Reconstitution of cytochrome bc_1 complex into lipid vesicles and the restoration of uncoupler sensitivity

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Received 30 August 1982

Beef heart mitochondrial bc_1 complex (ubiquinone–cytochrome *c* oxidoreductase) has been assayed by its ability to catalyse the reaction of duroquinol reduction of ferricytochrome *c*. When the isolated complex is reincorporated into lipid vesicles, the enzyme-catalysed electron transfer rate becomes uncoupler-sensitive. Initial experiments suggest that a protonmotive force is generated across the vesicles when electron transfer is initiated. Both $\Delta\Psi$ and ΔpH components of this protonmotive force then influence an internal rate constant of the bc_1 complex.

Mitochondrial bc *complex* *Lipid vesicle* *Respiratory control* *Uncoupler sensitivity*

1. INTRODUCTION

The reincorporation of hydrophobic proteins into lipid vesicles has found widespread uses [1]. In coupled bioenergetic systems reincorporation of proton-pumping cytochrome oxidase [2,3], bacteriorhodopsin [4] and ATPase [5] and of calcium-pumping ATPase [6,7] may be mentioned. This report describes the reincorporation of beef heart cytochrome bc_1 complex (ubiquinone–cytochrome *c* oxidoreductase) into lipid vesicles. When electron transport is initiated through the complex from a non-physiological quinol to ferricytochrome *c*, an uncoupler-sensitive flux of reducing equivalents is observed.

2. METHODS

Beef heart mitochondria bc_1 complex was prepared as in [8]. The final preparation was resuspended to ~25 mg/ml in 5 mM sodium–Tricine at pH 8.0 and stored at 77 K until required. Horse heart cytochrome *c* was obtained from Sigma (horse heart, type III). Duroquinol was prepared from duroquinone as in [9]. A stock solution of 20 mM in 96% ethanol with 10 mM HCl was generally used.

Abbreviations: Tricine, *N*-tris (hydroxymethyl) methylglycine; FCCP, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; DNP, dinitrophenol; $\Delta\Psi$, the electrical component of the protonmotive force; ΔpH, the pH component of the protonmotive force

2.1. *Preparation of coupled vesicles*

The method used to prepare vesicles was the lipid dilution technique [1,10]. A mixture of purified dioleoylphosphatidyl choline (DOPC) and purified potassium cholate (we are grateful to Drs J.C. Metcalfe and G. Smith at this department for their gifts of purified lipid and potassium cholate) were mixed together in 50 mM potassium phosphate, 2 mM EDTA buffer at pH 7.0. The ratio used was 50 mg DOPC : 25 mg potassium cholate : 1 ml buffer. The whole was rendered homogeneous by mixing in a vortex mixer for 10–15 min; the product was a cloudy suspension. This was then added to purified bc_1 complex in the ratio of 1 mg protein:30 mg lipid and the mixture was left at 4°C for 15 min. Vesicle formation occurred when an aliquot of this mixture (usually 2–10 μl) was injected into the reaction buffer (total vol. 2.5 ml). This was the generally used method. An alternative method involved dialysis of the protein/vesicle mixture against 1000 vol. 50 mM potassium phosphate, 2 mM EDTA at pH 7.0 and

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4°C for ≥3 h. The vesicles produced by the 2 methods were similar in their uncoupler sensitivities.

2.2. *Enzymatic assay*

Most reactions were followed at 550 nm or at 422 nm with a Cary 219 split beam instrument. For more complex kinetic analyses and derivation of rate constants, reactions were followed with an Applied Photophysics single beam spectrophotometer. This was linked via a Datalab DL902 transient recorder to an Apple microcomputer. Reaction buffer was 50 mM potassium phosphate, 2 mM EDTA at pH 7.0 and 25°C. The order of addition of the components was: (a) KCN; (b) vesicles (followed by mixing); (c) uncoupler (if required); (d) ferricytochrome *c*. The reaction was started by addition of an appropriate amount of duroquinol. A non-enzymatic rate of duroquinol reduction of ferricytochrome *c* which was concentration and pH dependent was always present; this was subtracted from the measured rate to give the enzymatic rates of flux.

3. RESULTS

3.1. *The preparation of uncoupler-sensitive quinol–cytochrome* c *oxidoreductase*

With the isolated bc_1 complex [8] (without incorporation into phospholipid membranes) addition of uncoupler (1 μM FCCP) had no effect on the catalytic activity of electron transfer from duroquinol to cytochrome *c* (fig.1A); in fact, higher concentrations of FCCP actually inhibited the reaction. In the vesicle reconstituted system, however, a marked stimulation of electron transfer rate occurred (fig.1B). Under the particular conditions of fig.1 (78 μM duroquinol, 9.3 μM cytochrome *c*, pH 7.0; turnover number of complex under coupled conditions = 2.5 s^{-1}) a stimulation of enzymatic rate of 2.5–4.5 was commonly observed. Identical results could be obtained with the uncoupler 2,4-dinitrophenol (at 1 mM) and hence the stimulatory effect was not specific to FCCP.

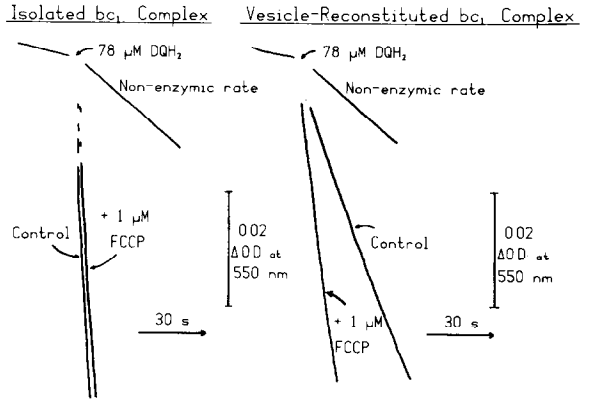

Fig.1. The effect of FCCP on isolated and vesicle-reconstituted bc_1 complex. In both cases the final concentration of bc_1 complex was 18 nM in 50 mM potassium phosphate, 2 mM EDTA at pH 7.0 and 24°C. Other conditions were: KCN, 1 mM; ferricytochrome *c*, 9.3 μM; duroquinol, 78 μM.

3.2. *The pH dependence of coupling*

Fig.2 illustrates the effect of pH on the coupled and uncoupled electron flux rates under conditions of reasonably high concentrations of duroquinol and ferricytochrome *c*. The coupled flux is fairly constant over pH 6–8.5. The uncoupled rate exhibits a pH optimum around 7–7.5 and therefore the coupled to uncoupled flux ratio is maximal in this pH range. A pH of 7 was routinely used for assay since the non-enzymatic rate increased rapidly with increasing pH.

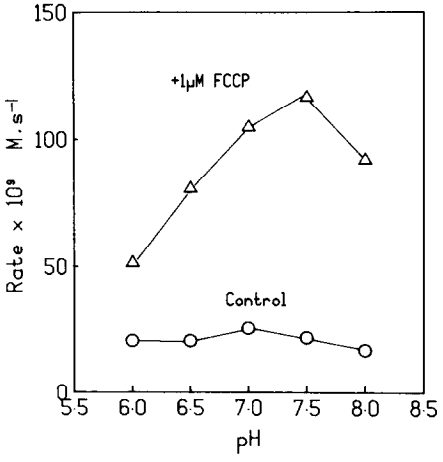

Fig.2. The effect of pH on uncoupler-sensitive flux through the vesicle-reconstituted bc_1 complex. Buffers were 50 mM potassium MES (pH 6, 6.5), 50 mM potassium phosphate (pH 7, 7.5) or 50 mM potassium Tricine (pH 8), all at 24°C and with 1 mM KCN added. Other conditions were: bc_1 vesicles, 25 nM; ferricytochrome *c*, 4.9 μM; duroquinol, 78 μM.

Table 1

Addition to vesicle system	Conc.	Initial rate of cytochrome *c* reduction ($M \cdot s^{-1} \cdot 10^9$)
Control	–	50
FCCP	1 μM	145
DNP	1 mM	142
Gramicidin	100 ng/ml	50
	4 μg/ml	83
Valinomycin	20 ng/ml	88
	200 ng/ml	93
Nigericin	40–200 ng/ml	35
	1 μg/ml	78

Conditions were: 18 nM bc_1 complex dissolved in 50 mM potassium phosphate, 2 mM EDTA at pH 7.0; KCN, 1 mM; ferricytochrome *c*, 9.3 μM; duroquinol, 78 μM

3.3. *The effects of ΔΨ and ΔpH*

Experiments were performed with gramicidin, nigericin and valinomycin to determine if the membrane potential or the ΔpH component of protonmotive force was the dominant controlling influence on the coupled vesicle preparation. The results are summarized in table 1. Gramicidin caused stimulation of flux through the complex, presumably because of its ability to act as an uncoupler. Valinomycin at > 10 ng/ml also caused a stimulation of electron flux, although not to the uncoupled rate of electron transfer. It is probable that valinomycin is causing an increased ΔpH at the expense of decreased membrane potential at low concentrations [11] and hence it is provisionally concluded that the ΔΨ is at least partly responsible for the observed control of electron flux in the coupled state. This conclusion is supported by the slight inhibitory effect of nigericin (over 10–200 ng/ml) since nigericin is presumably causing an increased ΔΨ and decreased ΔpH in this concentration range [11]. The finding that valinomycin was unable to cause stimulation to the fully uncoupled rate suggests that both ΔΨ and ΔpH can play a role in the control of electron flux.

3.4. *Investigation of the rate-controlling step in coupled electron flux*

By modulating the concentrations of duroquinol and ferricytochrome *c*, the rate limiting step for the

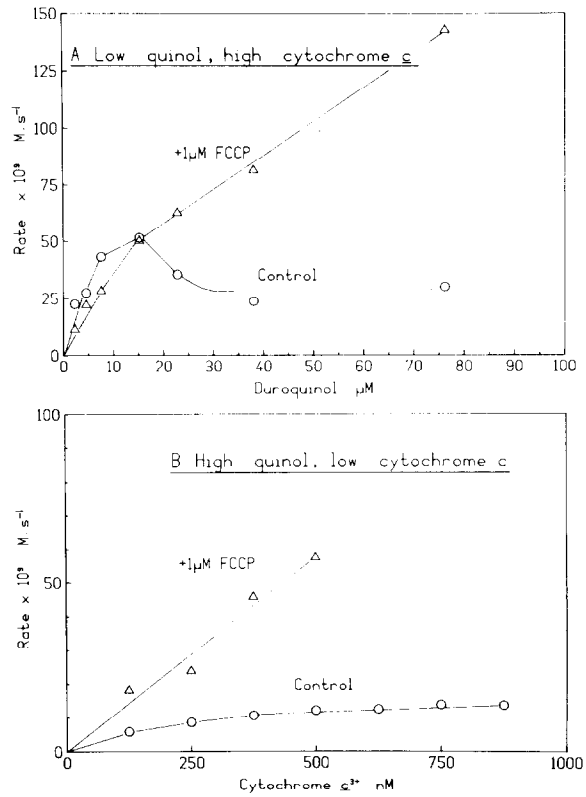

Fig.3. Uncoupler stimulation of flux under different conditions of maximum rate limitation. Both experiments used 50 mM potassium phosphate, 2 mM EDTA at pH 7.0 and 24°C with 1 mM KCN. (A) Other conditions were: bc_1 complex, 17.5 nM; ferricytochrome *c*, 20.4 μM. In the uncoupled state the overall rate would be mostly limited by rate of donation into the complex. (B) Other conditions were: bc_1 complex, 7.8 nM; duroquinol, 157 μM. In the uncoupled condition rate would be limited by donation out of the complex to ferricytochrome *c*.

donation of reducing equivalents into the complex can be obtained with low concentration of duroquinol and high ferricytochrome *c* (i.e., rate = $k_{IN} \cdot [QH_2] \cdot [bc_1]$). Alternatively, the rate-limiting step of flux of reducing equivalents out of the complex can be obtained with high duroquinol and low cytochrome *c* (i.e., rate = $k_{OUT} \cdot [\text{cyt } c^{3+}] \cdot [bc_1]$). The equations for this effect have been presented in [12,13]. For the present vesicle system, prepared by lipid dilution with duroquinol as donor the value for steady state flux of k_{IN} (duro-

quinol, 24°C, pH 7) = $1.6 \pm 0.3 \times 10^5$ $M^{-1} \cdot s^{-1}$ and of k_{OUT} (ferricytochrome *c*, 24°C, pH 7) = $1.5 \pm 0.5 \times 10^7$ $M^{-1} \cdot s^{-1}$.

Experiments were performed to determine whether the coupling control could be exerted under the two experimental conditions where rate in the uncoupled condition would be limited in one condition by donation of reducing equivalents by duroquinol into the complex and in the other condition by acceptance of reducing equivalents by ferricytochrome *c* from the complex. The results of these experiments are presented in fig.3. The rate in the uncoupled state is limited by donation into the complex, as may be seen from the nearly linear dependence of the uncoupled rate on duroquinol (fig.3A). In the coupled system, however, the rate rapidly becomes zero order with quinol with a turnover number at maximum coupled rate of around 2 s^{-1}. It is concluded that quinol donation into the complex is not rate limiting in the coupled state. Fig.2A also shows that under very low flux conditions in this experiment the FCCP (and DNP) actually inhibited the rate. The reason for this is not yet known.

Fig.3B presents a similar experiment performed under conditions where acceptance of reducing equivalents from the complex by ferricytochrome *c* was rate limiting. In the coupled system, the rate rapidly becomes saturated with ferricytochrome *c*, again at a turnover number at maximum coupled rate of around 2 s^{-1}, and so it appears that donation out of the complex to ferricytochrome *c* is also not rate limiting in the coupled state.

The general conclusion, therefore, of the above two experiments is that the control of flux through the complex in the coupled state is exerted at an internal rate constant of the bc_1 complex. Under coupled conditions it then becomes fairly easy to saturate the reaction with substrates such that a maximum turnover number of the coupled complex is reached. The range for this coupled turnover number was 1–3 s^{-1} at pH 7.0.

4. DISCUSSION

The cytochrome bc_1 complex may be reincorporated into lipid vesicles such that the reaction of duroquinol reduction of ferricytochrome *c* may be catalysed. It appears that electron flux through the complex produces an uncoupler-sensitive protonmotive force and this feeds back on the complex so that further electron flux is inhibited. Evidence is presented to suggest that both $\Delta\Psi$ and ΔpH components of the protonmotive force may act to control the complex and that this control is exerted on an internal process within the complex so that neither donation into the complex nor donation out of the complex to added cytochrome *c* is rate limiting. In [14], evidence that cytochrome *b*-562 reoxidation and cytochrome c_1 re-reduction may be the rate-limiting event in a hybrid bacterial reaction center bc_1 complex system in solution was presented. The halftime of this process is around 20 ms under optimal conditions. Electron transfer through the vesicle-reconstituted system may easily approach a turnover number equivalent to this halftime at sufficient duroquinol and ferricytochrome *c* concentrations under uncoupled conditions. In the coupled state, however, the turnover number is very much lower. One might tentatively speculate that the reoxidation of cytochrome *b*-562 by a 'Q_Z' species [14], which in this system may be the quinone product of the initial electron transfers from duroquinol to Rieske center and cytochrome *b* [13] at 'center o' [15], is the reaction which is modulated by the protonmotive force in the coupled system.

Two possible mechanisms by which a protonmotive force is produced by this system may be envisaged, if one makes the reasonable assumption that cytochrome *c* remains outside the vesicles. In the first of these, duroquinol reduces the bc_1 complex from the inside of the vesicle and protons are liberated inside. This would cause acidification of the internal solution and would also produce a $\Delta\Psi$ when the electron traversed the membrane to reduce cytochrome *c* in the outside medium. The second and more interesting possibility is that the duroquinol donates to the complex from the outside so that the protons liberated would also remain outside. The outside buffering would prevent a pH change and no $\Delta\Psi$ would be produced. The protonmotive force would then have to be produced by a proton pump, either by a 'Q-cycle' type of mechanism [15] or by a 'conformational proton pump' of some sort [16]. In these cases the inside of the vesicle would become alkaline and control would be of particular interest since it would be exerted on the major physiological energy transducing function of the complex. These possibilities are being actively investigated.

Entirely analogous results of restoration of uncoupler sensitivity to bc_1 complex electron transport have been presented in [17–19]. Indeed these authors have demonstrated that proton pumping is actually occurring from inside to outside in their systems. The combination of these results with the present demonstration of the control being exerted on an internal process within the complex makes the system ideal for further studies on coupling and its control.

ACKNOWLEDGEMENTS

We would like to thank Dr D.S. Bendall for his encouragement and the use of the facilities of his laboratory. The vesicle work would not have been possible without the advice and gifts of samples from Drs J. C. Metcalfe and G. Smith of this department. The work was financed by the Venture Research Unit of the British Petroleum Company PLC.

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