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Mitochondria catalyze the reduction of NAD by reduced methylviologen

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Mitochondria from beef heart and yeast catalyze the reduction of NAD to NADH at the expense of reduced methylviologen (MV^{+}). Based on protein the specific activity of mitochondria for this reaction is about 10–20-times higher than the consumption of oxygen in the presence of succinate or NADH. In ^2H_2O buffer (4*S*)-[4-^{2}H]NADH is formed in high enantiomeric excess if the reduced methylviologen is electrochemically regenerated.

Mitochondria; Methylviologen; NADH formation; Stereospecificity; NADH deuteration

1. INTRODUCTION

Recently we observed [1–3] that many different cells catalyze the reaction

$$NAD(P) + 2MV^{\cdot+} + H^{+} \longrightarrow NAD(P)H + 2MV^{2+}$$

This means NAD or NADP is enzymatically reduced at the expense of reduced methylviologen ($MV^{\cdot+}$) which is oxidized to MV^{2+}. However, among different organisms, the activity varies by several orders of magnitude and for the same organism it may depend heavily on growth conditions [3].

This type of reaction is of interest from several points of view: (i) what is the nature of the enzymes catalyzing this electron transfer? (ii) the reaction can be applied to the regeneration of NAD(P)H in the form of electro-enzymatic or electro-microbial reductions aiming at the preparation of chiral products [1–3]; and (iii) due to the fact that $MV^{\cdot+}$ seems to be able to enter into intact cells there may be a chance to influence the redox status of a cell by a defined external redox potential in the form of a fixed ratio $MV^{\cdot+}/MV^{2+}$ without offering the cell a metabolizable substrate. During our studies it turned out that the above mentioned reaction is also catalyzed by mitochondria isolated from yeast as well as from beef heart. In ^2H_2O buffer (4*S*)-[4-^{2}H]NADH is formed. To the best of our knowledge until now this reaction of mitochondria has not been described.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

Pyridine nucleotides, bovine serum albumin, enzymes and most other biochemicals were obtained from Boehringer, Mannheim; Zymolyase-5000 from Seikagaku Kogyo Co. Ltd., Tokyo; and methylviologen from Ega-Chemie, Steinheim. All other chemicals were purchased from Merck, Darmstadt. Solutions of 20 mM $MV^{\cdot+}$ were prepared in 0.1 M Tris-acetate buffer, pH 7.0, by the electrochemical reduction described [2,3] and kept under strict exclusion of oxygen.

2.2. *Mitochondria*

Candida utilis DSM 70167 was grown in a 16 l fermentor aerated with 2 l air per min (Eschweiler,

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Kiel) at 30°C in a medium containing 20 g glycerol, 15 g peptone (from meat; pept.), 3 g yeast extract (Difco), 5 g NaCl, 40 mg $FeSO_4$, 4 mg riboflavin/l deionized water, pH 6.0. The cultures were grown up to late log phase with a doubling time of 16 h and harvested at 48000 × *g* at 4°C. Mitochondria from beef heart prepared according to Smith [4] were a gift from Dr Engel, University of Munich. Mitochondria from *C. utilis* were prepared by the protocol of Daum et al. [5]. Both types of mitochondria were suspended in a buffer containing 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 0.5 mM EDTA and 0.1 mM phenylmethanesulfonyl chloride.

2.3. *Enzyme assay*

Unless otherwise stated the 0.2 cm cuvettes for the standard assay contained in a total volume of 0.325 ml of 0.1 M Tris-acetate buffer, pH 7.0, 2.5 mM NAD(P), 2.6 mM $MV^{\cdot+}$ and 0.02–0.05 mg protein of the mitochondrial preparations. The tests were performed in an anaerobic chamber (Coy Laboratories, Ann Arbor) under an atmosphere of 5% hydrogen in nitrogen by measuring the change of absorption at 730 nm. At this wavelength a solution of 2.6 mM $MV^{\cdot+}$ showed a millimolar extinction coefficient of 1.53. All solutions were made oxygen-free by repeatedly evacuating and gassing with nitrogen. One unit of enzyme activity is defined as the reoxidation of 2 μmol $MV^{\cdot+}$ per min and corresponds to the formation of 1 μmol NADH.

Protein was determined by the method of Read and Northcote [6].

2.4. *Measurement of respiration*

The oxygen consumption of mitochondria was measured with a Clark-type electrode in a water-jacketed chamber at 30°C according to [7]. The test solution contained 0.225 M sucrose, 10 mM potassium phosphate buffer (pH 7.5), 5 mM $MgCl_2 \cdot 6H_2O$, 20 mM KCl, and 10 mM Tris-HCl (pH 7.5) and 5 mM succinate or 0.56 mM NADH as the substrate.

The total cytochrome content of beef heart mitochondria was determined according to Azzone et al. [8] and that of *Candida utilis* as described by Ohnishi et al. [9].

3. RESULTS

3.1. *The methylviologen-dependent NAD reductase activity*

The applied mitochondria from beef heart and *C. utilis* showed an oxygen consumption of 0.2 and 0.3 μmol/min per mg protein if succinate was applied and 0.3 and 0.2 μmol, respectively if NADH was added to the preparations. The total cytochrome content was 1.3 and 1.8 nmol/mg protein.

A typical experiment with $MV^{\cdot+}$ is shown in fig.1. Under strict oxygen exclusion there is no disappearance of $MV^{\cdot+}$ in the presence of mitochondria. A small but fast decrease of absorption is observed if $MV^{\cdot+}$ and NAD are mixed followed by a very slow diminution of absorption. In the presence of NAD and mitochondria corresponding to 0.04 mg protein $MV^{\cdot+}$ disappears continuously. In separate experiments it had been confirmed that a corresponding amount of NADH is formed.

Table 1 reveals the initial rates of the reaction catalyzed by mitochondria from *C. utilis* and beef heart. The rates are 10–30-times higher than the respiration activities of mitochondria and about 100-fold of those for the methylviologen-dependent NAD reductase activities found in a series of crude extracts of yeasts and bacteria [1–3]. The mitochondria are fairly specific for NAD; NADP is hardly reduced (table 1). After the disintegration of beef heart mitochondria the activity increased about 3-fold. This effect may be due to a diffusion barrier of the mitochondrial membrane for either $MV^{\cdot+}$ and/or NAD. Thus the kinetic parameters of the reaction were determined. The dependency of the activities on the concentration of $MV^{\cdot+}$ as well as that of NAD followed simple Michaelis-Menten kinetics (not shown). For ultrasonically disintegrated beef heart mitochondria, the K_m value obtained from Lineweaver-Burk plots for $MV^{\cdot+}$ turned out to be 1.5 and for NAD 0.4 mM. The K_m values for $MV^{\cdot+}$ did not differ much for whole or disintegrated mitochondria. Nevertheless, in the preparation of beef heart mitochondria the V_{max} value increased 2–3-fold as a result of the disintegration.

From a practical point of view the mitochondria were tested under conditions of continuous

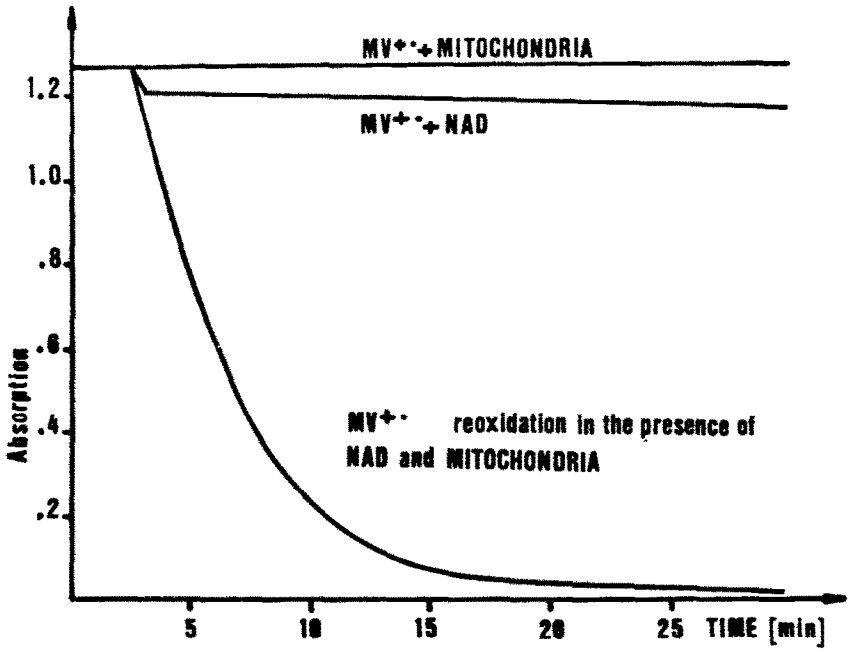

Fig.1. Reoxidation of 3.6 mM methylviologen ($MV^{\cdot+}$) in the presence of 2.5 mM NAD catalyzed by beef heart mitochondria corresponding to 0.04 mg protein and two control experiments.

regeneration of a constant concentration of the methylviologen cation radical in an electrochemical cell [1,2]. In Tris-acetate buffer, the activity of beef heart mitochondria was relatively stable. Under working conditions for 20 h, 35% of the original activity remained. In potassium phosphate buffer, however, the activity was not stable.

Table 1

Activity of the NAD-dependent reoxidation of reduced methylviologen by mitochondria from beef heart and *C. utilis* in the presence of different buffers

Buffer (pH 7.0)	Mitochondria from			
	Beef heart		*C. utilis*	
	Without U.D.	With U.D.	Without U.D.	WithU.D.
100 mM Tris-acetate	2.7[a]	8.4	2.1	4.0
10 mM Tris-acetate	3.7	8.0	1.2	3.0
100 mM Tris-sulfate	3.0	7.6	1.1	n.d.
10 mM Tris-sulfate	3.7	6.0	0.8	n.d.
100 mM potassium phosphate	2.0	7.0	n.d.	n.d.
10 mM potassium phosphate	3.0	8.0	n.d.	n.d.

[a] With NADP as electron acceptor the activity was < 0.1

Values expressed as μmol NADH formed/mg protein per min. U.D., ultrasonic disintegration; n.d., not determined

3.2. *Stereochemical course of the NADH formation and inhibitor studies*

In order to determine the stereochemical course of the NAD reduction NADH was prepared electro-enzymatically in ^2H_2O buffer. The deuterated NADH, obtained after 4 h (118 μmol), was isolated and purified according to Haid et al. [10]. As can be seen from fig.2 according to the assignment of Arnold et al. [11] reduction of NAD in ^2H_2O buffer catalyzed by beef heart mitochondria at the expense of electrochemically regenerated $MV^{\cdot+}$ led to almost stereochemically pure (4*S*)-[4-^{2}H]NADH. This is in agreement with Von Jagow and Klingenberg [12] who found *S*-specificity in the dehydrogenation of stereospecifically ^{3}H-labelled NADH.

Fig.2. NMR spectra of the hydrogen atoms of the dihydropyridine ring at C-4 of an authentic sample of NADH and that obtained by methylviologen-dependent reduction of NAD catalyzed by an extract of beef heart mitochondria. An electrochemical cell [1] contained in a volume of 7 ml, 0.1 M Tris-acetate buffer, p^{2}H 7.0, 3.3 mM methylviologen, 2.8 mM NAD and 100 μl extract (1.8 mg dry wt). According to Arnold et al. [11] the signals at 2.67 and 2.71 ppm represent the pro-*S* and pro-*R* proton, respectively.

The electron transfer from $MV^{\cdot+}$ to NAD in mitochondria may be catalyzed by some of the electron carriers of the respiratory chain. To get some insight into the components involved, experiments with known electron transport inhibitors were performed. Rotenone and amytal block the electron transfer from NADH to coenzyme Q [13]. Antimycin A inhibits the transfer of electrons from cytochrome *b* to *c* [14]. Using beef heart mitochondria none of these inhibitors blocks the electron transfer from $MV^{\cdot+}$ to NAD more than 10–20% at concentrations which inhibit the electron transport from NADH to oxygen by more than 90% (not shown). Thus, it seems that the site of the component of the electron transport chain of mitochondria which catalyzes the methylviologen-dependent NAD reduction is positioned before the blocks caused by the above mentioned inhibitors. However, it has to be established whether enzymes of the respiratory chain are involved or other enzymes of the mitochondria.

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