Effect of 2,4-dinitrofluorobenzene on the enzymatic properties of the b-c_1 complex isolated from beef heart mitochondria

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A study is presented on the effect of 2,4-dinitrofluorobenzene (DFNB) on the enzymatic properties of mitochondrial b-c_1 complex. The chemical modification by DNFB strongly inhibits the reductase activity of the complex, this being accompanied by labelling by [^{3}H]DNFB of core protein I, the apoprotein of b cytochromes and the 12 kDa subunit. Chemical modification by DNFB appears to alter, in particular, the domain of heme b-562.

Cytochrome b-c_1 *complex* *Chemical modification* *Amino acid residue* *Antimycin binding*

1. INTRODUCTION

Chemical modifiers of amino acid residues have been recently used in studying the mechanism of electron transport and proton translocation in respiratory complexes. *N,N'*-Dicyclohexylcarbodiimide treatment of intact mitochondria or purified b-c_1 complex reconstituted into phospholipid vesicles results in inhibition of vectorial proton translocation with little, if any, effect on the rate of electron flow in the complex [1–5]. This indicates a direct involvement of apoproteins in the proton-motive activity of the complex [6,7].

A decoupling effect was also effected by partial digestion of polypeptide subunits of the complex with papain [8], this further suggesting that coupling between electron flow and proton translocation in the reductase depends critically on the proper arrangement and interactions of various polypeptide components.

Other amino acid modifiers were found to inhibit to the same extent both electron transport and proton translocation, with no change in the H^+/e^- stoichiometry. Thus the amino acid modifiers, ethoxyformic anhydride (EFA) and tetranitromethane (TNM) revealed an essential role of histidyl and tyrosyl residues in the catalytic activity of the complex [1,9]. The utilization of these reagents could, indeed, contribute to identification of the pathways for transmembrane proton translocation and electron transfer in the complex between redox centers (electron tunnelling).

Here, we report on the effect of DNFB on the redox activities of the b-c_1 complex. This reagent, which can lead to the modification of amino groups and other basic nucleophilic groups in amino acid residues, decreased the rate of both electron transfer and proton translocation in b-c_1 vesicles. Our data indicate that chemical modification by DNFB inhibits intramolecular electron transfer between heme b-566 and b-562 and suppresses the binding of antimicin A to the complex.

Abbreviations: DNFB, 2,4-dinitrofluorobenzene; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; DQH_2, durohydroquinone

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2. MATERIALS AND METHODS

The b-c_1 complex was isolated according to Rieske [10] and characterized as in [11]. Reconstitution of the b-c_1 complex into phospholipid vesicles was performed by the cholate dialysis method as described by Leung and Hinkle ([12]; see also [11]).

2.1. *DNFB treatment*

b-c_1 complex at 32–40 mg protein/ml in 100 mM potassium phosphate (pH 8) was incubated with a freshly prepared ethanolic solution of DNFB at 22°C (ethanol in the control). The incubation was carried out in foil-wrapped vessels to exclude light. After incubation aliquots were diluted 30 times with the same buffer and immediately assayed for enzymatic activity. For reconstitution into phospholipid vesicles samples of the control and treated enzyme were added to sonicated phospholipid suspension.

2.2. *Electrophoretic analysis*

4 mg b-c_1 complex were incubated at 22°C with 2 mM [^{3}H]DNFB at a specific activity of 45 μCi/μmol. After 6 min incubation the reaction was stopped by the addition of ice-cold acetone to a final concentration of 90%. After centrifugation the resulting pellet was carefully dried with a nitrogen stream and solubilized for SDS-PAGE [13]. Fluorography of the gel was performed according to [14].

2.3. *Spectroscopy and measurement of proton translocation*

Cytochrome c reductase activity was measured as described in [11] with duroquinol as substrate. Measurement of redox-linked proton translocation was carried out as in [11]. Oxidoreduction of cytochromes was monitored with a dual-wavelength spectrophotometer at 566–575 and 562–575 nm for b and 552–540 nm for c cytochromes. The respective absorbance changes of cytochrome b-566 and b-562 were obtained from the absorbance traces monitored at the mentioned wavelength couples, by applying a mathematical procedure reported in [15]. Antimycin-induced red shift in the dithionite-reduced b-c_1 complex was carried out according to [16].

Durohydroquinone was from K & K Laboratories, horse heart ferricytochrome c from Sigma (USA), and DNFB from Merck (Darmstadt). All other reagents were of the highest purity grade commercially available.

3. RESULTS

Fig.1 shows the time course of the inhibition of the quinol-cytochrome c reductase activity of purified b-c_1 complex by various concentrations of DNFB. Both the rate and extent of inhibition increased with the concentration of DNFB. The half-time for inhibition was 4 min at 4 mM DNFB (34 mol DNFB per mol cytochrome c_1). This experimental condition was then generally used in subsequent experiments. Kinetic analysis of DNFB inhibition of duroquinol-cytochrome c reductase showed a mixed-type inhibition.

When the DNFB-treated enzyme was reconstituted into phospholipid vesicles, electron flow and proton translocation resulted in being equally depressed (see table 1). Fig.2 shows the effect of DNFB on electron flow and proton translocation

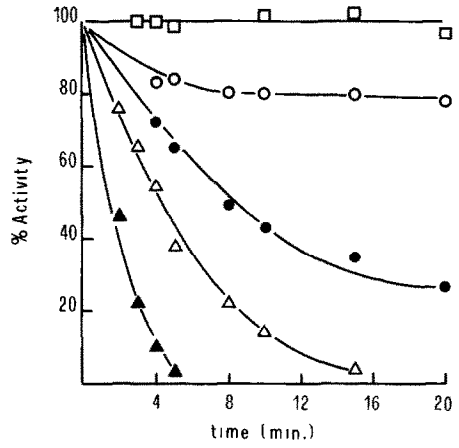

Fig.1. Effect of DNFB on the DQH_2-cytochrome c reductase activity of b-c_1 complex. b-c_1 complex at 32 mg protein/ml in 100 mM potassium phosphate (pH 8) was incubated with 0.0 (□), 0.5 (○), 2.0 (●), 4.0 (△), or 8.0 (▲) mM DNFB. After dilution, the enzyme was suspended at 8.2 μg protein/ml in the assay medium containing 50 mM phosphate (pH 7.2), 50 μM EDTA, 1 mM NaN_3, 9 μM ferricytochrome c and pulsed with 20 μM DQH_2. The specific reductase activity in the control was 1.2 μmol·min^{-1}·mg^{-1} protein.

Table 1

Effect of treatment with DNFB on redox and proton pumping activity in b-c_1 vesicles

	Rate of H^+ release	Rate of cytochrome c reduction	H^+/e^-
Control	602.2	301.8	1.99
+ DNFB	240.6	123.0	1.95

8 mg protein b-c_1 complex in 100 mM K phosphate (pH 8.0) were incubated 4 min with 4 mM DNFB and then added to sonicated phospholipid suspension for reconstitution. For other experimental details see legend to fig.2 and section 2. Values expressed as $nmol \cdot min^{-1} \cdot mg^{-1}$ protein

in b-c_1 complex treated with the modifier after reconstitution into phospholipid vesicles. Also in this case DNFB caused marked inhibition of duroquinol-cytochrome c reductase activity and equivalent depression of coupled proton translocation, with no change in the H^+/e^- ratio. However, it can be noted that the concentration of DNFB required to cause 50% inhibition was higher when the chemical modification was performed after reconstitution of the enzyme into the vesicles with respect to the enzyme treated in the soluble state. Thus phospholipids appear to protect the b-c_1 complex from modification by DNFB, probably due to dilution into the lipid phase of the hydrophobic reagent.

The Coomassie-blue stained gel of b-c_1 complex treated with [^{3}H]DNFB (fig.3A) exhibited the 8 major bands of the complex. The apparent molecular masses of the various polypeptide bands were unaffected by DNFB as judged from the electrophoretic mobilities [1]. Fluorography after 3 days exposure showed (fig.3B) a definite radioactivity in core protein I, the band corresponding to the apoprotein of b cytochromes and the 12 kDa band.

Further experiments were directed to the effect of DNFB on the pre-steady-state reduction kinetics of the b-c_1 complex. In fig.4 an experiment is shown in which the fully oxidized soluble enzyme was pulsed with duroquinol. By increasing the incubation time with DNFB both the rate and the extent of b-566 reduction increased, whereas the reduction of b-562 clearly decreased. The total

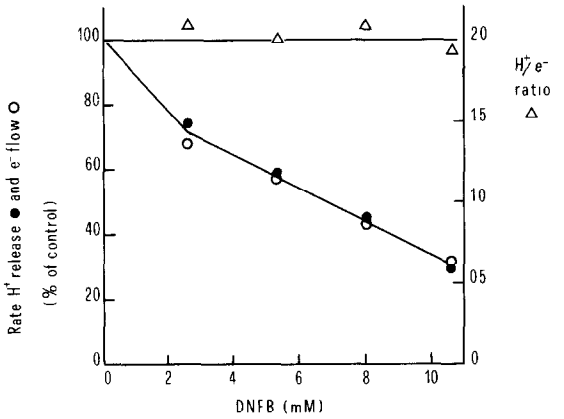

Fig.2. Effect of DNFB on energy-linked proton translocation in b-c_1 vesicles. 0.15 ml (2 mg protein/ml) b-c_1 vesicles after 4 min incubation with the indicated concentration of DNFB were diluted with 1.5 ml assay mixture containing 150 mM KCl, 5 mM $MgCl_2$, 1 mM NaN_3, 9 μM ferricytochrome c, 1 μg/ml valinomycin and pulsed with 10 μM DQH_2. For other experimental conditions see section 2. The initial rates of electron flow (○—○) and proton translocation (●—●) are expressed as percentage of the rates measured in the control; (△—△) H^+/e^- ratio.

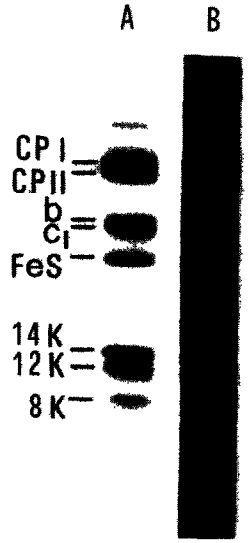

Fig.3. SDS-PAGE of b-c_1 complex treated with [^{3}H]DNFB. (a) Coomassie blue-stained gel dried under vacuum. (b) Fluorography of the same gel after 3 days exposure. For other conditions see section 2.

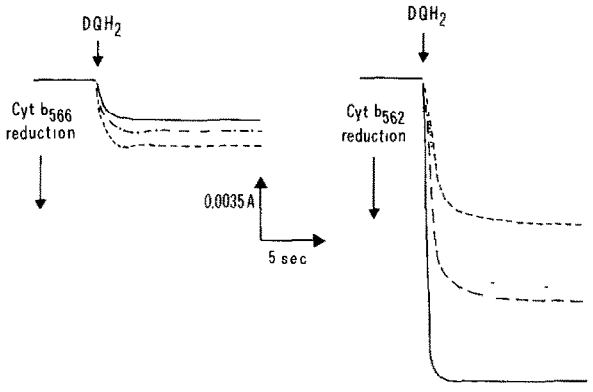

Fig.4. Kinetics of *b* cytochrome reduction induced by DQH_2 addition to *b*-c_1 complex. *b*-c_1 complex (0.4 mg protein) was incubated for 0.0 (——), 2.0 (– – –), 4.0 (···) and 6.0 (- - -) min with 4 mM DNFB. It was then diluted in 1.3 ml assay medium containing 50 mM K phosphate (pH 7.2), 50 μM EDTA, 1 mM NaN_3 and pulsed immediately with 40 μM DQH_2. The absorbance traces of the controls were similar to those for zero time incubation with DNFB and did not vary from 0 to 6 min incubation. For other experimental conditions, details and calculations see section 2.

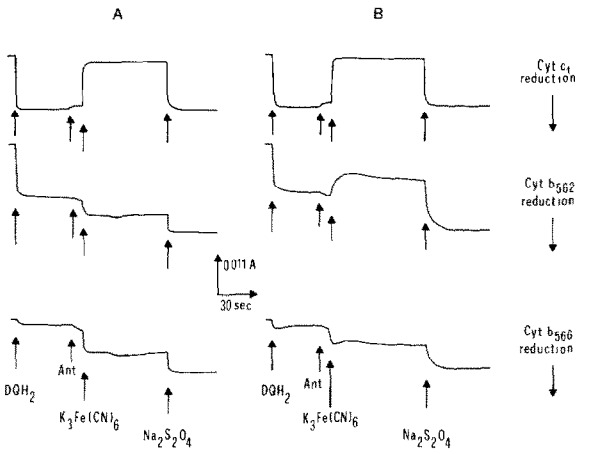

Fig.5. Effect of ferricyanide addition to antimycin-supplemented duroquinol-reduced *b*-c_1 complex. The enzyme was preincubated 4 min with 4 mM DNFB (traces B) and then suspended in the reaction mixture described in the legend to fig.4 supplemented with 0.2 μM ferricytochrome *c*. Additions were 40 μM DQH_2, 1 μg/ml antimycin, 10 μM ferricyanide. For other experimental conditions, details and calculations see legend to fig.4 and section 2.

dithionite-reducible amounts of the 2 hemes, corresponding to 0.013 and 0.032 *A* respectively, were unaffected by DNFB treatment, this indicating that chemical modification did not alter the spectral properties of the cytochromes in the α-region.

Fig.5 shows the effect of DNFB (traces B) on the oxidant-induced reduction of *b* cytochromes in presence of antimycin [15]. *b*-c_1 complex was first reduced with excess duroquinol, antimycin was then added, followed by ferricyanide addition. As expected, the latter caused oxidation of *c* cytochromes and extra-reduction of *b* cytochromes. In the enzyme treated with DNFB ferricyanide caused a large reduction of *b*-566, which was only slightly smaller than that observed in the control. In contrast, in the DNFB-treated enzyme ferricyanide caused a definite oxidation of cytochrome *b*-562 as compared to the reduction observed in the control. No difference was observed in the behaviour of cytochrome c_1.

The ferricyanide-induced oxidation of cytochrome *b*-562 observed in the DNFB-treated enzyme raises the question as to whether DNFB modification affects the binding of antimycin to the *b*-c_1 complex. In fig.6 a titration of the in-

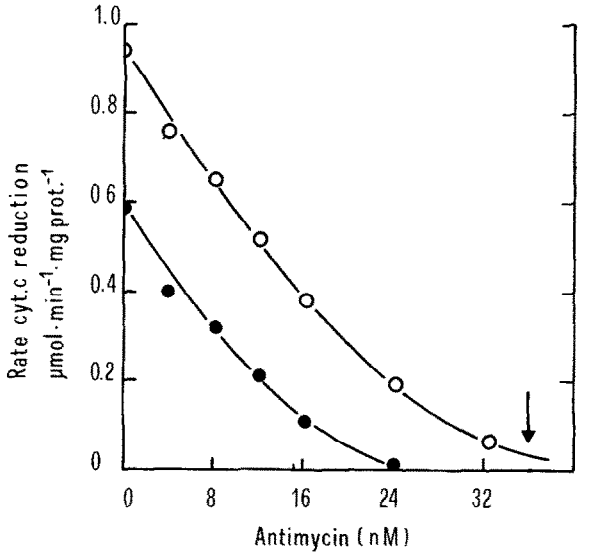

Fig.6. Titration of the effect of antimycin on duroquinol-cytochrome *c* reductase activity of DNFB-treated *b*-c_1 complex. The enzyme was incubated 4 min with 4 mM DNFB as described in section 2. (○—○) Control, (●—●) DNFB-treated *b*-c_1 complex. For other experimental conditions see legend to fig.1. The arrow indicates the stoichiometric equivalence between added antimycin and cytochrome c_1.

hibitory effect of antimycin on the duroquinol-cytochrome *c* reductase is presented. The modified enzyme shows a decreased antimycin titer with respect to the control enzyme. It is thus apparent that the molecules of the complex modified by DNFB lose the capacity to bind antimycin. This is directly supported by the finding that DNFB modification substantially decreased the red shift induced by antimycin in the dithionite-reduced b-c_1 complex (not shown).

4. DISCUSSION

The present results show that DNFB modification of the b-c_1 complex strongly inhibits the reductase activity, this being accompanied by labelling by [^{3}H]DNFB of core protein I, the apoprotein of *b* cytochromes and the 12 kDa subunit.

Although the role of core protein I and the 12 kDa protein remains to be defined, the effects described seem to derive from chemical modification of the apoprotein of *b* cytochromes.

Chemical modification by DNFB appears to alter the environment of *b*-562. This is shown by the induction of ferricyanide oxidation of *b*-562 observed in the presence of antimycin and by the depression of the antimycin-induced red shift of this prosthetic group. The small decrement of the ferricyanide-induced reduction of *b*-566, observed after treatment with DNFB, could be due to limited perturbation of the domain of heme *b*-566 but it also could be an indirect consequence of the modification of heme *b*-562 which undergoes, after DNFB treatment, significant oxidation by ferricyanide. At any rate, the persistence of the ferricyanide-induced reduction of heme *b*-566 after treatment of the enzyme with DNFB, under conditions where heme *b*-562 undergoes net oxidation, and the observed enhancement of duroquinol-induced reduction of heme *b*-566 as compared to depression of reduction of *b*-562 after treatment by DNFB suggest that the chemical modification results in inhibition of electron flow from heme *b*-566 to heme *b*-562.

Experiments with various inhibitors of the b-c_1 complex may help to verify further the effect of DNFB modification on the domain of hemes *b*-562 and *b*-566.

On the basis of the intrinsic dissociation constants and of the behaviour in polar and hydrophobic solvents [17], cysteine and histidine residues are the most likely candidates for modification by DNFB. Studies of amino acid sequences in several mitochondrial *b* cytochromes have revealed 4 invariant histidines, which are supposed to represent the axial ligands of the 2 hemes *b* and 2 more histidines which may be located at the membrane surface [18]. It would be interesting to verify whether these residues are involved in the effects caused by DNFB modification.

REFERENCES

[1] Lorusso, M., Gatti, D., Boffoli, D., Bellomo, E. and Papa, S. (1983) Eur. J. Biochem. 137, 413–420.
[2] Degli Esposti, M. and Lenaz, G. (1985) J. Bioenerg. Biomembranes 17, 109–121.
[3] Clejan, L., Bosh, L.G. and Beattie, D.S. (1984) J. Biol. Chem. 259, 11169–11172.
[4] Nalecz, M.J., Casey, R.P. and Azzi, A. (1983) Biochim. Biophys. Acta 724, 75–82.
[5] Price, B.D. and Brand, M.D. (1983) Eur. J. Biochem. 132, 595–601.
[6] Papa, S. (1982) J. Bioenerg. Biomembranes 14, 69–86.
[7] Papa, S. and Lorusso, M. (1984) in: Biomembranes: Dynamics and Biology (Burton, R.M. and Carvalho Guerra, F. eds) vol.76, pp.257–290, Plenum, New York.
[8] Lorusso, M., Gatti, D., Marzo, M. and Papa, S. (1985) FEBS Lett. 182, 370–374.
[9] Yagi, T., Vik, S.B. and Hatefi, Y. (1982) Biochemistry 21, 4777–4782.
[10] Rieske, J.S. (1967) Methods Enzymol. 10, 239–245.
[11] Papa, S., Lorusso, M., Boffoli, D. and Bellomo, E. (1983) Eur. J. Biochem. 137, 405–412.
[12] Leung, K.H. and Hinkle, P. (1975) J. Biol. Chem. 250, 8467–8471.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Bonner, W.H. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83–88.
[15] Papa, S., Lorusso, M., Izzo, G. and Capuano, F. (1981) Biochem. J. 194, 395–406.
[16] Von Jagow, G., Engel, W.D., Shagger, H. and Becker, W.F. (1982) in: Function of Quinones in Energy-Conserving Systems (Trumpower, B.L. ed.) pp.351–364, Academic Press, New York.
[17] Zahler, P. and Sigrist, H. (1984) in: Biomembranes: Dynamics and Biology (Burton R.M. and Carvalho Guerra, F. eds) vol.76, pp.159–178, Plenum, New York.
[18] Saraste, M. (1984) FEBS Lett. 166, 367–372.