Redox State of Peroxy and Ferryl Intermediates in Cytochrome *c* Oxidase Catalysis†

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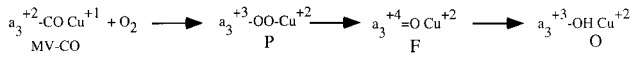
ABSTRACT: The redox states of the "peroxy" (P) and "ferryl" (F) intermediates formed during reoxidation of reduced bovine cytochrome *c* oxidase have been probed by reduction with both ferrocytochrome *c* and acetylpyridine NADH under anaerobic conditions using optical spectroscopy. The reduction of the P and F forms revealed that both are in very similar redox states. One-electron reduction of either the P or F form yields an optical spectrum primarily due to oxidized enzyme implying that the heme iron of cytochrome a_3 is in the ferryl state in both forms. The F and P forms were found to be 1 and less than 1.3 oxidizing equiv, respectively, above the oxidized enzyme. The slightly higher oxidation state in the P form is interpreted as being due to an optically undetectable redox center presumably located in the binuclear cavity.

Cytochrome *c* oxidase belongs to a superfamily of terminal oxidases that contain a heme−copper binuclear catalytic center (*1*, *2*). The binuclear center in the bovine enzyme is composed of cytochrome a_3 and Cu_B. In this center dioxygen is reduced to water and exogenous ligands are bound. Electrons for the conversion of O_2 to water are transferred intramolecularly from two other redox centers designated cytochrome *a* and Cu_A.

The reduction of O_2 to water proceeds through several discrete oxy intermediates (*3*−*15*). Two of these intermediates, the "peroxy" (P) and "ferryl" (F) forms, were first observed in mitochondria during the reversal of electron transfer from water to cytochrome *c* (*16*−*18*). It was suggested that in the P form the binuclear center contains an intact peroxy O−O bond (e.g., Fe_3^{3+}−O−O−Cu_B^{2+}), while in the F form the peroxy bond is cleaved by two-electron reduction during which the iron of cytochrome a_3 is converted into the ferryl state (Fe_3^{4+}═O Cu_B^{2+}) (*16*−*18*).

The same two intermediates are observed following addition of hydrogen peroxide to oxidized enzyme (oxidized CcO,[1] O) (*19*−*23*). The P form can also be produced by the reaction of oxygen with the mixed-valence CO complex (a^{+3}-$Cu_A^{+2}a_3^{+2}$CO Cu_B^{1+}) (*23*, *24*). Recently we have shown that binding of one molecule of hydrogen peroxide to the binuclear center (*23*) is sufficient for the formation of both the P and F forms. A similar result was obtained for the F form of the *bo* oxidase from *Escherichia coli* (*25*). These data indicate that the P and F forms might be in the same redox state, both 2 oxidizing equiv above oxidized oxidase. However, photoreduction of the P and F forms, a process assumed to involve one electron, showed that the P form is converted to the F form and the F form to oxidized CcO (Scheme 1) implying that the P form is 2 oxidizing equiv and the F form 1 oxidizing equiv above oxidized enzyme (*26*).

Scheme 1



Most surprising, however, is the result that in both the P and F forms a Raman mode characteristic of Fe^{IV}═O is present, an observation which implies that the O−O bond is broken (*27*−*30*). This result is consistent with our observation that the elements of hydrogen peroxide cannot be detected in the P form (*31*).

The presence of the oxo−iron structure in the P form suggests four possible states which differ in the location of the additional oxidative equivalent:

(i) Fe_3^{V}═O

(ii) Fe_3^{IV}═O + porphyrin π cation radical

(iii) Fe_3^{IV}═O + amino acid radical

(iv) Fe_3^{IV}═O + Cu_B^{3+}

In this study we have reacted the P and F forms of cytochrome oxidase with graded amounts of reduced cytochrome *c* and the P form with acetylpyridineNADH (apNADH) under anaerobic conditions. The data show that addition of one electron to either the P form or the F form is sufficient to convert the optical spectrum to that characteristic of oxidized enzyme, indicating that the two species have the heme iron of cytochrome a_3 in the same ferryl state. As expected the redox state of the F form was found to be 1 equiv more oxidized than resting enzyme; however the redox state of the P form was found to be less than 1.3 oxidative equiv above that of the resting enzyme.

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[1] Abbreviations: CcO, cytochrome *c* oxidase; P form (CcO-607), peroxy form of cytochrome *c* oxidase; F form (CcO-580), oxoferryl form of cytochrome *c* oxidase; MV-CO, mixed valence cytochrome *c* oxidase; bc_1, respiratory complex III; Tris, tris(hydroxymethyl)-aminomethane; DM, *N*-dodecyl-D-maltoside; TX-100, Triton X-100; apNADH, 3-acetylpyridine NADH; PMS, phenazine methosulfate.

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EXPERIMENTAL PROCEDURES

Cytochrome *c* oxidase (CcO) was isolated by the method of Soulimane and Buse (*32*) with small modifications. During the isolation of enzyme, K_2SO_4 was used instead of chloride salts, and for the extraction of bc_1 and CcO by TX-100, the mitochondrial protein concentration was 20 and 10 mg/mL, respectively. Enzyme concentration was determined at pH 8.0 from the absorbance at 424 nm using $A = 158$ mM^{-1} cm^{-1}. Acetylpyridine NADH (apNADH) was purchased from Sigma and phenazine methosulfate (PMS) from Aldrich. The concentration of apNADH was determined using an absorbance coefficient of 9.1 mM^{-1} cm^{-1} at 364 nm (*33*) and that of PMS determined using an absorbance coefficient of 26.3 mM^{-1} cm^{-1} at 387 nm (*34*).

Two oxy intermediates of CcO, designated "ferryl" (F or CcO-580) and "peroxy" (P or CcO-607), were prepared from oxidized enzyme at pH 8.0. The F form was formed by the addition of 2 mM H_2O_2 to oxidized enzyme in a 2-mm path length, Thunberg-style, anaerobic optical cuvette. When the enzyme was maximally converted to F, as gauged by optical spectra at room temperature, the sample was cooled to −12 °C, catalase added (22 000 units/mL), reduced cytochrome *c* placed in the sidearm, and the sample made anaerobic at this temperature by the gas-exchange technique. Typically the sample was exposed to a short vacuum; then argon was admitted followed by gentle shaking for about 2−3 min. The cycle was repeated at least five times with each sample. The yield of F form after anaerobiosis was usually about 80−85%. To maintain the sample as a fluid at −12 °C, we used a buffer (60 mM Tris, pH 8.0, 0.1% DM) containing 7% 1,2-propanediol. The −12 °C cold bath was a slush of solid CO_2 and ethylene glycol. Significant perturbations to the optical spectrum of enzyme by the cryo-protectant at room temperature were only observed using concentrations above 10%.

The P form was prepared from the mixed-valence CO complex by rendering the enzyme anaerobic under an argon atmosphere in a 2-mm path length, Thunberg-style, anaerobic optical cuvette and then replacing the argon with CO. The buffer was 100 mM Tris buffer, pH 8.0, 0.1% DM, 100 mM K_2SO_4, and catalase (160 units/mL). Reduced cytochrome *c* was placed in the sidearm of the cuvette before anaerobiosis was begun. The MV-CO complex was formed in 100% yield within 3−5 min. The CO was then removed to minimize over-reduction; this was accomplished using two cycles of evacuation and exposure to argon. The argon was then evacuated and the enzyme exposed to air and subjected to a flash of light from a Sunpak Auto 544 camera flash operating at full output. The sample was immediately cooled on ice and made anaerobic once more. The yield of the P form was never less than 90% and frequently approached 95%.

The concentrations of the P and F forms were determined from the difference spectra of P or F *minus* oxidized CcO using $\Delta A_{607-630} = 11$ mM^{-1} cm^{-1} and $\Delta A_{580-630} = 5.3$ mM^{-1} cm^{-1}, respectively (*18*). Both argon (99.999% purity) and CO (99% purity), used for the preparation of anaerobic samples or the mixed-valence CO complex, were passed through oxygen scrubbing columns (Oxisorb, GM Industries) before use.

Reduced cytochrome *c* was prepared by reduction with dithionite, desalted on a P6 column, and dialyzed overnight against buffer (50 mM Tris, pH 7.8, plus catalase) in an N_2 atmosphere at 4 °C. The ferrocytochrome *c* concentration was calculated from the reduced *minus* oxidized absorbance difference using $\Delta A_{550-540} = 19.1$ mM^{-1} cm^{-1} (*35*). The concentration of the stock solution of hydrogen peroxide was obtained from absorbance at 240 nm by using $A_m = 40$ M^{-1} cm^{-1} (*36*).

The reduction of the P or F form by ferrocytochrome *c* in anaerobic conditions was complete in less than 1 min when the initial ratio of c^{2+} to P or c^{2+} to F was less than 1. At ratios of c^{2+} to P or c^{2+} to F larger than 1, it took about 1.5−2 min to reach equilibrium, as judged by optical spectra.

In an alternative approach a known quantity of the P form was converted to oxidized CcO and the oxidized CcO subsequently converted to fully reduced CcO by addition of an excess of apNADH under anaerobic conditions; the amount of fully reduced enzyme produced was measured optically from the (reduced *minus* oxidized) difference spectrum using $\Delta A_{446-420} = 217$ mM^{-1} cm^{-1} (*37*). The reaction with apNADH was mediated by 0.4 μM PMS present in the reaction mixture. The quantity of apNADH consumed in converting P to oxidized enzyme could then be calculated from the total amount of apNADH consumed *minus* the amount consumed in converting oxidized CcO to reduced enzyme; the consumption of apNADH was followed at 364 nm which is its absorbance maximum and also isosbestic for the conversion of oxidized CcO to reduced CcO. The data were corrected for the amount of apNADH consumed in reducing the small amount of oxygen (0.8 μM) diffusing into the anaerobic apparatus over the 25 min needed to carry out the series of measurements.

All optical measurements were performed with a Hewlett-Packard diode array spectrometer (HP 8452A). The temperature in the thermostated cuvette holder was 15 °C.

RESULTS AND DISCUSSION

In characterizing the redox reactions of the P and F forms, we were interested in answering two questions: (i) How many electrons are needed for the conversion of the P or F form to fully oxidized enzyme? (ii) What is a product of the one-electron reduction of the P and F forms? Because oxidized enzyme and the P and F forms have characteristic optical spectra, we have used optical spectroscopy to monitor the reaction products.

To determine the number of electrons needed for the conversion of either the P form or the F form to oxidized CcO, we have assumed that the reduction of P or F by ferrocytochrome *c* under anaerobic conditions will only lead to the formation of partially reduced cytochrome *a* when the amount of electron donor exceeds that needed for the conversion of the P or F form to oxidized CcO. When the extent of reduction of cytochrome *a* was plotted against the initial molar ratio of c^{2+} to F (Figure 1A) the formation of reduced cytochrome *a* could not be detected until the ratio was greater than 1, signifying that the conversion of the F form to oxidized CcO is a one-electron reaction. However in the case of the P form a similar plot did not reveal any a^{+2} until the ratio of c^{2+} to P was greater than 1.3. For comparison, when reduced cytochrome *c* is added to the oxidized enzyme under the same experimental conditions, a readily measured amount of reduced cytochrome *a* could be

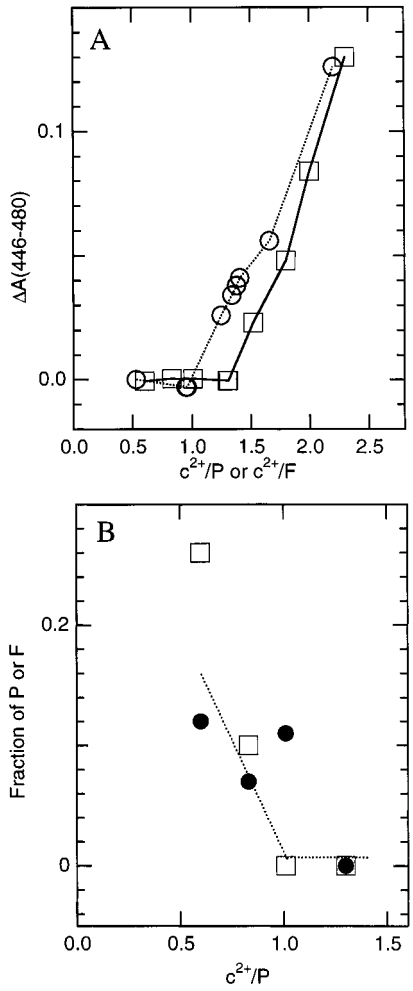

FIGURE 1: Reduction of the P or F form with various amounts of cytochrome c^{2+} in anaerobic conditions. (A) Dependence of extent of reduced cytochrome *a* (ΔA(446−480)) on the initial molar ratio of c^{2+} to P (□) or c^{2+} to F (○). (B) Remaining fraction of the P (□) and F (○) forms after reduction of P by varying amounts of c^{2+}. These quantities were obtained by first subtracting the amount of oxidized cytochrome *c* added to the enzyme from the final spectrum and then subtracting the spectrum of 100% fully oxidized enzyme. The resulting difference spectrum was then analyzed for the P and F forms using the individual spectra of P and F. The total concentration of enzyme was 25 μM. The broken lines have no theoretical significance and are intended to guide the eye.

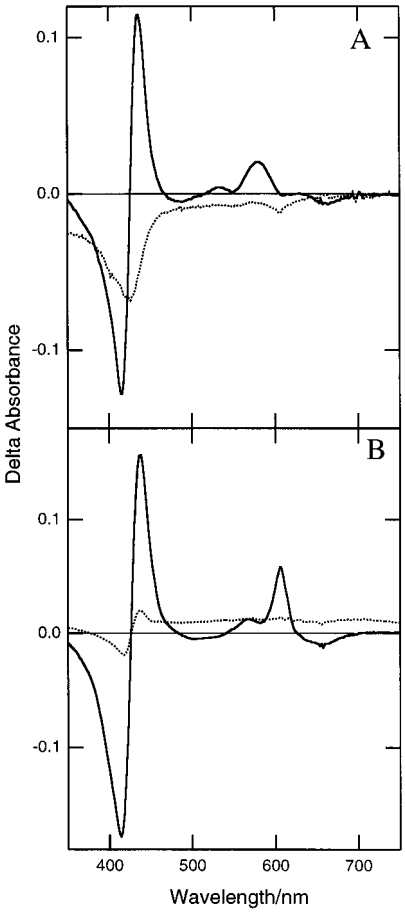

FIGURE 2: Optical difference spectra of the F and P forms (with respect to oxidized enzyme) before and after reaction with 1 equiv of cytochrome c^{+2}: (A) F (—), before addition of c^{+2}; (•••), after addition of c^{+2}. (B) P (—), before addition of c^{+2}; (•••), after addition of c^{+2}. The absolute spectrum of oxidized cytochrome *c* was subtracted from the data recorded after addition of c^{+2}. The total concentration of enzyme was 25 μM.

detected when the molar ratio of c^{2+} to oxidized CcO was 0.13 (not shown).

Analysis of the spectra following reduction of the P form with varying amounts of c^{2+} shows that the quantity of P decreases linearly with increasing amounts of c^{+2} and at a ratio of c^{2+} to P of 1 no P form could be detected in the spectral data (Figure 1B); some F, estimated to be between 7% and 10% of the initial amount of P, was present at this point.

The difference spectra (P or F) *minus* oxidized CcO before and after reaction with 1 equiv of c^{2+} are presented in Figure 2. In the case of the P form there is almost quantitative conversion to oxidized enzyme; the small residual spectrum reflects the 7% of the F form shown in Figure 1B. In both cases the spectra following reduction are those of oxidized oxidase and account for about 90% of enzyme. With the F form there is also almost complete loss of the characteristic difference in the Soret region; we interpret the residual spectral difference as evidence for bleaching of about 8% of the enzyme (Figure 2A) which arises as a consequence of the exposure of the enzyme to 2 mM hydrogen peroxide for several minutes during the preparation of F. This bleaching reflects destruction of the heme and consequently differs from the spectral changes associated with the reactions being studied.

With a ratio of c^{2+} to P of 1.3 it was not possible to detect any P, F, or reduced cytochrome *c* in the spectrum of the reaction mixture. EPR measurements on parallel samples showed that Cu_A was fully oxidized, while the absence of the $g = 6$ signal of oxidized cytochrome a_3 implies that Cu_B was also completely oxidized.

These titration and spectral data show that the redox state of cytochrome a_3 in the F form is 1 oxidized equiv above that of the oxidized enzyme. This observation is in agreement with earlier conclusions that in this species the iron of cytochrome a_3 is in a ferryl state (*27*−*30*, *38*, *39*) and verifies the observation (*26*) that no other oxidizing species needs to be reduced to return the F form to the resting enzyme.

However our observation that the redox state of the P form appears to be 1.3 oxidative equiv above that of oxidized oxidase came as a surprise. If the P form were a peroxy species, then it should be 2 oxidative equiv above oxidized enzyme and one-electron reduction of P should give stoi-

chiometric conversion of P to F. However the optical spectra clearly show that the reaction product following one-electron reduction of the P form is approximately 90% oxidized enzyme plus 7−10% of the F form.

To examine whether this result was a consequence of the choice of ferrocytochrome *c* as reductant, we have used an alternative approach in which the P form was converted to fully reduced enzyme using an excess of apNADH with PMS as mediator (apNADH was employed because its absorbance at 364 nm is almost 50% larger than that of NADH at 340 nm). In three separate experiments using 23, 32, and 41 μM P, the number of reducing equivalents in excess of that needed to reduce fully oxidized enzyme to the reduced state ranged from 1.03 to 1.3 with a mean value of 1.13. We interpret this value as the number of electrons needed to convert P form to oxidized CcO prior to conversion of oxidized CcO to reduced enzyme.

These data imply the presence of the ferryl state for Fe_{a3} in both the P form as well as the F form. This in turn implies that the peroxy bond presumed to be present in the P form must be broken, a reaction which requires the one-electron oxidation of a redox center other than Fe_{a3}. Because the optical spectrum of the P form following one-electron reduction is almost the same as that of oxidized enzyme, we conclude that this second center cannot be a porphyrin π cation radical. Moreover this second oxidized center should be relatively unstable so that after about 10−15 min, the time needed to make the P form, no more than 0.3 equiv of this species remains.

The obvious possibilities for this novel oxidized center are Cu_B or an aromatic amino acid present in the binuclear cavity. If Cu_B is involved, then it must be via its Cu(III) oxidation state.

From the crystal structures of both the bovine enzyme (*40*) and that from *Paracoccus denitrificans* (*41*), it was concluded that there is a covalent bond between N_ϵ of His-240 (one of the ligands to Cu_B) and C_ϵ Tyr-244 (bovine numbering), and it is reasonable to believe that this adduct could serve as an electron donor. Density functional calculations (*42*) show that the neutral radical of such a tyrosine (Y°) would place spin density of N_δ of the attached histidine, and thus a mechanism exists for coupling the organic radical and the copper such that the species $Y°-Cu^{II}$ and $Y-Cu^{III}$ might be formally equivalent.

A scheme summarizing the events is:

$$\underset{\text{MV-CO}}{[a_3{}^{+2}\text{-CO Cu}^{+1}\,(Y)]} + O_2 \xrightarrow{h\nu} \underset{\text{P(Y°)}}{[a_3{}^{+4}\text{=O Cu}^{+2}\,(Y°)]} \longrightarrow \underset{\text{P(Y)}}{[a_3{}^{+4}\text{=O Cu}^{+2}\,(Y)]}$$

Thus the initial reaction is a four-electron oxidation involving the two-electron oxidation of a_3 and the one-electron oxidation of both Cu_B and Tyr-244 to give the neutral radical (Y°). With time Y° decomposes more rapidly than the ferryl form. This enhanced instability of Y° relative to the ferryl form has precedent in the behavior of such radicals generated by treatment of myoglobin with H_2O_2 (*43*−*46*) which typically disappear completely within 20 min with little or no loss in the ferryl form. The mechanism for this phenomenon is not clear; one proposal is that the radical migrates to the surface of the protein and is quenched by reaction with a second protein molecule (*47*, *48*; see Note Added in Proof). Our data on the redox state of the P form are in contrast to those recently published based on the photoreduction of the P and F forms (*26*), where it was apparently shown that one-electron reduction of P led to the quantitative formation of F.

There are several obvious differences between these two sets of experiments. First, in our study the reactions were conducted under conditions in which there was almost quantitatively reaction of the P form; consequently, the data are representative of all of the enzyme. In the photoreduction experiments only a few percent of P is converted to the F form (*26*), and thus the observed behavior may be atypical of the enzyme as a whole.

Second, our measurements are performed some 10−15 min after P formation; in the photoreduction experiments the time delay between preparation of the P form and the first measurement was about 30 s. It is conceivable that the P form is originally 2 oxidative equiv above oxidized enzyme, but with time the locus of the second oxidizing equivalent spontaneously decays. If this is the case this second center should be the first to be reduced by photoreduction because the product of reaction was found to be in the ferryl state. This center cannot be the same as that which titrates last in our experiments.

This explanation implies that the reduction of the second center by the external donor causes the conversion of the P form to the F form found by Verkhovsky et al. (*26*), but our data show that the P form is still present when the second center is spontaneously quenched. Thus we have to conclude that the conversion of the optical spectrum of P to that of F is not dependent on the redox state of this second center but how it is reduced. We suggest that when the reduction of the binuclear center is achieved by the "physiological" path, i.e., via Cu_A and cytochrome *a*, the reduction of cytochrome *a* is the driving force which induces the relaxation of P to the F form. This explanation is supported by earlier data showing that the extent of reduction of cytochrome *a* affects the redox potential of the binuclear center (*49*).

Recently Michel stressed (*50*) that there is an equal probability that the reduction of cytochrome *a* or cytochrome a_3 be accompanied by proton uptake via the D channel. We have found that the endogenous decay of the P form to the F form is pH-dependent, and the dependence of the rate constant on pH indicates that approximately one proton is taken up from the medium during this conversion (M. Fabian and G. Palmer, unpublished results). So it seems likely that the optical relaxation of the P form to the F form is coupled to the uptake of one proton, and the difference between these two ferryl forms after the second center is endogenously quenched is not a redox difference but a change in the state of protonation of the binuclear center.

Third, photoreduction of the P form was conducted anaerobically using a camera flash, a photosensitive dye (tris-(2,2′-bipyridyl)ruthenium), and a sacrificial electron donor (aniline). However, in conditions very similar to those used for photoreduction of P, the reduction of Cu_A takes place in less than 1 μs and the slowest phase of cytochrome *a* reduction requires about a tenth of a millisecond (*51*). Because the duration of the camera flash is typically in the range of several milliseconds, it is possible that, with a ratio of photosensitive dye to P close to 4, one flash might produce more than one-electron reduction, and observed production

of the F form could be the reaction product of multiply reduced oxidase with dioxygen.

Finally, we note that the P form used in the earlier experiments (*26*) is significantly less stable than our preparation. We have found that the stability of the P form depends on at least three factors: namely, pH, temperature, and the presence of CO (which can lead to full reduction of the enzyme if allowed to remain in contact with P). The data of Verkhovsky et al. (*26*) suggest that the half-time for spontaneous decay of the P form is about 5 min; our preparation has a half-time of about 40 min at similar temperature and pH. It therefore seems that the increased lability of the preparation studied by Verkhovsky et al. can be traced to the residual CO present in solution, though we cannot exclude that there is an oxidizable group accessible in the enzyme which is spectroscopically invisible and that this group is less readily oxidized in our preparation.

There still remains the curious fact that the optical spectra of P and F forms are so dissimilar. The narrowness of the α-band of P at 607 nm suggests that this species is diamagnetic (*52*). This could be accomplished by an uniaxial distortion of the heme plane sufficient to lower the energy of the d_{xz}-orbital by the amount required to cause complete pairing of the four d-electrons of the heme iron. This possibility could be tested by recording Mossbauer spectra of the P and F forms of ^{57}Fe-substituted cytochrome aa_3 from bacteria.

NOTE ADDED IN PROOF

Chen et al. (*53*) have reported that treatment of soluble bovine cytochrome *c* oxidase with hydrogen peroxide gave rise to a protein-centered radical which could be trapped with 5,5-dimethyl-1-pyrroline. Production of the radical could be blocked by pretreatment of the enzyme with cyanide, thus implicating the binuclear center as the initial site of reaction with hydrogen peroxide. The EPR parameters of the trapped species indicate that the radical was located on a cysteine residue. Subunit I contains only one cysteine residue (Cys498); this residue is close to the surface of the subunit and more than 3 nm distant from the binuclear center. These data provide direct evidence for the migration of oxidizing equivalents within the enzyme to a site not believed to function in catalysis.

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