

“PEROXIDATIC” FORM OF CYTOCHROME OXIDASE AS STUDIED BY X-RAY ABSORPTION SPECTROSCOPY

BRITTON CHANCE AND CHELLAPA KUMAR

Johnson Research Foundation, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

L. POWERS AND YUAN-CHIN CHING

Bell Telephone Laboratories, Murray Hill, New Jersey 07974

ABSTRACT X-ray absorption spectroscopy shows pulsed oxidase to be similar to resting oxidase but to lack the sulfur bridge between iron and copper of active sites (Powers, L., Y. Ching, B. Chance, and B. Muhoferac, 1982, *Biophys. J.*, 37[2, Pt. 2]: 403a. [Abstr.]) The first shell ligands and bond lengths of the pulsed oxidase active site heme most clearly fit the ferric peroxidases from horseradish and yeast, and the pulsed oxidase cyanide compound resembles the low spin hemoprotein cyanide compounds. The structural results are consistent with an aquo or a peroxy form for pulsed oxidase as is also observed by optical studies. These structural and chemical data are consistent with a role for the pulsed forms in a cyclic peroxidatic side reaction in which the pulsed and pulsed peroxide compounds act as peroxide scavengers. The peroxidatic role of cytochrome oxidase in the nonsulfur bridged form suggests the renaming of the “oxygenated” or “pulsed” forms on a functional basis as “peroxidatic” forms of cytochrome oxidase.

INTRODUCTION

The possibility that the fully oxidized cytochrome oxidase could exist in multiple forms has been stressed by Antonini et al. (1), who coined the term “pulsed,” and the possible existence of other forms due to radiation damage was pointed out by Chance et al. (2). In addition, EPR studies suggest a variety of forms of the resting enzyme (3). The pulsed form, an anomalous state of the enzyme formed following oxidation of the reduced enzyme by oxygen pulses, is characterized by an increased rate of oxidation of cytochrome *c*, is acquired rapidly and persists for some time after the completion of the substrate oxidation (1). EPR and optical data indicate that heme *a* and Cu_a are substantially unaltered by conversion to the pulsed form. In a much earlier series of studies, the oxidation product of the dithionite-reduced enzyme is termed an “oxygenated” compound (4). The role of H_2O_2 in the formation of the “oxygenated” oxidase has been studied by Orii, Okunuki, and co-workers (4,5) and by Lemberg and Mansley (6), the former showing a reaction of the “oxygenated” oxidase with H_2O_2 , and the latter emphasizing the requirement for H_2O_2 in the formation of the “oxygenated” oxidase.

In experiments with the EPR technique, anomalous resonances (particularly at $g = 5$ [7] attributable to changes in heme a_3)¹ have been found to be associated with

¹ a_3 heme and Cu_a are identified with the active site of the oxidase, whereas Fe_a refers specifically to the iron atom of a_3 heme. Correspondingly, *a* heme and Cu_a refer to the electron reservoir component of the oxidase.

the procedure for forming the pulsed oxidase, yet none of these species has been demonstrated to exist at a significant level under steady state turnover of the enzyme (8). In fact, one of the key results of the kinetic studies of Gibson and co-workers on the purified oxidase (9, 10) and of Chance and Erecinska on the membrane bound oxidase (11) was a lack of evidence that substantial amounts of a form other than the fully oxidized resting form could be found in the reaction of the reduced form with excess O_2 . Thus, any additional species would seem to be present in low concentrations. Under certain transient conditions, however, a species differing from the fully oxidized was observed (4, 5, 12) and termed “oxygenated.” Using the same conditions, this compound was observed by Antonini et al. (1) and termed the “pulsed” form. Thus, multiple forms of the oxidized oxidase are postulated (1, 3, 13). We shall retain the term “pulsed” in the experimental part of the paper, meaning thereby “pulsed” or “oxygenated” form.

Because these functional differences are most likely to be caused by structural changes of the active site, an examination of the metal atom centers by x-ray absorption spectroscopy was appropriate. This technique has recently been successful in identifying the structure of the reduced CO, resting oxidized and mixed valence forms of the oxidase (14). We have now investigated the pulsed oxidase and present here evidence for structural differences between the resting oxidized and pulsed oxidase forms that have to do with the presence of a bridging atom in the former, and its absence in the latter (15). We further find

the active site heme of pulsed oxidase to be very similar in structure to the peroxidases and to form stable derivatives with hydrogen and ethyl hydrogen peroxide as well as cyanide. For these reasons, we term the pulsed form to have "peroxidatic" and "catalatic" activities (6).

PREPARATIONS

Cytochrome oxidase was prepared by the method of Yonetani (16), and it was found to have a purity of 9.6 nmol heme *a*/mg protein. Furthermore, the preparation could be concentrated to ~1 mM and remain fluid. The Soret band was at 416 nm as shown in Fig. 1 *A* (trace 1). The homogeneity of these preparations was tested by a kinetic method

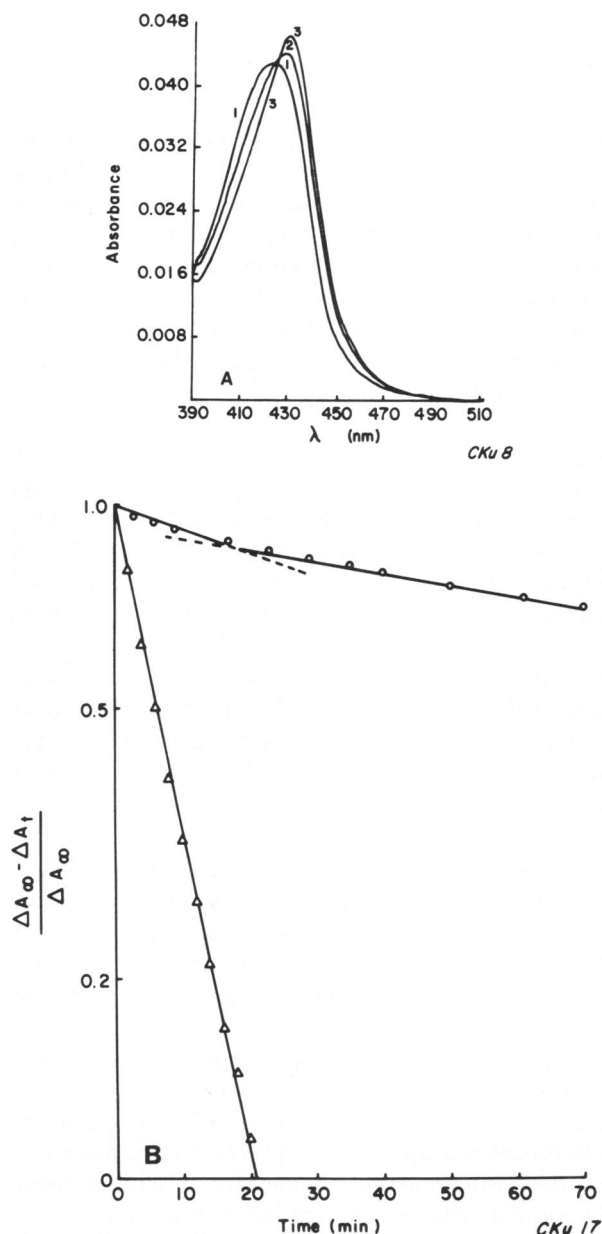


FIGURE 1 (A) Soret bands of resting (1), pulsed (2), and pulsed peroxide (3) compounds of cytochrome oxidase, prepared as described in the text (2.3 μ m oxidase, 2 mm pathlength). (B) Reaction of cyanide with resting (O) and pulsed (Δ) oxidase as prepared in this case by the reaction of 7 μ M oxidase with 1 mM cyanide.

described elsewhere (17) in which the reaction with cyanide was found to be over 90% fitted to a single exponential (in contrast to other types of preparations that show triple exponents of nearly equal amplitude), as illustrated by Fig. 1 *B*. The top trace of Fig. 1 *B* represents the reaction rate data with cyanide of the resting form of cytochrome oxidase. The small initial phase (~10%) was slightly faster than the rest of the reaction (first-order plot not shown). The kinetic constants for the initial and final phases of this reaction were 0.8 and 0.05 $M^{-1} s^{-1}$ for the conditions of measurement (1 mM cyanide pH 7.4).

To prepare the pulsed oxidase, 1 mM cytochrome oxidase (14) containing a small concentration of added catalase was reduced by titration with dithionite in a redox vessel (18) to a potential of -200 mV and was allowed to equilibrate for ~4 h to ensure reduction $\geq 90\%$. A sample was then withdrawn and placed in an extended x-ray absorption fine structure (EXAFS) sample holder (14). Oxygenation of the sample was obtained by the breakdown of 10-15 mM H_2O_2 , which reached a saturation oxygen concentration of 1.2 mM in a fraction of the half time required to decompose the total amount of H_2O_2 (3.5 s) (19). The sample was thoroughly oxygenated and the reduced oxidase converted to the oxygenated form. After an interval of between 1 and 2 min, the sample was retrapped in liquid nitrogen. The spectrum of the pulsed form is illustrated in Fig. 1 *A* (trace 2) having peaks at 426-427 nm. Its conversion to the peroxide compound by the addition of excess H_2O_2 is illustrated by Fig. 1 *A* (trace 3).

We have utilized a kinetic criterion, illustrated in Fig. 1 *B* for characterizing the oxygenated state (17). In short, based on independent studies on a large number of samples prepared by different methods, it has been shown that oxygenated oxidase shows a homogeneous, single-exponential, first-order reaction with cyanide under our conditions of measurement, with a second-order rate constant of 2 $M^{-1} s^{-1}$. The presence of significant quantities of any form other than the oxygenated, e.g., small quantities of the slower-reacting cyanide form, show up clearly in the form of nonlinearity in the first-order plots of the oxidase-cyanide reaction. This method is found in practice to be more sensitive than EPR or optical criteria. As seen from Fig. 1 *B* (triangles), the reaction of our oxygenated oxidase preparations with cyanide is homogeneously first order within experimental error, showing the oxygenated oxidase samples made by our method to be homogeneous, as required for examination by x-ray absorption spectroscopy.

The choice of oxygenating by stirring in dioxygen or generating it in situ depends upon the amount of oxygen required. For the concentrated preparations, the oxygen requirement is very nearly equal to the solubility of oxygen in water and the catalase + H_2O_2 method seems essential. However, for spectroscopic studies, oxygen can be stirred into a more dilute cytochrome oxidase preparation.

Optical spectra were run on a Johnson Research Foundation dual wavelength (room temperature) or double beam (low temperature reflectance spectra) spectrophotometer. EPR spectra were run at helium temperatures on either a Varian E-4 or E-109 spectrometer (Varian Associates, Inc., Palo Alto, CA).

The kinetic test is difficult to apply directly to the highly concentrated samples because of the difficulty of mixing cyanide with the highly concentrated cytochrome oxidase in the sample holder. However, comparison of spectroscopic data for the low and the highly concentrated systems indicates full conversion of the sample to the pulsed form.

$Cu(N-t\text{-butyl thiosemicarbazone})_2$ was prepared by J. Peisach, Albert Einstein College of Medicine, Bronx, New York. Di-*t*-butyl ketone was condensed with thiosemicarbazide to make *N-t*-butyl thiosemicarbazone. The ligand was recrystallized from a 1:1 mixture of dimethyl sulfoxide and benzene. A 10-fold molar excess of ligand was mixed then with copper acetate in dimethylformamide and the mixture frozen in a few seconds in the plexiglas EXAFS sample holder to prevent autoreduction. The EPR spectrum of this sample gives homogeneous peaks uniquely identifying this compound and quantitatively gives $g \parallel = 2.156$ $A \parallel = 18.6$ mT. Comparison of these results with those of similar complexes (two nitrogen, two sulfur ligated) (20, 21) for which the crystal structures are known and that have similar preparation procedures, indicate that a

single complex having two nitrogen and two sulfurs as ligands with a distorted tetrahedral geometry (20) was produced. EXAFS measurements agreed, giving two nitrogens at an average distance of 1.99 ± 0.02 Å and two sulfurs at 2.30 ± 0.03 Å. Autoreduction has been readily detected in a previous sample, and the Cu data differ significantly in both edge and EXAFS as the Cu apparently changes ligands under conditions of reduction (L. Powers, unpublished observations).

X-RAY ABSORPTION METHODS

EXAFS measurements were recorded at the Stanford Synchrotron Radiation Laboratory (SSRL), Stanford University, Stanford CA, during dedicated operation of the SPEAR storage ring at 3.0–3.5 GeV and 30–70 mA.

The data analysis procedures are described in detail in our previous publications (14, 22, 23) and both a two-atom type-fitting procedure (22, 23) and site modeling (14) were used. While the former method gives average distances, the number of ligands and changes in Debye-Waller and threshold contributions for two atom types with respect to model compounds containing the same atom type, such procedures have pitfalls and can give ambiguous results (23). Because the site modeling approach has no variables, these pitfalls and mathematical problems are avoided. Neither procedure gives a unique solution: the best fit is chosen by minimizing the sum of the residuals. In the site modeling approach, however, all known biochemical and physical data must be considered in choosing a model for the site, a requirement that imposes further restrictions on the solution.

The site modeling method is sensitive to changes in the geometry (distances) or one ligand of the model, as has been demonstrated by the differences in the edge data and first shell filtered data of both heme and copper complexes (14, 22–25). Further, the model must represent the site in all redox states (e.g., oxidized model = oxidized site, reduced model = reduced site) as previously applied to both edge and EXAFS cytochrome oxidase data (14, 24); hence, all observed states must be self-consistent. Thus the sum of model data with no fitting procedures was compared with the cytochrome oxidase data discussed below and was considered good only when the sum of the residuals squared was at least a factor of 2 better than that of the two-atom type-fitting procedure with all parameters variable. In this manner, models for each site of cytochrome oxidase were determined from the edge and EXAFS data that are consistent with all known biochemical and physical parameters.

RESULTS

Pulsed Oxidase

Copper Studies. Previous edge and EXAFS studies have shown that the “blue” or Type I copper protein stellacyanin (14, 23, 26) is a good model for Cu_a in both redox states. In addition, Cu_a contains one (or two) nitrogen(s) or oxygen(s) and three (or two) sulfurs as ligands (14, 26) that change slightly in distance on reduction. The edge data for pulsed oxidase are compared with those of the fully oxidized resting state in Fig. 2. Although the pulsed oxidase contained both coppers in the oxidized state and previously reported studies indicate that the Fe_a and Cu_a sites are unchanged from that in the resting oxidized form (27–29), the edge data could not be superimposed with those of the resting oxidized form. The intensities of the lower energy region (8,978–8,993 eV) were different but little if any changes in energy are observed. Comparisons with our previously published systematic study of copper model compounds (26) also suggest that it is the structure of Cu_a in the pulsed state that may be

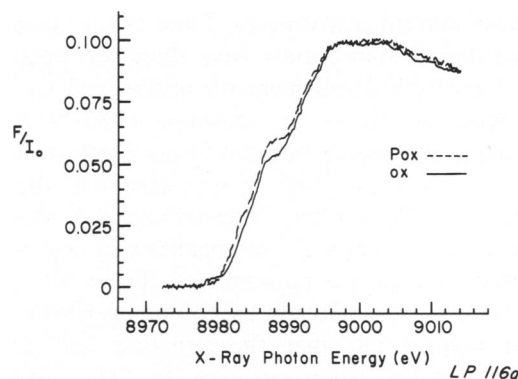


FIGURE 2 Copper x-ray absorption edge spectra of pulsed oxidase (broken line) and resting oxidized form (14, 26) (solid line).

different from that in the resting oxidase. Other changes occur in this conversion, for example as shown in optical (Fig. 1 A, trace 2) and EPR data (4, 7). The latter showed the distinctive $g = 5$ signal and its split higher field components, but showed no significant change in the $g = 3$ component or in the portion of the optical spectra attributable to heme a or Cu_a .

In fact, the edge data of the pulsed oxidase can be reproduced from the sum of the model edge for Cu_a in the oxidized state found in our previous studies of the resting oxidized and mixed valence formate forms (26) and the model compound $\text{Cu}(N-t\text{-butyl thiosemicarbazone})_2$ representing the Cu_a contribution to the pulsed form. The proportions of each, reported previously (26) as 35% Cu_a and 55% Cu_a , with a 10% attribution to adventitious copper, were here found to be 36 and 60%, respectively, with a 4% contribution from adventitious copper charac-

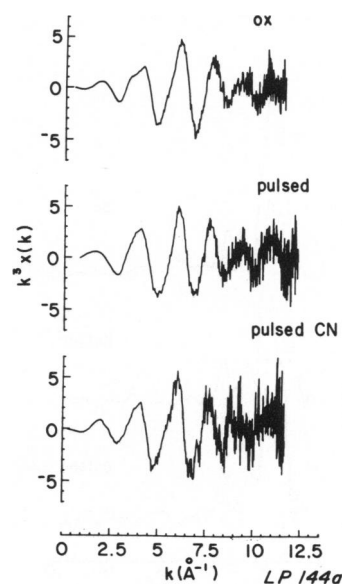


FIGURE 3 Copper EXAFS data after background removal, k^3 multiplication, and normalization to one copper atom for the resting oxidized (14) (top), pulsed (middle), and pulsed cyanide (bottom) forms of cytochrome oxidase.

teristic of current preparations. These best fit proportions did not deviate significantly from those corresponding to the 1:1 theoretical stoichiometry of Cu_a and Cu_{a_3} . Note that while the $1s \rightarrow 3d$ transition ($\sim 8,979$ eV) was observed in the resting oxidized form (which is similar to that of stellacyanin [14]), it was absent in the pulsed oxidase [and $\text{Cu}(N-t\text{-butyl thiosemicarbazone})_2$]. This is not unusual for covalent Cu^{+2} compounds and does not indicate that the copper is reduced (26). These data indicate that Cu_a is the same in both the resting oxidized form and pulsed oxidase but that the structure and geometry changed for Cu_{a_3} , from one like the "blue" copper of stellacyanin to one like $\text{Cu}(N-t\text{-butyl thiosemicarbazone})_2$.

The copper EXAFS data (after background subtraction and k^3 multiplication [14]) are shown in Fig. 3 and the Fourier transforms of these data are given in Fig. 4. It is useful to note that the signal-to-noise ratio in the pulsed oxidase data are comparable to that of the resting oxidized form (14). The Fourier transforms show clearly structural differences between the pulsed oxidase and resting oxidized form (14). Not only is the broad first coordination shell of the resting oxidized form split into two distinct contributions indicating ligand distances are different by ~ 0.3 Å, but second and third shells are also at different distances in the two forms of the oxidase. The filtered data of the pulsed oxidase first shell are shown in Fig. 5 together with the sum of the first shell data of the Cu_a contribution (14) and $\text{Cu}(N-t\text{-butyl thiosemicarbazone})_2$ representing the Cu_{a_3} contribution in the same proportions found for the edge data. The agreement is excellent—better than that obtained with a two-atom type (nitrogen and sulfur) fitting procedure (14, 23) and in complete agreement with the analysis of the edge data (Fig. 2).

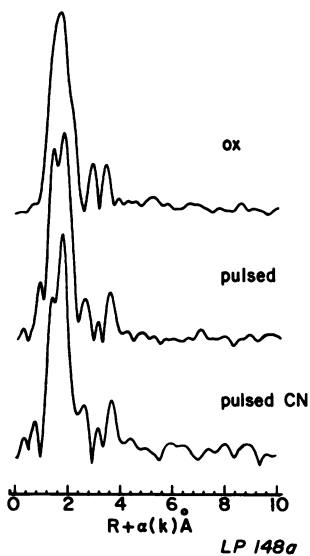


FIGURE 4 Fourier transforms of the copper EXAFS data shown in Fig. 3. Resting oxidized form (14) (top), pulsed oxidase (middle), and pulsed oxidase cyanide (bottom).

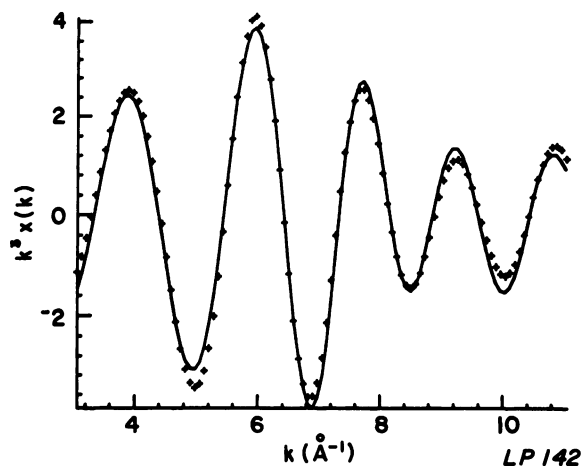


FIGURE 5 Copper first shell filtered data normalized to one copper atom of the pulsed oxidase (—) and sum of $\text{Cu}(N-t\text{-butyl thiosemicarbazone})_2$ and oxidized Cu_a contribution (+) (14).

The structural differences in the active site copper, Cu_{a_3} , between the resting oxidized form and the pulsed oxidase can be determined by comparison of the model compounds stellacyanin (Cu_a in the resting oxidized form) and $\text{Cu}(N-t\text{-butyl thiosemicarbazone})_2$ (Cu_{a_3} in the pulsed oxidase). The edge data are similar in the $1s \rightarrow 4p$ transition region (8,994–9,008 eV), but the energy differences between the resolved transitions are different, stellacyanin having three (14,23) and $\text{Cu}(N-t\text{-butyl thiosemicarbazone})_2$ two resolved transitions. Because these transitions have p character (30), this indicates $p_x \neq p_y \neq p_z$ for tetragonal (or distorted tetrahedral) symmetry for stellacyanin but $p_x \approx p_y \neq p_z$ or a more planar symmetry for $\text{Cu}(N-t\text{-butyl thiosemicarbazone})_2$. In addition, the shoulder between the $1s \rightarrow 3d$ and $1s \rightarrow 4p$ transition (8,982–8,992 eV), which is likely a mixed transition ($1s \rightarrow 4s, 4p$), is more pronounced and shifts 3 eV to lower energy in $\text{Cu}(N-t\text{-butyl thiosemicarbazone})_2$ and no $1s \rightarrow 3d$ transition ($\sim 8,979$ eV) is observed. These differences (28) indicate that $\text{Cu}(N-t\text{-butyl thiosemicarbazone})_2$ is more planar than stellacyanin (22, 26). This is supported by the EPR results for $A \parallel$ (3 and 18.6 mT [23], respectively, for stellacyanin and $\text{Cu}(N-t\text{-butyl thiosemicarbazone})_2$), which also indicate a more planar geometry for $\text{Cu}(N-t\text{-butyl thiosemicarbazone})_2$ (20). The structure of stellacyanin has been determined using x-ray absorption spectroscopy by Peisach and co-workers (23) and measurements were made under identical conditions as described in the X-ray Absorption Methods section. They conclude that stellacyanin contains two nitrogens at an average distance of 1.97 ± 0.02 Å and one (or possibly two) sulfur at 2.19 ± 0.03 Å. The same fitting procedure analysis of $\text{Cu}(N-t\text{-butyl thiosemicarbazone})_2$ gives two nitrogens at an average distance of 1.99 ± 0.02 Å and two sulfurs at 2.30 ± 0.03 Å. Apparently the more planar nature of $\text{Cu}(N-t\text{-butyl thiosemicarbazone})_2$ causes the shorter Cu—S distance observed in stellacyanin to be lengthened to a

more typical bond length in $\text{Cu}(N\text{-}t\text{-butyl thiosemicarbazone})_2$ and in the pulsed oxidase. This is the sulfur that forms the bridge to the active site Fe (Fe_{a_3}) in the resting oxidase form. This bridge is missing in the pulsed oxidase.

Iron Studies. The iron EXAFS data of the pulsed oxidase are given in Fig. 6, and comparison with the resting oxidized form shows significant differences, while the signal-to-noise ratios are comparable. These differences were also apparent in the Fourier transformed data (Fig. 7). The resting oxidase has a split first shell: the shorter distance portion contains heme nitrogens from both the active site (Fe_{a_3}) and Fe_a while the longer distance portion contains a single sulfur atom that comprises the sulfur bridge at the active site (between Fe_{a_3} and Cu_{a_3}) (14). The pulsed oxidase has no sulfur bridge contribution and the average distance of the observed first shell is at a longer distance than the nitrogen contributions of the resting oxidase. The contribution of Fe_a was shown to be that of *bis*(imidazole)- $\alpha,\beta,\delta,\gamma$ -tetraphenylporphinatoiron (III) Cl (Im_2 FeTPP) in the resting oxidized and mixed valence formate forms of our previous studies, which did not change on reduction (14), while other previous studies indicate that this contribution is unchanged in the pulsed form (27–29). Subtraction of this Fe_a contribution from the first-shell filtered data of the pulsed oxidase gave the active site (Fe_{a_3}) contribution shown in Fig. 8 A. Chemical intuition would suggest that this site might be a deoxy or met structure similar to those of hemoglobin (or myoglobin), since this site is identical to oxy (or CO) hemoglobin in the reduced form (14). In addition, the sulfur bridge observed in the resting oxidized form is broken during the

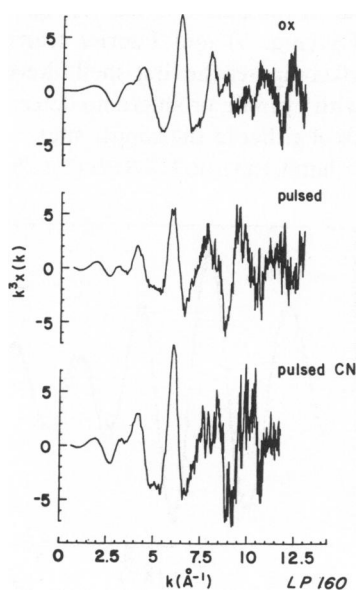


FIGURE 6 Iron EXAFS after background removal, k^3 multiplication, and normalization to one iron atom for the resting oxidized (14) (top), pulsed (middle), and pulsed cyanide (bottom) forms of cytochrome oxidase.

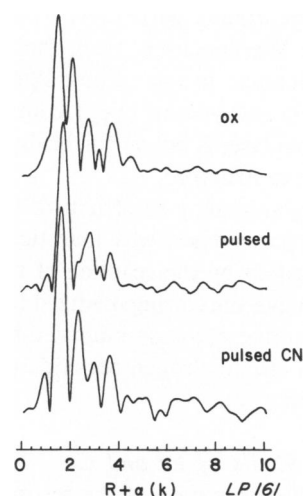


FIGURE 7 Fourier transforms of the iron EXAFS data shown in Figure 6: resting oxidized form (14) (top), pulsed oxidase (middle), and pulsed oxidase cyanide (bottom).

resting to pulsed transition, and consistent with this, the pulsed oxidase reacts much more readily with cyanide than the resting form (31). Comparison of these hemoglobin derivatives with the Fe_{a_3} contribution in the pulsed oxidase showed marked differences in both the phase and amplitude. However, the first-shell data of horseradish peroxidase (32), cytochrome *c* peroxidase (33), catalase (34), and cytochrome *o* (35) shown in Fig. 8 are identical within the error. Fitting procedures (14, 22, 23) clearly show the similarity of the peroxidases (15) and the cytochrome oxidase Fe_{a_3} contribution with the latter having pyrrole nitrogens at an average distance of $2.03 \pm 0.02 \text{ \AA}$, and axial ligand(s) (nitrogen, oxygen) at an average distance of $2.13 \pm 0.03 \text{ \AA}$ (C. Powers, B. Chance, C. Kumar, and Y.-C. Ching, manuscript in preparation). These results are in good agreement with those recently reported by Poulos

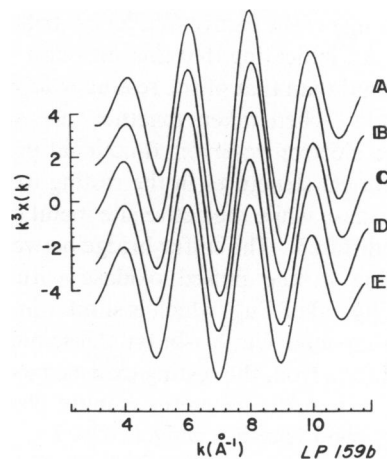


FIGURE 8 Iron first-shell filtered data normalized to one-half iron atom for the Fe_{a_3} contribution of pulsed oxidase (A) and peroxidases: horseradish peroxidase (B), cytochrome *c* peroxidase (C), catalase (D), and cytochrome *o* (E).

et al. (36) for cytochrome *c* peroxidase from x-ray crystallography studies. We conclude, then, that while the Fe_a contribution is identical in the resting oxidized form and pulsed oxidase, the active site (Fe_a) contribution is different in pulsed oxidase, is not sulfur bridged to Cu_a , and has a peroxidaselike structure.

This surprising similarity in structure between pulsed oxidase and the peroxidases was investigated biochemically and was verified by the reaction of the pulsed form with peroxide. Unlike the resting oxidized form, the pulsed oxidase forms a stable peroxide intermediate with hydrogen peroxide and ethylhydrogen peroxide (15) and reacts faster with cyanide (37).

Higher Shells of Fe and Cu. Higher shells in both the copper and iron data of the resting oxidase form were shown to contain contributions from the active site pair; i.e., Fe_a was found in the third shell of the copper data and Cu_a in the third shell of the iron data, together with outer shell heme contributions (14) at a separation of $3.75 \pm 0.05 \text{ \AA}$. This, together with the sulfur bridge, was verified by copper depletion of the resting enzyme (25). These assignments (14) involved several criteria in addition to phase and filtered data comparisons: the contribution must be uniquely identified as iron or copper and not possibly as nitrogen, carbon, oxygen, sulfur or any combination of these, and each must be observed in the data of the other at the same distance and similar Debye-Waller contribution ($\Delta\sigma^2$) (14). Proceeding to analyze the pulsed form in this manner with the same models as used previously (14), the third shell of the copper data of the pulsed oxidase (Fig. 4) contains a single iron atom at $3.83 \pm 0.05 \text{ \AA}$ while the iron third shell contains a single copper atom at this distance, together with outer shell heme contributions that are nearly identical to the outer shell heme contributions found in the resting oxidized (14) and copper-depleted forms (25). Amplitude comparisons of these contributions with that of the resting oxidized form show little change in the Debye-Waller contribution, $\Delta\sigma^2 = -1.5 \times 10^{-3} \text{ \AA}^2$, indicating that this structure may be only slightly less rigid than that of the resting oxidized form.

These results, when taken together, are sufficient to show that the electron reservoir functional pair, Cu_a and Fe_a , have identical structures in the resting oxidized form and pulsed oxidase while the active site metal centers, Cu_a and Fe_a , are different. The sulfur bridge between Cu_a and Fe_a is missing in the pulsed oxidase with the sulfur retained as a ligand of Cu_a , which is similar in structure to the model compound $Cu(N-t\text{-butyl thiosemicarbazone})_2$. This model differs from the resting oxidase model for Cu_a , stellacyanin, in that the geometry is more planar and the copper-sulfur bond length is longer. The Fe_a heme has a structure like that observed for peroxidase (15), having longer N(pyrrole) distances and axial ligand(s), and the $Fe_a - Cu_a$ separation is slightly ($<0.1 \text{ \AA}$) larger than that

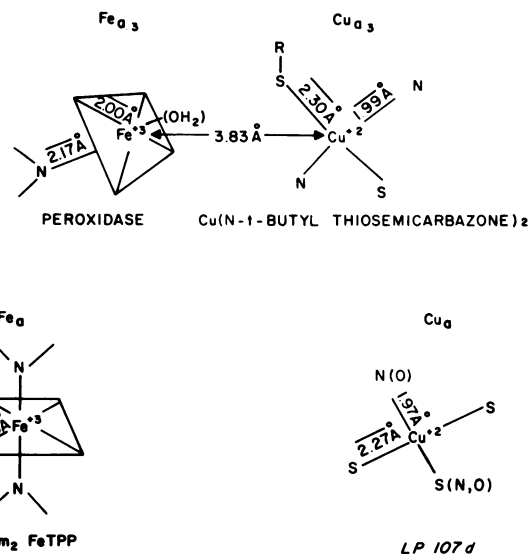


FIGURE 9 Pictorial representation of x-ray absorption results for the pulsed oxidase. Other ligand possibilities are given in parentheses and bars over distances indicate average distance for all the same type of ligands in that center except Fe_a , where the axial ligands are represented by an average distance. Model compounds are identified where appropriate and error bars are omitted but given in the text.

of the resting oxidized form. Fig. 9 summarizes these results.

Pulsed Oxidase Cyanide

Our preparations of the pulsed oxidase react readily and homogeneously with cyanide and this was also studied by x-ray absorption spectroscopy. Because the cyanide binds the iron, it is of interest to determine if Cu_a is involved. The copper edge data, however, are identical within experimental error to that of the pulsed oxidase (Fig. 2), as are the copper EXAFS (Fig. 3) and Fourier transformed data (Fig. 4). Fig. 10 compares the first-shell filtered data. Thus this reaction with cyanide produces no detectable changes in the structure of either of the copper sites.

On the other hand, the iron EXAFS (Fig. 6) and Fourier

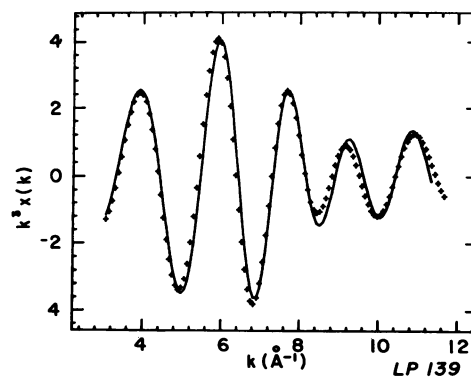


FIGURE 10 Copper first-shell filtered data normalized to one copper atom for pulsed oxidase (—) and pulsed-oxidase cyanide (---).

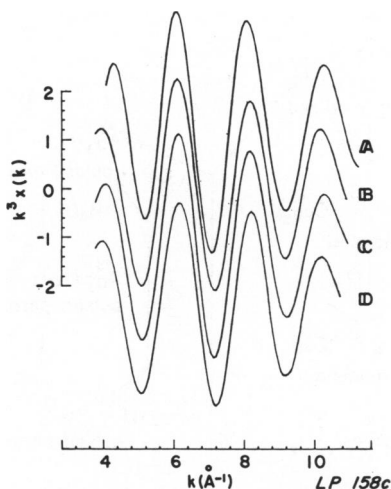


FIGURE 11 Iron first-shell filtered data normalized to one-half iron atom for the Fe_{a_2} contribution of pulsed oxidase cyanide (A) and the low-spin heme complexes: cytochrome *c* peroxidase cyanide (B); methemoglobin hydroxide (C) and metmyoglobin cyanide (D).

transformed data (Fig. 7) show significant differences. Subtraction of the Fe_a contribution (Im_2 FeTPP) from the first-shell filtered data leaves the active site (Fe_{a_2}) contribution shown in Fig. 11 A. Unlike the pulsed oxidase, the first-shell filtered data for the cyanide compound (Fig. 11) is identical within experimental error to that of cytochrome *c* peroxidase and metmyoglobin cyanide, as well as other low-spin derivatives such as methemoglobin hydroxide. These structures have pyrrole nitrogens at an average distance of 1.99 ± 0.02 Å, proximal nitrogen at 2.07 ± 0.03 Å, and cyanide (oxygen) at 1.85 ± 0.03 Å.

This result is not surprising since our previous studies of the resting oxidized form (14) showed the Fe_{a_2} contribution in the reduced CO form to be similar to that of oxyhemoglobin (carbonmonoxy hemoglobin). Hence, the rapid reaction of the pulsed oxidase with cyanide is consistent with a peroxidase-like structure that reacts rapidly with cyanide (38) as does yeast cytochrome *c* peroxidase and methemoglobin and metmyoglobin to form low-spin complexes (39).

Higher shell contributions (Figs. 4 and 7) from the active site pair, showed the Fe_{a_2} — Cu_{a_2} distance to be the same within the error for the cyanide product as for the pulsed oxidase.

DISCUSSION

The Lack of the Bridging Atom

The remarkable finding of the EXAFS data, that the sulfur bridge between the iron and the copper is absent in the pulsed oxidase, underlines the importance of this ligand in defining structural states and reactivity of the protein. The pulsed form and the resting form may coexist in the purified resting oxidase. Although the former reacts rap-

idly with peroxide, it forms a stable intermediate in contradistinction to Compound B that is very labile. Evidence for the function of pulsed or pulsed-peroxide forms of the oxidase in the very rapid turnover of oxygen by oxidase should be signalled by the accumulation of the pulsed or pulsed-peroxide forms as the end product of the cyclic function of Compound B, which seems not to occur in our studies. Indeed, the edge data show that the end product of oxidation of Compound B, as observed under cryogenic conditions (-20° , 7 min) contains the bridging atom between the two metals (Powers et al., in preparation). Another possibility is that the pulsed oxidase cycle involves a different set of intermediates (40) in a pathway parallel to that described recently, involving bridged structures (14). This hypothesis also implies a steady state concentration of pulsed or pulsed-peroxide forms of the oxidase in the oxidation-reduction cycle. Three experiments bear upon this hypothesis: (a) that the $g = 5$ signal identified with the pulsed oxidase has been found to be present only in a low concentration in steady state redox kinetics (9), (b) that a strong 655 nm band is observed in the steady state kinetics, and (c) that an inhibitor such as cyanide added to the steady state oxidizing system does not cause immediate inhibition (41). None of these experiments support the existence of a significant amount of the pulsed form in steady state.

The Pulsed, Oxygenated, and Peroxidatic Forms

The identification of pulsed or oxygenated oxidase as having a peroxidase structure affords a better understanding of the reaction mechanism of cytochrome oxidase, and the role of the pulsed or oxygenated oxidase in this reaction affords a unique example of the prediction of biochemical function from a structural study. To this point we will trace the historical role of peroxide in the reactions leading to the concept of oxygenated or pulsed oxidase. In the early work of Okunuki and co-workers, peroxide was surely present; indeed, from their own work (4, 5) the oxygenated form was only observed when sufficient oxygen had been added to the reduced cytochrome oxidase-dithionite solution to exhaust the latter and to oxidize the former to form their so-called oxygenated state. Lemberg and Mansley (6), however, advanced cogent arguments, based on chemical approaches, against the presence of O_2 in the oxygenated compound and instead proposed a peroxidatic activity of the oxygenated enzyme, but made no special claim to changing the nomenclature. Wainio put forward the idea that such an oxygenated intermediate might contain O_2 bound to the reduced enzyme (42). However, subsequently we found that the ferrous-oxy compound (14) is stable only at -130°C and has a very short lifetime at room temperature (43).

The appearance of the oxygenated form of the oxidase in

the reaction with oxygen pulses was observed in 1969 (12). A series of papers by Antonini et al. (1) used the word "pulsed" to describe an activated state of cytochrome oxidase as an early product of the oxygen reaction by a procedure similar to that used to identify the oxygenated or peroxidatic forms (6, 12). Because H_2O_2 is overtly present in the preparation of the oxygenated species (due to the reaction of dithionite and oxygen), and may be present in the reaction with O_2 pulses used in the formation of the pulsed form, it is possible that the ligand present in the peroxidatic oxidase is H_2O_2 and not H_2O , as would be expected of a ferric aquo peroxidase in the presence of peroxide. The formation of the pulsed form by reaction with ferricyanide was intended to have been carried out in the absence of oxygen, yet no tests were made for the presence of free or bound peroxide in the reaction mixtures (44). In fact, the situation is reminiscent of that in identifying the presence H_2O_2 in peroxidase Compound I or II (45).

Orii and King (13) suggest multiple forms of the pulsed oxidase, one of which could be a peroxide compound. In fact, Orii and Okunuki had already identified a peroxide compound (5). When excess H_2O_2 is added to pulsed oxidase, in our case ethyl hydrogen peroxide as well, a shift of the Soret band to 430 nm is recorded. These results speak in favor of an aquo form converted to a peroxo form and strongly support Lemberg and Mansley's (6) idea of a peroxidatic role of cytochrome oxidase, setting the stage for the kinetic study of Antonini (1), Brunori (40), and others. It is appropriate, however, to emphasize the peroxidatic or peroxidase-like function of cytochrome oxidase in the ferric-cupric form lacking the S bridge in the active site.

The fast reaction of the pulsed form with cyanide (38) and its structural similarity to peroxidase and metmyoglobin cyanide strongly suggest that the aquo form is involved, and that cyanide and peroxide compete for the aquo form (46), and indeed, the x-ray absorption data will not distinguish between the O atom of H_2O and H_2O_2 as a ligand of the Fe. Also, the observed red shift of the Soret band in the formation of the pulsed form may be further enhanced in the reaction with peroxide. Both these changes are suggestive of the formation of a peroxide compound similar to the red Compound II intermediate of horseradish peroxidase (47) and of yeast peroxidase (48). Thus, Fig. 12 gives a structure appropriate to an aquo or a peroxo form.

Pulsed Oxidase Reactions with Peroxide

The rapid formation of the peroxide compound is observed here, and was previously by Orii and Okunuki for the pulsed form (5). Bickar (49), unaware of the previous work used the resting form and obtained a small occupancy (cf. his Fig. 1 with Fig. 5 of reference 4) that was probably due to the small amounts of the pulsed form present in his preparations. These results suggest the existence of a

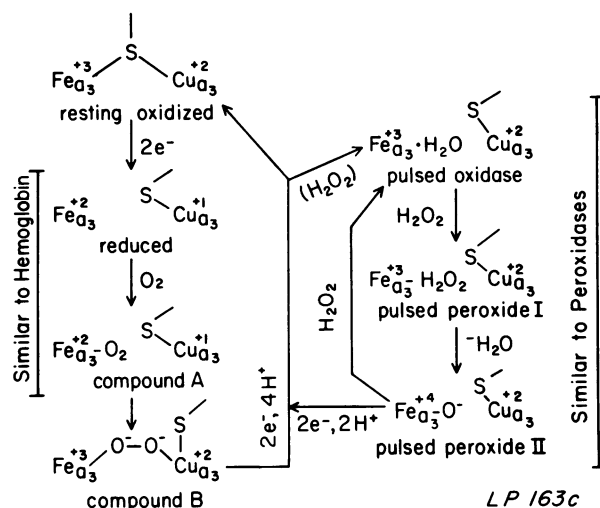


FIGURE 12 Proposed cyclic mechanism of oxygen reduction of cytochrome oxidase including role of pulsed oxidase as a peroxidase.

peroxidatic form lacking the sulfur atom bridging the iron and copper atoms of the active site that can bind cyanide and peroxide with ease. Thus, a further study of the chemistry of the multiple forms of cytochrome oxidase (3) seems useful, especially in view of the following considerations. Three possibilities are considered for the function of the pulsed oxidase, a peroxidatic (50) or a catalytic role (51), or, indeed, a mixture of both (52). In favor of a peroxidatic role on the one hand, are the studies of Lemberg et al. (12) in which many of the donors reacting with horseradish peroxidase Compound I and II (e.g., pyrogallol) were found to cause the disappearance of the oxygenated or pulsed oxidase. Their preparations may have also contained the pulsed-peroxide forms as well due to the reaction of O_2 and dithionite. On the other hand, the reaction of the pulsed form may involve reactions with H_2O_2 in both the formation and decomposition of the pulsed-peroxide species.

As a result of the structural homology of the first-shell structure of the pulsed oxidase, and that of peroxidase from horseradish and yeast and of the pulsed peroxidase cyanide with that of yeast peroxidase cyanide, similarities of chemical reactivities with peroxidase are to be expected. It is appropriate to take note of the similarities, since they impact upon the mechanism shown in Fig. 12 in a highly significant way (19, 53, 54). Reaction of the pulsed (oxygenated) form with peroxide was studied early by Okunuki and a partial reaction of the resting state with peroxide was recently reported upon by Bickar (49, 55). We have preferred ethyl hydrogen peroxide as a reagent for forming the peroxide compound, since artifacts produced by the dual function of H_2O_2 as a reductant and oxidant are obviated. A reaction with 20 mM ethyl hydrogen peroxide is readily observed and optical changes in the region of the Soret band characteristic of the shift of a resting peroxidase to Compound II are observed, i.e., a red shift of the Soret

band of ~4 nm. This shift is smaller than that of the peroxidases (~20 nm) that contain a single heme of fully reactive H₂O₂ (56), suggestive of partial occupancy by peroxide in the pulsed oxidase.

Characteristically, one would expect the lifetime of the peroxide intermediates to be shortened by donors, and we find that the disappearance of the pulsed-oxidase peroxide is accelerated by characteristic peroxidase donors, phenols, amines, etc., in agreement with Lemberg's classic study (6) of the reaction of pulsed (oxygenated) oxidase and peroxide (formed from the reaction of oxygen and dithionite). In addition, H₂O₂ appears to shorten the lifetime of the pulsed peroxide intermediate formed from ethyl hydrogen peroxide. (This is similar to the key reaction observed in the deciphering the catalase mechanism [14, 57].) In this case, H₂O₂ acts as a reductant, suggesting possibilities for catalytic reaction of the pulsed oxidase peroxide compound, classifying the enzymatic action as both peroxidatic and catalytic. Full details are being reported elsewhere; those supportive of the mechanism shown in Fig. 12 are reported here.

This is a typically catalytic reaction, which involves a primary peroxide or Compound I type of intermediate (47). Thus, it appears that both peroxidatic and catalytic reactions involve primary and secondary peroxide intermediates of structure such as Fe—H₂O₂ that may rearrange to the ferryl ion structure [Fe⁺ · O⁻²]⁺². These intermediates may react with either H₂O₂ or with electron donors (2e⁻, 2H⁺) to reenter the peroxidatic cycle if H₂O₂ is still present or the oxidative cyclic if H₂O₂ is absent.

The possibility that further forms of the peroxide compound may be formed is not ruled out by these data, and it is characteristic of the reactions of catalases with peroxide to form at least two and usually three or four intermediate compounds (56, 58). The reduction of the pulsed peroxide intermediate is slow but may occur with electrons donated from cytochrome *c*, cytochrome *a*, Cu_z, to cycle back to the pulsed form if H₂O₂ is available and, if it is not, to the oxidized bridged resting form (Fig. 12).

Possible Reaction Cycles

Fig. 12 illustrates the cycle of cytochrome oxidase in its usual, or resting configuration as previously proposed (14, 43) involving the S-bridged form in the resting state and the hemoglobin-like form in the reduced and reduced CO states, and recycling back to the resting form as found experimentally. On a side pathway is the non-S-bridged peroxidase-like species formed as we proposed with peroxide present from the reaction of O₂ with exogenous or endogenous reductants. Further reaction with peroxide gives the H₂O₂ adduct and a higher oxidation state of iron [Fe⁺ · O=]⁺² proposed on the basis of studies of peroxidase intermediates by volume magnetic susceptibility (59) and by analogy with myoglobin peroxide (60). Presumably, electrons can be accepted in pairs, as shown

here, or one at a time, to give the characteristic Compound II of the reaction of the horseradish or yeast enzymes (57, 58) with peroxide.

The enzymatic cycle of Fig. 12 shown here is essentially one of a protective or scavenger system that maintains any peroxide produced either from the cytochrome oxidase-oxygen reaction or from other systems as, for example, in the reaction of ubiquinone radicals with oxygen as studied together with Boveris and co-workers (52), leading to superoxide anion formation and its dismutation to H₂O₂ by superoxide anion dismutase (52). This portion of the cytochrome oxidase activity can be termed a peroxidatic or, indeed, a catalytic activity. The catalytic activity of cytochrome oxidase has been studied by Orii and Okunuki (5), who showed the activity to be ~10⁻⁴ of that of an equimolar concentration of catalase, and nonnegligible activity (10³/s as compared with 10⁷/s for catalase). This suggests that the cycle of the peroxidatic activity of cytochrome oxidase may also be catalytic and involve a second molecule of H₂O₂ (56, 58). This result is of considerable physiological importance as a correction system or a "proofreader," against peroxide effusion from which the cytochrome oxidase system must maintain the mitochondrial membrane that does not contain catalase-rich microbodies or peroxisomes itself and the catalytic or peroxidatic activity due to cytochrome oxidase may safeguard against the ubiquinone-linked production of H₂O₂. Thus, the pulsed and pulsed peroxide compounds can be renamed, on a structural basis, a non-S-bridged oxidized form and, on a functional basis, a peroxidatic form of cytochrome oxidase (6).

In summary, nature's way of guarding against radicals by the tight binding of peroxide to Compound B formed from oxygen and a side catalytic cycle for the elimination of further amounts of H₂O₂ that would come from mistakes of cytochrome oxidase in O₂ reduction or from the ubiquinone O₂⁻¹ and H₂O₂ generator indicates the perfection of years of development of an appropriate enzymatic activity.

The authors acknowledge the kindness of Dr. Tom Poulos for participation in the experiment on and for the preparation of yeast peroxidase, Dr. K. G. Paul for horseradish peroxidase, Dr. Michael Rossmann for beef-liver catalase, and Dr. Dale Webster for cytochrome *o* from *Vitreoscilla*. In addition, the assistance of Mr. Robert Fischetti and Dr. Ted J. Stazeski in data accumulation at SSRL is greatly appreciated.

This research was partially supported by National Institutes of Health grants GM-27476, GM-27308 and GM-31992, HL-18708, RR-01633; National Science Foundation grants PCM-80-26684; and SSRL Project 632 supported by the National Science Foundation through the Division of Materials Research and the National Institutes of Health through the Biotechnology Resource Program in the Division of Research Resources in cooperation with the Department of Energy.

Received for publication 26 July 1982 and in final form 22 August 1983.

REFERENCES

- Antonini, E., M. Brunori, A. Colosimo, C. Greenwood, and M. T. Wilson. 1977. Oxygen "pulsed" cytochrome *c* oxidase: Functional properties and catalytic relevance. *Proc. Natl. Acad. Sci. USA*. 74:3128-3132.
- Chance, B., P. Angiolillo, E. Yang, and L. Powers. 1980. Identification and assay of synchrotron radiation-induced alterations on metallo-enzymes and proteins. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 112:178-182.
- Brudvig, G., T. H. Stevens, R. H. Morse, and S. I. Chan. 1981. Conformations of oxidized cytochrome oxidase. *Biochemistry*. 20:3912-3921.
- Okunuki, K., B. Harihara, I. Sekuzu, and T. Horio. 1958. Studies on cytochrome system. In *Proceedings of the International Symposium on Enzyme Chemistry*. Maruzen, Tokyo. 264-272.
- Orii, T., and K. Okunuki. 1963. Studies on cytochrome *a*. X. Effect of hydrogen peroxide on absorption spectra of cytochrome *a*. *J. Biochem. (Tokyo)*. 54:207-213.
- Lemberg, R., and G. E. Mansley. 1966. Cytochrome oxidase and its derivatives. V. The reaction of ferrous cytochrome *c* oxidase with oxygen and hydrogen peroxide in the presence of sodium dithionite. *Biochem. Biophys. Acta*. 118:19-35.
- Shaw, R. W., R. E. Hansen, and H. Beinert. 1978. A novel electron paramagnetic resonance signal of oxygenated cytochrome *c* oxidase. *J. Biol. Chem.* 253:6637-6640.
- Wilson, M. T., P. Jensen, R. Aasa, B. G. Malmstrom, and T. Vanngard. 1982. An investigation by e.p.r. and optical spectroscopy of cytochrome oxidase during turnover. *Biochem. J.* 203:483-492.
- Greenwood, C. and Q. H. Gibson. 1969. The reaction of reduced cytochrome *c* oxidase with oxygen. *J. Biol. Chem.* 242:1782-1787.
- Gibson, Q. H., C. Greenwood, D. C. Wharton, and G. Palmer. 1965. The reaction of cytochrome oxidase with cytochrome *c*. *J. Biol. Chem.* 240:888-894.
- Chance, B., and Erecinska, M. 1971. Flow-flash kinetics of cytochrome *a*₃-oxygen reaction in coupled and uncoupled mitochondria using the liquid dye laser. *Arch. Biochem. Biophys.* 143:675-687.
- Lemberg, M. R., M. Gilmour, and B. Chance. 1969. Cytochrome oxidase and its derivatives. IX. Spectrophotometric studies on the rapid reaction of ferrous cytochrome *c* oxidase with molecular oxygen under conditions of complete and partial oxygenation. *Biochim. Biophys. Acta*. 172:37-51.
- Orii, Y., and T. E. King. 1972. New species of the "oxygenated compound" of cytochrome oxidase. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 21:199-202.
- Powers, L., B. Chance, Y. Ching, and P. Angiolillo. 1981. Structural features and the reaction mechanism of cytochrome oxidase. Iron and copper x-ray absorption fine structure. *Biophys. J.* 34:465-498.
- Powers, L., Y. Ching, B. Chance, and B. Muhoberac. 1982. Cytochrome oxidase: structure and mechanisms, redox states, oxygen intermediates, pulsed, and Cu-depleted forms—a synchrotron x-ray study. *Biophys. J.* 37(2, Pt. 2):403a. (Abstr.)
- Yonetani, T. 1960. Studies on cytochrome oxidase. I. Absolute and difference absorption spectra. *J. Biol. Chem.* 235:845-852.
- Kumar, C., A. Naqui, B. Chance, Y. Ching, L. Powers, and C. R. Hartzell. 1983. Differences in structure and reactivity of different cytochrome oxidase preparations. *Biophys. J.* 41(2, Pt. 2):409a. (Abstr.)
- Chance, B., J. Moore, L. Powers, and Y. Ching. 1982. A redox equilibrators for the preparation of cytochrome oxidase of mixed valence states and intermediate compounds for X-ray synchrotron studies. *Anal. Biochem.* 124:248-257.
- Bonnischen, R. K., B. Chance, and B. Theorell. 1947. Catalase activity. *Acta. Chem. Scand.* 1:685-709.
- Peisach, J., and W. E. Blumberg. 1974. Structural implications derived from the analysis of electron paramagnetic resonance spectra of natural and artificial copper proteins. *Arch. Biochem. Biophys.* 165:691-708.
- Blumberg, W. E., and J. P. Peisach. 1968. Bis (thiosemicarbazone) and other nitrogen and sulfur ligated complexes of Cu(II). *J. Chem. Phys.* 49:1793-1802.
- Brown, J. M., L. Powers, B. Kincaid, J. A. Larrabee, and T. G. Spiro. 1980. Structural studies of the hemocyanin active site. I. Extended x-ray absorption fine structure (EXAFS) analysis. *J. Am. Chem. Soc.* 102:4210-4216.
- Peisach, J., L. Powers, W. E. Blumberg, and B. Chance. 1982. Stellacyanin. Studies of metal-binding site using x-ray absorption spectroscopy. *Biophys. J.* 38:277-285.
- Scott, R. A., S. P. Cramer, R. W. Shaw, H. Beinert, and H. B. Gray. 1981. Extended x-ray absorption fine structure of copper in cytochrome *c* oxidase: direct evidence for copper-sulfur ligation. *Proc. Natl. Acad. Sci. USA*. 178:664-667.
- Powers, L., B. Chance, Y. Ching, B. Muhoberac, S. Weintraub, and D. Wharton. 1982. Structural features of the copper-depleted cytochrome oxidase from beef heart: iron EXAFS. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 138:245-248.
- Powers, L., W. E. Blumberg, B. Chance, C. Barlow, J. S. Leigh, Jr., J. Smith, T. Yonetani, S. Vik, and J. Peisach. 1979. The nature of the copper atoms of cytochrome *c* oxidase as studied by optical and x-ray absorption edge spectroscopy. *Biochim. Biophys. Acta*. 546:521-538.
- Karlsson, B., and L. E. Andreasson. 1981. The identify of a new copper (II) electron paramagnetic resonance signal in cytochrome *c* oxidase. *Biochim. Biophys. Acta*. 635:73-80.
- Armstrong, F., R. W. Shaw, and H. Beinert. 1983. Cytochrome *c* oxidase. Time dependence of optical and EPR spectral changes related to the "oxygen-pulsed" form. *Biochim. Biophys. Acta* 722:61-71.
- Brudvig, G. W., T. H. Stevens, R. H. Morse, and S. I. Chan. 1981. Conformations of oxidized cytochrome *c* oxidase. *Biochemistry*. 20:3912-3921.
- Kutzler, F., C. R. Natali, D. K. Misemer, S. Doniach, and K. Hodgson. 1980. Use of one-electron theory for the interpretation of near edge structure in K-shell x-ray absorption spectra of transition metal complexes. *J. Chem. Phys.* 73:3274-3288.
- Brittain, T., and C. Greenwood. 1976. Kinetic studies on the binding of cyanide to oxygenated cytochrome *c* oxidase. *Biochem. J.* 155:453-455.
- Paul, K. G. 1958. Isolation of horse radish peroxidase. *Acta Chem. Scand.* 12:1312.
- Yonetani, T., B. Chance, and S. Kajiwar. 1966. Crystalline cytochrome *c* peroxidase and complex ES. *J. Biol. Chem.* 21:2981-2982.
- Evantoff, W., N. Tanaka, and M. G. Rossmann. 1976. Crystalline bovine liver catalase. *J. Mol. Biol.* 103:799-801.
- Tyree, B., and D. A. Webster. 1979. Intermediates in the reaction of reduced cytochrome *o* (Vitreoscilla) with oxygen. *J. Biol. Chem.* 254:176-179.
- Poulos, T. L., S. T. Freer, R. A. Alden, S. L. Edwards, U. Sokogland, K. Takia, B. Eriksson, N. H. Xuong, T. Yonetani, and J. Kraut. 1980. The crystal structure of cytochrome *c* peroxidase. *J. Biol. Chem.* 255:575-580.
- vanGelder, B. F., and H. Beinert. 1969. Studies of the heme components of cytochrome *c* oxidase by EPR spectroscopy. *Biochim. Biophys. Acta*. 189:1-24.
- Chance, B. 1943. The effect of cyanide on the kinetics of the enzyme-substrate compound and overall reaction of peroxidase. *J. Cell Comp. Physiol.* 22:33-41.
- Griffiths, J. S. 1964. *The Theory of Transition Metal Ions*. Cambridge University Press, Cambridge, England.
- Brunori, M., E. Antonini, A. Colosimo, P. Sarti, and M. T. Wilson.

1981. A two-state model for cytochrome oxidase. Seventh International Biophysical Congress and Third Pan-Am Biochemical Congress, Mexico City. 159.
41. Chance, B. 1952. Spectra and reaction kinetics of respiratory pigments of homogenized and intact cells. *Nature (Lond.)*. 169:215-230.
 42. Davison, A. J., and W. W. Wainio. 1954. Possible functional significance of "oxygenated" cytochrome oxidase. *Fed. Proc.* 23:1332a. (Abstr.)
 43. Chance, B., C. Saronio, and J. S. Leigh, Jr. 1975. Functional intermediates in the reaction of cytochrome oxidase with oxygen. *Proc. Natl. Acad. Sci. USA*. 72:1635-1640.
 44. Brunori, M., A. Colosimo, P. Sarti, E. Antonini, and M. T. Wilson. 1981. 'Pulsed' cytochrome oxidase may be produced without the advent of dioxygen. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 126:195-198.
 45. Chance, B. and L. Smith. 1952. Biological oxidations. *Annu. Rev. Biochem.* 21:687-726.
 46. Chance, B. 1949. The reaction of catalase and cyanide. *J. Biol. Chem.* 179:1299-1309.
 47. Chance, B. 1949. The properties of the enzyme-substrate compounds of horseradish peroxidase and peroxides. IV. The effect of pH upon the rate of reaction complex II with several acceptors and its relation to their oxidation-reduction potential. *Arch. Biochem.* 24:410-421.
 48. Yonetani, T. 1976. Cytochrome *c* peroxidase. In *The Enzymes*. P. Boyer, editor. Academic Press, Inc., New York. 345-361.
 49. Bickar, D., J. Bonaventura, and C. Bonaventura. 1982. Cytochrome *c* oxidase binding of hydrogen peroxide. *Biochemistry*. 21:2661-2666.
 50. Chance, B. 1943. The kinetics of the enzyme-substrate compound of peroxidase. *J. Biol. Chem.* 151:553-577.
 51. Chance, B. 1947. An intermediate compound in the catalase-hydrogen peroxide reaction. *Acta Chem. Scand.* 1:236-267.
 52. Chance, B., H. Sies, and A. Boveris. 1979. Hydroperoxide metabolism in mammalian organs. *Phys. Rev.* 59:527-605.
 53. Yoshikawa, S., M. G. Choc, M. C. O'Toole, and W. S. Caughey. 1977. An infrared study of CO binding to heart cytochrome *c* oxidase and hemoglobin A. *J. Biol. Chem.* 252:5498-5508.
 54. Chance, B., C. Kumar, A. Naqui, L. Powers, and Y. Ching. 1983. Structural basis for the peroxidatic and catalatic activities of cytochrome oxidase. *Biophys. J.* 41(2, Pt.2):321a (Abstr.)
 55. Bickar, D., J. Bonaventura, and C. Bonaventura. 1980. Hydrogen peroxide binding by cytochrome *c* oxidase. *Fed. Proc.* 39:2061.
 56. Chance, B. 1952. The spectra of the enzyme-substrate complexes of catalase and peroxidase. *Arch. Biochem. Biophys.* 41:404-415.
 57. Chance, B. 1949. The primary and secondary compounds of catalase and methyl or ethyl hydrogen peroxide. IV. Reactions with hydrogen peroxide. *J. Biol. Chem.* 180:947-959.
 58. Chance, B. 1952. The kinetics and stoichiometry of the transition from the primary to the secondary peroxidase peroxide complexes. *Arch. Biochem. Biophys.* 41:416-424.
 59. George, P., and D. H. Irvine. 1951. Reaction of metmyoglobin with hydrogen peroxide. *Nature (Lond.)*. 168:164-165.
 60. Theorell, H., A. Ehrenberg, and B. Chance. 1952. Electronic structure of the peroxidase-peroxide complexes. *Arch. Biochem. Biophys.* 37:237-239.