Mendez, E., & Lai, C. Y. (1975) *Anal. Biochem. 68*, 47.
Morse, D., & Horecker, B. L. (1966) *Anal. Biochem. 14*, 429.
Pal, P. K., Wechter, W. J., & Colman, R. F. (1975) *J. Biol. Chem. 250*, 8140.
Paulos, T. L., & Price, P. A. (1974) *J. Biol. Chem. 249*, 1453.
Seto, S., Ogura, K., & Nishiyama, Y. (1963) *Bull. Chem. Soc. Jpn. 36*, 332.
Travis, J., & McElroy, W. D. (1966) *Biochemistry 5*, 2170.
Wyatt, J. L., & Colman, R. F. (1977) *Biochemistry 16*, 1333.
Zoller, M. J., & Taylor, S. S. (1979) *J. Biol. Chem. 254*, 8363.

Cobalt Tyrosinase: Replacement of the Binuclear Copper of *Neurospora* Tyrosinase by Cobalt†

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ABSTRACT: The antiferromagnetically spin-coupled copper(II) pair in *Neurospora* tyrosinase was substituted by cobalt, yielding a stoichiometry of 2 mol of Co/mol of protein. The low magnitude of the high-spin Co(II) EPR signal indicates spin coupling of the two Co(II) ions similar to that observed in the native enzyme. The absorption spectrum with four transitions in the visible region of intermediate intensity (ϵ_{607} 670, ϵ_{564} 630, ϵ_{526} 465), a shoulder at 635 nm, and the near-infrared bands at 1180 (ϵ 30) and 960 nm (ϵ 15) indicate tetrahedral coordination around the Co(II) center. The cobalt(II) tyrosinase is enzymatically inactive, and there is no evidence that it binds molecular oxygen. Upon addition of cyanide or the competitive tyrosinase inhibitors L-mimosine, benzoic acid, or benzhydroxamic acid the absorption spectrum changes in a characteristic manner. This optical perturbation shows that binding of these inhibitors (and presumably of the substrates) occurs at or near the metal site. One Co(II) ion can be removed preferentially by incubation with KCN at high pH, indicating the two ions not to be in an identical environment.

Tyrosinase (EC 1.14.18.1) is a copper-containing monooxygenase which catalyzes the oxidation of monophenols to *o*-diphenols (cresolase activity) and of *o*-diphenols to *o*-quinones (catecholase activity) (Mason, 1965). The enzyme was purified from different microorganisms, plants, and animals, and it is involved in the formation of melanins and other polyphenolic compounds (Vanneste & Zuberbühler, 1974; Nicolaus, 1968). *Neurospora* tyrosinase is a single-chain protein with a molecular weight of 46 000 containing 2 mol of copper/mol of protein (Lerch, 1976, 1978). The copper was found to be EPR[1] nondetectable, attributable to an antiferromagnetically spin-coupled copper pair (Deinum et al., 1976) as was demonstrated earlier for mushroom tyrosinase from *Agaricus bispora* (Schoot Uiterkamp & Mason, 1973). Resonance Raman studies showed that the oxygen in *Neurospora* oxytyrosinase is bound to the protein as peroxide (Eickman et al., 1978), as was previously reported for oxyhemocyanin (Freedman et al., 1976). In addition, these two proteins share other properties: both contain type 3 copper (Fee, 1975), display virtually the same absorption spectra, and have similar EPR-sensitive NO- and half-met derivatives (Schoot Uiterkamp & Mason, 1973; Eickman et al., 1979; Himmelwright et al., 1980). The two proteins, however, fulfill quite different functions (monooxygenase vs. oxygen transporting function). This must be reflected in their active centers which in the case of tyrosinase must contain a substrate binding site (lacking in hemocyanin) in addition to the oxygen binding site. From photooxidation (Pfiffner et al., 1980) and active-site-directed modification experiments (Dietler & Lerch, 1979) in conjunction with amino acid sequence data of *Neurospora* tyrosinase (Lerch, 1978), it was suggested that histidyl residues 188, 193, 289, and 306 represent possible ligands to the copper site in this enzyme. For more information on the active site of this monooxygenase, the copper of the native enzyme was chemically replaced by cobalt. Complexes of cobalt(II) exhibit characteristic d–d transitions which are dependent on the ligand geometry and which are very sensitive to changes in the immediate environment of the metal. Thus Co(II) substitution can be used to study the overall geometry and the interaction of ligands with the metal site in metalloproteins. Cobalt(II) derivatives have been prepared of carboxypeptidase A, carbonic anhydrase, alkaline phosphatase (Vallee & Wacker, 1970), phosphoglucomutase (Ray et al., 1972), hemoglobin (Hoffman & Petering, 1970), horseradish peroxidase (Wang & Hoffman, 1977), liver alcohol dehydrogenase (Sytkowski & Vallee, 1976), superoxide dismutase (Calabrese et al., 1972), stellacyanin (McMillin et al., 1974), rubredoxin (May & Kuo, 1978), and *Rhus* laccase type 1 (Larrabee & Spiro, 1979). In these Co derivatives cobalt forms a mononuclear complex with the protein moiety. Tyrosinase, however, contains a binuclear copper site, and hence a binuclear cobalt site is expected in cobalt(II) tyrosinase. Substitution of the oxygen-binding copper atoms of native tyrosinase with cobalt is also of special interest since a large number of low molecular weight Co(II) complexes are known to bind oxygen reversibly as a binuclear complex (Wilkins, 1971). In contrast, information on copper model complexes fulfilling a similar function is still severely limited.

This report describes the preparation and some properties of cobalt(II)-substituted tyrosinase from *Neurospora crassa*. The relationship of cobalt(II) tyrosinase to the binuclear cobalt model complexes as well as to the mononuclear cobalt substituted proteins is discussed.

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[1] Abbreviations used: EPR, electron paramagnetic resonance; CD, circular dichroism.

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Materials and Methods

Organism and Preparation of the Enzyme. *Neurospora crassa* wild-type strain (Fungal Genetic Stock Center No. 321, Arcata, CA) was grown on Vogel N medium according to Horowitz et al. (1970). The enzyme from cycloheximide-derepressed cultures was isolated as described by Lerch (1976). Enzymatic activity was measured according to Fling et al. (1963). Protein concentration was determined spectrophotometrically at 280 nm by using an absorption coefficient $A^{1\%}_{1cm}$ = 22.0 (Lerch, 1976). Freshly isolated tyrosinase has a specific activity of 1200 ± 100 U/mg.

Preparation of Cobalt(II) Tyrosinase. Native tyrosinase (10–30 mg) was incubated with 0.1 M potassium cyanide (a reagent successfully used to remove copper in many copper proteins) in 60 mM sodium phosphate, pH 9, for at least 4 h. The apoenzyme was obtained by removal of low molecular weight compounds by using gel filtration on Sephadex G-25 equilibrated with 50 mM Tris-HCl, pH 7.5. A solution of Co(II) (45 mg of $CoCl_2 \cdot 6H_2O$/mL) was added at a rate of approximately 0.1 mL/min until the final concentration of 2 mg/mL was reached (500-fold molar excess over apotyrosinase) and incubated for another 4 h. Excess cobalt was removed by gel filtration on a Sephadex G-25 column equilibrated with 10 mM sodium phosphate, pH 7.2. Cobalt(II) tyrosinase was concentrated by hydroxylapatite chromatography or by vacuum dialysis (Collodion bags from Sartorius Membranfilter GmbH).

Spectroscopic Techniques. Absorption spectra in the ultraviolet, visible, and near-infrared region were recorded on a Perkin-Elmer EL 350 spectrophotometer. The samples for measurements in the near-infrared regions were dialyzed 3 days against 10 mM sodium phosphate + 0.5 M NaCl pH 7.2, in D_2O (99.5%) with two buffer changes. Circular dichroism spectra were run on a Cary 61 spectropolarimeter. EPR measurements in the X-band were carried out on a Varian E-line spectrometer with a flow cryostat from Oxford Co. Quantitation of the EPR signals was accomplished by double integration according to Palmer (1967) by using the signal of 1 mM Co^{2+}EDTA as a reference. Metal analyses were performed on a IL 157 atomic absorption spectrophotometer after hydrolysis of 0.5–1.5 mg of protein in 6 N HCl at 110 °C for 20 h. The glassware used for the preparation of cobalt(II) tyrosinase was soaked in a 1:1 mixture of concentrated sulfuric and nitric acid for at least 24 h and extensively rinsed with triply distilled water. Buffer solutions were rendered metal free by passage through Chelex 100.

All chemicals used were of analytical grade. L-Mimosine was purchased from Sigma Chemical Co.; benzhydroxamic acid was from Koch Light Laboratories. An affinity matrix for tyrosinase was prepared according to Cuatrecasas (1970) using benzoic acid as a ligand. For this purpose *p*-aminobenzoic acid was covalently linked to CH-Sepharose 4B (Pharmacia) according to Gutteridge & Robb (1973). Dye-sensitized photooxidation of a 2-mL sample of cobalt(II) tyrosinase (0.55 mg/mL) was performed according to Pfiffner et al. (1980).

Results

Metal Substitution with Co(II). Native tyrosinase was found to be resistant toward direct substitution of Cu(II) by Co(II). Less than 0.1 mol of cobalt/mol of protein (Table I) was bound after 5-h incubation of 0.9 mg/mL native tyrosinase with 5 mg of $CoCl_2 \cdot 6H_2O$/mL (1000-fold excess). On the other hand, apotyrosinase binds 2.0 ± 0.2 mol of cobalt/mol of protein when it is exposed to Co(II) solutions. Cobalt(II) tyrosinase in turn does not bind more than 0.1 mol of copper/mol of protein when it is exposed to Cu(II), again in contrast to apotyrosinase which can be reconstituted with Cu(II). Thus these two metal ions are tightly bound to the protein and cannot be replaced by one another directly.

Table I: Metal Binding Behavior and Enzymatic Activity of *Neurospora* Tyrosinase

sample	mol of Cu/mol of protein	mol of Co/mol of protein	spec act. (units/mg)
native enzyme	2.0 ± 0.1[a]		1200
native enzyme after Co(II) incubation	2.0 ± 0.1	<0.1	1200
cobalt(II) tyrosinase	0.05–0.18	2.0 ± 0.2	<25
cobalt(II) tyrosinase after Cu(II) incubation	<0.1	2.0 ± 0.1	<25

[a] Lerch (1976).

Cobalt(II) tyrosinase displays only weak enzymatic activity (Table I) which can be explained by the residual copper in cobalt(II) tyrosinase. K_M values for the substrates L-Dopa and L-tyrosine ethyl ester are the same for native and cobalt(II) tyrosinase, and both proteins are inhibited to the same extent by fluoride, thiocyanate, thiourea, or cyanide. These kinetic measurements are in agreement with cobalt(II) tyrosinase being devoid of enzymatic activity. The amount of residual copper in the Co(II)-substituted enzyme varies somewhat from preparation to preparation. It depends mostly on the time of incubation with potassium cyanide. After 3 h of incubation 0.13–0.18 mol of copper/mol of protein were measured whereas after 15–20 h exposure to potassium cyanide the residual copper content is less than 0.1 mol of copper/mol of protein.

Spectral Properties of Co(II)-Substituted Tyrosinase. Co(II)-substituted tyrosinase is violet colored and its absorption spectrum is shown in Figure 1A. In the visible region there are three prominent absorption maxima at 526 (ϵ 465 L mol^{-1} protein cm^{-1}) 564 (ϵ 630), and 607 nm (ϵ 670), respectively, and a shoulder at 635 nm. The charge-transfer band at around 340 nm (ϵ 18 000) typical for oxytyrosinase and oxyhemocyanin is present in cobalt(II) tyrosinase only as a small shoulder (ϵ < 100) at ca. 340 nm. In the near-infrared region cobalt(II) tyrosinase (Figure 1C) shows weak absorption bands at 960 and 1180 nm with molar extinction coefficients of 15 and 30, respectively. The spectral features of cobalt(II) tyrosinase remained unchanged when the pH of the enzyme solution was varied between 4.9 and 8.0.

The circular dichroism spectrum of cobalt(II) tyrosinase is presented in Figure 1B. It shows three maxima of positive and one of negative ellipticity in the visible and near-ultraviolet region of relatively low intensity. They are centered at 640 (600 deg cm^2 $dmol^{-1}$), 570 (2000), 520 (800), and 360 nm (−250). The positions of the three bands with positive ellipticity correspond to those of three absorption bands. The absorption band at 610 nm is optically inactive.

Influence of Oxygen. A cobalt(II) tyrosinase sample (2.3 mg/mL) was evacuated and flushed with repurified analytical nitrogen several times to remove molecular oxygen completely. The spectrum of this sample was found to be identical with that obtained under aerobic conditions. Furthermore, a sample of cobalt(II) tyrosinase prepared under strict anaerobic conditions showed the same spectral features, suggesting no oxygen binding to the Co(II) derivative.

Effects of Other Ligands. Addition of chloride, *o*-phenanthroline, or inhibitors of native tyrosinase such as fluoride and azide does not affect the absorptive properties

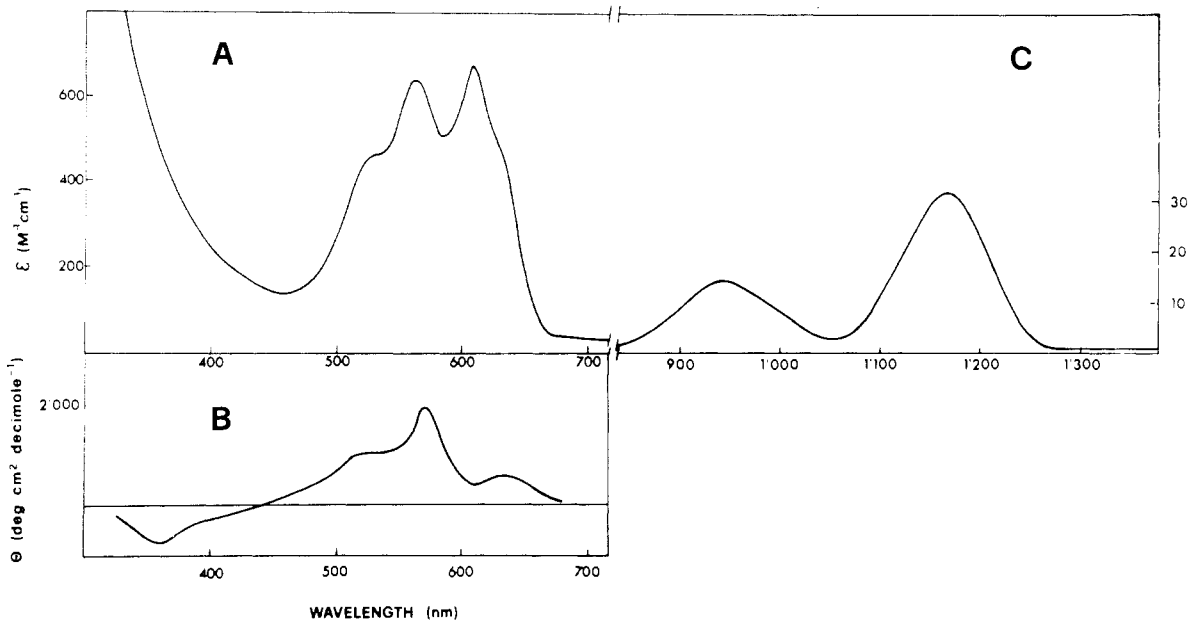

FIGURE 1: Optical spectra of cobalt(II) tyrosinase at 4 °C. (A) Ultraviolet–visible absorption spectrum of 80 μM protein in 10 mM sodium phosphate, pH 7.2, + 0.5 M NaCl (buffer A). (B) Near-infrared spectrum of 200 μM protein in 10 mM sodium phosphate, pD 7.2, + 0.5 M NaCl in D_2O. (C) Circular dichroism spectrum of 420 μM protein in buffer A.

of cobalt(II) tyrosinase. In contrast, a number of noncompetitive and competitive inhibitors of tyrosinase bring about remarkable changes of the absorption spectrum of cobalt(II) tyrosinase.

Cyanide. When cyanide—a noncompetitive inhibitor of native tyrosinase, K_i = 0.1 mM (Gutteridge & Robb, 1975)—was added stepwise to cobalt(II) tyrosinase, the shoulder at 630 and the band at 607 nm disappear while the band at 564 nm increases with a shift of the absorption maximum to 570 nm. Two shoulders at 540 and 595 nm are formed simultaneously. From the spectroscopic titration of cobalt(II) tyrosinase with cyanide, a binding stoichiometry of 1 mol of cyanide/mol of protein was calculated. Furthermore, a new absorption band is formed at 360 nm with an extinction coefficient of about 900 (Figure 2A). Extensive dialysis of the KCN-treated enzyme vs. cyanide free buffer does not result in any spectral changes, implying strong binding of the cyanide ion. The CD spectrum of cobalt(II) tyrosinase is also affected by cyanide binding. It displays only two ellipticity bands, but their intensities are larger than those of uncomplexed cobalt(II) tyrosinase (Figure 2B). Their maxima are located at 480 (4750 deg cm^2 $dmol^{-1}$) and 355 nm (−3800). The latter corresponds to the new absorption band at 360 nm.

Benzoic Acid. The effect of benzoic acid—a competitive inhibitor of tyrosinase, K_i = 14 μM (Gutteridge & Robb, 1975)—on the absorption spectrum of cobalt(II) tyrosinase is shown in Figure 3A. Stepwise addition of benzoic acid to an 80 μM cobalt(II) tyrosinase solution at pH 4.5 reduces the absorption of the Co(II) chromophore by more than 30% and shifts the bands toward lower wavelength (Figure 3A). The shoulder at 635 nm disappears simultaneously. The spectral changes are characterized by an isosbestic point at 510 nm, and they are virtually complete after addition of a stoichiometric amount of benzoic acid. The strong binding of cobalt(II) tyrosinase to benzoate ligands is also manifested by the fact that the cobalt enzyme, like native tyrosinase, is retained by a Sepharose matrix containing covalently linked benzoate. Under the same conditions apotyrosinase is not bound by this affinity matrix.

L-*Mimosine.* Somewhat different changes in the absorption features occur on binding of L-mimosine, another competitive

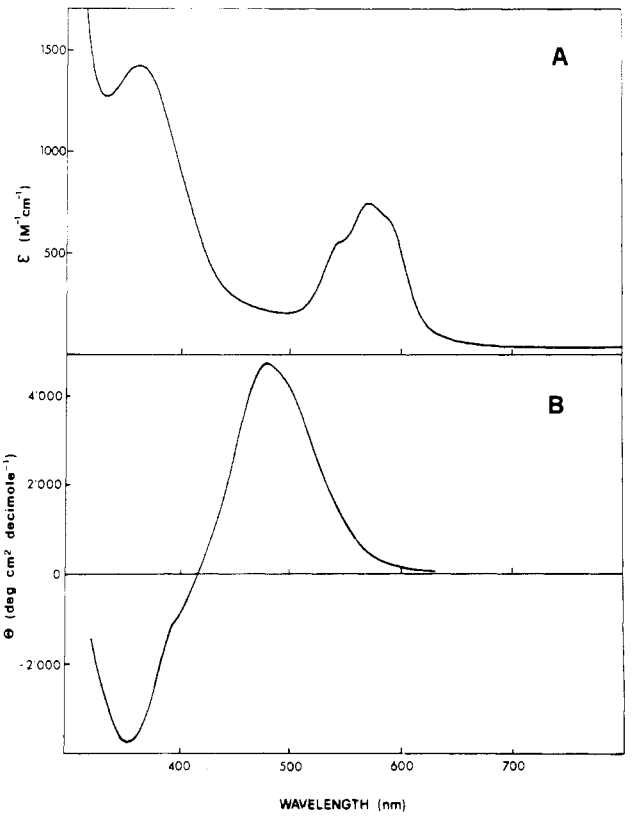

FIGURE 2: Influence of KCN on the optical spectra. (A) Visible absorption spectrum of 130 μM protein in buffer A + 10 mM KCN. (B) CD spectrum of 200 μM protein in buffer A + 10 mM KCN.

inhibitor (K_i = 10 μM). Addition of mimosine completely abolishes the shoulder at 635 nm and diminishes the intensity of the other bands to about half of its original value (Figure 3B). After an equimolar amount of L-mimosine is added no further changes of the visible spectrum are observed, indicating strong binding of 1 mol of L-mimosine/mol of protein.

Benzhydroxamic Acid. Still other modifications of the spectrum of cobalt(II) tyrosinase are brought about by binding of benzhydroxamic acid, another competitive inhibitor of tyrosinase [K_i = 0.6 μM; Rich et al. (1978)]. The addition

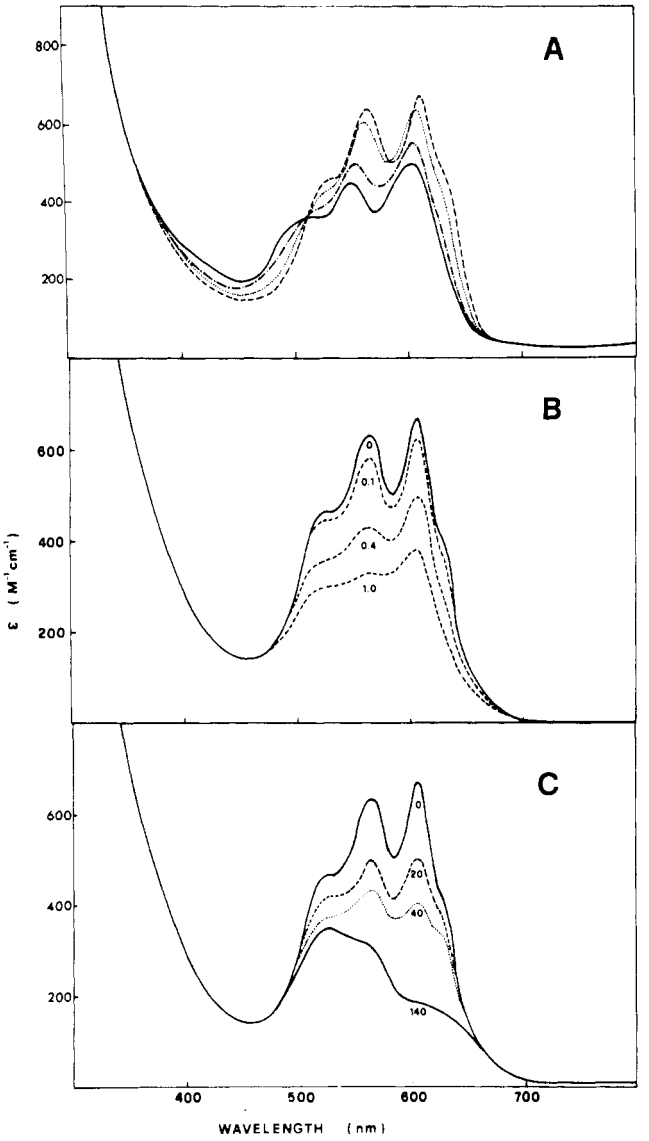

FIGURE 3: Optical perturbation of cobalt(II) tyrosinase by competitive inhibitors of native tyrosinase. (A) Benzoic acid: 80 μM protein in 0.1 M sodium phosphate + 0.05 M sodium citrate, pH 4.5. Benzoic acid concentrations were 0 (---), 10 (···), 50 (-·-), and 140 μM (—). (B) L-Mimosine: 70 μM protein in buffer A; L-mimosine was added at concentration ratios of 0.1, 0.4, and 1.0 mol of L-mimosine per mol of enzyme, respectively. (C) Benzhydroxamic acid: to 50 μM cobalt(II) tyrosinase were added 0, 20, 40, and 140 μM benzhydroxamic acid, respectively.

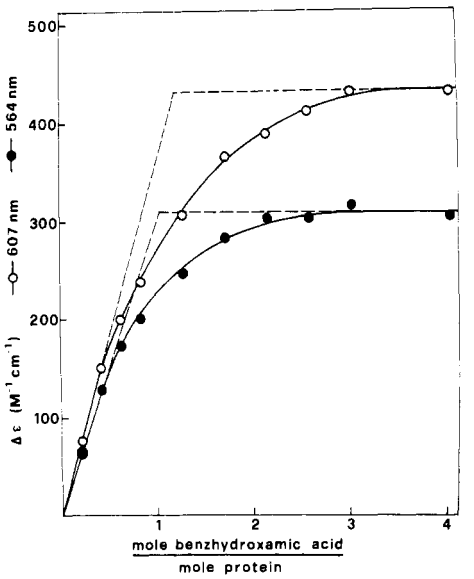

FIGURE 4: Spectroscopic titration of cobalt(II) tyrosinase with benzhydroxamic acid; absorption differences at 607 and 564 nm are plotted vs. the ratio of benzhydroxamic acid to protein.

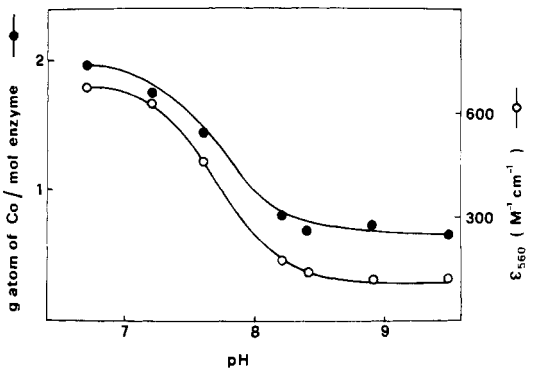

FIGURE 5: Removal of cobalt by KCN; cobalt(II) tyrosinase (15–35 μM) was dialyzed for 15 h against 0.1 M KCN in 50 mM sodium phosphate at different pH values; excess KCN was removed by dialysis. Residual cobalt content and molar absorptivity is plotted as a function of pH.

of equimolar amounts of this compound to cobalt(II) tyrosinase results in intensity reductions of the visible d–d bands (Figure 3C, two-thirds for the band at 607, one-half for the one at 564, and one-quarter for the band at 520 nm). The spectrophotometric titration of cobalt(II) tyrosinase with this inhibitor (Figure 4) shows binding of 1 mol of benzhydroxamic acid/mol of enzyme. The dissociation constant evaluated from the course of the titration curve is 60 μM.

Electron Paramagnetic Resonance Measurements. The EPR spectrum of a concentrated solution of cobalt(II) tyrosinase (1.7 mM) was measured at 4 K and 9.09 GHz. At low magnetic field strength (1190 G) it shows resonances typical for high-spin Co(II) complexes. At higher field strength (3190 G) weak copper resonance lines due to adventitiously bound copper (vide supra) are observed. Double integration of the Co(II) EPR signal reveals that only about 3% of the total cobalt present in cobalt(II) tyrosinase is EPR active, and hence is assumed to represent a small amount of unspecifically bound Co(II). The cobalt signal does not saturate at 20-mW incident power, but the intensity of the signal depends strongly on the temperature. Above 50 K, the EPR signal vanishes completely. Cobalt(II) tyrosinase treated with 10 mM KCN shows a spectrum with the same g parameters as cobalt(II) tyrosinase but of greater intensity [11% of the Co(II) present].

Chemical Reactivity of Cobalt(II) Tyrosinase. Unlike apotyrosinase, which is susceptible to dye-sensitized photooxidation, the holoenzyme is not (Pfiffner et al., 1980). Similarly cobalt(II) tyrosinase is resistant toward this treatment. Exposure of the cobalt derivative (0.55 mg/mL to methylene blue and intensive irradiation produced no change in histidine and cobalt content as measured by amino acid and metal analysis.

Differential Removal of Co(II) from Cobalt(II) Tyrosinase. Dialysis of cobalt(II) tyrosinase (0.7–1.6 mg/mL) against 0.1 M potassium cyanide in 50 mM sodium phosphate at different pH values demonstrates that above pH 8 about 50% of the metal is lost (Figure 5). A comparison of the absorption spectra recorded after extensive dialysis vs. the same buffer without KCN shows also typical changes in the absorption spectrum. At the low pH values where Co(II) is retained, the spectrum corresponds to that shown in Figure 2A. In the samples exposed to KCN at high pH, the absorption profile is much lower and also changed in shape (Figure 6). This derivative was also found to be EPR nondetectable, suggesting

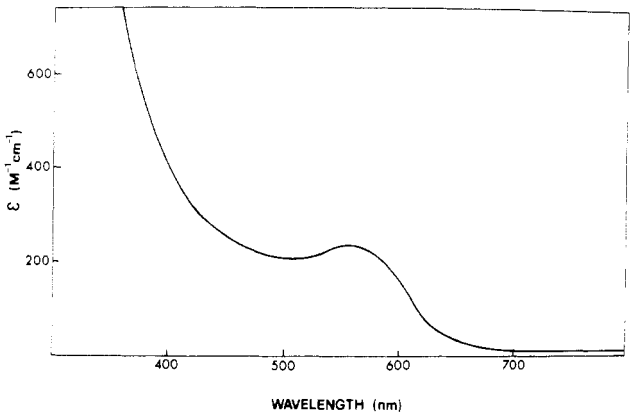

FIGURE 6: Absorption spectrum of 40 μM cobalt(II) tyrosinase in buffer A after exposure to 0.1 M KCN at high pH.

oxidation of Co(II) to Co(III).

Discussion

The present study shows that the contiguous copper pair—referred to as type 3 copper—of *Neurospora* tyrosinase can be substituted by cobalt. Because of the strong binding of copper in *Neurospora* tyrosinase, however, even large excesses of cobalt could not displace the metal in the native enzyme directly (Table I). Thus, as a prerequisite for metal substitution, copper had to be removed first and was subsequently replaced by Co(II) as was reported for many other Co-substituted metalloproteins. The metal:protein ratio was the same in native and cobalt(II) tyrosinase, suggesting that the cobalt ions bind to the same site as copper in the native enzyme. Further support for this conjecture comes from the fact that less than 0.1 mol of Co(II) binds to the native enzyme and that the Co(II) ions in cobalt(II) tyrosinase protect the histidine residues—previously shown to be susceptible toward dye-sensitized photooxidation (Pfiffner et al., 1980)—from destruction during this modification. The EPR results too suggest that the Co(II) ions occupy the copper site. From the present data, however, it cannot be ruled out that the ligands for the cobalt are different than those of the copper.

Spectral Properties. The visible absorption spectrum shows three major bands and a shoulder indicating four distinguishable electronic d–d transitions. The band positions and intensities of cobalt(II) tyrosinase are similar to those of human cobalt(II) carbonic anhydrase (Coleman & Coleman, 1972), cobalt(II) phosphoglucomutase (Ray et al., 1972), and cobalt(II) alkaline phosphatase (Simpson & Vallee, 1968). These three proteins have been assigned tetrahedral or distorted tetrahedral coordination around the active site cobalt(II) on the basis of visible absorption data for cobalt(II) phosphoglucomutase (Ray et al., 1972) and cobalt(II) alkaline phosphatase (Anderson et al., 1976) and by visible absorption and magnetic circular dichroism data for cobalt(II) carbonic anhydrase at low pH, respectively (Coleman & Coleman, 1972). X-ray analysis of native carbonic anhydrase reveals also a distorted tetrahedral geometry (Kannan et al., 1971). All these proteins contain one metal ion at the active site of the native enzyme and one Co(II) ion in the derivative. In contrast, tyrosinase contains two metal ions and the derivative 2 mol of cobalt/mol of enzyme, presumably as a contiguous pair (vide infra). The energies and intensities of the cobalt(II) tyrosinase absorption bands suggest tetrahedral coordination geometry of the cobalt(II) chromophore by comparison both with the Co(II) enzymes mentioned above and with tetrahedral low molecular weight complexes such as the Co(II) complex formed in 50% NaOH (Cotton et al., 1961) or Co(benzimidazole)$_4$(ClO$_4$)$_2$ (Goodgame & Cotton, 1962). The near-infrared spectrum of cobalt(II) tyrosinase is also in accordance with this view since the two bands ($\epsilon_{960} \sim 15$, $\epsilon_{1180} \sim 30$) of low intensity are in good agreement with the bands of $[Co(OH)_4]^{2-}$ [$\epsilon_{1230} \sim 40$, $\epsilon_{1390} \sim 40$, $\epsilon_{1590} \sim 40$; Cotton et al. (1961)], $[Co(benzimidazole)_4]^{2+}$ [$\epsilon_{1110} = 60$, shoulders at 1200 and 1000 nm, Goodgame & Cotton (1962)], and other tetrahedral Co(II) complexes (Hush & Horbs, 1968).

Neglecting spin–orbit interaction, the strength of the ligand field Δ_t of cobalt(II) tyrosinase can be calculated by

$$\Delta_t^2 - 0.529(\nu_2 + \nu_3)\Delta_t + 0.29\nu_2\nu_3 = 0$$

(Lane et al., 1977). By use of 17400 ± 600 cm^{-1} for ν_3 ($^4A_2 \rightarrow {}^4T_1(P)$) and 9200 ± 400 cm^{-1} for ν_2 ($^4A_2 \rightarrow {}^4T_1(F)$) the above equation yields for Δ_t a value of 5300 ± 200 cm^{-1}. Thus the tetrahedral ligand field of cobalt(II) tyrosinase is rather strong. Model complexes with ligand fields above 5000 cm^{-1} have been reported (Rosenberg et al., 1975). They contain nitrogen ligands, mostly in imidazole or benzimidazole groups. Thus, the deductions made from photooxidation (Pfiffner et al., 1980) and reaction inactivation studies (Dietler & Lerch, 1979) that histidine residues are ligands to the active site copper in tyrosinase are further supported. The ν_3 transition is split into four components whose separations (1280, 1260, and 730 cm^{-1}) are too large to arise from spin–orbit interactions alone and, hence, may reflect distortions from tetrahedral symmetry.

The active-site structures of met- and oxytyrosinase are proposed to be tetragonal (Himmelwright et al., 1979, 1980), which contrasts to the tetrahedral structure of cobalt(II) tyrosinase. Since Cu(I) complexes often show tetrahedral coordination (Cotton & Wilkinson, 1972) the Co(II) derivative of tyrosinase may thus reflect the coordination geometry of deoxytyrosinase [Cu(I)] rather than those of the oxy or met forms.

The CD spectrum of cobalt(II) tyrosinase is also consistent with tetrahedral coordination geometry. Three of the four absorption bands in the visible region are optically active, exhibiting positive ellipticity of relatively low intensity. This CD spectrum is in the range of other CD spectra of tetrahedrally coordinated cobalt metalloproteins like human cobalt carbonic anhydrase B (Coleman & Coleman, 1972) and tetracobalt alkaline phosphatase (Anderson & Vallee, 1977). The remarkable change in the CD of cobalt(II) tyrosinase upon potassium cyanide treatment has its correspondence in the similar changes of the carbonic anhydrase CD spectra (Coleman & Coleman, 1972).

A large number of low-molecular-weight Co(II) complexes capable of binding molecular oxygen reversibly have been characterized (Wilkins, 1971). In most instances cobalt is coordinated octahedrally in such complexes, and never tetrahedrally. Furthermore these cobalt–oxygen complexes display strong charge-transfer bands around 350 nm with ϵ values in the range of 10^3–10^4 M^{-1} cm^{-1} (Wilkins, 1971). However, such a band is not observed in cobalt(II) tyrosinase, and it cannot be produced by addition of H_2O_2 or NH_2OH, both agents known to convert mettyrosinase into its oxy form which is characterized by an intense charge-transfer band at 345 nm [ϵ 18000 M^{-1} cm^{-1}; Jolley et al. (1974); Lerch (1976)]. Under all conditions explored so far, no evidence for an oxygenated form of cobalt(II) tyrosinase could be obtained. This finding is in sharp contrast to the ease of many model Co(II) complexes to bind oxygen reversibly as a binuclear complex. However, all these complexes exhibit octahedral coordination (Wilkins, 1971) whereas cobalt(II) tyrosinase is tetrahedral. This difference in coordination geometry may explain the lack

of oxygen binding in cobalt(II) tyrosinase.

Optical Perturbations. The absorption spectrum of Co(II) is sensitive to the nature, number, and coordination geometry of its ligands. Therefore perturbations of the spectrum should be observed upon changing the coordination sphere of the Co(II) chromophore.

Spectral perturbations have been observed for Co(II)-substituted enzymes like cobalt carboxypeptidase (Latt & Vallee, 1971), superoxide dismutase (Calabrese et al., 1976), and especially cobalt carbonic anhydrase where it was found that inhibitors or H^+ compete with a protein ligand for a metal coordination site (Lindskog, 1963). Inhibitors of native tyrosinase like azide and fluoride or changing the pH do not give rise to spectral changes indicating that these agents do not bind to the metal chromophore. Other inhibitors of native tyrosinase like cyanide, benzoic acid, benzhydroxamic acid, and L-mimosine, however, cause spectral perturbations when incubated with cobalt(II) tyrosinase. This suggests that these inhibitors, which all but one are substrate analogues, interact with the protein either at the metal or at a nearby site. A direct coordination to the metal center is favoured in the case of benzoic acid since cobalt(II) tyrosinase binds to an affinity column with benzoate as a ligand whereas apotyrosinase does not.

In addition to the spectral changes in the visible region, KCN-treated cobalt(II) tyrosinase exhibits another band in the near-ultraviolet region ($\epsilon_{360} = 900$). An interpretation of this band, however, is difficult since no similar spectra of low-molecular-weight cobalt(II) cyanide complexes have been reported. When the binuclear character of the cobalt site and the tight binding of one cyanide ion/mol of protein are taken into account, this band could be interpreted to originate from a cyanide bridge between the two metal ions similar to the one reported for the half-met cyanide derivative of hemocyanin (Himmelwright et al., 1979, 1980; Eickman et al., 1979).

Electron Paramagnetic Resonance. About 97% of the cobalt present in cobalt(II) tyrosinase is EPR nondetectable. Since two Co(II) ions are bound to the protein on the ground of stoichiometry, this result strongly suggests that the two cobalt ions form a pair of antiferromagnetically spin-coupled ions. Antiferromagnetical interaction of unpaired electrons is only possible if the interacting ions are either juxtaposed to each other or linked via a bridging ligand which permits a superexchange mechanism. Similar results were obtained with native tyrosinase which displays less than 3% EPR detectable copper (Deinum et al., 1976). In another copper protein, bovine superoxide dismutase, reduction of the copper EPR signal was observed upon Co(II) substitution of Zn(II). This reduction has been interpreted by electronic interactions of the two paramagnetic ions (Fee, 1973; Rotilio et al., 1974) mediated by a bridging histidine residue. A bridging ligand has been postulated also for hemocyanin (Himmelwright et al., 1979) and tyrosinase (Himmelwright et al., 1980) and therefore might be also present in the cobalt derivative.

Removal of Co(II). Incubation of cobalt(II) tyrosinase with 0.1 M KCN above pH 8.2, i.e., above 10 mM of free cyanide, results in the selective removal of one Co/protein. Simultaneously with the removal of cobalt the visible absorption, characteristic for KCN-treated cobalt(II) tyrosinase, is greatly reduced (Figure 6). The intensity and position of the absorption band at 560 nm and the lack of an EPR signal of the high-pH sample suggest a change of the oxidation state from Co(II) to Co(III). Because Co(III) complexes are always octahedral with only few exceptions in the solid state, one can assume an octahedral coordination also for this derivative. This implies that, in addition to the oxidation, a rearrangement of ligands must occur. Such concomitant changes of coordination geometry upon oxidation of Co(II) to Co(III) have been observed in alkaline phosphatase (Anderson & Vallee, 1977) and carboxypeptidase A (Van Wart & Vallee, 1978). As already pointed out, cobalt(II) tyrosinase is not susceptible to oxidation by molecular oxygen, probably because of a restriction to tetrahedral coordination by the protein moiety. If, however, one Co(II) is removed, the steric constraints for the remaining one are relieved, and it becomes susceptible to oxidation. Removal of only one cobalt ion indicates that the two metal ions differ in their binding behavior. A similar finding was reported for mollusc hemocyanins (Himmelwright et al., 1979) whose copper sites can be selectively depleted of one copper ion. However, in arthropod hemocyanins and in native tyrosinase such a half-apo derivative cannot be isolated. Nevertheless, the data of cobalt(II) tyrosinase support the idea that in native tyrosinase too the binding sites for the two copper ions are slightly different.

Added in Proof

Since submission of this paper, the incorporation of one Co(II) ion per active site in squid hemocyanin has been reported by Suzuki et al. (1980). This cobalt(II) hemocyanin displays an optical spectrum similar to that of cobalt tyrosinase. The absorption intensities per Co(II) ion are virtually the same for both cobalt derivatives, hence supporting our view that both Co(II) ions in cobalt tyrosinase contribute to the optical spectrum.

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References

Anderson, R. A., & Vallee, B. L. (1977) *Biochemistry 16*, 4388–4393.

Anderson, R. A., Kennedy, F. S., & Vallee, B. L. (1976) *Biochemistry 15*, 3710–3716.

Calabrese, L., Rotilio, G., & Mondovi, B. (1972) *Biochim. Biophys. Acta 263*, 827–829.

Calabrese, L., Cocco, D., Morpurgo, L., Mondovi, B., & Rotilio, G. (1976) *Eur. J. Biochem. 64*, 465–470.

Coleman, J. E., & Coleman, R. V. (1972) *J. Biol. Chem. 247*, 4718–4728.

Cotton, F. A., & Wilkinson, G. (1972) *Advanced Inorganic Chemistry*, 3rd ed., pp 875, 904, Interscience Publishers, New York.

Cotton, F. A., Goodgame, D. M. L., & Goodgame, M. (1961) *J. Am. Chem. Soc. 83*, 4090–4099.

Cuatrecasas, P. (1970) *J. Biol. Chem. 245*, 3059–3065.

Deinum, J., Lerch, K., & Reinhammar, B. (1976) *FEBS Lett. 69*, 161–164.

Dietler, C., & Lerch, K. (1979) in *Proceedings of the Third International Symposium on Oxidases and Related Redox Systems* (Mason, H. S., King, T., & Morrison, M., Eds.) Academic Press, New York (in press).

Eickman, N. C., Solomon, E. I., Larrabee, J. A., Spiro, T. G., & Lerch, K. (1978) *J. Am. Chem. Soc. 100*, 6529–6531.

Eickman, N. C., Himmelwright, R. S., & Solomon, E. I. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2094–2098.

Fee, J. A. (1973) *J. Biol. Chem. 248*, 4229–4234.

Fee, J. A. (1975) *Struct. Bonding 23*, 1–60.

Fling, M., Horowitz, N. H., & Heinemann, S. F. (1963) *J. Biol. Chem. 238*, 2045–2053.

Freedman, T. B., Loehr, J. S., & Loehr, T. M. (1976) *J. Am. Chem. Soc. 98*, 2809–2815.
Goodgame, M., & Cotton, F. A. (1962) *J. Am. Chem. Soc. 84*, 1543–1548.
Gutteridge, S., & Robb, D. A. (1973) *Biochem. Soc. Trans. 1*, 519–520.
Gutteridge, S., & Robb, D. A. (1975) *Eur. J. Biochem. 54*, 107–116.
Himmelwright, R. S., Eickman, N. C., & Solomon, E. I. (1979) *J. Am. Chem. Soc. 101*, 1576–1586.
Himmelwright, R. S., Hepp, A. F., Eickman, N. C., Lerch, K., LuBien, C. D., Schoeniger, L. O., & Solomon, E. I. (1980) in *Comparative Study and Recent Knowledge on Quaternary Structure and Active Sites of Oxygen Carriers and Related Proteins*, Tours, France, Marcel Dekker, New York (in press).
Hoffman, B. M., & Petering, D. H. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 637–643.
Horowitz, N. H., Fling, M., & Horn, G. (1970) *Methods Enzymol. 17A*, 615–620.
Hush, N. S., & Horbs, R. J. M. (1968) *Prog. Inorg. Chem. 10*, 259–486.
Jolley, R. L., Evans, L. H., Makino, N., & Mason, H. S. (1974) *J. Biol. Chem. 249*, 335–345.
Kannan, K. K., Liljas, A., Waara, I., Bergstên, P.-C., Lövgren, S., Strandberg, B., Bengtsson, U., Carlbom, U., Fridborg, K., Järup, L., & Petef, M. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 221–231.
Lane, R. W., Ibers, J. A., Frankel, R. B., Papaefthymiou, G. C., & Holm, R. H. (1977) *J. Am. Chem. Soc. 99*, 84–98.
Larrabee, J. A., & Spiro, T. G. (1979) *Biochem. Biophys. Res. Commun. 88*, 753–760.
Latt, S. A., & Vallee, B. L. (1971) *Biochemistry 10*, 4263–4270.
Lerch, K. (1976) *FEBS Lett. 69*, 157–160.
Lerch, K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3635–3639.
Lindskog, S. (1963) *J. Biol. Chem. 238*, 945–951.
Mason, H. S. (1965) *Annu. Rev. Biochem. 34*, 595–634.
May, S. W., & Kuo, J. (1978) *Biochemistry 17*, 3333–3338.
McMillin, D. R., Rosenberg, R. C., & Gray, H. B. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4760–4762.
Nicolaus, R. A. (1968) *Melanins*, Hermann, Paris.
Palmer, G. (1967) *Methods Enzymol. 10*, 594–609.
Pfiffner, E., Dietler, C., & Lerch, K. (1980) in *Comparative Study and Recent Knowledge on Quaternary Structure and Active Sites of Oxygen Carriers and Related Proteins*, Tours, France, Marcel Dekker, New York (in press).
Ray, W. J., Goodin, D. S., & Ng, L. (1972) *Biochemistry 11*, 2800–2804.
Rich, P. R., Wiegand, N. K., Blum, H., Moore, A. L., & Bonner, W. D. (1978) *Biochim. Biophys. Acta 525*, 325–337.
Rosenberg, R. C., Root, C. A., & Gray, H. B. (1975) *J. Am. Chem. Soc. 97*, 21–26.
Rotilio, G., Calabrese, L., Mondovi, B., & Blumberg, W. E. (1974) *J. Biol. Chem. 249*, 3157–3160.
Schoot Uiterkamp, A. J. M., & Mason, H. S. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 993–996.
Simpson, R. T., & Vallee, B. L. (1968) *Biochemistry 7*, 4343–4350.
Suzuki, S., Mori, W., Kino, J., Nakao, Y., & Nakahara, A. (1980) *J. Biochem. (Tokyo) 88*, 1207–1209.
Sytkowski, A. J., & Vallee, B. L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 344–348.
Vallee, B. L., & Wacker, W. E. C. (1970) *Proteins 5*, 94–128.
Vanneste, W. H., & Zuberbühler, A. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., Ed.) pp 371–404, Academic Press, New York.
Van Wart, H. E., & Vallee, B. L. (1978) *Biochemistry 17*, 3385–3394.
Wang, M. R., & Hoffman, B. M. (1977) *J. Biol. Chem. 252*, 6268–6275.
Wilkins, R. G. (1971) *Adv. Chem. Ser. No. 100*, 111–134.

Manganese Ion Dependent Adenylate Cyclase Activity in Rat Testes: Purification and Properties†

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ABSTRACT: Testicular, soluble adenylate cyclase has been purified by anion-exchange chromatography, gel filtration, and isoelectric focusing. Upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis, peak fractions from the latter purification step showed only one polypeptide band with an apparent molecular weight of about 69 000. The following hydrodynamic and molecular parameters have been established for this enzyme: sedimentation constant, 4.3 S; Stokes radius, 3.95 nm; partial specific volume, 0.74 mL·g^{-1}; molecular weight, 74 000; frictional ratio, 1.4.

Regulation of adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1], the enzyme that catalyzes the synthesis of adenosine cyclic 3′,5′-phosphate (cyclic AMP), has been the subject of studies documented in profuse literature. However, a better understanding of the function of this enzyme would require an appropriate knowledge of both its structural and molecular properties. Progress in the purification of adenylate cyclase from mammalian cell membranes has been hindered mostly by two facts: first, the enzyme constitutes a very small proportion of the total cell protein; second, enzymatic activity is extremely unstable under the reasonably mild conditions of some standard protein purification procedures.

Adenylate cyclase from brain (Stellwagen & Baker, 1976)

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