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Carbon Monoxide Binding Studies of Cytochrome a_3 Hemes in Intact Rat Liver Mitochondria*

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ABSTRACT: Cytochrome a_3 of intact rat liver mitochondria has been titrated anaerobically with carbon monoxide. A new technique has been used which allows titrations to be completed in less than 10 min. The equilibrium constant (K) between cytochrome a_3 and carbon monoxide was found to be $(2.15 \pm 0.3) \times 10^6$ M^{-1} with a Hill coefficient (n) of 1.1 ± 0.1. Both of these values are not affected by a variation in pH from 6.1 to 7.8 and by the presence of uncoupling concentrations of uncouplers. The results demonstrate that there is no functional interaction between reduced a_3 proteins. The lack of effect of the very tightly bound uncoupler molecules on the K suggests that the binding site of the uncoupler is not on the a_3 protein. When adenosine triphosphate is added to anaerobic coupled mitochondria in the presence of N,N,N',N'-tetramethyl-p-phenylenediamine, ascorbate, and succinate, the K changes to $(1.1 \pm 0.1) \times 10^6$ M^{-1} while the n value remains unchanged.

The fact that carbon monoxide is an uncharged molecule and does not respond to potential and proton gradients set up by the adenosine triphosphate and that the K between CO and a_3 is independent of pH, demonstrates an energy-linked structural change in the a_3 which is not due to a pH change resulting from a proton gradient set up by ATP across the inner mitochondrial membrane and which results in a reduced affinity for carbon monoxide. We postulate the reduction of O_2 by a_3 to consist of two basic processes: (1) a pH-independent binding of O_2 to a_3 and (2) a pH-dependent oxidation of a_3 by O_2. We estimate a lower limit for the favorable free-energy change associated with the first process to be -560 mV.

Cytochrome a_3 is of primary importance to the process of energy-linked mitochondrial electron transport. Cytochrome a_3 is not only generally accepted to be the terminal cytochrome, *i.e.*, the cytochrome directly involved in the initial binding of oxygen before its reduction, but it has also been implicated to be the cytochrome directly involved in site III phosphorylation (Ramirez and Mujica, 1961; Muraoka and Slater, 1969; Wilson and Dutton, 1970a).

Carbon monoxide behaves in many respects like molecular oxygen except for its lack of participation in cytochrome redox reactions. We have utilized the analogous behavior of CO and O_2 to probe the environment of the a_3 heme with carbon monoxide. Our experimental design extends the equilibrium binding studies of Wald and Allen (1957) to intact mitochondria and evaluates the relation between the oligomeric state of purified cytochrome oxidase (Okunuki *et al.*, 1968) and the functional interaction of reduced a_3's in the mitochondrial membrane.

We also probed the a_3 heme environment as a function of mitochondrial metabolic state. We present in this communication the first direct evidence of an energy-linked structural change in the a_3 heme environment which is not due to an ATP-induced proton gradient.

Materials and Methods

Rat livers were carefully removed from decapitated animals and immediately perfused with MSE.[1] The mitochondria were prepared essentially according to the method of Schneider

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[1] Abbreviations used are: MSE, 0.22 M mannitol–0.07 M sucrose–200 μM Na_2EDTA (pH 7.2); Tes, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Mes, 2-(N-morpholino)ethanesulfonic acid; S-13, 5-Cl-3-*tert*-butyl-2′-Cl-4′-NO_2-salicylanilide; LEM, anaerobic, coupled mitochondria in the presence of reducing substrates and in the absence of ATP; HEM, anaerobic, coupled mitochondria in the presence of reducing substrates and ATP.

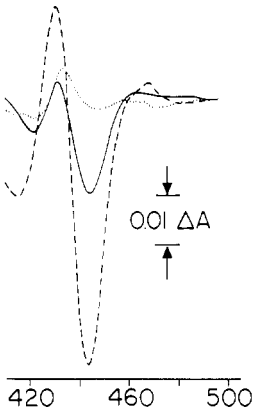

FIGURE 1: Difference absorption spectra of rat liver mitochondria. Rat liver mitochondria were suspended in MSE + 20 mM Tes at pH 7.2 in the closed cuvets. Both cuvets contained 10 mM sodium succinate and 20 μM NADH. The mitochondria were suspended at a concentration of 0.18 μM cytochrome oxidase. The following successive additions were made to the anaerobic cuvets to obtain the difference spectra: (———) 10 mM ATP to sample cuvet, (---) 20 μM CO to sample cuvet, (· · · · · ·) 20 μM CO to reference cuvet.

(1948). The mitochondrial pellet of the third wash in MSE is covered by a fluffy and pale-colored layer of microsomes that can easily be removed by creating a gentle vortex in the supernatant fluid which lifts this layer off the more tightly packed mitochondrial pellet. The mitochondrial pellet is washed once more with MSE and then once with 0.15 M KCl to remove any remaining hemoglobin (Jacobs *et al.*, 1965). Difference spectra of reduced mitochondria with and without carbon monoxide yielded an absorption peak at 429–430 nm (Figure 1) indicating the absence of hemoglobin.

The concentration of mitochondria was determined on the basis of their cytochrome *c* oxidase content (Wohlrab, 1969). Carbon monoxide at a concentration of 0.333 μM was prepared by mixing two volumes of helium-saturated distilled water with one volume of CO-saturated water in the closed system of two syringes. The titration was carried out in the following way. A 1-cm optical cuvet was filled with buffer solution and then covered with a needle-puncture stopper through which a No. 26 hypodermic needle was inserted to ensure that the system was not completely closed. More buffer was injected into the cuvet to displace the remaining gas phase. Two glass beads were put inside the cuvet to make mixing of the contents of the cuvet in the absence of a gas phase possible. Substrates were injected into the cuvet, and, finally, mitochondria were injected near the bottom of the cuvet to prevent mitochondria from becoming part of the overflow during injection. The contents of the cuvet were then carefully mixed, and the first optical density reading for the titration was determined.

After having reached anaerobiosis, microliter quantities of the CO solution were injected, and optical density changes were monitored at 430–415 nm. To correct for dilution effects, a parallel titration was carried out with distilled water instead of CO solution. These optical density changes were subtracted from the corresponding CO optical density changes.

Optical density measurements were carried out in a Hitachi-Perkin Elmer Model 356 dual wavelength spectrophotometer. S-13 was generously supplied by the agricultural division of Monsanto Chemical Co., St. Louis, Mo. Tes and Mes were obtained from Sigma Chemical Co., St. Louis, Mo. NADH was obtained from Boehringer Mannheim Corp., New York, N. Y.

TABLE I: Titration of Cytochrome a_3 of Intact Mitochondria with Carbon Monoxide.

Experiment			
Type[a]	Number of Repeats	K[b]	n[b]
I	3	2.05 ± 0.4	1.2 ± 0.1
II	3	2.4 ± 0.2	1.2 ± 0.1
III	1	2.0	1.1
IV	1	2.0	1.0
V	1	1.8	1.05
VI	3	1.1 ± 0.1	1.1 ± 0.1
VII	1	2.2	0.95

[a] The experiments were of the following types: I, rat liver mitochondria were suspended in MSE plus 20 mM Tes at pH 7.4, at a concentration of 0.75 μM cytochrome oxidase and in the presence of 10 mM sodium succinate, 50 μM NADH; II, conditions were as in I, however, 3 μM S-13 was present; III, conditions as in II, however, the buffered medium was MSE plus 20 mM Mes, final pH 6.1; IV, conditions as in II, however, the buffered medium was MSE plus 20 mM Mes, final pH 6.5; V, conditions as in II, however, the buffered medium was MSE plus 20 mM Tes, final pH 7.8; VI, conditions as described in Figure 2, no S-13 present; VII, conditions as in VI, 1.5 μM S-13 is present. [b] The numbers in these two columns are the equilibrium constant (K) between a_3 and CO and the Hill coefficient (n). The values are the arithmetic means with the statistical standard errors.

The numerical values are given as arithmetic mean of the experimental values with their corresponding statistical standard errors.

Results

Titration of Coupled and Uncoupled Mitochondria with Carbon Monoxide. Coupled mitochondria that have reached anaerobiosis in the presence of succinate are in a low-energy state since these mitochondria have spectra identical with those of the mitochondria that reach anaerobiosis in the presence of succinate and uncouplers (H. Wohlrab, submitted for publication). The mitochondria suspended in MSE plus 20 μM NADH were added to the suspension. The NADH was added to keep cytochrome b_5 reduced during the titration. The anaerobic mitochondria were then titrated with carbon monoxide, and the resulting dilution-corrected optical density changes were converted into CO–a_3 concentrations. We let y be the per cent of a_3 that is complexed with CO. The free CO concentration was calculated from the difference between the total CO injected into the cuvet and the CO–a_3 concentration. The slope of the line in the plot of $\log y/(100-y)$ *vs.* $\log(CO_{free})$ yields the Hill coefficient (n) (Hill, 1910). $\text{Log}^{-1}(CO_{free})$ when $\log y/(100-y)$ is zero, yields the equilibrium constant (K) between a_3 and carbon monoxide.

We repeated this titration after having added 4 moles of S-13 per mole of cytochrome *c* oxidase. The results are shown in Table I. We find a K of $(2.05 \pm 0.4) \times 10^6$ M^{-1} in the absence of S-13 and $(2.4 \pm 0.2) \times 10^6$ M^{-1} in the presence of S-13. The Hill coefficient in both cases is 1.2 ± 0.1.

Titration of a_3 in Uncoupled Mitochondria with Carbon

Monoxide at Different pH Values. Mitochondrial suspending medium was prepared at different pH values. The pH as listed in Table I is the final pH of the suspension at the end of the carbon monoxide titration. All media were MSE plus the following buffers: 20 mM Tes (pH 7.8) and 20 mM Mes (pH 6.1 and 6.5). The pH values were adjusted with KOH. We found no change in K or n as the pH of the suspending medium was varied from 6.1 to 7.8.

Effect of Exogenous ATP on the Equilibrium Constant Between Carbon Monoxide and a_3 of Coupled and Uncoupled Mitochondria. When ATP is added to coupled mitochondria in the presence of succinate and NADH, spectral changes as shown in Figure 1 occur with respect to the anaerobic, succinate- and NADH-reduced, coupled mitochondria. The addition of saturating amounts of carbon monoxide to the sample cuvet yields the second spectrum with a much larger trough at 444 nm and a peak at about 430 nm (CO–a_3 complex). The addition of saturating amounts of CO to the reference cuvet eliminates completely the trough at 444 nm and reduces the magnitude of the 430-nm peak and shifts it slightly to the red. We attribute the remaining 430-nm peak to a form of high-energy cytochrome b since this same peak alone can be obtained by the addition of micromolar quantities of ATP to an anerobic mitochondrial suspension in the absence of CO and can be removed in the presence or absence of CO by nanomolar quantities of uncoupler (H. Wohlrab, submitted for publication). These spectra show that (a_3 + CO) has a spectrum that is identical with the spectrum of (a_3 + ATP + CO).

We titrated the coupled mitochondria with CO in the presence of ATP. Glutamate, malate, and NADH were present. We also added TMPD and potassium ascorbate to eliminate ATP-induced reverse electron transfer at site III. The results are plotted on the Hill plot in Figure 2. The equilibrium constant K decreased to (1.1 ± 0.1) × 10^6 M^{-1} while the n value remained the same.

When we added S-13 in addition to the ATP and the reducing substrates, the K increased to that of uncoupled mitochondria and again, the n value did not change (Table I).

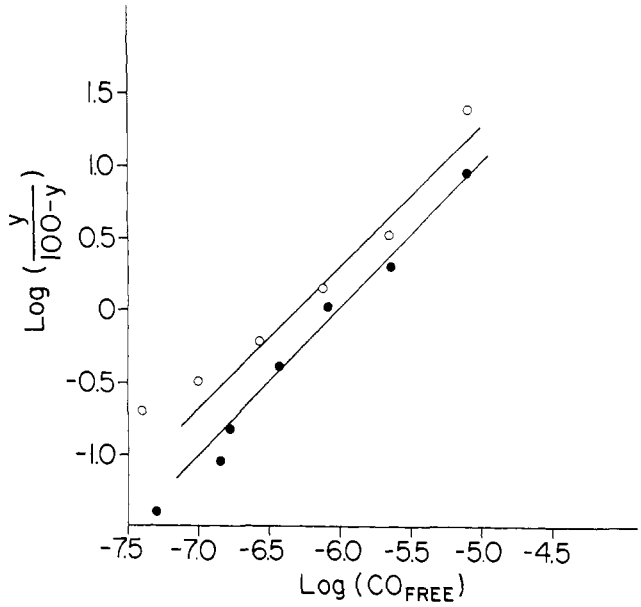

FIGURE 2: Titration of cytochrome a_3 of coupled rat liver mitochondria in the presence of ATP (●) and ATP plus S-13 (○), with carbon monoxide. Rat liver mitochondria were suspended in MSE plus 20 mM Tes at pH 7.2 at a concentration of 1.15 μM cytochrome oxidase. The following substrates were present: 10 mM glutamate, 10 mM malate, 100 μM TMPD, 10 mM ascorbate, and 20 μM NADH; (●) was carried out in the presence of 5 mM ATP; (○) was carried out in the presence of 5 mM ATP and 1.5 μM S-13.

Discussion

The ultimate goal of workers with submitochondrial and solubilized preparations is to be able to define the structure–activity correlation of the cytochrome components within the intact mitochondrial membrane. Various doubts exist in our mind as to how much of the reactivity of the different cytochrome components are modified in such processes. We therefore set out to study the carbon monoxide binding of cytochrome a_3 of intact rat liver mitochondria and to evaluate the structural states of the a_3 components with respect to each other and with respect to the rest of the electron-transporting system.

Wald and Allen (1957) titrated beef heart submitochondrial membrane preparations, which had been extracted with petroleum ether and solubilized with sodium desoxycholate, with carbon monoxide and found a Hill coefficient (n) which was greater than one, as might be expected in the presence of functional interaction between a_3 molecules. They found an n value of 1.26 and concluded that two a_3 molecules are sufficiently interlinked so as to contribute to the reduction of one oxygen molecule.

Gibson *et al.* (1965) found that each a_3 binds a CO molecule. Okunuki *et al.* (1968) found that active, purified monomeric oxidase consists of two a hemes, *i.e.*, a plus a_3. This evidence tends to exclude the possibility of one oxygen molecule binding two a_3's before its reduction.

Cytochrome oxidase is purified from mitochondria in an oligomeric state (Okunuki *et al.*, 1968) or as a membrane (Jacobs *et al.*, 1966) and only after special treatment does it become a monomer. This raises the possibility that in intact mitochondria the binding of oxygen to one a_3 will affect the reactivity of a neighboring a_3 toward the reduction of oxygen. Chance and Pring (1968), indeed, have suggested that low concentrations of oxygen modify the rate constants between cytochromes of many respiratory chains. In other words, cooperative interaction between neighboring oxidase molecules could be triggered either by the oxidation of one a_3 by oxygen or by the binding of oxygen to an a_3 molecule.

We have titrated coupled, low-energy state, intact mitochondria with carbon monoxide. We find a Hill coefficient (n) of 1.1 ± 0.1 and an equilibrium constant (K) of (2.15 ± 0.3) × 10^6 M^{-1}. These values are a reflection of all our experimental results of Table I except those of experiment type VI. The equilibrium constant agrees with the value determined with purified oxidase of 2.5 × 10^6 M^{-1} (Gibson *et al.*, 1965). The Hill coefficient (n) of 1.1 ± 0.1 shows that the a_3 molecules do not functionally interact in binding ligands.

S-13 did not affect the K or n value. This uncoupler binds rather specifically and quite tightly to the mitochondrial membrane. However, since no change in K was observed, we like to conclude that the active uncoupler binding is not on the a_3 protein.

It has been suggested that cytochrome a_3 is directly involved in oxidative phosphorylation at site III (Ramirez and Mujica, 1961; Muraoka and Slater, 1969; Wilson and Dutton, 1970a). In order to observe a possible energy-linked structural change in cytochrome a_3, we have added ATP to intact anaerobic mitochondria which had been reduced with gluta-

mate, malate, ascorbate, and TMPD and titrated the a_3 component with CO. The equilibrium constant between a_3 and CO was only about half of that of the uncoupled or low-energy mitochondria (LEM). We found a K of $(1.1 \pm 0.1) \times 10^6$ M^{-1}. The n value did not change upon addition of ATP to LEM. The addition of S-13 to high-energy mitochondria (HEM) changed the K to that of the LEM. The low K value demonstrates a lower affinity of a_3 of HEM (a_3*) for CO and implies by analogy a lower affinity of a_3* for O_2.

In view of the controversy as to whether a proton gradient is a primary intermediate in oxidative phosphorylation (Mitchell, 1966), we determined the effect of pH on the reactivity of a_3 hemes toward CO. We titrated uncoupled mitochondria with CO at pH values ranging from 6.1 to 7.8. We found no change in K or in n value as we altered the final pH of the suspension. Our result demonstrates that the change in K induced by ATP is not due to a pH change resulting from a proton gradient induced by ATP. This is the first evidence for an ATP-linked structural change in cytochrome a_3 which is not due to a proton gradient.

The observed decrease in the equilibrium constant K can be explained by the following hypothesis: ATP pushes the equilibrium between a reduced high-energy a_3 (a_3*) and the reduced low-energy a_3 toward the a_3* where (a) a_3* has a lower affinity for CO or (b) CO, by combining with a_3, pulls the a_3* completely toward a_3 with which it then combines.

a_3* is not an oxidized a_3 because ascorbate and TMPD as well as succinate easily reduce normal, oxidized a_3. One could still assume that the a_3* is an oxidized a_3 with a ligand which prevents the reduction of a_3* heme [*cf.* the a_3^{+3}–HCN complex (Yong and King, 1970)] by ascorbate and TMPD. The a_3^{+3}–HCN complex cannot be readily converted into a_3^{+2}–CO in the presence of reducing agents and CO. We observed, however (see Figure 1), that a_3*–CO has a spectrum identical with that of a_3–CO. We thus like to postulate that the a_3* is a reduced high-energy form of reduced a_3.

The primary reason for using CO is to probe the site of oxygen reduction on the cytochrome a_3 and to help elucidate the cytochrome oxidase–oxygen reaction. In relating our work to the mechanism of electron transport from oxygen at the terminal cytochrome a_3 through the cytochrome chains, we postulate that the mechanism of oxygen reduction can be split up into a two-part process: (1) binding of oxygen to the terminal end of the respiratory chain and (2) the oxidation of the ferrous heme oxygen complex to ferric heme with the eventual complete reduction of oxygen to water. Extrapolating our data to oxygen, we conclude that part 1 is pH independent, as shown by our data, while 2 is pH dependent, as shown by Wilson and Dutton (1970b).

We report some calculations on the thermodynamics of the first part of our postulated oxygen reduction mechanism to which our experimental results are relevant. The binding of oxygen to a_3^{+2} occurs faster than the rate of oxidation of a_3^{+2} by oxygen. Gibson *et al.* (1965) found the rate of a_3^{+2} oxidation by low amounts of oxygen to be 6×10^7 M^{-1} sec^{-1}. The reduction of oxygen by a_3^{+2} is essentially an irreversible process, and, therefore, the rate of dissociation of O_2 from a_3^{+2} must be slower than that of CO from a_3^{+2}, *i.e.*, 0.023 sec^{-1} (Gibson *et al.*, 1965). Using these two rate constants as (a) a lower limit for the forward rate constant for the binding of oxygen to a_3 and (b) an upper limit for the dissociation of O_2 from a_3, we obtain an equilibrium constant greater than 3×10^9 M^{-1}, which corresponds to a change in free energy of about -560 mV. The addition of ATP changes the equilibrium constant for CO by a factor of two and results in a change in the change in the free energy of binding of CO to a_3 of about 20 mV. Assuming that ATP changes the apparent equilibrium constant for oxygen by the same amount, the free-energy change would be altered to approximately -540 mV.

Carbon monoxide, in our experience, has provided a valuable probe of the site of oxygen binding in electron transport. From our data and thermodynamic reasoning, we have postulated that the primary event of site III oxidative phosphorylation is synonymous with oxygen binding and is not influenced by a proton gradient. It is a process that occurs with favorable free energy offsetting the necessary free energy of reduction of molecular oxygen to water by 560 mV. Control of electron transport by ATP at site III occurs in the primary event with an energy barrier of $+20$ mV.

References

Chance, B., and Pring, M. (1968), *in* 19th Colloquium der Gesellschaft für Biologische Chemie, April 1968, Mösbach/Baden, Berlin, Springer Verlag, p 102.

Gibson, Q. H., Palmer, G., and Wharton, D. C. (1965), *J. Biol. Chem. 240*, 915.

Hill, A. V. (1910), *J. Physiol. (London) 40*, 4.

Jacobs, E. E., Andrews, E. C., and Crane, F. L. (1965), *in* Oxidases and Related Redox Systems, King, T. E., Mason, H. S., and Morrison, M., Ed., New York, N. Y., Wiley, p 784.

Jacobs, E. E., Andrews, E. C., Cunningham, W., and Crane, F. L. (1966), *Biochem. Biophys. Res. Commun. 25*, 87.

Mitchell, P. (1966), *Biol. Rev. 41*, 445.

Muraoka, S., and Slater, E. C. (1969), *Biochim. Biophys. Acta 180*, 227.

Okunuki, K., Sekuzu, I., Orii, Y., Tsudzuki, T., and Matsumura, Y. (1968), *in* Structure and Function of Cytochromes, Okunuki, K., Kamen, M. D., and Sekuzu, I., Ed., Tokyo, University of Tokyo Press, p 35.

Ramirez, J., and Mujica, A. (1961), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 20*, 49.

Schneider, W. C. (1948), *J. Biol. Chem. 176*, 259.

Wald, G., and Allen, D. W. (1957), *J. Gen. Physiol. 40*, 593.

Wilson, D. F., and Dutton, P. L. (1970a), *Arch. Biochem. Biophys. 136*, 583.

Wilson, D. F., and Dutton, P. L. (1970b), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 403.

Wohlrab, H. (1969), *Biochem. Biophys. Res. Commun. 35*, 560.

Yong, F. C., and King, T. E. (1970), *Biochem. Biophys. Res. Commun. 38*, 940.