Pulse-radiolysis studies on the interaction of one-electron reduced species with blue oxidases

Reduction of Type-2-copper-depleted ascorbate oxidase

Peter O'NEILL,* E. Martin FIELDEN,* Luciana AVIGLIANO,† Giordana MARCOZZI,†
Amleto BALLINI† and Alessandro FINAZZI AGRÒ†

*Division of Molecular Processes, M.R.C. Radiobiology Unit, Harwell, Didcot, Oxon OX11 ORD, U.K., and

†Institute of Biological Chemistry and Institute of Applied Biochemistry, University of Rome and C.N.R. Centre for Molecular Biology, Rome, Italy

(Received 30 January 1984/Accepted 24 April 1984)

The interactions of one-electron reduced metronidazole ($\text{ArNO}_2^{\cdot-}$) with native and Type-2-copper-depleted ascorbate oxidase were studied in buffered aqueous solution at pH 6.0 and 7.4 by using the technique of pulse radiolysis. With $\text{ArNO}_2^{\cdot-}$, reduction of Type 1 copper of the native enzyme and of the Type-2-copper-depleted ascorbate oxidase occurs via a bimolecular step and at the same rate. Whereas the native protein accepts, in the absence of O_2 , 6–7 reducing equivalents, Type-2-copper-depleted ascorbate oxidase accepts only 3 reducing equivalents with stoichiometric reduction of Type 1 copper. On reaction of $\text{O}_2^{\cdot-}$ with ascorbate oxidase under conditions of $[\text{O}_2^{\cdot-}] \gg [\text{ascorbate oxidase}]$, removal of Type 2 copper results in reduction of all the Type 1 copper atoms, in contrast with reduction of the equivalent of only one Type 1 copper atom in the holoprotein. From observations at 610 nm, the rate of reduction of ascorbate oxidase by $\text{O}_2^{\cdot-}$ is not dependent on the presence of Type 2 copper. For the holoprotein, no significant optical-absorption changes were observed at 330 nm. It is proposed that electrons enter the protein via Type 1 copper in a rate-determining step followed by a fast intramolecular transfer of electrons within the protein. For the Type-2-copper-depleted protein, intramolecular transfer within the protein, however, is slow or does not occur. In the presence of O_2 , it is also suggested that re-oxidation of the partially reduced holoprotein occurs at steady state, as inferred from the observations at 330 nm and 610 nm. The role of Type 2 copper in ascorbate oxidase is discussed in terms of its involvement in redistribution of electrons within the protein or structural considerations.

Ascorbate oxidase (AAO) is a copper-containing enzyme that *in vitro* catalyses the oxidation of ascorbate by O_2 with the production of dehydro-ascorbate and water (Mondoví & Avigliano, 1984). It contains 8 atoms of copper per molecule (three Type 1, one Type 2 and four Type 3) and, by analogy with other blue oxidases, they are divided into three different types (Malmström *et al.*, 1975; Fee, 1975). Type 1 copper, which absorbs at 610 nm, and Type 2 copper are e.p.r.-detectable, and therefore their reduction may be directly monitored. Type 3 copper is believed to be associated with an absorption band at 330 nm, the

bleaching of which should, presumably, be associated with Type-3-copper reduction (Malmström *et al.*, 1975; Fee, 1975). Owing to these spectroscopic features it is possible to study the kinetics of and the position of the equilibria of the redox processes involving the different copper types.

It has previously been proposed that the different types of copper atoms in AAO represent an array of redox centres that may undergo alternate reduction and oxidation. The 'blue' copper atoms (Type 1) seem to be on the substrate side of the redox chain, whereas Type 3 copper (the diamagnetic pair) may be more associated with the O_2 side (Avigliano *et al.*, 1978), as previously proposed for caeruloplasmin (De Ley & Osaki, 1975) and laccases (Malmström *et al.*, 1969; Andréasson *et*

Abbreviations used: AAO, ascorbate oxidase; $\text{ArNO}_2^{\cdot-}$, radical anion of metronidazole.

al., 1973; Holwerda & Gray, 1974; Andréasson & Reinhammar, 1976). More problematic is the location and role of the Type 2 Cu^{2+} , since its oxidation state relative to those of the other copper atoms in AAO depends on the experimental conditions (Holwerda & Gray, 1974; Goldberg *et al.*, 1980). We have previously shown (O'Neill *et al.*, 1983) that one-electron reduced nitroaromatics reduce AAO in a bimolecular process and that a rapid equilibration of the electrons to the copper centres occurs in the absence of O_2 .

Since there is a lack of structural information concerning the spatial relationship of the various copper atoms, the mechanism of intramolecular electron transfer within the protein is not fully understood. The possibility of selective removal of Type 2 Cu^{2+} from ascorbate oxidase offers further scope for investigation of the role of the different types of copper atoms within the electron-transfer processes. In the present paper we report studies on the reduction of Type-2-copper-depleted AAO by a one-electron reduced nitroaromatic and by superoxide and compare the findings with those obtained with the native protein, using the technique of pulse radiolysis to generate the various reducing species. From the information obtained, more detailed aspects of electron transport within the protein are presented.

Materials and methods

AAO was prepared as previously described (Avigliano *et al.*, 1972). Two batches of enzyme (pooled different preparations with identical catalytic and spectroscopic features) were found to be homogeneous with respect to ultracentrifugation and disc-gel electrophoresis and were used throughout this investigation. Ultracentrifugation was performed at 56000 rev./min with a Spinco model E ultracentrifuge equipped with a temperature-control unit at 8–10°C. Gel electrophoresis was performed at pH 8.9 according to the method of Davis (1964). The pooled protein showed an A_{280}/A_{610} ratio of 26 and an A_{330}/A_{610} ratio of 0.9 and contained 7.8 g-atoms of copper/mol as determined chemically (Brumby & Massey, 1967) after dialysis against 0.5 mM-EDTA. Double integration of e.p.r. spectra (Varian E9 spectrometer operating at 9 GHz and 77 K) gave 3.8 g-atoms of e.p.r.-detectable copper/mol. Type 2 copper was removed from the two batches as previously described (Avigliano *et al.*, 1979). The Type-2-depleted AAO samples exhibited similar properties (sample 1: A_{280}/A_{610} ratio 28, 5.6 g-atoms of copper/mol, 2.7 g-atoms of which are e.p.r.-detectable; sample 2: A_{280}/A_{610} ratio 26, 5.8 g-atoms of copper/mol, 3.0 g-atoms of which are e.p.r.-detectable). The activity of the Type-2-

copper-depleted AAO was 0.5% of that of the holoprotein. This loss of activity on removal of Type 2 copper has previously been reported (Kroneck *et al.*, 1983). Reconstituted samples of AAO were obtained as previously described (Avigliano *et al.*, 1979). They exhibited spectroscopic and kinetic parameters identical with those of the native protein. In particular, they contained 7.6 g-atoms of copper/mol, 3.8 g-atoms of which are e.p.r.-detectable. The enzymic activity was measured spectrophotometrically by monitoring the decrease of optical absorption at 265 nm due to the oxidation of ascorbate.

The 4.3 MeV linear accelerator and the optical-detection and data-storage systems were described previously (McAdam *et al.*, 1977). All solutions were prepared from triple-distilled water, buffered at pH 6.0 or 7.4 with sodium phosphate buffer (10 mM), and contained EDTA (0.1 mM), formate (0.1 M) and AAO, the concentration of which was generally expressed in terms of protein concentration. Irradiation conditions and dosimetry details were as previously reported (O'Neill *et al.*, 1983).

Total conversion of the radicals produced on the radiolysis of water (reaction 1):

into one-electron reduced nitroaromatics has previously been described (O'Neill *et al.*, 1983). In order to study the reactions of $\text{O}_2^{\bullet -}$ with AAO, solutions contained formate and were aerated or oxygenated in order to convert all the radiolytically produced radicals of water (reaction 1) into $\text{O}_2^{\bullet -}$, as shown in reactions (2)–(4):

Metronidazole [2-(2-methyl-5-nitroimidazole-1-yl)ethan-1-ol] was supplied by May and Baker. All other reagents used were of AnalaR grade from BDH Chemicals.

Results

Reduction of AAO by $\text{ArNO}_2^{\bullet -}$

As previously reported, AAO may interact with one-electron reduced nitroaromatic radical anions produced on pulse irradiation (O'Neill *et al.*, 1983). We have confirmed that 6–7 mol of radical/mol of holoprotein is required to bleach completely the absorption, at 610 nm, of AAO, despite the

presence of only 3 blue (Type 1) copper atoms/molecule of enzyme.

On extension of our study to the Type-2-copper-depleted AAO, it was determined that only 2.9 ± 0.3 reducing equivalents are needed to achieve full bleaching of the absorption at 610 nm, assigned to Type 1 copper, in the absence of O_2 . This stoichiometry was determined in the absence of O_2 from the changes in absorbance at 410 nm, a wavelength at which the radical anion of metronidazole ($ArNO_2^{\cdot-}$, produce $17.5 \mu M$ /pulse) absorbs, in the absence and in the presence of the Type-2-copper-depleted protein (1.4 – $2.4 \mu M$) at pH 6.0 or 7.4. The second-order rate constants for the redox reaction between the Type-2-copper-depleted AAO and $ArNO_2^{\cdot-}$, as determined from the absorbance changes with time at 610 nm, are $2.2 (\pm 0.5) \times 10^7 M^{-1} \cdot s^{-1}$ and $3 (\pm 1) \times 10^7 M^{-1} \cdot s^{-1}$ at pH 6.0 and 7.4 respectively. These values are very similar to those determined under equivalent conditions with the native AAO protein (O'Neill *et al.*, 1983).

Previous treatment of Type-2-copper-depleted AAO with H_2O_2 , in an attempt to obtain a fully oxidized protein as reported by Lubien *et al.* (1981) for Type-2-copper-depleted laccase, did not influence the rate or the stoichiometry of reduction by $ArNO_2^{\cdot-}$. Furthermore, the rate and the stoichiometry of reduction of reconstituted samples of the Type-2-copper-depleted protein by

$ArNO_2^{\cdot-}$ are equivalent to those determined for the native protein. After a train of six radiation pulses, less than 10% bleaching of optical absorption as compared with the extent of bleaching at 610 nm was observed at 330 nm on interaction of $ArNO_2^{\cdot-}$ with Type-2-copper-depleted AAO (Fig. 1). This extent of bleaching at 330 nm is slightly less than that observed with the holoprotein of less than 20% (O'Neill *et al.*, 1983). It should be noted that at 330 nm the absorption coefficients of metronidazole and $ArNO_2^{\cdot-}$ are similar; in fact, the slight increase of optical absorption after each pulse in Fig. 1 is probably due to the absorption of $ArNO_2^{\cdot-}$. From Fig. 1, it is apparent that significant bleaching of optical absorption at 330 nm does not occur within approx. 0.18 s even though full bleaching of the absorption at 610 nm of Type 1 copper requires only two radiation pulses under these conditions. The quality of the traces only allow qualitative examination at 330 nm.

Reaction of AAO with $O_2^{\cdot-}$

The reaction of holo-AAO with $O_2^{\cdot-}$ was studied under various conditions. Most of the experiments were performed at pH 7.4 instead of pH 6.0 owing to the increased lifetime of $O_2^{\cdot-}$ at pH 7.4. Holo-AAO (1.4 – $5.2 \mu M$) was found to interact with $O_2^{\cdot-}$, as determined from the bleaching of the optical absorption at 610 nm (ΔA_{610}) (see Fig. 2). ΔA_{610} was found to be

Fig. 1. Absorbance changes with time observed at 330 nm (a) and 610 nm (b) on pulse irradiation of a Type-2-copper-depleted AAO solution ($3.7 \mu M$) containing metronidazole ($1 mM$) and formate ($0.1 M$) and saturated with N_2O at pH 7.5. A train of six irradiation pulses at a repetition rate of 50 pulses/s was delivered to the solution as indicated by the arrows. Each pulse produced approx. $8 \mu M$ - $ArNO_2^{\cdot-}$.

independent of $O_2^{\cdot-}$ concentration (train of 10–15 radiation pulses each producing $[O_2^{\cdot-}]$ of either 20, 30 or $40\mu M$), the concentration of O_2 (aerated and oxygenated) and showed only a slight variation with [AAO], as presented in Table 1. It is thought that these variations in ΔA_{610} are not significant. The ΔA_{610} determined is equivalent to the reduction of only one of the Type 1 copper-atoms.

As shown in Fig. 2, approx. 0.15 s (e.g. ten pulses) is required to establish equilibrium conditions; this time scale is similar to that for the natural decay of $O_2^{\cdot-}$. Therefore, under the experimental conditions used, the natural decay of $O_2^{\cdot-}$ is still important and is probably the reason why no significant bleaching of AAO ($2\mu M$), in the presence of $O_2^{\cdot-}$, was observed under single-pulse conditions (O'Neill *et al.*, 1983). One experiment was performed in which the rate of bleaching of absorption at 610 nm was determined on interaction of $26\mu M$ -AAO with $18\mu M$ $O_2^{\cdot-}$ at pH 7.4. Since the determined first-order rate constant of

$141 s^{-1}$, under these conditions, is about one order of magnitude greater than that estimated from the rate of bleaching in Fig. 2, it is estimated that the rate constant for initial interaction of $O_2^{\cdot-}$ with AAO is $< 5 \times 10^6 M^{-1} \cdot s^{-1}$. After completion of the interaction of $O_2^{\cdot-}$ with AAO, approx. 30% restoration of optical absorption at 610 nm was observed within 1 s.

On interaction of Type-2-copper-depleted AAO with $O_2^{\cdot-}$ at pH 7.4, at least 90% bleaching of the total bleachable absorption at 610 nm was observed (see Table 1). The extent of bleaching at 610 nm was not dependent on the $[O_2]$, $[O_2^{\cdot-}]$ or [AAO] (2 – $3\mu M$). The time scale of the reduction process, as shown in Fig. 2, is similar to that observed with the holoprotein. In contrast with the observations with holo-AAO, no increase in optical absorption was observed within 1 s after completion of the interaction of Type-2-copper-depleted AAO with $O_2^{\cdot-}$. On interaction of $O_2^{\cdot-}$ with the holoprotein at pH 7.4, no bleaching of the

Table 1. Percentage bleaching of optical absorption at 610 nm determined on interaction of native and Type-2-copper-depleted (apo) AAO with $O_2^{\cdot-}$ produced at pH 7.4 on pulse irradiation of oxygenated aqueous solutions containing 1.4 – $5.2\mu M$ -protein

$\Delta A'_{610}$ was determined at pH 7.4 with $ArNO_2^{\cdot-}$ as the reductant in the absence of O_2 . $[O_2^{\cdot-}]$ was 50 – $180\mu M$.

[AAO] (μM)	$100 \times \Delta A_{610} / \Delta A'_{610}$	[Apo-AAO] (μM)	$\Delta A_{610} / \Delta A'_{610}$
2.8	34.6, 33.1*	2.4	86, 84*
5.2	26.3, 25.2*	2.0	90, 87.8*
1.4	33.7, 36.2*		

* Determined on irradiation of aerated solutions of AAO.

Fig. 2. Absorbance changes with time observed at 610 nm on pulse irradiation of an AAO solution containing formate ($0.1 M$) and saturated with O_2 at pH 7.4

A train of ten radiation pulses at a repetition rate of 50 pulses/s was delivered to the solutions containing (a) $1.4\mu M$ -holo-AAO and (b) $2.3\mu M$ Type-2-copper-depleted AAO. Each pulse produced approx. $30\mu M$ - $O_2^{\cdot-}$.

optical absorption at 330nm was observed during or after completion of the reaction on the basis of the observations at 610nm (within approx. 0.15s). However, owing to the poor quality of the traces, only qualitative information is obtained. This apparent lack of bleaching of optical absorption at 330nm is consistent with the small extent of bleaching observed at 330nm with $\text{ArNO}_2^{\cdot-}$ as the source of reducing equivalents whereby complete reduction of Type 1 copper is inferred from the observations at 610nm (see Fig. 1) on this time scale.

Static reduction of Type-2-copper-depleted AAO by ascorbate

Type-2-copper-depleted AAO may be reduced under anaerobic conditions by ascorbate. Whereas the holoprotein is fully reduced by 4mol of ascorbate/mol of AAO (Avigliano *et al.*, 1978), 3mol/mol of protein is required for the static bleaching of Type-2-copper-depleted AAO. In other words both the native AAO and Type-2-copper-depleted AAO may accept as many electrons as copper atoms present within the protein, consistent with the findings reported by Kroneck *et al.* (1983).

Discussion

AAO is one of the few oxidases able to reduce O_2 directly to water. It has been suggested that this property is related to the presence of four electron acceptors per functional unit of the oxidases, as is the case with cytochrome *c* oxidase and laccase (Malmström *et al.*, 1975). It has previously been assumed that caeruloplasmin and AAO are dimers of laccase; however, this has been disproved, on the basis of finding that they show Type 1/Type 2 ratios of copper atoms of 2 and 3 respectively (Deinum *et al.*, 1974; Van Leeuwen *et al.*, 1975; Ryden & Björk, 1976; Marchesini & Kroneck, 1979; Avigliano *et al.*, 1979). Furthermore, the catalytic efficiency of AAO and caeruloplasmin does not correlate with the ratio of Type 2/Type 3 copper, since AAO is a powerful catalyst, whereas caeruloplasmin sluggishly oxidizes the substrates as yet studied (Fee, 1975; Frieden & Hsieh, 1976). A further problem associated with the understanding of the catalytic mechanism of oxidases is the pathway by which the electrons are transferred from the substrate to O_2 via the protein. Much evidence indicates that the electrons initially enter blue oxidases via Type 1 copper (Andréasson & Reinhammar, 1976).

In the present study, we took advantage of the reversible removal of Type 2 copper from AAO to obtain information concerning the role of Type 2 copper in the intramolecular electron-transfer

processes. We have previously demonstrated that total reduction of the Type 1 copper of AAO is observed when 6–7 reducing equivalents are accepted by AAO and that AAO is reduced in a rate-limiting bimolecular process by $\text{ArNO}_2^{\cdot-}$ (O'Neill *et al.*, 1983). Since the rate of reduction of Type-2-copper-depleted AAO by $\text{ArNO}_2^{\cdot-}$ is similar to that for reduction of the native protein, it is inferred that the initial interaction of $\text{ArNO}_2^{\cdot-}$ with AAO is independent of the presence of Type 2 copper. Type-2-copper-depleted AAO accepts, in the absence of O_2 , only 3 reducing equivalents, with stoichiometric reduction of Type 1 copper. Hence it is inferred that the electrons enter the protein via Type 1 copper. Furthermore, subsequent intramolecular electron transfer to the other copper types within the protein does not occur or the rate of the process(es) is much slower when Type 2 copper is removed. From the observations at 330nm with $\text{ArNO}_2^{\cdot-}$ as the reducing agent, ΔA_{330} for the holoprotein is slightly greater than that for the Type-2-copper-depleted protein. This difference may be indicative of the fact that Type 2 and/or Type 3 copper atoms are only reduced in the holoprotein, as implied from the observations at 610nm together with the known stoichiometries of the reaction of $\text{ArNO}_2^{\cdot-}$ with both proteins. In contrast with the stoichiometry of reduction determined on reaction of Type-2-copper-depleted AAO with $\text{ArNO}_2^{\cdot-}$, about six electrons are required for complete bleaching at 610nm of the Type-2-copper-depleted protein under static conditions with ascorbate.

On reaction of $\text{O}_2^{\cdot-}$ with AAO, removal of Type 2 copper resulted in the three Type 1 copper atoms being reduced as compared with only about the equivalent of one Type 1 copper atom in the holoprotein. In fact, with native AAO the reduction process with $\text{O}_2^{\cdot-}$ reaches an equilibrium (equivalent to reduction of one Type 1 copper atom), the position of which is not influenced by $[\text{O}_2]$ or $[\text{O}_2^{\cdot-}]$. Whether the position of the equilibrium is influenced by $[\text{AAO}]$ is, as yet, not known, since high concentrations of protein would be required and therefore very high concentrations of $\text{O}_2^{\cdot-}$ are necessary to establish equilibrium conditions. Since, in the presence of O_2 , an equilibrium may be established, it is inferred that electrons flow into the native protein from $\text{O}_2^{\cdot-}$ via Type 1 copper followed by a fast, non-rate-determining, intramolecular transfer of electrons to the Type 2 and 3 copper atoms. A fast intramolecular process is consistent with the very high catalytic activity of AAO. It is further inferred from the position of the equilibrium on reaction with $\text{O}_2^{\cdot-}$, on the basis of ΔA_{610} , that the protein is also rapidly re-oxidized by O_2 . A rapid re-oxidation of the reduced protein by O_2 is consistent with the observed lack of

bleaching at 330 nm for the holoprotein. In fact, re-oxidation of ascorbate-reduced AAO by O_2 has been shown to occur within less than 8 ms (Kroneck *et al.*, 1983), a time wholly consistent with the above mechanistic aspects proposed, whereby it is inferred that Type 2 and Type 3 copper atoms are predominantly in the oxidized form at equilibrium in this system. The rate-limiting step, under the conditions used, is suggested to be flow of electrons into the protein via Type 1 copper atoms.

For the Type-2-copper-depleted protein, all the Type 1 copper atoms are reduced on interaction with $O_2^{\cdot-}$. From the observations made with $ArNO_2^{\cdot-}$ (see above), subsequent intramolecular electron transfer from Type 1 to the Type 3 copper atoms is also probably less efficient or does not occur even with $O_2^{\cdot-}$ as the source of electrons. If, however, electron transfer to Type 3 copper atoms does occur, it is inferred that the Type 3 copper atoms would be in the reduced state, since re-oxidation of ascorbate-reduced Type-2-depleted AAO takes several minutes (Kroneck *et al.*, 1983), in contrast with the rapid re-oxidation of the reduced holoprotein. Reduction of Type 3 copper atoms by $O_2^{\cdot-}$ in Type-2-copper-depleted AAO would be in contrast with the observations made with $ArNO_2^{\cdot-}$ whereby the three reducing equivalents accepted by Type-2-copper-depleted AAO are accounted for in terms of reduction of all the Type 1 copper atoms. From observations at 330 nm, further information concerning the redox state of the Type 3 copper atoms cannot be obtained owing to the poor quality of the traces. However, it should be noted that even when Type 3 copper atoms are reduced, as implied on reaction of the holoprotein with $ArNO_2^{\cdot-}$, the extent of bleaching at 330 nm is only 20% or less of that observed at 610 nm (O'Neill *et al.*, 1983).

It is concluded that a rapid equilibration of the 6–7 electrons/molecule accepted by AAO occurs between all the types of copper atoms. Type 2 copper plays an essential role in the intramolecular electron-transfer processes involved in redistribution of the electrons to the different types of copper. In fact, removal of Type 2 copper greatly lowers or prevents the ability of electrons to be redistributed within the protein. Intramolecular processes within the native protein are fast compared with the electron-exchange process between substrate and the protein, and this is in contrast with the observations with laccases (P. O'Neill, E. M. Fielden, L. Morpurgo & E. Agostinelli, unpublished work). It is not possible to state whether Type 2 copper acts as a 'gate' for the transfer of electrons from Type 1 to Type 3 copper or whether it plays a structural role in the native protein whereby its removal from

AAO brings about a structural change that makes redistribution of the electrons within the protein unfavourable.

We are very grateful to Mr J. Currant for technical assistance. This work was funded by the Medical Research Council/Cancer Research Campaign Joint Committee. We are grateful to the British Council/Consiglio Nazionale delle Ricerche for financial assistance towards travel costs. A. F. A. thanks the European Molecular Biology Organization for a short-term fellowship.

References

- Andréasson, L. E. & Reinhammar, B. (1976) *Biochim. Biophys. Acta* **445**, 579–597
- Andréasson, L. E., Malmström, B. G., Strömberg, C. & Vänngård, T. (1973) *Eur. J. Biochem.* **34**, 434–439
- Avigliano, L., Gerosa, P., Rotilio, G., Finazzi Agrò, A., Calabrese, L. & Mondovì, B. (1972) *Ital. J. Biochem.* **21**, 248–255
- Avigliano, L., Rotilio, G., Urbanelli, S., Mondovì, B. & Finazzi Agrò, A. (1978) *Arch. Biochem. Biophys.* **185**, 419–422
- Avigliano, L., Desideri, A., Urbanelli, S., Mondovì, B. & Marchesini, A. (1979) *FEBS Lett.* **100**, 318–320
- Brumby, P. E. & Massey, V. (1967) *Methods Enzymol.* **10**, 473–474
- Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–422
- Deinum, J., Reinhammar, B. & Marchesini, A. (1974) *FEBS Lett.* **42**, 241–244
- De Ley, M. & Osaki, S. (1975) *Biochem. J.* **151**, 561–566
- Fee, J. A. (1975) *Struct. Bonding (Berlin)* **23**, 1–60
- Frieden, E. & Hsieh, H. S. (1976) *Adv. Enzymol. Relat. Areas Mol. Biol.* **44**, 187–236
- Goldberg, M., Farver, O. & Pecht, I. (1980) *J. Biol. Chem.* **255**, 7353–7361
- Holwerda, R. A. & Gray, H. B. (1974) *J. Am. Chem. Soc.* **96**, 6008–6022
- Kroneck, P. M. H., Jakob, W., Merkle, H. & Marchesini, A. (1983) *Inorg. Chim. Acta* **79**, 125
- Lubien, C. D., Winkler, M. E., Thamann, T. J., Scott, R. A., Co, M. S., Hodgson, K. O. & Solomon, E. I. (1981) *J. Am. Chem. Soc.* **103**, 7014–7016
- Malmström, B. G., Finazzi Agrò, A. & Antonini, E. (1969) *Eur. J. Biochem.* **9**, 383–391
- Malmström, B. G., Andréasson, L. E. & Reinhammar, B. (1975) *Enzymes 3rd Ed.* **12**, 507–579
- Marchesini, A. & Kroneck, P. M. H. (1979) *Eur. J. Biochem.* **101**, 65–76
- McAdam, M. E., Fox, A. R., Lavelle, F. & Fielden, E. M. (1977) *Biochem. J.* **165**, 71–79
- Mondovì, B. & Avigliano, L. (1984) in *Copper Proteins* (Lontie, R., ed.), vol. 3, C.R.C. Press, Boca Raton, in the press
- O'Neill, P., Fielden, E. M., Finazzi Agrò, A. & Avigliano, L. (1983) *Biochem. J.* **209**, 167–174
- Ryden, L. & Björk, I. (1976) *Biochemistry* **15**, 3411–3417
- Van Leeuwen, F. X. R., Wever, R., Van Gelder, B. F., Avigliano, L. & Mondovì, B. (1975) *Biochim. Biophys. Acta* **403**, 285–291