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CONTROL OF RESPIRATION IN PROTEOLIPOSOMES CONTAINING CYTOCHROME aa_3

II. INHIBITION BY CARBON MONOXIDE AND AZIDE

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Summary

1. Carbon monoxide (CO) acts competitively towards oxygen when the latter is taken up in respiration by cytochrome aa_3-containing proteoliposomes, both in the presence of *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone and valinomycin (deenergized state) and in their absence (energized state). At high levels of CO, the double reciprocal plots ($1/v$ vs. $1/[O_2]$) in the energized and deenergized states are parallel, i.e. energization acts "anti-competitively" towards oxygen, and the "respiratory control ratio" decreases as the oxygen concentration decreases.

2. Azide acts non-competitively towards cytochrome *c* when the latter is oxidized by cytochrome aa_3-containing proteoliposomes both in the energized and deenergized (plus *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone and valinomycin) conditions. At low azide concentrations the apparent K_i for azide is unaffected by energization, but at high azide levels the K_i increases in energized liposomes, i.e. the "respiratory control ratio" decreases as the azide concentration increases.

3. It is concluded that the inhibitor experiments are consistent with but do not prove the concept that the oxidase molecules in a single vesicle are responding to a single "energization state" or set of electrochemical gradients. This and other models are discussed.

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Abbreviation: FCCP, *p*-trifluoromethoxy carbonyl cyanide phenylhydrazone.

Introduction

Carbon monoxide was identified by Warburg and Kubowitz [1] as a respiratory inhibitor competitive towards oxygen. Azide, introduced by Keilin [2] as a more reversible inhibitor than the classical cyanide, is non-competitive towards cytochrome *c* [3] and anti-competitive towards oxygen [4]. Carbon monoxide reacts with the ferrous form and azide (as hydroazoic acid) with the ferric form of the terminal member of the respiratory chain, cytochrome a_3 [5].

More recently, studies have been carried out on the action of these inhibitors on coupled (energized) as well as uncoupled (isolated) oxidase systems. Carbon monoxide binds to both fully reduced enzyme (cytochrome $a^{2+}a_3^{2+}$) and half-reduced enzyme (cytochrome $a^{3+}a_3^{2+}$) with similar dissociation constants, 0.33 μM for the former and 0.6 μM for the latter [6]. The midpoint potential of cytochrome *a*, measured when cytochrome a_3 is liganded to CO, is a function of the energy state [7,8]. Energization by ATP addition to intact mitochondria also seems to produce changes at the cytochrome a_3^{2+}-CO centre itself [8,9], perhaps involving some decrease in the apparent affinity of a_3^{2+} for CO.

Azide was reported by Wilson and Chance [10,11] to inhibit state 3 (ADP-stimulated) resporation much more effectively than state 4 or state 3u (uncoupled) respiration; this did not involve any simple correlation with energy state. Palmieri and Klingenberg [12] reported that the mitochondrial respiratory state most sensitive to azide inhibition was the "ion-pumping" (valinomycin-K^+-stimulated) state. Nicholls and Kimelberg [13], however, stated that these effects did not involve changes in oxidase sensitivity to azide, and these authors later [14] claimed that all the azide effects might be due to changes in the steady-state reductions of the cytochromes under different metabolic conditions.

Although the binding of CO may be energetically dependent, it seems clear that the accumulation of a neutral gas in the mitochondrion should not be energy dependent. We therefore carried out the experiments described in the present paper to examine the effects of CO on the activity of cytochrome *c* oxidase in reconstituted vesicular preparations [15,16], and especially to determine: (a) whether the responses were the same as those seen in mitochondria and/or the isolated oxidase system [4,6]; and (b) whether reduction of the flux by means of the inhibitor would reduce the apparent "respiratory control ratio" (ratio of the rate of respiration in presence of FCCP plus valinomycin to the rate in absence of ionophores or uncouplers).

We also carried out experiments with the other type of inhibitor, azide, which may be energetically accumulated [12], to see if any of the reported anomalies of azide inhibition in mitochondria can be reproduced in the artificial proteoliposome system. Wrigglesworth and his coworkers [17,18] have indicated that respiration by cytochrome aa_3-containing vesicles is more sensitive to azide in the presence than in the absence of valinomycin.

Materials and Methods

Cytochrome *c* oxidase was isolated from beef heart submitochondrial particles by the method of van Buuren [19] as described previously [16]. Cyto-

chrome aa_3-containing proteoliposomes were prepared according to the methods described in the preceding paper [16]. All data except for the data shown in Fig. 5 were obtained with proteoliposomes prepared by alternative procedure "a" (see ref. 16, Materials and Methods).

In addition to the other reagents, described previously [16], sodium azide was obtained from Merck and Co., salmine was from B.D.H., and carbon monoxide as compressed gas from "Dansk Ilt og Brint".

Methods of measurement were described in the previous paper [16] except that carbon monoxide was maintained at a constant partial pressure by means of the extra gas mixer as described by Petersen [4].

Results

Fig. 1 shows the effect of carbon monoxide at three concentrations on the kinetics of the cytochrome oxidase vesicles with respect to oxygen, in the fully deenergized state (in the presence of valinomycin and FCCP). As reported for the isolated enzyme alone [4], carbon monoxide behaves as a purely competitive inhibitor towards oxygen, altering the slope of the double reciprocal plots

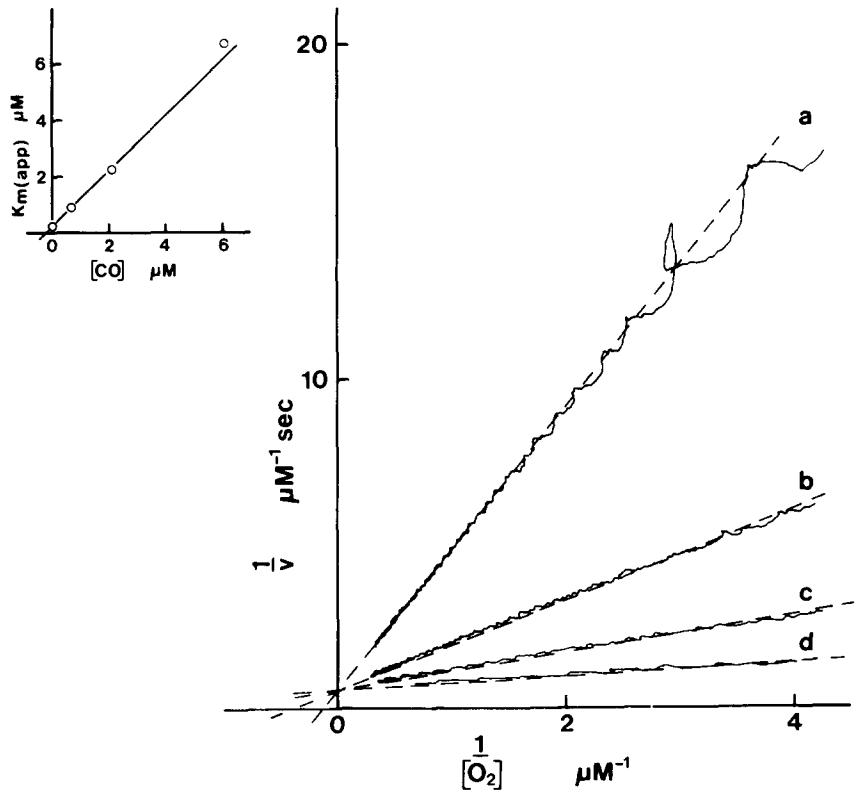

Fig. 1. Reciprocal plots of the rate of oxygen uptake versus the oxygen concentration with liposomal cytochrome oxidase in the presence of valinomycin and FCCP. The effect of carbon monoxide. The medium contained: 22.2 mM sodium ascorbate, 44.4 μM cytochrome *c*, 65 nM liposomal cytochrome aa_3, 0.53 μg/ml valinomycin, 4.4 μM FCCP, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 25°C. (a) 6.3 μM, (b) 2.1 μM, (c) 0.7 μM and (d) 0 μM carbon monoxide.

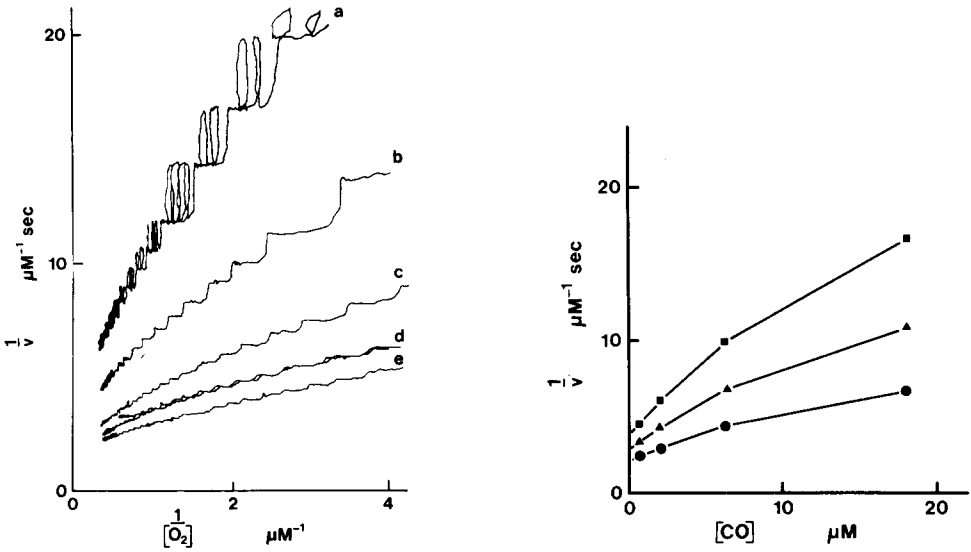

Fig. 2. (A) Reciprocal plots of the rate of oxygen uptake versus the oxygen concentration with liposomal cytochrome oxidase in the absence of valinomycin and FCCP. The effect of carbon monoxide. The medium contained: 22.2 mM sodium ascorbate, 133 nM liposomal cytochrome aa_3, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 25°C. (a) 19 μM, (b) 6.3 μM, (c) 2.1 μM, (d) 0.7 μM and (e) 0 μM carbon monoxide. (B) Plot of the rate of oxygen uptake versus the carbon monoxide concentration at varying fixed oxygen concentrations. Data taken from A. 0.5 μM (■) 1.0 μM (▲) and 2.7 μM oxygen (●).

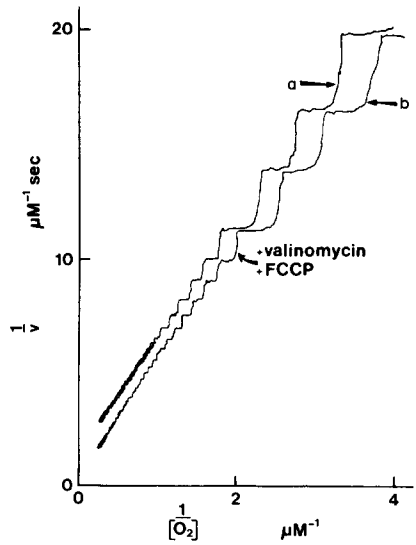

Fig. 3. Reciprocal plots of the rate of oxygen uptake versus the oxygen concentration with liposomal cytochrome oxidase in the presence of carbon monoxide. The effect of valinomycin plus FCCP. The medium contained: 22.2 mM sodium ascorbate, 44.4 μM cytochrome *c*, 133 nM liposomal cytochrome aa_3, 13 μM carbon monoxide, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 25°C. a, no further additions; b, plus 0.53 μg/ml valinomycin, 4.4 μM FCCP.

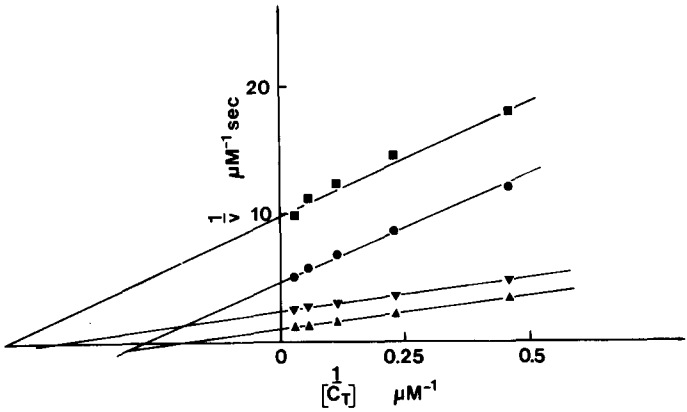

Fig. 4. Reciprocal plots of the rate of oxygen uptake versus the cytochrome *c* concentration with liposomal cytochrome oxidase. The effects of valinomycin plus FCCP and carbon monoxide. The medium contained: 24 mM sodium ascorbate, varying amounts of cytochrome *c*, 133 nM liposomal cytochrome aa_3, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 26°C. No further additions (●). Plus 0.51 μg/ml valinomycin, 4.2 μM FCCP (▼, ▲). Plus 1.0 μM carbon monoxide (■, ▼). The experiments were performed in the "oxygen-clamp" mode (cf. ref. 16) at 0.44 μM oxygen.

while leaving the intercept on the $1/v_R$ axis ($1/V$) unchanged. The inset of Fig. 1 shows the apparent K_m for oxygen plotted against CO concentration, and the calculated K_i value from this plot is 0.3 μM, identical with the experimental value obtained by Petersen [4] and the calculated values reported by Nicholls and Chance [21].

In the absence of ionophore and uncoupler the picture is more complicated (Fig. 2). The results shown in Fig. 2A cannot be analysed in the same way as those in Fig. 1. However, if $1/v_R$ is plotted vs. the CO concentration at varying values of oxygen concentrations (Dixon plots), the curves shown in Fig. 2B are obtained. A simple competitive reaction mechanism should yield straight lines having one common intersection in the second quadrant ($-K_i$, $1/V$). In this case the tangents to these curves do have common intersection points in the second quadrant, but such intersection points move towards higher K_i values as the CO concentration is increased. That is, energized liposomes are less sensitive to CO than deenergized liposomes, and their sensitivity diminishes as CO concentration increases. Wohlrab and Ogunmola [22] have reported a similar increase in K_d for CO when ATP was added to anaerobic mitochondria.

Fig. 3 compares the behaviour of cytochrome aa_3-containing liposomes blocked by 13 μM CO in the presence and absence of uncoupler plus ionophore. At low oxygen concentrations, and hence low respiratory rates, the respiratory control ratio is small; as the oxygen concentration increases, uncoupler sensitivity is restored. That is, energization in this inhibited system is "anti-competitive" towards oxygen.

When the kinetics at varying cytochrome *c* concentrations are examined in the presence of a constant amount of CO and oxygen ("oxygen-clamp" [16]), the results shown in Fig. 4 are obtained. Carbon monoxide is an anticompetitive inhibitor with respect to cytochrome *c*, both in the energized and in the deenergized state; but in this case energization has little effect on the apparent

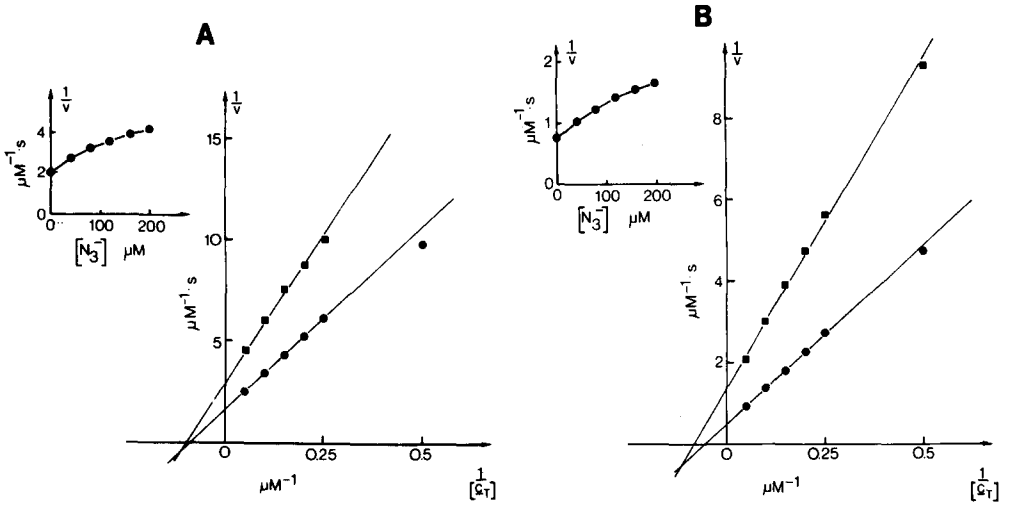

Fig. 5. Reciprocal plots of the rate of oxygen uptake versus the cytochrome *c* concentration with liposomal cytochrome oxidase. The effect of azide. (A) The medium contained: 22.2 mM sodium ascorbate, varying amounts of cytochrome *c*, 58.6 nM liposomal cytochrome aa_3, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 30°C. No further additions (•). Plus 100 μM NaN_3 (▪). Inset: Identical conditions except that the cytochrome *c* concentration was 44.4 μM and the azide concentration was varied. (B) The medium contained: 22.2 mM sodium ascorbate, varying amounts of cytochrome *c*, 29.3 nM liposomal cytochrome aa_3, 0.53 μg/ml valinomycin, 4.4 μM FCCP, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 30°C. No further additions (•). Plus 100 μM NaN_3 (▪). Inset: Identical conditions except that the cytochrome *c* concentration was 44.4 μM and the azide concentration was varied. The experiments were performed in the "oxygen-clamp" mode (cf. ref. 16) at 17.7 μM oxygen.

K_i for CO, which remains close to the 1.0 μM concentration actually used in both states.

In contrast to carbon monoxide, azide tends to be a non-competitive inhibitor with respect to cytochrome *c* both in the energized (Fig. 5A) and the deenergized (Fig. 5B) states, as it is toward the isolated enzyme [3]. The K_i values obtained from the Dixon plots (insets to Figs. 5A and 5B) were closely similar to those for the isolated enzyme under the "oxygen-clamp" conditions. The plots do, however, suggest some deviation from linearity, with slight increases in apparent K_i as the azide concentration increases, both in presence and absence of ionophore plus uncoupler. This is further seen in experiments involving the first-order oxidation of ferrocytochrome *c* by proteoliposomes, measured spectrophotometrically at 550—540 nm and high oxygen concentrations (Fig. 6). Under these conditions we also see a difference in curvature of the Dixon plots in the energized and deenergized states, indicating a decline in "respiratory control ratio" as the azide concentration increases, as with carbon monoxide (above). The addition of valinomycin in the absence of FCCP appears to decrease the K_i for azide, as reported by Wrigglesworth et al. [17,18] for proteoliposomes and by Palmieri and Klingenberg [12] for mitochondria. With proteoliposomes showing "control ratios" in excess of 5, however, this effect of valinomycin alone was negligible.

Polycations, which are thought to compete with cytochrome *c* for the latter's binding site in cytochrome aa_3 [20], were expected to be useful in finding a relation between flux and control ratio since their action would not be com-

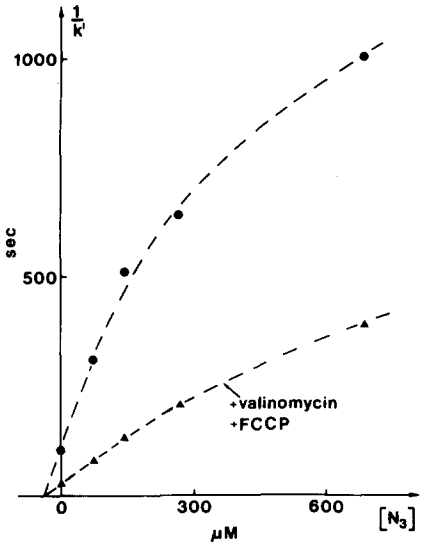

Fig. 6. Plot of the apparent first-order rate constant for the oxidation of ferrocytochrome *c* by liposomal cytochrome oxidase versus the azide concentration. The medium contained: 22.2 μM ferrocytochrome *c*, 5.5 nM liposomal cytochrome aa_3, 67 mM potassium phosphate, 1 mM EDTA, pH 7.4, 29.5°C. No further additions (●). Plus 0.3 μg/ml valinomycin, 0.37 μM FCCP (▲). The experiments were performed in a Perkin-Elmer 356 dual-wavelength spectrophotometer using the wavelength pair 550 — 540 nm. The rate constants were calculated by plotting ln (c_{tot}/c^{2+}) vs. time.

plicated by energy-linked accumulations. However, salmine, the most "reversible" of these compounds, was ineffective, as it caused aggregation of the negatively changed proteoliposomes.

Discussion

Petersen [4] has been distinguished three classes of cytochrome *c* oxidase inhibitors on the basis of their action on the kinetics with respect to oxygen: (a) the carbon monoxide-type, competitive towards oxygen; (b) the azide and formate-type, anti-competitive towards oxygen; and (c) the cyanide and sulphide-type, non-competitive towards oxygen. Our results show that carbon monoxide also acts competitively towards oxygen in both energized and deenergized cytochrome oxidase-containing proteoliposomes, with a dissociation constant similar to that found for both mitichondria [21,22] and soluble enzyme [4]. Similarly, azide, a non-competitive inhibitor with respect to cytochrome *c*, retains this essential kinetic characteristic when blocking the proteoliposome oxidase activity, despite some of the reported complexities in its inhibitory action on coupled systems [11,12,17].

Baum et al. [23] described experiments with submitochondrial particles involving titrations with oligomycin and rotenone which showed that such particles do not become less sensitive to oligomycin inhibition when respiratory flux is reduced by rotenone. That is, the two inhibitors act independently. They interpreted this as indicating that each energy-conserving unit can be controlled separately, and that such units are therefore not linked to a common pool of high energy intermediates. Such an interpretation would, of course,

conflict with the predictions made by the simpler versions of the chemiosmotic theory.

The effect of energization on the apparent K_i for CO (Fig. 2B), the effect of low oxygen concentrations on respiratory control in the presence of CO (Fig. 3), and the effect of high azide concentrations on the respiratory control ratio (Fig. 6) all suggest that, contrary to the results of Baum et al. [23] with submitochondrial particles, our cytochrome aa_3-containing proteoliposomes could be responding to a single energized or deenergized state, presumably the proton-motive force across the proteoliposome membrane.

However, the models put forward to explain the earlier data obtained with intact mitochondria [24] and mitoplasts [25], could also account for the present results with carbon monoxide. That is, the energized state promotes a back-reaction within the cytochrome c complex, governed by k_{-3} in Eqn. 1:

$$e^{3+}c^{2+} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} e^{2+}c^{3+} \xrightarrow{k_2} e^{2+}c^{3+*} \xrightarrow[O_2]{k_1} \text{products} \qquad (1)$$

$$\text{CO} \Big\downarrow \Big\uparrow K_i$$

$$e^{2+}c^{3+*}\text{CO}$$

When the subsequent reaction of "$e^{2+}c^{3+}$" is no longer part of the rate-limiting step (e.g. because CO, or low oxygen concentration, is limiting the oxidation rate of "$e^{2+}c^{3+*}$"), any effect of energization on k_{-3} will have less influence on the overall kinetics. That is, the effect of energization will be "anti-competitive" towards oxygen whether: (i) its action is a general one on the whole population of oxidase molecules; or (ii) its action is an individual one on the kinetics of each molecule.

Further experiments will be needed to distinguish more precisely these two possibilities.

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