the nucleophilic and electrophilic roles considered for active site residues.

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# Polarized Single-Crystal Absorption Spectra of $Co^{2+}$-Carboxypeptidase A[1a]

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**Abstract:** Recent X-ray diffraction studies from this laboratory have shown that the coordination structure of $Co^{2+}$-reconstituted carboxypeptidase A is essentially identical with that of the native enzyme. The protein donor-ligand atoms are the $N_{\delta_1}$ atoms of His-69 and His-196 and the carboxylate oxygens of Glu-72 equidistantly positioned from the metal ion. A water molecule acts as the only nonprotein ligand in the free enzyme. On the basis of NMR proton relaxation enhancement studies of $Co^{2+}$-carboxypeptidase A, Bertini and co-workers have suggested that two exchangeable water molecules are bound within the inner coordination sphere of the $Co^{2+}$-enzyme in solution. Here, we compare the polarized single-crystal absorption spectrum of $Co^{2+}$-carboxypeptidase A to that of the enzyme in solution and show that the crystal spectrum is almost identical in band shape with the spectrum of $Co^{2+}$-carboxypeptidase A in solution. We conclude that the coordination environment of the metal is the same in solution and in the crystals elongated along the $a$ axis ($a$ = 51.60 Å, $b$ = 60.27Å, $\beta$ = 97.27°).

In the study of carboxypeptidase A, it is often assumed that the $Zn^{2+}$-bound water molecule is displaced when a substrate or inhibitor binds. However, a recent 1.54-Å X-ray structure of the complex between the native carboxypeptidase A and the ketonic substrate analogue (-)-3-(*p*-methylbenzoyl)-2-benzylpropionate shows that the ketone carbonyl does not coordinate to the zinc ion and the $Zn^{2+}$-bound water remains on the metal.[2] Also, electron paramagnetic studies of $Co^{2+}$-carboxypeptidase A show that a water molecule is retained on the metal in the acyl enzyme reaction intermediate when the ester (*p*-chlorocinnamoyl)-L-phenyllactate is the substrate.[3,4]

The coordination structure of the metal ion in $Co^{2+}$-carboxypeptidase A is essentially identical with that of the native $Zn^{2+}$-enzyme.[5,6] The donor-ligand atoms in the free enzyme are the $N_{\delta_1}$ atoms of His-69 and His-196, the carboxylate oxygens of Glu-72, and a single water molecule. On the basis of NMR proton relaxation enhancement studies of $Co^{2+}$-carboxypeptidase A, Bertini and co-workers[7] have suggested that two exchangeable water molecules are bound within the inner coordination sphere of the $Co^{2+}$-enzyme in solution. In this paper, we compare the polarized single-crystal absorption spectrum of $Co^{2+}$-carboxypeptidase A to that of the enzyme in solution. The crystal spectrum is almost identical in band shape with the spectrum of $Co^{2+}$-carboxypeptidase A in solution, indicating that the coordination environment of the metal remains unchanged.

## Experimental Procedures

$Co^{2+}$-carboxypeptidase A was prepared by treatment of the $\alpha$-form of the native enzyme (Sigma) in solution with *o*-phenanthroline[8] followed by dialysis against buffered solutions of $CoCl_2$ (Specpure, Johnson & Matthey, Inc.). Single crystals were obtained at pH 7.5 in 0.02 M sodium cacodylate in microdialysis tubings (Spectropor) by reducing the concentration of NaCl from 1.0 to 0.3 M. All glassware and quartz cuvettes were washed in acid, and dialysis tubing and plastic laboratory ware were prewashed with buffer containing *o*-phenanthroline. Twice-distilled deionized water was used throughout. All other materials were of ultrapure or analytical grade.

Polarized single-crystal absorption spectra were determined with a custom-built microspectrophotometer.[9] For data collection, crystals were suspended in 0.1 M NaCl solution buffered to pH 7.5 with 0.02 M cacodylate and mounted between quartz slides and coverslips. Identification of the (001) crystal plane was made by microscopic examination of crystals and measurement of interfacial angles based on a description of the $P2_1$ crystals of the $\alpha$ form of $Zn^{2+}$- and $Co^{2+}$-carboxypeptidase A provided by Dr. Karl Hardman. We have previously evaluated the effects of scattered light and crystal misalignment on polarization data. For the polarization properties of $Co^{2+}$-carboxypeptidase A in $P2_1$ crystals, it is readily shown that errors introduced by these factors are less than the experimental uncertainty associated with single-crystal optical density and dichroic ratio measurements.

The solution spectrum of $Co^{2+}$-carboxypeptidase A in the visible wavelength region was determined at room temperature with a Cary 210 spectrophotometer.

## Results and Discussion

Figure 1 illustrates the polarized single-crystal spectrum of $Co^{2+}$-carboxypeptidase A. The solution spectrum of the enzyme is also shown for comparison. In the upper part of the diagram is illustrated the change in polarization ratio (PR) with wavelength. The PR is defined as the ratio of single-crystal optical densities measured with incident light polarized in two orthogonal directions defined by crystal symmetry. Fluctuations in the PR with wavelength reveal transition moment directions and the symmetry of perturbing influences on degenerate states.[9,10] The absorption

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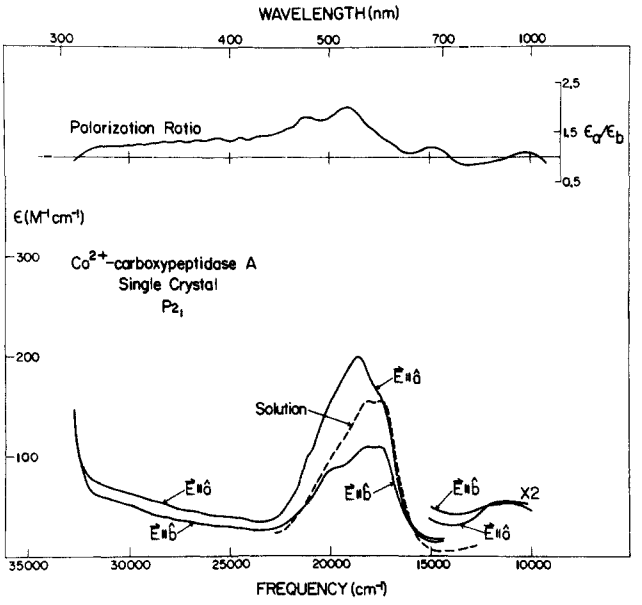


**Figure 1.** Polarized single-crystal absorption, polarization ratio, and solution spectra of $Co^{2+}$–carboxypeptidase A. The solution spectrum of the enzyme is shown in a dashed line. The polarization ratio (PR) is plotted in the upper part of the figure; the horizontal line corresponds to a PR value of 1.0. The crystal spectra shown here are constructed from data collected from a single crystal of approximate dimensions 3 × 2 × 2 mm. Identical spectra were also obtained for three other single crystals of smaller dimensions but with higher background light-scattering contributions. The crystal extinction coefficients are based on measurement of the *a*- and *b*-polarized absorbancies in the near-infrared region. The value of unity for the PR at the absorption maxima in this region requires that $\epsilon_a = \epsilon_b = \epsilon_{c^*}$. The crystal extinction coefficients at the maximum are then assigned according to the maxima value of $\epsilon_{soln}$ determined by Latt and Vallee[8] for the enzyme in solution and the oriented gas approximation $\epsilon_{soln} = {}^1/_3(\epsilon_a + \epsilon_b + \epsilon_{c^*})$.

**Table I.** Comparisons of Absorption Maxima ($cm^{-1}$) of the Single-Crystal and the Solution Spectra of $Co^{2+}$–Carboxypeptidase A

| spectm | $\nu_1$ | $\nu_2$ | $\nu_3$ | $\nu_4$ |
|---|---|---|---|---|
| $\epsilon_a$ | 11530 | 17620 | 18640 | 19790 |
| $\epsilon_b$ | 11020 | 17600 | 18200 | 19640 |
| $\epsilon_{soln}$ | 10638[a] | 17515 | 18135 | 19713 |

[a] From ref 8.

maxima for each spectrum are listed in Table I. The spectrum is characterized by diffuse absorption in the visible and near-infrared regions due to overlapping contributions of several transitions. For a spherically symmetric high-spin $Co^{2+}$ ion, as may be expected in a site of exact $T_d$ symmetry, the ${}^4A_2(F) \rightarrow {}^4T_1(P)$ transition is orbitally threefold degenerate. Lowering of the symmetry will lift the degeneracy of the three orbital components, and the observation that the average PR deviates markedly from a value of unity throughout the visible spectrum indicates that the absorption arises from a site of less than $T_d$ symmetry. The three components are readily identified by maxima and shoulders in the absorption profiles with corresponding local maxima or minima in the PR spectrum. The near-infrared region exhibits only a broad and essentially isotropically polarized band, and the three orbital components of this transition are closely overlapping and not resolved. It has been shown by Latt and Vallee[8] that lowering the temperature to ~77 K resolves this absorption into two components.

The habit of the $P2_1$ crystal of $Co^{2+}$–carboxypeptidase A prevents the collection of polarization and intensity data along the third orthogonal ($c^*$) direction. Thus, comparison of single-crystal and solution extinction coefficients according to the relation $\epsilon_{soln} = {}^1/_3(\epsilon_a + \epsilon_b + \epsilon_{c^*})$ cannot be made directly. However, for all of the intensely absorbing chromophoric prosthetic groups in crystalline proteins and enzymes studied to date,[9-12] it has been observed that there are no interchromophore electronic interactions and that the polarized crystal spectrum adheres to the "oriented gas" approximation.[13] We have recently demonstrated that this holds for active-site-specific $Co^{2+}$-reconstituted liver alcohol dehydrogenase.[12a] This circumstance is due to the large interchromophore separations in the crystal because of the inert protein matrix and, thus, applies similarly to the polarized crystal spectrum of $Co^{2+}$–carboxypeptidase A. On this basis, the polarized crystal spectrum of $Co^{2+}$–carboxypeptidase A is determined entirely only by local molecular properties of the active-site region containing the $Co^{2+}$ ion and not by the crystal environment. Several features of the polarized single-crystal spectrum suggest that the coordination environment of the $Co^{2+}$ ion in the crystalline enzyme has remained essentially unchanged from that in solution. The characteristic absorption maxima of the $Co^{2+}$–enzyme are retained in the crystal spectra in both the visible and the near-infrared regions (Table I). In fact, the *b* crystal spectrum and the solution spectrum are identical in band shape in the 16000–21000-$cm^{-1}$ range, and two maxima of equal extinction at 17600 and 18200 $cm^{-1}$ are seen in both the *b*-polarized crystal spectrum as in the solution spectrum. In the infrared region, maxima are seen near 11500 and 11000 $cm^{-1}$ in the *a*- and *b*-polarized spectra, similar to that of the enzyme in frozen solutions at 77 K described by Latt and Vallee.[8] Since these absorption maxima are sensitive to changes in coordination environment, agreement of the polarized single-crystal and solution spectra according to band shape and absorption maxima indicates that the metal ion environment is not altered by conversion of the enzyme from crystalline to solution states.

The proposal[7] of two water molecules coordinated to the active-site metal ion of free $Co^{2+}$–carboxypeptidase A is based on NMR data showing an enhancement in the water proton relaxation of the $Co^{2+}$–enzyme complexed with L-β-phenylpropionate. Since the inhibitor was assumed to have displaced a metal-linked water molecule in binding to the enzyme, it was suggested[7] that the free enzyme must contain more than one metal-bound water molecule. However, alternate interpretations of the NMR results are possible. For example, a distant (outer-sphere) water molecule, near the metal ion or hydrogen bonded to an inner-sphere ligand of the metal complex and in rapid exchange with the bulk solvent, can account for the NMR results.[14] Furthermore, in the X-ray structure of the complex between $Zn^{2+}$–carboxypeptidase A and the ketonic substrate analogue (−)-3-(*p*-methoxybenzoyl)-2-benzylpropionic acid, the 1.54-Å data reveal that the ketone carbonyl is hydrogen bonded to the guanidinium moiety of Arg-127 and does not coordinate to the zinc ion.[2] The $Zn^{2+}$-bound water molecule remains on the metal in this enzyme–inhibitor complex. Also, the zero-field splitting of $Co^{2+}$ ion in $Co^{2+}$–carboxypeptidase A is 8.3 $cm^{-1}$ and is compatible only with a tetraliganded metal ion.[15] This result is consistent with the X-ray structure provided Glu-72 is regarded as a single ligand. We conclude on the basis of these results that the coordination environment of the active-site metal ion of $Co^{2+}$–carboxypeptidase A remains unchanged in solution and in crystals. On the basis of high-resolution X-ray diffraction studies,[5] this requires only one (inner-sphere) water molecule coordinated to the active-site metal ion.

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