BBA 40258

HEME–HEME INTERACTION IN CYTOCHROME OXIDASE

DAVID F. WILSON, J. GORDON LINDSAY AND E. S. BROCKLEHURST

Department of Biophysics and Physical Biochemistry, Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pa. 19104 (U.S.A.)

(Received August 16th, 1971)

SUMMARY

The cytochromes *a* and a_3 of intact pigeon heart mitochondria are shown to contribute equally to the α band of cytochrome oxidase in the absence of added ligands. The oxidation–reduction midpoint potentials of the cytochromes *a* and a_3 at pH 7.2 are -220 mV and $+380$ mV, respectively. The oxidation of cytochrome a_3 but not cytochrome *a* is coupled to a proton dissociation from a group with a pK of 7.

Strong heme–heme interaction between these two cytochromes is expressed in the interdependence of their absorption spectra and half-reduction potentials. An increase of more than 50 % in the extinction coefficient of reduced cytochrome *a* occurs when reduced cytochrome a_3 binds CO and a similar increase in the extinction coefficient of reduced cytochrome a_3 occurs when oxidized cytochrome *a* binds azide. The total absorbance change on reduction of both cytochromes is unchanged by the addition of azide. Heme–heme interaction is also observed in a 30-mV increase in the half-reduction potential of cytochrome *a* when the measurements are carried out in the presence of CO and a 30-mV decrease in the half-reduction potential of cytochrome a_3 when the measurements are carried out in the presence of high concentrations of azide.

INTRODUCTION

Cytochrome *c* oxidase is a very important part of the mitochondrial respiratory chain. It is responsible both for electron transport leading to the reduction of O_2 to water[1–3] and for the conservation of the energy required for ATP synthesis[4–7]. In order to study these two functions it is extremely important to know the spectral properties of the two *a* cytochromes which are part of this hemoprotein. The spectral properties have previously been measured by techniques which utilize the ability of inhibitors to trap one of the two heme *a* (usually that of cytochrome a_3) in the oxidized or reduced form[8–12]. Several lines of evidence have suggested that the spectral properties measured in this way are correct only for these special conditions. For example, the EPR spectrum of the ferric cytochrome *a* is reported to be dependent on the degree of reduction of the cytochrome a_3[13,14], and it has been suggested that the visible light absorption spectrum of cytochrome a_3 is dependent on the degree of reduction of one of the copper atoms[12].

In this paper we will present evidence that a strong interaction exists between cytochromes *a* and a_3. This interaction makes it necessary to regard them as mutually

dependent species for which the measured properties of each component depend on the chemistry of the companion component.

METHODS

Pigeon heart mitochondria were prepared by the method of HAGIHARA AND CHANCE[15]. The oxidation–reduction titrations were carried out at room temperature by the method of DUTTON *et al.*[7, 16, 17]. A dual wavelength spectrophotometer designed and built in the Johnson Research Foundation was utilized to measure the optical changes. The band width at half height of the measuring light beams were always less than 1.6 nm.

The reagents were the same as previously used[7, 16].

RESULTS

The oxidation–reduction potential dependence of the absorbance change at 605–630 nm

Two components can be shown to contribute approximately equally to the absorbance change at 605–630 nm in intact pigeon heart mitochondria. When the oxidation–reduction potential dependence of this absorbance change was measured at pH 7.2. and the logarithm of the ratio of the oxidized form to reduced form (assuming that the increase in absorbance on reduction is proportional to the reduced form) is plotted against the oxidation–reduction potential in Fig. 1A, the titration curve is sigmoid. This is the expected behavior for a mixture of two components with quite different half-reduction potentials[17]. The sigmoid curve can be mathematically resolved into two individual components as shown in Fig. 1B. The resultant components have n values of 1.0 and half-reduction potentials of +375 mV and +230 mV for the high- and low-potential components, respectively. The curve analysis shows that approximately 40 % of the absorbance change is contributed by the high potential component and 60 % by the low-potential component. In 13 titrations of separate mitochondrial

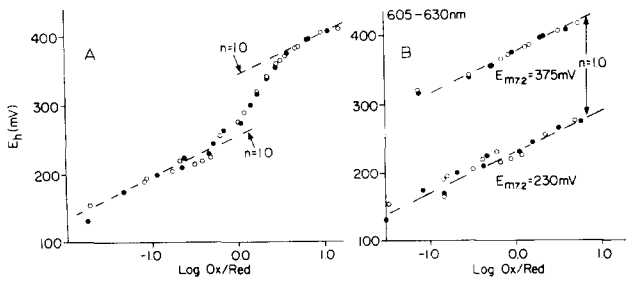

Fig. 1. The oxidation–reduction potential dependence of the 605 nm *minus* 630 nm absorbance change of cytochrome oxidase. Pigeon heart mitochondria were suspended at 4 mg protein per ml in a medium containing 0.22 M mannitol, 0.05 M sucrose and 50 mM morpholinopropane sulfonate, pH 7.2. Phenazine methosulfate (30 μM) and diaminodurene (40 μM) were added and anaerobiosis was attained by adding aliquots of NADH. After anaerobiosis the absorbance change was titrated oxidatively by ferricyanide addition (●) and then reductively by NADH addition (○). The resulting data are plotted with the logarithm of the ratio of the oxidized to reduced form on the abscissa and the measured oxidation–reduction potential relative to a hydrogen electrode on the ordinate. In A the absorbance change is treated as a single component while in B the sigmoid curve of A is resolved into its two-component parts.

suspensions the fraction of the absorbance change contributed by the high potential component had an average value of 0.48 and extreme values of 0.40 and 0.57.

The variability is much less (less than ± 0.02) for duplicate experiments using a single mitochondrial suspension. The value of ± 0.08 includes not only the use of different mitochondrial preparations but also different pH values (6.5–8.5), different spectrophotometers and different experiments. As such it includes biological and experimental errors as well as possible small pH effects.

The pH dependence of the half-reduction potentials of the high- and low-potential components

The pH dependence of the half-reduction potential of an oxidation–reduction component provides evidence for or against a direct coupling of a proton dissociation to the oxidation of the component[18]. In Fig. 2 the values of the measured half-reduction potentials of the high- and low-potential components are plotted as a function of pH. To permit direct comparison the half-reduction potentials of the corresponding components of rat liver mitochondria[14] (as measured at 445–455 nm) are plotted on the same scale. It is apparent that the two sets of values are experimentally indistinguishable with respect to both their numerical values and the pH dependence of these values. The low-potential component has a pH dependence of less than 30 mV/pH from pH 6.5 to 8.5 while the high-potential component is pH independent from pH 6.5 to 7.0 but becomes approx. 60 mV more negative with each unit that the pH increases from pH 7.0 to 8.5. A reasonable interpretation of this behavior is that the oxidation of the high-potential component is coupled to the dissociation of one proton per electron from a chemical group with a pK of 7.0. This coupling is not present in the low-potential component.

The absorption spectra of the high- and low-potential components from 575 to 630 nm

The sigmoid titration curve obtained for the absorbance change at 605–630 nm and its resolution into its two component parts are shown in Fig. 1. The spectra of the components can be accurately measured by repeated oxidation–reduction cycles for which different measuring wavelengths are used with the reference wavelength unchanged. The spectra for the high-potential ($+425$ to $+285$ mV) and the low-potential

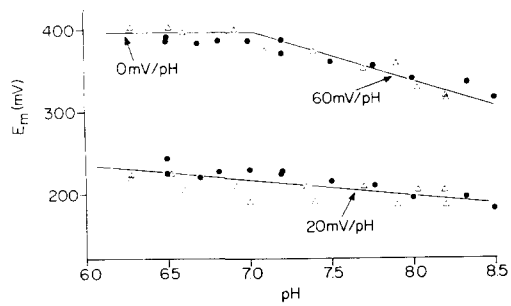

Fig. 2. The pH dependence of the half-reduction potentials of the components of cytochrome oxidase as measured at 605 nm *minus* 630 nm and at 445 nm *minus* 455 nm. The half-reduction potentials (E_m) were measured for pigeon heart mitochondria (●) at 605–630 nm as given in the legend of Fig. 1, while the values for rat liver mitochondria (△) were measured at 445–455 nm (see ref. 14). The pH was obtained by using 50 mM morpholinopropane sulfonate (pH 6.3–7.8) or 50 mM Tris (pH 7.5–8.5) as buffers.

(+285 to +125 mV) components are shown in Fig. 3. The high-potential component has a symmetric absorption band with a maximum at 604 nm while the low-potential component has a somewhat narrower absorption band with a maximum at 605 nm. The sum of the two is a typical α band for cytochrome oxidase.

The effect of CO on the oxidation–reduction potential of the components contributing to the 605–630-nm absorbance change

When the absorbance change at 605–630 nm is measured as a function of the oxidation–reduction potential and the gas phase is 50 % CO, 50 % argon, the absorbance change titrates as a single component (Fig. 4). The titration is presented from 99 % oxidized to 99 % reduced but there is no evidence for a deviation from an n value of 1.0. The experimental data shows two anomalies. First, the midpoint potential of the component is 30 mV more positive than that of the low-potential component in the absence of CO (see also ref. 19) and second, although the addition of CO decreases the absorbance (oxidized – reduced) only 6 %, the remaining absorbance is 90 % due to the low-potential component and is 10 % due to a component which cannot be oxidized by ferricyanide.

The half-reduction potential of the low potential component in the presence

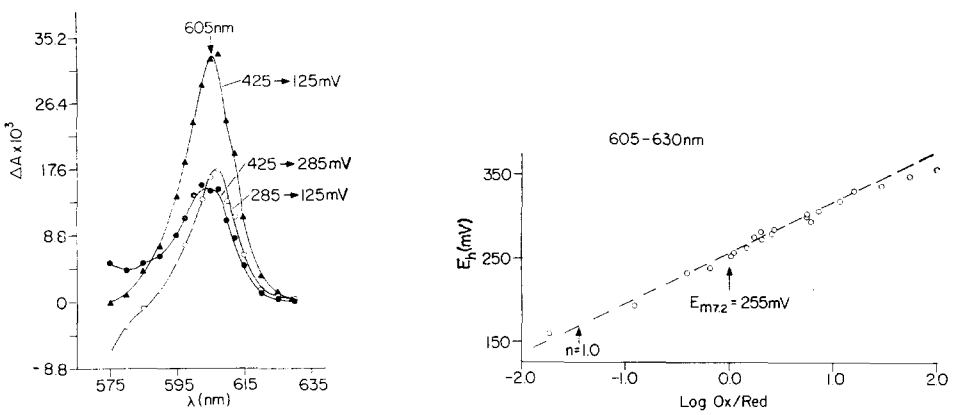

Fig. 3. The spectra of the α bands of the high- and low-potential components of cytochrome oxidase. The conditions employed were the same as in Fig. 1 except that the mitochondria were suspended at 2.7 mg protein per ml and the phenazinemethosulfate and diaminodurene were each 20 μM. The reference wavelength was 630 nm and the measure wavelength was the value given on the abscissa. The absorbance changes were measured using successive cycles of oxidation (ferricyanide addition) and reduction (NADH addition). ●—●, the absorbance change from +425 to +285 mV; ○—○, the absorbance change from +285 to +125 mV. ▲—▲, the complete absorbance change from +425 to +125 mV.

Fig. 4. The oxidation–reduction potential of the low potential component of cytochrome oxidase in the presence of CO. Pigeon heart mitochondria were suspended at 4.5 mg protein per ml in a medium containing 0.22 M mannitol, 0.05 M sucrose and 50 mM morpholinopropane sulfonate, pH 7.2. Phenazine methosulphate (30 μM) and diaminodurene (30 μM) were added and anaerobiosis attained by adding aliquots of NADH. After anaerobiosis the gas phase was changed to 50% CO, 50% argon, a 5-min period allowed for the CO to enter the ligand phase, and then the absorbance change at 605 nm *minus* 630 nm titrated both oxidatively with ferricyanide and then reductively with NADH. The data are plotted with the logarithm of the ratio of the oxidized to reduced form on the abscissa and the oxidation–reduction potential relative to a hydrogen electrode on the ordinate.

of CO is 250 mV, a value nearly identical to that reported for cytochrome *a* in isolated cytochrome oxidase in the presence of CO[19,20].

The effect of azide on the oxidation–reduction potential of the components contributing to the 605–630 nm absorbance change

The oxidation–reduction potential dependence of the absorbance change at 605–630 nm was measured for samples in the presence of various concentrations of azide. A typical titration curve at pH 7.2 (for 10 mM NaN_3) is shown in Fig. 5. Approx. 87 % of the absorbance change is titrated as a single component with an *n* value of 1.0 and a half-reduction potential of 350 mV. This half-reduction potential is 30 mV more negative than the value obtained for the high-potential component in the absence of azide. The absorbance change due to the component with a half-reduction potential of 350 mV is approx. 87 % of the absorbance change observed for the sum of the high- and low-potential components in the absence of azide.

The effect of increasing the azide concentration in the suspending medium from 0 to 20 mM is presented in Table I. As the azide concentration is increased there is a systematic change in several of the measured parameters. The most striking change is in the absorbance which is attributable to the high-potential and low-potential components respectively. Although the total reduced *minus* oxidized absorbance change is not dependent on the azide concentration, the fraction of the absorbance due

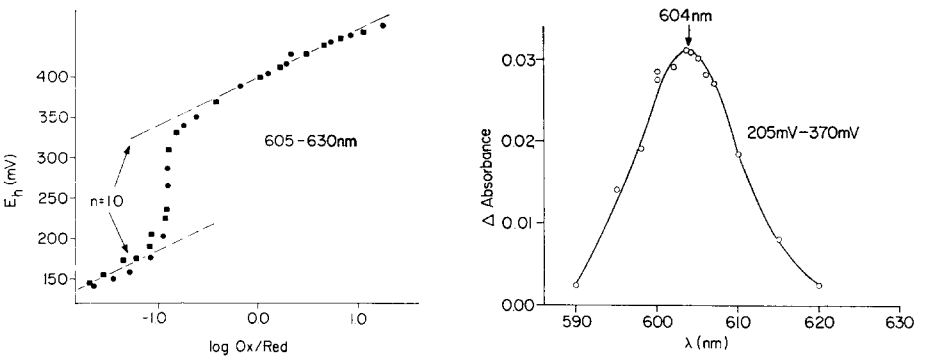

Fig. 5. The oxidation–reduction potential of the components of cytochrome oxidase in the presence of azide. Pigeon heart mitochondria were suspended at 2.5 mg protein per ml in a medium containing 0.22 M mannitol, 0.05 M sucrose and 50 mM morpholinopropane sulfonate, pH 7.2. Phenazine methosulfate (30 μM) and diaminodurene (30 μM) were added and anaerobiosis attained by adding aliquots of NADH. After anaerobiosis 10 mM NaN_3 was added and the absorbance change measured at 605 *minus* 630 nm titrated both oxidatively by ferricyanide additions and reductively by NADH additions. The data are plotted with the logarithm of the ratio of the oxidized to reduced form on the abscissa and measured oxidation–reduction potential relative to a hydrogen electrode on the ordinate. ●, oxidative titration; ■, reductive titrations.

Fig. 6. The spectrum of the high potential component of cytochrome oxidase in the presence of azide. Pigeon heart mitochondria were suspended at 2.5 mg protein per ml in a medium containing 0.22 M mannitol, 0.05 M sucrose and 0.05 M morpholinopropane sulfonate, pH 7.2. Phenazine methosulfate (30 μM) and diaminodurene (30 μM) were added and anaerobiosis attained by adding aliquots of NADH. The spectrum was obtained by measuring the absorbance change which occurred on changing the potential from +370 to +205 mV with NADH or from +205 to +370 mV with ferricyanide. The wavelength of the reference light was left at 630 nm and the wavelength of the measure light was set at the values indicated on the abscissa. The ferricyanide (+ ferrocyanide) concentration varied from 50 μM to more than 2 mM at the end of the experiment.

to the high-potential component increases from approx. 0.5 to 0.88. This increase in the absorbance is accompanied by a half-reduction potential shift from +380 to +350 mV in the high-potential component and from +220 to approx. +105 mV in the low-potential component. These changes are consistent with the azide binding to the ferric form of the low-potential component with a dissociation constant of approx. 0.25 mM at pH 7.2.

TABLE I

THE AZIDE CONCENTRATION DEPENDENCE OF THE PROPERTIES OF THE HIGH- AND LOW-POTENTIAL COMPONENTS

The half-reduction potentials (E_m) and the fraction of the absorbance change of the high- and low-potential components were determined as shown in Figs. 1 and 4. The E_m values are given in mV and are reproducible to within ± 10 mV. The total absorbance change on reduction of both components is not changed by the addition of azide.

Azide (mM)	E_m		E_m* *Low*	*Fraction of Absorbance Change*	
	High	*Low*		*High*	*Low*
0.0	380	220	220	0.5	0.5
0.1	370	210	210	0.54	0.46
0.5	360	190	190	0.74	0.26
1.0	350	185	180	0.74	0.26
2.7	350	155	155	0.83	0.17
5.0	350	145	140	0.84	0.16
10	350	130	125	0.87	0.13
20	345	105	105	0.88	0.12

* This column presents the theoretical half-reduction potentials of a heme which in the oxidized form binds azide with a dissociation constant of 0.25 mM but which in the reduced form has a very low affinity for azide. The half-reduction potential of the heme in the absence of azide is assumed to be 220 mV and the calculated values are given to the nearest 5 mV.

The spectrum of the major component of the 605–630-nm absorbance change in the presence of azide

The technique of measuring the spectrum of a component reduced in a specific oxidation–reduction potential range allows us to measure the spectra of the high- and low-potential components in the absence of added ligands. As shown in Fig. 6 the reduced *minus* oxidized spectrum of the high-potential component in the presence of azide has an α maximum at 604 nm, the position observed for either fully reduced cytochrome oxidase or reduced cytochrome *a* in the presence of CO, cyanide or sulfide. By contrast the α maximum of the reduced *a* cytochrome in the aerobic azide-inhibited state is at 601 nm.

DISCUSSION

On the identification of cytochromes a and a_3

KEILIN AND HARTREE[1,32] first concluded that two cytochromes of the *a* type were present in the respiratory chain of yeast and heart muscle. They defined the cytochrome which did not combine with cyanide, CO or oxygen as cytochrome *a* and the cytochrome which did combine with these ligands as cytochrome a_3. The cytochrome

a_3 was then identical to the "Atmungsferment" of WARBURG AND NEGELEIN[21,22]. This definition of cytochrome a_3 clearly identifies the high-potential component as measured either in the Soret region[7,14] or the α region as cytochrome a_3. CO is known to react with a high affinity[21,22] with the reduced form of cytochrome a_3 and to have a very low affinity for the oxidized form. Thus, CO would be expected to shift the half-reduction potential to much more positive values[18]. This is consistent with the observation that in the presence of excess CO the cytochrome a_3 is not oxidized by ferricyanide[23]. Moreover, the reaction rate for the oxidation of the reduced high-potential component by molecular oxygen is independent of the degree of reduction of the low-potential component[24]. The high-potential component is then cytochrome a_3 and the low-potential component is cytochrome a. For the rest of the discussion this identification will be used.

The identification of cytochromes a and a_3 on the basis of their spectral properties has always been made with the assumption that there is essentially no interaction between the two cytochromes. The techniques utilizing external ligands such as cyanide, azide and CO to obtain the spectra of the cytochromes a and a_3[8–12,26] suffer from the obvious limitation that a modifier (ligand) is always present and its effect cannot be evaluated. The potentiometric measurements, however, allow the properties of two chemically distinct species to be measured in the absence of added ligands. The concentration dependence of the ligand effect may then be accurately measured both on the component to which the ligand binds and on the component to which it does not bind. Moreover the identification of the component binding the ligand is precise because an accurate theoretical description is available[18].

On the interaction of cytochromes a and a_3

The concept of cytochromes a and a_3 as independent cytochromes, each with its own particular properties, is unable to fit the data presented in this paper. The half-reduction potential of cytochrome a becomes more positive when the cytochrome a_3 is liganded with CO. Because CO is highly specific for binding to reduced heme, this shift in the half-reduction potential must result from a heme–heme interaction and not from a copper–heme interaction.

The interaction between cytochromes a and a_3 is also expressed in the extinction coefficients for the reduced *minus* oxidized transition of the cytochromes. The addition of CO increased the absorbance change (reduced *minus* oxidized as measured at 605–630 nm) due to the cytochrome a from 50 % to 88 % of the aerobic to fully reduced transition (a 70 % increase in extinction coefficient[25]).

The failure of other techniques[8–12,26] to demonstrate the heme–heme interaction arises in part from the fact that the prosthetic group of both cytochromes a and a_3 is heme a. The differences between the two cytochromes are imposed on the heme a by the heme environment. A symmetric interaction between the two hemes would not be measurable using techniques which require one cytochrome to be bound to an external ligand such as cyanide, azide or CO in order to measure the spectral properties of the other.

The interaction of azide with cytochrome oxidase

The change in the half-reduction potential which occurs on addition of a ligand

to heme has been comprehensively discussed by CLARK[18]. The equation which is applicable to the azide binding (see CLARK[18] for the derivation used) is:

$$E_m = E_1 + 0.06 \log \frac{K_0}{K_r} + 0.06 \log \frac{K_r + [N_3^-]}{K_0 + [N_3^-]} \quad (1)$$

where K_0 and K_r are the dissociation constants for azide binding to the oxidized and reduced forms of the heme, respectively, and E_1 is the half-reduction potential in the absence of azide. The application of this equation to the data in Table I shows that azide binds much more strongly to oxidized cytochrome *a* than to reduced cytochrome *a*. The dissociation constant for azide from ferricytochrome *a* can be calculated to be 0.25 mM. If azide does bind to ferricytochrome *a* with this dissociation constant, the changes in half-reduction potential and extinction coefficient of reduced cytochrome a_3 at 605–630 nm are proportional to the fraction of ferricytochrome *a* bound to azide. The possibility that azide also binds to cytochrome a_3 does not seem viable. If the azide binds to ferricytochrome a_3 with a dissociation constant comparable to its dissociation constant from the inhibitory site (less than 20 μM) then from Eq. 1, the dissociation constant from the reduced form is less than 60 μM, a value without precedent in heme chemistry. In any case, the azide does not bind to ferricytochrome a_3 with an affinity more than 3-fold greater than the affinity for the ferrous form because the half-reduction potential changes by only 30 mV.

EPR measurements on samples which were frozen at defined oxidation–reduction potentials show that in the presence of azide the reduction of a component with a half-reduction potential of +350 mV is accompanied by the appearance of the low spin ferric heme *a* azide signal[13,27]. It is, therefore, likely that the reduction of the high-potential component (cytochrome a_3) results in a structural modification which permits azide to bind to ferricytochrome *a*. Although azide binding to ferricytochrome *a* in the ferricytochrome *a*–ferrocytochrome a_3 can be demonstrated, this is probably not the cause of azide inhibition. In intact mitochondria at pH 7.2 the azide inhibitor constant has been reported to be less than 20 μM for the inhibition of *N*,*N*,*N'*,*N'*-tetramethyl-*p*-phenylenediamine oxidase activity[28] and less than 80 μM for the inhibition of succinate oxidation[29]. In both cases the inhibition is uncompetitive with respect to substrate. The inhibitor constant must, therefore, be greater than the actual dissociation constant of azide from the inhibitory site and a reasonable estimate for the maximum possible value of the azide dissociation constant for the inhibitory site is 20 μM. The dissociation constant for the binding of azide to ferricytochrome *a* (0.25 mM) much larger than the 20 μM estimated for the inhibitory site. In addition, the spectum of the high-potential component in the presence of azide has an α maximum at 604 nm, not 601 nm as is observed for azide inhibition. The equilibrium data on azide binding thus cannot be readily related to the inhibition of cytochrome oxidase by azide.

A hypothetical model of cytochrome oxidase

Two concepts of cytochrome oxidase have existed for many years. The concept of two essentially independent cytochromes (*a* and a_3) is based on the original work of KEILIN AND HARTREE[1] and has been supported by many laboratories. The alternate concept of a single cytochrome *a* originated in the laboratory of Okunuki and has been

supported by other laboratories. Discussions of the two hypotheses are extensive and are summarized along with the general properties of cytochrome oxidase in the excellent review by LEMBERG[3] and in refs. 30 and 31. Neither of these hypotheses can explain the available data without modification. The behavior of the cytochromes *a* and a_3 is best fit by the original model of KEILIN AND HARTREE[32] who suggested that the cytochromes *a* and a_3 are intimately connected if not interconvertible and the "Siamese twins" hypothesis of KING[33].

In general, our concept of cytochrome oxidase calls for the two heme *a* molecules to be very close to each other and in very similar environments. In the fully oxidized state the sixth ligand positions of the iron atoms are shielded and there is no high affinity binding site for ligands such as azide. Reduction of one of the heme groups (the high potential cytochrome a_3) opens the structure in such a way that either the ferrocytochrome a_3 or the ferricytochrome *a* (but not both) can accept a ligand. If CO or O_2 are added, they react with ferrocytochrome a_3 but if azide is added, it reacts with ferricytochrome *a*. In either case the presence of a ligand on one of the two cytochromes changes the chemistry of, and prevents the addition of a ligand to, the other cytochrome.

ACKNOWLEDGEMENTS

This work was supported by grant No. NSF-GB-28125 from the U.S. National Science Foundation and by grant GM-12202 from the U.S. National Institutes of Health. The authors are indebted to Dr. John S. Leigh, Jr. for his many helpful suggestions both during the work and in the preparation of this manuscript.

REFERENCES

1 D. KEILIN AND E. F. HARTREE, *Proc. R. Soc. London, Ser. B*, 125 (1938) 171.
2 O. WARBURG, *Heavy Metal Prosthetic Groups and Enzyme Action*, Clarendon Press, Oxford, 1949, p. 144.
3 R. LEMBERG, *Physiol. Rev.*, 49 (1969) 48.
4 E. E. JACOBS, *Biochem. Biophys. Res. Commun.*, 3 (1960) 536.
5 D. D. TYLER, R. W. ESTABROOK AND D. R. SANADI, *Arch. Biochem. Biophys.*, 114 (1966) 236.
6 L. PACKER AND G. M. MUSTAFA, *Biochim. Biophys. Acta*, 113 (1966) 1.
7 D. F. WILSON AND P. L. DUTTON, *Arch. Biochem. Biophys.*, 136 (1970) 583.
8 T. YONETANI, *J. Biol. Chem.*, 235 (1960) 845.
9 S. HORIE AND M. MORRISON, *J. Biol. Chem.*, 238 (1963) 2859.
10 D. F. WILSON AND M. V. GILMOUR, *Biochim. Biophys. Acta*, 143 (1967) 52.
11 M. V. GILMOUR, D. F. WILSON AND R. LEMBERG, *Biochim. Biophys. Acta*, 143 (1967) 487.
12 T. E. KING AND F. C. YONG, in T. E. KING, H. S. MASON AND M. MORRISON, *Oxidases and Related Redox Systems II*, Wiley, New York, 1971, in the press.
13 B. F. VAN GELDER AND H. BEINERT, *Biochim. Biophys. Acta*, 189 (1969) 1.
14 D. F. WILSON, J. S. LEIGH, JR., J. G. LINDSAY AND P. L. DUTTON, in T. E. KING, H. S. MASON AND M. MORRISON, *Oxidases and Related Redox Systems II*, Wiley, New York, 1971, in the press.
15 B. CHANCE AND B. HAGIHARA, in A. N. M. SISSAKIAN, *Proc. 5th Int. Congr. Biochem., 1961, Moscow*, Vol. 5, Pergamon Press, New York, 1963, p. 3.
16 P. L. DUTTON, *Biochim. Biophys. Acta*, 226 (1971) 63.
17 D. F. WILSON AND P. L. DUTTON, *Biochem. Biophys. Res. Commun.*, 39 (1970)59.
18 W. M. CLARK *Oxidation–Reduction Potentials of Organic Systems*, Williams and Wilkins, Baltimore, Md., 1960.
19 T. TSUDZUKI AND D. F. WILSON, *Arch. Biochem. Biophys.*, 145 (1971) 149.
20 A. TZAGOLOFF AND D. C. WHARTON, *J. Biol. Chem.*, 240 (1965) 2628.
21 O. WARBURG AND E. NEGELEIN, *Biochem. Z.*, 233 (1931) 486.

22 O. WARBURG AND E. NEGELEIN, *Biochem. Z.*, 238 (1931) 135.
23 S. HORIE AND M. MORRISON, *J. Biol. Chem.*, 238 (1963) 1855.
24 B. CHANCE, M. ERECINSKA AND E. M. CHANCE, in T. E. KING, H. S. MASON AND M. MORRISON *Oxidases and Related Redox Systems II*, Wiley, New York, 1971, in the press.
25 B. F. VAN GELDER, *Biochim. Biophys. Acta*, 131 (1966) 36.
26 Y. P. MEYER AND T. E. KING, *Biochem. Biophys. Res. Commun.*, 34 (1969) 170.
27 D. F. WILSON AND J. S. LEIGH, JR., *Arch. Biochem. Biophys.*, in the press.
28 D. F. WILSON, *Biochemistry*, 8 (1969) 2475
29 D. F. WILSON AND B. CHANCE, *Biochim. Biophys. Acta*, 131 (1967) 421.
30 T. E. KING, H. S. MASON AND M. MORRISON, *Oxidases and Related Redox Systems*, Vol. 2 Wiley, New York, 1965.
31 T. E. KING, H. S. MASON AND M. MORRISON, *Oxidases and Related Redox Systems II*, Wiley New York 1971, in the press.
32 D. KEILIN AND E. F. HARTREE, *Proc. R. Soc. London, Ser. B*, 127 (1939) 167.
33 T. E. KING, M. KUBOYAMA AND S. TAKEMORI, in T. E. KING, H. S. MASON AND M. MORRISON *Oxidases and Related Redox Systems*, Vol. 2, Wiley, New York, 1965, p. 707.