INFLUENCE OF UNCOUPLERS ON SUCCINATE–CYTOCHROME *C* REDUCTASE

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1. Introduction

Succinate–cytochrome *c* reductase (SCR)* has been isolated from mitochondria and purified in several laboratories [1–3]. The preparation has been studied, both as an entity [3, 4] and as starting material for further purification and reconstitution [2]. Preparations of this kind contain, among other components, succinate dehydrogenase, the cytochrome *b*'s and cytochrome c_1, and antimycin-sensitive electron flow spans the carriers associated with the second phosphorylation site. Recent observation of changes in the midpoint potential of cytochrome b_T upon passage of electrons through succinate–cytochrome *c* reductase suggests that the preparation retains some portion of the primary energy-conserving mechanism [5]. This observation, together with that of uncoupler-induced stimulation of reconstituted cytochrome oxidase [6] encouraged us to examine the effect of uncouplers on purified succinate–cytochrome *c* reductase activity.

2. Materials and methods

Succinate–cytochrome *c* reductase was purified from phosphate-washed beef heart mitochondria by the method of Yamashita and Racker [2] which is essentially a cholate extraction followed by ammonium sulfate fractionation. The reductase preparation was then dialyzed for 20 hr at 0°C against 20 vol of 10 mM potassium phosphate buffer at pH 7.4 containing 0.5 mM EDTA. SCR activity was measured at 20°C with a Perkin–Elmer Model 350 spectrophotometer in the split-beam mode employing a reaction medium containing 60 mM sodium succinate, 1 mM EDTA, potassium phosphate 60 mM pH 7.4 and cytochrome *c* (Sigma, Type II) 1.5 mg in a final vol of 1.0 ml. The amounts of reductase employed were 0.06 mg and 0.3 mg in the experiments reported in tables 1 and 2.

Table 1
Stimulation of succinate–cytochrome *c* reductase by uncouplers.

Additions	Cytochrome *c* reduced (nmoles/min)
None	4.8
CCCP 20 μM	17.7
FCCP 20 μM	8.0
DNP 125 μM	4.9
Octyl DNP 25 μM	6.2
$CaCl_2$ 20 mM	4.8

Table 2
Release of hydrolapachol inhibition on addition of uncouplers.

Additions	Cytochrome *c* reduced (nmoles/min)
None	4.9
Hydrolapachol 8.2 nmoles	1.1
Hydrolapachol 8.2 nmoles + CCCP 20 μM	3.2
Hydrolapachol 8.2 nmoles + FCCP 20 μM	3.1
Hydrolapachol 8.2 nmoles + DNP 125 μM	2.5
Hydrolapachol 8.2 nmoles + cholate 0.5%	4.9

* *Abbreviations*: SCR, succinate–cytochrome *c* reductase; CCCP, carbonylcyanide m-chlorophenylhydrazone; FCCP, carbonylcyanide p-trifluoromethoxyphenylhydrazone; NQNO, 2-nonyl-4-hydroxyquinoline-N-oxide; DNP, 2,4-dinitrophenol; octyl-DNP, 6-octyl-2,4-dinitrophenol; hydrolapachol, 2-hydroxy-3-(3'methylbutyl)-1,4-naphthoquinone.

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respectively. Difference spectra were obtained using the same instrument with SCR suspended in a medium containing 60 mM potassium phosphate pH 7.4.

3. Results

Addition of uncouplers of oxidative phosphorylation gives rise to stimulation of succinate oxidation by SCR (table 1). The stimulation is most dramatic with CCCP and FCCP, while addition of Ca^{2+}, an uncoupler in the case of intact mitochondria, is without effect. Likewise, in other experiments (not shown) addition of valinomycin in the presence of K^+ did not give rise to stimulation of electron transport. Stimulation by uncouplers was lessened when dialysis was omitted perhaps owing to increased cholate in the preparation which would give rise to less vesicular character [7]. That a vesicular preparation is required for stimulation by uncouplers is also consistent with the observation that inclusion of 0.5% sodium cholate in the reaction medium renders SCR insensitive to addition of CCCP.

The failure to observe substantial stimulation of electron flow on addition of DNP (while octyl-DNP is effective) appears to result from a secondary inhibition of the reductase by the somewhat higher concentrations of DNP required. We have found that the rapid electron transport measured in the presence of CCCP is inhibited about 40% by 50 μM DNP. It appears that inhibition by DNP occurs at a concentration comparable to that at which its uncoupling effect commences, so that uncoupling effects are diminished. Octyl-DNP uncouples at a lower concentration than DNP itself, owing to its increased solubility in lipid and, thus, remains an effective stimulator of electron transport. This result suggests that uncoupling may reflect the compound's concentration in lipid, while inhibition reflects that in the aqueous phase.

We showed earlier that inhibition of mitochondrial succinoxidase by hydroxynaphthoquinones and hydroxyquinoline-N-oxides was reversible upon addition of uncoupling compounds [8]. Reversibility appears due to the energy-linked translocation of the inhibitor to its locus of action and it is noteworthy that it does not occur with inside-out submitochondrial particles [9]. Thus, it is of interest to examine SCR for similar reversibility of inhibition, in part because of the bearing that such information may have on the sidedness of the reductase vesicles. Table 2 demonstrates substantial release of hydrolapachol inhibition with CCCP, FCCP, DNP, octyl-DNP and cholate, and it should be pointed out that the degree of stimulation of the inhibited rate is much greater than the stimulation of uninhibited electron flow (table 1).

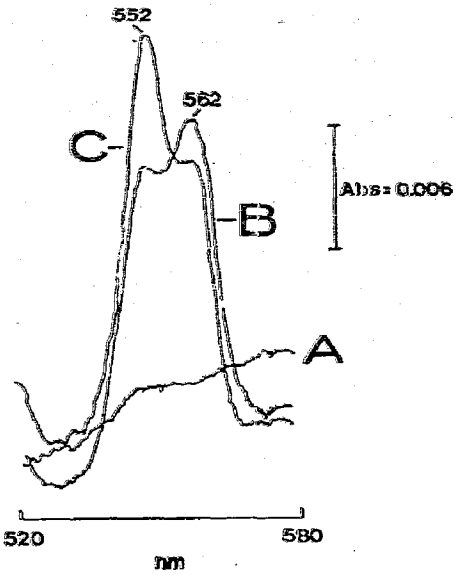

Fig. 1. Change in the oxidation–reduction difference spectrum of SCR upon addition of CCCP. The cuvettes contained 60 mM potassium phosphate pH 7.4 and 2.0 mg of the reductase preparation in a vol of 1.2 ml. Cytochrome *c* was not present in the medium: A) Baseline (oxidized–oxidized; B) Reduced (8 mM sodium succinate)–oxidized; C) CCCP (30 μM) added to test cuvette. Spectrum C was unaltered on addition of CCCP to the reference cuvette, as well. In this preparation, about one-half of the dithionite reducible cytochrome *b* was reduced by succinate

Uncoupler-induced stimulation of respiration in intact mitochondria is accompanied by changes in the oxidation–reduction state of the individual carriers of the respiratory chain [10]. Fig. 1 shows that addition of CCCP to SCR, in the presence of succinate, causes an oxidation of cytochrome(s) *b* and a reduction of cytochrome c_1.

4. Discussion

The fact that uncouplers of oxidative phosphorylation stimulate electron transport and release respiratory

inhibition in purified SCR indicates that some features of energy-coupling remain in this partially resolved, vesicular preparation. If, as widely supposed, uncouplers act by conducting protons across the coupling membrane [11], uncoupler-induced stimulation of SCR activity probably reflects collapse of a proton gradient (or an equivalent potential difference) which opposes electron flow. Since it is improbable that a purified segment of the respiratory chain would contain a specific proton pump, it is likely that electron transport, itself, produces the gradient, perhaps by occurring in a vectorial fashion as suggested by Mitchell [12].

If, as we have suggested [9], release of naphthoquinone inhibition by uncouplers is due to failure of concentrative transport of the inhibitor to its site of action, then that site must be inaccessible to the exterior both in intact mitochondria [8, 9] and in SCR. Thus, these two systems appear to exhibit the same sidedness in contrast to sonic submitochondrial particles, where inhibition is not released by uncoupling and where, presumably, the site of inhibition is exposed.

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