INVOLVEMENT OF THE IRON–SULFUR PROTEIN OF THE MITOCHONDRIAL CYTOCHROME b–c_1 COMPLEX IN THE OXIDANT-INDUCED REDUCTION OF CYTOCHROME b

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1. Introduction

The iron–sulfur protein of the mitochondrial cytochrome b–c_1 complex, which is characterized by an EPR signal at $g = 1.90$ [1], has recently been purified in a reconstitutively active form from bovine heart mitochondria [2]. The resolution and reconstitution of this iron–sulfur protein has established unequivocally that it is required for electron transfer within the b–c_1 complex [3].

Several observations have provided insight into how the iron–sulfur protein may function in the electron-transfer reactions within the b–c_1 complex. Preliminary experiments with succinate–cytochrome c reductase complex depleted of iron–sulfur protein indicated that the iron–sulfur protein is required for reduction of cytochrome c_1 by succinate and, specifically in the presence of antimycin, is required for reduction of both cytochromes b and c_1 [4,5].

The effect of depletion of iron–sulfur protein on reduction of cytochrome b by succinate in the presence of antimycin is mimicked in intact succinate–cytochrome c reductase if cytochrome c_1 is reduced before addition of succinate. Under these conditions, if antimycin is present, succinate is unable to reduce cytochrome b [6,7]. On oxidation of cytochrome c_1, cytochrome b goes reduced, a phenomenon known as the oxidant-induced reduction of cytochrome b [8–10].

A synthetic analogue of ubiquinone, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT), inhibited the oxidant-induced reduction of cytochrome b in mitochondrial succinate-cytochrome c reductase complex [11]. In photosynthetic bacteria UHDBT prevents electron transfer from the iron–sulfur cluster to cytochrome c_2, apparently by binding to the iron–sulfur protein [12].

The above results suggest that the iron–sulfur protein of the b–c_1 complex may be required for the oxidant-induced reduction of cytochrome b. Here we demonstrate this requirement by showing that oxidant-induced reduction of cytochrome b can not be demonstrated in reductase complex depleted of iron–sulfur protein, but can be demonstrated after the complex is reconstituted with iron-sulfur protein.

2. Methods

Succinate–cytochrome c reductase [13] and cytochrome c oxidase [14] were isolated from bovine heart mitochondria. Reductase complex depleted of iron–sulfur protein and reconstitutively active iron–sulfur protein were prepared as in [2]. UHDBT was synthesized as in [15] with minor modification [16].

Depleted reductase was reconstituted with iron–sulfur protein by mixing at 4°C, in sequence, 35 μl 100 mM sodium phosphate, 5 mM EDTA (pH 7.4); 10 μl 20 mM sodium succinate (pH 7.4); 1.2 nmol depleted reductase complex (based on c_1 content); 15 μg succinate dehydrogenase; variable amounts of iron–sulfur protein as indicated in the figure legends, and distilled water to 175 μl final vol. The samples were incubated for 60 min at 35°C and then stored at 4°C for up to 3 h.

Prior to each spectroscopy experiment it was neces-

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sary to remove the succinate (which was necessary to stabilize succinate dehydrogenase during the incubation) from the reconstituted samples. This was done by centrifugal gel filtration chromatography [17]. For each sample a 3 ml disposable hypodermic syringe cylinder was filled with Sephadex G-25 (medium), equilibrated at 4°C with 100 mM sodium phosphate, 5 mM EDTA (pH 7.4). Just before use the syringe columns were suspended inside 1.5 × 10 cm test tubes and centrifuged at 1100 rev./min for 8 min in an IEC clinical centrifuge to expel excess buffer from the resin. The syringe column was transferred to a clean test tube; 150 μl reconstituted reductase was then applied to the resin surface, and the column was centrifuged for 8 min as above. Reconstituted reductase, free of succinate, was recovered in the resulting effluent, diluted to 1.5 ml with 100 mM sodium phosphate, 0.5 mM EDTA (pH 7.2) and placed in an open stirred cuvette at 30°C.

The oxidation–reduction state of the cytochromes in the complex was determined spectrophotometrically and titrated to the fully oxidized state by addition of potassium ferricyanide (≤10 μM). To assay for oxidant-induced reduction of cytochrome *b*, cytochrome c_1 was first reduced by addition of 80–200 μM ascorbate; 1 μM antimycin and 5 mM succinate were then added as indicated in the figure legends and as in [11]. Reduction of cyt. *b* was initiated by addition of a mixture of 30 μM cytochrome *c* + 36 μM cytochrome *c* oxidase in 100 mM sodium phosphate, 1% Tween 80 (pH 7.4). The volume of cytochrome *c* + cytochrome *c* oxidase was adjusted to maintain a ratio of cytochrome *c* oxidase: cytochrome c_1 of ~0.45. Reduction of cytochrome *b* and spectra of the cytochromes were measured by dual wavelength spectroscopy [11].

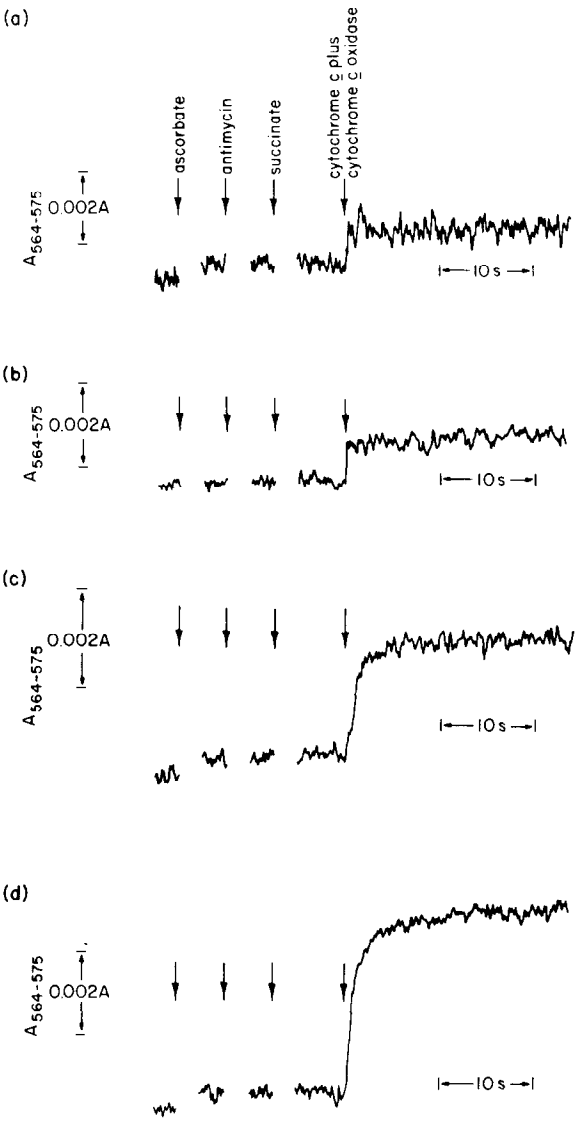

Fig.1. Effect of reconstitution with iron–sulfur protein on the ability to elicit oxidant-induced reduction of cytochrome *b*. Details of the procedure used to elicit oxidant-induced reduction of cytochrome *b* are in section 2: (a) experiment performed with depleted reductase complex, lacking the iron–sulfur protein of the b–c_1 segment; (b) the depleted reductase was omitted from the reaction mixture to demonstrate the absorbance increment resulting from addition of cytochrome *c* plus cytochrome *c* oxidase; (c,d) experiments performed with depleted reductase pre-incubated with 0.43 and 1.51 mol iron–sulfur protein/mol c_1, respectively. The concentrations of depleted reductase in the cuvettes in (a,c) and (d) varied from 0.27–0.52 μM, due to variable recovery from the centrifugal chromatography. To permit a direct comparison, the kinetics traces and the absorption spectra in fig.2 were normalized to identical concentrations of cytochrome c_1.

3. Results

In intact isolated succinate–cytochrome *c* reductase complex it is possible to demonstrate an oxidant-induced reduction of cytochrome *b* involving both *b*-562 and *b*-566 [6,11]. The tracing in fig.1a shows an attempt to demonstrate this reaction in reductase complex lacking the iron–sulfur protein of the b–c_1 segment [2]. After addition of ascorbate, antimycin, and succinate, an absorption spectrum was obtained as shown in fig.2a to confirm that cytochrome c_1 was reduced and the *b* cytochromes were oxidized.

A mixture of cytochrome *c* plus cytochrome *c* oxi-

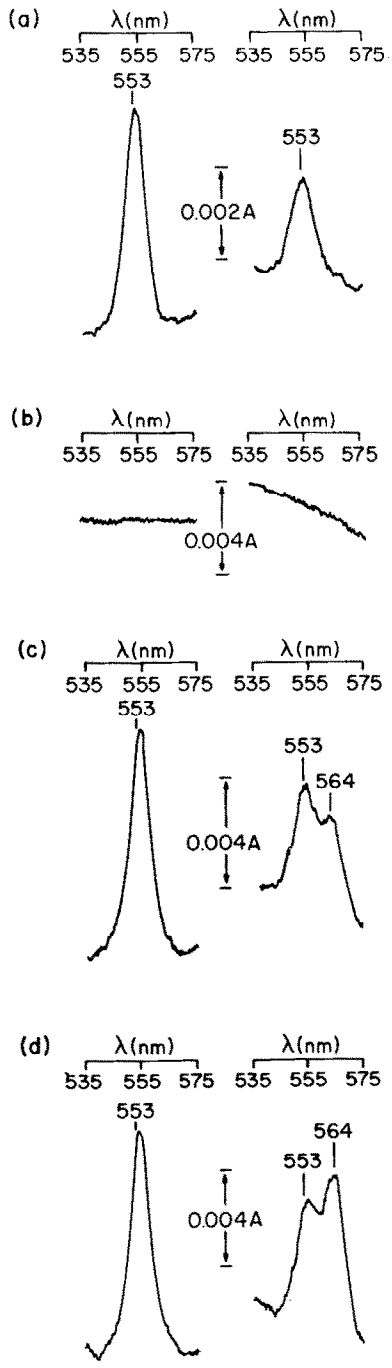

Fig.2. Absorption spectra of depleted and reconstituted reductase complex before and after addition of cytochrome *c* plus cytochrome *c* oxidase. The spectra in (a)–(d) correspond to the experiments in fig.1 (a)–(d). In each case the spectrum to the left was obtained after addition of ascorbate and that to the right was obtained after addition of cytochrome *c* plus oxidase.

dase was then added (fig.1a) and this resulted in oxidation of 60% of the cytochrome c_1 in the depleted complex, based on $\Delta A_{553-539}$, as shown in fig.2a. It is significant that in the depleted complex there is no reduction of cytochrome *b* as c_1 is oxidized (fig.2a). This is in marked contrast to the intact reductase, in which oxidation of c_1 elicits a kinetically linked reduction of *b* (see fig.1 in [11]).

Addition of cytochrome *c* + oxidase does cause a small absorbance increment at the 564 *vs* 575 nm wavelength pair used to monitor *b* reduction (fig.1a). However, this increment is due to a turbidity change as shown by the control experiment in fig.1b, in which a comparable increment was obtained when cytochrome *c* + oxidase was added to the reaction mixture from which the depleted reductase was omitted. Absorption spectra from this control are shown in fig.2b.

The trace in fig.1c shows that with depleted reductase which has been reconstituted with iron–sulfur protein (0.43 mol/mol c_1) there is a detectable oxidant-induced *b* reduction in addition to the non-specific absorbance change and, as shown in fig.1d, the magnitude of the oxidant-induced reduction increases with increasing amounts of iron–sulfur protein reconstituted to the depleted complex.

The spectra in fig.2c,d show this proportional increase in oxidant-induced *b* reduction. The spectra indicate a greater amount of *b* reduction, calculated from $\Delta A_{564-575}$, than is reflected in the kinetics traces owing to slow *b* reduction occurring while switching from kinetics to scanning mode. The absorption maximum of the reduced *b* is at 564 nm, characteristic of *b*-562 in the presence of antimycin [18,19].

In [3] it was noted that the efficacy of the reconstitution of iron–sulfur protein is incomplete, and under optimal conditions ~40% of the catalytic activity is restored. The incompleteness of the reconstitution is probably partly due to some aggregation of the depleted complex as indicated by the incomplete oxidation of cytochrome c_1 by cytochrome *c* plus oxidase (fig.2a). In these experiments, the amount of *b* which was rapidly reduced by succinate in the presence of antimycin in the maximally reconstituted samples (fig.1d) corresponded to 30% of that in the native reductase. However, when depleted reductase was incubated with a limiting amount (fig.1c) or a molar excess (fig.1d) of iron–sulfur protein, the amount of *b* reduced in the rapid phase of oxidant-induced reduction (<4 s) was ~80% of the *b* reduced by succinate

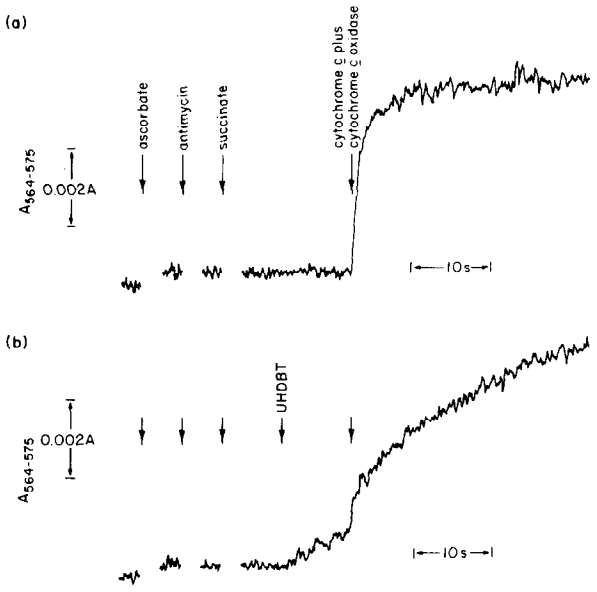

Fig.3. Effect of UHDBT on the oxidant-induced reduction of cytochrome *b* after reconstitution of depleted reductase with iron–sulfur protein. Depleted reductase was incubated with 1.51 mol iron-sulfur protein/mol cytochrome c_1 and oxidant-induced reduction of cytochrome *b* was elicited as in fig.1. The reconstituted complex was suspended at 0.50 μM in (a) and 0.46 μM in (b). Where indicated, 2 μM UHDBT was added.

in the presence of antimycin, after reconstitution with the same amounts of iron–sulfur protein. In native reductase ~85% of the succinate reducible *b* is reduced by the oxidant-induced pathway [11]. Thus, by this criterion the reconstitutively active portion of the depleted complex is quantitatively comparable to the native complex.

In native reductase, the inhibitory analogue of ubiquinone, UHDBT, inhibits oxidant-induced reduction of cytochrome *b* [11]. The tracings in fig.3 demonstrate that UHDBT also inhibits this reaction in the reconstituted system. It is also noted that UHDBT leads to a slow reduction of *b* prior to addition of oxidant and incomplete inhibition thereafter (fig.3b). A similar effect was observed with native reductase [11] and appears to be due to UHDBT acting as a slowly reacting mediator under the conditions of these experiments.

4. Discussion

The above results show that the iron–sulfur protein of the b–c_1 segment is required to elicit oxidant-induced reduction of cytochrome *b* which is normally linked to cytochrome c_1 oxidation. We have demonstrated this role of the iron–sulfur protein by using the physiologically relevant cytochrome *c* oxidase system to oxidize cytochrome c_1, thus minimizing the possibility that the reconstituted reductase complex might react in a misleading fashion with artificial oxidants. At the same time, the results in fig.2 indicate that the iron–sulfur protein is not required for oxidation of c_1 by cytochrome *c* plus oxidase, in agreement with evidence that cytochrome *c* is the direct oxidant for c_1 [20].

It is not yet possible to deduce a unique electron-transfer mechanism to account for oxidant-induced reduction of cytochrome *b*. Oxidant-induced reduction of *b* was proposed as a heme–heme interaction in which oxidation of cytochrome c_1 leads to an increase in midpoint potentials of the *b* cytochromes, thus enabling their reduction by ubiquinol [8,10]. Our results indicate that such a mechanism is probably not applicable since, in the absence of iron–sulfur protein, oxidation of c_1 does not cause reduction of *b*.

An analogous mechanism, which would require a structural interaction between the iron–sulfur protein and cytochrome *b* has been proposed in [22]. In this case oxidation of the iron–sulfur cluster increases the midpoint potential of cytochrome *b*. This mechanism also seems unlikely since, in the absence of antimycin, reduction of cytochrome *b* is independent of both the presence of iron–sulfur protein [5] and the oxidation–reduction state of cytochrome c_1 [6].

These results do not rule out a mechanism in which, specifically in the presence of antimycin, oxidation of cytochrome c_1 is required to raise the midpoint potential of cytochrome *b*, and the iron–sulfur protein is required for the delivery of the electrons which reduce the cytochrome. However, considered with our observations on the inhibitory effects of a ubiquinone analogue [11] and the effects of depletion of iron–sulfur protein [5], the results are at present most simply interpreted in terms of cyclic mechanisms of electron transfer in the b–c_1 complex, such as the protonmotive Q cycle [5,21] and the *b* cycle proposed in [23]. A common feature of these mechanisms is that oxidation of the iron–sulfur protein by ferricytochrome c_1 and its subsequent rereduction by ubiquinol are required for formation of a bound ubisemiquinone which acts as the reductant for cytochrome *b* in the presence of antimycin.

Acknowledgements

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