OXIDATION OF EXTERNAL DPNH BY MITOCHONDRIA FROM HUMAN AND RAT SKELETAL MUSCLE *

Rune Hedman, Eva M.Suranyi, Rolf Luft, and Lars Ernster

Wenner-Gren Institute, University of Stockholm, and Department of Endocrinology and Metabolism, Karolinska Sjukhuset, Stockholm, Sweden

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The reaction mechanism by which the oxidation of extramitochondrial reduced diphosphopyridine nucleotide (DPNH) is linked to the mitochondrial respiratory chain has been the subject of many investigations (Lehninger, 1951, 1955; Ernster et al., 1955; Ernster, 1956, 1959; Maley, 1957; Devlin and Bedell, 1960; Jacobs and Sanadi, 1960; Borst, 1962). Several alternative pathways have been considered (Bücher and Klingenberg, 1958; Estabrook and Sacktor, 1958; Dickens et al., 1959; Conover, 1961; Boxer and Devlin, 1961; Borst, 1961), but the question of their physiological relevance has not yet been settled. In this paper we wish to report preliminary studies relating to this problem, carried out with isolated rat and human skeletal muscle mitochondria. It is demonstrated that addition of cytochrome c greatly enhances the aerobic oxidation of external DPNH by these mitochondria, and gives rise to a respiratory rate which exceeds that observed with Krebs cycle intermediates or with glycerol-1-phosphate. Amytal, antimycin A and the fish-poison, Rotenone (which acts on mitochondrial electron transport in an Amytal-like manner [Lindahl and Öberg, 1961; Öberg, 1961; Ernster et al., 1962]), exert an inibition of the cytochrome c-induced DPNH oxidation which is partial in the case of human muscle and almost complete in the case of rat muscle mitochondria. In both cases the respiration is virtually devoid of coupled phosphorylation.

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A stimulating effect similar to that of cytochrome c is obtained with certain coenzyme Q homologues. Below the relevant data are presented briefly.

Mitochondria from skeletal muscle were prepared in the Chappell and Perry (1954) "Tris-KCl" medium as described elsewhere (Azzone et al., 1961). Respiration was recorded polarographically using a stationary platinum electrode with a rotating cuvette at 22°C. The composition of the reaction mixtures is indicated in the legends. Phosphate uptake was estimated by the P^{32} distribution method described by Lindberg and Ernster (1955). Protein was determined by the biuret method. A sample of coenzyme Q_0 was obtained by the courtesy of Dr. K. Folkers.

In Table 1 data are summarized on the respiratory rates with pyruvate + malate, glycerol-1-phosphate and DPNH as substrates in the absence and

Table 1.

Respiratory rates of skeletal muscle mitochondria with pyruvate + malate, glycerol-1-phosphate and DPNH as substrates in the absence and presence of added cytochrome c.

The polarographic cuvette contained 20 mM Tris buffer (pH 7.4), 40 mM KCl, 6.4 mM $MgCl_2$, 5 mM P_i (pH 7.4), 2 mM ADP, rat muscle mitochondria (1.8 mg protein) or human muscle mitochondria (3.0 mg protein), and, when indicated, 10 mM pyruvate + 10 mM l-malate, 5 mM glycerol-1-phosphate, 1 mM DPNH and 0.015 mM cytochrome c. Total volume 1 ml.

Substrate	Cyt.c	RAT	HOMO
		µatoms of oxygen/min/g protein	
Pyruvate + malate	-	39	17
" "	+	63	17
Glycerol-1-phosphate	-	47	9
"	+	54	10
DPNH	-	9	3
"	+	97	28

presence of cytochrome c. The oxidation of the first two substrates was only slightly stimulated by cytochrome c with the rat mitochondria, and not at all with the human. By contrast, an almost ten-fold respiratory stimulation by cytochrome c occurred with DPNH as substrate in both cases. The rate of oxygen uptake so obtained markedly exceeded those observed with the two other substrates. It may be added that pyruvate + malate gave the highest respiration among Krebs cycle metabolites.

Table II shows data concerning respiratory control and P/O ratio of rat skeletal muscle mitochondria for the oxidation of DPNH in comparison with other substrates. Respiration was greatly stimulated by inorganic phosphate (P_i) and ADP in the case of pyruvate + malate, and the P/O ratio was close to the generally accepted maximum of 3. With glycerol-1-phosphate, there was a less pronounced but still well-marked respiratory stimulation by P_i and ADP, and the P/O ratio was 1.5. These data are in agreement with those of Klingenberg and Schollmeyer (1960) obtained with the same material, except that in our experiments no serum albumin or ATP was required to demonstrate a respiratory control with glycerol-1-phosphate. In contrast with the findings with the above two substrates, no effect of P_i and ADP was observed on the oxidation of DPNH measured in the presence of cytochrome c, and the P/O ratio was only 0.2. When cytochrome c was omitted, however, the low residual respiration gave rise to a P/O ratio of 1.2. Yet, omission of cytochrome c resulted in a definite decrease in the absolute amount of phosphate esterified, indicating that the cytochrome c-induced respiration was not completely devoid of coupled phosphorylation.

Fig. 1 illustrates the effects of Amytal, Rotenone and antimycin A on the oxidation of DPNH in the presence of cytochrome c. With the rat mitochondria the three agents inhibited the respiration by about 90%, whereas with the human mitochondria an inhibition of not more than 30% was observed. In complementary experiments it was found that with pryuvate + malate as substrate respiration was completely inhibited in both cases by

Table II.

Respiratory control and P/O ratio of rat skeletal muscle mitochondria with various substrates.

Conditions as in Table 1.

Substrate	Cyt. c	Additions: none		P_i		P_i + ADP		
		oxygen, μatoms /min /g protein		oxygen, μatoms /min /g protein		oxygen, μatoms /min /g protein	P_i uptake, μmoles /min /g protein	P/O
Pyruvate + malate	+	9		14		53	145	2.7
Glycerol-1-phosphate	+	28		29		36	53	1.5
DPNH	+	81		84		81	19	0.2
DPNH	−					9	11	1.2

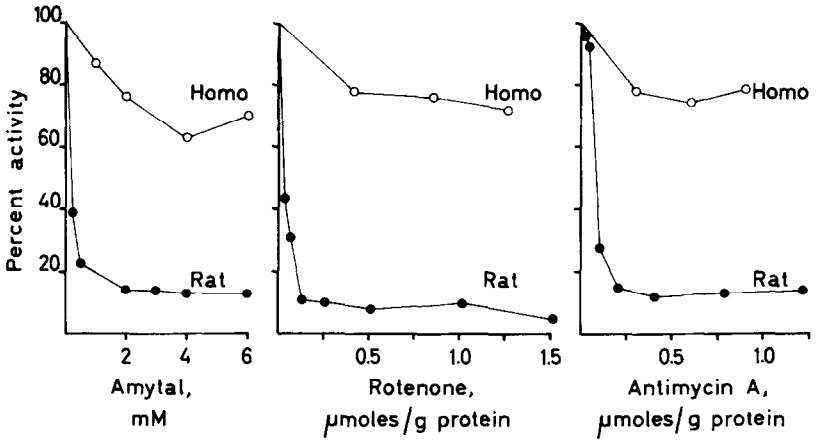

Fig. 1. Effect of Amytal, Rotenone and antimycin A on cytochrome c-stimulated DPNH oxidation in skeletal muscle mitochondria.

Conditions as in Table 1. The desired amount of each inhibitor was added to the medium prior to the addition of the mitochondria. Rotenone and antimycin A were added in the form of 0.01 ml ethanol solution.

all three agents, whereas with glycerol-1-phosphate antimycin A was inhibitory but not Amytal and Rotenone.

Attempts were made to replace cytochrome c by various quinones. As shown in Table III, coenzyme Q_2 stimulated DPNH oxidation with both the rat and the human mitochondria to a level of about one-half of that reached with cytochrome c. Coenzyme Q_0 gave a markedly less but still a definite stimulation, whereas the higher coenzyme Q homologues tested were inactive. Vitamin K_3, vitamin $K_{2(20)}$ and α-tocopherol also gave negative results. Similar observations with the coenzyme Q homologues have been reported by Jacobs and Crane (1960) with mitochondria from rat liver.

Table III.

Stimulation of DPNH oxidation in skeletal muscle mitochondria by coenzyme Q homologues.

The coenzyme Q homologues were added in a final concentration of 10^{-5}M. Other conditions as in Table I.

Additions	RAT	HOMO
	Stimulation of DPNH oxidation, times	
Cytochrome c	9.2	9.3
Coenzyme Q_0	3.3	2.0
" Q_2	5.5	4.0
" Q_6	no stim.	-
" Q_9 or Q_{10}	no stim.	no stim.

In further attempts to stimulate the oxidation of DPNH, catalytic amounts of acetoacetate, oxaloacetate (+ crystalline malic dehydrogenase), or glycerol-1-phosphate (+ purified glycerol-1-phosphate dehydrogenase) were added. The rationale of these additions was to test whether the oxidation of

external DPNH by the mitochondria could be promoted by the acetoacetate-β-hydroxybutyrate (Devlin and Bedell, 1960), oxaloacetate - malate (Borst, 1962), or dihydroxyacetone phosphate - glycerol-1-phosphate (Bücher and Klingenberg, 1958; Estabrook and Sacktor, 1958) cycles, as has been suggested in the literature. Thus far, however, we have obtained no indication of an operation of these pathways under the conditions employed.

It is concluded that skeletal muscle mitochondria contain a highly active oxidation pathway for external DPNH whose operation is dependent on added cytochrome c. This pathway differs from that involved in the oxidation of intramitochondrial DPNH in that it lacks both respiratory control, and, to a large extent, coupled phosphorylation. It also differs from the earlier described pathway for the oxidation of external DPNH with rat liver mitochondria (Lehninger, 1955; Ernster et al., 1955,1962; Ernster, 1956,1959), in that it is sensitive to Amytal, Rotenone and antimycin A, a feature which is especially striking with the skeletal muscle mitochondria from rat. The difference between the rat and human skeletal muscle mitochondria with respect to the action of these inhibitors is puzzling and requires further studies.

Because all cytochrome c in muscle, and in animal tissues in general, appears to be located in the mitochondria (Schneider, 1959; Schollmeyer and Klingenberg, 1962), the physiological significance of the pathway here described in linking the oxidation of extramitochondrial DPNH to the mitochondrial respiratory chain remains unclear. However, the finding that certain coenzyme Q homologues can substitute for cytochrome c, together with the recent demonstration of extramitochondrial coenzyme Q by Leonhäuser et al. (1962), may open new lines of approach to this problem.

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