ACTIVATION OF MITOCHONDRIAL DPN KINASE FROM YEAST

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1. Introduction

It was previously shown [1,2] that mitochondria from aerobic yeast contain a unique isozyme of DPN kinase which catalyzes the phosphorylation of DPN and DPNH to form TPN and TPNH, respectively. In contrast with the behavior of DPN kinases from other sources [3–4], the mitochondrial enzyme from yeast exhibits a marked specificity for the phosphorylation of DPNH. We now report that this enzyme is activated either by a particulate fraction derived from mitochondria or, more effectively, by high concentrations of certain salts such as sodium acetate. Only the ability of the DPN kinase to phosphorylate DPNH is influenced by these activators; the rate of phosphorylation of DPN is unaffected.

2. Methods

A strain of *Sacharomyces cerevisiae* isolated from commercial yeast was cultured aerobically for 12 hr [6], and intact mitochondria were obtained by osmotic lysis of yeast spheroplasts prepared according to the two-step method of Duell et al. [6].

DPN kinase was extracted by suspending the mitochondria in 20 mM tris-Cl, pH 7.8, 0.1 M $MgCl_2$, 1 mM EDTA, and 0.5 M sucrose and then treating the mixture with diethylstilbestrol (DES) essentially as described by Byington et al. [7]. The supernatant fraction resulting from centrifugation at 54,000 *g* for 20 min contained 80–90% of the original DPN kinase activity. The enzyme was then precipitated with ammonium sulfate and further purified by gel filtration. Details of the procedure will be presented elsewhere [8].

A particulate fraction which can stimulate the soluble DPN kinase was prepared by suspending the insoluble residue from the DES-treated mitochondria in 20 mM tris-Cl, pH 7.8, 1 mM EDTA, and 0.5 M sucrose and then centrifuging the mixture at 30,000 *g* for 30 min. The lightly-packed upper layer was next resuspended, collected by centrifugation at 30,000 *g* for 60 min, and finally suspended in 20 mM tris-Cl, pH 7.8.

The standard assay system for DPN kinase consisted of 90 mM tris-Cl, pH 7.8, 3 mM ATP, 2 mM DPNH, 10 mM $MgCl_2$, 0.1% bovine serum albumin, and enzyme in a final volume of 0.5 ml. Reactions were conducted at 30° and were linear for 30 min. For measurement of DPN kinase in intact or DES-treated mitochondria, or when the insoluble fraction was present, an ATP-generating system (10 mM phosphoenolpyruvate, 10 mM KCl, and 10 μg of rabbit muscle pyruvate kinase) was incorporated, and the assays were conducted in a N_2 atmosphere. Assays with DPN as the substrate were carried out in the presence of air.

For convenience, DPN kinase activity was measured as the sum of the TPN and TPNH synthesized. Separate experiments with the purified enzyme showed that TPN and TPNH were the actual products formed when DPN and DPNH were the substrates, respectively. At the end of each incubation, 0.1 ml of 3 mM 5-ethylphenazinium ethyl sulfate was added, and the mixture was vortexed in air for 90 sec to ensure complete oxidation of all pyridine nucleotides. The

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reaction mixture was then treated with 0.2 ml of 2 N $HClO_4$ and incubated for 2 min at 30°. It was next chilled in ice, neutralized with 0.2 ml of 2 N KOH in 0.5 M tris-maleate, pH 7.4, and centrifuged at 30,000 *g* for 15 min. The clear supernatant fraction was assayed for TPN by the cycling procedure described by Slater et al. [9] using aliquots no greater than 50 μl. Under these conditions, the components of the DPN kinase assay mixture and the salts tested for activation of the enzyme had no effect on the TPN determination.

3. Results

During our initial attempts to purify the mitochondrial DPN kinase, an apparent loss of activity was encountered whenever the solubilized enzyme was separated from the DES-treated mitochondria by centrifugation. The lost activity could be recovered by adding a small quantity of the insoluble residue to the assay. The insoluble fraction, which itself possesses no DPN kinase activity, also has a marked effect on the activity of partially purified DPN kinase (fig. 1). The ability of the insoluble fraction to activate DPNH phosphorylation is destroyed by heating at 80° for 1 min.

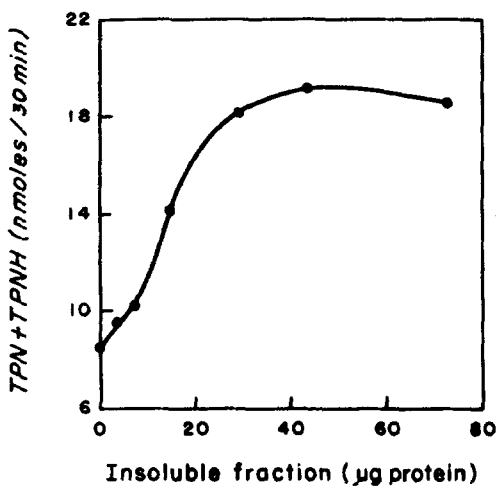

Fig. 1. Effect of the insoluble fraction derived from mitochondria on the activity of DPN kinase. Assays are carried out as described in Methods and contain 90 μg of protein from a 7.8-fold purified enzyme preparation. Reactions are initiated by addition of DPNH.

No activation is observed when DPN is the substrate.

Further study of the activation led to the finding that an even greater stimulation of DPNH phosphorylation could be achieved by the presence of high concentrations of sodium acetate in the assay. The degree of stimulation increases as the enzyme is purified. The effect of sodium acetate and of several other salts on the activity of partially purified DPN kinase is shown in fig. 2. Formate is less effective than acetate in stimulating DPNH phosphorylation, but propionate is at least as effective as acetate. Sodium chloride has only a slight effect, whereas ammonium sulfate, which stabilizes DPN kinase at low protein concentrations, has no effect when added to the assay. The activation thus appears to involve the interaction of DPN kinase with a carboxylate anion. The activation is not specific for acetate, nor is it a non-specific salt effect. When DPN kinase is assayed in the presence of saturating levels of both acetate and the insoluble fraction, the rate of DPNH phosphorylation is no greater than when the assay is carried out with saturating levels of acetate

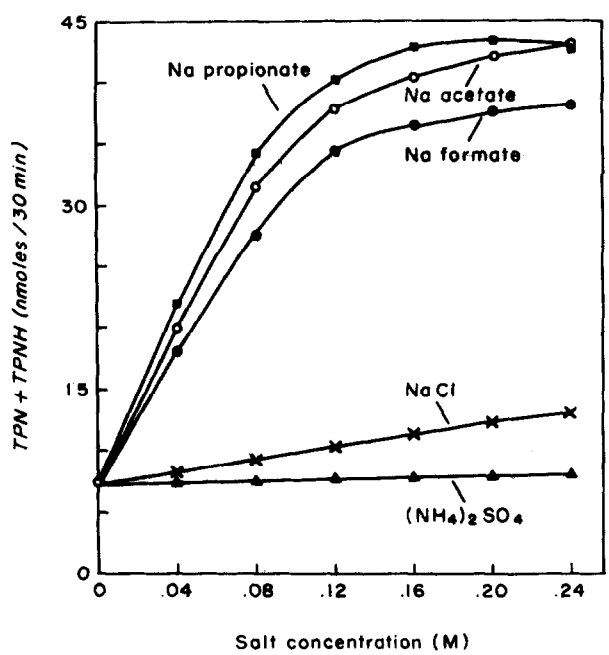

Fig. 2. Effect of salts on the activity of DPN kinase. Assays are performed with DPNH under standard conditions in the presence of various concentrations of the salts indicated. Each assay contains 86 μg of protein from a 7.8-fold purified enzyme preparation.

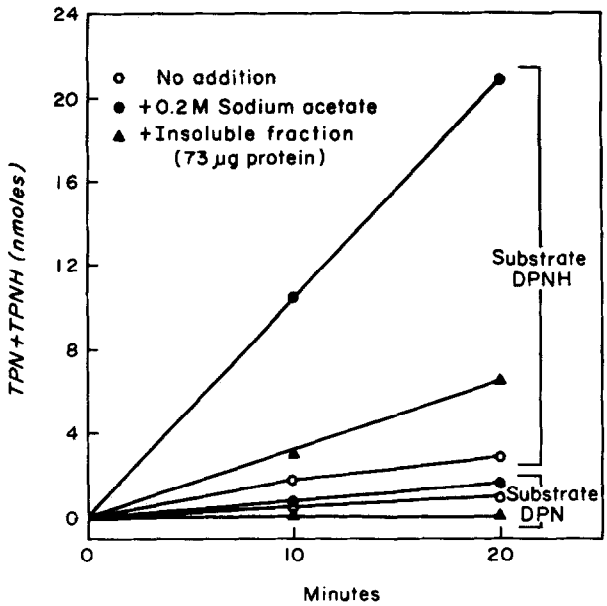

Fig. 3. Effect of saturating concentrations of sodium acetate and of the insoluble fraction on the activity of DPN kinase using DPNH and DPN as substrates. Assays are carried out as described in Methods and contain 9.8 μg of protein from a 58-fold purified enzyme preparation.

alone. No activation by acetate is observed when DPN is the substrate.

As the DPN kinase is purified, its activity with DPNH in the absence of activators decreases until it approaches the activity with DPN. Fig. 3 shows that, in the absence of activators, the activity of a partially purified DPN kinase preparation is only slightly higher when DPNH rather than DPN is used as a substrate. However, the phosphorylation of DPNH is activated both by acetate and the insoluble fraction, whereas the phosphorylation of DPN is only slightly affected by these substances (fig. 3). It thus appears that during the course of purification, some factor is lost which is responsible for conferring DPNH specificity upon the DPN kinase, and that this factor can be replaced either by a particulate fraction derived from the mitochondria or, more effectively, by high concentrations of certain salts such as acetate or propionate.

4. Discussion

In the present communication, the assumption is made that we are dealing with a single DPN kinase. Nevertheless, our results do not exclude the possibility that there are two enzymes [10] : one specific for DPN and a second specific for DPNH. If two enzymes are indeed present, then the activation which we have reported relates only to the enzyme which is specific for DPNH. Thus far, however, two distinct DPN kinases of mitochondrial origin have not been separated.

Although yeast mitochondria contain very small amounts of TPN and TPNH [1,11], the action of the mitochondrial DPN kinase can potentially raise the intramitochondrial level of TPN + TPNH 5-fold in 2 min [1,2]. Such an elevation would greatly influence the activities of metabolic pathways which are linked to TPN and TPNH, such as isocitrate oxidation and fatty acid synthesis. The activity of DPN kinase would in turn be expected to be regulated in some manner by a mechanism which reflected the requirement for the latter reactions in mitochondria. The strong dependence of the activity of the isolated mitochondrial DPN kinase on the assay conditions suggests that this enzyme is likely to be subject to some kind of metabolic control *in vivo*.

Regulation of the overall level of TPN and TPNH in mitochondria also implies that there is a means for lowering the concentrations of these pyridine nucleotides. This function could be fulfilled by a mitochondrial phosphatase which can convert TPN and TPNH to DPN and DPNH, respectively. The presence of such a phosphatase in yeast mitochondria has already been reported [1]. It therefore seems likely that maintenance of the intramitochondrial levels of TPN and TPNH, as a consequence, control over TPN-linked metabolic pathways in yeast mitochondria is determined at least in part by the combined actions of mitochondrial DPN kinase and phosphatase.

Acknowledgement

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References

[1] C.Bernofsky and M.F.Utter, Science 159 (1968) 1362.
[2] M.M. Griffiths and C. Bernofsky, Federation Proc. 29 (1970) 344.
[3] T.P. Wang and N.O. Kaplan, J. Biol. Chem. 206 (1954) 311.
[4] A.E. Chung, J. Biol. Chem. 242 (1967) 1182.
[5] H. Oka and J.B. Field, J. Biol. Chem. 243 (1968) 815.
[6] E.A. Duell, S. Inoue, and M.F. Utter, J. Bacteriol. 88 (1964) 1762.
[7] K.H. Byington, J.M. Smoly, A.V. Morey, and D.E. Green, Arch. Biochem. Biophys. 128 (1968) 762.
[8] M.M. Griffiths and C. Bernofsky, in preparation.
[9] T.F. Slater, B. Sawyer, and U. Sträuli, Arch. Intern. Physiol. Biochem. 72 (1964) 427.
[10] D.K. Apps, European J. Biochem. 13 (1970) 223.
[11] C. Bernofsky, unpublished data.