Spectral Properties of Cobalt Carboxypeptidase. The Effects of Substrates and Inhibitors*

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ABSTRACT: Cobalt carboxypeptidase is enzymatically active and exhibits visible and near-infrared spectra indicative of an unusual environment of the cobalt atom. There is a shoulder near 500 nm and maxima at 555 and 572 nm (150 cm^{-1} M^{-1}) and 940 and 1570 nm (20 cm^{-1} M^{-1}) at room temperature. The visible spectrum is resolved into four bands of undiminished intensity at 4.2°K. Three bands in the visible region are optically active, while a fourth, at higher wavelength, becomes optically active in a coaxial magnetic field. The spectra of cobalt carboxypeptidase in solution indicate that the geometry of the metal atom at the active site is irregular, consistent with distorted tetrahedral coordination of the cobalt based on data from cobalt complex ions. Perturbation by substrates, inhibitors, and hydrogen ions relate these spectra to catalytic properties of the enzyme in a manner consistent with the kinetic effects of such agents. Characteristic responses are found for different inhibitors, and when employed over a wide concentration range, individual inhibitors induce dissimilar spectral alterations, corresponding to complex inhibition modes. Slowly hydrolyzed substrates effect the largest spectral changes, possibly reflecting the role of the metal in peptide-bond hydrolysis. Glycyl-L-tyrosine inverts the sign of the circular dichroic spectrum which increases several times in magnitude, while the above coordination appears to be maintained. Spectral characteristics of the cobalt atom, an integral participant in the catalytic mechanism, reflect events of this process. The cobalt spectra of a number of cobalt-substituted metalloenzymes are considered in regard to the catalytic properties of these systems.

Chromophoric metal atoms at the active sites of metalloenzymes are well known both to participate in enzymatic catalysis and to be capable of serving as optical probes (Vallee and Williams, 1968a,b; Vallee and Wacker, 1970). In zinc metalloenzymes, other chromophoric and paramagnetic metals can often be substituted for zinc, which is diamagnetic and does not absorb radiation in the visible spectrum. Such metal exchange results in spectrally distinctive and catalytically active derivatives (Vallee and Latt, 1970; Vallee and Wacker, 1970). Cobalt carboxypeptidase was shown early to be enzymatically active (Vallee *et al.*, 1958) and to exhibit a characteristic absorption spectrum, unlike that of known cobalt complex ions (Coleman and Vallee, 1960). Similar substitutions were performed subsequently on carbonic anhydrase (Lindskog and Nyman, 1964), alkaline phosphatase (Simpson and Vallee, 1968), and yeast aldolase (Simpson *et al.*, 1971), among others.

The improved sensitivity and resolution of present instrumentation has allowed more detailed characterization of the absorption spectrum of cobalt carboxypeptidase than was possible previously, and the circular dichroic and magneto circular dichroic spectra have also been examined (Latt and Vallee, 1969; Latt, 1971). They indicate that the cobalt environment in the enzyme is asymmetric. Moreover, the spectra are responsive to factors such as hydrogen ion concentration, inhibitors, and substrates. Such spectra and the extensive information available on the primary sequence, function, and three-dimensional structure of carboxypeptidase have been related to the catalytic properties of the enzyme (Bradshaw *et al.*, 1969; Lipscomb *et al.*, 1969, 1970; Neurath *et al.*, 1970; Vallee *et al.*, 1970). The results provide a specific example of the general relationships between unusual spectra of chromophoric metals and the existence of an irregular active-site geometry in some metalloenzymes (Vallee and Williams, 1968a,b).

Materials and Methods

Carboxypeptidase A (Cox) was isolated by DEAE-cellulose chromatography from acetone powder of bovine pancreas (Princeton Laboratory Products Co.) by the method of Cox *et al.* (1964). The average proteolytic coefficient, *C* (Davies *et al.*, 1968), of the preparations employed was 1.8×10^2, pH 7.5, 25° corresponding to a turnover number, V_0/e = 8.5×10^3/min, when assayed using Z-Gly-L-Phe,[1] 0.02 M. The crystals were washed with deionized, distilled water before being dissolved in the appropriate buffers.

Apocarboxypeptidase was prepared by dialysis of 10^{-3} M carboxypeptidase A *vs.* at least four changes of a 50-fold volume excess of 2×10^{-3} M 1,10-phenanthroline in 1.0 M NaCl, 0.10 M Tris-Cl, pH 7.5, followed by dialysis *vs.* four changes of dithizone-extracted buffer.

Cobalt Carboxypeptidase. Apocarboxypeptidase was dialyzed twice *vs.* a 50-fold volume excess of 10^{-3} M cobaltous ion in 1 M NaCl, 0.005 M Tris-Cl, pH 7.5, 4° followed by dialysis *vs.* 2×10^{-4} M cobaltous ion, 2.5 mM NaCl, 0.3 mM

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[1] The abbreviations used for blocking groups will be: Z, benzyloxycarbonyl; Dns or dansyl, 5-dimethylaminonaphthalene-1-sulfonyl. The abbreviations used for buffers will be: ammediol, 2-amino-2-methyl-1,3-propanediol; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2′-ethanesulfonic acid.

Tris-Cl, pH 7.5, for crystallization. The cobalt carboxypeptidase thus prepared had an apparent proteolytic coefficient, C, of 4.0×10^2 at pH 7.5, 25° when assayed using Z-Gly-L-Phe, 0.02 M, and on the average contained 1 g-atom of cobalt and 0.01 g-atom of zinc per mole of enzyme as determined by atomic absorption analysis (Fuwa and Vallee, 1963; Fuwa *et al.*, 1964; R. Erbe and B. L. Vallee, in preparation).

For optical studies, cobalt carboxypeptidase crystals were washed with water, dissolved in 1 M NaCl, 0.005 M Tris-Cl, pH 7.5 to concentrations ranging from 20 to 100 mg per ml, and centrifuged at high speed for at least 30 min. The pH of the solutions so prepared was 7.1 ± 0.1.

Carboxypeptidase concentrations were measured by the absorbance at 278 nm, based on a molar absorptivity at 278 nm of $6.42 \times 10^4\ M^{-1}\ cm^{-1}$ (Simpson *et al.*, 1963).

Chemicals were of reagent grade or better and were used without further purification except as described. Thiobenzoate was recrystallized by acidification of the anion at 0°. β-Iodopropionate was extracted with CCl_4 prior to use. Recrystallization of β-phenylpropionate was achieved by cooling a solution saturated at 60° to 0°. D_2O (Bio-Rad Corp.) was freed of heavy metals by passage over a Chelex-100 (Bio-Rad Corp.) column.

All buffers used in experiments involving the measurement of metals, their removal from protein, or metal-substituted enzymes were freed of trace metal contaminants either by extraction with dithizone in carbon tetrachloride (Thiers, 1957) or by passage over a Chelex-100 column.

Cobalt used for reconstitution of apoenzyme was either spectrographically pure cobalt sulfate, or cobalt chloride, obtained by dissolving cobalt sponge (Johnson Matthey Co., Ltd.) with metal-free 6 N HCl followed by HCl removal by evaporation. Standards used in metal determination were the chlorides prepared by dissolving spectrographically pure metals (Johnson Matthey Co., Ltd.) in metal-free HCl.

Glassware and cuvets utilized in experiments in which trace metal contamination was of concern were cleaned by soaking in 1:1 nitric and sulfuric acids, followed by rinsing in water freed of trace metal contaminants by passage over a mixed-bed ion-exchange column. This water was purified further by distillation from an all-glass still before use in the preparation of buffers.

Z-Gly-L-Phe was purchased from Miles Laboratories. Dansyl peptide synthesis from Dns-Cl and Gly-L-Trp will be described in a future publication.

Peptidase Activity. Assays using Z-Gly-L-Phe at 0.02 M were performed at 25° by a modification of the ninhydrin method (Snoke and Neurath, 1949) in 0.02 M Veronal, 1 M NaCl, pH 7.5 (Coleman and Vallee, 1960), using a Technicon Auto-Analyzer (Auld and Vallee, 1970a). Assay mixtures containing cobalt carboxypeptidase were 10^{-4} M with respect to cobaltous ion to ensure at least 99% formation of the cobalt enzyme as judged by its stability constant (Coleman and Vallee, 1961).

Fluorescence assay of the substrate Dns-Gly-L-Trp at low ratios of enzyme to substrate was based on the increase in tryptophan fluorescence subsequent to scission of the bond between glycine and tryptophan (Latt *et al.*, 1970). For determination of peptidase activity as a function of pH, Hepes and ammediol buffers were used in contiguous pH ranges.

Absorbance measurements at discrete wavelengths were obtained with a Zeiss PMQ II spectrophotometer.

Absorption spectra were obtained usually with a Cary Model 14R spectrophotometer, equipped with a 0–0.1 absorbance slide-wire. Absorption spectra of cobalt carboxypeptidase were obtained *vs.* a zinc carboxypeptidase blank or corrected for this blank, with identical results. In addition, some spectra between 700 nm and 1800 nm were obtained with a Cary Model 17 spectrophotometer kindly made available by Dr. David E. Drum.

Because of the high absorptivity of H_2O above 1300 nm, measurements in this region were performed in solutions prepared from D_2O. Apocarboxypeptidase, prepared as described above, was dialyzed six times *vs.* a fivefold excess of the D_2O buffer. The solution was clarified by centrifugation and equal volumes were used to obtain a spectral base line. Following formation of cobalt carboxypeptidase in the sample cuvet by addition of a small volume of a solution of $CoSO_4$ in D_2O buffer, and addition of an equal volume of D_2O buffer to the reference cuvet, the near-infrared spectrum was obtained.

Low-temperature spectra were obtained with a cryogenic dewar flask (Andonian Associates, Inc., Waltham, Mass.). A copper–constantan thermocouple, calibrated *vs.* air, ice water, liquid nitrogen, and liquid helium was used for temperature measurement. Spectra were obtained at room temperature against a blank of zinc carboxypeptidase and partially corrected for scattering by setting absorption at 700 nm as the base-line zero. In experiments carried out between 4°K and 244°K, temperature was increased by passing current through a heating wire near the sample. Samples diluted 40–50% with glycerol were frozen in a 6 mM Lucite cuvet in liquid nitrogen prior to introduction into the dewar flask maintained at liquid helium temperature.

Circular dichroism measurements were performed with a Cary Model 60 recording spectropolarimeter equipped with a Model 6001 circular dichroism accessory. Some of the measurements were repeated with a Cary Model 61 recording spectropolarimeter with identical results. Repeated measurements on a standard 1-mg/ml aqueous solution of camphorsulfonic-*d*-10 acid gave a value of (+)0.31 ± 0.01° at 290 nm in a 1-cm cell. Ellipticity is expressed as molecular ellipticity; $[\theta]^{25} = (\theta/10)(M/lc)$ with units of (deg cm²)/dmole, where θ = observed ellipticity in degrees, M = molecular weight, 34,600 for carboxypeptidase A, l = path length in centimeters, and c = concentration in grams per milliliter. Molecular ellipticities are not corrected for the refractive index of the solvent.

Magneto circular dichroism measurements were performed with a Cary Model 61 recording spectropolarimeter using a Cary sample dewar flask and a superconducting magnet operated at fields up to 47×10^3 G.

Spectral titrations with inhibitors were performed by adding microliter volumes of concentrated inhibitor solutions to cobalt carboxypeptidase in a cuvet. Results were corrected for small enzyme dilutions due to inhibitor addition. The spectral dissociation constants of inhibitors were determined as the concentration of free inhibitor for 50% maximal spectral perturbation.

The spectral effects of substrate addition were measured by adding a 0.1 volume of substrate to a cuvet containing cobalt carboxypeptidase and recording the spectrum within 5–10 min. By rapidly repeating the scan an identical spectrum could be obtained under these conditions. pH spectral titrations utilized the Auld–French titration cell (Auld and French, 1970). pH was measured with a radiometer pH meter equipped with a general purpose glass electrode.

Results

Spectra of Cobalt Carboxypeptidase. The absorption spectrum of cobalt carboxypeptidase between 350 and 700 nm

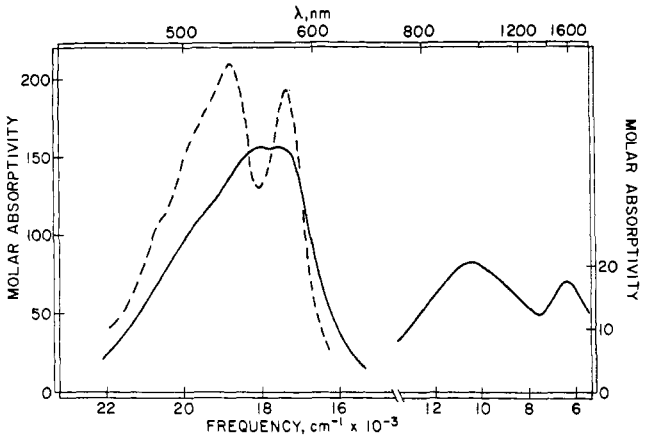

FIGURE 1: Absorption spectra of cobalt carboxypeptidase for (a) visible region. The enzyme, 1 mM, was dissolved in 1 M NaCl, 0.005 M Tris-Cl, pH 7.1, 20° (——). Another enzyme solution, about 3 mM, was diluted with glycerol to 45% v/v and cooled to 4.2°K (- - - -) for spectral measurements. (b) Near-infrared region. Apocarboxypeptidase, 1.5 mM, was dissolved in 1 M NaCl, 0.005 M [D]Tris-Cl, D_2O, pH 7.2. The sample cuvet contained 1.5 mM enzyme plus $Co(SO_4)$ in D_2O buffer to yield a final total cobalt concentration of 2.0 mM, and, hence, a 0.5 mM excess of free Co(II) ions; the reference cell contained 1.5 mM apoenzyme, brought to volume with buffer.

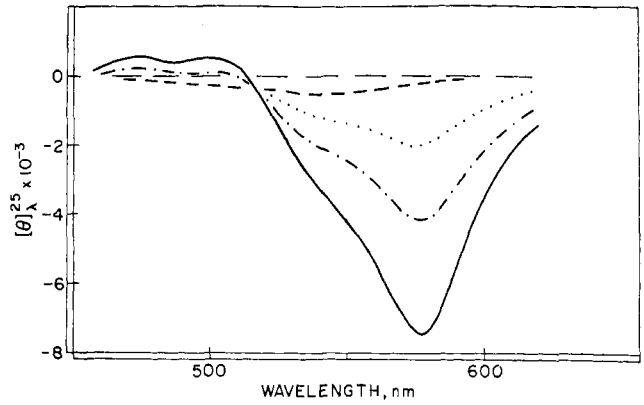

FIGURE 2: Circular and magneto circular dichroism of cobalt carboxypeptidase. The enzyme was dissolved in 1 M NaCl, 0.005 M Tris-Cl, pH 7.1. Circular dichroism (- - - -) and magneto circular dichroism were measured, the latter with coaxial fields of 13 (· · · ·), 27 (-·-·-·-·), and 47 (——) × 10^3 G.

obtained at neutral pH has a broad shoulder near 500 nm and maxima at 555 and 572 nm, both with molar absorptivity (ϵ) slightly greater than 150, and a total oscillator strength, f, of 3×10^{-3} (Figure 1). The apoenzyme does not exhibit any absorption over this spectral range, and the stoichiometry of its interaction is followed readily when $CoCl_2$ is added incrementally while observing the amplitude of the absorption spectrum generated. Thus, on addition of from 0 to 1 g-atom of Co^{2+} per mole of apocarboxypeptidase, absorption at 572 nm increases linearly, while an excess of Co^{2+} does not induce further changes. There are also two bands in the near-infrared region, an especially broad band, centered at 940 and a second at 1570 nm, each of $\epsilon \simeq 20$ (Figure 1). Exposure of the enzyme to 5 M guanidinium·HCl abolishes the absorption spectrum.

The resolution of the spectra at 4.2°K, the temperature of liquid helium, is markedly increased (Figure 1). A maximum at 532 nm and two shoulders at lower wavelength apparently correspond to the 555-nm peak and the broad shoulder found at room temperature. Compared with the room-temperature spectrum, the position of the maximum near 575 nm changes little and the overall intensity remains essentially unaltered. The spectral changes apparent at 4.2°K are reversed gradually when the temperature is increased to 224°K; beyond this the sample becomes opaque. Upon return to room temperature, the original spectrum is observed.

The cobalt carboxypeptidase spectrum in the visible region is optically active (Figure 2). A negative circular dichroic extremum at 538 nm corresponds to the position of the maximum in the spectrum at 4.2°, rather than to that of the spectrum at room temperature, but the maximum at 572 nm is optically inactive. The ultraviolet circular dichroic spectrum of cobalt carboxypeptidase above 210 nm is identical with that of zinc carboxypeptidase.

In addition to natural optical activity, ellipticity of a different origin can be induced in bands by a magnetic field (Figure 2). The magneto circular dichroic spectrum of cobalt carboxypeptidase, observed as the field-dependent portion of the ellipticity at 13 × 10^3, 27 × 10^3, and 47 × 10^3 G, increases linearly up to the limit of the applied field studied. The extrema of the magneto circular dichroic spectrum at room temperature correspond to all four absorption bands, at 478, 503, 537, and 577 nm, resolved in the absorption spectrum at liquid helium temperature. At zero field, the first three bands are optically active, while the fourth, at 577 nm, is optically inactive (Figure 2), but exhibits the greatest magneto circular dichroism. The amplitude of this band at 47 × 10^3 G is almost 15 times that of the extremum at 538 nm in the absence of an applied magnetic field (Figures 1 and 2).

These spectra were obtained in solutions containing chloride as the anion, though the possibility of specific anion effects was also examined. However, neither replacement of 1 M NaCl by NaBr or NaF, nor the addition of other anions ranging from SO_4^{2-} to NO_2^- displaces the cobalt carboxypeptidase spectrum.

Spectral Perturbants. The spectra of cobalt carboxypeptidase are sensitive to hydrogen ion concentration, inhibitors, activators, and substrates.

Hydrogen Ions. An increase of pH from 7 to 9 progressively induces shoulders near 510 and 625 nm in the absorption spectrum while the two maxima coalesce to become a single peak at 560 nm. In addition, in D_2O there are small bathochromic shifts of the near-infrared bands. The difference spectrum of cobalt carboxypeptidase at alkaline relative to that at neutral pH has a maximum at 625 nm, and pH titration at 625 nm results in a sigmoid curve with a point of inflection at about pH 8.8 (Figure 3). Peptidase activity of cobalt carboxypeptidase, with 5 × 10^{-6} M Dns-Gly-L-Trp serving as the substrate ($K_m \sim 10^{-4}$ M at pH 7.5) (Latt *et al.*, 1970), is shown for comparison. It is apparent that the pH dependence of the peptidase activity of the cobalt enzyme and the spectral changes at 625 nm are superimposable.

Inhibitors. The cobalt carboxypeptidase spectra are similarly responsive and sensitive to inhibitors. Titrations of cobalt carboxypeptidase with inhibitors of the enzyme change the absorption and circular dichroic spectra in characteristic manner (Table I). Addition of L-phenylalanine shifts the cobalt carboxypeptidase absorption bands to 510, 555, 574, 610, and 1000 nm. The amplitude of the circular dichroic spectrum decreases, resulting in small positive extrema at 510 and 580 nm and a negative extremum at 550 nm (Figure 4). Optical activity corresponding to the 610 nm absorption band is not observed. The amplitudes of the spectral changes follow

TABLE I: Inhibitor Effects on Cobalt Carboxypeptidase Absorption and Circular Dichroic Spectra.[a,b]

Inhibitor	Spectral K_I[mM] or I (mM)	Absorption (ϵ, cm^{-1} M^{-1})		Circular dichroism [θ] ((deg cm^2)/dmole)
β-Phenylpropionate	(0.5)	505(110), 555(160), 575(155), 610(80)	1040	490(−250), 543(−650)
	(2)	510(125), 555(150), 590(120)	1100	490(−450), 536(−900), 580(−800)
β-Iodopropionate	(0.5)	505(110), 558(178), 575(165)	1030	490(−200), 551(−650)
	(5)	520(140), 555(160), 590(130)	1090	495(−375), 555(−850), 590(−550)
Indole-3-acetate	(0.8)	510(120), 557(180), 573(180), 610(90)	1010	505(50), 555(−175)
	(25)	520(130), 565(160), 590(130)	1070	492(−300), 537(−500)
Phenylacetate	(2)	510(125), 555(155), 575(165), 605(95)	1030	495(−200), 539(−400)
	(20)	515(160), 575(180)	1090	510(100), 536(−275), 600(125)
Butyrate	(1)	505(105), 555(165), 570(170), 605(100)	1010	495(−150), 573(−450)
	(20)	515(130), 575(150)	1090	495(−150), 585(−700)
Acetate	(1000)	515(135), 575(155)	1050	510(100), 555(−300)
D-Phe	(0.5)	545(165), 580(205), 602(180)	1030	510(100), 575(100)
L-Phe	[3]	510(130), 555(200), 574(205), 610(140)	1000	510(200), 545(0), 580(100)
Benzoate	[20]	505(105), 560(165), 575(160), 610(90)	1000	485(−50), 505(25), 560(−400)
Thiobenzoate	[5]	505(115), 555(275), 574(300), 625(125)[c]	1080	475(−100), 510(0) 570(−700); 370(−2000)
L-β-Phenyllactate	[<0.5]	510(135), 560(195), 572(190)	970	495(−300), 527(−325) 585(100)
L-Lys-L-Tyr-NH_2	[7]	515(175), 555(175)	1030	505(1500), 550(1050)

[a] 1 M NaCl, 0.005 M Tris-Cl, pH 7.1, 25°. [b] The effects of inhibitors on the cobalt spectra were examined in H_2O. Hence, in the infrared only effects on the band at 940 nm could be examined. [c] High background has prevented resolution of absorption below 390 nm.

simple hyperbolic titration curves with a K_I of about 3 mM L-phenylalanine.

In contrast, butyrate, phenylacetate, β-phenylpropionate, β-iodopropionate, and indole-3-acetate each generate characteristic spectral shifts, the positions of which also depend on inhibitor concentration (Table I). Thus, addition of β-phenylpropionate up to about 0.7 mM, an approximately stoichiometric amount, slightly alters the cobalt carboxypeptidase absorption spectrum (Figure 5), increasing the maximum absorptivity. Further increase in the concentration of this inhibitor results in another pattern, near maximal at 3 mM β-phenylpropionate, with band positions at 510, 555, and 590

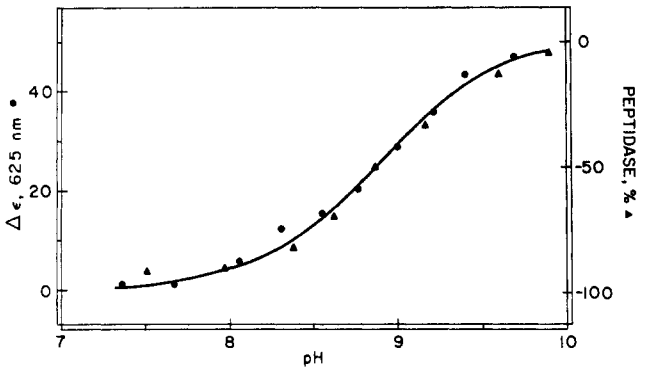

FIGURE 3: Dependence on pH of the absorptivity and peptidase activity of cobalt carboxypeptidase. Increase in molar absorptivity at 625 nm (●) and per cent of Dns-Gly-L-Trp hydrolysis (▲), relative rate to values at pH 7.1, are plotted *vs.* pH where spectral and kinetic measurements were performed as described in the text.

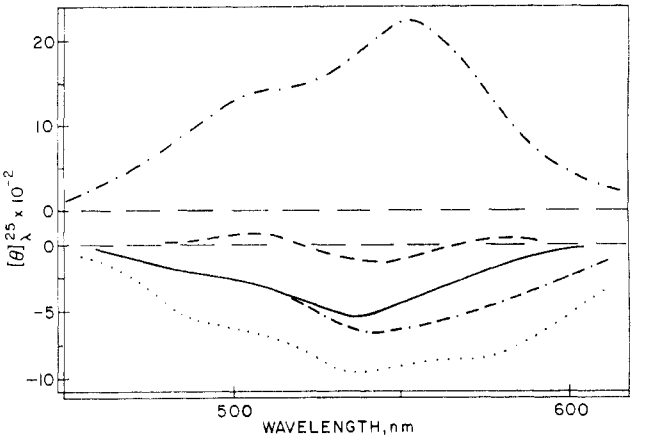

FIGURE 4: Effect of glycyl-L-tyrosine, β-phenylpropionate, and L-phenylalanine on the circular dichroic spectrum of cobalt carboxypeptidase. Conditions are as in the zero-field spectrum of Figure 2 for the control (——). Spectra of the enzyme were also obtained in the presence of glycyl-L-tyrosine, 10 mM (·····), L-phenylalanine, 9 mM (– – –), of β-phenylpropionate, 2 mM (–·–·–), or 9 mM (····).

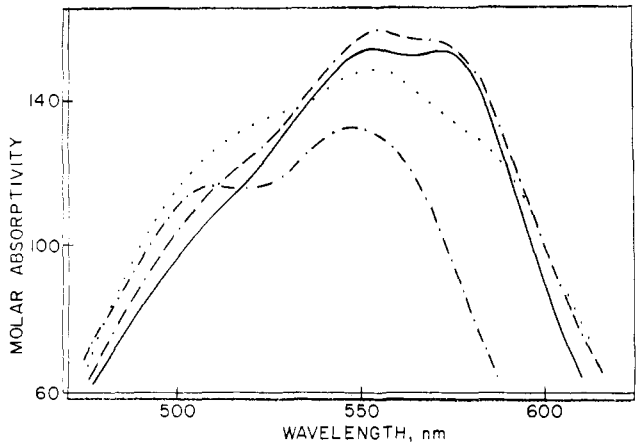

FIGURE 5: Effect of glycyl-L-tryrosine and β-phenylpropionate on the absorption spectrum of cobalt carboypeptidase. Spectra of the enzyme, 0.7 M, were obtained under the conditions of Figure 4 for the control (——). Spectra were also obtained in the presence of glycyl-L-tyrosine, 10 mM (- · - · - · - · -), or β-phenylpropionate, 0.7 mM (- - - · - - - · -), or 3.1 mM (· · · · ·).

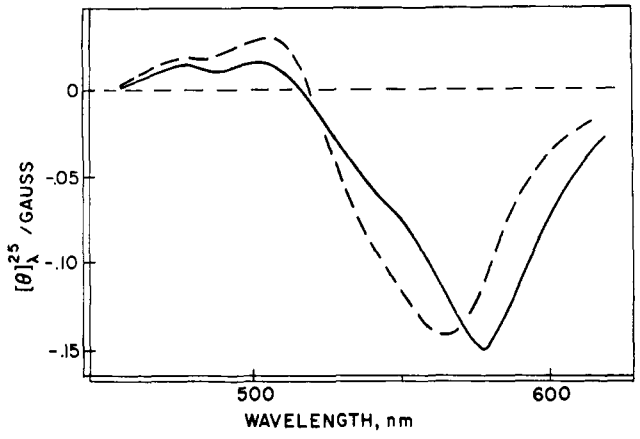

FIGURE 6: Effect of glycyl-L-tyrosine on the magneto circular dichroic spectrum of cobalt carboxypeptidase. Conditions for the enzyme (——) as in Figure 3; for the second spectrum, glycyl-L-tyrosine, 15 mM (- - - -), was added. The zero-field circular dichroism has been subtracted from the total circular dichroism at 47×10^3 G, and is then normalized per unit field.

nm and a concomitant decrease in maximal absorptivity. Incremental shifts are also observed in the near-infrared region (Table I).

Similarly, different concentrations of β-phenylpropionate cause at least two types of circular dichroic perturbations (Figure 4). At a stoichiometry of inhibitor to enzyme of approximately 1:1, the extremum shifts to 543 nm and increases in amplitude. Further increases in concentration of β-phenylpropionate generate a new shoulder at 580 nm, corresponding to the 575-nm absorption band at 4°K (Figure 1). Similarly, following incremental addition of β-phenylpropionate, magneto circular dichroism of cobalt carboxypeptidase exhibits two spectral perturbations.

Spectral titrations were also performed using thiobenzoate. The absorption intensity in both the visible and infrared regions approximately doubles, and a transition in the circular dichroic spectrum is observed near 370 nm. This band is not seen with the inhibitors bearing a free carboxyl group (Table I).

Unlike these agents, L-lysyl-L-tyrosinamide causes different spectral perturbations. The visible absorption exhibits a peak at 510 nm in addition to one at 555 nm (Table I). The infrared maximum at 940 shifts to 1020 nm. The circular dichroism undergoes a sign inversion and a moderate increase in amplitude, with bands at 505 and 550 nm. The amplitudes of both these spectral effects can be described by simple hyperbolic inhibitor titrations with an apparent K_I of about 7 mM.

Substrates. The effects of substrate addition on the spectra of cobalt carboxypeptidase are more extensive than those brought about by inhibitors, but were limited experimentally to substrates which are hydrolyzed slowly. On addition of glycyl-L-tyrosine to cobalt carboxypeptidase, the two maxima at 555 and 572 nm coalesce into a single broad one at 550 nm, while a shoulder near 505 nm remains (Figure 5). The 940-nm maximum splits into two bands peaking at about 850 and 1150 nm, and in D_2O the maximum at 1570 nm shifts to 1420 nm (not shown). Spectra below 1300 nm have also been measured following addition of glycyl-L-phenylalanine, with results identical with those on glycyl-L-tyrosine addition.

The difference spectrum of the cobalt carboxypeptidase·glycyl-L-phenylalanine complex *vs.* cobalt carboxypeptidase exhibits a prominent trough at 585 nm which allows the examination of the kinetics of interaction of glycyl-L-phenylalanine with cobalt. Stopped-flow absorption measurements at 585 nm indicate that the spectral changes due to interaction of cobalt carboxypeptidase with this substrate, 5×10^{-4} M, occur in a time too short to be resolved with the available instrumentation used, placing a lower limit of about 10^6 sec^{-1} M^{-1} on the second-order rate constant.

Glycyl-L-tyrosine induces circular dichroic bands at 505 and 550 nm (Figure 4) corresponding in wavelength to those apparent in the visible absorption spectrum. Identical changes follow addition of glycyl-L-phenylalanine or glycyl-L-isoleucine. Remarkably, the optical activity in the absorption band at 550 nm has the sign opposite to that of the control and the magnitude of this ellipticity, +2100°, is four times that of any band observed in the absence of substrate. Other slowly hydrolyzed substrates of carboxypeptidase, such as Z-sarcosyl-L-phenylalanine and sarcosyl-L-tryptophan, substituted at their N terminus, also perturb the spectrum of the cobalt enzyme. While the circular dichroism does not change sign for those examined, the band at 550 nm again becomes optically active, and with an amplitude in some cases even greater than that observed for the dipeptides with free N-terminal groups.

In striking contrast to the natural circular dichroism, the magneto circular dichroism of cobalt carboxypeptidase does not change markedly following the addition of glycyl-L-tyrosine (Figure 6). Compared with the control, the amplitude is altered little though band positions shift, to correspond with those observed in the absorption and circular dichroic spectra of this enzyme–substrate complex.

Discussion

The cobalt analogs of a considerable number of zinc metalloenzymes are catalytically active (Vallee and Latt, 1970). Both cobalt(II) and zinc(II) are known to accept unusual coordination geometries (Vallee and Williams, 1968a), but cobalt(II) is paramagnetic and its derivatives absorb radiation in the visible spectrum, rendering this metal a good environmental probe in active centers of such metalloenzymes.

While spectral characteristics may reflect the number of cobalt ligands, as well as their geometry, bonding, and vicinal

features, their detailed evaluation for the purpose of specific assignment has proved difficult even for cobalt(II) complex ions (Cotton *et al.*, 1963; Carlin, 1965).

In an enzyme, where cobalt could be bound by a number of different ligands comprising a potentially highly anisotropic environment, interpretations are even more conjectural. However, comparison of spectral data of cobalt carboxypeptidase and of other cobalt enzymes with those of cobalt complex ions might give first indications of the features of their metal–protein interaction both to reveal similarities and to specify characteristic differences.

The intensity of the cobalt carboxypeptidase spectra, undiminished at liquid helium temperature, 4.2°K (Figure 1), and the optical activity (Figure 2) are indicative of low symmetry in the environment of the metal (Ballhausen, 1962). The band intensities are greater than those normally associated with octahedral (Carlin, 1965), close to those reported for certain trigonal bipyramidal (Dori and Gray, 1968) and are within the range observed for tetrahedral (Cotton and Soderberg, 1962) cobalt(II) complexes. The band energies are high, consistent with strong metal–ligand interactions. The overall band pattern of the cobalt carboxypeptidase spectra, especially as seen in magneto circular dichroism (Figures 2, 6) is similar to that of tetrahedral cobalt(II) complexes (Denning and Spencer, 1969) though the energy span of the enzyme visible spectrum is a few thousand cm^{-1} greater, conceivably due to asymmetry of coordination. In basically tetrahedral cobalt complexes, the near-infrared spectrum can be especially diagnostic of the geometry of coordination (Goodgame and Goodgame, 1965). The two-banded pattern observed here (Figure 1), split by about 4000 cm^{-1}, is consistent with distortion of coordination, possibly axial in nature.

The spectra of cobalt carboxypeptidase resemble those of pseudotetrahedral cobalt(II) complexes with oxygen and nitrogen ligands (Sacconi *et al.*, 1962; O'Conner *et al.*, 1968; Morris and Martin, 1970) or imidazolyl nitrogen ligands (Eilbeck *et al.*, 1967; Dobry-Duclaux and May, 1968; Taylor and Underhill, 1969). Differences in intensity and band energies among the spectra of these complexes and that of cobalt carboxypeptidase may reflect differences in the covalent character of metal–ligand bonding, ligand asymmetry, or influences of solvent (Ballhausen and Liehr, 1958, 1960; Cotton and Soderberg, 1962; Owen and Thornley, 1966).

Based on data from cobalt complex ions (Latt and Vallee, 1969; Vallee and Latt, 1970) the cobalt spectra of the enzyme in solution are consistent with irregular tetrahedral geometry as indicated by the visible and infrared absorption spectra and the magneto circular dichroism in agreement with the nature and number of protein ligands, and the coordination of one water molecule to the zinc atom (Lipscomb *et al.*, 1969; Neurath and Bradshaw, 1970).[2] The addition of inhibitors and substrates does not appear to change this basic geometry of coordination again consistent with X-ray studies of crystalline carboxypeptidase (Lipscomb *et al.*, 1969).

The spectra of cobalt carboxypeptidase are similar to those of cobalt yeast aldolase (Simpson *et al.*, 1971) and the low pH form of cobalt carbonic anhydrase (Lindskog and Nyman, 1964), possibly reflecting common features of their active-site environments. In contrast, the spectrum of cobalt alkaline phosphatase (Simpson and Vallee, 1968), and that of the alkaline form of cobalt carbonic anhydrase (Lindskog and Nyman, 1964) resemble one another, while differing strikingly from those of the first group. Remarkably, solely protonation or the addition of simple anions change the alkaline spectrum of carbonic anhydrase into that of the low pH form, which resembles that of cobalt carboxypeptidase. It will be of great interest to learn whether or not the spectral changes are characteristic of ligands and specific metal coordination geometries characteristic of the above enzymes and their potential solvent-dependent isomers.

Importantly, agents affecting activity perturb the spectra of cobalt carboxypeptidase in a manner which seemingly depends on the catalytic role of the particular agent. The effects of hydrogen ion and inhibitors on carboxypeptidase activity have been studied (Auld and Vallee, 1970a,b, and references therein). Both simple and mixed inhibition modes exist, and the latter can be resolved by stopped-flow assay (Latt *et al.*, 1970). At any given concentration of perturbant, the effects of hydrogen ions (Figure 3) and inhibitors (Table I) on spectral alterations and activities of the enzyme correlate closely. Competitive inhibitors such as L-phenylalanine perturb the cobalt spectrum as a simple hyperbolic function of inhibitor concentration. The shifts in energy of the absorption bands are small in response to the competitive inhibitors L-phenylalanine and D-phenylalanine (Table I) and to pH change. However, inhibitors such as β-phenylpropionate,[3] whose kinetic effects are complex and concentration dependent, induce multiple spectral changes which are also concentration dependent, apparently corresponding to different modes of inhibitor binding. Indeed, these kinetics and spectral changes correlate (Latt, 1971).

At low concentrations, this agent acts noncompetitively (Auld and Vallee, 1970a), and brings about small spectral changes. At higher concentrations, different and more extensive shifts are induced (Figures 4, 5). Moreover, under these conditions the absorption band at 590 nm becomes optically active. These second effects are manifest at β-phenylpropionate concentrations comparable to those at which the agent is thought to displace water bound to manganese of this carboxypeptidase derivative, as judged by nuclear magnetic resonance data (Shulman *et al.*, 1966). Further, at this concentration inhibition contains a competitive component (S. A. Latt, D. S. Auld, and B. L. Vallee, in preparation), consistent with direct interaction of this inhibitor with the active site. Multiple enzyme–inhibitor interactions are consistent with the previous observation that β-phenylpropionate could protect two active site tyrosyl residues of carboxypeptidase from acetylation (Simpson *et al.*, 1963). Similarly X-ray data indicate two metal-dependent binding sites for the *p*-iodo derivative of β-phenylpropionate in the crystalline enzyme (Steitz *et al.*, 1967).

The largest spectral perturbations result from addition of peptide[4] substrates, potentially reflecting participation of the metal in catalysis. Glycyl-L-tyrosine and glycyl-L-phenylalanine effect hypsochromic shifts in the visible absorption spectrum (Figure 5), and a displacement in average position to higher energy in the near infrared. All components of the visible absorption spectrum become optically active, and there is an

[2] Small anions of the spectrochemical series (Phillips and Williams, 1966) do not perturb the spectra of the cobalt enzyme in solution as expected for an additional coordination site of cobalt carboxypeptidase, as proposed for manganese carboxypeptidase (Navon *et al.*, 1970).

[3] Preliminary observations that this inhibitor affects the cobalt carboxypeptidase absorption spectrum were made by Dr. R. C. Davies in this laboratory.

[4] Up to now the lack of suitable ester substrates has prevented a similar examination of the function of metals in their hydrolysis by carboxypeptidase.

inversion in sign and a fourfold increase in rotational strength compared with that of the control (Figure 4). Similar but less extensive changes are induced by L-lysyl-L-tyrosinamide, which is not hydrolyzed and does not bring about the large conformational changes in the crystalline enzyme seen with substrate (Steitz *et al.*, 1967). The slowly hydrolyzed substrates Z-sarcosyl-L-phenylalanine and sarcosyl-L-tryptophan perturb the visible absorption spectrum and increase the maximum amplitude of the circular dichroism five- and sixfold, respectively. The absorption band at 572 nm, which is optically inactive in the free cobalt enzyme, becomes active in the presence of substrate and dominates the magneto circular dichroic spectrum (Figure 2).

The dramatic changes in the circular dichroism of cobalt carboxypeptidase in the presence of substrates might reflect the role of the metal in peptide hydrolysis. The metal is considered to polarize the carbonyl group of the peptide bond to be split by coordination with the carbonyl oxygen (Vallee *et al.*, 1963; Lipscomb *et al.*, 1969; Vallee *et al.*, 1970). The spectral data suggest that this interaction profoundly influences the metal environment. The need for electronic methods to detect these events is underscored by the apparent constancy of the metal coordination number. The absorption and magneto circular dichroic spectra (Figures 5, 6) can be interpreted to indicate that the basic metal coordination geometry is unchanged upon formation of the enzyme–substrate complex in solution, an interpretation which is consistent with crystallographic data of the zinc enzyme (Lipscomb *et al.*, 1969).

While the spectra of cobalt carboxypeptidase indicate an asymmetric active-site environment sensitive to catalytic events, it remains a matter of speculation precisely how the metal–ligand interactions, which might generate the unusual spectra, relate to catalytic potential. It has been suggested that the metal and its ligands might constitute part of a system poised to act in peptide hydrolysis (Vallee and Williams, 1968a,b). The data here presented are consistent with such a hypothesis.

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References

Auld, D. S., and French, T. C. (1970), *Anal. Biochem. 34*, 262.

Auld, D. S., and Vallee, B. L. (1970a), *Biochemistry 9*, 602.

Auld, D. S., and Vallee, B. L. (1970b), *Biochemistry 9*, 4352.

Ballhausen, C. J. (1962), Introduction to Ligand Field Theory, New York, N. Y., McGraw-Hill.

Ballhausen, C. J., and Liehr, A. D. (1958), *J. Mol. Spectrosc. 2*, 342.

Ballhausen, C. J., and Liehr, A. D. (1960), *J. Mol. Spectrosc. 4*, 190.

Bradshaw, R. A., Ericsson, L. H., Walsh, K. A., and Neurath, H. (1969), *Proc. Nat. Acad. Sci. U. S. 63*, 1389.

Carlin, R. L. (1965), *in* Transition Metal Chemistry, Vol. 1, Carlin, R. L., Ed., New York, N. Y., Marcel Dekker, Chapter 1.

Coleman, J. E., and Vallee, B. L. (1960), *J. Biol. Chem. 235*, 390.

Coleman, J. E., and Vallee, B. L. (1961), *J. Biol. Chem. 236*, 2244.

Cotton, F. A., Goodgame, D. M. L., and Soderberg, R. H. (1963), *Inorg. Chem. 2*, 1162.

Cotton, F. A., and Soderberg, R. H. (1962), *J. Amer. Chem. Soc. 84*, 872.

Cox, D. J., Bovard, F. C., Bargetzi, J. P., Walsh, K. A., and Neurath, H. A. (1964), *Biochemistry 3*, 44.

Davies, R. C., Riordan, J. F., Auld, D. S., and Vallee, B. L. (1968), *Biochemistry 7*, 1090.

Denning, R. G., and Spencer, J. A. (1969), *Symp. Faraday Soc. 3*, 84.

Dobry-Duclaux, A., and May, A. (1968), *Bull. Soc. Chim. Biol. 50*, 2053.

Dori, A., and Gray, H. B. (1968), *Inorg. Chem. 7*, 889.

Eilbeck, W. J., Holmes, F., and Underhill, A. E. (1967), *J. Chem. Soc. A*, 751.

Fuwa, K., Pulido, P., McKay, R., and Vallee, B. L. (1964), *Anal. Chem. 36*, 2407.

Fuwa, K., and Vallee, B. L. (1963), *Anal. Chem. 35*, 942.

Goodgame, D. M. L., and Goodgame, M. (1965), *Inorg. Chem. 4*, 139.

Latt, S. A. (1971), Ph.D. Thesis, Harvard University, Cambridge, Mass.

Latt, S. A., Auld, D. S., and Vallee, B. L. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 666.

Latt, S. A., and Vallee, B. L. (1969), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 28*, 601.

Lindskog, S., and Nyman, P. O. (1964), *Biochim. Biophys. Acta 85*, 462.

Lipscomb, W. N., Hartsuck, J. A., Quiocho, F. A., and Reeke, G. N., Jr. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 28.

Lipscomb, W. N., Reeke, G. N., Jr., Hartsuck, J. A., Quiocho, F. A., and Bethge, P. H. (1970), *Phil. Trans. Roy. Soc. London, Ser. B 257*, 177.

Morris, P. H., and Martin, R. B. (1970), *J. Amer. Chem. Soc. 92*, 1543.

Navon, G., Shulman, R. G., Wyluda, B. J., and Yamane, T. (1970), *J. Mol. Biol. 51*, 15.

Neurath, H., and Bradshaw, R. (1970), *Accounts Chem. Res. 3*, 249.

Neurath, H., Bradshaw, R. A., Petra, P. H., and Walsh, K. A. (1970), *Phil. Trans. Roy. Soc. London, Ser. B 257*, 159.

O'Connor, M. J., Ernst, R. E., and Holm, R. H. (1968), *J. Amer. Chem. Soc. 90*, 4561.

Owen, J., and Thornley, J. H. M. (1966), *Rep. Progr. Phys. 29*, 675.

Phillips, C. S. G., and Williams, R. J. P. (1966), Inorganic Chemistry, Vol. 2, New York, N. Y., Oxford University Press.

Sacconi, L., Ciampolini, M., Maggio, F., and Cavasino, F. P. (1962), *J. Amer. Chem. Soc. 84*, 3246.

Shulman, R. G., Navon, G., Wyluda, B. J., Douglass, D. C., and Yamane, T. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 39.

Simpson, R. T., Kobes, R. D., Erbe, R. W., Vallee, B. L., and Rutter, W. J. (1971), *Biochemistry 10*, 2466

Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.

Simpson, R. T., and Vallee, B. L. (1968), *Biochemistry 7*, 4343.

Snoke, J. E., and Neurath, H. (1949), *J. Biol. Chem. 181*, 789.

Steitz, T. A., Ludwig, M. L., Quiocho, F. A., and Lipscomb, W. N. (1967), *J. Biol. Chem. 242*, 4662.
Taylor, E., and Underhill, A. E. (1969), *J. Chem. Soc. A*, 368.
Thiers, R. E. (1957), *Methods Biochem. Anal. 5*, 273.
Vallee, B. L., and Latt, S. A. (1970), *Proc. Int. Symp. Structure–Function Relationships Proteolytic Enzymes*, 144.
Vallee, B. L., Riordan, J. F., Auld, D. S., and Latt, S. A. (1970), *Phil. Trans. Roy. Soc. London, Ser. B 257*, 215.
Vallee, B. L., Riordan, J. F., and Coleman, J. E. (1963), *Proc. Nat. Acad. Sci. U. S. 49*, 937.
Vallee, B. L., Rupley, J. A., Coombs, T. L., and Neurath, H. (1958), *J. Amer. Chem. Soc. 80*, 4750.
Vallee, B. L., and Wacker, W. E. C. (1970), *Proteins 5*.
Vallee, B. L., and Williams, R. J. P. (1968a), *Proc. Nat. Acad. Sci. U. S. 59*, 498.
Vallee, B. L., and Williams, R. J. P. (1968b), *Chem. Brit. 4*, 397.

Baker's Yeast Cytosine Deaminase. Some Enzymic Properties and Allosteric Inhibition by Nucleosides and Nucleotides*

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ABSTRACT: Enzymological studies on partially purified preparations of baker's yeast cytosine deaminase are described. The enzyme has a molecular weight of about 34,000, shows a strict specificity for cytosine and 5-methylcytosine (K_m = 2.5 mM for both substrates), and is inhibited by a number of nucleosides and nucleotides, including cytidine, CMP, CDP, CTP, thymidine, TTP, guanosine, GMP, GDP, and GTP. The concentrations required for 50% inhibition range between 0.385 mM for GMP and 0.690 mM for thymidine. Plots of kinetic data for most of the inhibitors take the form of sigmoidal inhibition curves; however, first-order kinetics are observed in substrate saturation curves, suggesting the absence of interaction between substrate binding sites. The inhibition is markedly dependent on hydrogen ion concentration: thus the per cent inhibition, which is maximal below pH 6, decreases to a minimum near pH 7. The inhibition data, together with other observations showing that crude yeast extracts catalyze the biosynthesis of UMP in the presence of cytosine and 5-phosphoribosyl 1-pryophosphate, suggest an anabolic, rather than catabolic role of yeast cytosine deaminase. It is postulated that the enzyme is involved in a "salvage pathway" for pyrimidine nucleotide biosynthesis in yeast, leading to the formation of UMP from cytosine, *via* the combined action of cytosine deaminase and UMP pyrophosphorylase.

Cytosine deaminase (cytosine aminohydrolase, EC 3.5.4.1) was originally found by Hahn and Schäfer (1925) in yeast and *Escherichia coli*, and has since been identified in other microorganisms (see O'Donovan and Neuhard, 1970, for review).

In yeast, the hydrolytic deamination of cytosine and 5-methylcytosine, catalyzed by a fraction precipitated with ammonium sulfate from crude extracts, has been described by Kream and Chargaff (1952). The properties of the enzyme, however, have never been reported in purified systems. Yeast cytosine deaminase appears to be distinct from cytidine deaminase, since after gel filtration on G-100 Sephadex, two separate peaks are obtained for the two enzyme activities (Ipata *et al.*, 1970).

The present paper describes a procedure for the purification of cytosine deaminase from yeast plasmolysates. The final preparation shows a strict specificity for cytosine and 5-methylcytosine.

The kinetic data show that the enzyme is allosterically inhibited by a number of nucleosides and nucleotides, when tested with cytosine or 5-methylcytosine as substrate.

Experimental Section

Materials. Purine and pyrimidine bases, nucleosides, and nucleotides were obtained from Sigma Chemical Co. Whale skeletal myoglobin was obtained from Seravac Laboratories. Pancreatic ribonuclease was obtained from Sigma Chemical Co. Adenosine deaminase from calf intestinal mucosa was obtained from Boehringer and Soehne. Tris (Sigma) was used as a buffer. Other chemicals were of reagent grade or of the highest quality available.

Cytosine Deaminase Assay Procedure. Cytosine deaminase was assayed according to Ipata *et al.* (1970), by a spectrophotometric method based on the differential absorption of cytosine and uracil and of 5-methylcytosine and thymine

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