INHIBITION OF THE OXIDANT–INDUCED REDUCTION OF CYTOCHROME *b* BY A SYNTHETIC ANALOGUE OF UBIQUINONE

John R. BOWYER and Bernard L. TRUMPOWER
Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755, USA

Received 16 April 1980

1. Introduction

The mechanism of electron transfer through the cytochrome (cyt.) b–c_1 segment of the mitochondrial respiratory chain is not understood. An especially enigmatic aspect of this electron transfer process is the phenomenon known as the 'oxidant-induced reduction of cyt. b' whereby addition of an oxidant to mitochondria in which cyt. c_1 is reduced and the b cytochromes are oxidized results in transient reduction of cyt. b coincident with cyt. c_1 oxidation [1–3]. The oxidant-induced reduction of cyt. b requires a source of reducing equivalents (succinate, NADH, or ubiquinol), depends on the presence of ubiquinone [1], and involves both cyt. b-562 and b-566 [2]. Although the reduction of cyt. b is most readily demonstrated in the presence of antimycin, a comparable oxidant-induced reduction can be observed at low temperatures [4], which indicates this reaction is intrinsic to the mechanism of electron transfer in the cyt. b–c_1 segment and not an aberrant response elicited by this inhibitor.

The oxidant-induced reduction of cyt. b is not readily explained by a classical linear mechanism of electron transfer through the cyt. b–c_1 segment. Wikström and Berden [2] suggested that reduction of the b cytochromes is obligatorily linked to reduction of cyt. c_1 via a common ubisemiquinone intermediate. Mitchell incorporated features of the Wikström-Berden mechanism into the protonmotive Q cycle [5] and thus provided the simplest explanation to date for the oxidant-induced reduction of cyt. b. According to the Q cycle hypothesis, ubiquinol is oxidized at the cytoplasmic side of the inner mitochondrial membrane by transfer of an electron to cyt. c_1, and the low potential ubisemiquinone which is formed then reduces cyt. b. Thus, a central premise of the Q cycle hypothesis is that high potential oxidants, such as ferricyanide, generate the prerequisite ubisemiquinone reductant for cyt. b by oxidizing ubiquinol via cyt. c_1 and the iron–sulfur protein of the cyt. b–c_1 segment [6,7].

The proposed protonmotive Q cycle therefore assigns a uniquely important role to the oxidation–reduction reactions of ubiquinone in the oxidant-induced reduction of cyt. b. This paper reports that a synthetic analogue of ubiquinone, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT), inhibits the oxidant-induced reduction of cyt. b in isolated succinate–cyt. c reductase complex.

2. Methods

Succinate–cyt. c reductase complex was isolated from bovine heart mitochondria [8]. To demonstrate oxidant-induced reduction of cyt. b, reductase complex was suspended at 0.36 μM, based on cyt. c_1 content, in 0.1 M sodium phosphate, 0.5 mM EDTA, 0.5% cholate, 0.25 mM KCN (pH 7.2) and placed in an open, stirred cuvette at 27°C. Cytochrome c_1 was first reduced by addition of 30–70 μM ascorbate. Antimycin and succinate were then added as indicated in the figure legends. Under these conditions the cyt. b remains fully oxidized, due to the inability of succinate to reduce cyt. b in the presence of antimycin when cyt. c_1 is reduced by ascorbate [9], and this was checked spectrally at the beginning of each experiment.

Reduction of cyt. b was then initiated by addition of ferricyanide as indicated in the figure legends. Kinetics of cyt. b reduction were monitored with an Aminco DW 2a dual-wavelength spectrophotometer using the wavelength pairs 563 versus 575 nm and a

2 nm bandpass to include contributions from cyt. *b*-562 and *b*-566. Oxidation–reduction of cyt. c_1 was monitored at 553 versus 539 nm. To establish the oxidation–reduction status of the cytochromes before and after each kinetics experiment, spectra were scanned from 535–585 nm in the dual-beam mode. Absorption difference spectra as shown in fig.2 were obtained on a Cary 118 recording spectrophotometer. UHDBT was synthesized as in [10] with minor modifications [11].

3. Results

When succinate is added to isolated succinate–cyt. *c* reductase complex in the absence of inhibitors, ~75% of the dithionite-reducible cyt. *b* is reduced [8,9,12]. This cyt. *b* consists of cyt. *b*-562 and a portion of the low potential cyt. *b*-566 [13], while the residual dithionite-reducible cyt. *b* consists mainly of cyt. *b*-566 [13]. If antimycin is added to isolated reductase complex and cyt. c_1 and the iron–sulfur protein of the cyt. b–c_1 segment [14] are reduced by addition of ascorbate, there is no significant reduction of cyt. *b* for several minutes after succinate addition, reflecting a 14 000-fold increase in the half-time for cyt. *b* reduction under these conditions [13]. However, if ferricyanide is added during this time interval when there is otherwise no appreciable reduction of cyt. *b*, the oxidant induces a rapid reduction of cyt. *b* [9].

The tracing in fig.1a shows this oxidant-induced reduction of cyt. *b* in isolated reductase complex. The amount of cyt. *b* reduced is ~60% of the dithionite reducible cyt. *b* monitored at 563–575 nm. Addition of 1 μM UHDBT almost completely abolishes the oxidant-induced reduction as shown in fig.1b. As shown in [15], a similar oxidant-induced reduction of cyt. *b*, although smaller in magnitude, can be demonstrated with ubiquinol-2 as a source of reducing equivalents instead of succinate. This oxidant-induced reduction is also inhibited by UHDBT (not shown).

The kinetics of the oxidant-induced cyt. *b* reduction (fig.1a) are very similar to the kinetics of cyt. c_1 oxidation which results from addition of ferricyanide (fig.1c). Increasing the concentration of ferricyanide accelerates the initial rates of both cyt. c_1 oxidation and cyt. *b* reduction, although the final extent of the latter is diminished (results not shown),

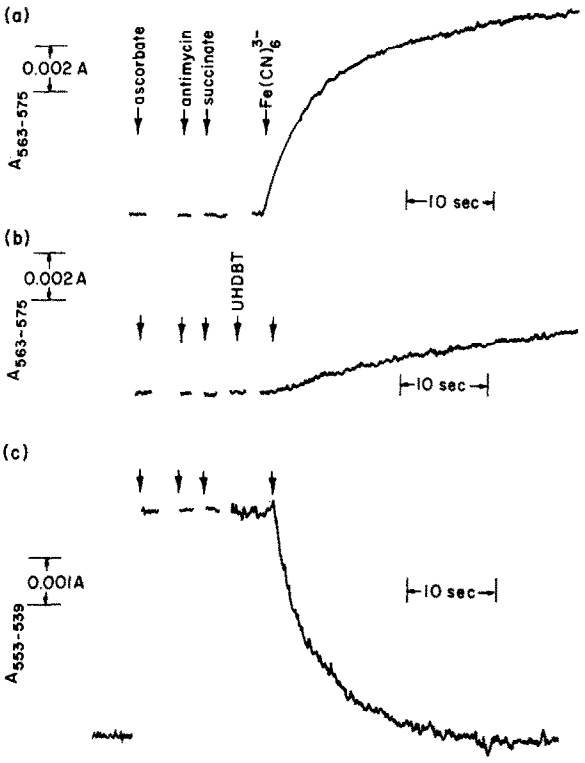

Fig.1. Inhibition of the oxidant-induced reduction of cyt. *b* in isolated succinate–cyt. *c* reductase complex by 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole. Reductase complex was suspended as in section 2. For the reaction shown by tracing (a) ascorbate was added to reduce cyt. c_1, followed by 3 μM antimycin and 5 mM succinate. Reduction of cyt. *b* was then induced by addition of 4 μM ferricyanide 2 min after addition of the succinate. The reaction shown in tracing (b) was performed under the same conditions, except that 1 μM UHDBT was added prior to addition of ferricyanide. Tracing (c) shows the oxidation of cyt. c_1 during the oxidant-induced reduction of cyt. *b* in the absence of UHDBT. The reaction was carried out under conditions identical to those of fig.1a.

presumably owing to a direct reaction between ferricyanide and ferrocytochrome *b* and/or its physiological reductant. UHDBT does not inhibit the initial phase of cyt. c_1 oxidation by ferricyanide, but the subsequent rereduction is inhibited (not shown).

A spectrum of the cyt. *b* which is reduced by oxidant-induced reduction is shown in fig.2a. The A_{564} max indicates that this cyt. *b* population consists primarily of cyt. *b*-562, the absorption maximum of which is shifted slightly to the red by antimycin [16], and probably a small contribution from cyt. *b*-566.

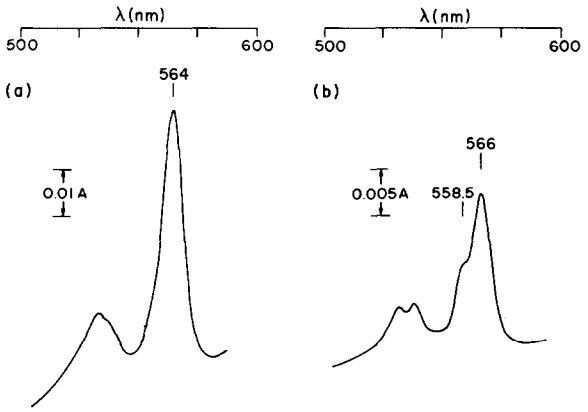

Fig.2. Absorption difference spectra of the *b* cytochromes reduced by oxidant-induced reduction. The spectrum in (a) is of cyt. *b* reduced by addition of ferricyanide to a sample in which cyt. c_1 was reduced and the *b* cytochromes were completely oxidized prior to addition of oxidant. Reductase complex was suspended at 1.62 μM in the reference and sample cuvettes of a split beam spectrophotometer, and cyt. c_1 was reduced in both cuvettes by addition of ascorbate. To the sample cuvette, 11.2 μM antimycin was added followed by 15 mM succinate. A spectrum was then recorded to insure that the cyt. *b* was initially oxidized. Reduction of cyt. *b* was then induced by addition of 15 μM ferricyanide. The spectrum of reduced cyt. *b* was scanned after ~2 min to allow re-reduction of cyt. c_1 (see fig.1). The spectrum in (b) shows the oxidant-induced reduction of the low potential cyt. *b*-566 which results when oxidant is added after reduction of cyt. *b*-562 by succinate. The protocol was similar to that in (a), except that succinate was added prior to antimycin. A spectrum was then recorded to confirm that succinate had reduced ~70% of the total cyt. *b*. The reference cuvette was similarly treated, and a spectrum recorded to show that the redox states of the cytochromes in the two cuvettes were identical, after which 7.5 μM ferricyanide was added to the sample. After an interval to allow re-reduction of cyt. c_1, the difference spectrum of succinate-reduced plus oxidant-reduced cyt. *b* versus succinate-reduced cyt *b* was recorded.

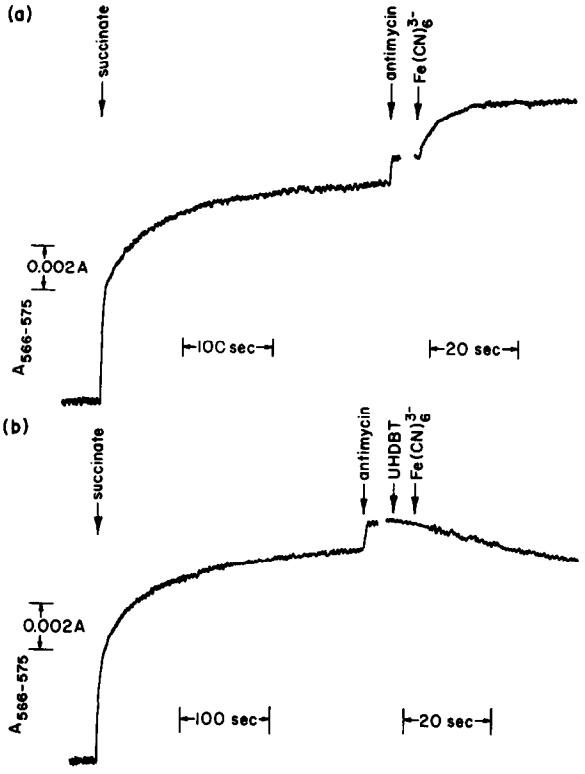

Fig.3. Inhibition of oxidant-induced reduction of low potential cyt. *b*-566 by UHDBT. Succinate was added to reductase complex to reduce cyt. c_1 and ~70% of the cyt. *b*. After reduction of cyt. *b* attained an almost constant level, as monitored at 566–575 nm, 3 μM antimycin was added. Oxidant-induced reduction of the cyt. *b* not pre-reduced by succinate was then initiated by addition of 2 μM ferricyanide. Where indicated, 1 μM UHDBT was added.

When succinate is added to isolated reductase complex in the presence or absence of antimycin, but without prior reduction of cyt. c_1 by ascorbate, the cyt. *b* which is reduced consists mostly of cyt. *b*-562 [8,9,12]. The low potential cyt. *b*-566 which remains oxidized can subsequently be reduced by an oxidant-induced reduction. The spectrum of this low potential reduced cyt. *b*, shown in fig.2b, has a maximum at 566 nm and a shoulder at 558 nm, characteristic of cyt. *b*-566. The tracing in fig.3a shows the selective oxidant-induced reduction of the low potential cyt. *b*. Addition of UHDBT in the presence of antimycin results in a slow reoxidation of a fraction of the reduced cyt. *b* (not shown). However, this oxidation rate is considerably slower than the rate of the ferricyanide-induced reduction of cyt. *b*-566, and UHDBT clearly inhibits the latter process (fig.3b). These results show that UHDBT inhibits oxidant-induced reduction of both cyt. *b*-562 and *b*-566.

4. Discussion

There is now extensive evidence, from experiments with yeast mitochondria [17] and bovine heart mitochondrial succinate–cyt. *c* reductase complex [11], that UHDBT is a highly potent and specific inhibitor

of electron transfer in the cyt. b–c_1 segment of the respiratory chain. This compound also inhibits rereduction of photo-oxidized cyt. c_2 and photo-reduction of cyt. b in purple non-sulfur photosynthetic bacteria [18,19], and recent results indicate that the inhibitor blocks electron transfer between the Rieske-type iron–sulfur cluster and cyt. c_2 in these organisms, possibly by binding to the iron–sulfur protein (J. R. B., P. L. Dutton, R. C. Prince, A. R. Crofts, submitted.

The results presented here show for the first time that UHDBT inhibits the oxidant-induced reduction of cyt. b. Because UHDBT is structurally similar to ubiquinone, it seems likely that this inhibitor interferes with normal ubiquinone function. If this rationale is correct, our findings provide evidence that oxidation–reduction of ubiquinone is involved in the oxidant-induced reduction of cyt. b.

Inhibition of the oxidant-induced reduction of cyt. b by UHDBT is also of interest because of the hypothesis that the iron–sulfur protein of the cyt. b–c_1 segment functions as a ubiquinol/cyt. c_1–ubisemiquinone/cyt. b oxidoreductase in a protonmotive Q cycle mechanism [6,7]. According to this proposal, the iron–sulfur protein would participate in the oxidant-induced reduction of cyt. b by transferring an electron from ubiquinol to cyt. c_1, thus generating the ubisemiquinone reductant for cyt. b. The finding that UHDBT appears to interact with the Rieske-type iron–sulfur protein in photosynthetic bacteria (see above) is noteworthy in this regard. The availability of reconstitutively active iron–sulfur protein from mammalian mitochondria [20] will permit experimentation to test whether UHDBT and ubiquinol interact with the isolated iron–sulfur protein.

Acknowledgements

This work was supported by an NIH research grant GM 20379. B. L. T. is an Established Investigator of the American Heart Association.

References

[1] Erecinska, M., Chance, B., Wilson, D. F. and Dutton, P. L. (1972) Proc. Natl. Acad. Sci. USA 69, 50–54.
[2] Wikström, M. K. F. and Berden, J. (1972) Biochim. Biophys. Acta 283, 403–420.
[3] Chance, B. (1974) in: Dynamics of energy-transducing membranes (Ernster, L. et al. eds) pp. 553–578, Elsevier/North-Holland, Amsterdam, New York.
[4] Erecinska, M. and Wilson, D. F. (1972) FEBS Lett. 24, 269–272.
[5] Mitchell, P. (1976) J. Theor. Biol. 62, 327–367.
[6] Trumpower, B. L. (1976) Biochem. Biophys. Res. Commun. 70, 73–80.
[7] Trumpower, B. L. and Edwards, C. A. (1979) FEBS Lett. 100, 13–16.
[8] Trumpower, B. L. and Simmons, Z. (1979) J. Biol. Chem. 254, 4608–4616.
[9] Trumpower, B. L. and Katki, A. (1975) Biochem. Biophys. Res. Commun. 65, 16–23.
[10] Friedman, M. D., Stotter, P. L., Porter, T. H. and Folkers, K. (1973) J. Med. Chem. 16, 1314–1316.
[11] Trumpower, B. L. and Haggerty, J. G. (1980) J. Bioenerg. Biomembr. in press.
[12] Erecinska, M. and Wilson, D. F. (1972) FEBS Lett. 24, 269–272.
[13] Trumpower, B. L. and Katki, A. (1979) in: Membrane proteins in energy transduction (Capaldi, R. A. ed) pp. 89–200, Marcel Dekker, New York.
[14] Rieske, J. S., MacLennan, D. H. and Coleman, R. (1964) Biochem. Biophys. Res. Commun. 15, 338–344.
[15] Rieske, J. S. (1971) Arch. Biochem. Biophys. 145, 179–193.
[16] Berden, J. A. and Opperdoes, F. R. (1972) Biochim. Biophys. Acta 267, 7–14.
[17] Roberts, H., Choo, W. M., Smith, S. C., Marzuki, S., Linnane, A. W., Porter, T. H. and Folkers, K. (1978) Arch. Biochem. Biophys. 191, 306–315.
[18] Bowyer, J. R. and Crofts, A. R. (1978) in: Frontiers of biological energetics (Dutton, P. L. et al. eds) vol. 1, pp. 326–333, Academic Press, New York.
[19] Bowyer, J. R., Tierney, G. V. and Crofts, A. R. (1979) FEBS Lett. 101, 207–212.
[20] Trumpower, B. L. and Edwards, C. A. (1979) J. Biol. Chem. 254, 8697–8706.