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

I. KINETIC STUDIES OF THE REACTION

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SUMMARY

The succinate-linked NAD^+ reduction in sonic particles derived from beef-heart mitochondria has been described. The reaction showed an absolute requirement for Mg^{2+}. K_m values for succinate, Mg^{2+} and NAD^+ have been determined. A lag phase in NAD^+ reduction was observed which has been related to the link of energy and electron-transfer reactions. The pH optimum of the reaction was found to be at pH 8.5–9.0.

INTRODUCTION

Considerable attention has been paid in recent years to the reversal of oxidative phosphorylation, the succinate-linked NAD^+ reduction. CHANCE AND HOLLUNGER[1–7], CHANCE AND HAGIHARA[8,9], KLINGENBERG *et al.*[10,11] and AZZONE *et al.*[12], have studied this reaction in intact mitochondria of various origin, while LÖW *et al.*[13,14], CHANCE AND FUGMAN[15], SANADI *et al.*[16–18] and HOMMES[19,20] studied the reaction in submitochondrial particles. In a previous communication[19] some properties of the energy-linked reduction of NAD^+ in submitochondrial particles derived from beef-heart mitochondria have been described. Compared to the same reaction in intact mitochondria some differences were observed, namely, a pronounced lag phase in NAD^+ reduction, also observed by SANADI AND FLUHARTY[17] and by LÖW AND VALLIN[14], and an absolute requirement for Mg^{2+} for this reaction.

This communication describes an extension of these studies.

EXPERIMENTAL

Beef-heart mitochondria and submitochondrial particles were prepared as described earlier[20]. The succinate-linked NAD^+ reduction was measured fluorometrically in a medium containing 80 mM KCl, 10 mM triethanolamine·HCl and 3 mM $MgCl_2$ (pH 7.4). NADH and succinate oxidation were inhibited by addition of Na_2S to a

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final concentration of 1 mM. The succinate and protein concentrations were, respectively, 5 mM and about 0.5 mg/ml. NAD^+ and ATP concentrations are indicated in the figure legends.

ATPase measurements were carried out employing the pH method described by NISHIMURA *et al.*[21]. Adenine nucleotides were determined by the fluorometric method of ESTABROOK AND MAITRA[22].

O_2 consumptions were measured polarographically according to the method of CHANCE AND WILLIAMS[23]. Protein determinations were carried out by the biuret method using bovine serum albumin as a standard.

RESULTS

Lag phase

As reported earlier[19,20] a pronounced lag phase in the energy-linked NAD^+ reduction was observed. This lag phase is defined as the time between addition of ATP, added as last component of the reaction system to start the reaction and the

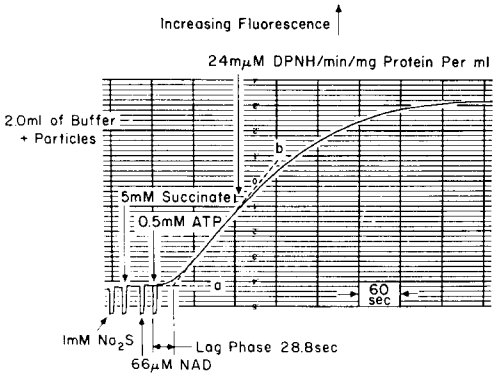

Fig. 1. Kinetics of the succinate-linked NAD^+ reduction in sonic particles from beef-heart mitochondria and definition of the lag phase. The base line *a* is extrapolated and the time between addition of ATP and the intersection of the extended base line (a) and the extrapolated linear part of the fluorescence trace (b) is taken as the lag phase.

intersection of the extended base line and the extrapolated linear part of the fluorescence trace (Fig. 1).

This lag phase was dependent on ATP. Table I shows the results of experiments in which the order of the addition of the different components of the reaction system was varied. As can be seen no lag phase was observed when the system was preincubated with ATP. Similar results were observed when *p*-phenylenediamine was used as electron donor. AZZONE AND ERNSTER[24] have suggested that succinate oxidation needs high-energy intermediates of oxidative phosphorylation for maximal activity. By the use of *p*-phenylenediamine as electron donor in the energy-linked NAD^+ reduction the succinate dehydrogenase (EC 1.3.99.1) is bypassed. Nevertheless, a lag phase was observed when *p*-phenylenediamine was used as electron donor[14]. This ruled out the location of the lag phase at succinate dehydrogenase

TABLE I

Submitochondrial particles suspended in 2.0 ml of buffer to a final protein concentration of 0.75 mg/ml. The different components of the reaction system were added in the order as indicated to the following final concentrations: succinate 5 mM; Na_2S, 1 mM; NAD^+, 66 μM; ATP, 0.5 mM. A lag phase of 0 sec means that within experimental error (1 sec) no lag phase is detectable.

Order of additions				*Lag phase (sec)*	*Rate (mμmoles NADH per min/mg/ml)*
1	*2*	*3*	*4*		
S^{2-}	Succinate	NAD^+	ATP	45	30
Succinate	S^{2-}	ATP	NAD, 2 min after ATP	0	19.5
S^{2-}	NAD^+	ATP	Succinate, immediately after ATP	40	27
S^{2-}	NAD^+	ATP	Succinate, 2 min after ATP	0	20
S^{2-}	ATP		NAD^+ + succinate, immediately after ATP	38	24
S^{2-}	ATP		NAD^+ + succinate, 2 min after ATP	0	20

and suggested that its location had to be sought at energy or electron-transfer reactions between cytochrome *b* and NAD^+. Further evidence for this hypothesis was obtained by the use of the coenzyme Q_1–ascorbate system as electron donor recently described by SANADI[18]. Fig. 2A shows the kinetics of this reaction when ATP was added as last component to start the reaction. A lag phase of 38 sec was observed. When the reaction was repeated with preincubation with ATP, no lag phase was apparent (Fig. 2B).

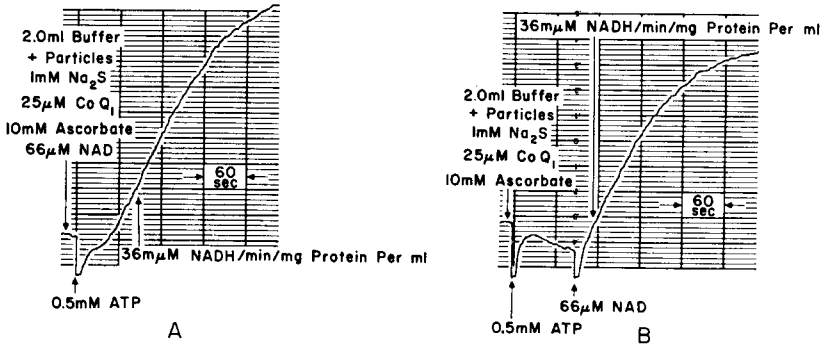

Fig. 2. Kinetics of the energy-linked NAD^+ reduction with coenzyme Q_1–ascorbate as electron donor. Protein concentration, 0.45 mg/ml. A: ATP added as last component to start the reaction. B: NAD^+ added as last component to start the reaction after 1.5 min of preincubation with ATP.

No lag phase was observed when the particles were pretreated with NADH. To the particles suspended in buffer in the presence of 5 mM succinate, 66 μM NADH were added. At the moment that this NADH was oxidized, 1 mM sulfide was added and immediately afterwards 0.5 mM ATP. NAD^+ reduction proceeded without a lag at a rate of 47 mμmoles NADH/min/mg protein/ml. When the reaction was carried out in the usual way, *i.e.*, with ATP added as last component to start the

TABLE II

Experimental conditions similar as in Table I, except for the Mg^{2+} concentration. Samples were withdrawn at the times indicated and deproteinized by trichloroacetic acid. After centrifugation of the precipitated protein, the neutralized samples were analyzed for adenine nucleotides according to ESTABROOK AND MAITRA[22].

Time after ATP addition to a final concentration of 0.5 mM	*Percentage of adenine nucleotide*											
	ATP	*ADP*	*AMP*	*ATP*	*ADP*	*AMP*	*ATP*	*ADP*	*AMP*	*ATP*	*ADP*	*AMP*
30 sec	81.5	17.4	1.1	76.8	17.3	5.9	81.3	11.8	6.8	84.2	15.8	0
2 min	54.3	34.3	10.4	60.5	29.6	9.9	64.2	23.9	11.9	78.8	16.4	0.8
4 min	39.3	35.3	25.5	42.9	35.1	22.0	53.7	31.9	14.3	53.4	37.1	9.5
6 min	27.5	36.1	36.8	30.6	42.1	27.4	39.2	42.2	18.6	43.6	41.0	15.4
8 min	22.0	33.0	45.1	25.8	39.6	34.6	31.8	43.4	24.4	36.1	41.9	22.0
15 min	13.5	24.3	62.2	10.6	29.8	59.6	20.3	41.1	34.4	23.5	49.7	26.8
Mg^{2+} concentration (mM)	1			3			10			20		
Lag phase in NAD^+ reduction (sec)	19.2			14.4			9.5			9.0		
Rate of NAD^+ reduction (mμmoles NADH/min/mg protein/ml)	12			24			26			26		
Percentage of NAD^+ reduced after 15 min reaction time	26			49			78			72		

reaction, a lag phase of 22 sec was observed, while the reaction proceeded at a rate of 45 mμmoles NADH/min/mg protein/ml. When sulfide was added 2 min after complete oxidation of NADH, and then ATP (0.5 mM) a lag phase of 13 sec was observed in NAD reduction, while the rate remained unchanged.

Magnesium

The influence of the Mg^{2+} concentration on the lag has been described earlier[19]. Not only the lag decreased but the rate of NAD^+ reduction increased with increasing Mg^{2+} concentration. The K_m of the reaction in the presence of Mg^{2+} was found to be 3 mM (Fig. 3).

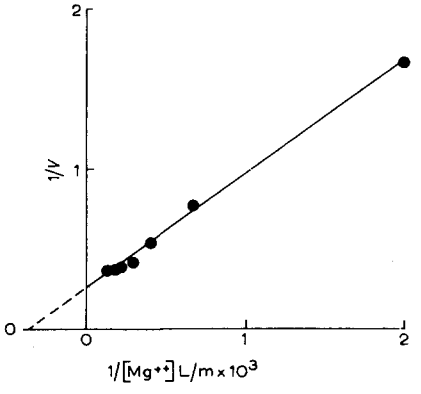

Fig. 3. Lineweaver–Burk plot of the maximal velocities observed during the succinate-linked NAD^+ reduction *versus* Mg^{2+} concentration. Experimental conditions as in Table I.

The ATP concentration in this experiment was 0.5 mM. One function of Mg^{2+} may be to complex ATP, presumably at a 1:1 ratio. Maximum activities were, however, observed at a much higher $[Mg^{2+}]$:[ATP] ratio. In order to investigate this phenomenon, further samples were withdrawn at different times during the reaction and analyzed for adenine nucleotides. The results are presented in Table II. The appearance of AMP indicates the presence of a rather strong adenylate kinase (EC 2.7.4.3) reaction, which is apparently inhibited by a high Mg^{2+} concentration (see DISCUSSION). The demonstration of the presence of the adenylate kinase reaction confirms the suggestion by Löw *et al.*[13], who found that in some submitochondrial preparations ATP could be replaced by ADP, although the rates were much lower than with ATP.

K_m of succinate

The K_m of the reaction for succinate was found to be 0.2 mM (Fig. 4). It is noteworthy, as is indicated in Table III, that the lag phase was dependent on the succinate concentration.

K_m of NAD^+

A K_m of the reaction of $4 \cdot 10^{-5}$ M for NAD^+ was found. This value was constant

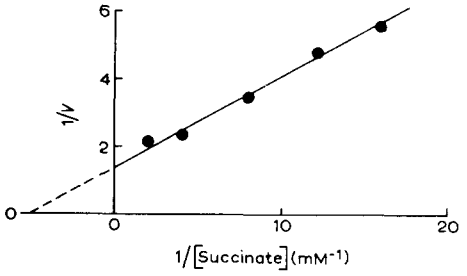

Fig. 4. Lineweaver–Burk plot of the maximal velocities observed during the succinate-linked NAD^+ reduction *versus* succinate concentration. Experimental conditions as in Table I.

TABLE III

EFFECT OF SUCCINATE CONCENTRATION ON THE LAG PHASE IN NAD^+ REDUCTION IN SUBMITOCHONDRIAL PARTICLES

Experimental conditions as in Table I. The definition of the lag phase is given in the text and in Fig. 1.

Succinate concentration (mM)	*Lag phase (sec)*
0.063	30.4
0.083	25.6
0.125	16.0
0.250	12.8
0.500	11.2

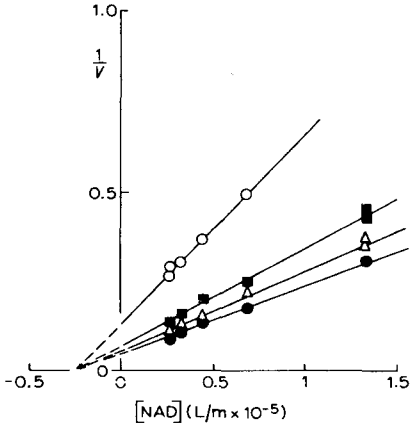

Fig. 5. Lineweaver–Burk plot of the maximal velocities observed during the succinate-linked NAD^+ reduction *versus* NAD^+ at different Mg^{2+} concentrations. ○—○, 0.25 mM $MgCl_2$; ■—■, 1.25 mM $MgCl_2$; △—△, 2.25 mM $MgCl_2$; ●—●, 3.25 mM $MgCl_2$. Experimental conditions as in Table I.

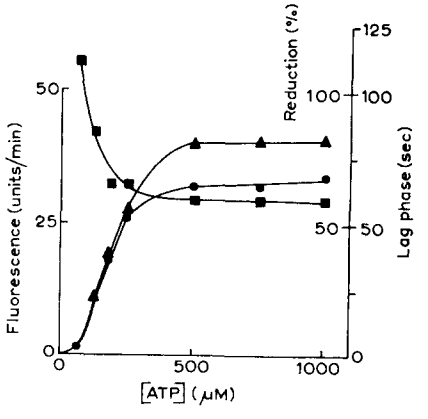

Fig. 6. Effect of ATP on the succinate-linked NAD^+ reduction. Experimental conditions as in Table I. Fluorescence unit per min corresponds to 0.3 mμmole NADH/ min/mg protein/ml. ●—●, rate of NAD^+ reduction; ■—■, lag phase in NAD^+ reduction; △—△, percentage of NAD^+ reduced.

within experimental error when determined with different samples of submitochondrial particles. As shown in Fig. 5, the K_m of NAD^+ was independent of the Mg^{2+} concentration. In these experiments succinate (5 mM) was used as electron donor.

Effect of ATP

A sigmoid curve was obtained for the rate as well as for the extent of NAD^+ reduction when increasing concentration of ATP were used (Fig. 6). The lag phase proved to be dependent on the ATP concentration. As the Mg^{2+} concentration had a pronounced influence on the rate of the energy-linked NAD^+ reduction, the ex-

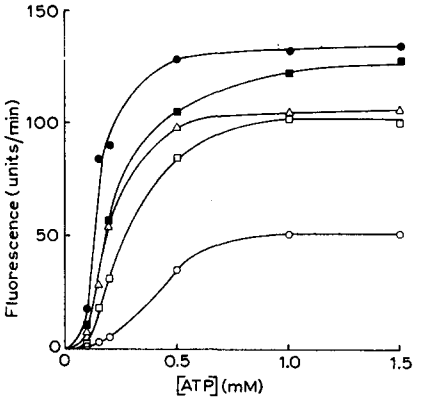

Fig. 7. Effect of the ATP concentration on the rate of the succinate-linked NAD^+ reduction at different Mg^{2+} concentrations. Experimental conditions as in Table I. ○—○, 0.25 mM $MgCl_2$; □—□, 1.25 mM $MgCl_2$; △—△, 2.25 mM $MgCl_2$; ■—■, 3.25 mM $MgCl_2$; ●—●, 6.25 mM $MgCl_2$.

periments with different ATP concentrations were repeated at different Mg^{2+} concentrations. The results are presented in Fig. 7. At all Mg^{2+} concentrations sigmoid curves were obtained.

Effect of pH

The pH–activity curve of the succinate-linked NAD^+ reduction in these submitochondrial particles showed a broad optimum at pH 8.5–9.0 (Fig. 8). ATPase measured under the same conditions is also included in the figure. The lag phase is plotted only to pH 8.9 but lag phases up to 6 min have been observed at pH 9.4. The activities of the pH–activity curve are the maximum rates observed during the succinate-linked NAD^+ reduction and ATPase reaction (initial velocities). The acid side of this pH–activity curve represents a true pH dependency of the reaction. Incubation of the particles in the pH-6.5 buffer for 10 min at 25° and readjustment of the pH to pH 7.5 did not result in a decrease in activity as measured at pH 7.5.

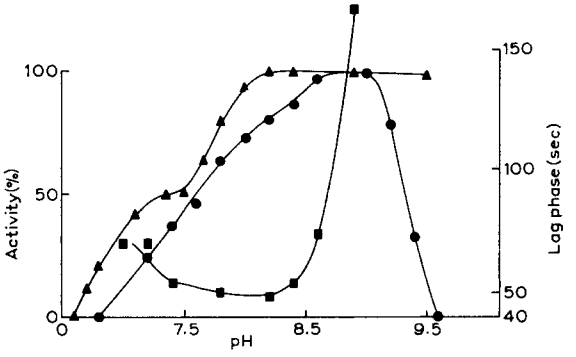

Fig. 8. pH–activity curve of the succinate-linked NAD^+ reduction (●—●), ATPase (▲—▲) and lag phase in NAD^+ reduction (■—■). Experimental conditions as in Table I. For the succinate-linked NAD^+ reduction the maximum velocities observed during the reaction are plotted. For the ATPase, measured under the same conditions except that 3 mM triethanolamine·HCl was used, initial velocities are plotted.

After incubation at pH 9.5, however, only 22% of the activity at pH 7.5 could be recovered. The alkaline side of the pH–activity curve is therefore more complex. For comparison the rates of oxidation of NADH and succinate at different pH values are given in Table IV.

TABLE IV

RATE OF SUCCINATE AND NADH OXIDATION BY SUBMITOCHONDRIAL PARTICLES AT DIFFERENT pH VALUES

Succinate concentration, 5 mM; NADH concentration, 1 mM. Protein concentration, respectively, 0.48 and 0.24 mg/ml. O_2 consumption was measured polarographically. The buffer contained 80 mM KCl, 10 mM triethanolamine·HCl and 3 mM $MgCl_2$.

pH	*Rate of oxidation (μmoles O_2/min/mg protein/ml)*	
	NADH	*Succinate*
6.5	793	61.4
7.0	915	90.1
7.5	973	149.6
8.0	1096	221.4
8.5	1200	223.4
9.0	1210	200.4
9.5	529	96.9

DISCUSSION

The finding of a relatively strong adenylate kinase reaction in these submitochondrial particles makes a study of the phosphate potential[7] more or less impractical because these particles cannot maintain a phosphate potential. At low Mg^{2+} concentration ADP formed by an ATPase reaction will be converted to ATP and AMP. The end product will, therefore, be predominantly AMP, as shown in Table II. At high Mg^{2+} concentration the end product is predominantly ADP, indicating an inhibition

of the adenylate kinase reaction. The extent of NAD reduction is, however, much larger at high Mg^{2+} concentration. To explain this one has to assume that the ATPase is also inhibited by these Mg^{2+} concentrations. The data of Table II are not in disagreement with this assumption. An experimental approach to this assumption is almost impossible as both reactions occur together. Fluoride, well known to be an inhibitor of adenylate kinase[25], interfered also with the succinate-linked NAD^+ reduction. Extrapolation of these results to intact mitochondria—if permissible—poses special problems as to a possible compartmentation of adenylate kinase in intact mitochondria.

The question arises, however, if the effect of Mg^{2+} is sufficiently explained by assuming an inhibition of ATPase and adenylate kinase at high Mg^{2+} concentration. The finding that the K_m of NAD^+ was independent of the Mg^{2+} concentration indicates that it does not participate in the binding of NAD^+.

CHANCE AND HOLLUNGER[7] have described the succinate-linked NAD^+ reduction by the following reactions:

$$ATP + X \rightleftharpoons X \sim P + ADP$$
$$X \sim P + I \rightleftharpoons X \sim I + P_i$$

$$X \sim I + NAD^+ + \text{reduced flavoprotein} \rightleftharpoons X + I + NADH + H^+ + \text{flavoprotein}$$
$$\text{succinate} + \text{flavoprotein} \rightleftharpoons \text{fumarate} + \text{reduced flavoprotein}$$

As these submitochondrial particles demonstrate a rather strong ATPase two reactions have to be added, namely:

$$X \sim P \rightarrow X + P_i$$
$$X \sim I \rightarrow X + I$$

Table II indicates that at high Mg^{2+} concentration ATPase is inhibited. Nevertheless, the rate of NAD^+ reduction increases with increasing Mg^{2+} concentration. This may be due to an increased stabilization of either $X \sim P$ and/or $X \sim I$ by high Mg^{2+} concentration. This would result in a higher steady-state level of $X \sim P$ and/or $X \sim I$, in turn resulting in a higher rate of NAD^+ reduction. The sigmoid titration curves for ATP at different Mg^{2+} concentrations presented in Fig. 7 could be interpreted as meaning a build up of $X \sim P$ or $X \sim I$, as has been suggested by CHANCE AND HOLLUNGER[7].

The optimum for the succinate-linked NAD^+ reduction was found at pH 8.5–9.0. CHANCE AND CONRAD[26] have shown that the pH optimum for glutamate oxidation in the presence of phosphate and phosphate acceptor in rat-heart sarcosomes is around pH 7. The reverse reaction shows apparently a quite different pH dependency than the forward reaction. Furthermore, CHANCE AND CONRAD[26] showed that the lower activity at pH 8 was due to irreversible inactivation. Irreversible inactivation of the succinate-linked NAD^+ reduction does, however, not take place below pH 9. Table III shows that the maximum of either NADH or succinate oxidation in these submitochondrial particles occurs at pH 8.5, well above pH 8.0. The differences in experimental conditions and enzyme systems might, however, account for these differences.

An intriguing feature of the succinate-linked NAD^+ reduction in these sub-

mitochondrial particles is the lag phase in NAD^+ reduction. The fact that no lag phase was observed in succinate oxidation[19] and that the lag was observed when *p*-phenylenediamine or the coenzyme Q_1–ascorbate[18] system were used as electron donor, locates the lag phase between NAD^+ and cytochrome *b* or in the energy-transfer reactions. Preincubation with ATP did take away the lag phase and a high Mg^{2+} concentration decreases the lag phase, suggesting the location of the lag phase in the energy-transfer reactions. In agreement with this assumption is the finding of a minimum lag phase at maximum ATPase activity (Fig. 8). However, the succinate concentration had a pronounced influence on the lag phase (Table III), indicating the influence of electron flow. Both processes are, however, closely related as electron flow from succinate to NAD^+ requires energy from ATP. It has been demonstrated earlier[20] that a soluble factor which decreases the lag phase and stimulates the rate of the succinate-linked NAD^+ reduction can be isolated from mitochondria. As will be demonstrated in the following paper this enzyme must react very near the electron-transport chain. It is therefore not unlikely that the lag phase is located at the link of energy and electron-transfer reactions.

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