THE ENERGY REQUIREMENT FOR ACTIVATION OF SUCCINATE METABOLISM IN INTACT HEART MITOCHONDRIA

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1. Introduction

The so-called energy-linked reduction of NAD(P)* by succinate in mitochondria and submitochondrial particles has been extensively studied (e.g. [1–7], for review see [8]). The activation mechanism of succinate dehydrogenase (SDH) has been studied from a very different point of view [9–11]. It seems possible, however, to correlate these phenomena because the rate of NAD(P) reduction must somehow be determined by the SDH activity which in turn is controlled by the extent of NAD(P) reduction [12]. This control was explained by a transformation of SDH from a deactivated to an activated form as a result of NAD(P)H binding to the enzyme. The activation was a reversible function of the redox level under very different conditions [12].

It is generally accepted that NAD(P) reduction by succinate is energy-linked and that it occurs by a reversal of the respiratory chain through site 1 of oxidative phosphorylation [8]. This view has, however, been questioned by Krebs and coworkers [13], because uncouplers did not prevent the reduction of NAD(P) by succinate in the presence of amytal. The activation of succinate oxidation by ATP led also to the assumption that oxidation required formation of an energized redoxcarrier (flavoprotein) which was oxidized in the respiratory chain [14, 3] or was able to reduce NAD(P) [15]. ATP could furthermore activate succinate oxidation by causing removal of oxaloacetate via different reactions [16–18]. Most recently Gutman et al. [11] reported a direct activation of SDH by ATP.

The present paper describes data which show that the NAD(P) reduction by succinate is not energy requiring, that SDH is not activated by ATP but by a combination of NAD(P)H and ATP, and that the activation requires a finite, but very small amount of ATP (1.1 μmole/g protein). The "energy-linked" NAD(P) reduction by succinate is explained as a result of the ATP requiring activation of SDH, which does not involve reversal of oxidative phosphorylation, and the NAD(P) reduction which does not involve energy.

2. Methods

Pigeon heart mitochondria were prepared as previously described [19] and stored (about 15 mg protein/ml) in a 225 mM mannitol, 75 mM sucrose, 1 mM Tris, 0.05 mM EDTA medium pH 7.4 at 0°. Oxygen concentration was recorded amperometrically, simultaneously with the fluorescence of NAD(P)H as previously described [19]. The differentiated output signal from the oxygen electrode amplifier was recorded

* *Abbreviations*

NAD(P) is used as a symbol for the oxidized nicotinamide adenine dinucleotide pool in order to stress that the detection method does not distinguish between NADH and NADPH. The use of the term does not infer that the biochemical reactions are nonspecific with respect to NAD and NADP. Succinate dehydrogenase (EC 1.3.99.1): SDH; Carbonylcyanide-*p*-trifluoromethoxy phenylhydrazone: FCCP.

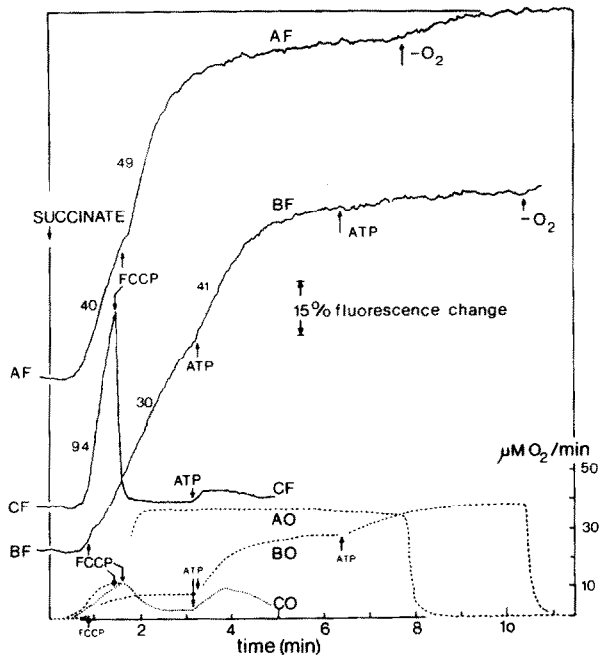

Fig. 1. Recordings of fluorescence (F) and respiratory rates (0, averaged curves) from 3 experiments. Heart mitochondria (500 μg protein/ml) were suspended in a buffer (pH 7.37, 23°) containing 225 mM mannitol, 75 mM sucrose, 20 mM Tris, 0.5 mM EDTA and 10 mM phosphate. The metabolic changes were initiated by 7 mM succinate. FCCP (8.6×10^{-8} M) was added when the NAD(P) was 36% reduced (exp. A), 4% reduced (exp. B), and 54% reduced (exp. C). 1.37 μM rotenone was present in exps. A and B. Each addition of ATP corresponded to 140 μM. The rates marked at the fluorescence curves were calculated as %/min of the maximal fluorescence change. The FCCP concentration employed was the optimal with respect to acceleration of the succinate respiration.

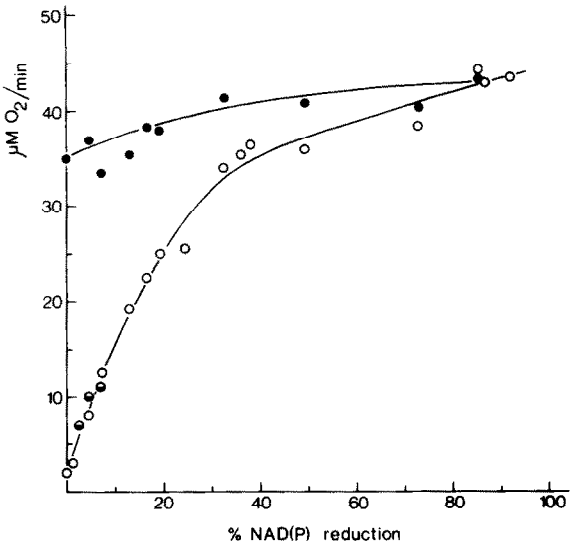

Fig. 2. Correlation between the degree of reduction at the time of FCCP addition and the final respiratory rates without ATP (o—o—o) or with ATP addition (•—•—•). The points below the curve are obtained after 1 addition of ATP, the others after 2 additions. Additions were made in steady states of respiration. The values corresponding to 0% NAD(P)reduction were obtained by addition of FCCP prior to succinate. The half filled circles (◒) indicate corresponding values of actual degree of reduction and respiratory rate in experiments where ATP was added, when NAD(P) was mainly on the oxidized form due to FCCP addition in absence of rotenone (cf. exp. C, fig. 1). Experimental conditions as described for fig. 1.

simultaneously with a peak-to-peak noise of about 2 μM O_2/min (time constant about 1 sec) [20]. ATP and ADP were analyzed in neutralized perchloric acid extracts by a luciferase method [21].

The fluorescence increase (366/450 nm) was used as a measure of NAD(P) reduction. The oxygen consumption rate with succinate was used as a measure of SDH activity when the respiratory chain was not rate limiting, i.e. in the presence of inorganic phosphate and ADP or uncouplers. The NAD(P) reduction by succinate involved SDH[1] and the rate of reduction could therefore be used as a measure of SDH activity provided the enzyme was rate limiting. This was a serious limitation because the mechanism of the reduction is unknown and because of possible reoxidation. The assay was used when the respiratory rate assay was inapplicable and only semiquantitatively, i.e. to evaluate changes in activities under almost identical conditions.

3. Results

NAD(P) reduction by succinate occurred in the presence of uncouplers (fig. 1), i.e. in the absence of ATP and high-energy intermediates. The reduction was completed to 100% in the presence of FCCP independent of the time of addition. The increased rate of reduction upon FCCP addition (exp. A) might be explained by the increased turnover of the respiratory chain. In this respect the effect of the uncoupler was completely analogous with that of ADP previously

described [12], but it was in obvious disagreement with an energy requirement of the reduction. The maximal respiratory activity recorded in the presence of FCCP was, within ± 5%, equal to the rate measured with ADP. Rotenone was present in exps. A and B to prevent reoxidation (via the respiratory chain) of the NAD(P) reduced by succinate. In the absence of rotenone (exp. C) the reoxidation in the presence of uncoupler was considerably faster than the simultaneously occurring reduction as indicated by the shift in steady state concentration.

The activation of SDH by NAD(P)H mentioned in the introduction could account for the different rates of respiration following addition of FCCP in exps. A and B. In exp. A the NAD(P) was 36% reduced prior to FCCP addition and SDH should, according to the previous data, be almost completely activated [12] and the respiration limited only by the respiratory chain. Addition of uncoupler relieved the respiration to 82% of the maximal activity. In exp. B where NAD(P) was only 4% reduced prior to FCCP addition, SDH should be only little activated, as was also observed when the respiration was relieved by FCCP. Further activation corresponding to the continuing NAD(P) reduction did not occur in the presence of FCCP probably because ATP disappeared very rapidly in the uncoupled state. Activation to the same value as in exp. A was obtained when ATP was added. Much ATP was needed to get complete activation due to the high activity of the "ATP-ase" induced by the uncoupler. Activation of SDH apparently needed energy, but ATP alone was not sufficient as shown by exp. C. In this experiment ATP was added in the uncoupled oxidized state of NAD(P) and caused only a small transient activation of SDH. The activation corresponded to a small transient reduction of NAD(P), probably due to reversal of site 1 of oxidative phosphorylation by ATP.

The rate of respiration attained upon addition of FCCP was maintained until all oxygen was consumed (fig. 1, curve AO). Thus there was no indication of deactivation of SDH upon removal of ATP or of any inhibitory effect of the uncoupler (in contrast to [22]). SDH was, however, instantaneously deactivated to the level determined by the degree of reduction if reoxidation was allowed (exp. C).

The data of fig. 2 were derived from experiments like those shown in fig. 1. The steady state rates of succinate respiration in the presence of FCCP (o) and the steady state rates of respiration attained upon ATP addition to the system with NAD(P) completely reduced (●) were plotted as a function of the degree of reduction at the moment FCCP was added. ATP was apparently able to cause almost complete activation of SDH independent of the prior activation and the period with FCCP present.

The curve indicated by the open circles showed half maximal activity when FCCP was added after 16% reduction of NAD(P). It was consequently much alike the previously published activation curve for SDH measured at steady state values of NAD(P) reduction [12], although it showed somewhat lower values at the high degrees of activation, probably because the reduction was faster than the activation (cf. [12]). The identity of the 2 curves supports the interpretation that the activation of SDH was ceased almost instantaneously upon addition of FCCP unless ATP was supplemented. Determinations verified that the concentration of uncoupler used removed the endogenous ATP completely (i.e. to the basal value, see below) with a half time of about 10 sec. Other uncouplers (dicoumarol and 4-isooctyl-2, 6-dinitrophenol) gave activation curves similar to that of FCCP.

The experiments indicated by half filled circles (⊖) corresponded to exp. C (fig. 1), i.e. rotenone was absent and the NAD(P)H was oxidized by FCCP addition prior to addition of ATP. The resulting respiratory rates fitted the activation curve, when plotted as a function of the actual degree of reduction, indicating that the ATP was unable to cause even minor activation of SDH beyond that determined by the NAD(P) level.

The ATP requirement for the activation process was demonstrated qualitatively by the cessation of SDH activation at the moment of uncoupler addition, and by the ability of ATP to restore complete activation. SDH activity increased from 2 to 44 μM O_2/min for increasing NAD(P)H without addition of ATP to the system (fig. 2). The ATP required for this activation could be either ATP present initially or ATP formed during the simultaneous oxidative phosphorylation. Quantitative determination of the ATP requirement was possible: fresh heart mitochondria contained 2.5 μmoles ATP/g protein or 57% of the total ATP + ADP (table 1). The ATP content declined to a minimum value (0.77 μmoles/g protein) after about 30 min aging of the mitochondria at 0° (15 mg protein/ml).

Table 1
Changes of endogenous adenine nucleotides in pigeon heart mitochondria during aging and during activation of succinate metabolism.

	Age of preparation (min)	
	10–15	60
(ATP + ADP) content	4.42 ± 0.17	3.86 ± 0.14
ATP content	2.50 ± 0.13	0.77 ± 0.05
ATP consumed during SDH activation	1.10 ± 0.12	
Oligomycin (5.3 μM) inhibition of rate of NAD(P) reduction by succinate	30%	70%

All amounts are given as μmoles/g protein ± S.E.M. (7 different preparations). The age of a preparation was measured from the moment of suspension of the final pellet.

This remaining ATP appeared metabolically inactive and could not be depleted by any means, e.g. uncouplers. Most of the ATP which disappeared during aging was recovered as ADP. Addition of succinate to the fresh mitochondria caused consumption of 1.1 μmole ATP/g protein during the activation of respiration. 5.3 μM oligomycin was present in order to prevent recycling due to oxidative phosphorylation. This estimate could be too low, although the reproducibility supported the value. At the actual ADP concentration 1% remaining oxidative phosphorylating activity would correspond to the formation of 0.10 μmole ATP/g protein in the approx. 2 min which preceded sampling.

The ATP requirement for the activation was also reflected in the sensitivity of the initial rate of reduction of NAD(P) to oligomycin. The rate of reduction upon succinate addition was inhibited 30% by oligomycin in fresh mitochondria and 70% in aged mitochondria (table 1). 80% inhibition was recorded in other experiments. The degree of inhibition by oligomycin of the rate of reduction of NAD(P) was a function of the ATP content, and the 2.5 μmoles ATP/g protein in the experiments of table 1 were probably of the order of the K_m value of the activation process as indicated by the 30% inhibition. At higher initial ATP concentrations the inhibition decreased, eventually to zero. The very effective inhibition in aged preparations indicated that high-energy compounds other than ATP were of minor importance in providing energy for the SDH activation. Added ITP, UTP, CTP or GTP were unable to replace ATP in the activation process.

4. Discussion

The experiments presented above are interpreted by the hypothesis that ATP was needed to obtain activation of SDH by NAD(P)H. Reduction of 16–18% of the NAD(P) was sufficient to obtain half maximal activity with sufficient ATP supply. NAD(P)H was not oxidized during the activation while ATP was hydrolyzed to ADP in very constant proportion to the protein content. The activation by ATP was insensitive to oligomycin (cf. [11]) and ATP could not be replaced by other nucleoside triphosphates.

Gutman et al. [10, 11] have recently described an activation of SDH by reduced coenzyme Q and by ATP. No inconsistency, however, appears to exist between the data supporting these 2 activations and the present hypothesis: the reduced coenzyme Q activation might have been activation by NAD(P)H (as discussed in [12]) and was observed under conditions where ATP was not depleted (uncoupler absent); and the ATP activation was observed under conditions where the NAD(P) of the system most likely was reduced.

The activation of SDH observed was very unlikely due to removal of oxaloacetate from the enzyme. None of the reactions which remove oxaloacetate require the combination of ATP and NAD(P)H observed to be necessary for activation. Either NAD(P)H or ATP are theoretically able to remove oxaloacetate. Control by NAD(P)H mediated through the oxaloacetate–malate equilibrium was ruled out because very different activities of SDH were recorded for complete reduction of NAD(P) (fig. 2). The possible removal of oxaloacetate in processes requiring ATP was investigated in collaboration with Drs. H.N. Rasmussen and S. Vangbæk and will be described elsewhere. Phosphoenolpyruvate was formed in exceedingly small amounts (nmoles/g protein) but not correlated with the observed effects of ATP. The possibility of fatty acid activation in turn leading to formation of acetyl-CoA and then citrate [16] seemed also ruled out, as no AMP was formed during the SDH activation.

The data presented above show that the NAD(P) reduction by succinate per se was not an energy re-

quiring reaction, but that the rate of reduction was correlated with the degree of activation of SDH which in turn was dependent upon ATP. An eventual energy requirement for the NAD(P) reduction by succinate must therefore be studied at constant SDH activity and preferably with maximally activated SDH. Under these conditions uncouplers did not prevent the NAD(P) reduction by succinate. The rate of NAD(P) reduction in the presence of FCCP was actually about twice the rate with ADP, although the same respiratory rates were recorded. The lack of energy requirement for NAD(P) reduction by succinate agreed with the observations by Krebs and Eggleston [13], but was in obvious disagreement with the commonly accepted hypothesis involving reversal of site 1 of oxidative phosphorylation [8]. The present experiments leave the reduction mechanism unexplained. A reversal of the NAD-branch of the respiratory chain may be questioned because the reduction occurred in the presence of rotenone concentrations which apparently inhibited the NADH-oxidase completely.

A consequence of the present hypothesis is that the energy demand of the NAD(P) reduction by succinate as studied earlier has been the energy demand of the SDH activation, i.e. ATP. The earlier data showed that the NAD(P) reduction by succinate was insensitive to oligomycin and this was explained by assuming that the energy was supplied from high-energy intermediates formed prior to ATP. The present data showed that the SDH activation was oligomycin insensitive provided sufficient ATP was present, but that oligomycin sensitivity increased as ATP formation became necessary. The energy demand of 1.1 μmole ATP/g protein for the activation was low, and mitochondria containing more endogenous substrates than heart mitochondria probably always contain sufficient endogenous ATP and therefore exhibit no oligomycin inhibition of the SDH activation and the NAD(P) reduction by succinate. High-energy intermediates were consequently unnecessary to explain the oligomycin insensitivity. In the presence of oligomycin the rate of NAD(P) reduction was inhibited 70–80% in aged preparations. The correlation between the rate of reduction and the SDH activity was, however, rather involved, and the SDH activity might have been considerably more inhibited than expressed by these values. When FCCP was added prior to succinate (fig. 2) the rate of NAD(P) reduction was still 24%/min, although the SDH activity was only 2 μM O_2/min. The activity remaining in the presence of oligomycin might be either the activity of the deactivated enzyme, or the succinate activated enzyme [9], or the ATP activated enzyme formed because of a leakage in the oligomycin block, or activated by high-energy intermediates. The last possibility was, however, improbable as the insensitivity to oligomycin of the ATP activation reaction implied that reversal of oxidative phosphorylation was not involved.

The effects of ATP upon the various aspects of succinate metabolism described in the literature [1–8, 11, 13–18] may all be reflections of the mechanism suggested: SDH is converted from a deactivated to an activated form with NAD(P)H as a positive modifier at the expense of ATP.

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