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# THE EFFECT OF MITOCHONDRIAL ENERGIZATION ON CYTOCHROME *c* OXIDASE KINETICS AS MEASURED AT LOW TEMPERATURES

## I. THE REACTION WITH CARBON MONOXIDE

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## Summary

The kinetics of CO binding by the cytochrome *c* oxidase of pigeon heart mitochondria were studied as a function of membrane energization at temperatures from 180 to 280°K in an ethylene glycol/water medium. Samples energized by ATP showed acceleration of CO binding compared to those untreated or uncoupled by carbonylcyanide *p*-trifluoromethoxyphenylhydrazone but only at relatively low temperatures and high CO concentrations. Experiments using samples in a "mixed valency" (partially oxidized) state showed that the acceleration of ligand binding is not due to the formation of a partially oxidized state via reverse electron transport.

It is concluded that in the deenergized state one CO molecule can be closely associated with the cytochrome $a_3$ heme site without actually being bound to the heme iron; in the energized state, two or more ligand molecules can occupy this intermediate position.

The change in the apparent ligand capacity of a region near the heme iron in response to energization is evidence for an interaction between cytochrome oxidase and the ATPase system. Furthermore, these results suggest a control mechanism for $O_2$ binding.

## Introduction

Cytochrome *c* oxidase is a complex enzyme that functions via a complicated mechanism. The enzyme minimally requires four components to function: two heme groups and two copper atoms. In the role of terminal oxidase it catalyzes

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Abbreviation: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

the reduction of dioxygen to water. This reaction is coupled to the synthesis of ATP from ADP + $P_i$ at site III in the intact mitochondrial membrane. Thus, a complicated enzymatic process interacts with and is controlled by another equally complex and relatively unknown enzymatic process. This interaction between electron transport and energy transfer has been the object of intensive investigation for over a quarter century.

Intermediate complexes of cytochrome *c* oxidase with oxygen have been observed and described by Chance and colleagues [1,2] using low temperature spectroscopic techniques, and are believed to be involved in the physiological reduction of $O_2$. Because of the interaction of electron transport and oxidative phosphorylation mechanisms, it is of great interest to investigate the effects of an induced "energized" state (state 4 [10]) of the mitochondria on the formation of intermediate oxygen compounds. This energized state is the resting state of the mitochondria with high levels of ATP present. Energized states of the mitochondrial cytochrome oxidase have been observed in the presence and absence of a ligand on cytochrome oxidase [3—9] and have been detected as small but significant shifts in the absorption spectrum of the oxidase. The interaction of ATP and ADP + $P_i$ with the electron transport chain has previously been documented as has the concept of "cross over points" indicative of the position of the energy transfer interaction with the respiratory carriers [10].

Preliminary reports of the effect of membrane energization on the formation of oxygen intermediate compounds have indicated that in the "energized" state (state 4), the formation of the compounds is accelerated, resulting in an increased rate of oxidation of the enzyme [11,12]. The acceleration may be due to an increased affinity of the enzyme for the substrate (oxygen) or to an increased rate of reduction of oxygen by the oxidase, or both. Oxidation-reduction titrations of the oxidase in the presence and absence of ATP indicate shifts in the midpoint potentials of the two heme components to less positive values in the presence of ATP [13,14], corresponding to a decreased affinity of the enzyme (or enzyme · ligand complex) for electrons from cytochrome *c* and, correspondingly, indicative of an increased rate of electron transfer from cytochrome $a_3$ to $O_2$. The potentiometric evidence, however, reflects the results of a change in the enzyme by energization and does not shed light on the causative mechanism, nor does it provide information on the affinity of the enzyme for ligand.

The low temperature kinetic approach to ligand binding to heme proteins such as myoglobin [15] and soluble oxidase [16—19] offers the opportunity to explore the nature of the binding site of CO or $O_2$ and the region surrounding the site in terms of the number of ligand molecules involved at each point in the binding "pathway" and the thermodynamic parameters that control movement of the ligand to and from the binding site. Employment of these techniques to determine the binding characteristics of CO and $O_2$ to the enzyme under energized/deenergized conditions indicates that differences in ligand binding do indeed exist.

## Materials and Methods

Pigeon heart mitochondria were isolated by the method of Chance and Hagihara [20].

Medium containing succinate, glutamate, mannitol, and Tris/Cl (pH 7.4) buffer and ethylene glycol was bubbled continuously with CO or with a mixture of CO in $N_2$ for 10 min. An equal volume of pigeon heart mitochondria was then added to the medium such that a final concentration of 10 mM succinate, 10 mM glutamate, 30 mM Tris/Cl, 75 mM mannitol, and 40% (by vol.) ethylene glycol was reached. This mixture was then loaded into quartz EPR tubes (0.6 ml/tube) and allowed to incubate 5 min in the dark at room temperature before being frozen at −78° C until use.

At the time of use, an aliquot of mitochondria was warmed to 0° C in an ice bath. The mitochondrial membrane was energized by the addition of ATP to a final concentration of 8 mM and 10 μl of 100% ethanol followed by mixing with a tight fitting stainless steel spiral with minimal introduction of air. Deenergized samples were prepared by the addition of ATP and the uncoupler carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) to final concentrations of 8 mM and 4 μM, respectively. The sample was then warmed to 17° C during a 45 s incubation and then either frozen in EPR tubes for use in the multichannel spectrophotometer or transferred to a special cuvette for single-beam measurement. The transfer was done using a syringe that had been previously flushed with the desired $CO/N_2$ mixture.

The recombination of CO with the oxidase was measured spectrophotometrically following photodissociation of the oxidase · CO complex with a rhodamine liquid dye laser pulse (585 nm) or with an electronic strobe light as described previously [16]. The rebinding of CO was detected by measuring the light absorption at 448 nm as described previously [16,23] or by measuring the change in intensity of the 594—653 nm signal of a multi-channel spectrophotometer as described by Chance et al. [1,2,21].

Partially oxidized cytochrome oxidase was prepared by redox poising at +312 mV with dithionite and ferricyanide in the absence of $O_2$ and the presence of 100% or 1% CO atmosphere. The mixed valency samples [22] were then frozen at −78° C until assayed.

## Results

Experimental curves for the recombination of CO with reduced cytochrome oxidase following photodissociation are shown in Fig. 1. The fraction of the heme sites that are dissociated from CO is plotted against time after the flash. Logarithmic scales are used to facilitate showing on one plot data taken over a large range of time. The time dependence of most of these curves is very nearly exponential; i.e., to a good approximation,

$$f(t) = e^{-kt} \tag{1}$$

where $f(t)$ is the fraction of the originally photodissociated cytochrome $a_3$ not recombined with CO at time $t$ after the flash. For some data, particularly those below about 200 K, Eqn. 1 is not obeyed very well; for these the expression

$$f(t) = (1 + t/t_0)^{-n}, \quad k = n/t_0 \tag{2}$$

is used [15,16,19]. This is a more general expression; in either case, $k$ is proportional to the rate of recombination of reduced cytochrome $a_3$ sites with CO

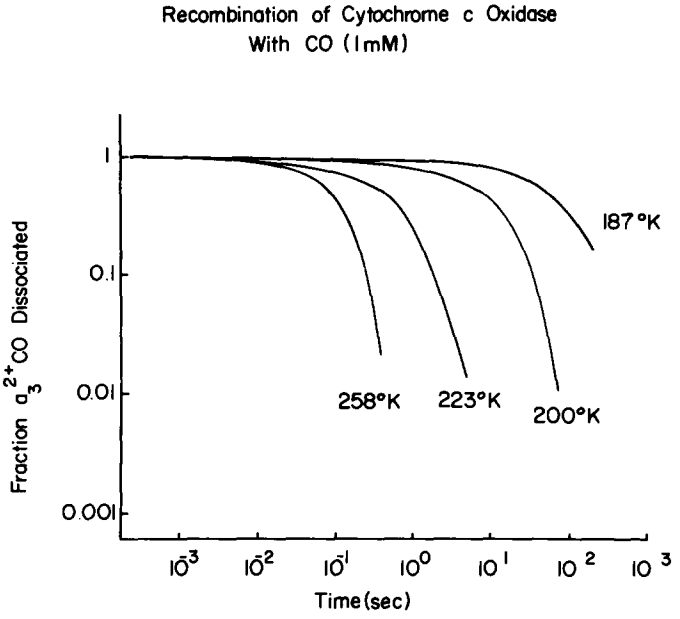


Fig. 1. Plot of photodissociated (fully reduced) cytochrome oxidase · CO complex vs. time. The curves shown are experimental curves obtained at the temperature indicated. These normalized data were obtained by measuring the light absorption at 448 nm in the absence of oxygen using the computer-interfaced apparatus described by Sharrock [23]. 4 μM FCCP and 1 atm. CO were present in all samples.

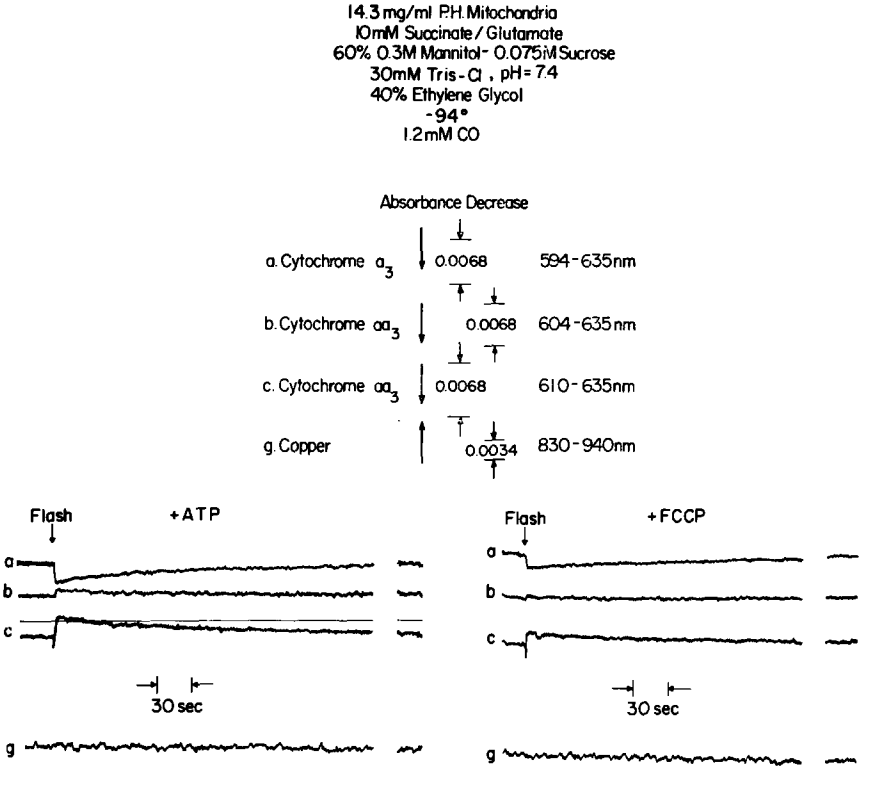


Fig. 2. Recombination of CO with cytochrome oxidase in energized and deenergized pigeon heart mitochondria (P.H. Mitochondria) at −94° C. The half-time in the presence of ATP is 15 s while the half-time in the presence of FCCP is 80 s. These data were measured with the multichannel spectrophotometer described by Chance et al. [1,2].

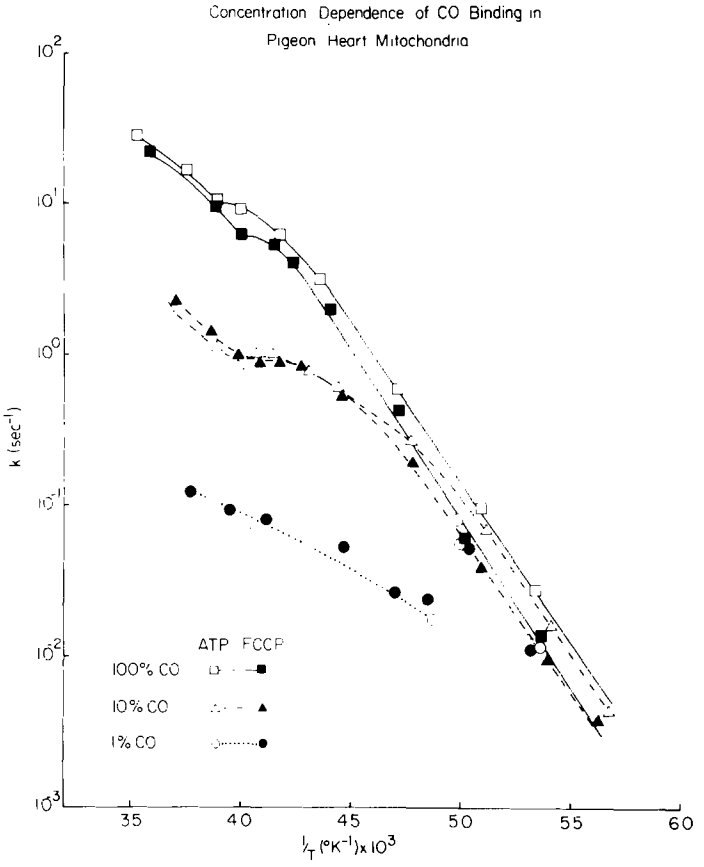


Fig. 3. Plot of log $k$ of CO recombination vs. inverse temperature in ATP-energized and FCCP-uncoupled samples as a function of CO concentration. The samples were prepared as described in Materials and Methods, saturating the buffer/ethylene glycol mixture with 100, 10, or 1% CO gas mixtures. The $k$ values were obtained by measuring the light absorption at 448 nm and least-square fitting the experimental curves as explained in the text. The activation energy for the linear portion of the curves is approximately 9.5 kcal/mol. The kinetics for 1% CO consist of two phases of which the faster predominates below 200 K. Above this temperature, the slower phase increases in magnitude, comprising about 50% of the total response at 205 K and about 90% at 240 K.

immediately after the dissociating flash.

The recombination of the photolyzed oxidase with CO at approximately −94° C is shown in Fig. 2. The half-time of the reaction may be calculated from either the increase in 594—635 nm signal intensity (trace a) or the decrease in intensity of the 612—635 nm signal (trace c) following photolysis. The apparent half-time in the energized (ATP supplemented) sample is 15 s and 80 s in the uncoupled (with FCCP) preparation. These data indicate substantially increased kinetic rates as a result of membrane energization. In the absence of any addition, untreated samples give the same results as obtained with FCCP-treated samples, indicating that the difference in rates is not due to an FCCP-induced alteration of the enzyme or its environment. Since ATP is present in the FCCP-uncoupled samples, the increased rates are due to energization and not due solely to the presence of ATP or the presence of 8 mM salt.

The data shown in Fig. 3 show the value of $k$ as a function of temperature in energized and deenergized samples at three CO concentrations. It is seen that the 100% (1 atm) CO data in the presence of ATP and FCCP are consistent with that shown in Fig. 2, although the difference between the two states is less pronounced in Fig. 3. At colder temperatures the acceleration in rate of CO recombination upon ATP addition is observed in both 100% (1 atm) and 10% (0.1 atm) CO samples. The ATP-induced acceleration is not observed in the 1% (0.01 atm) CO samples, however.

Under the conditons in which the experiments are performed, all components of the electron transport system are reduced during the 5 min room temperature incubation prior to freezing and storage. Upon anaerobiosis, CO is bound to the fully reduced oxidase. The hydrolysis of exogenous ATP results in the oxidation of cytochrome $c$ is shown in Fig. 4. This oxidation is not observed if FCCP is added prior to ATP addition. Cytochrome $c$ ($E_m$ = 275 mV) is essentially equipotential with cytochrome $a$ ($E_m$ = 240 mV) and the detectable copper moiety ($E_m$ = 245 mV) [14]. One thus expects ATP hydrolysis to result in the net oxidation of cytochromes $a$ and $c$ and the "visible" copper protein. The oxidation of cytochrome $a$, as well as cytochrome $c$, by ATP hydrolysis has been reported [24]. Thus, the state of the oxidase following energization by ATP hydrolysis (with only 45 s incubation time insufficient for hydrolysis of all added ATP and reversal of the cytochrome oxidation [24], as seen from the time scale of Fig. 4) is essentially that of the "mixed valency" (partially oxidized or partially reduced) $a^{3+}Cu^{2+}Cu^{+}a_3^{2+}CO$ oxidase obtained by the oxidation of CO-bound cytochrome oxidase with ferricyanide and not that of the fully reduced enzyme found in the presence of uncoupler or following a 10 min incubation in the presence of ATP at room temperature ( in the absence of FCCP). It now becomes imperative to investigate the kinetics of the partially oxidized "mixed valency" oxidase.

Mixed valency oxidase can be obtained in mitochondria either by oxidizing fully reduced CO-bound oxidase by ferricyanide addition or by poising the mitochondria anaerobically at +312 mV with redox mediators prior to CO

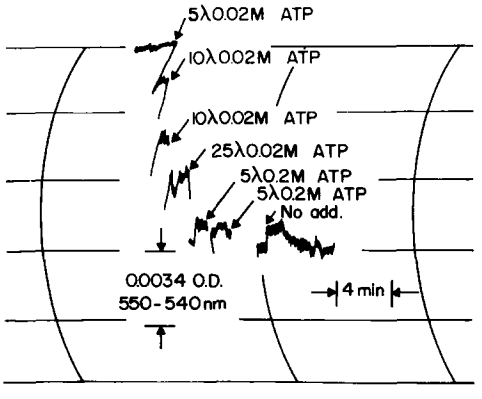


Fig. 4. Oxidation of cytochrome $c$ in response to ATP addition to pigeon heart mitochondria. The samples were prepared as described in Materials Methods in the presence of 40% ethylene glycol. The EPR tube containing the sample was flushed with $N_2$ gas during the ATP additions to prevent the introduction of oxygen.

binding. Care must be exercised during the formation of the mixed valency CO compound that oxygen is not added, since upon photolysis, compound C [1,2], the mixed valency "peroxy" compound is formed, exhibiting absorbance changes in the 594 nm region that are superficially similar but substantially faster than the 594 nm changes seen in CO recombination or oxygen binding. CO recombination rate is not altered by the creation of the mixed valency state.

## Discussion

The object of these studies is to examine the interaction between the electron transport and oxygen reduction systems and the mechanism of oxidative phosphorylation at site III. To do so we must consider the terminal portion of the mitochondrial respiratory chain as a single unit, since under "coupled" conditions one system affects the other. To this end it is useful to examine the energy-linked effects that one can observe in the oxidase. The most prominent effect is that of induction of the oxidation of cytochromes *a* and *c* by ATP hydrolysis. This effect has been catalogued previously using $S^{2-}$ as the terminal oxidase inhibitor and has been shown to be sensitive to uncouplers and inhibitors of oxidative phosphorylation [24]. ATP-induced shifts in midpoint potentials of cytochrome *a* and $a_3$ have been described by Dutton and Wilson [13,14], resulting in the former being oxidized by cytochrome *c*. Since the detectable copper moiety is nearly isopotential with cytochrome *a*, one would expect that it would also be oxidized upon ATP hydrolysis. Data in Fig. 4 indicate that the addition of ATP results in the oxidation of cytochrome *c* as well. It is significant that these ATP-induced oxidations are not observed in the presence of uncouplers such as FCCP.

It is apparent then that the state of the oxidase in energized mitochondria ($a^{3+}Cu^{2+}Cu^{+}a_3^{2+}CO$) is not identical to that in the non-energized or non-treated samples ($a^{2+}Cu^{+}Cu^{+}a_3^{2+}CO$). But since CO binding occurs via cytochrome $a_3$, it is not readily apparent that differences in the binding of ligands should exist in these species since the $a_3$ heme and its associated (invisible) copper are reduced in both states. Experimental observations indicate that the partially oxidized oxidase binds CO at the same rate as the fully reduced enzyme.

*Nature of the ligand binding site* *

The curves shown in Fig. 3 are complex and contain a large amount of information. Because the binding of CO is not a readily reversible reaction at low temperatures, treatment of the data with steady-state kinetic concepts is not valid. We will, however, interpret them in terms of the model shown in Fig. 5, which is similar to that of Sharrock and Yonetani [16–19] for soluble cytochrome oxidase. Considering first the data for FCCP-treated samples, at temperatures below 200 K the rates of recombination obey an Arrhenius rela-

* While the data presented in this paper exclusively involve CO, the results are expanded to include $O_2$ binding by analogy. Evidence in support of these findings for $O_2$ is provided in the companion communication [27].

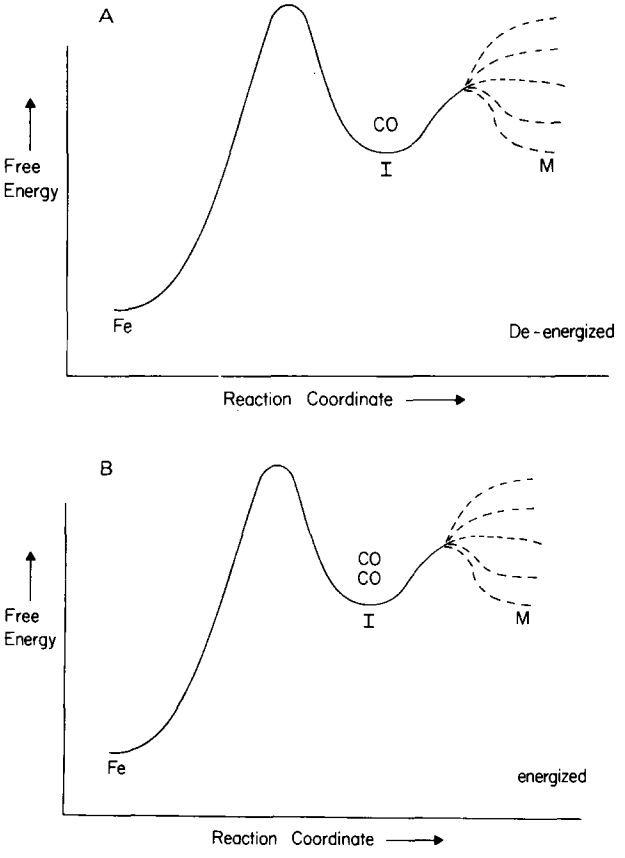


**Fig. 5. Model of cytochrome $a_3$ heme site showing the rate processes involved in ligand binding. A shows that one ligand (CO) molecule can occupy region I in the deenergized or untreated mitochondria while B illustrates that more than one CO molecule can occupy this region in the energized state. The size of the free energy barriers between region I and the iron (Fe) site are the same in the two states. Region I is an intermediate pocket near the heme iron. M denotes the medium; changes that corresponds to melting, as the temperature is increased, are shown by the broken lines. The height of the M barrier increases with decreasing temperature.**

tionship with inverse temperature and are independent of CO concentration. The lack of CO dependence indicates that only one CO molecule per heme site is involved in the binding process. In terms of the model (Fig. 5), the low temperature binding is the movement of CO from the region labelled "I" to the heme iron. I is assumed to have a maximum occupancy of only one CO molecule and, at low temperature, to be continuously occupied after photodissociation until rebinding occurs. As the temperature is raised above 200 K, the outer region "M" begins to be accessible to the photodissociated ligand molecule. Below 200 K, the amount of NMR-detectable protons is extremely small, indicating that region M may correspond to the bulk medium of the sample. The probability of I being occupied after photodissociation depends on the equilibrium of ligand molecules between I and M. Thus, in Fig. 3, between 250 K and 200 K, the rates for 1.0 atm (100%) CO essentially lie on a straight line with the low temperature points (I being occupied continuously) while with

0.1 atm (10%) CO the rates are decreased (I is empty much of the time after dissociation). For 0.01 atm (1%) CO, I is basically empty of ligand molecules and binding takes place by migration from the surrounding solvent. At temperatures below approximately 200 K region M is frozen and represents a significant barrier to CO moving I → M or M → I.

The explanation of the ATP effect upon ligand binding lies in the energization process increasing the capacity of the region I for CO without appreciably affecting the I-M equilibrium or the binding rate of a single CO molecule to the heme iron from region I. Thus, ATP affects the rate only if the CO concentration is high enough and the temperature low enough to produce multiple occupancy of I. All data in Fig. 3 are explained by these assumptions. Other models involving more barriers are possible, but are functionally identical to that of Fig. 5.

The addition of ATP changes the concentration dependence of the CO binding to cytochrome oxidase at low temperatures. As shown in Fig. 3, energization has little or no effect on the rates in the presence of 0.01 atm (1% CO), while for higher CO concentrations the rates are enhanced compared to values found for the FCCP-uncoupled state. It should be stressed that the low temperature kinetics are not truly second-order, since the rates change only slightly over a 100-fold range of CO concentration.

The molecular basis for the changes of ligand-binding rates upon energization is not readily apparent. Conformation changes in the molecule may be responsible for these effects. These changes may be the same as those suggested by the spectral shifts observed in the ferric or fully ferrous oxidase as already explained [3—9] and those involved in the decreased affinity of the oxidase for $CN^-$ in the presence of ATP [25].

The fact that the deenergized samples show kinetics at very low temperatures that are independent of ligand concentration, while those of energized samples are concentration dependent may indicate that the innermost regions of the $a_3$ heme site may be capable of containing more than one CO or $O_2$ molecule in the energized enzyme. As explained previously for cytochrome oxidase [16, 19] and myoglobin [15], the rate of CO (or $O_2$) movement from one region to another is a function of the concentration of ligand in the first region and the height of the free energy barrier that must be overcome. While it is inconceivable that addition of ATP alters the energy barrier of the frozen solvent phase (totally frozen at roughly −80° C) (Leigh, Jr., J.S., personal communication), one way of inducing a concentration dependence, it is possible that in the energized state an inner region(s) could hold more molecules than in the presence of FCCP. Since the energization/deenergization procedure occurs at room temperature (cf. Materials and Methods), the occupancy of the inner regions will be a function of the bulk concentration in the solvent. The equilibration of these regions with the solvent occurs while the solvent is liquid and cannot occur in the frozen state. CO binding involves multiple ligand molecules that entered from the liquid phase to the inner region that is now capable of containing an increased number of molecules. These molecules are trapped in regions that are unaffected by the energy barrier of the frozen solvent at low temperatures.

The data shown in Fig. 3 indicate that the energy barriers and the kinetic constants describing them are unaffected by energization. Changes either in the

ligand concentration in region I or in the barrier kinetic constants can result in increased CO binding rates. The uncoupled samples at all CO concentrations show the same rate of recombination as does the energized sample in 1% CO (at a given temperature below 200 K). With 1% CO, both the ATP- and FCCP-treated samples contain approximately one CO molecule per oxidase (bound to the heme). Since under these conditions the observed rates of recombination are the same in the presence or absence of ATP, the barrier constants are unaffected. Although the stoichiometry of bound CO to enzyme is unaffected by energization [26], the amount of CO associated with (but not bound by) the oxidase is greater in ATP-treated samples (provided the CO concentration is sufficiently high). A similar conclusion is obtained for $O_2$ binding [27].

The increased rate of ligand binding due to the increased occupancy of an intermediate region near the heme is an effect of energization of the mitochondrial (oxidase) and not a result of the induction of a partially oxidized state of the oxidase by reverse electron transport. The exact mechanism of the interaction of the ATP hydrolyzing-synthesizing system and cytochrome oxidase is currently unknown, as is the molecular basis for the apparent occupancy increase. These results do, however, indicate substantial interaction between the two energy systems and demonstrate a possible means of controlling the rate of electron transfer through the oxidase by controlling the oxygen affinity of the oxidase.

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## References

1 Chance, B., Saronio, C. and Leigh, Jr., J.S. (1975) J. Biol. Chem. 250, 9226—9237
2 Chance, B., Saronio, C. and Leigh, Jr., J.S. (1975) Proc. Natl. Acad. Sci. U.S. 72, 1635—1640
3 Lindsay, J.G. and Wilson, D.F. (1972) Biochemistry 11, 4613—4621
4 Erecinska, M., Wilson, D.F., Sato, N. and Nicholls, P. (1972) Arch. Biochem. Biophys. 151, 188—193
5 Wilson, D.F. Erecinska, M. and Nicholls, P. (1972) FEBS Lett. 20, 61—65
6 Wikström, M.K.F. (1975) in Electron Transfer Chains and Oxidative Phosphorylation (Quagliariello, E., Papa, S., Palmieri, F., Slater, E.C. and Siliprandi, N., eds.), pp. 97—103, North-Holland, Amsterdam
7 Wikström, M.K.F. (1972) Biochim. Biophys. Acta 283, 385—390
8 Wikström, M.K.F. and Saris, N.-E.L. (1970) in Electron Transport and Energy Conservation (Tager, J.M., Papa, S., Quagliariello, E. and Slater, E.C., eds.), pp. 77—88, Adriatica Editrice, Rome
9 Wikström, M.K.F. and Saari, H.T. (1975) Mol. Cell. Biochem., 11, 17—34
10 Chance, B. and Williams, G.R. (1955) J. Biol. Chem. 217, 409—427
11 Harmon, H.J. Chance, B. and Wikström, M.K.F. (1976) Biophys. J. 16, 135a
12 Chance, B., Harmon, J. and Wikström, M. (1976) in Electron Transfer Chains and Oxidative Phosphorylation (Quagliariello, E., Papa, S., Palmieri, F., Slater, E.C. and Siliprandi, N., eds.), pp. 81—95, North-Holland, Amsterdam
13 Wilson, D.F. and Dutton, P.L. (1970) Arch. Biochem. Biophys. 136, 583—624
14 Dutton, P.L. and Wilson, D.F. (1974) Biochim. Biophys. Acta 346, 165—212
15 Austin, R.H., Beeson, K.W., Eisenstein, L., Frauenfelder, H. and Gunsalus, I.C. (1975) Biochemistry 14, 5355—5372

16 Sharrock, M. and Yonetani, T. (1976) Biochim. Biophys. Acta 434, 333—344
17 Sharrock, M. and Yonetani, T. (1976) Biophys. J. 16, 86a
18 Sharrock, M. and Yonetani, T. (1976) Biophys. J. 17, 241a
19 Sharrock, M. and Yonetani, T. (1977) Biochim. Biophys. Acta, 462, 718—730
20 Chance, B. and Hagihara, B. (1963) Proc. V Int. Congr. Biochem. V, 3—33
21 Chance, B., Graham, N. and Legallais, V. (1975) Anal. Biochem. 67, 552—579
22 Greenwood, C., Wilson, M.T. and Brunori, M. (1974) Biochem. J. 137, 205—215
23 Sharrock, M. (1977) Rev. Sci. Instrum. 48, 1202—1206
24 Chance, B. (1961) J. Biol. Chem. 236, 1544—1554
25 Wilson, D.F. and Fairs, K. (1974) Arch. Biochem. Biophys. 163, 491—497
26 Wohlrab, H. and Ogunmola, G.B. (1971) Biochemistry 10, 1103—1106
27 Harmon, H.J. and Wikström, M.K.F. (1978) Biochim. Biophys. Acta, 503, 67—77