Proc. Natl. Acad. Sci. USA
Vol. 75, No. 11, pp. 5245–5249, November 1978
Chemistry

Reductant-dependent electron distribution among redox sites of laccase

(reductive titrations/blue copper oxidases/uncoupling of copper pair/driving force/nonequilibrium states)

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Communicated by Harry B. Gray, July 26, 1978

ABSTRACT ***Rhus*** **laccase (monophenol monooxygenase, monophenol,dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) an O_2/H_2O oxidoreductase containing four copper ions bound to three redox sites (type 1, type 2, and type 3 Cu pair), was titrated anaerobically with several reductants having various chemical and thermodynamic properties. The distribution of electron equivalents among the redox sites was found to be reductant dependent. When the data for titration by various reductants of the type 3 site were plotted against those of the type 1 site according to the Nernst formalism, the slope *n* varied from 2.0 to 1.0. The redox potential of the reductant's first oxidation step is qualitatively correlated with the value of *n* and is suggested as the factor that modulates the electron distribution. Such a behavior implies a nonequilibrium situation. A very good simulation of the data was provided by an analysis assuming a formally variable cooperativity between the two type 3 copper ions. This apparent variability is suggested to result from a process whereby sufficiently strong reductants induce a transition of the type 3 site from a cooperative two-electron acceptor to a pair of independent one-electron acceptors. This uncoupled state of the type 3 site is considered metastable. Other possible models were also investigated. Summarizing the available data, we conclude that the two-electron accepting behavior of the 330-nm chromophore is the exception rather than the rule.**

Native laccase (monophenol monooxygenase, monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) from the Japanese lacquer tree *Rhus vernicifera* contains four Cu(II) ions in three distinct sites (1, 2). Earlier spectrophotometric and potentiometric titrations of native laccase with reductants such as $Fe(CN)_6^{4-}$ (3), ascorbate in the presence of $Fe(CN)_6^{3-/4-}$ as mediator (4), and 1,4-benzohydroquinone (BhQ) (5) have led to the conclusion that the type 1 and 2 copper ions act as one-electron acceptors, while the type 3 copper ions act as cooperative two-electron acceptors. We have recently shown that in oxidative titrations of reduced laccase with O_2 or H_2O_2 a different pathway is followed, in which the type 3 site seems to act like two single-electron donors (6).

BhQ was shown to serve as a one-electron donor for laccase (7). Consequently, the relevant redox potential is that of the semiquinone-hydroquinone couple, $E_2°'$ (480 mV, Table 1). With ascorbate as reductant in the presence of $Fe(CN)_6^{3-/4-}$, the relevant potential is the one of the mediator [420 mV under the conditions used (4)]. In two previous studies (4, 5) the potentials of the enzyme's redox sites were found to be 395 (420) mV, 365 (390) mV, and 435 (460) mV for the type 1, type 2, and type 3 sites, respectively (values in parentheses from ref. 5). Thus, the reductants that have been used previously have similar or even higher potentials than the redox sites of the protein. In contrast, the electron acceptors that were used in the oxidative titrations of reduced laccase, O_2 and H_2O_2, are both multi-electron acceptors and also have much higher oxidation potentials than the electron donor sites of the enzyme (8).

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In order to identify the reason for the variable electron distribution pattern and resolve between specific structural features, redox potential, or reactivity of the reductants, reductive titrations were carried out with electron donors spanning a wide range of redox potentials and differing markedly in their structure and mechanism of reaction. We found that the course of the titration is controlled by the driving force of the reduction, a phenomenon that to the best of our knowledge has not been observed before for multicenter redox proteins.

MATERIALS AND METHODS

Laccase was prepared from acetone powder of the lacquer of *Rhus vernicifera* according to a modified version of Reinhammar's procedure (9, 10). Its spectroscopic, electron paramagnetic resonance (EPR), and enzymatic properties were in agreement with those reported earlier (11). $A_{280}/A_{615} = 14.6$. The concentration of the protein was determined by using $\epsilon = 5700\ M^{-1}\ cm^{-1}$ (11). The reductants used were prepared as described elsewhere (10). All other chemicals were of analytical grade and used without further purification.

Absorption spectra were recorded on a Cary 118 at 20°C and EPR spectra on a Varian E-3 at liquid nitrogen temperature. The titration technique and the method of data analysis have been described earlier (6). All calculations, simulations, and plots were produced by a BASIC program running on a Hewlett–Packard 2100 computer.

RESULTS

Reduction of Laccase by BhQ. Anaerobic reductive titrations of laccase with BhQ (Figs. 1 and 2) gave results in excellent agreement with data reported earlier (5).

Reduction of Laccase by $Ru(NH_3)_6^{2+}$. Oxidation of this strong single electron donor (17) with either Cu(II)-bathocuproinesulfonate, O_2, or oxidized laccase led to the stoichiometric formation of a Ru(III) compound. A molar extinction coefficient of $310\ M^{-1}\ cm^{-1}$ was used for correction of the measured absorbance at 330 nm in the laccase titrations.

Fig. 1 shows the results of a reductive anaerobic titration of laccase with $Ru(NH_3)_6^{2+}$. It is obvious that the electron distribution among the sites is rather different from that obtained in the BhQ titration. This difference becomes even more pronounced in the double logarithmic plots (Fig. 2). In order to examine whether the electron distribution can be modified by using benzoquinone as mediator, a reductive titration of 79 μM laccase with $Ru(NH_3)_6^{2+}$ was performed in the presence of 39 μM benzoquinone. However, the results were identical to those obtained without the mediator. Laccase was also titrated sequentially by two different reductants, starting with BhQ and,

Abbreviations: BhQ, 1,4-benzohydroquinone; XhQ, xylohydroquinone (2,5-dimethyl-1,4-benzohydroquinone); DhQ, durohydroquinone (2,3,5,6-tetramethyl-1,4-benzohydroquinone); EPR, electron paramagnetic resonance.

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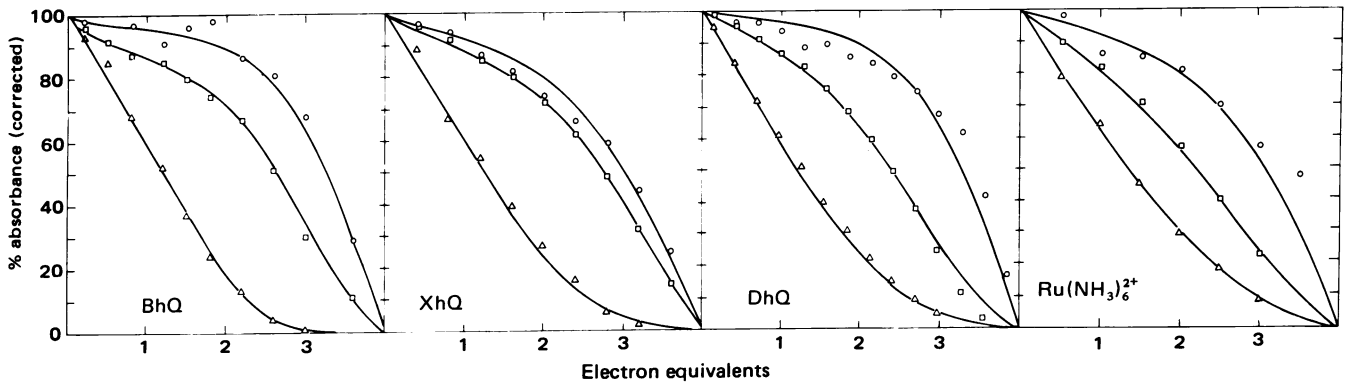

FIG. 1. Anaerobic spectrophotometric titrations of *Rhus* laccase with various reductants at 25°C. BhQ, 1,4-benzohydroquinone; XhQ, xylohydroquinone (2,5-dimethyl-1,4-benzohydroquinone); DhQ, durohydroquinone (2,3,5,6-tetramethyl-1,4-benzohydroquinone). The initial solutions contained 30–120 μM laccase in 0.1 M potassium phosphate buffer (pH 7.0, ionic strength I = 0.22 M). EDTA concentrations varied from 0 to 400 μM without having any effect on the results. △, Corrected fractional absorbance at 330 nm; □, at 614 nm; ○, fraction of oxidized type 2 sites, calculated from the overall stoichiometry and the measured amounts of reduced type 1 and type 3 copper ions. (All experimental errors accumulate in the computed values for the type 2 site, causing the enhanced data scatter.) The fractional absorbances at 330 and 614 nm were calculated from $(A - A_{red})/(A_{ox} - A_{red})$, in which A_{ox} is the initial absorbance of the oxidized chromophore, A_{red} is the absorbance at full reduction, and A is the measured absorbance. The latter two were corrected for oxidized titrant absorption at 330 nm [xyloquinone, ϵ = 100 M^{-1} cm^{-1} (12); duroquinone, ϵ = 240 M^{-1} cm^{-1} (12), Ru(III), ϵ = 310 M^{-1} cm^{-1} (see text)]. The lines represent simulated titration curves. The simulations were generated for the general case of 16 possible species (0–4 electrons in 4 sites) linked by 15 equilibrium constants. These were defined via reduction potentials, assuming the Nernst formalism. First the concentrations of each of the species were obtained by assuming a certain electrochemical solution potential, then the reduction equivalents taken up were calculated from the concentrations weighted by the number of electrons in each species. Care was taken to maintain path independence of the free energy change for total reduction. In all simulations the potential of the type 1 site, E_I, was kept constant and the potentials of the other sites, E_{II} (type 2 site) and E_{III} (type 3 site), were chosen relative to E_I. Thus, together with ΔE_{III}, the interaction potential of the type 3 site (see text), actually three adjustable parameters were available for the simulation. The values used are indicated in Table 1. As to the absorption properties of the type 3 copper ions, it is assumed that they contribute equally to the absorbance changes at 330 nm.

when the laccase was about half reduced, continuing to completion with $Ru(NH_3)_6^{2+}$ (Fig. 3). The shape of the titration curve as well as the logarithmic plot clearly demonstrate that the pattern of the electron distribution drastically changes with the reductant.

In order to check whether laccase molecules that have first been through a reduction–reoxidation cycle behave differently, a series of experiments with BhQ and $Ru(NH_3)_6^{2+}$ as reducing agents was performed, in which the laccase solutions contained a known initial amount of O_2. However, as soon as the oxygen had been consumed, the reduction proceeded as in the anaerobic experiments.

Another set of experiments was carried out in which laccase was titrated with $Ru(NH_3)_6^{2+}$ and then reoxidized by O_2. The solution was deaerated again, and a second reductive titration with $Ru(NH_3)_6^{2+}$ was performed. Within experimental error, there was no difference between the first and the second cycle.

Reduction of Laccase by DhQ. DhQ has a two-electron redox potential of 65 mV at pH 7.0 and 25°C (13). However, assuming that the mechanism of electron transfer to laccase is the same as that for BhQ, the relevant potential, $E_2°'$, is 355 mV under the same conditions (16). The results (Figs. 1 and 2) are clearly different from those found with BhQ, and it is seen that

Table 1. Reductant-dependent electron distribution parameters

Reductant	$E°'$,[a] mV	$E_2°'$,[b] mV	n[c]	E_I,[d] mV	E_{II},[d] mV	E_{III},[d] mV	ΔE_{III},[d] mV
BhQ	285[e]	480[f]	2.0	395	365	438	±100[g]
XhQ	175[e]	415[f]	1.4	395	375	440	±30
DhQ	65[e]	355[h]	1.1	395	355	435	±10
$Ru(NH_3)_6^{2+}$	—	51[i]	1.0	395	365	420	±5
$Fe(CN)_6^{4-}$ [j]	—	420[k]	2.0	—	—	483	±65

[a] Two-electron redox potential at pH 7.0 and 25°C.
[b] Redox potential of first oxidation step, at pH 7.0 and 25°C [except for $Fe(CN)_6^{4-}$].
[c] Slope of the central part of the line in the double logarithmic plot (Fig. 2).
[d] Best simulation parameters for type 1, type 2, and type 3 sites. For the choice of E_I see legend of Fig. 1. ΔE_{III} is the interaction potential as defined in the text.
[e] Ref. 13.
[f] Calculated from $E_2°' = E°' - 0.030 \log K_i$, in which K_i is the semiquinone formation constant at pH 7 (14, 15).
[g] A good simulation is also obtained for ΔE_{III} = +70 mV.
[h] Ref. 16.
[i] At I = 0.1 M and 25°C (17).
[j] Best simulation of the data reported in ref. 4. Because binding of hexacyanoferrate ions to the enzyme strongly modulates the redox behavior of the latter (ref. 4; M. Goldberg, unpublished results), one has to be cautious in drawing conclusions from redox titrations of laccase in the presence of ferro- or ferricyanide.
[k] In sodium phosphate buffer, pH 7.5, I = 0.2 M (18).

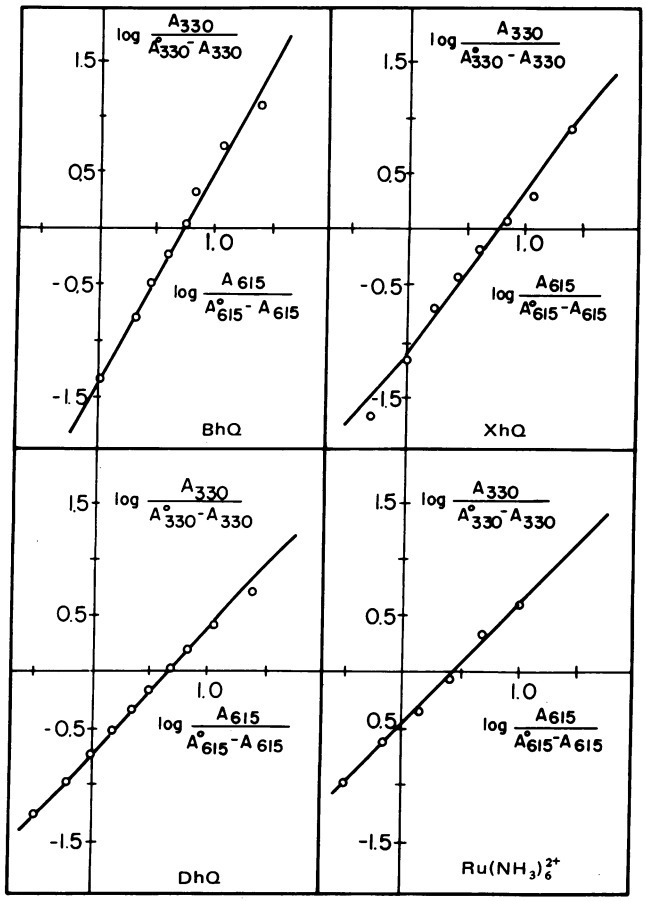

FIG. 2. Plots of log $[A_{330}/(A^{\circ}_{330} - A_{330})]$ against log $[A_{615}/(A^{\circ}_{615} - A_{615})]$. A_{330} and A_{614}, respectively, are equal to $A - A_{red}$ as defined in the legend of Fig. 1, A°_{330} and A°_{614} are equal to $A_{ox} - A_{red}$ at their respective wavelengths. The values are taken from the titrations presented in Fig. 1. The lines represent simulations using the same parameters as in Fig. 1.

a straight line through the experimental points gives a slope of about 1, as with $Ru(NH_3)_6^{2+}$.

Laccase reduction by DhQ and by $Ru(NH_3)_6^{2+}$ is fast, stable absorbance values at 615 and 330 nm being established within the time of mixing. Only towards the end of the titration did it take several minutes before the reading became constant.

Because the slope of 1 might suggest a participation of the type 3 Cu(II) ions as one-electron acceptors in equilibrium with the type 1 site (see *Discussion*), an EPR experiment was carried out. An aliquot of 410 μM laccase was reduced anaerobically with 1.0 electron equivalents of DhQ. The EPR spectrum at 95 K did not reveal any new feature. However, this result was found to be inconclusive. Both from the intensity of the type 1 and type 2 signals and from the absorbance of the type 1 and type 3 chromophores measured just before freezing the partly reduced laccase, it became obvious that the electron distribution sharply differed from the one obtained at protein concentrations one order of magnitude lower (cf. Fig. 1). The type 3 site was only 28% reduced, instead of 42% as expected, and the type 2 site was 30% reduced, which even exceeded the value measured for the type 1 site (15%). Further investigation of the role of protein concentration on the electron distribution proved difficult, because laccase solutions at concentrations $\gtrsim$0.5 mM (55 mg/ml) are rather viscous and tend to undergo gelation.†

Reduction of Laccase by XhQ. The titration curves and the double logarithmic plot are shown in Figs. 1 and 2, respectively. The slope of a straight line through the experimental points lies clearly between those of BhQ and DhQ (Fig. 2), just as the two-electron redox potential of XhQ and the potential of the xylosemiquinone/XhQ couple are intermediate between the corresponding values for BhQ and DhQ (Table 1).

Reduction by Ascorbate and Dithionite. These two reducing agents produced results of limited reliability. Ascorbate [two-electron potential $E^{\circ\prime} = +55$ mV (13); potential of first oxidation step $E_2^{\circ\prime} = +295$ mV, recalculated from data given in ref. 19] led to double logarithmic plots with slopes between 1.3 and 1.8. Replotted data from an earlier study (20) gave a slope of $\approx$1. These variations are probably due to experimental problems; the reduction by ascorbate is rather slow, so that it took up to 40 min to reach a constant absorbance. Furthermore, the oxidation product, dehydroascorbate, can also donate electrons to laccase, although in a very sluggish reaction (M. Goldberg, unpublished results; ref. 21). Dithionite [$E^{\circ\prime} = -527$ mV (22)] gave a slope of 1.2, but aerobic reoxidation of the fully reduced enzyme restored only 75% of the original absorbance at 615 nm, without further increase even after 24 hr, thus indicating some irreversible change. In all the other titrations

† Note that a concentration of 0.5 mM corresponds to a mean distance of 149 Å between the centers of neighboring laccase molecules, or, because these have a diameter on the order of 60 Å, to a mean distance of only $\approx$90 Å between their surfaces.

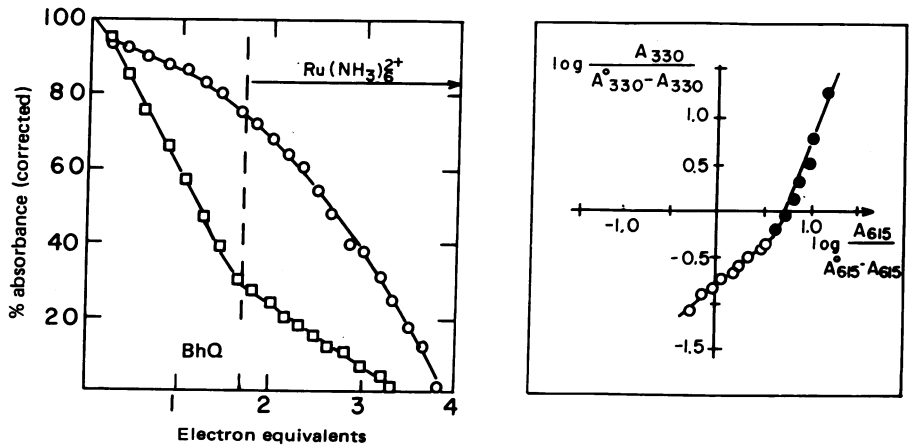

FIG. 3. Anaerobic titration of *Rhus* laccase by BhQ and $Ru(NH_3)_6^{2+}$. (*Left*) The broken vertical line at 1.7 electron equivalents represents the stage at which the titration was switched from the first to the second reductant. □, Corrected fractional absorbance at 330 nm; ○, corrected fractional absorbance at 614 nm. The plotted values were calculated as described in the legend of Fig. 1. (*Right*) Double logarithmic plot. ●, Value measured after addition of BhQ aliquot; ○, value measured after addition of $Ru(NH_3)_6^{2+}$ aliquot.

described above, about 95% of the native absorbance at 615 nm was recovered upon aerobic reoxidation.

DISCUSSION

All the above results lead to the conclusion that the pattern of electron distribution among the redox sites of laccase depends on the potential of the reductant's first oxidation step. This is borne out by the following observations: (*i*) Reductants that are as different chemically, structurally, and in their charge as $Ru(NH_3)_6^{2+}$ and DhQ lead to the same electron distribution pattern. By the same token, $Fe(CN)_6^{4-}$ (4) (see footnote [j] to Table 1) and BhQ, also very different from each other in the above respects, yield similar electron distributions that, however, are different from those produced by the above reductants. This excludes a rationalization in terms of either specificity between the enzyme and a given reductant (or its oxidation product), or differences in the mode of electron transfer by 1- or 2-equivalent reductants. (*ii*) Subjecting the enzyme to redox cycles prior to the reductive titration led to electron distributions identical to those obtained when starting with the native protein. Also, when reduction is commenced with BhQ up to about half reduction and then continued with $Ru(NH_3)_6^{2+}$, the pattern of the electron distribution in the second half is the same as that obtained in titrations in which only $Ru(NH_3)_6^{2+}$ is used.

It is important to realize that by whatever mode the redox potential affects the electron distribution in the protein, a nonequilibrium situation is involved. The assumption of full thermodynamic equilibrium involving *all* redox sites and redox-related conformational states of the protein will always predict a reductant-independent pattern of electron distribution (unless direct or allosteric binding effects take place). That this does not hold in the present case is most clearly demonstrated in the sequential reduction by BhQ and $Ru(NH_3)_6^{2+}$. Moreover, the existence of one or more metastable states of laccase is suggested by the switch from one pattern of electron distribution to another, because benzoquinone as a mediator is expected to promote equilibration via intermolecular electron exchange. A metastable conformational state in laccase has already been proposed to explain the results of oxidative titrations (23), and a recent study of the interaction of partially reduced laccase with O_2 also led to this conclusion (M. Goldberg, O. Farver, and I. Pecht, unpublished).

In general terms, there are two ways by which the redox potential of the reductant may have a controlling effect on the establishment of a nonequilibrium situation: (*i*) The electron distribution may be governed by the *free energy difference of the electron transfer* from the reductant to the enzyme; i.e. if a particular electron distribution can only be attained by a pathway involving the reduction of a low-potential site, only sufficiently strong reductants might be able to produce it. (*ii*) Alternatively, because the rate of electron transfer is expected to depend on, among other things, the free energy difference, the redox potential could affect the electron distribution via *kinetic control*. For example, if there are two competing reduction pathways, each involving a different rate-limiting step, the biomolecular electron transfer might prevail for one pathway, and the intramolecular transfer step for the second.

Several specific models were conceived in the attempt to rationalize our observations. The best simulation of the data was obtained with a model based on the positive cooperativity of the two-electron acceptor site, a concept used earlier (5) to explain the titration behavior of the type 3 site. This thermodynamic notion does not exclude the formation of the half- (i.e., singly) reduced form, but it means that the lower the redox potential of the "oxidized/half-reduced" couple relative to that of the "half-reduced/reduced" couple, the less stable the half-reduced form will be, hence the lower its equilibrium concentration and the higher the degree of cooperativity of the two-electron acceptor. This can be expressed quantitatively by defining an interaction potential ΔE_{III} that in the present context is equal to half the difference between the potential of the second and the first couple. ΔE_{III} will determine the degree of cooperativity. Now, in the present model, the type 3 site is treated as a two-electron acceptor with *formally* varying extent of positive cooperativity, depending on the reductant. This *apparent* variability is then rationalized in terms of a molecular mechanism.

A reduction pattern corresponding to the case of strong cooperativity ($\Delta E_{III} > 60$ mV) is found when the free energy difference between reductant and laccase is small (BhQ) and the converse for strong reductants [$Ru(NH_3)_6^{2+}$, DhQ] ($\Delta E_{III} = 5$ to 10 mV; Figs. 1, 2). In the limit of ($\Delta E_{III} \rightarrow 0$), the two type 3 copper ions behave as fully independent electron acceptors. The moderately strong reductant XhQ leads to an apparently intermediate cooperativity ($\Delta E_{III} = 30$ mV; Figs. 1, 2). However, a similar reduction pattern is obtained if 40% of the laccase molecules titrate with full and 60% with negligible type 3 cooperativity. For all titrants very good agreement between the simulation and the data was obtained (Figs. 1, 2). This is significant, because it means that each titration considered *separately* can be fully described in terms of a simple equilibrium model. It is also noteworthy that while the slope of the double-logarithmic plot changes from 2.0 to 1.0, the redox potential of the type 3 site, E_{III}, varies little (Table 1). The same holds for the type 2 site potential.[‡] The relationships among absorption properties, degree of coupling, and oxidation state of the type 3 copper ions were also analyzed by using different models. The most satisfactory one assumes equal extinction contributions by the two copper(II) ions, and this assumption was used for the simulations presented (Figs. 1, 2).

The apparent modulation of the type 3 site cooperativity may be explained by either of two tentative mechanisms, one assuming the predominance of the driving force, the other a kinetic control effect: (*i*) The type 3 copper pair is initially strongly coupled ($\Delta E_{III} > 60$ mV); i.e., the potential of the oxidized/half-reduced form is relatively low ($E_{III} - \Delta E_{III} < 375$ mV). Only electron donors with sufficiently low $E_2°'$ values (Table 1) will therefore be efficient in producing the half-reduced form, whereas for weaker reductants ($E_2°' \gg E_{III} - \Delta E_{III}$) the extent of this reaction will be insignificant. The half-reduced type 3 site is postulated to undergo *uncoupling*—i.e., a structural change that abolishes the cooperativity between the two copper ions. This process must be fast to be effective, because it is expected to be in competition with (*a*) accepting an additional electron to this type 3 site and (*b*) transferring the single electron to the other sites in the protein. This model also requires that for strong reductants the oxidized type 3 is the primary electron accepting site. From the data in Table 1 it can be inferred that the threshold value for $E_2°'$ leading to full uncoupling is around 370 mV. Interestingly, this is in the region of $E_{III} - \Delta E_{III}$, the redox potential calculated for the half-reduced type 3 site in the coupled state. (*ii*) In an *alternative* model, the type 3 site in the native molecule is assumed to exist in two different states: coupled and uncoupled. These are in fast equilibrium, the coupled state being strongly favored. Now, the strong reductants are proposed to react preferentially with the uncoupled type 3 site, whereas weak reductants will react with type 1 or type 2 Cu(II) as primary reduction site. However, the titration pattern observed for

[‡] The potential of the type 1 site was chosen *a priori* to be constant at 395 mV (cf. legend of Fig. 1).

strong reductants is obtained only if the singly reduced uncoupled type 3 site is locked into the uncoupled state—i.e., if the uncoupling is no longer reversible. Both mechanistic hypotheses imply a *metastable* uncoupled state of type 3 site, which does not relax within the usual time range of the titrations (15 to 20 min per addition). The uncoupling of the two type 3 Cu(II) ions may in principle lead to the appearance of a new EPR signal. However, no evidence for such a signal has yet been obtained. Such experiments may turn out to be difficult to perform, because according to the simulation of the DhQ titrations the signal from uncoupled type 3 Cu(II) ions will even at the best not exceed 12% of the total EPR intensity. Additional detection difficulties may arise because the EPR spectrum could be very much broadened (intermediary electron exchange range between the two type 3 ions) or split into seven hyperfine lines (fast exchange).

There is circumstantial evidence in support of the model discussed: When 1–3 electron equivalents of BhQ or ascorbate is added to an O_2-containing laccase solution, a relatively long-lived transient absorption is formed (λ_{max} around 340 nm, $k_{decay} = 4 \times 10^{-4}\ s^{-1}$; ref. 10). This absorption is attributed to a particular state of the type 3 site, induced by the interaction with O_2. When $Ru(NH_3)_6^{2+}$ is used as reducing agent, no such transient is observed. This may be taken as an indication for a different state of the type 3 site interacting with O_2. Undoubtedly, EPR or magnetic susceptibility evidence is required to verify the uncoupling hypothesis. It is noteworthy that the extent of *inter*molecular electron exchange between laccase molecules has not been determined unambiguously. An intermolecular equilibrium is, however, part of the general equilibrium assumption underlying the Nernst formalism used in the present simulations. Its absence would require treatment describing a nonequilibrium distribution of electrons between molecules in those cases in which no mediator is present.

As a general conclusion, it has become evident that the 330-nm chromophore can no longer be characterized as a simple cooperative two-electron acceptor as hitherto thought (1). Out of 11 redox agents—the 7 reductants mentioned in this study and the oxidants O_2, H_2O_2 (6, 20, 23), $Mo(CN)_8^{3-}$, and $Mn(EDTA)^-$ (M. Goldberg, O. Farver, and I. Pecht, unpublished)—only BhQ and $Fe(CN)_6^{4-}$ led to a fully cooperative behavior. In all the other cases the electron distributions were found to deviate from the pattern expected for a cooperative two-electron acceptor.

The question whether the 330-nm chromophore is an unambiguous indication of the redox state of the nonparamagnetic electron acceptors has already been raised, at least for ceruloplasmin (24). Their suggestion (24) that the reduction of type 2 Cu^{2+} could also lead to changes in the 330-nm band is particularly interesting because it can form an alternative rationale for our observations.

We thank Dr. Doron Lancet for many fruitful discussions. O.F. gratefully acknowledges a grant from the Danish Natural Science Research Council. S.W. acknowledges an Aharon Katzir-Katchalsky Fellowship.

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