A Crystalline Flavin Pyruvate Oxidase*

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Previous communications from this laboratory have reported the recognition and partial purification of a flavoprotein pyruvate oxidase which catalyzes the oxidative decarboxylation of pyruvate to acetate and CO₂ (1, 2). This enzyme is obtained from an acetate auxotroph of *Escherichia coli* and is formed in response to the accumulation of pyruvate in the growth medium (3). We now wish to report the isolation of this flavoprotein in crystalline form and to describe some of the properties of the purified enzyme.

The purified flavoprotein has the typical behavior of a globular protein. It is insoluble in aqueous solutions of low ionic strength but readily dissolves in solutions of ionic strength of 0.2 and higher. Purification and crystallization of the flavoprotein is accomplished by the following steps: (a) rupture of the *Escherichia coli* cells by grinding with glass beads in 0.02 M potassium phosphate buffer, pH 7, in a colloid mill (4); (b) preparation of a 0.25 to 0.75 saturated ammonium sulfate fraction; (c) protamine sulfate treatment to remove nucleic acids; (d) preparation of a 0.36 to 0.55 saturated ammonium sulfate fraction; (e) heat treatment at 60°C for 5 minutes to denature inactive protein; (f) chromatography on DEAE-cellulose* at pH 5.7 with a gradient elution between 0.02 M and 0.3 M potassium phosphate buffer; (g) protamine sulfate precipitation and elution of the flavoprotein with 0.2 M potassium phosphate buffer, pH 5.7; and (h) dilution and crystallization of the flavoprotein in 0.05 to 0.1 M potassium phosphate buffer. A typical purification procedure for preparation of the crystalline enzyme is given in Table I. A photograph of the crystalline protein showing the rhombohedral character of the crystals is shown in Fig. 1.

The crystalline flavoprotein has a molecular weight of approximately 265,000 as determined by the Archibald method (5). The sedimentation constant (s₂₀,ₚ) of the enzyme in 0.2 M potassium phosphate buffer, pH 5.7, is 11.5 S. Flavin analyses (6-8) performed on the enzyme indicate that FAD is the sole flavin component and is present in a ratio of 4 moles of FAD per mole of enzyme. The crystalline enzyme does not contain thiamine pyrophosphate but has an absolute thiamine pyrophosphate requirement for reduction of the enzyme-bound FAD.

The reduced flavoprotein-substrate complex is nonautoxidizable. However, in confirmation of previous reports (9, 10), it is air oxidizable in the presence of a cytochrome-containing particulate fraction or in the presence of a soluble cytochrome b₁ preparation derived from the particulate fraction. When fully saturated with soluble cytochrome b₁, the flavoprotein has a turnover number of 10,000 (moles of pyruvate oxidized per minute per mole of enzyme). The incubation of reduced flavoprotein with soluble cytochrome b₁ leads to the oxidation of the flavoprotein followed by the autoxidation of cytochrome b₁. These results suggest that oxidation of the flavoprotein in vivo is accomplished via a cytochrome pathway.

When supplemented with pyruvate as substrate and thiamine pyrophosphate as coenzyme, the purified flavoprotein will also react sluggishly with electron acceptors such as 2,6-dichlorophenolindophenol and ferricyanide (turnover number = 1800, ferricyanide). Maximal activity (turnover number = 53,000, ferricyanide) can be achieved in these reactions by a variety of methods. Partial hydrolysis of the flavoprotein by trypsin (11) or chymotrypsin (12) produces an altered protein which is approximately 25 times more active than the native protein with ferricyanide as electron acceptor. Exposure of the flavoprotein to certain ionic surface active agents, such as sodium lauryl sulfate, also converts the flavoprotein to an activated form in the ferricyanide reaction. Finally, the cytochrome containing particulate fraction (10), soluble cytochrome b₁ (10), or lipid extracts derived from the particulate fraction (13) activate the flavoprotein in the ferricyanide reaction. The mechanism of activation of the flavoprotein by these various agents is under investigation.
REFERENCES


Fig. 1. Crystals of flavin pyruvate oxidase (750 X)
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