COMPONENTS OF COUPLING SITE II ACCESSIBLE TO *p*-DIAZOBENZENESULFONATE FROM THE OUTSIDE OF MITOCHONDRIA

Irina V. GRIGOLAVA and Alexander KONSTANTINOV

Division of Bioenergetics, A. N. Belozersky' Laboratory of Bioorganic Chemistry and Molecular Biology, M. V. Lomonosov State University, Moscow 117234, USSR

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1. Introduction

Investigation of the redox carriers' spatial arrangement in the coupling mitochondrial membrane is of great importance for the elucidation of energy conservation mechanisms in the respiratory chain. While the cytochrome oxidase localization has been studied extensively (see [1] for review) and proved to be transmembranous, in accordance with the prediction of the chemiosmotic concept [2], little is known about the topography of the coupling sites I and II [1,3]. It has been reported by Packer and co-workers [4] that treatment of rat liver mitochondria with the membrane impermeable reagent DABS, that modifies certain protein amino acid residues [5], inhibited electron transfer from succinate to exogenous cytochrome c^{3+}. This observation suggested that some component of the mitochondrial succinate cytochrome *c* reductase is localized at the outer face of the coupling membrane. Since no further characterization of this DABS-inhibition site had been presented by the authors [4], we have attempted to study it, with a view to identify the redox carrier accessible to DABS from the outside of mitochondria.

We now report results consistent with the localization of the 'oxidation factor' [6,7] (which is possibly identical with the BAL-sensitive factor described by Slater [8]) and of cytochrome b_{566} at the outer face of the coupling mitochondrial membrane.

Abbreviations: BAL, 2,3-dimercaptopropanol; DABS, *p*-diazobenzenesulfonate, HEPES, *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DCIP, dichlorophenolindophenol; PMS, phenazine methosulfate; SDH, succinate dehydrogenase

2. Methods

Rat liver mitochondria were isolated according to [9]. DABS synthesis and treatment of mitochondria were performed as described by Packer and co-workers [4] with slight modifications. Mitochondria, subjected to the same treatment as the DABS-modified, but omitting DABS from the reaction mixture, were used as the control. SDH activity was measured as 50 μM DCIP reduction by KCN-inhibited mitochondria in the presence of 200 μM PMS and 20 mM succinate [10]. Reaction was monitored at 600 nm. PMS was omitted in the assays of the TTPA-sensitive succinate-DCIP reductase. Succinate-ferricyanide reductase activity of mitochondria was monitored at 430 nm minus 510 nm in the presence of 1 mM $K_3Fe(CN)_6$, 5 mM succinate and 5 mM cyanide. Reduced duroquinol was prepared [11] and the initial rate of its oxidation by mitochondria (zero-order in respect to duroquinol) was measured at 265 nm or 265 nm minus 283 nm, taking approximate extinction coefficients of 16 $mM^{-1} \cdot cm^{-1}$ and 13 $mM^{-1} \cdot cm^{-1}$, respectively. Optical measurements were carried out at room temperature. Oxidoreduction of cytochromes in mitochondrial suspensions was monitored with a Hitachi-356 spectrophotometer. A miniature magnetic stirrer was built into the cuvette holder of the instrument to prevent precipitation of mitochondria. Difference spectra were recorded at the split-beam/dual-wavelength spectrophotometer with an integrating sphere designed in our laboratory.

The basic incubation medium used throughout the experiments contained 0.2 M sucrose, 50 mM KCl, 20 mM HEPES buffer, pH 7.5, and also 5 μM rotenone

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and 1 μM the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Other additions are indicated in the text.

3. Results

In agreement with the observations of Packer and co-workers [4] we found the succinate-cytochrome *c* reductase activity of rat liver mitochondria to be greatly inhibited by DABS-treatment, while SDH activity was scarcely affected indicating that diazonium salt did not penetrate mitochondrial inner membrane under the conditions used. To obtain more precise location of the inhibition site a few more partial electron transfer reactions were studied (table 1, scheme I).

It may be visualized from the data presented in table 1 that the principal site of inhibition is localized between the sites of duroquinol oxidation and ferricyanide reduction by the mitochondrial respiratory chain. These sites are believed to be cytochrome(s) *b* [12] (or 'oxidation factor' [7]) and cytochrome c_1 [13,14], respectively.

In the following experiments reducibility of cytochromes $(c + c_1)$ and *b* in the control and DABS-modified mitochondria has been studied. It can be seen (fig.1) that DABS-inhibition brings about a decrease (about seven-fold) in the aerobic steady-state reduction level of cytochromes $(c + c_1)$ in the succinate-oxidizing mitochondria.

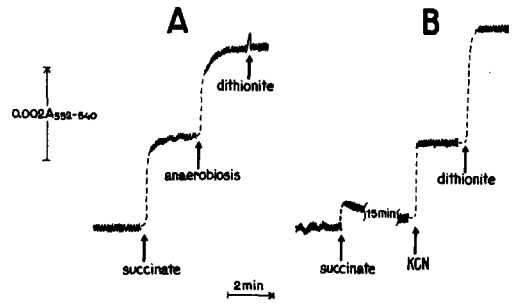


Fig.1. Effect of DABS on reducibility of cytochromes $(c + c_1)$ in rat liver mitochondria. (A) Control mitochondria, 2.2 mg protein/ml. (B) DABS-modified mitochondria, 2.5 mg protein/ml. Basic incubation medium, room temperature. Additions: succinate 5 mM, cyanide 2 mM, dithionite, a few grains.

In accordance with the above data on the severe inhibition of electron transfer by DABS, anaerobiosis was not reached in 20–30 min in the suspension of the DABS-modified mitochondria. One can also see that, in modified mitochondria, cytochromes $(c + c_1)$ fail to be completely reduced by succinate even in

Table 1
Effect of DABS on partial electron transfer activities in rat liver mitochondria

Reaction assayed	Specific activity (nequiv/min/mg protein)		Inhibition %[a]
	Control mitochondria	DABS-modified mitochondria	
Succinate dehydrogenase[b]	188	184	9 ± 7 (7)[c]
Succinate-DCIP reductase[d]	72	52	28 ± 5 (7)
Succinate-ferricyanide reductase			
total	77	19[e]	77 ± 11 (6)
antimycin sensitive	72	< 1	> 98
Duroquinol oxidase			
total	106	22	78 ± 6 (5)
antimycin sensitive	95	< 2	> 98

[a] Averages ±SD for the indicated number of experiments are given
[b] No special efforts were made to 'activate' SDH
[c] Stimulation of SDH observed in two experiments was treated as zero inhibition
[d] This was more than 95% sensitive to TTFA
[e] This was not further inhibited by TTFA and is probably due to increased permeability of DABS-modified mitochondria to ferricyanide

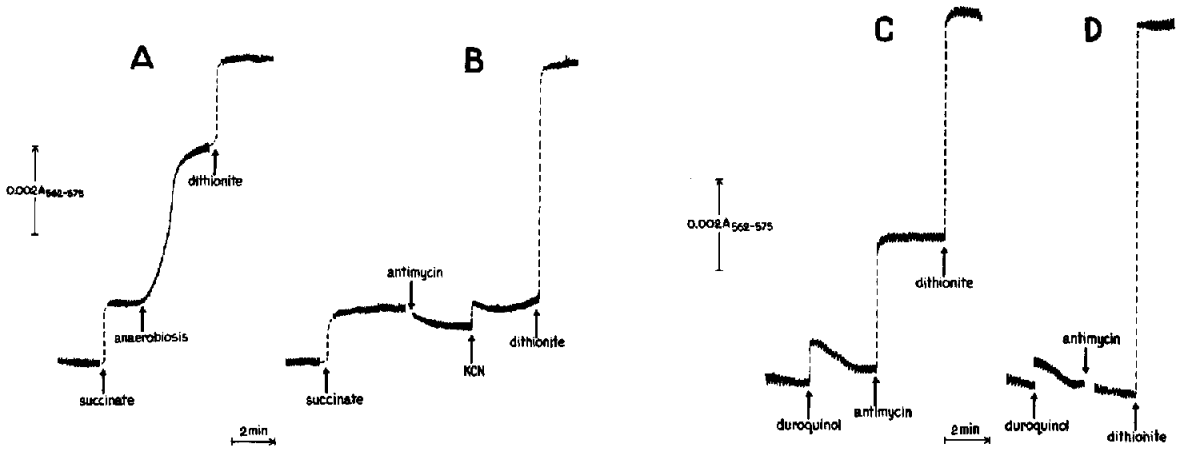


Fig.2. Oxidoreduction of cytochromes *b* in DABS-modified and control rat liver mitochondria. (A and C) Control mitochondria, 2.2 mg protein/ml. (B and D) DABS-treated mitochondria, 2.2 and 2.6 mg protein/ml, respectively. The basic incubation medium, room temperature. Additions: succinate 5 mM; duroquinol 0.3 mM; cyanide 5 mM; antimycin 3 γg/ml; dithionite, a few grains.

the presence of cyanide. A greatly increased $(c + c_1)$ reduction could be observed in the latter case upon addition of PMS (not shown), which was capable of shunting the inhibition site. These results indicate the modified component of the respiratory chain to operate on the substrate side of cytochromes $(c + c_1)$.

The effect of DABS on the oxidoreduction of cytochromes *b* in rat liver mitochondria is shown in fig.2. Addition of succinate to control mitochondria (fig.2A) resulted in a reduction of cytochromes *b* to a steady-state level, followed by an instantaneous increase in the absorbance at 562 nm minus 575 nm upon anaerobiosis. At this stage neither cyanide nor antimycin + oxygen could further reduce cytochromes *b* to any significant extent (not shown). The increase of the absorption induced by dithionite, (which varied with different batches of mitochondria: from approximately one-third to one-half of the total absorption changes) was therefore most probably due to cytochrome b_5 reduction. An essentially complete reduction of respiratory *b*-cytochromes could be also observed upon addition of succinate to the aerobic KCN-inhibited mitochondria (not shown).

The pattern of cytochromes *b* oxidoreduction behaviour was dramatically changed in DABS-modified mitochondria (fig.2B). The steady-state level of absorption at 561 nm minus 575 nm brought about by succinate addition to aerobic mitochondria, was stable for an indefinitely long period. This partial reduction of *b*-components could be increased neither by antimycin addition nor by cyanide but only with dithionite. The observed loss of reducibility by succinate was not overcome by insertion of redox

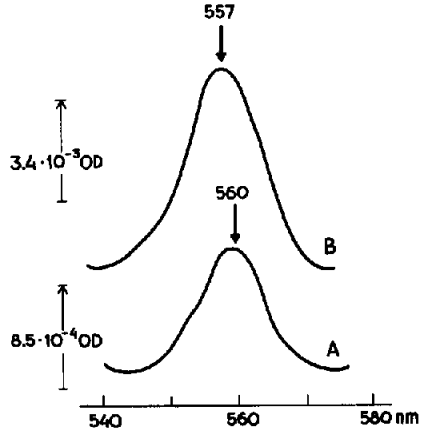


Fig.3. Difference spectra of the succinate-reducible and the dithionite-reducible cytochromes *b* in DABS-treated mitochondria. Both cuvettes contained the DABS-modified rat liver mitochondria, 4.6 mg protein/ml, in the basic incubation medium. Room temperature. (A) Reference: no additions; sample: 5 mM succinate added. (B) 5 mM ascorbate, 0.3 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine and 5 mM succinate added to both cuvettes and dithionite to the sample.

mediators into the reaction mixture. PMS, duroquinone, menadione and DCIP, tried either separately or in combination, were ineffective. The difference spectrum of the succinate-reducible cytochrome *b* in the modified mitochondria (fig.3A) showed α-maximum at 560–561 nm, which is typical of cytochrome b_K. The spectrum of the dithionite-reducible component(s) (fig.3B), dominated by the contribution of cytochrome b_5 was not very informative.

Similar results were obtained with duroquinol as substrate (fig.2 C,D). Antimycin greatly enhanced reducibility of cytochromes *b* in control mitochondria (fig.2C), but not in those treated with DABS (fig.2D).

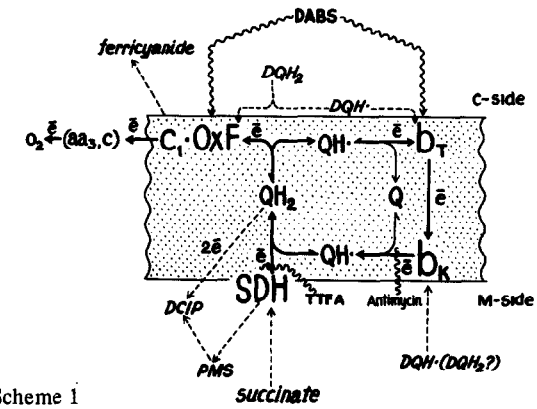
4. Discussion

There are three effects that DABS-treatment exerts on redox events within the coupling site II of rat liver mitochondria.

First, there is a complete inhibition of antimycin-sensitive electron flow through the $b-c_1$ segment of the respiratory chain. The inhibition site is clearly on the substrate side of cytochrome c_1, but on the oxygen side of the duroquinol oxidation site, which is reportedly cytochrome(s) *b* [12] or, probably, 'oxidation factor' [6,7]. Since in DABS-inhibited mitochondria all of the succinate-reducible cytochrome *b* (identified as b_K by its α-absorption band) is already reduced in the aerobic steady-state with succinate, it may be also concluded that inhibition occurs on the oxygen side of cytochrome b_K.

Second, in mitochondria treated with DABS a considerable part of the respiratory chain cytochromes *b* can no longer be reduced by succinate in a cyanide-inhibited or anaerobic state. This inhibition could not be reversed by a number of redox dyes, including PMS, menadione, DCIP, duroquinone, and therefore probably was not due to impairment of the electron transfer pathway from succinate to cytochrome *b*, but rather to the modification of cytochrome *b*, which resulted in the shift of its midpoint potential to the negative [15,16]. At present it is difficult to resolve the effects of DABS on the individual cytochromes 'b_T' and 'b_K' [17–19]. Since the component retaining reducibility by succinate in modified mitochondria has the spectral properties of 'b_K', one can suggest that 'b_T' is more susceptible to inactivation by DABS.

Redox interactions in coupling site II (after Mitchell–Trumpower [22,7]).



Scheme 1

Only electron flow is considered. Thick lines, electron transfer in the respiratory chain. Dashed lines, electron transfer to or from exogenous redox compounds. Wavy line, action of inhibitors. DQH_2, duroquinol; DQH·, durosemiquinol; OxF, 'oxidation factor'.

The third and probably the most striking effect of DABS treatment is that no antimycin-induced extra-reduction of cytochromes *b* [20,21] can be observed in DABS-modified mitochondria. Cytochrome 'b_T' modification does not account for this effect because antimycin also failed to increase reduction of enzymatically active 'b_K' when the latter was partially reduced either by duroquinol (fig.2 C,D) or by trace amounts of succinate (not shown). A plausible explanation for this effect would be the inhibition of the 'coenzyme *Q*-cycle' [19,22] at the step(s) of ubiquinol or ubisemiquinol oxidation: at the C-side of the mitochondrial membrane (scheme 1).

The inhibition of the respiratory chain by DABS with a crossover point between cytochromes *b* and c_1, accompanied by an impairment of the antimycin + oxidant-induced cytochromes *b* extra-reduction mechanism, is rather a specific mode of the inhibitory action previously observed in the cases of (1) modification of Slater's 'factor' by BAL [8,23,24] and (2) 'oxidation factor' removal [6,7]. We propose that DABS and BAL treatments may inactivate the same component in the $b-c_1$ locus of the respiratory chain which is possibly identical with the 'oxidation factor'. This component should be an essential part of the co-enzyme *Q* oxidation centre '*O*', introduced by

Mitchell in his recent formulation of the protonmotive Q-cycle [22].

Modification of cytochrome b can be the second effect of DABS, not related to the modification of the 'oxidation factor'.

DABS is known not to penetrate mitochondrial inner membrane [4,23, this communication]. Our results therefore indicate that BAL-sensitive 'factor' [8] or 'oxidation factor' [6] and possibly also cytochrome 'b_T' tentatively identified as the components of the coupling site II attacked by DABS in mitochondria are exposed at the C-side of the inner mitochondrial membrane, in accordance with the 'protonmotive Q-cycle' hypothesis of Mitchell ([19,22] see also [7]).

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