REGULATION OF SUCCINATE OXIDATION BY ENDOGENOUS REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDES IN INTACT HEART MITOCHONDRIA

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1. Introduction

It is well established that succinate may cause complete reduction of the endogenous pool of nicotinamide adenine dinucleotides (NAD(P))* in intact mitochondria [2, 3]. The reaction is usually called the energy-linked reduction of NAD by succinate, and a very labile mechanism is indeed involved. The reduction rate decreased 50% in the pigeon heart mitochondria used in these studies when they were kept for only 3 min at 35° before dilution. The response of the NAD(P)H to ADP was half maximal after 7–10 min at 35° [4] while the respiratory activities were not affected by this treatment. This paper provides evidence that the level of NAD(P)H serves as a direct controlling factor in the metabolism of succinate in intact mitochondria.

Pigeon heart mitochondria were very suitable for these studies for the following reasons: (i) endogenous substrates able to reduce NAD(P) in the presence of rotenone were absent. (ii) Experimental conditions could be obtained without use of inhibitors under which the NAD(P) reduction by succinate was completely prevented (i.e. no reduction observed in 50 min). (iii) Almost any redox level of the NAD(P) could be obtained by taking advantage of the large capacity and the tight coupling of the phosphorylating system.

* 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, however, infer that the biochemical reactions are unspecific with relation to NAD or NADP. In heart mitochondria NAD + NADH amounts to about 80% of the total nicotinamide adenine dinucleotides [1].

2. Methods

Pigeon heart mitochondria were prepared as previously described [4] and stored (about 15 mg protein per ml) in a mannitol (225 mM), sucrose (75 mM), Tris (1 mM), EDTA (0.05 mM) medium, pH 7.4. Oxygen concentration was recorded amperometrically and simultaneously with the fluorescence of NAD(P)H in the microfluorimeter as described [4].

3. Results and discussion

Succinate respiration in intact mitochondria can be inhibited by uncouplers (e.g. [2, 5–7]) which at the same time cause oxidation of NAD(P)H. The respiratory rates are plotted as a function of NAD(P) in fig. 1 which compiles data from 5 experiments with different concentrations of dicoumarol. NAD(P) was reduced 100% by succinate and reoxidized by the uncoupler to different levels with very different kinetics (cf. the legend of fig. 1). The amounts of oxygen consumed before a certain redox level was attained were consequently subject to large variations. For instance, in one experiment 120 μM O_2 and in another experi-

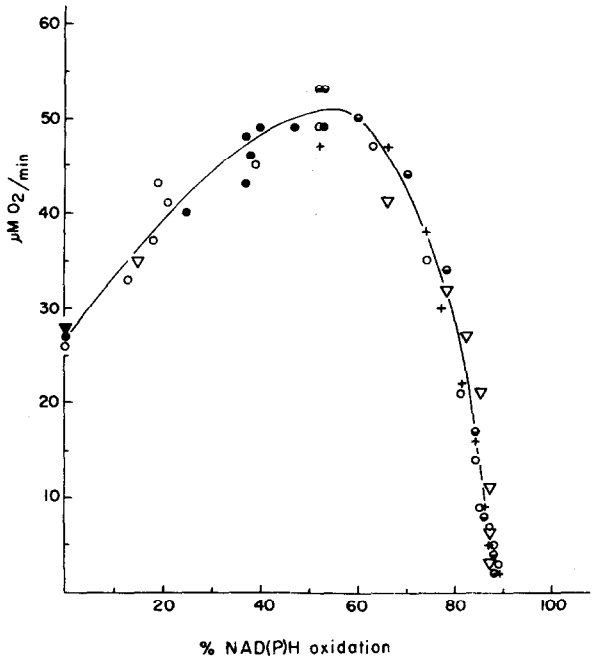

Fig. 1. Correlation between the redox level of the NAD(P) and the respiratory rate for succinate at different degrees of uncoupling. The mitochondria (515 μg protein/ml) were suspended in a medium containing phosphate (10 mM), mannitol (225 mM), sucrose (75 mM), Tris (20 mM) and EDTA (0.5 mM), pH 7.35. 7 mM succinate caused a respiration rate of 27 μM O_2/min and 100% reduction of NAD(P). Dicoumarol was then added and reoxidation of NAD(P)H followed in 5 different experiments. Dicoumarol, 2 × 0.86 μM (open circles), caused maximally 20% reoxidation after the first addition. Dicoumarol, 1.14 μM (solid circles), caused very slow reoxidation which was followed to only 50%. Dicoumarol, 1.43 μM (half filled circles), caused maximal reoxidation after about 4 min. Dicoumarol, 1.89 μM (crosses), caused maximal reoxidation in about 2 min. Dicoumarol, 2.86 μM (triangles), gave complete reoxidation in about 20 sec.

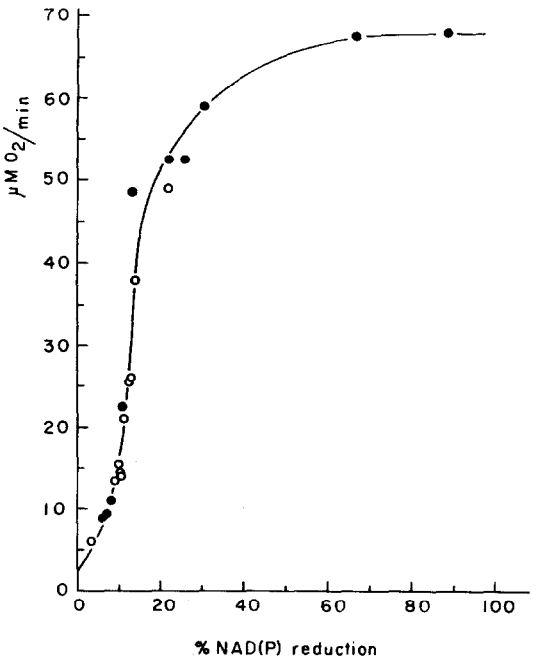

Fig. 2. Correlation between the steady state values of NAD(P)H levels and respiratory rates. The mitochondria (830 μg protein/ml) were suspended in a phosphate containing medium (cf. fig. 1) and ADP (950 μM) and succinate (7 mM) were present in all experiments. Rotenone, 0–1.37 μM, pH 7.36 (solid circles). In the absence of rotenone the respiratory rate was 9 μM O_2/min and the NAD(P) 7% reduced. Rotenone, 0–0.08 μM, pH 7.09–7.61 (open circles). The intersection of the curve and the ordinate represents the respiratory rate in the absence of ADP and phosphate for total oxidized NAD(P).

ment only 10 μM O_2 was consumed prior to 85% oxidation. Nevertheless one curve does express the correlation between NAD(P) and respiratory rate in all the experiments. Increase in respiratory rate due to uncoupling was observed at about 60% reoxidation. Uncoupling above this level caused increased oxidation, but the progressive inhibition of the respiratory rate indicated that the rate became limited by another factor. Half maximal activity was obtained for about 82% reoxidation of the NAD(P).

Addition of rotenone to mitochondria metabolizing succinate in the presence of excess ADP and phosphate caused NAD(P) reduction, as reoxidation via the respiratory chain was prevented. The correlation between steady states of the redox levels and of the respiratory rates is shown in fig. 2. Solid circles indicate experiments where the different redox levels were established exclusively by varying the rotenone concentration, for instance 50% reduction was maintained by 3.2×10^{-7} M rotenone. The correlation was almost identical with the one of fig. 1 in the interval where the uncoupler concentration was not rate limiting. 17% reduction (versus 18% in fig. 1) gave

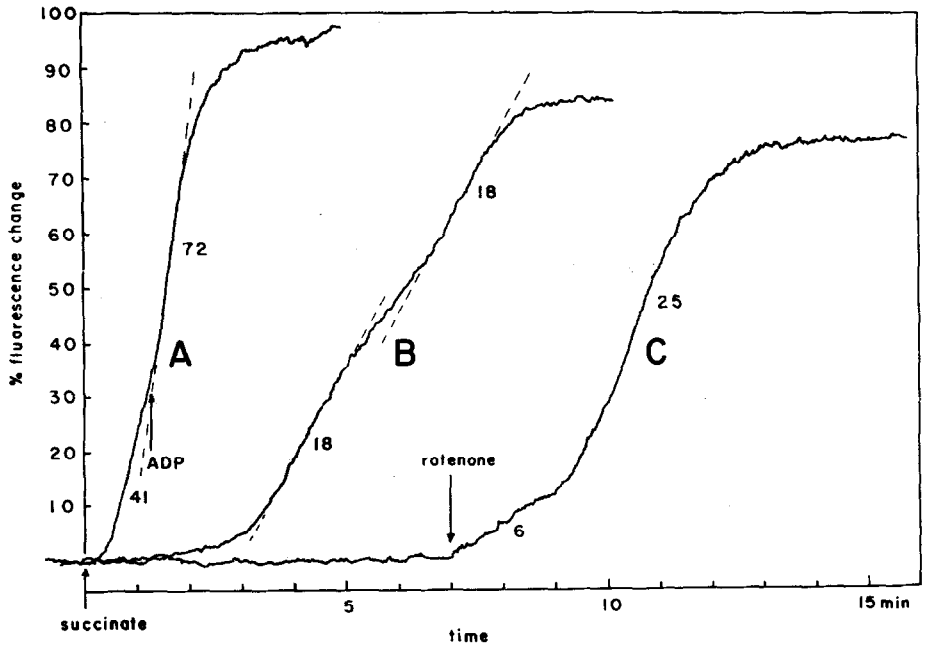

Fig. 3. Time course of fluorescence changes (NAD(P)H formation) upon succinate addition in the presence (expt A) and absence (expts B, C) of added phosphate. The medium was as in fig. 1 except for phosphate, pH 7.38, 730 μg protein/ml. Succinate addition was the starting point in all 3 experiments. The numbers on the curves represent rates of fluorescence change expressed as %/min of the maximal fluorescence change due to metabolic changes. Expt. A: 10 mM phosphate and 1.37 μM rotenone were added prior to succinate (10 mM). When the respiratory rate approached a steady state (24 μM O_2/min), 950 μM ADP were added resulting in a respiratory rate of 67 μM O_2/min. Expt. B: 96 mM ethanol was present before addition of succinate (6 mM). Expt. C: 24 mM ethanol was present before addition of succinate (6 mM). 1.37 μM rotenone was added at the time indicated (ethanol concentration increased to 48 mM). The final respiratory rate was 14 μM O_2/min in expts. B and C.

half maximal respiratory rate (6.6×10^{-8} M rotenone present). The redox level of NAD(P) was also controlled by the pH of the medium in a way that less rotenone was needed to obtain a certain redox level at a lower pH. The correlation between redox level and respiratory rate was, however, the same (open circles on fig. 2). It appears from these experiments that NAD(P)H controlled the succinate respiration and not *vice versa*, because the redox changes under most conditions were so fast that the changes in respiratory rate were delayed. Respiration was found to be maximally accelerated under conditions where the fluorescence changed at only 15% of the possible rate.

Addition of 7 mM succinate at pH 7.36 in the absence of phosphate to pigeon heart mitochondria did not cause NAD(P) reduction and the respiratory rate was only 2–3 μM O_2/min. At this very low turnover of succinate, NAD(P)H was presumably reoxidized faster than it was formed and no accumulation could occur. Addition of rotenone should therefore be able to trigger succinate respiration by preventing reoxidation of NAD(P)H and this was observed. An experimental difficulty was that rotenone was added in ethanol and that larger amounts of ethanol were themselves able to trigger succinate metabolism without rotenone being present (expt. B, fig. 3). The amounts of ethanol usually added with the rotenone were, however, insufficient to trigger succinate metabolism (cf. fig. 3C) and extrapolation to zero alcohol showed that rotenone *per se* had the expected triggering effect. Reduction of a small amount of NAD(P) by ethanol could be demonstrated in the presence of rotenone (no succinate) and the fact that ethanol can initiate succinate metabolism is further evidence for the assumption that this is controlled by small amounts of NADP(P)H.

The final respiratory rates attained in expts. B and

C (fig. 3) were not limited by NAD(P)H but by phosphate as shown in expt. A. The respiratory rate was increased from 14 to 24 μM O_2/min by phosphate, but was still limited by the phosphorylating system. ADP increased the respiratory rate to 67 μM O_2/min and the rate of reduction from 41 to 72%/min due to the increased turnover of the respiratory chain. Oxidative phosphorylation did increase succinate oxidation because the NAD(P) pool was sufficiently reduced to allow this (cf. fig. 2).

The data presented may be explained by assuming that succinate dehydrogenase is converted from an inactive to an active form in a rather slow process by NAD(P)H formed, for instance, during succinate metabolism. A positive feedback mechanism results, i.e. accumulation of even tiny amounts of NAD(P)H will accelerate succinate metabolism which in turn reduces more NAD(P) thereby further activating the process. This also explains why the reduction of the NAD(P) by succinate shows a lag in most cases prior to an "all or none" response.

Activation of succinate dehydrogenase by succinate has been described by Kearney et al. [8]. Recently Gutman et al. [9] reported activation of succinate dehydrogenase in submitochondrial particles by added NADH. This activation was, however, ascribed to the formation of endogenous reduced coenzyme Q because of inhibition experiments (rhein, piericidin A), pentane extraction and reduced coenzyme Q reactivation experiments which seems to exclude a direct action of added NADH. The data of the present paper are, however, in favor of a direct control by endogenous NAD(P)H or by some compound changing in parallel with this. It seems most unlikely that coenzyme Q can fulfill this requirement at the very different rotenone concentrations employed. The experiments of Gutman et al. may, however, also accord with the hypothesis that the actual activator is endogenous NAD(P)H reduced via a pathway involving coenzyme Q and not directly by added NADH. Coenzyme Q may even substitute for the physiological redox carrier considering the experiments of Albracht et al. [10] which showed that pentane extraction removed something, different from coenzyme Q, but necessary for succinate oxidase. Succinate dehydrogenase was recently shown to be composed of two subunits [11] and is therefore well suited for allosteric control. NAD(P)H is a likely positive effector of this enzyme. Less than about 40% of the amount of NAD(P) which could be reduced by succinate was involved in the activation (fig. 2). This might be due to strong binding of some NAD(P)H to the succinate dehydrogenase. Some evidence for the existence of two pools of NAD(P) which can be distinguished by succinate dehydrogenase has been obtained (cf. the two phases of reduction of about equal pools in curve B, fig. 3).

The effects of uncouplers upon succinate metabolism have usually been explained as being mediated through a shift in the equilibrium of malate dehydrogenase resulting in an increased concentration of oxaloacetate, which is a potent inhibitor of succinate dehydrogenase (cf. the discussion by Klingenberg, Slater and others [12]). Conditions where oxaloacetate is likely to control succinate respiration have been described for isolated mitochondria [13]. This paper suggests, however, that NAD(P)H might exert a direct effect upon succinate dehydrogenase and moreover that the indirect effect upon oxaloacetate concentration is of minor importance in heart mitochondria. The evidence is that an identical correlation between NAD(P)H and the rates of succinate respiration exists under most varied conditions, some of which are shown above. It is reasonable to assume that the amount of oxaloacetate formed is somehow related to the amount of oxygen consumed. For instance the oxaloacetate concentration would have to differ at least by an order of magnitude for a given redox level (and a given respiratory rate) in the 5 experiments of fig. 1. This, however, makes it very unlikely that oxaloacetate inhibition explains these results, unless compartmentation of oxaloacetate is assumed. The same conclusion was drawn from experiments in which chemical analyses of oxaloacetate formed during succinate metabolism was done [14]. A change in redox level from 10 to 15% reduction caused a several fold increase in the rate of succinate respiration, which is also difficult to explain by a shift in equilibrium at malate dehydrogenase. A hypothesis involving compartmentation of oxaloacetate becomes very complicated, especially if the initiation experiments (rotenone, ethanol) are also to be explained. The role of NAD(P)H suggested in this paper is simple and easily tested experimentally because NAD(P)H is easy to measure in the concentrations at which it is suggested to exert its control. Moreover, the same activation mechanism is suggested to function when the NAD(P)H

concentration can be considered limiting, for instance activation of succinate metabolism in the initial or later phases of an experiment or in the presence or absence of ADP and phosphate.

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