Role of phospholipid in the low affinity reactions between cytochrome *c* and cytochrome oxidase

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The steady-state oxidation of ferrocytochrome *c* by cytochrome oxidase monitored spectrophotometrically showed that: (1) the kinetics were strictly biphasic with purified enzyme, while mitochondrial membrane-bound enzyme exhibited multiphasic kinetics with extended low affinity phases; (2) the TN_{max} for the highest affinity phase was as slow as 5–10 electron $\cdot s^{-1}$ for both preparations, while for the low affinity phases it was about 45 electron $\cdot s^{-1}$ for the purified enzyme and 150 electron $\cdot s^{-1}$ for the mitochondrial membrane-bound enzyme; (3) reconstitution of purified enzyme into acidic phospholipid vesicles partially repleted the extended low affinity phases, while reconstitution into uncharged vesicles had no effect.

Polyphasic kinetics *Acidic phospholipid* *Mitochondrial membrane*

1. INTRODUCTION

Recent studies have indicated that the low affinity phase of the steady-state oxidation of ferrocytochrome *c* by purified beef cytochrome oxidase involves binding a second molecule of cytochrome *c* to phospholipid associated with the complex [1,2]. We have postulated that this binding is non-catalytic and serves only to decrease the affinity of cytochrome *c* for the catalytic site. Such non-catalytic binding provides an explanation for the occurrence of the multiphasic steady-state kinetics of cytochrome *c* reacting with cytochrome oxidase, without requiring more than one catalytic site on the enzyme [3]. It also accounts for the previously observed differences in the low affinity kinetic phases of the reactions with purified as compared with mitochondrial membrane-bound oxidase [4].

To characterize the role of phospholipid in the low affinity kinetics of electron transfer between cytochrome *c* and cytochrome oxidase more fully, the kinetic behavior of purified cytochrome oxidase was compared to that of inner mitochondrial membrane cytochrome oxidase, and to purified cytochrome oxidase reconstituted into phospholipid vesicles of different compositions. Under low ionic strength conditions at pH 7.8, it was observed that: (1) the kinetics as a function of cytochrome *c* concentration with purified cytochrome oxidase were strictly biphasic; in contrast, mitochondrial membrane-bound cytochrome oxidase exhibited multiphasic kinetics with extended low affinity phases; (2) the maximal turnover rate (TN_{max}) for the highest affinity phase was very slow with both preparations, ranging from 5–10 electron $\cdot s^{-1}$; (3) TN_{max} for the low affinity phase with purified cytochrome oxidase was about 45 electron $\cdot s^{-1}$, whereas with mitochondrial membrane-bound oxidase the reaction appeared to saturate at about 150 electron $\cdot s^{-1}$; and (4) reconstitution of purified cytochrome oxidase into acidic phospholipid vesicles was able to replete partially the extended low affinity phases observed

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with mitochondrial membrane-bound cytochrome oxidase, while reconstitution into uncharged vesicles had little effect on low affinity kinetics.

2. MATERIALS AND METHODS

Horse cytochrome *c* was prepared by the procedure in [5] as modified in [6]. Prior to enzymic assay the cytochrome *c* was fully reduced with minimal dithionite and chromatographed on Sephadex G-50 Superfine (Pharmacia) in 100 mM Tris–acetate (pH 7.5) to separate any polymeric material [4]. Beef cytochrome oxidase was prepared as in [7]. The enzyme was stored at a concentration of 0.6 mM in liquid nitrogen. Prior to assay, fresh enzyme was diluted into 100 mM Tris–acetate (pH 7.25) containing 0.2% dodecyl D-maltopyranoside (Calbiochem), 0.2% Tween 20 (Sigma), 1 mM EDTA and 50% (v/v) glycerol.

Mitochondrial particles depleted of endogenous cytochrome *c* were prepared from fresh beef hearts and stored as in [8,9]. The cytochrome content of these particles was estimated from the reduced-minus-oxidized difference spectrum as in [10]. The concentration of cytochrome oxidase available on the external surface of the mitochondrial particles was estimated by detergent stimulation of the activity, as in [8]; this value was checked independently by determining the amount of cytochrome c_1 that could be reduced by ascorbate in intact particles (1 mM ascorbate, 25 mM Tris–acetate (pH 7.8), 250 mM sucrose) as compared to disrupted particles (same buffer containing 2% sodium cholate).

Rat liver mitochondria were prepared by dispersing the tissue with a Teflon homogenizer in 0.30 M sucrose, and isolating the mitochondrial fraction by differential centrifugation [11]. The mitochondria were depleted of endogenous cytochrome *c* as in [12].

Cytochrome oxidase was incorporated into phospholipid vesicles by the cholate dialysis procedure [13,14]. The final lipid:protein ratio was 12.5:1 for all preparations. The lipid systems employed were: asolectin (Associated Concentrates); 4:1 molar ratio phosphatidylethanolamine (PE)/phosphatidylcholine (PC); 1–9 diphosphatidylglycerol (DPG)/PC; and 3:7 DPG/PC (Avanti Polar Lipids; PC and DPG from bovine heart, PE from bovine brain). Asolectin containing 95% soy phosphatides was partially purified by acetone extraction [14]. Incorporation of cytochrome oxidase into the liposomes was monitored by determining the ratio of cytochrome oxidase activities for uncoupled and coupled preparations [14]. Only preparations having coupling ratios of at least 2 were used.

The rate of oxidation of ferrocytochrome *c* was monitored spectrophotometrically with either an Aminco DW-2a dual-wavelength spectrophotometer, or a Hitachi model 557 dual-wavelength spectrophotometer, at 416 minus 410 nm ($\Delta\epsilon_{mM}$ = 40.3) or 550 minus 526.5 nm ($\Delta\epsilon_{mM}$ = 20). Assays were run in either 25 mM Tris–acetate (pH 7.8) or 10 mM sodium phosphate (pH 7.8) buffer. When purified, unreconstituted cytochrome oxidase was assayed, 0.25% Tween 20 was added to the buffer.

3. RESULTS AND DISCUSSION

A comparison of the kinetics of oxidation of ferrocytochrome *c* by purified and Keilin-Hartree particle cytochrome oxidase, monitored spectrophotometrically, is shown in fig.1. The data are depicted on an Eadie-Hofstee plot in which the velocities were converted to turnover numbers by dividing by the estimated available cytochrome oxidase concentration. However, it should be noted that determination of the effective concentration of cytochrome oxidase in Keilin-Hartree particles is prone to error, due to the large proportion of particles which are in an inverted orientation, namely with the cytochrome *c* electron accepting site of the oxidase facing the inside of the vesicle and thus incapable of reacting with externally added cytochrome *c* (see section 2). As shown in fig.1, both purified and Keilin-Hartree particle oxidases exhibit a high affinity phase. However, purified cytochrome oxidase exhibits only a single low affinity phase with a TN_{max} of about 45 electron $\cdot s^{-1}$, while Keilin-Hartree particle cytochrome oxidase exhibits extensive multiphasic low affinity kinetics with an estimated TN_{max} of 150 electron $\cdot s^{-1}$, confirming an earlier observation [4].

The high affinity activity of Keilin-Hartree particle cytochrome oxidase exhibits a slower turnover than that for purified beef cytochrome oxidase (fig.1). This may be the result of either an overestimation of the effective concentration of cytochrome oxidase, or of a difference in the high

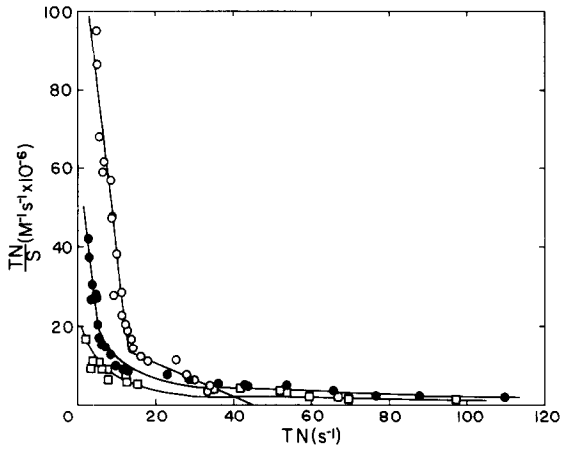

Fig.1. Oxidation of horse ferrocytochrome *c*, by beef purified and Keilin-Hartree mitochondrial particle cytochrome oxidases, and rat liver inner mitochondrial membrane cytochrome oxidase monitored spectrophotometrically. Assay conditions: 25 mM Tris–acetate (pH 7.8), 25°C, 0.25% Tween 20 included when assaying purified cytochrome oxidase, 0.05–55 M cytochrome *c*, 0.62×10^{-9} M purified cytochrome particles or 1.2×10^{-9} M rat liver inner mitochondrial membrane cytochrome oxidase. (○—○) Purified beef cytochrome oxidase; (●—●) Keilin-Hartree particle cytochrome oxidase; (□—□) rat liver inner mitochondrial membrane cytochrome oxidase.

affinity activity of the enzyme in the membrane-bound and detergent-purified states. The latter possibility is supported by the observation that cytochrome *c*-depleted rat liver inner mitochondrial membrane preparations exhibit high affinity kinetics with a TN_{max} even lower than that for the beef Keilin-Hartree particles (see fig.1). The procedure for preparing such inner membranes is mild and is unlikely to disrupt extensively the mitochondria, so it was assumed that all the cytochrome oxidase is in the correct orientation. In contrast, the rat liver inner mitochondrial membranes show multiphasic low affinity kinetics, confirming the observation with Keilin-Hartree particles that mitochondrial membrane-bound cytochrome oxidase does indeed exhibit more extensive low affinity activity than the purified enzyme.

To determine whether binding of cytochrome *c* to acidic phospholipid might be involved in the low affinity kinetics, cytochrome oxidase was reconstituted into phospholipid vesicles of various acidic phospholipid compositions. It has been shown that cytochrome oxidase preferentially orients itself correctly into liposomes [14], thus eliminating the problem of determining the effective cytochrome oxidase concentration. Incorporation of cytochrome oxidase into neutral PC/PE vesicles, as expected, had only a small effect on the low affinity kinetics of oxidation of ferrocytochrome *c* (fig.2). However, incorporation of cytochrome oxidase into asolectin, or PC vesicles containing varying amounts of cardiolipin, had a marked effect on the low affinity kinetics. The maximal velocity of the low affinity phase is approximately doubled for cytochrome oxidase reconstituted into asolectin or 30% DPG vesicles. This only accounts for about one half of the difference observed between purified and mitochondrial membrane-bound cytochrome oxidase, and undoubtedly reflects the inability to mimic adequately the complex composition of the inner mitochondrial membrane with its large proportion of protein.

These results support and extend the observation in [2], that phospholipid associated with cytochrome oxidase is involved in the low affinity

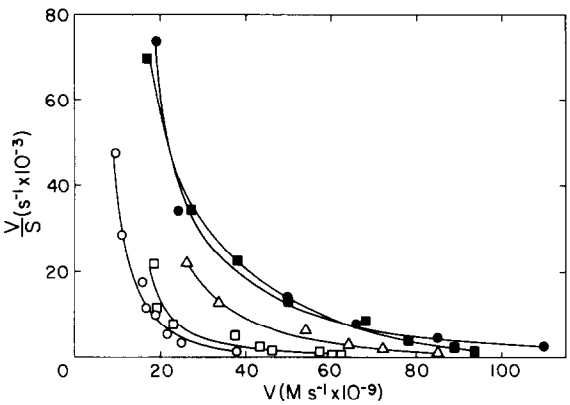

Fig.2. Low affinity steady-state kinetics of oxidation of horse ferrocytochrome *c* by purified beef cytochrome oxidase reconstituted into phospholipid vesicles of various compositions. Assay conditions: 10 mM sodium phosphate (pH 7.8), 25°C, 0.5–50 M cytochrome *c*, 1.4×10^{-9} M cytochrome oxidase. (○—○) Unreconstituted purified cytochrome oxidase, (□—□) reconstituted into neutral 4:1 PE/PC vesicles, (△—△) reconstituted into 1:9 DPG/PC vesicles, (●—●) reconstituted into 3:7 DPG/PC vesicles, and (■—■) reconstituted in asolectin.

reactions of ferrocytochrome *c* with cytochrome oxidase. The important question which remains unresolved is whether ferrocytochrome *c* bound to phospholipid closely associated with cytochrome oxidase can transfer electrons to cytochrome oxidase. While it is difficult to rule out completely the existence of more than one catalytic site on cytochrome oxidase to ferrocytochrome *c*, the results from several cross-linking studies argue strongly against two and three catalytic site models [14–17]. Indeed, all indications so far are that protein–protein electron transfer requires precise molecular alignment [18], which seems unlikely to occur with the binding of cytochrome *c* to phospholipid. Furthermore, it is not necessary to invoke the existence of more than one catalytic site to explain the multiphasic kinetics of oxidation of ferrocytochrome *c* by cytochrome oxidase. We have recently derived an initial rate equation for a single catalytic site model, which is based on a form of substrate inhibition [3]. In this model, binding of cytochrome *c* to phospholipid sites near the catalytic site is nonproductive, but does decrease the site binding constant for ferro- and ferricytochrome *c* at the catalytic site. This results in a decrease in the bimolecular association rate constant, an increase in the rate of dissociation of the ferricytochrome *c*–cytochrome oxidase complex, the rate-limiting step in steady-state turnover of electrons, as monitored in the spectrophotometric assay, and therefore an overall increase in the K_m (i.e., a lower affinity kinetic phase). Computer simulation of this model, using the wealth of presteady-state data which has been obtained with this system, yielded a kinetic profile very similar to that obtained experimentally [3].

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