Pulse-radiolysis Studies on the Oxidised Form of the Multicopper Enzyme Ascorbate Oxidase: Evidence for Two Intramolecular Electron-transfer Steps†

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Two intramolecular electron-transfer steps have been identified in pulse-radiolysis studies on the multicopper enzyme ascorbate oxidase, which has four Cu atoms in the catalytically active monomer form. The enzyme was initially in the fully oxidised Cu^{II}_4 state. Pulse radiolysis was carried out at 19 °C, pH 7.0 (40 mM phosphate), $I = 0.100$ M, in the first instance with formate to generate $CO_2^{\bullet -}$ as the only (reducing) radical present. When in addition appropriate amounts of methyl viologen (1,1'-dimethyl-4,4'-bipyridinium, $dmbipy^{2+}$), deazaflavin, or lumiflavin were present the $CO_2^{\bullet -}$ was rapidly converted into CO_2 with concomitant formation of the corresponding radical form (*e.g.* $dmbipy^{\bullet +}$) as the only reactive species. Reactions of all four radicals with ascorbate oxidase (reactant in excess) give a metastable type 1 copper reduced product. Contrary to earlier reports two intramolecular electron-transfer steps k_1 and k_2 follow in which the colour of the type 1 site is restored. Both are independent of the radical type used. Thus the first stage is assigned as electron transfer from the type 1 Cu' to the trinuclear combined type 3/type 2 site. Rate constants k_1 and k_2/s^{-1} are for $CO_2^{\bullet -}$ (120, 2.0), $dmbipy^{\bullet +}$ (127, 2.3), deazaflavin (121, 2.5) and lumiflavin (97, 2.4). Mechanistic assignments for the two stages are considered, and an apparent disagreement with a previous study is explained.

The blue copper proteins ascorbate oxidase, laccase and ceruloplasmin catalyse the four-electron reduction of dioxygen to water without the release of intermediates such as H_2O_2 or OH radicals.[1,2] Their highly specific and efficient catalytic function has been attributed to the synergistic use of three different types of Cu present in all members of this class of metalloprotein. These three types are classified according to their UV/VIS and EPR spectroscopic properties.[3] Thus the single type 1 site of the blue copper protein is found in *e.g.* plastocyanin and azurin, with His Cys His Met co-ordination in a distorted-tetrahedral arrangement. The S(Cys)→Cu^{II} charge-transfer band at ≈600 nm is responsible for the blue colour, and the EPR hyperfine coupling constant for the Cu^{2+} state is unusually small ($A_{\parallel} < 95 \times 10^{-4}$ cm^{-1}). The type 2 single site has variable co-ordination and exhibits 'normal' EPR characteristics for Cu^{2+} ($A_{\parallel} > 140 \times 10^{-4}$ cm^{-1}), but is not normally UV/VIS detectable. Finally, the type 3 site consists of two Cu atoms which, in the oxidised state, are antiferromagnetically coupled *via* an OH^- bridge to give an EPR-silent site. In haemocyanin, which might be regarded as having a typical type 3 site, each Cu is co-ordinated to three His residues, and the copper(II) form absorbs strongly at ≈330 nm.

A number of spectroscopic[4,5] and kinetic[6] studies have been carried out on the blue copper oxidases in an attempt to elucidate the spectral features and the catalytic mechanism. The X-ray crystal structure of ascorbate oxidase from zucchini squash (*Cucurbita pepo medulosa*) at 2.5 Å resolution,[7a] and more recently refined at 1.9 Å resolution,[7c] has given new insight into such studies, and is believed to be a prototype for all the blue oxidases. Amino-acid sequences for a number of these have been reported.[7b]

Ascorbate oxidase consists of two identical subunits each of molecular weight 70 000 (552 amino acids). The four Cu atoms in one subunit are well separated from those in the other, and there are no cross-interactions. Each monomer folds into three β-barrel type domains which provide the co-ordination environment for the Cu atoms. The co-ordination and proximity of the Cu atoms to each other in the oxidised form are indicated in Fig. 1. The type 1 Cu is in a plastocyanin like domain. A novel feature is the proximity of the remote type 2 and the binuclear type 3 coppers giving a trinuclear site, which possibly merits some alternative description.

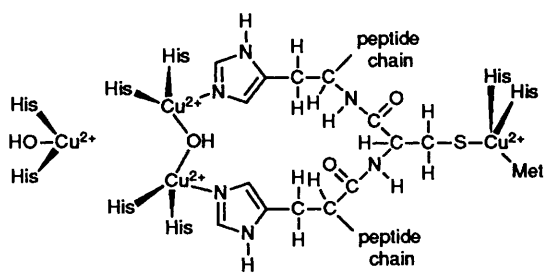

Fig. 1 The location of the four Cu atoms in fully oxidised ascorbate oxidase from the X-ray crystal structure coordinates of the zucchini squash enzyme, ref. 7*a*

It is now believed that the type 1 site is the primary acceptor of electrons from the substrate (ascorbate). Subsequently, intramolecular electron transfer between the type 1 and the trinuclear site provides electrons for the four-electron reduction of dioxygen bound at this site.[4b,d,7a] As can be seen from Fig. 1, possible pathways for electron transfer from the reduced type 1 Cu are by way of Cys 507 and either His 506 or His 508 to the trinuclear site. The reduction potentials for the type 1 and type 3 sites have been reported to be about the same at 345 mV (25 °C), whereas the type 2 site may have a lower potential.[2]

In vitro studies on electron transfer between the copper sites in ascorbate oxidase are relevant to the physiological function

† *Non-SI units employed:* M = mol dm^{-3}, eV ≈ 1.6×10^{-19} J, cal = 4.184 J.

of the enzyme. They also provide an example of long-range (> 10 Å) electron transfer.[8] At about the time we commenced our pulse-radiolysis studies on ascorbate oxidase two research groups reported quite different rate constants for intramolecular electron transfer from the type 1 to the trinuclear centre of the same protein. These were 160 s^{-1} from flash-photolysis studies with the lumiflavin radical as reductant,[9] and ≈1.0 s^{-1} from pulse-radiolysis studies with $CO_2^{\bullet -}$ as reductant.[10] In contrast our results provided evidence for two stages with (approximately) the same two rate constants as reported previously and independent of the identity of the reductant. Implications with regard to the catalytic function of the blue copper oxidases, and comparisons with existing intramolecular electron transfer kinetic data, are considered.

Materials and Methods

Enzyme Source.—Ascorbate oxidase was prepared from zucchini squash by the method of Marchesini and Kroneck.[11] Prior to pulse-radiolysis studies the enzyme was purified using a Mono S cation-exchange FPLC (Pharmacia) column, using 20 mM 2-morpholinoethanesulfonic acid (mes)–NaOH buffer, pH 6.2, ionic strength gradient of 0 to 1 M NaCl. Fractions with A_{330}/A_{610} ≈0.7 were used for kinetic experiments. Dialysis into phosphate buffer (40 mM) was carried out before pulse-radiolysis experiments.

Other Reagents.—Sodium formate hydrate (BDH, Analar), methyl viologen (1,1′-dimethyl-4,4′-bipyridinium) dichloride hydrate (7.8×10^{-4} M, Aldrich), deazaflavin (potassium 10-methyl-5-deazaisoalloxazine-3-propanesulfonic acid) (1.0×10^{-4} M, sample generously provided by Dr. P. M. H. Kroneck) and lumiflavin (40 μM; Sigma) were used, formulae as shown in Fig. 2. In the latter two cases flavin semiquinone radicals are generated in the pulse radiolysis.

Pulse-radiolysis Studies.—These were carried out at the Cookridge Radiation Research Centre, using a 1 cm light-path cell, and a beam of 2.5 MeV electrons. The yield of reducing radicals [R], for a given pulse, is given in (1). The secondary

$$[R] = V_{sec}S_{sec}G_r \quad (1)$$

emission chamber voltage, V_{sec}, was measured directly, and the sensitivity S_{sec} obtained by thiocyanate dosimetry.[12] Here G_r is the radiation chemical yield of radicals per joule of energy absorbed by the system.

Solutions were made up in water which had been distilled and ion-exchange Millipore filtered. All kinetic studies were carried out under strictly anaerobic conditions (N_2O-saturated solutions) at 19 ± 1.5 °C, in buffers containing 40 mM potassium phosphate and 0.010 M formate. Under these conditions the production of radicals was as in expression (2)[13]

$$4.25\ H_2O \longrightarrow$$
$$e_{aq}\ (2.75),\ HO\ (2.85),\ H\ (0.67),\ H_2\ (0.47),\ H_2O_2\ (0.75) \quad (2)$$

where the G values in parentheses (all × 10^{-7}) correspond to the number of moles of product obtained per joule of energy absorbed. Subsequent reactions are indicated in equations (3)–(5),

$$e_{aq}^- + N_2O + H_2O \longrightarrow N_2 + {}^-OH + OH^\bullet \quad (3)$$

$$OH\cdot/H + HCO^{\bullet -} \longrightarrow CO_2^{\bullet -} + H_2O/H_2 \quad (4)$$

$$CO_2^{\bullet -} + X \longrightarrow CO_2 + X^{\bullet -} \quad (5)$$

where X is in turn methyl viologen ($dmbipy^{2+}$), deazaflavin or lumiflavin, and radicals *e.g.* $dmbipy^{\bullet +}$ are produced. The formate radical $CO_2^{\bullet -}$ is no longer effective in the presence of excess of X.

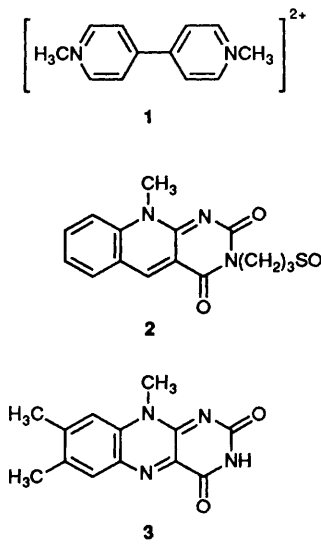

Fig. 2 Structures of the compounds that have been used to generate radicals for the reduction of ascorbate oxidase: methyl viologen **1**; deazaflavin **2**; lumiflavin **3**

Concentrations of ascorbate oxidase were calculated per monomer unit (each containing four Cu atoms) using ε = 4850 M^{-1} cm^{-1} at 610 nm.[11] The dose was adjusted so that the concentration of radicals produced was much smaller (≈5 μM) than the protein concentration (20–100 μM), thereby ensuring that the 4 Cu enzyme unit was only singly reduced. Pulse-radiolysis traces were recorded on a Gould 4072 100 MHz Transient Digitiser, which was used in conjunction with a DEC 11/73 computer for storage and processing. The traces were subjected to first-order kinetic analysis by a program TREAT.[14]

Results

Partial one-electron reduction of fully oxidised ascorbate oxidase by pulse radiolysis generated formate, methyl viologen, deazaflavin and lumiflavin radicals, as in equations (2)–(5), and was monitored at 610 nm. In all cases a fast bimolecular reduction of the type 1 Cu^{II} was observed (see inset to Fig. 3). A graph of k_{obs} *vs.* protein concentration for reduction by $dmbipy^{\bullet +}$ is shown in Fig. 3. At the same time, reduction at the trinuclear site may well occur, but was not monitored in these studies. Second-order rate constants for the four different reductants, along with their reduction potentials, are given in Table 1. The rate constant of 3.8×10^7 M^{-1} s^{-1} for bimolecular reduction with the lumiflavin radical is in satisfactory agreement with the value of 2.7×10^7 M^{-1} s^{-1} reported by Meyer *et al.*[9]

More significant is the electron transfer which follows from the type 1 to the trinuclear site monitored by recovery of the absorption at 610 nm. Kinetic traces observed for all four radicals are biphasic, *e.g.* Fig. 4(*a*) and 4(*b*). Rate constants k_1 and k_2 listed in Table 2 are independent of both protein and radical concentrations. Contributions to the recovery of absorption at 610 nm are respectively 45 ± 10 and 20 ± 10% of the total absorption change for the initial reduction (Fig. 4). With $dmbipy^{\bullet +}$ as reductant the pH was varied in the range 7.0–8.0, and rate constants obtained for the two stages are also given in Table 2.

Activation parameters for the two stages were determined by varying the temperature in the range of 5–30 °C, and carrying out Eyring plots of ln k/T against $1/T$. These rate constants are listed in Table 3. The first stage (k_1) gives $\Delta H_1^{\ddagger}$ = 7.1 ± 0.3 kcal mol^{-1} and $\Delta S_1^{\ddagger}$ = −25.8 ± 1.3 cal K^{-1} mol^{-1}. From a

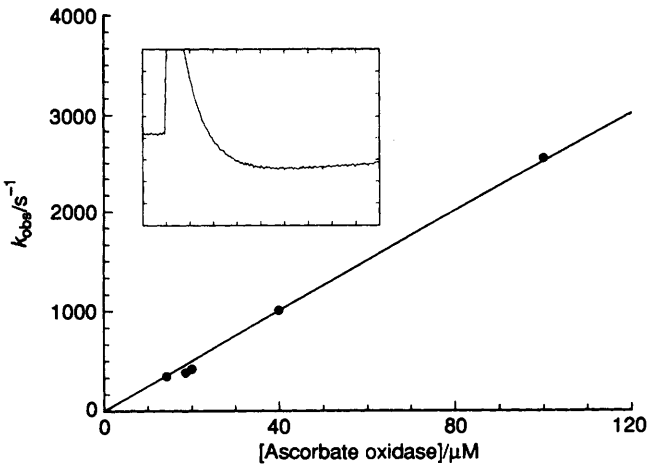

Fig. 3 Plot of first-order rate constant *vs.* protein concentration for reduction of type 1 Cu^{II} in ascorbate oxidase by $dmbipy^{\bullet+}$ and pH 7.0 (40 mM phosphate) with 0.010 M formate and 7.8 × 10^{-4} M $dmbipy^{2+}$, I = 0.100 M. Inset: pulse-radiolysis trace for the reduction of type 1 Cu^{II} by $dmbipy^{\bullet+}$ observed at 610 nm. Ordinate, 1.36% absorption per division; abscissa, 500 μs per division

Table 1 Second-order rate constants (19 °C) for the reduction of the type 1 Cu^{II} of 20–100 μM ascorbate oxidase ($E°$ 345 mV) by different radical reductants*, pH 7.0 (40 mM phosphate) with 0.010 M formate, I = 0.100 M

Reductant	$E°$/V	Ref.	k/M^{-1} s^{-1}
$CO_2^{\bullet-}$	−2.00	15	(1.1 ± 0.2) × 10^9
$dmbipy^{\bullet+}$	−0.450	16	(2.6 ± 0.1) × 10^7
Deazaflavin	−0.650	17	(1.5 ± 0.1) × 10^8
Lumiflavin	−0.240	18	(3.8 ± 0.1) × 10^7

* Concentrations of radical reductants ≈ 5 μM.

Table 2 First-order rate constants (19 °C) for the reoxidation of the type 1 Cu in ascorbate oxidase after initial reduction by the radical. The concentration of protein was varied between 20 and 100 μM and of radicals between 5 and 20 μM at pH 7.0 (40 mM phosphate), 7.5 and 8.0 with 0.010 M formate, I = 0.100 M. The number of runs averaged is indicated in parentheses

Radical	k_1/s^{-1}	k_2/s^{-1}
$CO_2^{\bullet-}$ (4)	120 ± 3	2.0 ± 0.1
$dmbipy^{\bullet+}$ (4)	127 ± 3	2.3 ± 0.5
pH 7.5	62 ± 2	0.7 ± 0.1
pH 8.0	31 ± 3	0.4 ± 0.1
Deazaflavin (3)	121 ± 5	2.5 ± 0.2
Lumiflavin (3)	97 ± 10	2.4 ± 0.2

similar analysis for the second stage (k_2), $\Delta H_2^{\ddagger}$ = 12.7 ± 0.6 kcal mol^{-1} and $\Delta S_2^{\ddagger}$ = −14.0 ± 1.3 cal K^{-1} mol^{-1}. For laccase Farver and Pecht[10b] have reported values of $\Delta H^{\ddagger}$ = 11.3 kcal mol^{-1} and $\Delta S^{\ddagger}$ = −20 cal K^{-1} mol^{-1} for their single intramolecular electron-transfer step.

Changes in absorbance at the trinuclear 330 nm peak were also monitored. These are first of all an increase and then a decrease in absorbance with rate constants of similar magnitude to k_1 and k_2 determined at 610 nm. It has been reported previously in studies on laccase that the 330 nm chromophore is a co-operative two-electron acceptor.[19] We have no independent information as to the absorbance change corresponding to formation of the half-reduced type 3 copper pair. Based on existing reduction potential information it seems unlikely that the type 2 centre is reduced. A further complication is that the radicals used absorb in the 330 nm region of the spectrum making monitoring at 610 nm preferable.

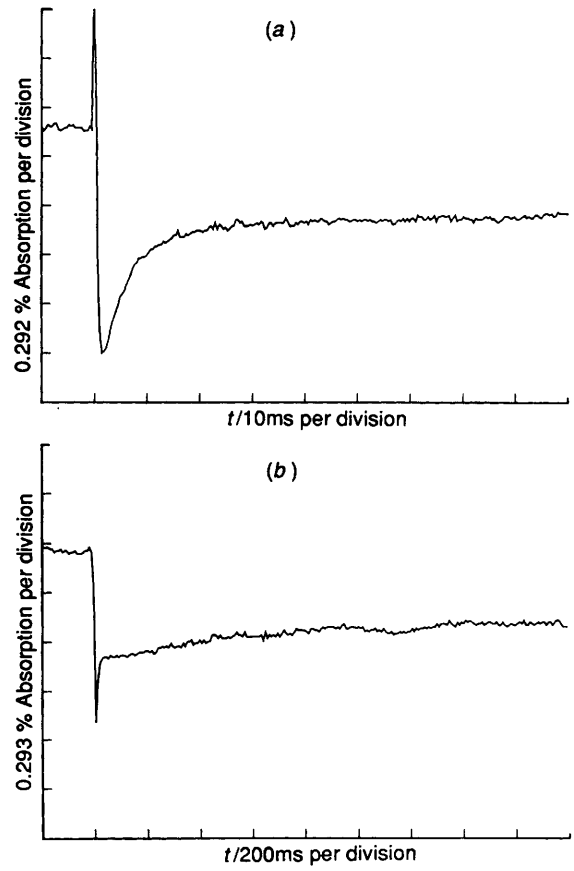

Fig. 4 Pulse-radiolysis traces for the recovery of the absorption due to type 1 Cu^{II} in ascorbate oxidase (50 μM) following reduction by $dmbipy^{\bullet+}$ at pH 7.0 (40 mM phosphate) in solutions containing 0.010 M formate and 7.8 × 10^{-4} M $dmbipy^{2+}$, I = 0.100 M. The traces have been fitted by a two-stage biphasic process

Discussion

Two quite different rate constants have been reported by Meyer *et al.*[9] (160 s^{-1}) and Farver and Pecht[10] (≈1.0 s^{-1}) for intramolecular electron transfer between the type 1 and trinuclear sites of ascorbate oxidase. In contrast our kinetic results indicate two intramolecular electron transfer processes k_1 and k_2 between the single type 1 and the trinuclear site, as summarised in Table 2.

The faster process (k_1) corresponds to that reported by Meyer *et al.*,[9] and is assigned as intramolecular electron transfer between the reduced type 1 copper site and an approximately equipotential electron-acceptor site. The equipotential nature of the two sites is supported by the observation that only ≈45% of the reduced type 1 is reoxidised in this process. The fact that the type 3 copper site has been reported to have the same reduction potential as the type 1 site (≈345 mV at room temperature)[2] supports such an interpretation. For such an equilibration process with K ($=k_f/k_b$) ≈ 1, where k_f and k_b are the forward and back rate constants for electron transfer, $k_1 = k_f + k_b$, and $k_f = k_b = k_1/2$. Therefore, the actual rate constant for intramolecular electron transfer between the type 1 and the trinuclear sites is approximately half the k_1 values listed in Table 2. The listed k_1/s^{-1} values for $CO_2^{\bullet-}$ (120), $dmbipy^{\bullet+}$ (127) and deazaflavin (121) average at 122 s^{-1}, and divided by two give an intramolecular rate constant of 61 s^{-1}. From the temperature dependence (Table 3), the rate constant k_1 of 122 s^{-1} at 19 °C increases to a value close to 160 s^{-1} at 25 °C in excellent agreement with 160 s^{-1} (25 °C) reported by Meyer *et al.*[9] for lumiflavin. We are uncertain as to why k_1 for lumiflavin should be ≈20% less than for other radicals. As compared to ref. 9, the different buffer concentrations (phosphate at 40 and

Table 3 Rate constants for the reoxidation of type 1 Cu, after reduction with dmbipy$^{\bullet+}$, at different temperatures at pH 7.0 (40 mM phosphate) with 0.010 M formate, I = 0.100 M

(*i*) Fast stage, k_1

T/°C	4.0	7.0	10.0	13.0	18.0	18.0	18.0	20.0	20.0	20.7	20.7	25.0	25.0	30.0
k/s^{-1}	57	65	81	95	126	120	121	124	129	135	127	166	154	177

(*ii*) Slow stage, k_2

T/°C	6.0	10.0	14.4	15.0	15.0	15.0	16.5	25.0	30.0	30.0	30.0
k/s^{-1}	0.52	0.60	0.92	1.10	1.03	1.21	1.12	2.8	4.5	4.1	3.7

20 mM) and techniques (flash photolysis in ref. 9) are not expected to have any influence.

Rate constants k_2 (s^{-1}) for the slower second stage for $CO_2^{\bullet-}$ (2.0), dmbipy$^{\bullet+}$ (2.3), deazaflavin (2.5) and lumiflavin (2.4) average at 2.3 s^{-1}, and are in satisfactory agreement, Table 2. This process very likely corresponds to that reported by Farver and Pecht[10] for ascorbate oxidase ($\approx$1.0 s^{-1}). Our interpretation has to be different however. The crystal structure of the fully reduced form of ascorbate oxidase is now known,[20] and draws attention to structural changes occurring at the trinuclear site. Thus on reduction the OH bridge, Fig. 1, is released and the two Cu atoms move towards their respective histidines and become three-co-ordinate, a perfectly acceptable stereochemistry for Cu^{I}. The Cu · · · Cu distance increases from 3.7 to about 4.8 Å. The structure of the fully reduced trinuclear copper site is quite different therefore from that of the fully oxidised rest-state enzyme.

Absorption changes at 330 nm are more difficult to interpret, but are consistent with earlier observations on laccase.[19] Ascorbate oxidase has three disulfide bridges and we checked the possibility of RSSR$^-$ radical formation, as in similar studies with azurin[21] and laccase.[10b] Under our conditions we saw no absorption changes at 410 nm (where RSSR$^-$ absorbs) with $CO_2^{\bullet-}$ as reductant.

The k_2 step corresponds to a further restoration of the 610 nm absorption. The most likely explanation is therefore that it represents further electron transfer from the type 1 to the trinuclear site following some (minor) structural changes at the latter. As a result of these changes a readjustment in electron distribution is required. It can be seen from the data in Table 2 that both k_1 and k_2 increase (factors of 4 and 6 respectively) as the pH decreases from 8 to 7. The possibility that there is at the outset an [H$^+$]-controlled dynamic equilibrium involving two forms of the fully oxidised trinuclear site (giving rise to k_1 and k_2) seems to be ruled out, since if this explanation were valid the dependencies of k_1 and k_2 on [H$^+$] would be expected to run counter to each other. Absorption changes for the two stages at pH 7.5 and 8.0 are within the ranges quoted for pH 7.0 and do not appear to be pH dependent.

For non-adiabatic electron transfer processes the entropy of activation $\Delta S^{\ddagger}$ includes both ΔS^*, the entropic term which appears in the thermodynamic equation, and an entropic contribution from the transmission coefficient, κ, as in equation (6),[8a] where κ decreases exponentially with the distance r

$$\Delta S^{\ddagger} = \Delta S^* + R \ln \kappa \quad (6)$$

separating the electron-donor and -acceptor sites, equation (7),

$$\kappa(r) = \exp[-\beta(r - r_o)] \quad (7)$$

where r_o is the closest contact distance, normally taken to be 3 Å (van der Waals contacts of the edge of the donor and acceptor groups), and β is the exponential term describing the electron-tunnelling barrier, which is likely to vary from system to system. By using a through-space distance between the S$^{\gamma}$ of Cys 507 and N$^{\gamma}$ of His 506 of 6.42 Å,[7a] which is the edge-to-edge distance between the two redox centres, the recently favoured β value of 1.4 Å^{-1},[22] and combining equations (6), (7) with the thermodynamic equation (8), $\Delta G^{\ddagger}$ is calculated to be 11.9 kcal mol^{-1} (50 kJ mol^{-1}) for k_1. From equation (9),[8a] assuming identical

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^* \quad (8)$$

$$\Delta G^{\ddagger} = \frac{\lambda}{4}\left(1 + \frac{\Delta G^{\circ}}{\lambda}\right)^2 \quad (9)$$

reduction potentials for the type 1 and the type 3 copper sites,[2] a reorganisation energy λ of 2.07 eV is obtained. The latter is significantly larger than the corresponding 1.2 eV estimated for azurin (intramolecular electron transfer from a disulfide radical ion RSSR$^{\bullet-}$ to Cu^{II}),[21] and cytochrome c [intramolecular electron transfer from His 33–Ru^{II}(NH$_3$)$_5$ to the haem Fe^{III}],[8b] suggesting that substantial structural changes are required at the copper sites prior to intramolecular electron transfer. Such changes are indicated in the structure of the trinuclear site from crystallographic studies on the fully reduced enzyme.[20]

As pointed out earlier, a direct pathway for electron transfer, consisting of Cys 507 and either His 506 or His 508, exists for intramolecular electron transfer between the two copper sites on ascorbate oxidase, Fig. 1. An interesting question is whether the two pathways are 'equivalent' with regard to electronic coupling between the two sites.[8a] Beratan and Onuchic[23] have recently developed an algorithm to help identify the most favourable long-range electron-transfer pathways in metalloproteins. Using their program, the electronic coupling between the S$^{\gamma}$ of Cys 507 and the imidazole rings of His 506 and His 508 has been examined. The most favoured route consists of four covalent bonds and a hydrogen bond between the carbonyl O of Cys 507 and the N$^{\delta}$ atom of His 506 (hydrogen-bond length 2.88 Å), Fig. 5, which gives an electronic coupling ε^2 value of 2.3 × 10^{-3}. The second most efficient pathway contains an extra covalent bond between the N$^{\delta}$ and C$^{\gamma}$ atoms of His 506, and gives an ε^2 of 8.28 × 10^{-4}. For the second imidazole ring the best pathway consists of seven covalent bonds between S$^{\gamma}$ of Cys 507 and C$^{\gamma}$ of His 508 with an ε^2 of 7.8 × 10^{-4}. Thus the hydrogen-bonded Cys 507-His 506 pathway gives approximately three times more efficient electronic coupling than the Cys 507-His 508 route. In the case of the type 1 blue copper protein plastocyanin it is believed that a similar electron-transfer pathway, consisting of the copper ligand Cys 84 and adjacent highly conserved Tyr 83 is relevant, and made use of in the reaction with cytochrome f.[24] Further evidence for this route has been obtained in recent work.[25]

As far as possible implications for the physiological function of ascorbate oxidase are concerned, we note that in cytochrome oxidase, where intramolecular electron transfer between Cu and haem Fe are part of the catalytic cycle, changes in the reorganisation energies are believed to control the efficient function of the enzyme as a proton-pump.[26] Possibly, similar changes in the structure of the trinuclear site of ascorbate oxidase may take place after the first electron-transfer process, which alters the λ requirements for the second one, thus slowing it down. Recent EPR studies on laccase have provided evidence for temperature-dependent changes in the magnetic coupling within the trinuclear cluster.[5b] The latter changes, although

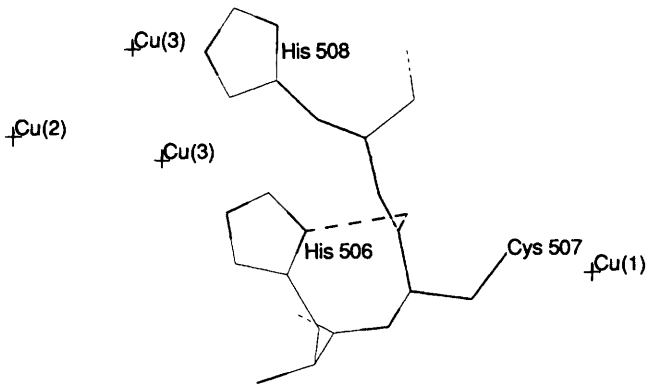

Fig. 5 Electron-transfer pathways between the single type 1 and the trinuclear sites based on the crystallographic data for ascorbate oxidase. The hydrogen bond between the O of Cys 507 and N^{δ} of His 506 is indicated. Two atoms, N^{δ} of His 506 and C^{γ} of His 508, give the most effective coupling between the respective imidazole rings and the S^{γ} of Cys 507 (see text)

observed under non-physiological conditions, indicate that significant reorganisation of the trinuclear copper site is feasible. The properties we have demonstrated for the trinuclear site again draw attention to the special nature of this site, and that the simplistic type 3/type 2 description may not be appropriate.

Acknowledgements

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