_2[a]	N_2Cl_2	4.48	14,600 (320), 15,070 (311) 17,250 (232), 18,200 (70 sh)
Co(Me$_4$en)Cl_2[b]	N_2Cl_2	4.65	15,200 (447), 17,300 (214)
Co(MOBenNEt$_2$)-Cl_2[c]	N_2Cl_2	4.50	15,400 (410), 15,750 (425), 17,150 (425)
Co(PA)$_2$[d]	N_4	4.67	16,000 (58), 17,100 (129) 19,610 (600), 20,830 (629)
Co(MBrPM)$_2$[e]	N_4	4.53	13,010 (498), 14,090 (383)
Co(Barb)$_2$(Im)$_2$[f]	N_4	4.54	17,270 (about 350), 18,867 (150 sh)
Five coordinate			
Co(Et$_4$dien)Cl_2[g]	N_3Cl_2	4.71	18,300 (60), 19,200 (58)
[Co(Me$_6$tren)Br]-Br[h]	N_4Br_2	4.47	15,600–16,100 (128) 19,200–19,800 (112)
Co(Me$_5$dien)Cl_2[i]	N_3Cl_2	4.60	16,100 (106), 18,800 (112)
β-Co(Paphy)Cl_2[j]	N_3Cl_2	4.84	15,700, 20,000
Co(Terpy)Cl_2[j]	N_3Cl_2	5.03	15,850, 20,000
Co(Me$_4$daeo)Cl_2[k]	N_2OCl_2	4.70	16,200 (225), 18,500 (151)
Co(Py(Cy)$_2$)Cl_2[l]	N_3Cl_2	4.85	16,500 (52), 17,900 (52)
Cobalt enzymes			
Co(II)CPA		4.77[m]	17,480–18,020 (150)[n]
Co(II)CA[o]		4.23	15,670 (250), 16,180 (250) 18,180 (400), 19,600 (240)

[a] Lott, A. L. III & Rasmussen, P. G. (1970) *J. Inorg. Nucl. Chem.*, **132**, 101–107. EDM, ethylenedimorpholine.

[b] Sacconi, L., Bertini, I. & Mani, F. (1967) *Inorg. Chem.* **6**, 262 267. Me$_4$en, *N,N,N'N'*-tetramethylethylenediamine.

[c] Sacconi, L. & Bertini, I. (1968) *Inorg. Chem.* **7**, 1178–1183. MOBenNEt$_2$, *N,N,*-diethyl-*N'*(*o*-methoxybenzylidene)ethylenediamine.

[d] Holm, R. H., Chakravorty, A. & Theriot, L. (1966) *Inorg. Chem.* **5**, 625–635. PA, *N-t*-butyl-pyrolle-2-aldimine.

[e] Fergusson, J. E. & Ramsay, C. A. (1965) *J. Chem. Soc. London*, 5222–5225. MBrPM, 5'-bromo-3,4',5-trimethyldipyrromethene-3'-4-dicarboxylate.

[f] Wang, B. C. & Craven, B. M. (1971) *Chem. Commun.* 290–291; R. C. Rosenberg, unpublished results. Barb, 5,5-diethylbarbituric acid; Im, imidazole.

[g] Dori, Z. & Gray, H. B. (1968) *Inorg. Chem.* **7**, 889–892. (Et$_4$dien), 1,1,7,7-tetraethyldiethylenetriamine.

[h] Ciampolini, M. & Nardi, N. (1966) *Inorg. Chem.* **5**, 41–44. Me$_6$tren, tris-(2-dimethylaminoethyl)amine.

[i] Ciampolini, M. & Speroni, G. P. (1966) *Inorg. Chem.* **5**, 45–49. Me$_5$dien, bis(2-dimethylaminoethyl)methylamine.

[j] Lions, S. F., Dance, I. B. & Lewis, J. (1967) *J. Chem. Soc. A* 565–572. Paphy, pyridine-2-aldehyde-2-pyridylhydrazone; Terpy, terpyridine.

[k] Ciampolini, M. & Nardi, N. (1967) *Inorg. Chem.* **6**, 445–449. Me$_4$daeo, bis(2-dimethylaminoethyl)oxide.

[l] Sacconi, L., Morassi, R. & Midolfini, S. (1968) *J. Chem. Soc. A* 1510–1515. Py(Cy)$_2$, *N,N'*-dicyclohexyl-2,-6-diacetylpyridinebisimine.

[m] This work.

[n] Latt, S. A. & Vallee, B. L. (1971) *Biochemistry* **10**, 4263–4270.

[o] Lindskog, S. & Ehrenberg, A. (1967) *J. Mol. Biol.* **24**, 133–137. CA, Carbonic anhydrase.

was performed for 24.4 mg of Co(II)CPA containing 29.7 μg of Co^{2+} over the temperature range 30–130°K in fields between 31 and 47 G. In each case the data were corrected for the temperature-independent susceptibility contribution and the magnetic moment was calculated from the slope of the χ versus 1/T plot.

RESULTS

A plot of χ against 1/T for Co(II)CPA is shown in Fig. 1. The magnetic behavior of the metalloprotein is strictly in accordance with the Curie law over the temperature range investigated. The magnetic moment (μ_{eff}) obtained of 4.77 ± 0.15 Bohr Magnetons (B.M.) confirms that the ground state of Co(II)CPA arises from a high-spin (spin-quartet) electronic configuration.

The visible absorption spectral maxima and magnetic moments of Co(II)CPA, Co(II) carbonic anhydrase, and selected Co(II) model complexes are given in Table 1. Only four- and five-coordinate Co(II) complexes are included in the comparison, as both the positions and the intensities of the visible absorption bands of Co(II)CPA rule out an octahedral active-site geometry (6).

The temperature dependence of the magnetic susceptibility of Ni(II)CPA is shown in Fig. 2. The μ_{eff} of 2.53 ± 0.10 B.M. conclusively establishes an orbitally nondegenerate, spin-triplet ground state. The observed nonlinearity in the χ against 1/T curve below 10°K is attributable to a zero-field splitting of the spin-triplet ground state (14).

DISCUSSION

The observed magnetic moment of Ni(II)CPA suggests very strongly that the metal center be assigned an octahedral geometry. The electronic ground state is not compatible with either a four- or five-coordinate high-spin Ni(II) configuration, as all such complexes have μ_{eff} values that exceed 3.2 B.M., due to substantial orbital contributions (15). Furthermore, the visible absorption spectrum of Ni(II)CPA, which exhibits band maxima at 14,600 (ϵ 9) and 24,270 cm^{-1} (ϵ 27), is consistent only with an octahedral ground state (Rosenberg, R. C., Root, C. A., and Gray, H. B., unpublished results). Apparently, in the Ni(II) enzyme, there are three H_2O molecules included in a distorted octahedral coordination core of the type Ni(II)N_2O_4.

The magnetic properties of Co(II)CPA can be accommodated more or less satisfactorily in terms of a five-coordinate or a distorted tetrahedral metal center. The μ_{eff} values

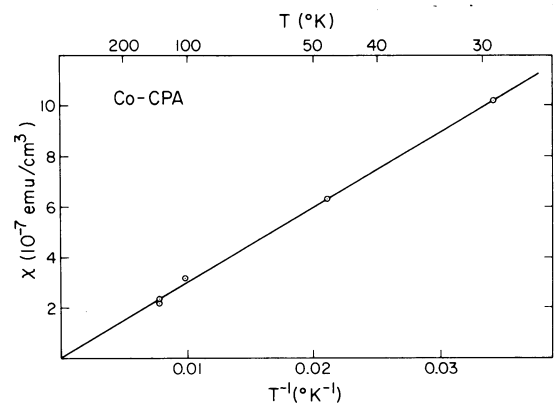

FIG. 1. Temperature dependence of the magnetic susceptibility (χ) of Co(II)CPA in the range 30–130°K.

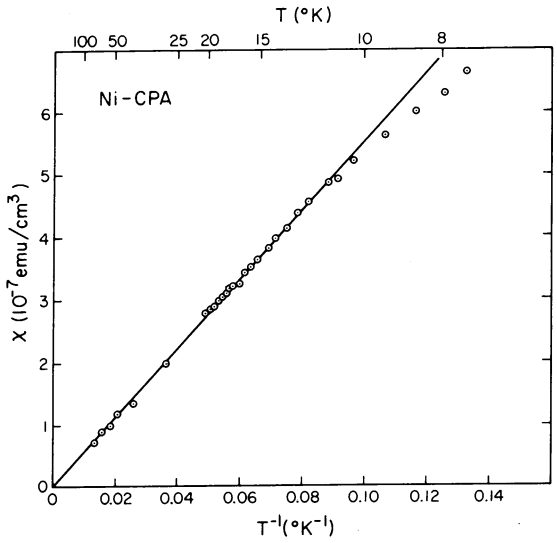

FIG. 2. Temperature dependence of the magnetic susceptibility (χ) of Ni(II)CPA in the range 8–75°K.

of the model complexes we have selected as being representative of Co(II) in distorted tetrahedral coordination are all less than 4.7 B.M., whereas all but one of the five-coordinate Co(II) complexes exhibit magnetic moments between 4.6 and 5.0 B.M. The μ_{eff} of 4.77 B.M. for Co(II)CPA thus suggests that five-coordinate Co(II) is the likely ground state, although distorted tetrahedral coordination cannot be completely eliminated as a possibility.

The intensities of the visible absorption bands attributable to electronic transitions from the ground state to the ligand field levels derived from the free-ion ^4P (Co^{2+}) excited state are often used as indicators of coordination structure for Co(II) complexes. The most intense visible bands in distorted tetrahedral Co(II) complexes generally have molar extinction coefficients that exceed 250, whereas the effective ϵ_{max} range for five-coordinate Co(II) is 80–225. The observed molar extinction coefficient of 150 for the principal visible band maximum of Co(II)CPA, thus, is in much better agreement with a five-coordinate ground state. In contrast, much higher visible absorption band intensities are observed for the enzyme Co(II) carbonic anhydrase, where a distorted tetrahedral active-site geometry, as suggested earlier (16), is probable. It is to be noted further that the large difference in the magnetic moments of Co(II)CPA and Co(II) carbonic anhydrase also suggests that the metal centers in the two enzymes are not analogously structured.

The magnetic and spectroscopic properties of Co(II)CPA and Ni(II)CPA show that the ligand environment of the enzyme is sufficiently flexible to allow changes in coordination number and geometry. The change from five or distorted tetrahedral to octahedral coordination does not drastically affect enzymatic reactivity, as the peptidase and esterase activities of Co(II)CPA and Ni(II)CPA toward several model substrates are of the same order of magnitude (7, 8), with a peptidase activity order Co(II)CPA > Ni(II)CPA = Zn(II)-CPA observed (8) in most cases. The fact that full peptidase activity can originate in M(II)CPA enzymes for which we have established very ordinary metal-ion ground states is not easily reconciled with the entatic state concept (9, 10) of catalytic activity.

Our finding of octahedral coordination for Ni(II)CPA and a probable five-coordinate structure for Co(II)CPA opens the possibility that at least one H_2O molecule remains bound to the metal center in the enzyme–substrate complex. Direct participation of coordinated H_2O in the active intermediate could be accommodated nicely in the recent mechanistic proposals of metallocarboxypeptidase A action (1, 3, 5, 17, 18), but has not been considered in any detail previously because of the generally accepted view of a four-coordinate metal center. Whether coordinated H_2O is of any importance at all in the catalytic activity of the enzyme is a question deserving of further study.

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