THE REACTION OF "BLUE" COPPER OXIDASES WITH O_2

A PULSE RADIOLYSIS STUDY

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The mechanism of the reduction of O_2 to H_2O by oxidase enzymes is of particular chemical interest due to the ability of these enzymes to solve efficiently a number of major energetic and mechanistic problems. These include: the unfavorable first reduction step $O_2 + e^- \rightarrow O_2^-$, which is largely responsible for the "kinetic inertness" of O_2; the production of a potentially harmful intermediate species such as the OH radical; and the multiequivalent nature of the O_2 reduction taken as a whole. The latter process, which catalyzed by blue copper oxidases or cytochrome oxidase, takes place without the release of intermediate products, and is believed to involve multielectron transfers. The first step in the reduction of the O_2 molecule is its binding to the enzyme. This raises the question as to the reduction state of the oxidase at which the interaction with O_2 takes place.

We have studied the blue copper oxidases tree laccase and human ceruloplasmin in this respect, using the large perturbation method of pulse radiolysis (Fig. 1). Blue copper oxidases contain four (laccase) or more (ceruloplasmin) Cu ions, bound in three different types of redox sites (type 1, "blue" copper absorbing around 600 nm, embedded deeply inside the protein; type 2, accessible to solvent and external ligands; type 3, pair of Cu ions, coupled in the native-oxidized state, accessible to external ligands). Controlled partial reduction of the oxidase in the presence of O_2 was achieved by pulse radiolysis of O_2-containing solutions, where within microseconds and in the presence of an OH scavenger (*t*-butanol) a part or all of the initially produced reducing species (e_{aq}^-, H) are converted into O_2^-. Both e_{aq}^- and O_2^- reduce the copper oxidases, as evidenced by the decrease of the type 1 Cu(II) absorption. The presence of O_2 then leads to the prompt reoxidation of this site.

Two distinct processes of reduction and reoxidation of type 1 Cu take place. This was most clearly demonstrated in experiments with laccase, where full reoxidation was also observed at low O_2 concentrations, conditions where most of the type 1 Cu(II)

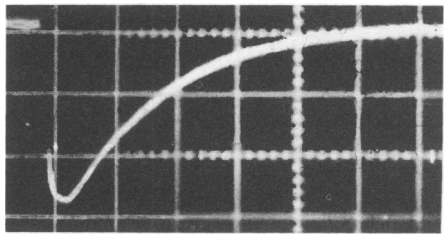

FIGURE 1

reduction is due to e_{aq}^-. However, when laccase or ceruloplasmin were reduced by e_{aq}^- or CO_2^- radicals under anaerobic conditions, no such reoxidation occurred. Nor was reoxidation observed with anaerobic solutions containing H_2O_2 (decay product of O_2^-), thus excluding this species as the oxidant.

The reduction of laccase type 1 Cu(II) by O_2^-. The absorption decrease was first-order under all conditions used. The observed rate constant, k_{red}, varied with the initial O_2^- concentration according to $k_{red} = 5 \times 10^1 + 3 \times 10^7 [O_2]_i \ s^{-1}$ (~25°C, pH ~6.6 – 6.8, $I < 0.001$ M). The small, but similar extent of reduction found for both O_2 and e_{aq}^-—≤3% of total type 1 Cu(II) reduced even at large excess of reductant ($I < 0.001$ M)—was explained in terms of most of the laccase being present in an "inactive state," which undergoes a transition to the "active" state at a slower rate than the self-decay of the reductants.

The reoxidation of laccase type 1 Ci(I) in the presence of O_2. (*a*) The absorption increase was first-order under all conditions used. The observed rate constant, k_{ox}, was independent of the O_2 or any other concentration; thus reoxidation is a true first-order process. (*b*) k_{ox} was 8.3 ± 0.4 s^{-1} (32 pulses) for a protein sample in all experiments where full reoxidation was obtained. In other protein samples where the relative extent of reoxidation varied from 60 to 100%, k_{ox} was 2.8 ± 1.0 s^{-1} (109 pulses). The degree of reoxidation did not depend on the O_2 concentration or on the amount of initially produced reducing equivalents. (*c*) In particular, full reoxidation was also obtained when $([e_{aq}^-] + [H])_i <$ [laccase], i.e. when almost no laccase molecules had accepted more than one electron. Furthermore k_{ox} was insensitive to the ratio $([e_{aq}^-] + [H])_i$/[laccase]. (*d*) F^- ions (2.5 mM), known to bind to type 2 Cu(II) with a concomitant effect on the redox properties of the type 3 site, strongly inhibited the reoxidation, lowering k_{ox} by more than one order of magnitude. Also $HCCO^-$ exerted an inhibitory effect on the reoxidation process. Essentially similar results were obtained with ceruloplasmin.

Discussion and conclusions. Since the type 1 site is reoxidized only in the presence of O_2, it follows from (*c*) that laccase in the singly reduced state is able to interact with O_2. This agrees with other findings showing that a long-lived structural change is induced in the enzyme by the reaction of singly reduced laccase with O_2 (to be published). Since under turnover conditions the laccase molecule assumes the same state,

as judged from spectral data, the catalytic pathway might involve binding of O_2 to singly reduced laccase.

It is widely accepted that the type 1 Cu is inaccessible to external ligands, and that there exist intramolecular redox equilibria between the different copper sites. Based on this and on (*a*), we propose that O_2 reacts with a solvent accessible redox site (type 2 Cu(I) or half-reduced type 3 site), in an electron exchange equilibrium with the type 1 site:[1]

$$\left\{\begin{matrix}\text{type 1 Cu(I)}\\ \text{X(ox)}\end{matrix}\right\} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \left\{\begin{matrix}\text{type 1 Cu(II)}\\ \text{X(red)}\end{matrix}\right\} + O_2 \xrightarrow{k_2} \left\{\begin{matrix}\text{type 1 Cu(II)}\\ \text{X(red):O}_2\end{matrix}\right\}$$

$$K_1 = k_1/k_{-1} \ll 0.1$$

X = type 2 or type 3 site.

The rate-determining step is probably the intramolecular electron transfer from the type 1 site to the site of O_2 interaction ($k_{ox} = k_1 \ll k_2[O_2]$). The fluoride effect is consistent with the type 2 site being involved directly or indirectly, e.g. via allosteric modulation of the type 3 electron acceptor properties.

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[1] A complex and as yet unresolved transient pattern was observed around 330 nm (type 3 band).

VOLTAGE-INDUCED CHANGES IN THE CONDUCTIVITY OF ERYTHROCYTE MEMBRANES

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Previous reports (1–3) have shown that exposure of an isotonic suspension of erythrocytes to an electric field of a few kilovolts per centimeter for a duration in microseconds dramatically increases the permeability of the cell membranes. As a result, the erythrocytes undergo hemolysis through colloid osmotic swelling. Since the enhanced permeability is limited to small ions or molecules, the effect has been attributed to the formation of aqueous pores in the membranes. These pores are formed when the transmembrane potential induced by the externally applied field exceeds 1 V. The effective radius of the pores is several Ångstroms and can be varied by the adjustment of field intensity, field duration, or the ionic strength of the medium. Here we report a study of the kinetics of the pore formation, where the increase in permeability was detected by conductivity measurements.

Erythrocytes suspended either in isotonic NaCl or in a 1:9 mixture of isotonic NaCl