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MENADIOL AS AN ELECTRON DONOR FOR REVERSED OXIDATIVE PHOSPHORYLATION IN SUBMITOCHONDRIAL PARTICLES

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SUMMARY

Dithiothreitol in the presence of menadione or *N,N,N',N'*-tetramethyl-*p*-phenylenediamine provides the reducing equivalents for oxidative phosphorylation and the ATP-dependent reduction of NAD^+ in submitochondrial particles. With menadione the reaction is nearly as fast as with succinate and it is insensitive to antimycin, indicating electron entry between the first and second sites of oxidative phosphorylation. The phenylenediamine-mediated reduction of NAD^+ is nearly as fast as succinate-linked reduction and is antimycin sensitive.

INTRODUCTION

Red–ox dyes and quinones have been used extensively to introduce electrons into or withdraw them from the mitochondrial respiratory chain. One of the widely used dyes is *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) which introduces electrons at the cytochrome *c* level[1,2]. It mediates the oxidation of ascorbate with a P/O of nearly 1 in rat liver mitochondria, and also the antimycin-sensitive reduction of NAD^+ *via* reversed electron flow in submitochondrial particles[2,3]. Energy for the latter reaction can be supplied either by ATP or by oxidation.

Artificial electron carriers that introduce electrons between oxidative phosphorylation Sites I and II have been explored[4,5]. Oxidation of such carriers by oxygen should be antimycin sensitive and reversed electron flow from these to NAD^+ should be antimycin insensitive when energy is supplied by ATP. The ATP-dependent reduction of NAD^+ by ascorbate or by dihydropyrogallol ($E'_0 = -270$ mV, ref. 6) when they are mediated by menadione or pyocyanine are only partially sensitive to antimycin indicating electron entry partly between Sites I and II and partly in the cytochrome *c* region[4,5].

It was noted recently[7] that dithiothreitol stimulated the ATP-dependent reduction of NAD^+ by succinate or ascorbate–TMPD. During these experiments,

Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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it became apparent that dithiothreitol could act as an electron donor in the presence of menadione or TMPD, which led to an examination of the use of dithiothreitol ($E'_0 = -0.33$ V at pH 7, ref. 8) as an electron source for energy-linked reactions in phosphorylating electron transport particles from heavy layer bovine heart mitochondria. A preliminary report of these findings has been presented[9].

EXPERIMENTAL PROCEDURES AND DISCUSSION

Table I compares the effectiveness of succinate and several artificial electron donor systems in providing the reducing equivalents for the ATP-dependent reduction of NAD^+ with phosphorylating electron transport particles from heavy layer bovine heart mitochondria. The advantage of dithiothreitol over ascorbate as an electron donor is apparent since dithiothreitol gave much higher reaction rates. The rates with menadione and TMPD were nearly the same as with succinate. The initial rates of reduction with dithiothreitol–pyocyanine was significant as seen from Table I, but it was not maintained, and the total NAD^+ reduced under these conditions was only about 10 % of that obtained with succinate or menadione. We have noted that with some electron transport particle preparations, dithiothreitol was effective in the absence of menadione or pyocyanine. However, the initial rate was low (< 20 nmoles NADH/min per mg of protein) and it ceased after a low level of NADH had been produced.

TABLE I

ENERGY-LINKED REDUCTION OF NAD^+ BY DIFFERENT REDUCING SUBSTRATES

The reaction medium contained 0.05 M Tris–sulfate at pH 7.8 (22 °C), 3.3 mM ATP, 3.3 mM $MgCl_2$, 1 mM KCN, 3 mM NAD^+, 0.5 mg electron transport particles and 2 mg crystalline bovine serum albumin in 3.0 ml. In addition 1.67 mM dithiothreitol, 5 mM ascorbate, 66 μM menadione, 300 μM TMPD, 6.7 mM succinate and 50 μM pyocyanine were present as shown above. The assays were carried out at 38 °C using the Gilford multiple absorbance recorder for measuring the absorbance change at 340 nm. The electron transport particles were prepared in the presence of Mg^{2+} and ATP[10].

Substrate	*nmoles NADH/min per mg protein*
Succinate	84
Succinate + dithiothreitol	121
Ascorbate–menadione	39
Dithiothreitol–menadione	95
Dithiothreitol–pyocyanine	44
Ascorbate–TMPD	22
Dithiothreitol–TMPD	72

The sensitivity of the reaction mediated by the artificial donors to various inhibitors is seen in Table II. NAD^+ reduction by dithiothreitol–TMPD was completely inhibited by antimycin while that by menadione and succinate was inhibited but little (less than 15 %), indicating that the sites of electron entry from menadione

TABLE II

EFFECT OF INHIBITORS ON ATP-DEPENDENT NAD^+ REDUCTION

The reaction conditions are described under Table I. The inhibitor concentrations were 0.2 μg antimycin A, 0.2 μg rotenone and 1 μg oligomycin per mg particle protein.

Substrate	*nmoles NADH/min per mg particle protein*				
	Control	*+Antimycin*	*Control*	*+Rotenone*	*+Oligomycin*
Succinate + dithiothreitol	99	88	103	4	0
Dithiothreitol–menadione	109	93	90	5	0
Dithiothreitol–TMPD	72	0	66	4	0

and succinate were on the substrate side of the antimycin block. However, it was noted that at higher levels of menadione, the extent of inhibition increased. The inhibition of the ascorbate–menadione system by antimycin amounted to 30 %[4,5]. Thus, consistent with earlier conclusions, menadione can provide electrons at two sites, on either side of the antimycin block, depending upon the experimental conditions.

The sensitivity of the dithiothreitol–menadione system to rotenone indicates that the electrons are entering on the oxygen side of the rotenone block, possibly around ubiquinone. The reactions are dependent upon energy from ATP for the reduction of NAD^+, as shown by the sensitivity to oligomycin.

In view of the comparable rates of NAD^+ reduction with dithiothreitol *plus* menadione, and succinate and their very low sensitivity to antimycin, it was of interest to determine their relative efficiency in oxidative phosphorylation (P/O). Since menadiol is oxidized non-enzymatically, the correction for the background rate of oxidation was determined using heat-denatured particles. The appropriate background, varying from 26 to 35% of the enzymatic rate with 20–100 μM menadione and 3 mM dithiothreitol, has been subtracted before calculation of P/O. The oxidation rate of dithiothreitol alone with electron transport particles was low, amounting to less than 11 nmoles oxygen/min per mg protein. Table III shows that

TABLE III

PHOSPHORYLATION OF ADP WITH DITHIOTHREITOL–MENADIONE

The reaction mixture consisted of 0.15 M sucrose, 10 mM Tris–HCl at pH 7.4 (22 °C), 10 mM KCl, 1.5 mM ADP, 0.2 mg bovine serum albumin (dialyzed, fat free from Pentex Co.) per ml, 50 μmoles glucose, 50 units of hexokinase (Sigma III), and 5 mM succinate or 1.67 mM dithiothreitol *plus* menadione or TMPD as shown. The assay was carried out at 38 °C in the chamber of the oxygen electrode as described previously[1].

Substrate	*–Antimycin*		*+Antimycin*	
	natoms oxygen	*P/O*	*natoms oxygen*	*P/O*
Succinate, 5 mM	290	1.21	36	0.19
Dithiothreitol– 20 μM menadione	115	1.52	110	0.17
Dithiothreitol– 50 μM menadione	142	1.31	146	0.21
Dithiothreitol–100 μM menadione	140	1.17	152	0.19
Dithiothreitol–180 μM TMPD	175	0.23		

the P/O with menadione varied with its concentration. At low levels, the P/O was significantly greater than one, indicating electron entry between Sites I and II. As the menadione concentration was increased, the oxidation rate was increased slightly and the P/O decreased. In the presence of 0.2 μg antimycin/mg particle protein, the P/O with succinate was reduced to 0.19. This level of antimycin, the same as that used in the ATP-dependent NAD^+ reduction (Table II) was chosen to have as little uncoupling effect as possible, although the inhibition of oxidation was incomplete. The P/O with increasing menadione was decreased to the same level as with succinate. It is interesting to note that the oxidation of dithiothreitol–menadione was unaffected in the presence of antimycin, but the P/O declined to values similar to those with succinate, possibly indicating that while electrons enter the respiratory chain primarily between phosphorylation Sites I and II, they can be diverted with undiminished rates to a carrier between Sites II and III in the presence of the inhibitor.

The P/O for the dithiothreitol–TMPD system, which also permits electron entry between Sites II and III, was 0.23, which is similar to the values reported previously for ascorbate–TMPD using submitochondrial particles[1].

The effectiveness of dithiothreitol in assays involving reactions associated with oxidative phosphorylation has been noted several times. Besides stimulating the ATP-dependent reduction of NAD^+ by reversed electron flow[7] (see Introduction), it eliminates a lag in the reaction[12]. The activities of energy transfer factors B[13], D[14] and of the soluble ATP synthetase[15] are inhibited by organic mercurials and the inhibition is reversed by dithiothreitol. Thus, one role of dithiothreitol might be in maintaining functionally active –SH groups in the proteins.

The advantage of dithiothreitol over the previously described use of ascorbate[4] for reversed electron flow experiments with menadione as the mediator is the more predominant entry of electrons between Sites I and II at equal menadione concentration as shown by the difference in antimycin sensitivity. This is probably related to the lower oxidation–reduction potential of dithiothreitol compared to ascorbate potential, an effect distinct from maintenance of –SH groups discussed above. Although dithiothreitol–TMPD is more effective for ATP-dependent NAD^+ reduction than ascorbate–TMPD (Table I), the P/O values associated with their oxidation are not different.

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REFERENCES

1 D. D. Tyler, R. W. Estabrook and D. R. Sanadi, *Arch. Biochem. Biophys.*, 114 (1966) 239.
2 E. E. Jacobs, *Biochem. Biophys. Res. Commun.*, 3 (1960) 536.
3 H. Low and I. Vallin, *Biochem. Biophys. Res. Commun.*, 9 (1972) 307.
4 J. B. Warshaw, K. W. Lam and D. R. Sanadi, *Arch. Biochem. Biophys.*, 115 (1966) 307.
5 D. R. Sanadi, *Biochim. Biophys. Acta*, 89 (1964) 367.
6 W. M. Clark, *Oxidation Reduction Potentials of Organic Systems*, William and Wilkins, Baltimore, Md., 1960.

7 C. K. R. Kurup and D. R. Sanadi, *Biochemistry*, 7 (1968) 4483.
8 W. W. Cleland, *Biochemistry*, 3 (1964) 480.
9 W. V. Taggart, *Fed. Proc.*, 29 (1970) 403.
10 A. W. Linnane and D. M. Ziegler, *Biochim. Biophys. Acta*, 29 (1958) 630.
11 F. J. Ruzicka and F. L. Crane, *Biochem. Biophys. Res. Commun.*, 38 (1970) 249.
12 F. M. A. H. Schuurmans Stekhoven, B. P. Sani and D. R. Sanadi, *Biochem. Biophys. Res. Commun.*, 39 (1970) 1026.
13 K. W. Lam, D. Swann and M. Elzinga, *Arch. Biochem. Biophys.*, 130 (1965) 175.
14 B. P. Sani and D. R. Sanadi, *Arch. Biochem. Biophys.*, 147 (1971) 351.
15 R. J. Fisher, J. C. Chen, B. P. Sani, S. S. Kaplay and D. R. Sanadi, *Proc. Natl. Acad. Sci. U.S.*, 68 (1971) 2181.