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Spectral Properties of Cobalt Carboxypeptidase A. Interaction of the Metal Atom with Anions†

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ABSTRACT: At pH >7 the absorption and magnetic circular dichroic spectra of cobalt carboxypeptidase A are insensitive to anions [Latt, S. A., & Vallee, B. L. (1971) *Biochemistry 10*, 4263–4270], but at pH <6 chloride and other anions perturb them in a manner specific for each anion. Lowering of the pH apparently facilitates the entry of an anion into the metal coordination sphere, suggesting that an acidic group normally stabilizes a metal-coordinated water molecule against displacement. The lack of sensitivity to anions at pHs between 7 and 9—when the enzyme is maximally active—and its evident abolition upon protonation of an active-site group are consistent with this interpretation. Selective modification of cobalt carboxypeptidase at Glu-270 using a carbodiimide affinity reagent generates sensitivity to anions at pH 7 very similar to that of the unmodified enzyme at pH ~5. This suggests that the group stabilizing the metal-coordinated water is the catalytically essential carboxylate of Glu-270. These and related results provide evidence for a mechanistically important interaction of Glu-270 with a metal-bound water molecule.

Replacement of the zinc atom of carboxypeptidase A with cobalt(II) provides a powerful spectral probe of its environment at the active site while maintaining enzymatic activity (Coleman & Vallee, 1960; Vallee, 1981). The accompanying electronic absorption, CD,[1] and MCD spectra (Latt & Vallee, 1971; Vallee & Holmquist, 1980) together with the X-ray structure analysis of the crystalline zinc enzyme (Lipscomb et al., 1968) have delineated the nature of the coordination of the active-site metal atom. Jointly, they suggest that the imidazolyl groups of His-69 and -196 and the carboxylate of Glu-72, together with the oxygen atom of either a water molecule or a hydroxide ion, constitute an irregular, tetrahedral-like environment of the metal atom.

The γ-carboxylate group of Glu-270 is also located close to the metal atom (Lipscomb et al., 1968). The results of chemical modifications implicate this group in the mechanism of catalysis (Riordan & Hayashida, 1970; Hass & Neurath, 1971; Pêtra & Neurath, 1971; Nau & Riordan, 1975), but the exact nature of its function remains to be determined. Chemical and kinetic studies with oligopeptide substrates and their ester analogues have failed to substantiate claims that Glu-270 forms a covalent anhydride link with the substrate in the course of catalysis (Breslow & Wernick, 1976; Galdes et al., 1983), although covalent intermediates are thought to occur in the hydrolysis of (chlorocinnamoyl)-L-phenyllactate (Makinen et al., 1976). Alternatively, it has been proposed that a residue such as Glu-270 could serve as a general base to deliver water as the nucleophile which attacks the substrate (Vallee et al., 1963; Vallee, 1964; Breslow & Wernick, 1976), but this, too, awaits verification.

The available evidence suggests that ligand exchange in the inner coordination sphere of the metal atom is an essential step in the hydrolysis of substrates catalyzed by zinc proteases, including carboxypeptidase A (Van Wart & Vallee, 1978). Consistent with this, previous studies of this enzyme employing the probe properties of cobalt substituted for zinc in the active center have shown that pseudosubstrates and specific inhibitors alter the symmetry of metal binding to the enzyme (Latt & Vallee, 1971; Vallee & Holmquist, 1980). However, simple anions such as halides and others of the spectrochemical series (Phillips & Williams, 1966) either fail to perturb its spectra

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[1] Abbreviations: CD, circular dichroism; MCD, magnetic circular dichroism; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; CMC, 1-cyclohexyl-3-[2-(*N*-methylmorpholino)ethyl]carbodiimide.

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at pH 7 or affect them only when added at relatively high concentrations (Latt & Vallee, 1971). The accessibility of the metal atom to large specific ligands together with its inaccessibility to small anions may indicate a metal binding site that is poised for ligand exchange or addition (Vallee & Williams, 1968a,b) yet also includes some elements of protein structure that protect the metal coordination sphere of carboxypeptidase A from disruption by anions. Spectral data presented here show that both acidification to pH ~5 and selective modification of Glu-270 with 1-cyclohexyl-3-[2-(*N*-methylmorpholino)ethyl]carbodiimide (CMC) (Riordan & Hayashida, 1970; Nau & Riordan, 1975) can remove the barrier to coordination of an anion with the cobalt atom.

Materials and Methods

Carboxypeptidase A, prepared according to Cox et al. (1964) and supplied by Sigma Chemical Co., was recrystallized and converted to cobalt(II) carboxypeptidase A by published procedures (Auld & Holmquist, 1974). The cobalt enzyme was modified with CMC *p*-toluenesulfonate (Aldrich) at pH 6.0 by the method described for the zinc enzyme (Riordan & Hayashida, 1970; Nau & Riordan, 1975), employing from 5 $\times 10^{-4}$ to 1 $\times 10^{-3}$ M enzyme. Inactivation of the enzyme was followed by monitoring the decrease of peptidase activity with furanacryloylglycyl-L-phenylalanine (Peterson et al., 1982) until the activity had fallen to <3% of its original value. Following modification, the enzyme was dialyzed against several changes of 0.05 M Hepes–1 M NaCl, pH 7.0, to remove excess reagent. Plastic labware was used whenever possible and precautions were taken to remove adventitious metal ions from all solutions (Thiers, 1957).

Absorption spectra of the cobalt enzyme were recorded with a Varian Cary 219 spectrophotometer. Absorptivities, ϵ, are given in units of M^{-1} cm^{-1}. To compensate for weakened metal binding by carboxypeptidase A at pH <6, 10 mM $CoCl_2$ or $Co(NO_3)_2$ was added to the samples whose spectra were recorded at pH 5.2 (Figures 1 and 2). These were corrected for the presence of the free cobalt(II) ions by placing a cobalt(II) solution of equal concentration in the reference cuvette.

CD and MCD spectra were recorded with a Cary 61 spectropolarimeter equipped with a superconducting magnet by using a cylindrical cell of 1-cm path length and, for MCD, a magnetic field of 4.0 T (40 kG). Values of the molar magnetic ellipticity $[\theta]_M$ in units of deg cm^2 $dmol^{-1}$ G^{-1} were calculated from the difference between the ellipticity in the presence and that in the absence of the magnetic field and normalized to 1 G. Enzyme concentrations were estimated from A_{278} by using a molar absorptivity for carboxypeptidase A of 6.42 $\times 10^4$ M^{-1} cm^{-1} or, for modified enzyme, on the basis of direct measurement of metal concentrations by conventional atomic absorption procedures (Perkin-Elmer Model 2280).

Results

Studies at Neutral pH. Earlier studies have described the visible absorption and MCD spectra of cobalt(II) carboxypeptidase A and their responses to pseudosubstrates, inhibitors, and alkaline pH (Coleman & Vallee, 1960; Latt & Vallee, 1971; Holmquist et al., 1975). The absorption spectrum is sensitive to subtle shifts in the symmetry of the metal coordination sphere, while the MCD, in contrast, provides an index of the overall metal coordination geometry (Vallee & Holmquist, 1980). The cobalt enzyme in 1 M NaCl, pH 7, exhibits an absorption spectrum with two equal maxima at 555 and 572 nm (155 M^{-1} cm^{-1}) and a shoulder near 500 nm (~100 M^{-1} cm^{-1}). The MCD spectrum displays a weak, positive band near 500 nm and a strong negative band with maximal ellipticity close to 572 nm. Replacing NaCl by NaBr or NaF or adding any one of several other anions does not alter these spectra (Latt & Vallee, 1971); they also remain virtually the same in the presence of up to 3 M NaCl, NaBr, or $NaNO_3$. Thus, they are not sensitive to anion perturbations.

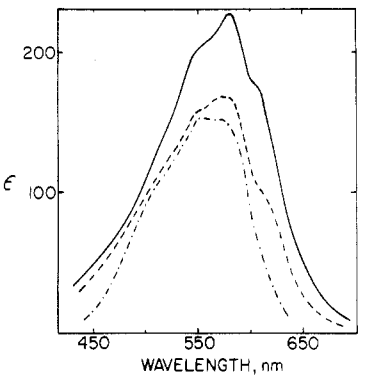


FIGURE 1: Visible absorption spectra of cobalt carboxypeptidase A (3.0×10^{-4} M) dissolved in 0.05 M Mes, pH 5.2, containing 3 M NaCl (—), 3 M NaBr (- - -), or 3 M $NaNO_3$ (-·-). Spectra are corrected for the presence of 0.01 M cobalt(II) which is added to prevent metal dissociation by the enzyme at this pH.

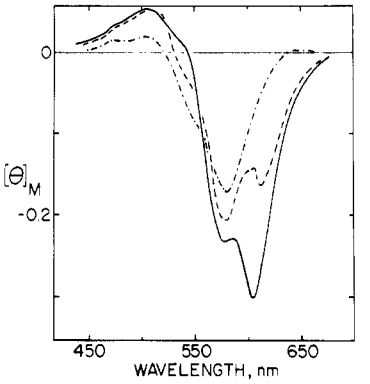


FIGURE 2: MCD spectra corresponding to the absorption spectra shown in Figure 1.

Spectra at Acid pH. Upon acidification to pH ~5, the absorption and MCD spectra of cobalt carboxypeptidase acquire anion sensitivity. In solutions containing 3 M NaCl,[2] the absorption spectrum changes slightly on decreasing the pH to 6 but changes markedly on decreasing it further. The instability of the enzyme below pH 5 precludes titration beyond this point. At pH 5.2, the absorption spectrum of the enzyme in 3 M NaCl has a maximum at 576 nm (~210 M^{-1} cm^{-1}) with shoulders at 548 and 604 nm (Figure 1). Replacing NaCl with 3 M NaBr (Figure 1) shifts the band of lowest energy to 614 nm and decreases the intensity of the spectral maximum. However, in the presence of 3 M $NaNO_3$ the absorption spectra of the enzyme at pH 5.2 and at pH 7.0 are identical (Figure 1).

The bands of the corresponding MCD spectra are well resolved (Figure 2). Again, the positions of the bands of lowest energy at 604 and 614 nm in the chloride and bromide spectra, respectively, are characteristic of each. As with the absorption spectra, the MCD spectra of the enzyme in 3 M $NaNO_3$ at pH 5.2 and at pH 7.0 are the same.

Addition of NaN_3, 80 mM, to the cobalt enzyme at pH 5.2 in the presence of 3 M NaCl, NaBr, or $NaNO_3$ further alters

[2] Solutions of the cobalt enzyme in 1 M NaCl rapidly become turbid at pH <6, making spectral analysis difficult. The problem is largely avoided by using higher concentrations of salt.

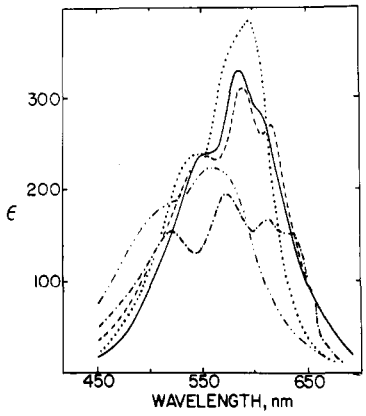


FIGURE 3: Absorption spectra of cobalt carboxypeptidase modified with CMC. Spectra of the modified enzyme (2.5×10^{-4} M) dissolved in 0.05 M Hepes, pH 7.0 (—·—), or 0.05 M Hepes, pH 7.0, also containing 1 M $NaNO_3$ (—··—), 1 M NaBr (---), 1 M NaCl (—), or 1 M NaCl/0.02 M NaN_3 (···).

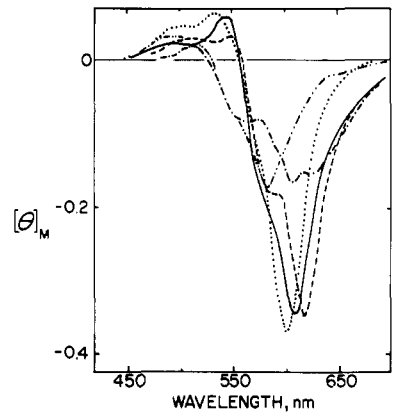


FIGURE 4: MCD spectra corresponding to the absorption spectra shown in Figure 3.

the spectra, converting each of these divergent spectra to a single form with increased maximal intensity, with $\lambda_{max} \sim 590$ nm for the absorption spectrum and 600 nm for the MCD spectrum (not shown).

CMC Modification. Selective modification of Glu-270 with the water-soluble carbodiimide, CMC, abolishes catalytic activity and renders the absorption spectrum of cobalt carboxypeptidase A sensitive to anions at pH 7.0. The CMC–enzyme exhibits anion effects at neutral pH analogous to those that could be observed with the unmodified enzyme only at acidic pH (Figures 3 and 4). Thus, the positions and relative intensities of absorption bands of the CMC–enzyme in 1 M NaCl, pH 7.0 (Figure 3), are very similar to those in the spectrum of the unmodified enzyme in 3 M NaCl, pH 5.2 (Figure 1). When NaBr replaces NaCl in both cases, the shoulder at 604 nm of each of the chloride spectra is shifted to 614 nm. The absorption spectra of the CMC–enzyme and pH 5 enzyme in $NaNO_3$ do not agree so well, since the spectrum of the CMC-modified enzyme is perturbed slightly while nitrate does not perturb that of the unmodified enzyme at pH 5.2.

On addition of sodium azide, 20 mM, the spectra of the CMC-modified cobalt enzyme in 1 M NaCl, NaBr, or $NaNO_3$, pH 7, all converge to a new common form, λ_{max} = 590 nm (Figure 3).

The MCD spectra of the CMC-modified enzyme are also sensitive to anions at pH 7 (Figure 4). The maximal ellipticity of the MCD spectrum at pH 7 coincides with the absorption band of lowest energy which is resolved for the CMC–enzyme

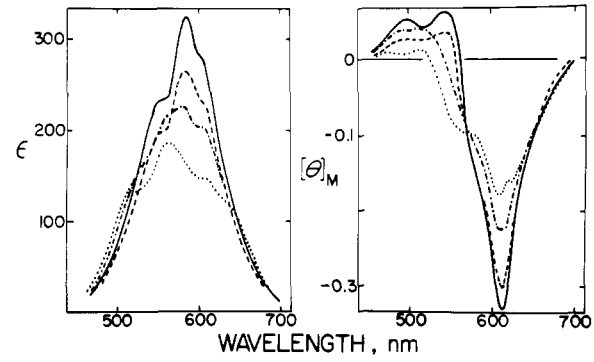


FIGURE 5: Effect of pH on the visible absorption (left) and MCD (right) spectra of CMC-modified cobalt carboxypeptidase A in the presence of 1 M NaCl. The spectra of the modified enzyme (3.0×10^{-4} M) in 1 M NaCl are at pH 7.0 (—), 8.6 (---), 9.5 (—·—), and 10.4 (···).

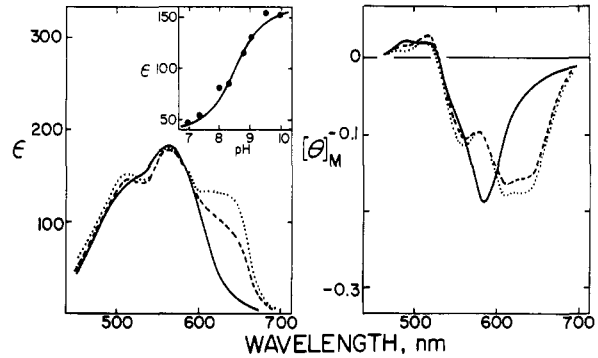


FIGURE 6: Effect of pH on the visible absorption (left) and MCD (right) spectra of CMC-modified cobalt carboxypeptidase A in the presence of 1 M $NaNO_3$. The spectra of the modified enzyme (3.0×10^{-4} M) in 1 M $NaNO_3$ are at pH 7.0 (—), 9.4 (---), and 9.9 (···). Inset: Spectral change at 620 nm as a function of pH for CMC-modified enzyme in 1 M $NaNO_3$.

in 1 M NaCl or NaBr, while that in 1 M $NaNO_3$ is virtually unaltered from that of the unmodified enzyme under the same conditions. Azide, 20 mM, also alters these MCD spectra to a common form with maximal ellipticity close to 600 nm.

The absorption and MCD spectra of the CMC-modified cobalt enzyme were obtained after dialysis against 20 mM Na Hepes buffer, pH 7.0, in the absence of additional anions. This "anion-free" enzyme exhibits peaks at 512, 569 (absorption maximum), 608 (MCD maximum), and 634 nm (Figures 3 and 4). Addition of various anions generates the expected specific spectra.

The spectra of the CMC-modified enzyme are sensitive to pH (Figures 5 and 6). Addition of NaOH to the enzyme in 1 M NaCl, NaBr, or $NaNO_3$, pH 7, progressively and reversibly alters the absorption and MCD spectra, which are characteristic of each anion, toward a common "alkaline form". Single-wavelength spectral pH titrations generate a pK_{app} = 8.6 in 1 M $NaNO_3$ (Figure 6, inset) but remain incomplete in 1 M NaCl even at pH 10.5. Higher pH rapidly denatures the enzyme. The limiting forms on which the absorption and MCD spectra of the anion-dependent CMC-enzyme converge at alkaline pH and those observed at pH 7 in the absence of anions (Figures 3 and 4) resemble each other closely.

Discussion

The spectra of certain cobalt(II)-substituted zinc metalloenzymes, notably carbonic anhydrase and *Escherichia coli* alkaline phosphatase, have long been known to be very responsive to anions. Thus, chloride, bromide, and a large number of anions of the spectrochemical series as well as

sulfonamides perturb the spectra of cobalt carbonic anhydrase [Lindskog, 1963; Bertini et al., 1978; for a review, see Pocker & Sarkanen (1978)], and phosphate and arsenate alter those of cobalt alkaline phosphatase (Simpson & Vallee, 1968; Holmquist et al., 1975). The spectral consequences of introducing such electron-donating ligands into the metal coordination sphere have led to inferences concerning the catalytic mechanisms of carbonic anhydrase. Among these, the proposal that hydroxide occupies the solvent position on the metal atom in the active enzyme to the exclusion of other anions has received particular attention (Pocker & Sarkanen, 1978).

In contrast, cobalt carboxypeptidase A and related cobalt(II)-substituted zinc enzymes fail to exhibit analogous spectral characteristics (Latt & Vallee, 1971; Breddam et al., 1979; Baldwin et al., 1980). The reasons for this difference have not been readily apparent and provided the incentive for the present work. Previous studies of unmodified cobalt carboxypeptidase A centered upon the pH range from 7 to 9 where its spectral response to anions is minimal (Latt & Vallee, 1971; Holmquist et al., 1975). The molecular basis of this behavior is not obvious from the crystal structure of carboxypeptidase A, since the metal binding site seems readily accessible and small anions might reasonably be expected to compete effectively with solvent for a site on the metal atom.

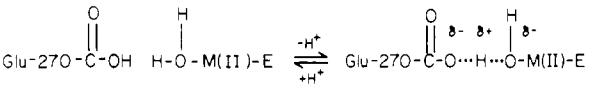
After adjustment of cobalt carboxypeptidase A to pH ~5 or after selective modification of Glu-270 with CMC, both of which markedly curtail catalytic activity (Auld & Vallee, 1970; Riordan & Hayashida, 1970), the spectrum of the enzyme becomes sensitive to anions (Figures 1–4). Moreover, for a particular anion, the spectral consequences of acidification and modification are similar. Thus, the similarity of the absorption and MCD spectra of the cobalt enzyme in 3 M NaCl, pH 5.2 (Figures 1 and 2), and of CMC-modified cobalt enzyme in 1 M NaCl, pH 7 (Figures 3 and 4), shows that ~50% of the cobalt enzyme at pH ~5 has been converted to a form much like that resulting from CMC modification.

Replacement of one anion with another induces significant and parallel spectral shifts in the pH 5 and CMC-modified forms of the cobalt enzyme, indicating direct coordination of the anion with the metal. The results of other studies support this interpretation. First, the spectra of CMC-modified cobalt carboxypeptidase in the presence of chloride, bromide, or azide resemble those of cobalt carboxypeptidase A inhibitor complexes in which the substrate-like structure of the inhibitor "site directs" an anionic group into the coordination sphere (Holmquist & Vallee, 1979). Second, the spectra of inner-sphere complexes of anions with cobalt carbonic anhydrase closely resemble those recorded here (Figure 3) (Lindskog, 1963). Further, in the zinc enzyme both decreasing the pH to less than 7 and modifying Glu-270 increase metal–chloride interaction (Stephens et al., 1974).

An anion could enter the coordination sphere either by displacing solvent or by filling a vacant site to expand the coordination number. Following modification with CMC, the MCD spectrum of cobalt carboxypeptidase remains essentially tetrahedral-like and resembles those of cobalt complex ions whose tetrahedral geometry has been established by X-ray diffraction analysis (Kaden et al., 1974). This indicates that displacement of solvent is the predominant mode of anion entry.

The metal-coordinated anion can, in turn, be displaced by yet another anion, as seen on addition of azide (Figures 3 and 4) or on increasing the pH from 7 to >10 (Figures 5 and 6). Significantly, increasing pH up to pH 9 with the unmodified cobalt enzyme induces a rather small change in the absorption spectrum (Latt & Vallee, 1971) while a more apparent change is seen with the CMC-cobalt enzyme, consistent with the view that modification of Glu-270 allows anions to coordinate to the metal atom. Spectra of the modified enzyme are largely unperturbed by chloride, bromide, and nitrate at pH >10. Thus, these anions do not coordinate at high pH, presumably as a result of competition with another negatively charged species supplied either by the solvent, i.e., hydroxide ion, or by the enzyme, e.g., the phenolate oxygen of a tyrosyl residue. Thus, there is a striking parallel between the pH dependence of the spectra of CMC–cobalt carboxypeptidase and that of cobalt carbonic anhydrase which is thought to be hydroxide-ion induced (Lindskog & Coleman, 1973). There is also unequivocal evidence that Tyr-248 can interact with the metal atom of carboxypeptidase A (Johansen & Vallee, 1975; Scheule et al., 1980; Bachovchin et al., 1982), an interaction which could generate spectral effects such as those reported above. However, at present there is no evidence that could distinguish between these two and yet other mechanisms that might be responsible for the alkaline spectral changes.

Scheme I



The transition between the anion-sensitive and -insensitive forms of unmodified cobalt carboxypeptidase occurs with a pK_{app} near pH 5. This value is consistent with that of the ionization which controls the acid limb of the pH–activity profile of the cobalt enzyme, $pK_a \sim 5.3$ (Auld & Vallee, 1970). This pK_{app} reflects the interconversion of the EH_2 form of the enzyme (which binds peptide substrates but does not hydrolyze them) and the catalytically competent EH form (Auld & Vallee, 1970). This ionization is dependent upon the particular metal atom present at the active site, linking it to a group very close to the metal (Auld & Vallee, 1970) and consistent with its also being the ionization that controls access of anions to the metal atom.

While the control of anion sensitivity in cobalt carboxypeptidase A could be linked to an ionization of the metal-coordinated water molecule $M(OH_2) \rightleftharpoons M(OH^-)$, this alone would not account for the apparent role of Glu-270 in controlling metal–anion interactions. A coupled function of Glu-270 and the metal-bound water would explain the data more satisfactorily. In such a scheme, the equilibrium $EH_2 \rightleftharpoons EH$ (Auld & Vallee, 1970) would be attributed to the structures shown in Scheme I.

Glu-270 is thought to assist in maintaining the enzyme in its catalytically competent EH form by stabilizing the metal-coordinated water molecule through hydrogen bonding. In so doing it would tend to block the access of monovalent anions to the coordination sphere, thereby resulting in a lack of spectral sensitivity to anions at neutral pH. It could, of course, do this merely by serving as an electrostatic barrier to approaching anions, but a direct, hydrogen-bonded interaction with the metal-bound water is more attractive in that it is consistent with a plausible catalytic mechanism (Vallee et al., 1983). Anion sensitivity can be conferred upon the cobalt absorption spectrum by protonating the EH form of the enzyme as shown in Scheme I. This would remove the negative charge, weaken the stabilizing hydrogen bond, and, thus, permit anion entry by displacement of the metal–bound water. Chemical modification of Glu-270 with CMC would also disrupt the metal-bound water–carboxylate interaction and allow anion entry at neutral pH. Such a scheme is in accord with the observation that carboxylate inhibitors of the enzyme,

e.g., 3-phenylpropionate and benzylsuccinate, coordinate to the metal most effectively at pH 6 or below (Auld et al., 1972; Byers & Wolfenden, 1973). Since metal binding of such inhibitors displaces solvent (Navon et al., 1970), the presence of unstabilized water at the coordination site for which they compete would favor their binding.

The present results clarify some aspects of structure and function of carboxypeptidase A. They show that the active-site structure, as depicted in Scheme I, protects the catalytically essential metal atom from complexation with potentially inhibitory anions. This structure would also be mechanistically significant, since it positions Glu-270 for a general base attack on the coordinated water to generate a nucleophilic metal hydroxide that could initiate peptide bond hydrolysis. The arrangement is consistent with the 4.5-Å distance between the carboxylate of Glu-270 and the metal (Lipscomb, 1973), with calculations of coordination geometry from perturbed angular correlation spectra and angular overlap theory (Bauer et al., 1979) and with ab initio molecular orbital calculations (Nakagawa et al., 1981). Evidence in favor of a hydrogen bond between the coordinated water molecule and Glu-270 also comes from a comparison of active center geometries of various zinc metalloenzymes (Argos et al., 1978) and from the pH dependence of ^{35}Cl NMR of zinc carboxypeptidase (Stephens et al., 1974).

Taken together these data suggest a general base catalyzed mechanism that involves the metal-coordinated water molecule. In addition, spectroscopic studies carried out with cobalt carboxypeptidase–substrate complexes at low temperatures reveal that transient changes in metal symmetry and coordination geometry are critical to the catalytic process as well (Vallee et al., 1983). Thus, the probe characteristics of the active center cobalt atom are helping to elucidate many details of the structure and dynamics of carboxypeptidase pertinent to its mechanism of action.

Registry No. Chloride, 16887-00-6; bromide, 24959-67-9; nitrate, 14797-55-8; carboxypeptidase A, 11075-17-5; Glu, 56-86-0.

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Kinetic and Electrophoretic Properties of Native and Recombined Isoenzymes of Human Liver Alcohol Dehydrogenase†

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ABSTRACT: Ten, electrophoretically distinct, molecular forms of alcohol dehydrogenase have been isolated from a single human liver by affinity and ion-exchange chromatography. The starch gel electrophoresis patterns after the dissociation–recombination of the forms are consistent with the hypothesis that they arise from the random combination of α, β_1, γ_1, and γ_2 subunits into six heterodimeric and four homodimeric isoenzymes. Large differences in kinetic properties are observed for the homodimeric isoenzymes, $\alpha\alpha$, $\beta_1\beta_1$, $\gamma_1\gamma_1$, and $\gamma_2\gamma_2$. At pH 7.5, the K_m value of $\beta_1\beta_1$ for ethanol is 0.049 mM and that of $\alpha\alpha$ is 4.2 mM. Forms $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ do not obey Michaelis–Menten kinetics at pH 7.5 but exhibit negative cooperativity with Hill coefficients of 0.54 and 0.55 and $[S]_{0.5}$ values of 1.0 and 0.63 mM, respectively. However, all isoenzymes display Michaelis–Menten kinetics for ethanol oxidation at pH 10.0 with K_m values ranging from 1.5 to 3.2 mM. The maximum specific activity of $\beta_1\beta_1$ is considerably lower than that of the other three homodimers at both pH 7.5 and 10.0. The K_m values of the four homodimers for NAD^+ at pH 7.5 range from 7.4 to 13 μM and those for NADH, from 6.4 to 33 μM. K_i values for NADH range from 0.19 to 1.6 μM. At pH 7.5, the kinetic properties of $\alpha\alpha$ and $\beta_1\beta_1$, prepared in vitro from dissociated and recombined $\alpha\beta_1$, are similar to those of the native homodimers. The forms $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$, prepared from dissociated and recombined $\alpha\gamma_1$ and $\beta_1\gamma_2$, respectively, exhibit negative cooperativity with Hill coefficients that are similar to those seen with the respective native homodimers.

Human liver alcohol dehydrogenase (EC 1.1.1.1), a dimeric enzyme, exhibits multiple, electrophoretically distinct, molecular forms (Smith et al., 1971; Schenker et al., 1971; Pietruszko et al., 1972; Harada et al., 1978; Bosron & Li, 1981) which can be divided into three classes according to their functional and structural properties (Vallee & Bazzone, 1983). Those isoenzymes formed by the random combination of subunits α, β, and γ, named according to the scheme of Smith et al. (1971), belong to class I. They migrate as a group toward the cathode on starch gel electrophoresis at pH 7.7–8.6 and are inhibited by micromolar concentrations of 4-methylpyrazole. The tryptic peptide elution profiles of these enzyme forms obtained with high-performance liquid chromatography are similar to one another, suggesting close structural homology within the group (Strydom & Vallee, 1982). The enzyme forms in class II, π-ADH, and class III, χ-ADH, differ substantially from class I isoenzymes with respect to electrophoretic mobility on starch gels, sensitivity to inhibition by 4-methylpyrazole, K_m values for ethanol, and tryptic peptide elution profiles (Vallee & Bazzone, 1982).

A system of nomenclature for the class I molecular forms was proposed some time ago, based on an isoenzyme model involving three structural genes.[1] It was postulated that a single subunit, α, is produced at the ADH_1 gene locus, two allomorphic subunits, β_1 and β_2, are produced at ADH_2, and two allomorphic subunits, γ_1 and γ_2, are produced at ADH_3 (Smith et al., 1971). These subunits would combine randomly to form hetero- and homodimers. Evidence in support of this hypothesis was obtained in studies of enzyme electrophoretic patterns following the dissociation–recombination of purified isoenzyme mixtures (Smith et al., 1973). For example, $\alpha\alpha$ and $\beta_1\beta_1$ were obtained from fetal liver and from lung, tissues that preferentially express these forms, and upon dissociation–recombination, a new heterodimer with intermediate electrophoretic mobility, $\alpha\beta_1$, was generated. However, because convenient and effective methods for the complete purification of alcohol dehydrogenase isoenzymes from human liver became available only recently, a systematic and more definitive study using the entire set of enzyme forms purified from the same liver had yet to be performed.

We report here the purification from a single liver of the ten class I alcohol dehydrogenase molecular forms postulated to arise from the combination of α, β_1, γ_1, and γ_2 subunits and the results of the dissociation–recombination of each of these enzyme forms. To substantiate that the subunits in the heterodimers are identical with those present in homodimers, we have isolated the homodimers produced in vitro from dissociated and recombined heterodimers and compared their kinetic properties with those of the native homodimers. Although prior studies of several partially purified isoenzymes of uncertain identity revealed only minor differences in substrate specificity or kinetic properties (Pietruszko et al., 1972), the

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[1] Gene loci and phenotypes are named according to Giblett (1976). Alcohol dehydrogenase loci are designated by a subscript, e.g., ADH_3. Polymorphic alleles are designated by a superscript above the gene locus, e.g., ADH_3^1. Phenotypes are identified by the subscript for the gene locus followed by the designation of the 2 alleles present on each copy of the gene. Hence, for the ADH_3 2-1 phenotype, both the ADH_3^2 and ADH_3^1 alleles are present, and the individual is heterozygous at the ADH_3 locus.

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