# Reconstitution of $Cu^{2+}$-depleted bovine serum amine oxidase with $Co^{2+}$*

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Two different $Cu^{2+}$-depleted derivatives of bovine serum amine oxidase (BSAO) have recently been prepared, which contain about 0.5 mol/dimer of phenylhydrazine-reactive topa quinone (TPQ) cofactor and, depending on the reagents used, about 0.2 or 0.7 residual $Cu^{2+}$/dimer [Agostinelli, De Matteis, Sinibaldi, Mondovì and Morpurgo (1997) Biochem. J. **324**, 497–501]. The benzylamine oxidase activity of both derivatives was $< 5\,\%$ and increased up to $\approx 20\,\%$ on incorporation of $Co^{2+}$, irrespective of the residual $Cu^{2+}$ content, which was unaffected by the treatment according to atomic absorption and ESR spectroscopy. The residual $Cu^{2+}$ ions appeared to be distributed one per dimer and to be bound to inactive subunits, whereas $Co^{2+}$ was bound to active subunits. The change in the active site had an appreciable influence on the kinetic behaviour. With several amines, the kinetic parameters, $K_m$ and $k_c$, measured for $Co^{2+}$-BSAO were different from those for native BSAO. This excludes the possibility that the catalytic activity was due to residual $Cu^{2+}$. Furthermore, $Co^{2+}$ restored to nearly native level the intensity of the TPQ 480 nm band and the reactions with phenylhydrazine or benzylhydrazine, which had been slowed down or abolished, respectively, in $Cu^{2+}$-depleted samples. The CD spectrum, measured for the derivative with low $Cu^{2+}$ content, was compatible with $Co^{2+}$ binding to the copper site. The amine oxidase activity of the $Co^{2+}$ derivative, which cannot form a semiquinone radical as an intermediate of the catalytic reaction, strongly suggests that the $Cu^{+}$-semiquinone is not an obligatory intermediate of BSAO catalytic pathway.

## INTRODUCTION

$Cu^{2+}$-containing amine oxidases are mostly homodimers with subunits of 75–90 kDa [1,2]. The active site also contains topa quinone (TPQ) [3], an organic cofactor derived from the copper-catalysed oxidation of a tyrosine residue [4,5]. One or two TPQ per dimer are titrated by phenylhydrazine and similar inhibitors in amine oxidases from different sources [6–10]. In bovine serum amine oxidase (BSAO), phenylhydrazine reacts with one TPQ per dimer, whereas other reagents rapidly bind one TPQ, then slowly bind a second one [8]. The latter reaction is restricted to a fraction of BSAO molecules and to ligands that either form a hydrophobic bond together with the covalent one, or form an adduct that changes conformation on standing [11]. A different reactivity of the subunits was recently reported by a X-ray study on *Escherichia coli* amine oxidase [12]. One particular subunit fully reacted with 2-hydrazinopyridine after the crystals were incubated for 24 h with the inhibitor, whereas the other one remained largely unreacted. Saturation of both subunits was achieved after 30 days incubation.

Similar to other $Cu^{2+}$-amine oxidases, BSAO operates by a Ping-Pong mechanism, via a reduction step, which converts quinone and amine into amino-quinol and aldehyde with a transamination-like mechanism, and a reoxidation step, in which molecular oxygen restores the quinone with release of ammonia and hydrogen peroxide [13,14]. Copper plays a role in both the formation of reduced species and their reoxidation [15] and, in particular, appears to control the transfer of substrates from a hydrophobic binding site near the protein surface [16] to the deeply buried active site [17,18]. In $Cu^{2+}$-depleted BSAO, the reaction of TPQ with amines and substrate analogues is exceedingly slow or does not occur at all [16], and the quinol reoxidation to TPQ is inhibited [19,20].

In anaerobic conditions, the reaction with amines gives rise to the equilibrium $Cu^{+}$-semiquinone $\rightleftharpoons$ $Cu^{2+}$-quinol, which depends on enzyme source and temperature [21]. For all examined proteins, the formation of $Cu^{+}$-semiquinone causes in the room-temperature ESR spectrum a decrease in the copper signal and the appearance of a radical with an identical hyperfine structure [21]. The same radical was detected during the copper-catalysed generation of TPQ from tyrosine [5]. This would imply the direct participation of copper in the two redox processes, namely tyrosine and substrate oxidation. However, a very small signal was observed in BSAO [21], and, moreover, $Co^{2+}$ was able to restore in the $Cu^{2+}$-depleted protein some amine oxidase activity [19,22]. This suggested that BSAO metal, whether Cu or Co, is not directly involved in amine oxidation, but has a structural function, later confirmed by the closely similar behaviour of $Cu^{2+}$- and $Co^{2+}$-BSAO in differential scanning calorimetry [23].

The conclusion that $Co^{2+}$-BSAO is catalytically competent was questioned by Dooley et al. [24], who correlated the activity of $Co^{2+}$-containing samples with the presence of residual or adventitious copper. The data from Table 1 in [24] are rather scattered, as they were collected in a variety of experimental conditions. When they are plotted as % activity versus % Cu, it becomes quite evident that many experimental points fall above the theoretical straight line. The activity in excess is especially significant, since $Cu^{2+}$-depleted BSAO is usually less active than predicted by the $Cu^{2+}$ content [16,20].

The present paper describes the results of a re-examination of the issue based on the study of the effects of $Co^{2+}$ incorporation into $Cu^{2+}$-depleted BSAO containing $\approx 0.5$ TPQ per dimer in oxidized state. Two such derivatives, with different residual $Cu^{2+}$ content, were prepared in the presence of cyanide by using different reducing agents. Most of the $Cu^{2+}$ was removed with a strong unspecific reductant such as dithionite; much less was removed with the substrate benzylamine, which mostly reacts with one subunit/dimer [16]. Both derivatives had low oxidase

Abbreviation used: BSAO, bovine serum amine oxidase.

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* This paper is dedicated to Professor Alessandro Ballio on the occasion of his 75th birthday.

activity, but the fraction of molecules having oxidized organic cofactor preserved some reactivity with substrates and inhibitors, different from the fully reduced $Cu^{2+}$-depleted BSAO previously available [22]. This allowed a more precise evaluation of $Co^{2+}$ catalytical efficiency and of the metal role in the reactions.

## EXPERIMENTAL

BSAO was purified from bovine blood as described previously [25]. The purified protein moved as a single band on SDS/PAGE, and all samples employed had a minimum specific benzylamine oxidase activity of 0.3 IU/mg ($\mu$mol of substrate oxidized/min), assayed at 25 °C by monitoring the formation of benzaldehyde from the 250 nm absorbance ($\epsilon$ 12500 $M^{-1}\cdot cm^{-1}$) [26]. The protein concentration was measured from the 280 nm absorbance, using $E$ 1.74 $l\cdot g^{-1}\cdot cm^{-1}$ [20].

$Cu^{2+}$-depleted BSAO containing $\approx 0.5$ mol of oxidized cofactor/dimer was prepared by the procedure described previously [16], namely by adding dithionite or benzylamine as reducing agents to solutions of the KCN-reacted protein in anaerobic conditions. The derivatives contained $\approx 10\,\%$ and $\approx 35\,\%$ residual $Cu^{2+}$ per dimer respectively. The content of reactive TPQ was measured with phenylhydrazine. Native BSAO was immediately inhibited by 1 mol/dimer with formation of a band at 447 nm, $\epsilon$ 41500 $M^{-1}\cdot cm^{-1}$ [8], whereas the $Cu^{2+}$-depleted protein required about 8 h incubation with phenylhydrazine in excess of 1 mol/dimer to form a band at 434 nm, $\epsilon$ 32000 $M^{-1}\cdot cm^{-1}$ [11]. Reconstitutions were carried out at 5 °C, either by 48 h incubation with stoichiometric $CuCl_2$ or by 48 h dialysis against 2.0 mM $CoCl_2$, in 0.1 M Tris/HCl, pH 7.2. In every case, the samples were eventually dialysed against 0.5 mM EDTA and two changes of 0.1 M potassium phosphate buffer. The treatment did not always remove unspecifically bound $Co^{2+}$, so the final overall metal content was $> 2$. However, small amounts added to native BSAO did only show minor inhibitory effects.

The content of $Cu^{2+}$ and $Co^{2+}$ was measured by atomic absorption spectrometry using a Perkin–Elmer apparatus equipped with an HGA-400 graphite furnace. The paramagnetic copper content was also calculated from X-band ESR spectra, with 1.81 mM Cu-EDTA as standard. The spectra were recorded on a Varian E9 spectrometer interfaced with a Stelar Prometheus Data Acquisition system. Optical spectra were recorded on a Lambda 9 Perkin–Elmer spectrophotometer equipped with a Peltier thermostat; CD spectra were recorded on a Jasco spectropolarimeter with thermostatted cell holder.

Steady-state kinetics were carried out at 25 °C, in 0.1 M potassium phosphate buffer, pH 7.2. Initial velocities were measured by the consumption of oxygen in air-saturated solutions (0.24 mM oxygen) with a YSI oxygraph model 53, equipped with a Clark electrode, or spectrophotometrically in the case of benzylamine. The data were fitted to the equation $v = V_{max}[S]/(K_m + [S])$ by non-linear regression analysis using Microcal Origin 3.5 software. Substrate solutions were freshly prepared before use. All chemicals were commercial reagents of analytical grade, used without further purification, except phenylhydrazine, which was recrystallized from ethanol.

## RESULTS AND DISCUSSION

### Reconstitution with $Co^{2+}$ of BSAO depleted of $Cu^{2+}$ by cyanide and dithionite

$Cu^{2+}$-depleted BSAO, prepared by the cyanide/dithionite method [16] and containing $\approx 0.5$ TPQ per dimer in oxidized form and $\approx 0.2$ $Cu^{2+}$ per dimer, recovered 95 % of metal content and activity on 48 h incubation at room temperature with 2 $Cu^{2+}$ per dimer, but recovered a maximum 11 % of benzylamine oxidase activity on incubation with 2 $Co^{2+}$ per dimer (Table 1). In these conditions 1.3 $Co^{2+}$ per dimer were bound. A larger amount of $Co^{2+}$, 1.9 per dimer, was detected after 48 h dialysis at 5 °C against 2.0 mM $Co^{2+}$, while the activity increased up to 19 %. This value is nearly twofold that expected on the basis of residual $Cu^{2+}$ content. In the derivative, the TPQ 480 nm band recovered almost native intensity (results not shown), and the reaction with phenylhydrazine, which was slow and partial in $Cu^{2+}$-depleted BSAO, recovered nearly native features (Figure 1 and Table 1). Subsequent incubation with $Cu^{2+}$ had no effect on the properties of samples in which $Co^{2+}$ had been incorporated by dialysis (results not shown), but caused a substantial increase of bound $Cu^{2+}$, enzymic activity, and reactivity with phenylhydrazine in samples previously incubated with 2 $Co^{2+}$ per dimer (Table 1). Nearly all of the $Cu^{2+}$ was bound on incubation with 1 $Cu^{2+}$ per

**Table 1 BSAO depleted of $Cu^{2+}$ by KCN and sodium dithionite (D) or benzylamine (B) and reconstituted with $Cu^{2+}$ or $Co^{2+}$**

The catalytic activity was measured with benzylamine, the content of $Cu^{2+}$ and $Co^{2+}$ per dimer was measured by atomic absorption spectrometry, and the reactive TPQ per dimer was measured from the band of the phenylhydrazine adduct at [a]447 nm, $\epsilon$ 41500 $M^{-1}\cdot cm^{-1}$, or [b]434 nm, $\epsilon$ 32000 $M^{-1}\cdot cm^{-1}$.

| BSAO | Activity (%) | $Cu^{2+}$ | $Co^{2+}$ | Reactive TPQ |
|---|---|---|---|---|
| Native | 100 | 2.0 | – | 1.00[a] |
| $Cu^{2+}$-depleted (D) | 2.5 | 0.22 | – | 0.48[b] |
| + 2 $Cu^{2+}$ | 95 | 1.9 | – | 1.00[a] |
| + 2 $Co^{2+}$ | 11 | 0.23 | 1.3 | 0.66[a] |
| + 2 $Co^{2+}$ + 1 $Cu^{2+}$ | 62 | 1.1 | 1.2 | 0.91[a] |
| + 2 $Co^{2+}$ + 2 $Cu^{2+}$ | 76 | 1.2 | 1.3 | 1.01[a] |
| + excess $Co^{2+}$ | 19 | 0.22 | 1.9 | 0.90[a] |
| $Cu^{2+}$-depleted (B) | 4.2 | 0.70 | – | 0.38[b] |
| + 0.5 $Cu^{2+}$ | 45 | 1.2 | – | 0.50[b] |
| + 1.0 $Cu^{2+}$ | 75 | 1.6 | – | 0.78[a] |
| + 1.4 $Cu^{2+}$ | 95 | 2.0 | – | 0.95[a] |
| + excess $Co^{2+}$ | 22 | 0.66 | 2.0 | 0.95[a] |

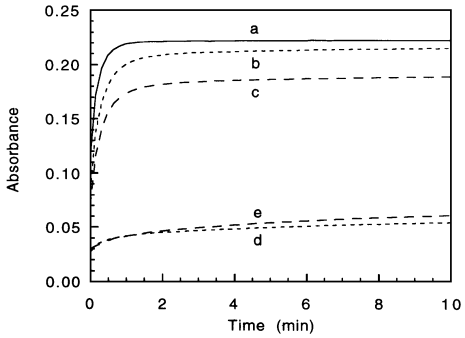


**Figure 1 Absorbance changes at 447 nm on reaction with phenylhydrazine of native, $Cu^{2+}$-depleted and $Co^{2+}$-reconstituted BSAO**

Protein (5.3 $\mu$M) was reacted at 25 °C with 9.0 $\mu$M phenylhydrazine: native BSAO (trace a); $Co^{2+}$-BSAO, reconstituted from the cyanide/benzylamine sample (trace b) or from the cyanide/dithionite sample (trace c); $Cu^{2+}$-depleted BSAO, with cyanide and benzylamine (trace d) or with cyanide and dithionite (trace e).

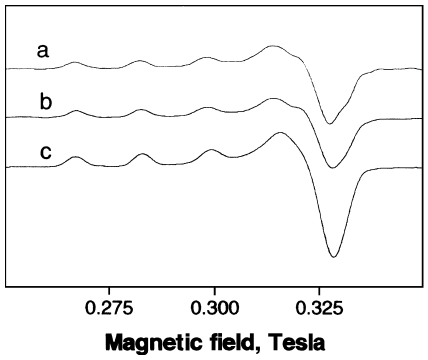


**Figure 2 X-band ESR spectra of native, $Cu^{2+}$-depleted (by cyanide and benzylamine) and $Co^{2+}$-reconstituted BSAO**

BSAO (50 $\mu$M): depleted of $Cu^{2+}$ by cyanide and benzylamine (trace a); reconstituted with $Co^{2+}$ by dialysis (trace b); native (trace c). Spectrum (c) was recorded at half-gain. Instrument settings: 9.15 GHz, 20 mW, 100 K.

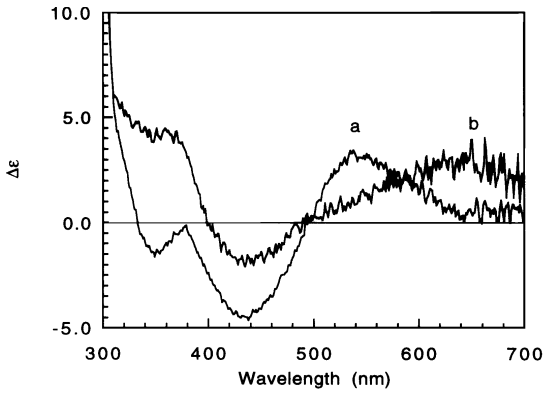


**Figure 3 CD spectra of native and $Co^{2+}$-reconstituted BSAO**

The spectra of $Co^{2+}$-BSAO reconstituted from the cyanide/dithionite-$Cu^{2+}$-depleted BSAO (trace a) and of native BSAO (trace b) were recorded at 25 °C on solutions containing 20 mg/ml protein in 0.5 cm pathlength cells. $\Delta\epsilon$ represents the ellipticity in degrees $M^{-1} \cdot cm^{-1}$.

dimer, with the same 50% increase in the enzymic activity as found in $Cu^{2+}$-depleted BSAO, whereas a second $Cu^{2+}$ caused a very small increase in both activity and bound $Cu^{2+}$ content (Table 1), being part of the sites occupied by $Co^{2+}$. The high $Co^{2+}$ excess necessary to saturate the sites shows that $Co^{2+}$ has lower affinity for the protein than $Cu^{2+}$ does. However, bound $Co^{2+}$ was not displaced on incubation with $Cu^{2+}$. Apparently metal exchange does not occur in the reoxidized protein.

### Reconstitution with $Cu^{2+}$ and $Co^{2+}$ of BSAO depleted of $Cu^{2+}$ by cyanide and benzylamine

Further information on the two metal sites relating to $Co^{2+}$ substitution was obtained from the $Cu^{2+}$-depleted BSAO prepared by using benzylamine as the reducing agent. This derivative had a high residual $Cu^{2+}$ content (0.7 ions/dimer); nevertheless, it was almost inactive [16]. On incubation with substoichiometric $Cu^{2+}$ amounts (Table 1), it recovered appreciably greater activity than dithionite-treated samples [16]. Increases of about 40, 70 and 90% were produced by addition of 0.5, 1.0 and 1.4 $Cu^{2+}$ per dimer respectively. This demonstrates that residual $Cu^{2+}$ was bound one per dimer to subunits either that were already inactive or that had been inactivated upon removal of the other $Cu^{2+}$.

Dialysis of benzylamine-treated BSAO against 2 mM $Co^{2+}$ induced 20% recovery of enzymic activity and a high recovery of the reactivity with phenylhydrazine (Table 1 and Figure 1). The close similarity of the ESR spectra before and after dialysis against $Co^{2+}$ (Figure 2) excludes the possibility that $Cu^{2+}$ displacement by excess $Co^{2+}$ or incorporation of adventitious $Cu^{2+}$ had occurred, and suggests that the $> 2$ total metal content (Table 1) is due to unspecifically bound $Co^{2+}$. The activity of $Co^{2+}$-dialysed samples apparently does not depend on the amount of residual $Cu^{2+}$, i.e. on whether benzylamine or dithionite was employed in the preparation. These data seem to imply that there are inactive subunits, which contain residual $Cu^{2+}$, and active subunits, where $Co^{2+}$ mostly binds. The lack of activity may be an intrinsic feature of a site or be induced by the change that occurred in the other subunit, $Co^{2+}$ substitution being equivalent to the absence of metal in this respect.

An almost inactive, half-$Cu^{2+}$-depleted, BSAO was previously obtained by means of dithionite and 1.0 mM *N*,*N*-diethyldithiocarbamate in anaerobic conditions [22,23]. This result was considered to be inconsistent with the linear relationship found between re-incorporated $Cu^{2+}$ and recovered native properties in fully $Cu^{2+}$-depleted BSAO. It was argued that linearity implies that $Cu^{2+}$ binding either is highly co-operative or occurs with identical affinity at the two sites [24,27]. This alleged inconsistency is disproved by the present results. The new procedure for the preparation of a similar inactive derivative clearly shows that the removal of only one $Cu^{2+}$ occurs in conditions that make the sites no longer identical. Benzylamine, as hydrazines [11], at most reacts with 1–1.3 TPQ per dimer, so that a large $Cu^{2+}$ amount is neither reduced nor removed [16]. When both $Cu^{2+}$ ions are removed, the subunits may no longer be different. Even in the case of a persisting difference, this is most likely related to TPQ and does not necessarily include the two metal-binding sites, since TPQ is not directly bound to $Cu^{2+}$ in BSAO [20,28] or in amine oxidases of known structure [17,18]. Therefore, active and inactive subunits may have the same high affinity for $Cu^{2+}$ and bind it in equal amount, reaching saturation at 2 ions per dimer. Co-operative binding of $Cu^{2+}$ is also ruled out by the above conclusion that the residual $Cu^{2+}$ of the derivative depleted of $Cu^{2+}$ by cyanide plus benzylamine is bound 1 per dimer.

### Spectroscopic properties of $Co^{2+}$-BSAO

The CD spectrum of $Co^{2+}$-BSAO, prepared by dialysis against excess $Co^{2+}$ of the protein depleted of $Cu^{2+}$ by cyanide and dithionite (Figure 3, spectrum a), is similar to the previously reported one, prepared from the fully reduced apo protein [29]. It shows a band at 540 nm, which, like the band at 640 nm of the native protein (Figure 3, curve b), was assigned as an $Me^{2+}$ d–d electronic transition [20,29]. The band at 440 nm, occurring in both spectra, was assigned as a TPQ transition [29]. The lack in spectrum (a) of appreciable intensity in the region where $Cu^{2+}$ absorbs confirms that the $Co^{2+}$ derivative does not contain more than 10% $Cu^{2+}$ (Table 1).

It was previously observed that BSAO and $Cu^{2+}$-substituted bovine carbonic anhydrase display similar spectroscopic properties, either in the unreacted form or in the adducts with *N*,*N*-diethyldithiocarbamate [24]. In particular, the ESR spectra of the latter adducts are typical of tetragonal copper with two nitrogens and two sulphurs as square planar ligands. This is quite reasonable, since available structural data have shown that the metal-binding site of carbonic anhydrase is made up of three histidyls and one or two water molecules with distorted tetrahedral or

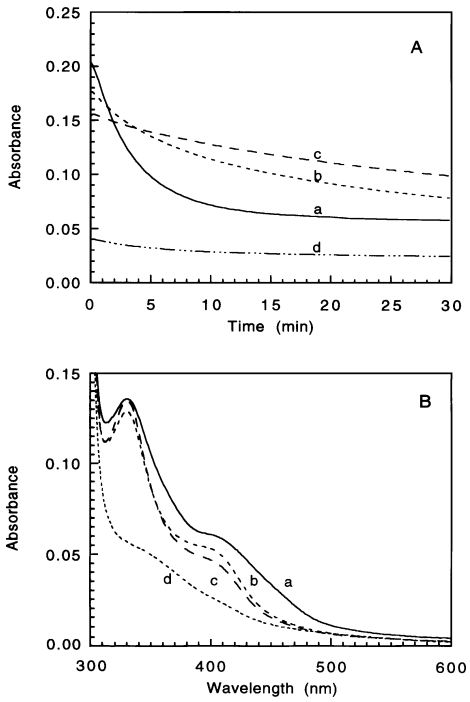


**Figure 4 Reaction with benzylhydrazine of native, $Cu^{2+}$-depleted and $Co^{2+}$-reconstituted BSAO**

(**A**) Absorbance changes at 405 nm on reacting 5.3 $\mu$M BSAO with 25 $\mu$M benzylhydrazine: native (trace a); $Co^{2+}$ reconstituted from the cyanide/benzylamine sample (trace b) or from the cyanide/dithionite sample (trace c); $Cu^{2+}$ depleted, with cyanide and benzylamine/dithionite (trace d). (**B**) Spectra recorded 30 min (traces a and d), 1 h (trace b) and 2 h (trace c) after benzylhydrazine addition.

pentaco-ordinate geometry [30], similar to that of *E. coli* amine oxidase active form [17] and of pea seedlings amine oxidase [18]. A close spectroscopic similarity also exists between the acidic form of $Co^{2+}$-substituted carbonic anhydrase [31] and $Co^{2+}$-BSAO (Figure 3, spectrum a). Both show a CD band at 540 nm, which strongly implies $Co^{2+}$ binding to the $Cu^{2+}$ sites of BSAO, since $Co^{2+}$ spectroscopic properties are quite sensitive to changes of co-ordination environment.

## Reactions with TPQ-binding inhibitors

$Co^{2+}$ incorporation by dialysis restored in both $Cu^{2+}$-depleted BSAO derivatives almost native protein reactivity with phenylhydrazine (Figure 1) and other BSAO inhibitors, most notably benzylhydrazine (Figure 4). It had previously been reported that this molecule binds $Cu^{2+}$-depleted BSAO in a spectroscopically silent form, detectable by the inhibitory effect on the reaction with other molecules, e.g. semicarbazide [16]. The bands of low intensity formed by this derivative in Figure 4 (A and B, traces d), can probably be attributed to residual active molecules. The reconstitution with $Co^{2+}$ restored the ability to form with benzylhydrazine, within mixing time, the benzylhydrazone, characterized by an intense band at 405 nm [32]. This decayed in air to the hydrazone at a slower rate than in native BSAO (Figure 4A). The spectra of $Co^{2+}$-BSAO hydrazones were slightly different (Figure 4B, traces b and c) from that of the BSAO hydrazone (trace a). Moreover, the last one decayed within several hours to native BSAO [32], whereas $Co^{2+}$-BSAO hydrazones were stable for days. The differences may be due to the presence in the $Cu^{2+}$ derivative of small amounts of the *para*-quinone conformer in equilibrium with the more stable *ortho*-quinone [28]. Similar reactions were previously reported for $Co^{2+}$-BSAO reconstituted from the fully reduced $Cu^{2+}$-depleted protein [22].

**Table 2 Steady-state kinetic parameters, at 25 °C, for the oxidative deamination of primary amines catalysed by BSAO and $Co^{2+}$-BSAO**

| | BSAO | | $Co^{2+}$-BSAO | |
|---|---|---|---|---|
| Substrate | $K_m$ (mM) | $k_c$ ($s^{-1}$) | $K_m$ (mM) | $k_c$ ($s^{-1}$) |
| Spermine | 0.087 ± 0.009 | 1.13 ± 0.04 | 0.018 ± 0.004 | 0.13 ± 0.02 |
| Spermidine | 0.42 ± 0.05 | 0.90 ± 0.03 | 0.085 ± 0.008 | 0.10 ± 0.01 |
| Benzylamine | 1.9 ± 0.1 | 0.82 ± 0.03 | 0.66 ± 0.04 | 0.14 ± 0.01 |
| Phenylethylamine | 0.63 ± 0.07 | 0.17 ± 0.04 | 0.45 ± 0.05 | 0.070 ± 0.003 |

## Steady-state kinetics

The steady-state kinetic parameters for the oxidation of some primary amines catalysed by native and $Co^{2+}$-substituted BSAO are compared in Table 2. The measurements were carried out on samples from the same stocks at pH 7.2, the pH of maximum activity for both proteins. $Co^{2+}$ substitution decreased the $k_c$ of all substrates tested, but the extent of decrease was variable. The $k_c$ of spermine and spermidine was 11% of the native value, 17% that of benzylamine, and 41% that of phenylethylamine. The same decrease should have occurred in all cases, were the activity of $Co^{2+}$-BSAO due to adventitious or residual copper. This possibility is also excluded by the significantly lower $K_m$ values of $Co^{2+}$-BSAO, an effect that cannot be ascribed to a lower concentration of active protein. A remarkable result was that, within the limits of experimental error, the kinetic parameters of $Co^{2+}$-BSAO were unaffected by the presence of 0.7 residual $Cu^{2+}$ per dimer. This confirms that this copper has no catalytic function.

The substitution of copper with cobalt had the peculiar effect of decreasing both $k_c$ and $K_m$ values (Table 2). This could imply that a high amine affinity for the protein inhibits the catalytic reaction, in agreement with a previous suggestion that amines bind a hydrophobic site near the protein surface, at some distance from the deeply buried active site [16]. However, considerable changes in the $K_m$ occurred when either native or $Co^{2+}$-substituted BSAO reacted with a series of different amines (Table 2), whereas the changes of $k_c$ were rather small. Analogous effects of other series of amine substrates were taken to imply that reaction steps subsequent to enzyme reduction, namely imine hydrolysis or enzyme reoxidation, are the rate-limiting processes [33,34]. The dramatic effect of metal removal on the reaction with oxygen strongly suggests that this reaction is affected by the change of metal and is responsible for $k_c$ decreases.

## Conclusions

The present paper reports the preparation of $Co^{2+}$-containing BSAO from two different $Cu^{2+}$-depleted proteins [16] and compares the properties of the new derivatives with those of native and $Cu^{2+}$-depleted BSAO. All the evidence is consistent with the conclusion that $Co^{2+}$ is catalytically competent and most likely occupies the same site as does $Cu^{2+}$, though it may have a somewhat different co-ordination. This excludes the possibility that the formation of a semiquinone radical is an essential step

in the BSAO-catalysed reaction. According to model studies, TPQ analogues do not require the presence of copper to catalyse amine oxidation [35–37]. It appears that copper does not play a unique role in this reaction. A different case is the reaction leading to tyrosine oxidation to TPQ, which cannot be induced by metal ions other than copper [38].

It has been shown previously that $Cu^{2+}$ removal decreases by a factor of 2.5 the benzylamine affinity for the protein and by a factor of $10^3$ the rate at which it reduces the cofactor [16]. These largely different effects were related to copper location, near TPQ and far from the hydrophobic benzylamine-binding site, most likely at the protein surface. The reaction with oxygen was completely inhibited [16]. The incorporation of $Co^{2+}$ in place of $Cu^{2+}$ restored and even increased the affinity for substrates. Subsequent steps involving reaction of the substrate with TPQ were restored to nearly native level as shown by the recovery of reactivity with substrate analogues such as phenylhydrazine and benzylhydrazine. Also, the access of oxygen to the active site was restored. Therefore, the lower $k_c$ values, by a factor between 2 and 9, may be attributable to changes in the oxygen affinity for the modified protein and/or its rate of reaction at the active site.

Present results clearly show that the two BSAO subunits are not equivalent. At least 0.7 $Cu^{2+}$ per dimer did not contribute to catalytic activity in any derivative, namely in the native protein from which they could not be removed on reduction with substrate, in $Cu^{2+}$-depleted and in $Co^{2+}$-BSAO. A not-fully-processed tyrosine or one TPQ with inactive conformation may be responsible for the difference, although half-of-the-site reactivity [11] cannot be excluded, since in any case one subunit was modified.

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