Endergonic Reduction of Mitochondrial Diphosphopyridine Nucleotide by Sarcosine

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SUMMARY

The aerobic dehydrogenation of the N-methyl group of sarcosine initiates an energy-requiring reduction of diphosphopyridine nucleotide in intact mitochondria. The formation of DPNH can be measured both spectrophotometrically and by the reduction of acetoacetate. Except in the presence of uncouplers, such as Dicumarol and arsenate, reduction of DPN by sarcosine does not require added adenosine triphosphate. The formation of DPNH is inhibited by Amytal, but not by oligomycin.

It is proposed that DPN is reduced by electron transfer flavoprotein(2H) via the Amytal-sensitive site of the respiratory chain, and that the energy for this endergonic process can be transferred from the two terminal sites of energy conservation without the participation of inorganic phosphate.

METHODS

The mitochondria of rat liver were isolated as described previously (8). DPN reduction was measured in the Cary spectrophotometer, model 14, with the model 1462 scattered transmission accessory, the 7664 photodetector, and the 0 to 0.2 and 0 to 2.0 slide wires, and also by the method of acetoacetate reduction described by Azzone, Emster, and Weinbach (10).

The spectrophotometric experiments were carried out aerobically under two sets of conditions: (a) in the presence of arsenate, Dicumarol, and ATP, according to the procedures of Azzone, Emster, and Klingenberg (11); and (b) in the absence of added ATP and with preincubation of the mitochondria with arsenite. The details of the procedures are given in the legends to tables and figures.

RESULTS AND DISCUSSION

ATP-dependent Reduction of Mitochondrial DPN by Sarcosine in Presence of Arsenate and Dicumarol—Both arsenate and Dicumarol function as uncouplers of oxidative phosphorylation (12, 13). In addition, arsenate can lower the level of endogenous ATP by interfering with substrate level phosphorylations, and Dicumarol inhibits oxidation of DPNH via the DPNH-TPNH diaphorase pathway (14, 15). Reduction of mitochondrial DPN by sarcosine in the presence of these agents, therefore, might be expected to require added ATP. A typical experiment showing such an ATP-dependent reaction is summarized in Table I. As shown by these data, the rate of DPN reduction by sarcosine plus ATP was twice the sum of their individual rates. Similar results, but with higher reduction rates (Table II) were obtained when the mitochondria had been pretreated with arsenite. This agent inhibits the oxidation of endogenous substrates (15) and, therefore, can be used to advantage in lowering the level of mitochondrial DPNH prior to the addition of the exogenous substrate.

During the course of the experiments described above, it became apparent that mitochondrial preparations, isolated and treated under identical conditions, were not uniformly sensitive...
to Dicumarol and arsenate. Frequently, the rate of DPN reduction in the presence of sarcosine plus ATP was found to be equal to the sum of the rates observed with sarcosine and ATP individually, and in some cases the rates with sarcosine alone were high enough to suggest that, under appropriate conditions, DPN could be reduced without the addition of ATP.

Energy-dependent Reduction of Mitochondrial DPN by Sarcosine in Absence of Exogenous ATP—As shown in Fig. 1 and Table III, the DPN of arsenite-treated mitochondria was reduced by sarcosine in the absence of Dicumarol, arsenate, and exogenous ATP. Under aerobic conditions, the rapid rate of reduction of DPN initiated by sarcosine was approximately equal to the rate at which the level of DPNH had been lowered in the preceding treatment with arsenite. Both the reduction of DPN and its reoxidation were strongly inhibited by Dicumarol, Amytal, and cyanide (Figs. 2 to 4). When ATP was added after sarcosine (Table III), the rate of DPN reduction was greatly decreased. Presumably, under these conditions there was a suppression of coupled respiration by excess ATP, which served also to suppress the endergonic reduction of DPN.

**Table I**

ATP-dependent reduction of mitochondrial DPN by sarcosine in presence of arsenate and Dicumarol

Freshly isolated mitochondria were resuspended in cold 0.25 M sucrose (30 mg of protein per ml). A portion of this suspension was added to a small conical flask containing Tris buffer, pH 7.5. Arsenate and Dicumarol were then added together, and the incubation mixture was shaken gently for 3 min. Portions (2.8 ml) of the reaction mixture were pipetted into sample and reference cuvettes. When balance of the cuvettes had been verified for several minutes, 0.1 ml of sarcosine was added to the sample cuvette, and an equal volume of water to the reference cuvette. After the change in optical density had been recorded 3 min, 0.1 ml of ATP was added to the sample cuvette and balanced with water in the reference cell as before. The new rate was then recorded. When ATP alone was used as a control, it was preceded by 0.1 ml of water. The final volume in each cell was 3.0 ml, and the concentrations of the sample cell components were: KCl, 240 μmoles; MgCl₂, 24 μmoles; Tris, 60 μmoles; MgCl₂, 24 μmoles; phosphate, 7.5 μmoles; mitochondria (3 mg of protein); arsenite, 6 μmoles; and sarcosine, 20 μmoles, in a final volume of 0.8 ml. The reference cuvette contained all components except arsenite and sarcosine. Mitochondria, isolated in cold 0.25 M sucrose, were resuspended in sucrose (30 mg of protein per ml). The suspension (0.1 ml) was added to sample and reference cuvettes and diluted with 2.5 to 2.8 ml of buffer, pH 7.5. After balancing in the spectrophotometer, arsenite was added to the sample and water to the reference. Changes in optical density at 340 mμ were recorded continuously, except for 30 sec intervals when substrate or inhibitors were added to the sample cuvette only.

**Table II**

ATP-dependent reduction of mitochondrial DPN by sarcosine in presence of arsenite, arsenate, and Dicumarol

The experimental conditions were the same as in Table I, except that arsenite (6 μmoles) was added to the reaction mixture 3 min before the other components.

**Table III**

Reduction of DPN by sarcosine in arsenite-treated mitochondria without arsenate and Dicumarol

Experimental conditions were the same as described in the legend to Table I, except that arsenate and Dicumarol were omitted.

<table>
<thead>
<tr>
<th>Additions to sample cuvette</th>
<th>Increase in ΔA₄₅₀/5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcosine</td>
<td>0.105</td>
</tr>
<tr>
<td>Sarcosine, followed by ATP</td>
<td>0.040</td>
</tr>
<tr>
<td>ATP only</td>
<td>0.090</td>
</tr>
</tbody>
</table>

In the experiments described in Fig. 5, the reduction of acetocetate to β-hydroxybutyrate was employed as a "trapping reaction" for the DPNH produced in the presence of sarcosine. When the rate of DPN reduction had become maximal, the addition of acetocetate resulted in a rapid reoxidation of the DPNH. Subsequent addition of Amytal to the system prevented further generation of DPNH.

The formaldehyde resulting from the dehydrogenation of the CH₂ group of sarcosine can be oxidized by a DPN-dependent dehydrogenase localized in the soluble compartment of the mitochondrion. However, the oxidation of formaldehyde is not responsible for the energy-linked reduction of DPN initiated by sarcosine. This conclusion is supported by several lines of evidence, including the kinetic data of Tables I to IV and Figs. 1 to 5.

W. R. Frisell, unpublished results.
Thus, it was found that the reduction of DPN begins immediately upon addition of sarcosine, and that the rate of reaction is maximal during the initial period, in which the quantity of formaldehyde derived from the oxidized methyl group is still negligible.

**Fig. 2.** Inhibition by Dicumarol of sarcosine-induced reduction of DPN. The sample cuvette contained: KCl, 390 μmoles; Tris, 60 μmoles; MgCl₂, 24 μmoles; mitochondria (3 mg of protein); arsenite, 6 μmoles; Dicumarol, 0.18 μmole; and sarcosine, 20 μmoles, in a final volume of 3.0 ml. The reference cuvette contained all components except arsenite, Dicumarol, and sarcosine. Other experimental conditions were as in Fig. 1. Dashed line shows how inhibitor prevented the changes recorded in its absence.

**Fig. 3.** Inhibition by Amytal of sarcosine-induced reduction of DPN. The sample cuvette contained: KCl, 240 μmoles; Tris, 60 μmoles; MgCl₂, 24 μmoles; mitochondria (3 mg of protein); arsenite, 9 μmoles; Amytal, 9 μmoles in 0.02 ml of ethanol; and sarcosine, 20 μmoles, in a final volume of 3.0 ml. The reference cuvette contained all components except arsenite, Amytal, and sarcosine. Other experimental conditions were as in Fig. 1. Dashed line as in Fig. 2.

**Fig. 4.** Inhibition by cyanide of sarcosine-induced reduction of DPN. The sample cuvette contained: KCl, 390 μmoles; Tris, 60 μmoles; MgCl₂, 24 μmoles; mitochondria (3 mg of protein); arsenite, 6 μmoles; KCN, 4 μmoles; and sarcosine, 20 μmoles, in a final volume of 3.0 ml. The reference cuvette contained all components except arsenite, KCN, and sarcosine. Other experimental conditions were as in Fig. 1. Dashed line as in Fig. 2.

**Fig. 5.** Reduction of DPN by sarcosine, reoxidation of DPNH by acetooacetate, and inhibition of DPNH formation by Amytal. The sample cuvette contained: KCl, 240 μmoles; Tris, 60 μmoles; MgCl₂, 24 μmoles; mitochondria (3 mg of protein); arsenite, 9 μmoles; sarcosine, 20 μmoles; acetooacetate, 10 μmoles; and Amytal, 9 μmoles in 0.02 ml of ethanol, in a final volume of 3.0 ml. The reference cuvette contained all components except arsenite, sarcosine, acetooacetate, and Amytal. Other experimental conditions were as in Fig. 1.

More direct evidence that the N-CH₃ group is the primary electron donor for DPN reduction was obtained in experiments with substrate quantities of formaldehyde. As shown in Table IV, addition of an aldehyde-trapping agent, such as semicarbazide, does not affect the rate of DPN reduction by sarcosine, but prevents the reduction by formaldehyde. It was also possible...
to exclude formaldehyde as the reductant of DPN in the sarcosine system, by the experiment summarized in Fig. 6. These data show that, as in the case of the other pyridine nucleotide-linked substrates (10, 16), the reduction by formaldehyde is not affected by Amytal, whereas the reduction by sarcosine under the same conditions is inhibited.

**Endergonic Reduction of Acetoacetate in Presence of Sarcosine or Succinate—Azzone, Ernesti, and Weinbach (10) and Ernesti et al. (16) have shown that the energy-dependent reduction of DPN by succinate can be linked to the reduction of acetoacetate. With this additional reaction, the DPNH can be continuously reoxidized by acetoacetate via the β-hydroxybutyrate dehydrogenase. Thus, reducing equivalents appearing as “catalytic” amounts of DPNH can be trapped in “substrate” quantity, and can be measured by the disappearance of the acetoacetate. Such a reductive reaction with sarcosine and its inhibition by Amytal are shown by the data of Table V. The requirement of energy for the reduction of acetoacetate under these conditions was confirmed by the additional observation that, in the presence of an uncoupling concentration of Dicumarol (0.08 mM), no acetoacetate was removed.

Confirming the results described in the previous section (Fig. 6), it was also possible, with the use of Dicumarol and Amytal, to exclude formaldehyde as the primary reductant in the sarcosine-acetoacetate system. Dicumarol, which does not interfere with the oxidation of sarcosine to formaldehyde (9) or with the reduction of DPN by formaldehyde, inhibited DPN reduction in the presence of sarcosine. Moreover, under circumstances in which the removal of acetoacetate by sarcosine was inhibited strongly by Amytal, there was no inhibition of acetoacetate reduction by added formaldehyde. It may be concluded, therefore, that the reducing equivalents appearing in DPNH originate from the initial dehydrogenation of the sarcosine methyl group and that

**Table IV**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Increase in A502 5 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcosine</td>
<td>0.040 0.040</td>
</tr>
<tr>
<td>Sarcosine, followed by ATP</td>
<td>0.110 0.110</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>0.0075 0.0075</td>
</tr>
<tr>
<td>Formaldehyde, followed by ATP</td>
<td>0.040 0.040</td>
</tr>
<tr>
<td>ATP only</td>
<td>0.040 0.040</td>
</tr>
</tbody>
</table>

**Table V**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Acetoacetate reduced*</th>
<th>Inhibition by Amytal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without Amytal</td>
<td>With Amytal</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>0.946</td>
<td>0.297</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.810</td>
<td>0.173</td>
</tr>
</tbody>
</table>

* Acetoacetate present initially was 2.6 μmoles/2.1 ml.
Table VI  
Intramitochondrial production of acetoacetate and its endergonic reduction by sarcosine or succinate

The conditions were the same as given in the legend to Table V, except that, prior to addition of oligomycin and Amytal, the acetoacetate was produced from \( \beta \)-hydroxybutyrate for 10 min in the presence of phosphate acceptor. Prior to addition of trichloracetic acid, the incubation mixtures contained: 0.2 ml of mitochondria (9 mg of protein), 1.5 ml of buffer (glycylglycine, pH 7.5, 28 \( \mu \)moles; \( \text{MgCl}_2 \), 11.2 \( \mu \)moles; KCl, 70 \( \mu \)moles; sucrose, 70 \( \mu \)moles; and phosphate, pH 7.5, 15 \( \mu \)moles), 0.02 ml of oligomycin (6 \( \mu \)g, in ethanol), 0.1 ml of phosphate acceptor (glucose + hexokinase + ATP), acetoacetate derived from 4 \( \mu \)moles of \( \beta \)-hydroxybutyrate, and, where indicated, 0.1 ml of sarcosine or succinate (20 \( \mu \)moles), and 0.02 ml of Amytal (4 \( \mu \)moles).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Acetoacetate removed*</th>
<th>Inhibition by Amytal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without Amytal</td>
<td>With Amytal</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>0.65 ( \mu )mole</td>
<td>0.196 ( \mu )mole</td>
</tr>
<tr>
<td>Succinate</td>
<td>1.00 ( \mu )mole</td>
<td>0.130 ( \mu )mole</td>
</tr>
</tbody>
</table>

*Acetoacetate present initially was 1.34 \( \mu \)moles/2.1 ml.

As was found by Azzone, Ernster, and Weinbach (10), the rate of acetoacetate removal in the presence of succinate is variable from one mitochondrial preparation to another, and may be dependent on the age of the animals. In our hands, the rate of reduction of exogenous acetoacetate with succinate never exceeded the lower values reported by Azzone, Ernster, and Weinbach (10). In an attempt to circumvent these difficulties, a system was devised in which acetoacetate was produced endogenously from \( \beta \)-hydroxybutyrate. Energy-dependent reduction of the acetoacetate with either sarcosine or succinate was then measured in the same preparation.

Under the conditions described in the legend to Table VI, it was first determined that the oxidation of both succinate and \( \beta \)-hydroxybutyrate, in the presence of phosphate, could be greatly stimulated by ADP and that the ADP-dependent respiration was completely sensitive to oligomycin. It was also shown that succinate respiration could be increased only 25% by the addition of acetoacetate, with or without oligomycin. These findings indicated that relatively little phosphate acceptor was present in the mitochondria and, also, that the rate of acetoacetate removal could be expected to be much lower than the rate of sarcosine or succinate oxidation.

To demonstrate the sarcosine-linked reduction of acetoacetate

\[
\text{Sarcosine} - \text{CH}_3 + 2e^- \xrightarrow{\text{Dehydrogenase}} \text{ETF}(2H) \xrightarrow{\text{DPNH Dehydrogenase}} \text{Cyto } b \xrightarrow{\text{(Amytal)}} \xrightarrow{\text{Carrier}} \text{DPNH} \xrightarrow{\text{AcAc}} \xrightarrow{\text{\( \beta \)-OH-B}} \xrightarrow{\text{Carrier}} \text{O}_2
\]

The concomitant reduction of acetoacetate proceeds via the Amytal-sensitive site of the respiratory chain.

**Fig. 7.** Proposed mechanism for the endergonic reduction of mitochondrial DPN by sarcosine. Cyto \( b \), cytochrome \( b \); AcAc, acetoacetate; \( \beta \)-OH-B, \( \beta \)-hydroxybutyrate.
in such preparations, \( \beta \)-hydroxybutyrate was oxidized in the presence of ADP and phosphate or in the presence of ATP, glucose, and hexokinase. After an appropriate quantity of acetoacetate had been formed, the trapping of energy by phosphate and phosphate acceptor was terminated by the addition of oligomycin. As summarized in Table VI, addition of sarcosine to this system resulted in reduction of 50% of the acetoacetate. During the same period, 80% of the acetoacetate was removed in the presence of succinate. In both cases, the acetoacetate reduction was inhibited by Amytal.

Proposed Mechanism for Energy-dependent Reduction of DPN by Sarcosine—Based on previous work in this laboratory (9) the relationship between the sarcosine dehydrogenase-ETF system and the terminal electron transport chain has been formulated as shown in Fig. 7. Since sarcosine oxidation is Amytal-insensitive, but exhibits the same sensitivity to antimycin A as do the succinate and DPNH oxidase systems, it can be concluded that the point of interaction of ETF with the respiratory chain is located between the Amytal- and antimycin A-sensitive sites. This scheme also accounts for the observed competition among the succinate, sarcosine, and DPNH oxidases (9), and indicates how the endergonic reduction of DPN with sarcosine and succinate can be expected to involve common high energy intermediates.

The pathways outlined in Fig. 7, similar to those postulated by Ernster (19) for the reduction of DPN with succinate, also provide a working hypothesis for the mechanism of energy-dependent reduction of DPN by sarcosine. Summarized below are the proposed relations between the concurrent reactions whereby electrons from the N-methyl group produce high energy intermediates via the respiratory chain as well as reduce DPN.

ETF reduction.

\[
\text{Sarcosine} + \text{dehydrogenase} \rightarrow \text{formaldehyde} + \text{glycine} + \text{dehydrogenase (2H)} \quad (1)
\]

\[
\text{Dehydrogenase(2H) + ETF} \rightarrow \text{dehydrogenase + ETF(2H)} \quad (2)
\]

Oxidation of ETF(2H) and production of high energy intermediates via respiratory chain (\( P \cdot O = 2 \)).

\[
\text{ETF(2H) + sites (II and III) + X} \rightarrow \text{ETF + sites (II and III) (2H) \sim X} \quad (3)
\]

\[
\text{Sites (II and III) (2H) \sim X + Y + 4O_2} \rightarrow \text{sites (II and III) + X \sim Y + H_2O} \quad (4)
\]

\[
X \sim Y + P, \quad Y \sim P + X \quad (5)
\]

\[ Y \sim P + \text{ADP} \Rightarrow \text{ATP} + Y \quad (6)
\]

Reduction of DPN by ETF(2H).

\[
\text{ETF(2H) + DPN + X \sim Y} \quad \text{DPNH dehydrogenase} \quad \text{ETF + DPNH + X + Y} \quad (7)
\]

In these reaction sequences, the energy-linked reduction of DPN may proceed both via high energy phosphate intermediates (Reactions 5 to 7) and, more directly, by way of nonphosphorylated forms (Reactions 3, 4, and 7).

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