TETRAHYDROPTERIN: REDUCTION OF CYTOCHROME C AND COUPLED PHOSPHORYLATION AT MITOCHONDRIAL SITE 3

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Received September 8,1975

Summary Incubation of rat liver mitochondria with tetrahydropterin results in ATP production with a P:O ratio of 0.85, consistent with the entry of reducing equivalents into the mitochondrial electron transport chain at cytochrome c. No evidence for an enzymatic reduction of cytochrome c was found. The reduction of either soluble or mitochondrial cytochrome c was not diminished by superoxide dismutase or anaerobic conditions, indicating that the reaction is not dependent on the autoxidation of the reduced pterin and the formation of an active species of oxygen. The experiments indicate a potential pathway for the production of ATP coupled to the oxidation of NADPH through the activity of NADPH-dependent pteridine reductases.

The ability of tetrahydropterins to stimulate mitochondrial respiration through the reduction of cytochrome c and, thus, cytochrome oxidase has been known for several years, but it was reported that the increased oxygen consumption was not coupled to ATP production (1). We have found, on the other hand, that the reduction of mitochondrial cytochrome c by 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropterin ($DMPH_4$) results in a P:O ratio of about 0.9, a value consistent with phosphorylation at site 3 of the electron transport chain (2). The details of these experiments are described herein.

The mechanism of tetrahydropterin-cytochrome c interaction has not been previously described. It is known that tetrahydropterins oxidize spontaneously to generate O_2^-, the superoxide anion (3). O_2^- can reduce cytochrome c in a reaction that is inhibited by superoxide dismutase (4). Therefore, it is of special interest that we have found that the reduction of cytochrome c by $DMPH_4$ takes place under anaerobic as well as aerobic conditions and that it is not inhibited by superoxide dismutase. Our experiments suggest that $DMPH_4$-dependent phosphorylation does not involve the intermediate formation of the superoxide anion.

METHODS

Mitochondria were isolated from the livers of male Sprague-Dawley rats by the method of Schnaitman and Greenwalt (5) except that the livers were perfused with the cold isolation medium before being removed from the animals. Mitochondrial respiration studies were carried out using a Clark electrode and 2.5-3 mg mitochondrial protein in an incubation volume of 1.0 ml at 23° C.

The medium for oxygen uptake studies was 5 mM K_2HPO_4, 10 mM tris, 20 µM EDTA, 250 mM mannitol, 10 mM $MgCl_2$, pH 7.5. Mitochondria were added to buffer, followed by the addition of 1 mM ADP. After the endogenous respiration rate was ascertained, a 25 µl sample was taken for ATP determination, and 0.4 mM $DMPH_4$ was added. A second sample was taken 1.5 min after addition of $DMPH_4$. Samples were added directly to cold 10% perchloric acid and precipitated protein was removed by centrifugation at 2°. The difference in ATP levels in the perchloric acid extracts of the first and second samples was taken to be the ATP production. ATP was measured by the method of Stanley and Williams (6).

Mitochondria were disrupted by sonicating a dilute (10 mg/ml) suspension in cold isolation medium for 10 sec. (3/8" tip, Biosonik III).

Superoxide dismutase (SOD) was prepared from bovine erythrocytes by the method of McCord and Fridovich (4). It was catalase free and had a specific activity of 1700 units/ml, a unit being defined as 0.18 absorbance units per min using the system of the above authors.

The $DMPH_4$ concentration was measured by its extinction in 0.1 M HCl at 265 nm (7). It was dissolved in 5 mM HCl or in the appropriate buffer saturated with N_2 and kept on ice. The pH of all solutions was adjusted to neutrality before use. Anaerobic spectrophotometric studies were carried out under N_2. The reduction of cytochrome c was followed at 550 nm.

RESULTS AND DISCUSSION

$DMPH_4$-dependent ATP production. Oxygen uptake by mitochondria in respiratory control states 1,2,3 & 4 increased when $DMPH_4$ was added. In all 4 respiratory control states, in the presence of $DMPH_4$, oxygen consumption ceased upon addi-

tion of 1.0 mM KCN. Figure 1 illustrates the stimulation of oxygen consumption of mitochondria by $DMPH_4$ in State 1 (respiration limited by ADP and substrate) and State 2 (respiration limited only by lack of substrate). It can be seen in the figure that the initial rate of oxygen uptake after addition of $DMPH_4$ was greater in the presence of ADP than in its absence, suggesting that this increased oxygen consumption was coupled to ATP formation. Rembold and Buff (1) also reported stimulation of mitochondrial respiration by tetrahydropterins and showed by low temperature difference spectrometry that these compounds will not reduce cytochrome b, but that the reducing equivalents enter the electron transport chain at cytochrome c. Thus, one would expect production of ATP to occur at the third phosphorylation site in the presence of $DMPH_4$. Indeed, the P:O ratio of 0.85 obtained by us (Table 1) is consistent with this concept. Under the same conditions, using α-ketoglutarate in place of $DMPH_4$, the P:O ratio was 2.2. It is not apparent why our results differ from those of Rembold and Buff (1) who report that $DMPH_4$ does not support oxidative phosphorylation.

Despite the inability of $DMPH_4$ to reduce cytochrome b (1), the presence of antimycin at concentrations of 0.05-0.12 μg per mg of mitochondrial protein in the incubation mixture resulted in decreased ATP production (Table 1) with

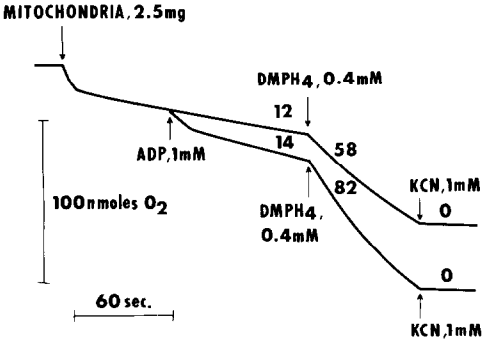

Figure 1. Uptake of oxygen by mitochondria in the presence of $DMPH_4$. Conditions are described in MATERIALS AND METHODS. The numbers represent nmoles of O_2/min; those following the addition of $DMPH_4$ refer to initial rates of O_2 consumption.

Table I. $DMPH_4$-Dependent Production of ATP in Mitochondria.

Antimycin (μg/mg protein)	ATP Produced (nmoles/mg protein)	Oxygen Consumed (nmoles/mg protein)	P:O
-	50 ± 12	59 ± 7	0.85
0.05	32 ± 4	67 ± 14	0.48
0.12	23 ± 3	70 ± 12	0.33

Conditions are described in MATERIALS AND METHODS

little or no change in oxygen consumption. Others have noted a similar effect of this inhibitor. For instance, Howland (8) reported a decrease of about 50% in the P:O ratio for rat liver mitochondria in the presence of 0.33 μg antimycin per mg of protein, and uncoupling of electron transport by antimycin has been noted in heart mitochondria and digitonin particles (9,10). Meyers and Slater (11) have also demonstrated that antimycin stimulates hydrolysis of ATP although at concentrations above 1 μM.

$DMPH_4$-dependent cytochrome *c* reduction. $DMPH_4$ has been shown to be a 2 electron donor, oxidizing first to an "active" dihydropterin reducible directly by such compounds as NADPH (12), dithiothreitol (13), or by an NADPH-dependent dihydropterin reductase (12). This "active" molecule rearranges rapidly to an "inactive" 7,8-dihydropterin, reducible, not by the above compounds but by dihydrofolate reductase (12). Table II illustrates the non-enzymatic reduction of soluble cytochrome *c* by $DMPH_4$. Since no system for regenerating reduced pteridine was present, a stoichiometric reduction would be expected, and, indeed, nearly 2 moles of cytochrome were reduced for each mole of $DMPH_4$ present. In every experiment, the reduction of cytochrome *c* was greater under anaerobic than aerobic conditions. Thus a species of active oxygen could not be participating in the reaction. These results are especially interesting because O_2^- has been reported as a product of the autoxidation of $DMPH_4$ (3), and because O_2^- can reduce cytochrome *c* (14). However, the production of O_2^- apparently de-

TABLE II. Reduction of Cytochrome c by $DMPH_4$.

Aerobic	Anaerobic	pH	SOD	Cyt c Reduced (nmoles)
+	-	7.5	-	97
+	-	7.5	+	96
-	+	7.5	-	100
+	-	8.5	-	97
+	-	8.5	+	94
-	+	8.5	-	100

The final volume of 1.0 ml contained 125 nmoles cytochrome c, 50 nmoles $DMPH_4$ and, when present, 25 units SOD. Buffer was 0.1 M tris-HCl, pH 8.5 or the oxygen uptake medium described in MATERIALS AND METHODS, pH 7.5. The reaction was started with $DMPH_4$.

Table III. The Reduction of Soluble Cytochrome c by by $DMPH_4$ in the Presence of Mitochondria.

Inhibitor KCN	Inhibitor N_2	Mitochondria	Sonicate	Seconds for 75% Reduction
+	-	-	-	29 ± 2
+	-	+	-	39 ± 3
+	-	-	+	>60
-	+	-	-	30 ± 4
-	+	+	-	40 ± 2
-	+	-	+	>60

The final volume of 2.0 ml of oxygen uptake medium contained 76 nmoles cytochrome c, 38 nmoles $DMPH_4$, 0.34 mg mitochondrial or mitochondrial sonicate protein, and 1 mM KCN. The reference cuvette contained the same reagents as the sample cuvette except that cytochrome c and $DMPH_4$ were omitted. The reaction was started with $DMPH_4$.

pends on pH and is increased at high pH. Our experiments were carried out at pH 7.5 when rapid production of O_2^- would not be expected. Confirmatory results were obtained when superoxide dismutase (SOD) was included with the cytochrome c and $DMPH_4$. Addition of the enzyme did not decrease the amount of cytochrome c reduced (Table II), nor was the rate of reduction found to be affected. Exam

ination of the spectra produced by the reduction of intramitochondrial cytochrome c with $DMPH_4$ also revealed no alterations after the addition of SOD.

We have found no evidence for an enzymatic reduction of cytochrome c by $DMPH_4$. The rate of reduction of added cytochrome c was not increased in the presence of mitochondria or mitochondrial sonicate, as seen in Table III. In fact, a decrease was found which was greater for the sonicate than for the intact mitochondria. The cause of this phenomenon is unknown. In these experiments, identical results were obtained whether cyanide or anaerobic conditions (N_2) were used to prevent the reoxidation of cytochrome c over cytochrome oxidase. Although not shown in the Table, the inclusion of 7,000 x g supernatant (post mitochondrial) fraction did not enhance cytochrome c reduction but, rather, decreased it. The results in this Table also illustrate that the increase in total reduction of cytochrome c by a given amount of $DMPH_4$ always observed under anaerobic conditions (Table II) is not a consequence of a faster initial rate of reduction, but rather an increased rate later in the reaction. This probably results from the lack of autoxidation, and hence conservation, of $DMPH_4$ under anaerobic conditions which would tend to manifest itself later in the reaction.

These experiments raise the question of the possible contribution of pterins as cofactors in the oxidation of cytoplasmic NADPH via mitochondrial cytochrome c and in the formation of ATP over mitochondrial site 3 in intact cells.

Acknowledgments: This work was supported, in part, by a research grant (HD 08159) and by Training grant (T01 HL 05536-12) from the NIH. We thank Ms. Gemma Seo for excellent technical assistance.

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