BBA 12246

THE SUCCINATE-LINKED NICOTINAMIDE-ADENINE DINUCLEOTIDE REDUCTION IN SUBMITOCHONDRIAL PARTICLES

II. STUDIES WITH INHIBITORS

F. A. HOMMES*

Johnson Foundation for Medical Physics, University of Pennsylvania, Philadelphia, Pa. (U.S.A.)

(Received January 21st, 1963)

SUMMARY

The influence of inhibitors of electron and energy-transfer reactions on the rate of and lag in the succinate-linked NAD^+ reduction in submitochondrial particles has been studied. An ATP-dependent cytochrome *b* reduction has been demonstrated. From selective inhibition of sites of oxidative phosphorylation with guanidine derivatives and the absence of a lag phase in the ATP-dependent cytochrome *b* reduction, it is concluded that this cytochrome *b* reduction preceeds NAD^+ reduction. The implications for the antimycin A sensitivity of the reaction are discussed.

It is suggested that a soluble mitochondrial factor, previously isolated from mitochondria, reacts very near the electron-transfer chain.

INTRODUCTION

In the preceeding communication some of the kinetic properties of the energy-requiring NAD^+ reduction in sonic particles from beef-heart mitochondria have been described. These studies dealt mainly with the interaction of the different components of the reaction system, succinate, Mg^{2+}, NAD^+ and ATP. In this communication the effect of energy and electron-transfer inhibitors is described.

EXPERIMENTAL

Beef-heart mitochondria, submitochondrial particles and soluble mitochondrial factor were prepared as described earlier[2].

The succinate-linked NAD^+ reduction was measured fluorometrically in the system described in the preceeding communication[1].

Difference spectra were recorded employing the wavelength scanning recording spectrophotometer described by YANG[3].

The kinetics of cytochrome *b* reduction were measured by the double-beam spectrophotometric technique as described by CHANCE[4].

* Fulbright Scholar 1961–1963. Permanent address: Department of Biochemistry, School of Medicine, University of Nijmegen, Nijmegen (The Netherlands).

Abbreviation: DBI, phenethylbiguanide.

RESULTS

The sensitivity of the succinate-linked NAD^+ reduction to the antibiotic antimycin A is controversial as far as the participation of the "classical" antimycin A-sensitive point is concerned.

LÖW *et al.*[5] and SANADI AND FLUHARTY[6] have found that the inhibition of the energy-linked NAD^+ reduction requires higher concentrations of the antibiotic than oxidation of succinate or NAD^+-linked substrates. LÖW AND VALLIN[7] and PACKER AND DENTON[8] compared the sensitivity of the reaction to antimycin A when succinate was used as electron donor with the reaction in which tetramethyl-*p*-phenylenediamine–ascorbate was used as electron donor. In our studies it was confirmed that in the latter case the reaction showed a much higher sensitivity to antimycin A than with succinate as electron donor (Fig. 1). It is noteworthy that the lag phase in NAD^+ reduction increased with reduced electron flow.

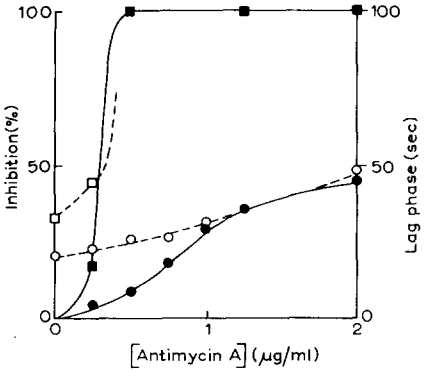

Fig. 1. Inhibition of the energy-linked NAD^+ reduction in submitochondrial particles by antimycin A. Full line: inhibition of energy-linked NAD^+ reduction; ●—●, succinate (5 mM) as electron donor; ■—■, *p*-phenylenediamine (1 mM) as electron donor. Dotted line: lag phase in sec. To the particles suspended in buffer (0.45 mg protein per ml) was added 1 mM sulfide, 5 mM succinate and 66 μM NAD^+. The reaction was started with 0.5 mM ATP, added 2 min after succinate.

This was also the case when malonate, DBI or 2,4-dibromophenol were used to inhibit the reaction, in contrast to Amytal and octylguanidine, which only inhibited the rate, without influencing the lag phase (Fig. 2).

The fact that the succinate-linked NAD^+ reduction is sensitive to antimycin A cannot be neglected and poses the problem as to whether cytochrome *b* is involved in the sequence of reactions or not. A difference spectrum is presented in Fig. 3. The reference and experimental cuvettes contained the particles suspended in buffer, containing 5 mM succinate and 1 mM Na_2S. Addition of ATP (0.5 mM) to the experimental cuvette caused the appearance of a peak at 430 mμ. A very small peak at 564 mμ is also observed, identifying the changes in absorbancy as due to cytochrome *b* (see refs. 9, 10). No NAD^+ was added in this experiment. Sensitive fluorometric assay under identical conditions did not show an increase in fluorescence indicating that no NAD^+ is present—at least in a form which can be reduced under these conditions.

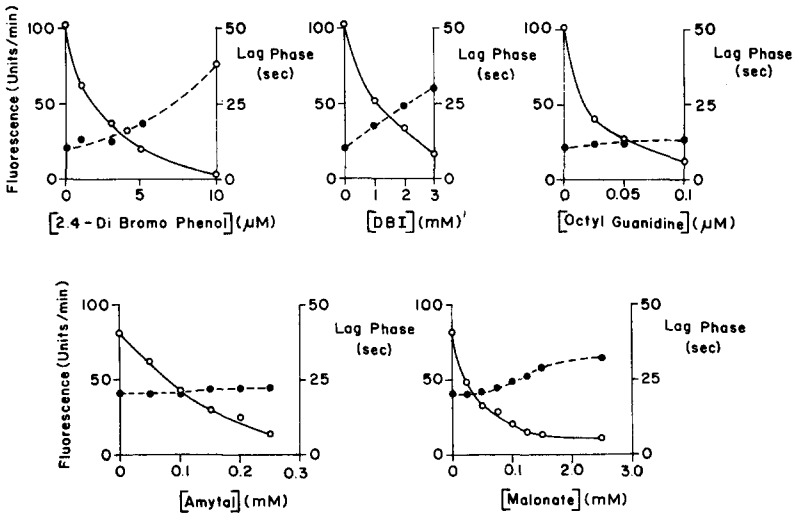

Fig. 2. Effect of 2,4-dibromophenol, DBI, octylguanidine, Amytal and malonate on the rate of the succinate-linked NAD^+ reduction. The full line (○—○) gives the rate, the dotted line (●- - -●) the lag phase. Experimental conditions as in Fig. 1.

The kinetics of cytochrome *b* reduction are shown in Fig. 4, where the sensitive double-beam spectrophotometric technique developed by CHANCE[4] was used to measure cytochrome *b* reduction at the wavelength pair 430–410 mμ. Addition of sulfide to the particles suspended in buffer caused a slight reduction of cytochrome *b*. The extent of this sulfide-reducible material varied somewhat in different preparations. However, it never exceeded more than 15% of the dithionite-reducible material. It was also observed in the presence of Amytal (1 mM) and malonate (3 mM).

Subsequent addition of succinate causes a further reduction of cytochrome *b* up to about 75% of the dithionite-reducible material.

Addition of ATP caused a further reduction without a lag phase. In contrast to the energy-linked NAD^+ reduction this ATP-dependent additional cytochrome *b*

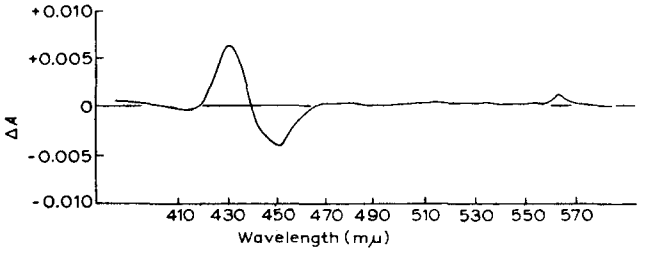

Fig. 3. Difference spectrum. Two cuvettes, each containing submitochondrial particles (protein concentration 4.2 mg/ml) suspended in 80 mM KCl, 10 mM triethanolamine, 8 mM $MgCl_2$ (pH 7.5), 5 mM succinate and 1 mM sulfide. To one cuvette was added ATP to a final concentration of 0.5 mM, to the other an amount of buffer solution which gave the same dilution.

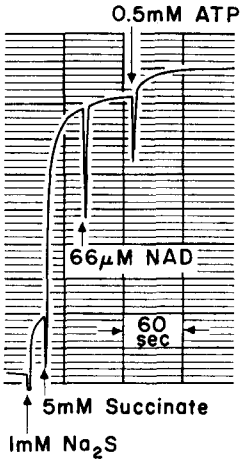

Fig. 4. Kinetics of the ATP-dependent additional cytochrome *b* reduction as measured at the wavelength pair 430–410 mμ. Protein concentration, 0.6 mg/ml.

TABLE I

EFFECT OF OCTYLGUANIDINE AND OLIGOMYCIN ON THE ATP-DEPENDENT ADDITIONAL CYTOCHROME *b* REDUCTION IN SUBMITOCHONDRIAL PARTICLES

Experimental conditions as in Fig. 4.

	Per cent inhibition	
	Additional cytochrome b reduction	*NAD^+ reduction*
Octylguanidine (μM)		
33	0	65
66	3.1	78
99	6.2	88
Oligomycin (μg/ml × 10^2)		
6.7	4.7	6.0
13.3	9.0	10.1
26.7	62.2	65.2
66.7	89.0	90.0

reduction was independent of the Mg^{2+} concentration (Fig. 5). Table I shows that this additional cytochrome *b* reduction is not inhibited by octylguanidine but that it is inhibited by oligomycin. An ATP-dependent reduction of cytochrome *b* in intact mitochondria has been observed by CHANCE[10,11], who found that this reduction was not inhibited by Amytal at concentrations which inhibited NAD^+ reduction.

DBI was shown earlier to inhibit the succinate-linked NAD^+ reduction. It also inhibited the ATP-dependent additional cytochrome *b* reduction. When measured as a function of the DBI concentration both reactions were inhibited to the same

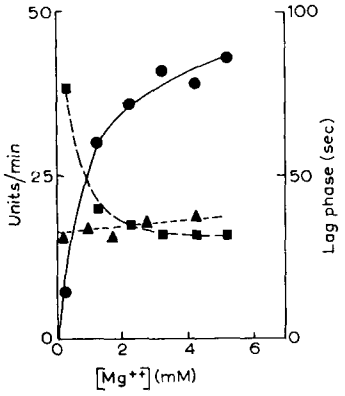

Fig. 5. Relation between the Mg^{2+} concentration and the rate of the succinate-linked NAD^+ (●—●), the lag phase in NAD^+ reduction (■— —■) and the ATP-dependent additional cytochrome *b* reduction (▲- - -▲) was measured at 430–410 mμ. Experimental conditions as in Table I.

extent (Fig. 6). Succinate oxidation was not inhibited by these concentrations of DBI.

The concentration which gave 50% inhibition of the succinate-linked NAD^+ reduction was 1.2 mM. This same concentration was found to give 50% inhibition when the system was supplemented with soluble mitochondrial factor which stimulates

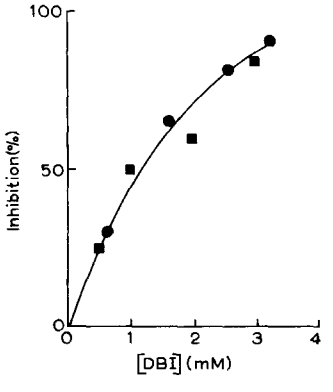

Fig. 6. Relation between DBI concentration and inhibition of succinate-linked NAD^+ reduction (■) and inhibition of the ATP-dependent additional cytochrome *b* reduction (●). Experimental conditions similar as in Table I. Cytochrome *b* reduction was measured at the wavelength pair 430–410 mμ.

the rate of the succinate-linked NAD^+ reduction[2]. In this and the following experiment with oligomycin 10 units of soluble mitochondrial factor were added to the particles (for definition of unit see ref. 2). Larger amounts of soluble mitochondrial factor did not stimulate the rate of NAD^+ reduction further. In the presence of this amount of soluble mitochondrial factor a concentration of oligomycin of $24 \cdot 10^{-2}$ μg/ml gave 50% inhibition, the same value as found in the absence of the factor (Table II).

TABLE II

INHIBITION OF THE SUCCINATE-LINKED NAD^+ REDUCTION IN THE PRESENCE AND ABSENCE OF SOLUBLE MITOCHONDRIAL FACTOR BY OLIGOMYCIN AND DBI

Experimental conditions as in Fig. 1.

Inhibitor	*Per cent inhibition*	
	No factor added	*10 units added*
Oligomycin ($\mu g/ml \times 10^{-2}$)		
12.5	25	30
25	48	54
50	86	89
75	100	100
DBI (mM)		
1.25	55	56
2.50	53	78
3.75	95	97

The effect of octylguanidine differed from those of DBI and oligomycin. Higher amounts of octylguanidine were found to be necessary in the presence of soluble mitochondrial factor for 50% inhibition. A plateau was reached which coincided with the plateau obtained in the stimulation of the succinate-linked NAD^+ reduction (Fig. 7).

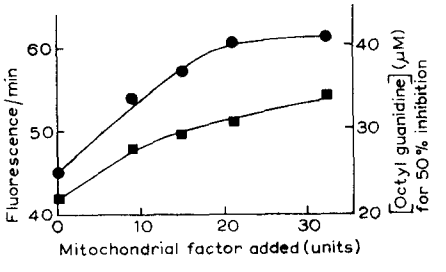

Fig. 7. Relation between stimulation of the succinate-linked NAD^+ reduction by soluble mitochondrial factor and concentration of octylguanidine needed for 50% inhibition. 1 fluorescence unit/min corresponds to 0.27 mμmoles NADH/min/mg protein/ml. Experimental conditions as in Table I.

DISCUSSION

FALCONE *et al.*[12] and PRESSMAN[13] have shown that DBI inhibits energy-transfer reactions. The first mentioned authors concluded that DBI prevents the reaction of a high-energy intermediate of oxidative phosphorylation located between the electron-transport chain and the 2,4-dinitrophenol-sensitive point. Subsequently PRESSMAN[12] showed that DBI preferentially inhibited succinate oxidation in tightly coupled mitochondria rather than the oxidation of NAD^+-linked substrates, thus demonstrating the site specificity of DBI.

The observation that DBI inhibited the energy-linked reduction of NAD^+ and cytochrome *b* to the same extent when measured as a function of the DBI concen-

tration, suggests the participation of the same site of oxidative phosphorylation in the succinate-linked NAD^+ reduction as is inhibited in succinate oxidation.

Octylguanidine on the other hand, as a member of the guanidine family shown by HOLLUNGER[14] to be an inhibitor of energy-transfer reactions, exerts its inhibition preferentially in oxidation of NAD^+-linked substrates, as shown by PRESSMAN[13,15].

Although octylguanidine inhibited the succinate-linked NAD^+ reduction, it did not inhibit the ATP-dependent additional cytochrome *b* reduction, in agreement with the site specificity of octylguanidine.

These experiments suggest strongly that the site of oxidative phosphorylation associated with cytochrome *b* is involved in the succinate-linked NAD^+ reduction. Cytochrome *b* reduction proceedes without a lag phase, which is observed in NAD^+ reduction. Cytochrome *b* reduction preceeding NAD^+ reduction is therefore not in disagreement with the kinetics. In fact, the lag phase in NAD^+ reduction may be partly due to the ATP-dependent additional cytochrome *b* reduction.

With *p*-phenylenediamine as electron donor for the energy-linked NAD^+ reduction, complete inhibition is obtained at about 0.2 μg antimycin A per ml, about the same concentration as is required for the inhibition of glutamate oxidation with potassium ferricyanide as electron acceptor in rat-liver mitochondria[16]. Forward and backward reaction are therefore equally sensitive towards antimycin when a complete passage of the antimycin-sensitive point is necessary. Despite extensive research[16,17], the exact location of antimycin A inhibition is not known. It can perhaps be best stated that it prevents the reoxidation of cytochrome *b* by cytochrome c_1, in the forward reaction and the reduction of cytochrome *b* by cytochrome c_1, in the reverse reaction. Forward and reverse reaction (with *p*-phenylenediamine as electron donor) are thus equally sensitive to antimycin A. When succinate is used as electron donor in the reverse reaction no complete passage of the antimycin A-sensitive point is necessary. Therefore, no equal sensitivity to the inhibitor in the forward and backward reaction needs to be expected. Reoxidation of cytochrome *b* by flavoprotein of the NADH dehydrogenase is not necessarily as equally sensitive to antimycin A as reoxidation by cytochrome c_1.

Figs. 1 and 2 show that both electron- and energy-transfer inhibitors influence the lag phase in the succinate-linked NAD^+ reduction. This suggests that both types of reaction contribute to the lag phase. Of considerable significance is the observation that the system needs more octylguanidine for 50% inhibition in the presence of a soluble mitochondrial factor which stimulates the rate of the succinate-linked NAD^+ reduction and takes away the lag phase in this reaction[2] (Fig. 7). This is considered as evidence that this factor reacts very near or at the octylguanidine-inhibited site. PRESSMAN[14] concluded from the slow and incomplete recovery of oxidation rate after addition of 2,4-dinitrophenol to octylguanidine-inhibited mitochondria, that octylguanidine reacts between the electron-transport chain and the dinitrophenol-sensitive point. The soluble mitochondrial factor must therefore react very near the electron-transport chain.

ACKNOWLEDGEMENTS

The author is deeply indebted to Professor Dr. B. CHANCE and Dr. R. W. ESTABROOK for their constructive criticism during the course of experimentation and preparation

of the manuscript. Octylguanidine and DBI were gifts from Dr. B. C. PRESSMAN.

REFERENCES

1 F. A. HOMMES, *Biochim. Biophys. Acta*, preceding paper.
2 F. A. HOMMES, *Biochim. Biophys. Acta*, 71 (1963) 595.
3 C. G. YANG, *Rev. Sci. Instr.*, 25 (1954) 807.
4 B. CHANCE, *Science*, 120 (1954) 767.
5 H. LÖW, H. KRUEGER AND D. M. ZIEGLER, *Biochem. Biophys. Res. Commun.*, 5 (1961) 231.
6 D. R. SANADI AND A. L. FLUHARTY, personal communication.
7 H. LÖW AND T. VALLIN, *Biochem. Biophys. Res. Commun.*, 9 (1962) 307.
8 L. PACKER AND M. D. DENTON, *Federation Proc.*, 21 (1962) 52.
9 B. CHANCE AND G. R. WILLIAMS, *Advan. Enzymol.*, 17 (1956) 65.
10 B. CHANCE, *Federation Proc.*, 21 (1962) 55.
11 B. CHANCE, *J. Biol. Chem.*, 236 (1961) 1544.
12 A. B. FALCONE, R. L. MAS AND E. SHRAGO, *J. Biol. Chem.*, 237 (1962) 904.
13 B. C. PRESSMAN, *Federation Proc.*, 21 (1962) 55.
14 G. HOLLUNGER, *Acta Pharmacol. Toxicol. Suppl. 1*, 11 (1955).
15 B. C. PRESSMAN, *J. Biol. Chem.*, 238 (1963) 401.
16 R. W. ESTABROOK, *Biochim. Biophys. Acta*, 60 (1962) 236.
17 A. M. PUMPHREY, *J. Biol. Chem.*, 237 (1962) 2384.