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Effect of Metal Substitution on the Chromophore of Bovine Serum Amine Oxidase†

Shinnichiro Suzuki,* Takeshi Sakurai, Akitsugu Nakahara, Takashi Manabe, and Tsuneo Okuyama

ABSTRACT: The spectral and chemical properties of the putative organic chromophore in bovine serum amine oxidase (BSAO) have been characterized by metal ion substitutions of the enzyme. Native BSAO is yellowish pink on account of a broad electronic absorption band at 476 nm. Copper(II)-depleted BSAO (Cu-depBSAO) was prepared by dialyzing pale yellow BSAO, which had been obtained by a reduction of native BSAO with sodium dithionite, against KCN-containing buffer solution. The Cu-depBSAO exhibited no electronic absorption extremum in the region 350–650 nm. Dialysis of Cu-depBSAO against pH 7.2 buffer containing Ni(II) ion gave pink Ni(II)-substituted BSAO (Ni(II)BSAO), which displayed an absorption extremum at 484 nm. Upon removal of Ni(II) from Ni(II)BSAO by dialyzing Ni(II)-BSAO against dimethylglyoxime and KCN-containing buffer solution, pink (instead of pale yellow) Ni-depBSAO was obtained, which showed an electronic absorption peak at 470 nm. The absorption maxima of Ni(II)BSAO and Ni-depBSAO immediately disappeared on addition of a substrate, benzylamine, and did not reappear even under aerobic conditions. This indicates that the chromophore in Ni(II)BSAO or Ni-depBSAO was reduced by benzylamine. It was suggested that yellowish pink or pink BSAO which exhibits an absorption maximum at 470–484 nm possesses the chromophore in oxidized form (resting state) whereas pale yellow or colorless BSAO which exhibits no absorption peak in the region 350–650 nm possesses the chromophore in reduced form. A comparative inspection of electronic absorption and CD spectra of native BSAO, Ni(II)BSAO, and Ni-depBSAO treated with phenylhydrazine revealed that the chromophore is located near the metal ion at the active site of BSAO. The close location of the chromophore to Cu(II) ion was also supported by ESR data. The role of the chromophore is considered to bind a substrate and to catalyze the subsequent deamination while the Cu(II) ion operates the transfer of electrons from the reduced chromophore to oxygen molecule. The cooperative catalysis of the chromophore and Cu(II) ion in BSAO may correspond to the function of flavin–cofactor of mitochondrial amine oxidases.

Amine oxidases [amine:oxygen oxidoreductase (deaminating), EC 1.4.3] are known to catalyze the oxidative deamination of amines by accepting two electrons from amines and transferring them to molecular oxygen. The reactions are expressed by the equation

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$$

They may be conveniently divided into two classes: copper-containing amine oxidases (Malmström et al., 1975) and FAD-containing ones. The copper enzymes are widely distributed in plasma of bovine (Yamada & Yasunobu, 1962a,b, 1963; Yamada et al., 1963; Inamasu & Yasunobu, 1974; Ishizaki & Yasunobu, 1976; Suva & Abeles, 1978; Berg & Abeles, 1980; Zeidan et al., 1980) and pig (Buffoni & Blaschko, 1964; Buffoni et al., 1968; Taylor et al., 1972; Lindström et al., 1973, 1974; Lindström & Pettersson, 1974; Yadav & Knowles, 1981), kidney tissue of pig (Mondovi et al., 1967, 1968; Yamada et al., 1967; Finazzi-Agrò et al., 1977), pea seedling (Hill & Mann, 1964; Hill, 1967; Kluetz et al., 1980), and fungus (Yamada et al., 1965, 1969; Adachi & Yamada, 1969). The copper(II) ion in these proteins is known to be type 2 copper [nonblue and electron spin resonance (ESR)[1] detectable] (Malkin & Malmström, 1970; Peisach & Blumberg, 1974) and to restore the activity of copper-depleted oxidases (Yamada & Yasunobu, 1962b; Hill

† From the Institute of Chemistry, College of General Education, Osaka University, Osaka 560, Japan, and the Department of Chemistry, Faculty of Science, Tokyo Metropolitan University, Fukazawa, Setagaya-Ku, Tokyo 158, Japan. *Received September 10, 1982.* This work was supported by Grants-in-Aid for Special Project Research (57102009) and Science Research (50740247) from the Ministry of Education, Science and Culture of Japan.

* Address correspondence to this author at the Institute of Chemistry, College of General Education, Osaka University.

[1] Abbreviations: BSAO, bovine serum amine oxidase; Ni(II)BSAO, Ni(II)-substituted BSAO; Co(II)BSAO, Co(II)-substituted BSAO; Zn(II)BSAO, Zn(II)-substituted BSAO; Cu-depBSAO, Cu(II)-depleted BSAO; Ni-depBSAO, Ni(II)-depleted BSAO; CHR, organic chromophore; CT, charge transfer; ESR, electron spin resonance; CD, circular dichroism; MCD, magnetic circular dichroism; Tris, tris(hydroxymethyl)aminomethane; TCNQ, tetracyanoquinodimethane.

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& Mann, 1964). The ESR parameters of amine oxidases are very much alike, suggesting that the geometry of Cu(II) center is tetragonal (Malmström et al., 1975). The ESR studies also indicated that no significant weakening of the ESR signal has been observed even after anaerobic treatment of the enzymes with substrates (Yamada et al., 1963, 1969; Buffoni et al., 1968; Lindström et al., 1973; Mondovi et al., 1967). Copper-containing amine oxidases are pink or yellowish pink because of a broad absorption band with a maximum between 420 and 500 nm. The color disappears on reduction with amines or sodium dithionite under anaerobic condition and reappears upon oxidation with oxygen (Yamada & Yasunobu, 1962a,b; Buffoni & Blaschko, 1964; Mondovi et al., 1967; Hill & Mann, 1964; Yamada et al., 1965). However, it was also reported that the absorption band of pig kidney diamine oxidase did not vanish on reduction with dithionite (Finazzi-Agrò et al., 1977). The visible absorption band at 420–500 nm is attributed to some organic cofactor and/or charge-transfer transition from ligating groups to the Cu(II) ion.

Amine oxidase isolated from bovine serum (BSAO) catalyzed the oxidative deamination of benzylamine, spermine, histamine, and especially several lysyl peptides (Oda et al., 1981). This enzyme exhibits the yellow-pink color which results from a broad absorption band near 480 nm. Our previous spectral studies (Suzuki et al., 1980, 1981, 1982) of native, Co(II) substituted, and diethyl dithiocarbamate treated BSAO have partly clarified the coordination chemistry of the copper(II) and the apparent presence of an unknown organic prosthetic group. This paper describes more profound spectroscopic and chemical properties of the chromophore, the roles of copper ion, and the intimate correlation between the two cofactors in native BSAO, and its derivatives.

Experimental Procedures

Materials. BSAO was isolated from bovine serum and crystallized by the method of Okuyama (Oda et al., 1981). The highly purified BSAO showed a specific activity of 580 units/mg at 25 °C according to the method of Tabor et al. (1954), and disc gel electrophoreses of the sample showed a single band on protein staining (above 95% purity). The enzyme has a molecular weight of 190 000 and contains 2.0 g-atoms of copper/mol of protein; the protein contains two electrophoretically equivalent subunits. The protein concentration was determined by measuring the absorptivity at 280 nm ($E^{1\%}_{1cm}$ = 17.4). Nickel chloride was prepared by dissolving nickel metal (Ni 99.99% purity) in hydrochloric acid. All other reagents used were of the highest grade commercially available.

Measurements of Metal Ion Concentration in BSAO and Its Derivatives. The concentrations of copper, nickel, cobalt, and zinc in BSAO and its derivatives were determined by use of an atomic absorption spectrophotometer and by employing the method of standard addition.

Preparations of Cu(II)-Depleted BSAO, Ni(II)-Substituted BSAO, and Ni(II)-Depleted BSAO. Copper(II)-depleted BSAO (Cu-depBSAO) was prepared by dialyzing pale yellow BSAO which had been reduced by treatment of native BSAO with sodium dithionite under anaerobic conditions against 0.2 M sodium phosphate buffer (pH 7.2) containing 10 mM KCN at 4 °C. The dialysis was carried out 3 times under anaerobic conditions. After removal of KCN by anaerobic dialysis against pH 7.2 phosphate buffer, the Cu-depBSAO was obtained as a pale yellow solution.

Nickel(II)-substituted BSAO (Ni(II)BSAO) was obtained as a pink solution by dialyses (twice a day) of Cu-depBSAO against 0.05 M Tris-HCl buffer (pH 7.2) containing 3 mM $NiCl_2$ and by subsequent dialyses against 2 L of 0.2 M phosphate buffer (pH 7.2). The concentration of Ni(II) in the product was determined to be 80% of the total sites for Cu in native BSAO.

Table I: Activities of Native and Modified BSAO

	units/mg	relative activity (%)	metal ion content (%)
BSAO	580	100	100 (Cu)
CHR	2	<1	2 (Cu)
Co(II)BSAO	68	13	73 (Co)
Ni(II)BSAO	13	2	80 (Ni)
Zn(II)BSAO	5	1	40 (Zn)

Nickel(II)-depleted BSAO (Ni-depBSAO) was obtained by dialyses of Ni(II)BSAO against pH 7.2 phosphate buffer solutions containing 1 mM dimethylglyoxime and the same buffer containing 10 mM KCN. The concentration of the residual Ni(II) ion in the Ni-depBSAO was below 5% of the total sites for Cu in native BSAO.

Preparation of Co(II)BSAO and Zn(II)BSAO. Cobalt(II)-substituted BSAO was prepared by four dialyses of Cu-depBSAO against 0.05 M Tris-HCl buffer (pH 7.2) containing 2 mM $CoCl_2$ (Co 99.99% purity) under argon gas, followed by dialyses against 2 L of Tris-HCl buffer alone and then by treatment with Chelex-100 resin (30% by volume, preequilibrated with pH 7.2 Tris-HCl buffer). The concentration of Co(II) in the product was determined to be 73% of the total sites for Cu in native BSAO.

Zinc(II)-substituted BSAO (Zn(II)BSAO) was obtained according to the similar method as employed in preparation of Ni(II)BSAO using $ZnCl_2$ in place of $NiCl_2$.

Instruments. The absorption, circular dichroism, and ESR spectra were measured with a Hitachi 323 spectrophotometer, a JASCO J-40A or J-500A spectropolarimeter attached with a data processor DP-500, and a JEOL JES-FE1X ESR spectrometer, respectively. The measurements of absorption and CD spectra were carried out at room temperature and those of ESR spectra at 77 K. The ESR spectra were recorded at 100 KHz modulation and approximately 9.5 GHz microwave frequency. Magnetic field and g values were calibrated with Mn(II) in MnO and Li-TCNQ, respectively.

Results

Metal Ion Substitution of BSAO. Metal ion content and activity of native BSAO, Cu-depBSAO, Ni(II)BSAO, Ni-depBSAO (CHR), Co(II)BSAO, and Zn(II)BSAO are listed in Table I.

Absorption and CD Spectra of Native BSAO and Cu(II)-Depleted BSAO. The electronic absorption and the CD spectra of native BSAO at pH 7.2 are illustrated in Figure 1, in which the values of ϵ and $\Delta\epsilon$ of electronic absorption and CD spectra, respectively, are given per mole of protein (dimer 190 000 daltons). The yellowish pink color of the protein results from the strong absorption band at 476 nm, of which the ϵ value was estimated as 3800 M^{-1} cm^{-1}.[2] No significant shift of the maximum wavelength was observed with variation of pH from 5 to 8, although a small corresponding variation of the absorptivity was observed. The CD spectrum in the

[2] In a previous communication (Suzuki et al., 1980), we reported that the wavelength and absorptivity of an extremum of the optical spectrum for BSAO were 460 nm and 6600 M^{-1} cm^{-1} (3300 M^{-1} cm^{-1} per mole of copper concentration), respectively. The purity of the sample was ascertained as a single band when examined by disc gel electrophoresis. However, the enzyme exhibited low specific activity [440 units/mg according to the method of Tabor et al. (1954)]. The specific activity of the present BSAO was estimated to be 580 units/mg.

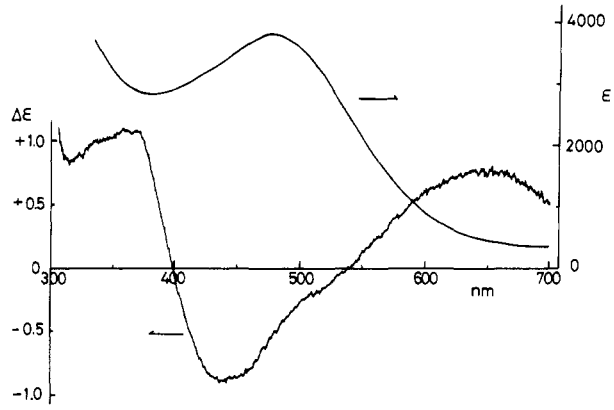


FIGURE 1: Electronic and CD spectra of native BSAO at pH 7.2. The protein concentration was 1.59×10^{-4} M/190 000-dalton polypeptide, and the solvent was 0.2 M phosphate buffer (pH 7.2). The ϵ and $\Delta\epsilon$ values are given per mole of protein.

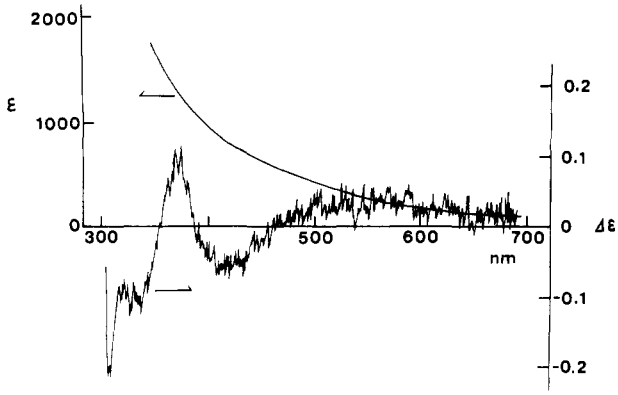


FIGURE 2: Electronic and CD spectra of Cu-depBSAO at pH 7.2. The protein concentration was 1.01×10^{-4} M.

range 300–700 nm displayed two positive extremum at around 370 and 650 nm, a negative extremum at 450 nm, and a negative shoulder at about 525 nm.

The yellowish pink BSAO turned pale yellow upon reduction of its solution with dithionite under anaerobic conditions, being restored to the original color with the consumption of dithionite ion under aerobic conditions. The recoloration of BSAO suggests the occurrence of an oxidation reaction of Cu(I) ion and the chromophore by O_2 molecule. On removal of copper from reduced BSAO, restoration of the original color of BSAO was not attained even if the solution was kept for 2 weeks under an oxygen atmosphere at 5 °C. The removal of 98% of total copper in native BSAO was assured by use of an atomic absorption spectrophotometer. The optical absorption and CD spectra of the pale yellow Cu-depBSAO are shown in Figure 2, which reveals that the CD spectrum involves two weak positive bands at around 370 and 550 nm and two weak negative bands at around 415 and 330 nm, despite the absence of any distinct optical absorption band in the corresponding region.

Preparation and Spectral Properties of Ni(II)-Substituted BSAO and Ni(II)-Depleted BSAO. Although the coloration of Cu-depBSAO under O_2 atmosphere was not attained, a dialytic incorporation of Ni(II) ion into the Cu-depBSAO easily gave rise to the restoration of the pink color.

The Ni(II) ion in Ni(II)BSAO was further removed by dialyzing the Ni(II)BSAO against dimethylglyoxime and KCN, and a depletion of above 95% of total Ni(II) ion was attained. The Ni(II)-depleted BSAO (Ni-depBSAO) also displayed a slight specific activity (2–3% of that of native BSAO). Figures 3 and 4 depict the optical absorption and

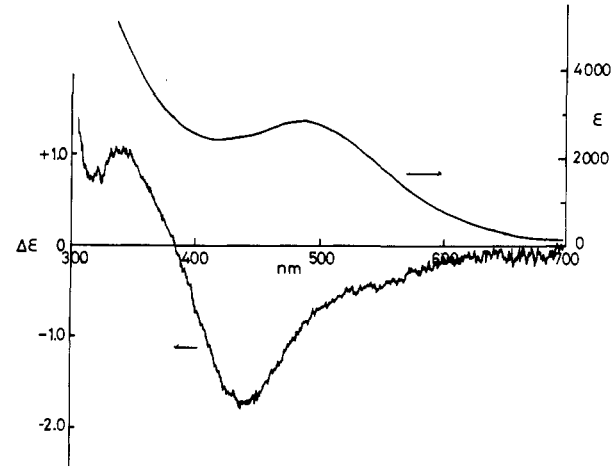


FIGURE 3: Electronic and CD spectra of Ni(II)BSAO at pH 7.2. The protein concentration was 1.13×10^{-4} M.

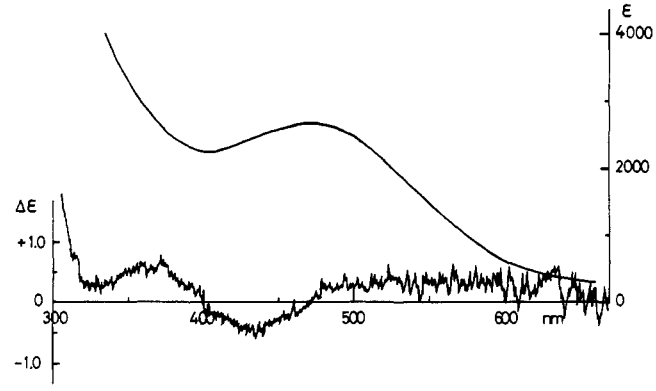


FIGURE 4: Electronic and CD spectra of Ni-depBSAO at pH 7.2. The protein concentration was 9.8×10^{-5} M (electronic absorption spectrum) and 3.1×10^{-5} M (CD spectrum).

the CD spectra of Ni(II)-BSAO and Ni-depBSAO, respectively. Each of the visible absorption spectra of Ni(II)BSAO and Ni-depBSAO exhibited a prominent maximum at 484 (ϵ = 2800 M^{-1} cm^{-1}) and 470 nm (ϵ = 2700 M^{-1} cm^{-1}), respectively. These visible bands which are responsible for the pink colors of Ni(II)BSAO and Ni-depBSAO are nearly identical with that of native BSAO and disappeared immediately upon addition of a substrate, benzylamine. They did not reappear readily even under aerobic conditions. The positive and the negative CD bands of Ni(II)BSAO and Ni-depBSAO at around 350 and 440 nm, respectively, are closely similar to those of native BSAO. However, the difference in CD bands of Ni(II)BSAO and Ni-depBSAO in the region 500–600 nm is indicative of the binding of Ni(II) with the protein in Ni(II)BSAO. No ESR signal was detected for the benzylamine-bound colorless Ni-depBSAO.

Spectral Properties of Reaction Mixtures of Phenylhydrazine and Native BSAO, Ni(II)BSAO, or Ni-depBSAO. Amine oxidases are well-known to be strongly inhibited by carbonyl reagents such as hydroxylamine, phenylhydrazine, and hydrazine (Yamada & Yasunobu, 1963; Lindström & Pettersson, 1973). At the same time, the exzyme exhibits an intense absorption band at around 450 nm in the presence of phenylhydrazine. Figure 5A represents the visible absorption and the CD bands obtained by adding 10 equiv of phenylhydrazine to native BSAO. The markedly yellow color of the solution stems from the strong absorption band at 448 nm (ϵ = 38 000 M^{-1} cm^{-1}). These electronic absorption and CD spectra are extraordinarily similar to those for Ni(II)BSAO in the presence of excess phenylhydrazine as illustrated in

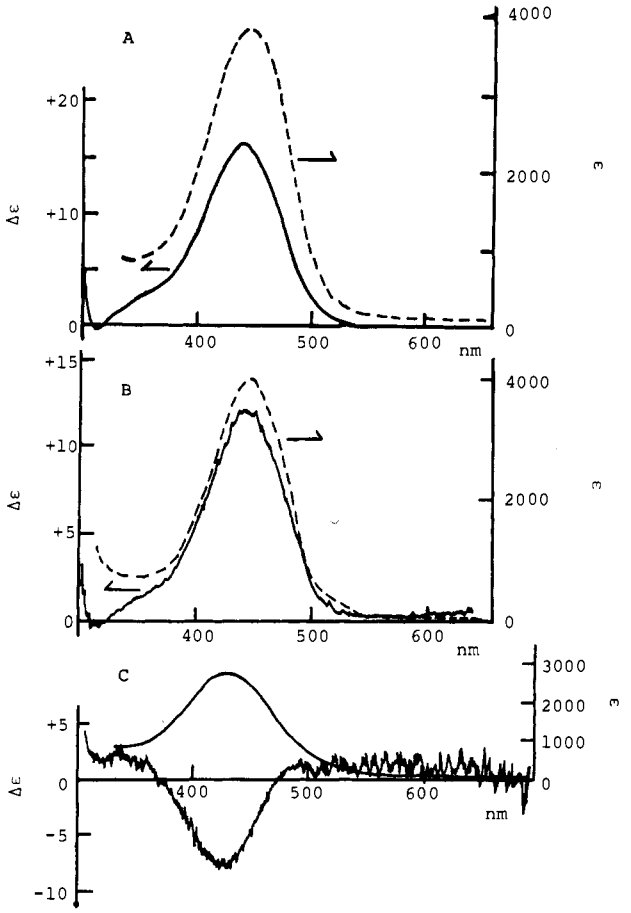


FIGURE 5: Electronic and CD spectra of reaction mixture of (A) native BSAO and phenylhydrazine at pH 7.2 (the protein concentration was 3×10^{-5} M and phenylhydrazine concentration was 3×10^{-4} M), (B) Ni(II)BSAO and phenylhydrazine at pH 7.2 (the protein concentration was 4.7×10^{-5} M and phenylhydrazine concentration was 3.2×10^{-3} M), and (C) Ni-depBSAO and phenylhydrazine at pH 7.2 (the protein concentration was 7.5×10^{-6} M and phenylhydrazine concentration was 8×10^{-5} M).

Figure 5B. The optical and CD spectra of Ni-depBSAO treated with phenylhydrazine are represented in Figure 5C. The pink color of Ni-depBSAO immediately turned deep yellow upon addition of phenylhydrazine. From a comparison of the absorption and CD spectra in Figure 5C and those in Figure 5A,B, it should be noted that the prominent electronic absorption band of the phenylhydrazine-treated Ni-depBSAO lies at a somewhat shorter wavelength region (ϵ_{max} = 423 nm) and the pattern of its CD spectrum is entirely different from that of either the phenylhydrazine-treated native BSAO or the same Ni(II)BSAO. On the other hand, the electronic absorption spectrum of Cu-depBSAO treated with 10 equiv of phenylhydrazine shows a weak absorption band at 417 nm, whose extinction coefficient was estimated to be 3300 M^{-1} cm^{-1}, and the resultant mixture was pale yellow. Yamada and Yasunobu reported that when phenylhydrazine is added to the copper-free bovine plasma amine oxidase, the band at 380 nm is replaced by a maximum at 410 nm (Yamada & Yasunobu, 1963).

X-Band ESR Spectra of Native BSAO, Colorless BSAO Treated with Benzylamine under N_2 Atmosphere, and Yellow BSAO Modified with Phenylhydrazine. The ESR spectra for these samples at pH 7.2 are illustrated in Figure 6. The spectrum of native BSAO (Figure 6A) can be interpreted as axial with spin Hamiltonian parameters, $g_{\parallel} = 2.30$, $g_{\perp} = 2.06$, and $A_{\parallel} = 164$ G. When 160 equiv of benzylamine was added to native BSAO under N_2 atmosphere, the mixture changed

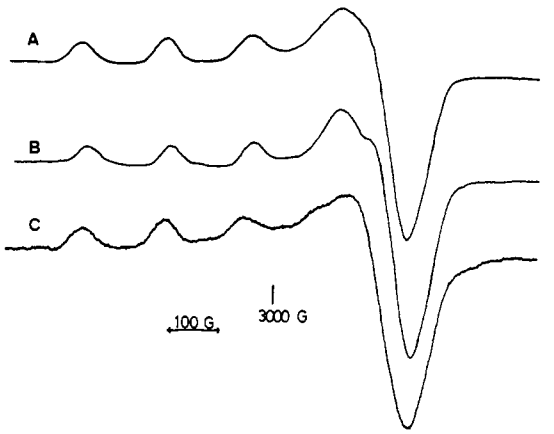


FIGURE 6: ESR spectra of native BSAO (A), native BSAO treated with benzylamine under anaerobic conditions (B), and native BSAO treated with phenylhydrazine (C) at 77 K. The solvent was 0.2 M phosphate buffer (pH 7.2). The protein concentrations were 1.59×10^{-4} (A), 2×10^{-4} (B), and 3.0×10^{-4} M (C). The concentrations of benzylamine (B) and phenylhydrazine (C) were 3.2×10^{-2} and 3.0×10^{-4} M, respectively.

gradually from yellowish pink to colorless, exhibiting a quite similar ESR signal with the spin Hamiltonian parameters of $g_{\parallel} = 2.31$, $g_{\perp} = 2.06$, and $A_{\parallel} = 162$ G (Figure 6B). The signal intensity calibrated by a Li-TCNQ marker of Figure 6A was not any different from that of Figure 6B. The ESR studies of native and amine-treated BSAO indicate that virtually no reduction of Cu(II) occurred other than a slight variation in the ESR parameters. Under aerobic conditions, the colorless reduced BSAO immediately turned pink and gave the ESR signal as represented in Figure 6A. This characteristic behavior of amine oxidase has also been reported by many investigators (Yamada et al., 1963; Buffoni et al., 1968; Lindström et al., 1973; Mondovi et al., 1967).

The ESR spectrum ($g_{\parallel} = 2.31$, $g_{\perp} = 2.08$, and $A_{\parallel} = 156$ G) of BSAO modified with 10 equiv of phenylhydrazine is given in Figure 6C. The yellow BSAO sample treated with 4 equiv of phenylhydrazine also exhibited the same kind of ESR signal for Cu(II). As is apparent from Figure 6, these ESR signals are axial with $g_{\parallel} > g_{\perp} \sim 2$, indicating that the Cu(II) ions in these proteins have tetragonal geometries.

Discussion

Prosthetic Groups. In previous papers (Suzuki et al., 1981, 1982), we pointed out that there is an organic chromophore (CHR) from which the pink color of BSAO stems in BSAO. Especially, the results of the spectroscopic studies on Co(II)-substituted BSAO suggested that CHR displays a positive CD peak at around 370 nm and a negative CD band at ~440 nm (Suzuki et al., 1981). As represented in Figures 1, 3, and 4, native BSAO, Ni(II)BSAO, and Ni-depBSAO also have similar optical absorption and CD bands in the region 300–500 nm, clearly indicating that these bands are not concerned with metal ions but with the organic chromophore. However, the absorption maximum of Ni-depBSAO (λ_{max} = 470 nm) slightly shifts to a longer wavelength with the incorporation of metal ions: Cu(II)BSAO (λ_{max} = 476 nm), Ni(II)BSAO (λ_{max} = 484 nm), and Co(II)BSAO (λ_{max} = 470 nm). Concomitantly, absorption magnitude also increases with the incorporation of each metal ion. The CD spectral behavior also seems to be in good accordance with the optical absorption behavior.

Thus Cu(II), Co(II), and Ni(II) ions are apparently closely related to the chromophore. These metal ions are supposed to facilitate oxidation of the chromophore. The red shift and

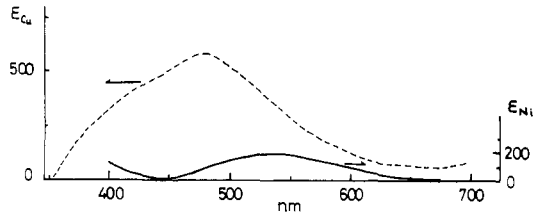


FIGURE 7: Difference spectra of native BSAO [broken line, native BSAO (Figure 1) minus Ni-depBSAO (Figure 4)] and Ni(II)BSAO [solid line, Ni(II)BSAO (Figure 3) minus Ni-depBSAO (Figure 4)]. The ϵ_{Cu} or ϵ_{Ni} was expressed per mole of copper ion or nickel ion, respectively.

increasing magnitude of the chromophore could be attributed to some conformational change in the presence of transition metal ions. After native BSAO, Ni(II)BSAO, and Ni-depBSAO were treated with benzylamine under N_2 atmosphere, the absorption peaks at around 470–480 nm completely disappeared. These spectral data indicate that colored BSAO has the oxidized form of CHR, while amine-treated colorless BSAO has the reduced one irrespective of the presence or absence of metal ion. The oxidized and the reduced CHR's are abbreviated hereinafter as CHR_{ox} and CHR_{red}, respectively. Therefore, the absorption and CD spectra of Cu-depBSAO shown in Figure 2 are actually those of CHR_{red}. The CHR_{red} in Cu-depBSAO does not change to a pink color (CHR_{ox} form) even under aerobic conditions, being easily oxidized upon the procedure of dialysis of Cu-depBSAO against buffer solution containing Cu(II), Co(II), or Ni(II) ion. On the other hand, the reaction of Cu-depBSAO with $[Fe(CN)_6]^{3-}$ is extremely slow and gave an unidentified ESR signal which is characteristic of an organic radical ($g = 2$). Since the oxidation of flavin with $[Fe(CN)_6]^{3-}$ is known to occur very rapidly, BSAO could not be a flavoprotein. Taking into account the fact that Ni(II) is not an effective oxidizing agent, we consider that the coloration of Cu-depBSAO results from a conformational change of CHR, which is accompanied by the incorporation of metal ion [Cu(II), Co(II), or Ni(II)] at the proper site to make easier the subsequent oxidation of CHR with O_2. In other words, the binding of the metal ion at the active site facilitates the oxidation of CHR. Figure 7 shows the difference between the electronic absorption spectrum of native BSAO (Figure 1) or Ni(II)BSAO (Figure 3) and that of Ni-depBSAO (Figure 4). The difference spectrum (broken line in Figure 7) between native BSAO and Ni-depBSAO exhibits an absorption maximum at ~480 nm (ϵ = 600 M^{-1} cm^{-1}), which might be assigned to a charge-transfer transition (CT transition) of ligand → Cu(II), as observed in the electronic absorption spectrum of galactose oxidase (Hamilton, 1981). Thus the CD peaks at 650 (Figure 1) and 800 nm (Suzuki et al., 1980) are considered to be due to the d–d transitions of Cu(II), while the negative shoulder band at ~500 nm may be attributed to the unknown CT transition described above. The absorption band at 540 nm in the difference spectrum of Ni(II)BSAO and Ni-depBSAO (solid line in Figure 7) is probably assigned to a d–d transition of Ni(II) ion, which was observed as a negative CD band at ~500 nm (Figure 3). The magnitude of the extinction coefficient per Ni(II) ion (ϵ_{Ni} = 175 M^{-1} cm^{-1}) at 540 nm is consistent with the tetrahedral geometry of Ni(II) ion (Johnson & Schachman, 1980; Rosenberg et al., 1975, and references cited therein).

The addition of phenylhydrazine to native BSAO, Ni(II)-BSAO, and Ni-depBSAO resulted in marked changes in the electronic absorption spectra of the proteins (Figure 5). Upon addition of phenylhydrazine to native BSAO and Ni(II)BSAO, the mixtures change immediately from pink to yellow to give quite similar optical and CD spectra (Figure 5A,B). However, the absorption and CD spectra (Figure 5C) of the phenylhydrazine derivative of Ni-depBSAO are distinctly different from those in Figure 5A,B. These results suggest that the Ni(II) ion may be located at the site for Cu(II) of BSAO and the presence of a metal ion such as Cu(II) and Ni(II) affects the spectroscopic behavior of phenylhydrazine-bound CHR. On the other hand, the reaction mixture of Cu-depBSAO (CHR_{red}) and phenylhydrazine shows a different absorption maximum (λ_{max} = 417 nm; ϵ = 3300 M^{-1} cm^{-1}) and reflects the structural differences between CHR_{red} and CHR_{ox} treated with phenylhydrazine. This spectral behavior induced by the reaction of phenylhydrazine again indicates that the electronic state of the CHR is greatly influenced by the presence of metal ions.

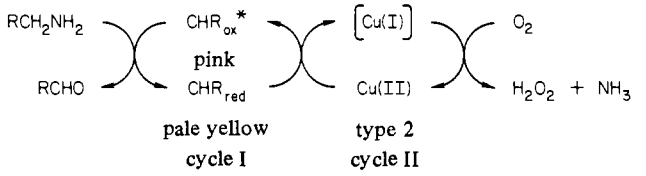
The structure of the chromophore has not yet been elucidated. Although the presence of pyridoxal phosphate as a chromophore in some amine oxidases was first postulated by many authors (Lindström & Pettersson, 1974; Mondovi et al., 1968; Finazzi-Agrò et al., 1977; Yamada & Yasunobu, 1963), very little information has been available concerning the characterization of this cofactor in the resting enzymes.

In addition, there appeared many investigations against the presence of pyridoxal phosphate (Inamasu & Yasunobu, 1974; Hill, 1967; Berg & Abeles, 1978), and the present spectral behavior of the CHR seems to be entirely different from that of pyridoxal phosphate whenever the environmental variation is taken into account. The ligating groups of Cu(II) in BSAO have been supposed to consist of at least two imidazole nitrogen atoms and some oxygen atoms (oxygens of water and/or carboxylate groups) according to the investigations of Co(II)BSAO (Suzuki et al., 1981) and copper-modified BSAO (Suzuki et al., 1982). The relationship between the ESR parameters $g_\parallel$ and $A_\parallel$ for BSAO also supports that the ligands to the Cu(II) ion might be both nitrogen and oxygen atoms, since these values fall in the region where the ligands around Cu(II) ion are two nitrogens and two oxygens in the $g_\parallel - A_\parallel$ profile reported by Peisach & Blumberg (1974). As to the coordination of sulfur atoms around the Cu(II) ion, the spectroscopic data of Co(II)BSAO (Suzuki et al., 1981) and the present result of the difference spectrum in Figure 7 diminish the relevancy of the previous evidence that the Cu(II) ion in BSAO might be bound by one or two sulfur atoms of cysteine or methionine because of the presence of some CD peaks between 350 and 600 nm (Suzuki et al., 1980). The magnetic circular dichroism spectrum of Co(II)BSAO suggested that no thiol group of cysteinyl residue is coordinated around Co(II) (Suzuki et al., 1981). In addition, the difference spectrum of native BASO and Ni-depBSAO (CHR_{ox}) as illustrated by the broken line in Figure 7 displayed no intense absorption band which is attributed to a S → Cu(II) CT transition in a square-planar Cu(II) system in the region 340–400 nm (Schugar et al., 1976; Amundsen et al., 1977; Nikles et al., 1979; Sakurai et al., 1981).

When benzylamine was added to native BSAO under anaerobic conditions, the intensity of the ESR signal was not significantly reduced, and only small changes in the shape of the spectrum and parameters were observed (Figure 6B). This finding means that the reduction of CHR_{ox} with benzylamine does not affect the copper valence but induces a perturbation at the copper(II) site. The environmental and the conformational changes of protein on binding of the substrate, which are observed as small changes of the ESR parameters, might promote the accessibility of an O_2 molecule to a Cu(II) ion.

The ESR signal shown in Figure 6C and its parameters indicate that they are slightly different from those of native BSAO. These phenomena seem to reflect the occurrence of conformational changes with the binding of phenylhydrazine to CHR_{ox} at the active site, rather than the direct coordination of phenylhydrazine to the Cu(II) ion. The ESR spectrum in Figure 6C is appreciably similar to that of pig plasma amine oxidase treated with phenylhydrazine (Lindström & Pettersson, 1973).

Reaction Mechanism of BSAO. On the basis of the previous kinetic studies (Malmström et al., 1975) and our present experiments, we propose the following reaction scheme for this enzyme:



where the asterisk indicates a resting state. Under aerobic conditions, two electrons transfer from substrate to oxygen molecule through the CHR (cycle I) and copper ion (cycle II), although it is not decided whether the reduction of O_2 occurs in a one- or two-electron mechanism, and the existence of Cu(I) ion in the reaction course remains unknown. In BSAO, the CHR and Cu(II) ion as a set may correspond to the flavin–cofactor of mitochondrial amine oxidase. Here, we point out the important contribution of Cu(II) in retaining the conformation of the active site to facilitate the oxidation of CHR_{red} with O_2. The extremely low activities of Ni(II)BSAO and Ni-depBSAO can be elucidated on the basis of the prohibited turnover of cycle I.

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Registry No. BSAO, 9001-53-0; Cu, 7440-50-8; Ni, 7440-02-0; Co, 7440-48-4; Zn, 7440-66-6.

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