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The role of copper in bovine serum amine oxidase

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Summary. The role of copper in bovine serum amine oxidase was investigated by studying the effect of copper-binding inhibitors on the reactions of the pyrroloquinoline quinone carbonyl and on the reaction with oxygen. Hydrazines and hydrazides were used as carbonyl reagents and one of the hydrazines, benzylhydrazine, which was found to behave as a pseudo-substrate, was used to probe the reaction with oxygen. The presence of *N,N*-diethyldithiocarbamate, a chelator that binds copper irreversibly, did not prevent the reactions at the carbonyl, but slowed down their rate and modified the conformation of the adducts. The same happened to the reaction with oxygen, which was slowed down but not abolished. Copper, which was never seen in the reduced state, thus appears to control all reactions without being directly involved in the binding of either hydrazines or oxygen. The enzyme functionality was in fact preserved upon substitution of copper with cobalt. The specific activity of the cobalt-substituted enzyme was only reduced to about 40% the native amine oxidase value. This is the first case so far in which the role of copper can be performed by a different metal ion.

Key words: Copper amine oxidase – Cobalt substitution – Pyrroloquinoline quinone (PQQ) – Copper-binding inhibitors – Carbonyl-binding inhibitors

Introduction

Bovine serum amine oxidase (BSAO; EC 1.4.3.6) catalyzes the two-electron oxidation of primary amines by molecular oxygen with the formation of the corresponding aldehyde, ammonia, and hydrogen peroxide. The enzyme molecule is made up of two subunits of identical M_r of 90000 (Achee et al. 1968) and contains as prosthetic groups two Cu(II) ions (Suzuki et al. 1986) and at least one pyrroloquinoline quinone (PQQ) molecule, independently identified by two research groups (Ameyama et al. 1984; Lobenstein-Verbeek et al. 1984). The enzyme shows highest oxidase activity towards biogenic amines (spermine, spermidine) and benzylamine (Tabor et al. 1954). It is inhibited by two types of molecules, one type reacting with a carbonyl group of PQQ and the other with the copper ions. Substances such as hydroxylamine, hydrazine, and semicarbazide belong to the first type, while copper ligands such as azide, cyanide, *N,N*-diethyldithiocarbamate (DCC) belong to the second one.

The similar enzyme from pig plasma was shown to react by a ping-pong mechanism (Pettersson 1985) involving in the first phase stepwise reduction of the enzyme by the substrate with transfer of the amino group to PQQ and production of the aldehyde. In the second phase the reduced enzyme is reoxidized by oxygen with formation of hydrogen peroxide and of the imino derivative, that subsequently hydrolyzes to native enzyme and ammonia. Copper was found to catalyze the redox steps of both phases, which are slowed down by copper-binding anions (Olsson et al. 1978) while only the reoxidation phase is abolished by the absence of metal ions (Suzuki et al. 1983) and even of only one of them (Suzuki et al. 1986; Morpurgo et al. 1987).

The way in which the copper ions interact with oxygen and with PQQ itself is, however, not yet known. The problem was approached in our laboratory through the study of the effect of modifications of the copper sites on the reactions at the PQQ site, i.e. of the effect of Cu-binding inhibitors and of Cu substitution with other metals, Co(II) in particular, on the binding to PQQ of inhibitors and of a pseudo-substrate.

Adducts of BSAO with hydrazines and hydrazides

The simplest inhibitors of the two types are hydrazine and semicarbazide, respectively, which react with BSAO in 1:1 ratio and like the other compounds listed

Abbreviations. BSAO, bovine serum amine oxidase; DDC, *N,N*-diethyldithiocarbamate; PQQ, pyrroloquinoline quinone

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Table 1. Absorption bands of BSAO-hydrazide and -hydrazine adducts

Adduct	λ (nm)	ε ($M^{-1}\,cm^{-1}$)
Semicarbazide	360	22700
Benzohydrazide	359	28000
Isoniazide	347	22500
4-(Pyrrol-1-yl)benzohydrazide	362	23700
Hydrazine	330	20200
	410	8400
Benzylhydrazine[a]	405	22000
Phenylhydrazine	446	38000
4-Chlorophenylhydrazine	450	42000
2,4-Dinitro-phenylhydrazine	470	44000
9-Acridino-hydrazine[b]	495	86000
	525	98000

Measurements were made according to Morpurgo et al. (1988)
[a] Measured immediately after addition
[b] Lamkin et al. (1988)

in Table 1, form an intense absorption band in the near-ultraviolet region (Morpurgo et al. 1988). The time of reaction varied greatly depending on the type of inhibitor. In particular, the great majority of hydrazines reported in Table 1 reacted within the mixing time, while the hydrazides had a longer reaction time. The data of Table 1 also show that the absorption band around 360 nm of the hydrazide adducts is almost independent of the reagent, from the simple semicarbazide to the more sophisticated compounds, while the band of the hydrazine adducts shows large variability of maximum wavelength and intensity, which increase together along the series of compounds. This was taken to mean that in the first group the transition is localized on the PQQ molecule, which is bound to the hydrazide with a C=N linkage, while in the second one the transition is delocalized on the hydrazine aromatic system because of conjugation to PQQ through an azo bridge. The latter situation implies a two-electron shift towards PQQ, namely its reduction to hydroquinone.

All above reactions became extremely slow when they were carried out in the presence of DDC, which binds the copper ions of BSAO firmly producing an absorption band at 380 nm and a typical EPR spectrum (Suzuki et al. 1982; Morpurgo et al. 1987). Moreover the presence of DDC greatly modified the shape and intensity of the absorption band at 445 nm of the BSAO-phenylhydrazine adduct (Morpurgo et al. 1987). These data provide two clear indications: (a) that the copper ions and PQQ carbonyls are not very close to each other since they can simultaneously bind the respective inhibitors; (b) that a modification of the copper site(s), such as DDC binding, affects the PQQ site, resulting in a change of conformation of the phenylhydrazine adduct.

Reactions of BSAO with benzylhydrazine

The absorption band at 405 nm, which was immediately formed on addition of benzylhydrazine to BSAO (Table 1), decayed within about 15 min both in air and in anaerobiosis. In air it was replaced by a band at 335 nm with a shoulder at 410 nm, in anaerobiosis by a band at 355 nm, which converted to the previous one on admission of air into the solution (Morpurgo et al. 1989). Also the latter species decayed within about 8 h, while the protein recovered enzymatic activity. The mechanism shown in Fig. 1 is based on the catalytic reaction pathway proposed by Pettersson (1985) for the pig plasma enzyme and on the observation that the intermediate absorbing at 335 nm and 410 nm was also formed in the direct reaction of BSAO with hydrazine (Table 1 and Fig. 1). The formation of a hydroperoxo intermediate in the reaction with oxygen was proposed on a speculative basis. Oxygen is expected to react at the copper site(s), as in other copper oxidases, and actually copper-binding inhibitors were found to be competitive against oxygen and uncompetitive against the amine (Olsson et al. 1978; Barker et al. 1979; Dooley and Cotè 1985). However, prior to oxygen binding, copper reduction is expected to occur (Al-Arab and Hamilton 1986) which was never observed, either in the present reaction (Morpurgo et al. 1989) or in the enzymatic reaction of the pig plasma enzyme (Grant et al. 1978). Furthermore the overall process was considerably slowed down, but not abolished, by the presence in the reaction medium of DDC, which binds copper irreversibly (Morpurgo et al. 1987). Direct copper-oxygen binding seemed therefore improbable. The reaction of BSAO with benzylhydrazine was stopped halfway, with the 355-nm-absorbing species as the final

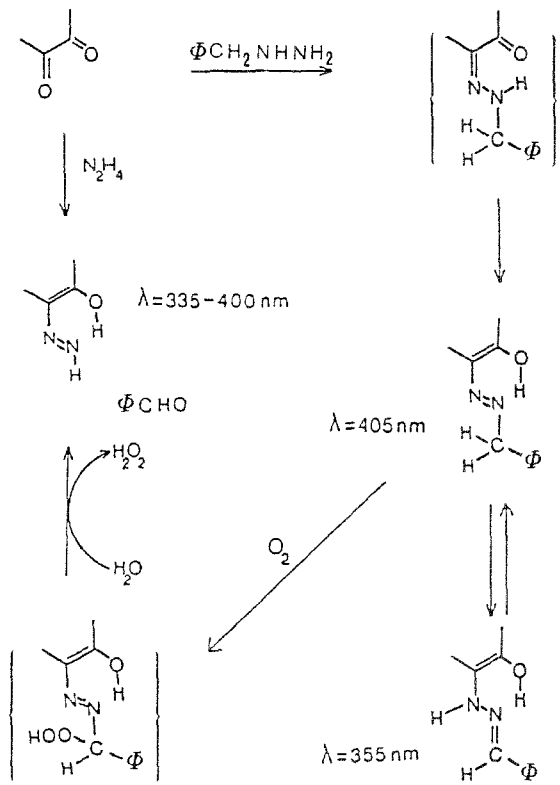

Fig. 1. Reactions of BSAO with benzylhydrazine and with hydrazine

Table 2. Properties of cobalt-substituted BSAO

Sample	Activity, A (% native)	Phenylhydrazine bound, PHy (mol/mol enzyme)	Cobalt (mol/mol enzyme)	ε_{405} ($M^{-1}\,cm^{-1}$)	A/PHy
1	13	0.33	—	31000	39
2	14	0.31	2.5	30000	45
3	24	0.50	2.0	30000	48

The enzymatic activity was measured spectrophotometrically at 25° C from the absorbance increase at 250 nm ($\varepsilon = 12500\ M^{-1}\,cm^{-1}$) produced by benzylamine oxidation. The specific activity of native samples was 0.28, 0.30, 0.42 μmol min^{-1} mg^{-1}, respectively. Enzyme concentration was measured at 280 nm using the absorption coefficient of the native sample of 1.74 mg^{-1} cm^{-1}. Cobalt was determined with a Perkin-Elmer spectrometer equipped with an HGA-400 furnace. ε_{405}, the absorption coefficient of the band at 405 nm formed on reacting Co-BSAO with benzylhydrazine, is referred to the concentration of active carbonyl groups given as A in the second column

reaction product, only by previous removal of at least one copper ion from the protein molecule (Morpurgo et al. 1989). The action of azide, a reversible inhibitor of BSAO, was qualitatively similar to that of DDC, in that it also stabilized the 355-nm-absorbing species. In the reaction scheme outlined in Fig. 1, azide binding is not competitive with the substrate, but stabilizes a form of the enzyme-substrate complex which is unreactive against oxygen and is in equilibrium with the reactive complex. A reaction mechanism for the amine oxidase enzymatic reaction, in which oxygen does not bind the copper ions, thus seems compatible with the behaviour of azide, competitive against oxygen (Olsson et al. 1978).

Cobalt-substituted BSAO

From the results described above, copper did not appear to have the usual redox role in the reactions catalyzed by BSAO. It rather seemed to affect the redox potential of PQQ and/or the pK of a group involved in the catalysis, a function that in principle could also be carried out by a different metal ion. For this reason the Co(II)-substituted BSAO prepared by Suzuki et al. (1983) and described by these authors to retain 13% of the native enzyme activity was re-examined in our laboratory. The procedure of Suzuki et al. proved to be reproducible. The apo-enzyme was first prepared by treatment with cyanide and it was found to be totally devoid of catalytic activity. Then it was reconstituted with $CoCl_2$, with 13–24% recovery of the native enzyme activity. These values are related to the amount of protein present as detected from the 280-nm absorbance. The copper content of the samples was $< 5\%$ and the cobalt content was about 2 mol ions/mol enzyme, as measured by atomic absorption spectroscopy (Table 2).

In order to determine the concentration of active carbonyl groups, Co(II)-reconstituted BSAO samples were titrated with phenylhydrazine. The reaction proceeded at a slower rate than in the native enzyme, but a similar absorption band was formed at 445 nm. A typical titration is reported in Fig. 2, which shows that only about 30% of the carbonyl groups expected on the basis of the protein absorbance at 280 nm were available for reaction. From this value an absorption coefficient of $38000\ M^{-1}\,cm^{-1}$ was calculated, which is identical with that of the native enzyme. In Table 2 are reported the results for samples from different protein batches.

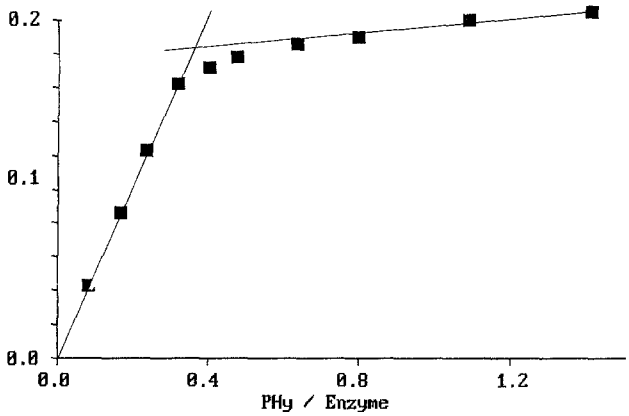

Fig. 2. Titration of Co-substituted BSAO with phenylhydrazine. 14 μM enzyme in 0.1 M phosphate pH 7.2. Values on the ordinate are absorbance at 445 nm

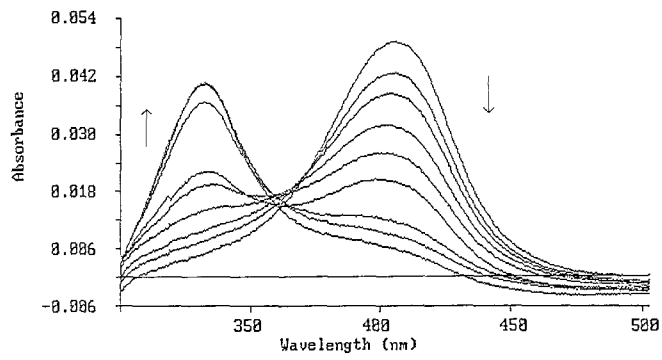

Fig. 3. Absorption difference spectra of Co-substituted BSAO during the reaction with benzylhydrazine. The spectra were recorded 0, 4, 8, 16, 27, 37, 80, 106, 128 min after benzylhydrazine addition. 5.2 μM enzyme in 0.1 M phosphate pH 7.2, 25° C

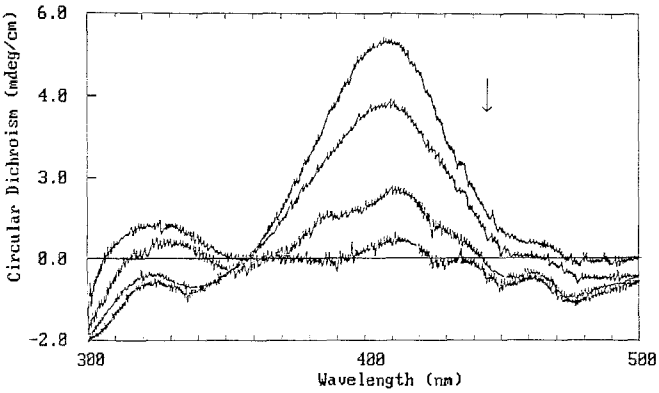

Fig. 4. CD difference spectra of Co-substituted BSAO during the reaction with benzylhydrazine. The spectra were recorded 0, 15, 45, 120 min after benzylhydrazine addition. 14 μM enzyme in 0.1 M phosphate pH 7.2, 25° C

When the samples were treated with benzylhydrazine a reaction pattern qualitatively similar to that of the native enzyme was found (Fig. 3). The band at 405 nm, which was formed immediately after benzylhydrazine addition, decayed in air to a species absorbing at 335 nm. As in the native enzyme, the lack of an isosbestic point indicates the presence of more than two species. The reaction was much slower than that of the native enzyme since the complete decay of the 405-nm band required more than 2 h, whilst 15–20 min were sufficient in native BSAO. The absorption coefficient of the band at 405 nm, as well as that of the positive CD band at 405 nm (Fig. 4), were comparable to the corresponding ones of the native copper enzyme, when referred to the concentration of active carbonyl groups obtained from the phenylhydrazine titration (Table 2).

Besides the lower reaction rate, substantial differences from native BSAO were found in the properties of the compound absorbing at 335 nm, which was previously assigned as the hydrazine-BSAO adduct. It showed higher intensity, but a less intense shoulder at 410 nm, a completely different CD spectrum (Fig. 4) and the subsequent decay was not followed by reactivation and recovery of the original absorbance in the 480 nm region, where oxidized PQQ absorbs. These same properties, however, were also peculiar to hydrazine-reacted Co-BSAO, so that the assignment is still pertinent. The reasons for the different behaviour are not clear.

Conclusions

Albeit preliminary, the data above show that the samples of Co(II)-reconstituted BSAO were heterogeneous and contained a substantial proportion of molecules (50–70%) in which the carbonyl group was not available for reaction with phenylhydrazine. When the low oxidase activity of the samples is related to the concentration of molecules carrying a reactive carbonyl, a quite high value is obtained, about half that of the copper enzyme. This confirms the above suggestion for a role of copper in the catalysis that is different from the usual one in copper enzymes. This is also the first case in which substitution of copper does not substantially impair the enzymatic activity.

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References

Achee FM, Chervenka CH, Smith RA, Yasunobu KT (1968) Amine oxidase. XII. The association and dissociation, and number of subunits of beef plasma amine oxidase. Biochemistry 7:4329–4335

Al-Arab MM, Hamilton GA (1986) Possible model reaction for some amine oxidases. Kinetics and mechanism of the copper(II)-catalyzed autoxidation of some diaminouracils. J Am Chem Soc 108:5972–5978

Ameyama M, Hayashi M, Matsushita K, Shinagawa E, Adachi O (1984) Microbial production of pyrroloquinoline quinone. Agric Biol Chem 48:561–565

Barker R, Boden N, Cayley G, Charlton SC, Henson R, Holmes MC, Kelly ID, Knowles PF (1979) Properties of cupric ions in benzylamine oxidase from pig plasma as studied by magnetic resonance and kinetic methods. Biochem J 177:289–302

Dooley DM, Cotè CE (1985) Copper(II) coordination chemistry in bovine plasma amine oxidase: azide and thiocyanate binding. Inorg Chem 24:3996–4000

Grant J, Kelly I, Knowles P, Olsson J, Pettersson G (1978) Changes in the copper centres of benzylamine oxidase from pig plasma during the catalytic cycle. Biochem Biophys Res Commun 83:1216–1224

Lamkin MS, Williams TH, Falk MC (1988) Excitation energy transfer study of the spatial relationship between the carbonyl and metal cofactors in pig plasma amine oxidase. Arch Biochem Biophys 261:72–79

Lobenstein-Verbeek CL, Jongejan JA, Frank J, Duine JA (1984) Bovine serum amine oxidase: a mammalian enzyme having covalently bound PQQ as prosthetic group. FEBS Lett 170:305–309

Morpurgo L, Agostinelli E, Befani O, Mondovi' B (1987) Reactions of bovine serum amine oxidase with *N,N*-diethyldithiocarbamate. Biochem J 248:865–870

Morpurgo L, Befani O, Sabatini S, Mondovi' B, Artico M, Corelli F, Massa S, Stefancich G, Avigliano L (1988) Spectroscopic studies of the reaction between bovine serum amine oxidase (copper-containing) and some hydrazides and hydrazines. Biochem J 256:565–570

Morpurgo L, Agostinelli E, Muccigrosso J, Martini F, Mondovi' B, Avigliano L (1989) Benzylhydrazine as a pseudo-substrate of bovine serum amine oxidase. Biochem J 260:19–25

Olsson B, Olsson J, Pettersson G (1978) Effects on enzyme activity of ligand binding to copper in pig-plasma benzylamine oxidase. Eur J Biochem 87:1–8

Pettersson G (1985) Plasma amine oxidase. In: Mondovi' B (ed) Structure and functions of amine oxidases. CRC Press, Boca Raton FL, pp 105–120

Suzuki S, Sakurai T, Nakahara A, Oda O, Manabe T, Okuyama T (1982) Copper binding site in serum amine oxidase treated with sodium diethyldithiocarbamate. Chem Lett 487–490

Suzuki S, Sakurai T, Nakahara A, Manabe T, Okuyama T (1983) Effect of metal substitution on the chromophore of bovine serum amine oxidase. Biochemistry 22:1630–1635

Suzuki S, Sakurai T, Nakahara T (1986) Roles of the two copper ions in bovine serum amine oxidase. Biochemistry 25:338–341

Tabor CN, Tabor H, Rosenthal SM (1954) Purification of amine oxidase from beef plasma. J Biol Chem 208:645–665

Yamada H, Yasunobu KT (1962) Monoamine oxidase - I. Purification, crystallization and properties of plasma monoamine oxidase. J Biol Chem 237:1511–1516