DIPHOSPHOPYRIDINE NUCLEOTIDE-NITRATE REDUCTASE FROM ESCHERICHIA COLI[1]

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Received for publication November 17, 1954

Neurospora nitrate reductase which catalyzes the reduction of nitrate to nitrite by reduced triphosphopyridine nucleotide (TPNH) is a metallo-flavoprotein with flavin-adenine dinucleotide (FAD) as the prosthetic group and molybdenum as the metal component (Nason and Evans, 1953; Nicholas and Nason, 1954). A comparable FAD-molybdenum-protein which can utilize reduced diphosphopyridine nucleotide (DPNH) or reduced triphosphopyridine nucleotide has also been characterized from soybean leaves (Evans and Nason, 1953; Nicholas and Nason, *in press*).

In *Escherichia coli*, however, the nature of the immediate electron donor as well as other properties of nitrate reductase has been less clear. Yamagata (1938, 1939) found that nitrate reductase in a cell-free preparation of *E. coli* failed to oxidize DPNH and was strongly inhibited by cyanide. Subsequently, others, using chemically or enzymatically reduced dyes as the electron source, have reported that the enzyme is a sulfhydryl flavoprotein with an iron component as indicated by light-reversible carbon monoxide inhibition (Egami and Sato, 1947, 1948*a*, *b*; Sato and Egami, 1949). Joklik (1950), however, was unable to demonstrate an effect of —SH reagents and carbon monoxide. The earlier statement that cytochrome b is identical with the enzyme has been withdrawn (Sato and Niwa, 1952).

It is the purpose of this paper to describe the purification and properties of a DPN-linked nitrate reductase in *E. coli* and its identification as a metallo-flavoprotein with molybdenum as the probable metal component.

[1] Contribution no. 104 of the McCollum-Pratt Institute. This investigation was supported in part by a research grant (no. 2332) from the National Institutes of Health, United States Public Health Service, and from the Climax Molybdenum Company.

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MATERIALS AND METHODS

Cell-free extracts were prepared from cells of *E. coli* strain B. The cells were grown on a basal medium consisting of the following per liter: Na_2HPO_4, 6 g; KH_2PO_4, 3 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.5 g; NH_4Cl, 0.1 g; $NaNO_3$, 1.0 g; glucose, 4 g. The medium was dispensed in 8 liter volumes in 20 liter pyrex carboys. These were inoculated and grown on a shaking machine at 23 C for 24 hours. The cells were harvested in a Sharples centrifuge at 30,000 ×G and were washed with 1 per cent saline to remove nitrite. They were then frozen at −15 C for 3 hours before homogenizing with a cold mortar and pestle using an equal weight of alumina powder (Alcoa A-301). After grinding for 10 minutes, three times their weight of cold 0.1 M K_2HPO_4 (pH 7) containing sodium versenate (10^{-4} M final concentration) was slowly added and grinding continued for another 5 minutes. After centrifugation for 15 minutes at 4 C, the supernatant solution which was cell-free and turbid was used as the crude extract for further purification. Preliminary experiments showed that at least 75 per cent of the nitrate reductase activity of the homogenate was present in the crude extract.

Cofactors and other substances. The preparation of the various cofactors, substrates, and other materials used in this study has already been indicated (Nason and Evans, 1953). The activity of the FAD-free apoenzyme of pig kidney D-amino acid oxidase using DL-alanine and added FAD (5×10^{-7} M) was 115 μL O_2 per ml per 30 minutes compared to 32 μL O_2 when FAD was omitted.

Determination of nitrate reductase. The assay procedure consisted of adding 0.1 ml enzyme, 0.1 ml of 0.1 M KNO_3, 0.05 ml of 10^{-4} M FMN or

TABLE 1

Summary of purification of Escherichia coli nitrate reductase

Fraction	Total Volume	Total Units	Total Protein	Specific Activity	Per Cent Recovery
	ml		*mg*	*units per mg protein*	
I Crude extract	10	473	43	11	—
II 0–70 per cent ppt ammonium sulfate	5	382	11.5	33	81
III 0–50 per cent ppt ammonium sulfate	2	376	4.7	80	79
IV Calcium phosphate gel eluate	2	156	0.9	173	33

boiled pig heart extract, 0.04 ml of 4×10^{-3} μM DPNH, and 0.1 M phosphate buffer pH 7.0, to give a final volume of 0.5 ml. After 10 minutes of incubation at 25 C the typical red azo dye was developed and measured in the usual manner (Nason and Evans, 1953). The definition for an enzyme unit and specific activity is the same as that for Neurospora (Nicholas, Nason, and McElroy, 1954). Protein was determined by the method of Lowry *et al.* (1951).

RESULTS

Enzyme purification. A summary of the purification steps is given in table 1. The 0–70 per cent ammonium sulfate fraction and subsequent 0–50 per cent fraction were prepared and collected in essentially the same manner as described for the Neurospora enzyme (Nason and Evans, 1953). Treatment of this fraction with an equal volume of calcium phosphate gel followed by two washings with equal volumes of 0.1 M phosphate buffer, pH 7.0, and finally elution with an equal volume of 0.2 M pyrophosphate, pH 7.0, at 4 C yielded a fraction (IV) with an over-all purification of 15-fold and representing 10 per cent of the units in the crude starting material.

Enzyme stability and pH optimum. The enzyme (Fraction IV) which stores well at −15 C, pH 7.0, loses more than 90 per cent of its activity after 5 minutes at 65 C. Addition of

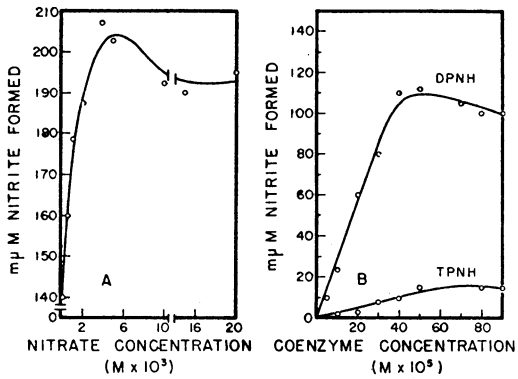

Figure 1a. Effect of nitrate concentration on nitrate reductase activity.

Figure 1b. Effect of concentration of reduced pyridine nucleotides on nitrate reductase activity. The coenzyme concentrations are the final concentrations in the test.

glutathione at 10^{-3} M final concentration further enhances storage of the enzyme, and the use of glutathione treated membranes markedly increases stability of *E. coli* nitrate reductase during dialysis (Nicholas and Nason, 1954). The maximal pH is between 7.0 and 8.0, the activity falling to 50 per cent at pH's 5.0 and 9.5.

Substrate saturation. The dissociation constant (Km) of the enzyme-nitrate complex estimated from the saturation curve (figure 1a) is approximately 0.1×10^{-3} moles per liter. The enzyme shows a marked specificity for DPNH as the electron donor whereas TPNH is relatively ineffective (figure 1b). One-half maximal activity with DPN was obtained at about 2.0×10^{-4} moles per liter. Glutathione and molecular hydrogen failed to replace DPNH as an electron donor. Enzymatic nitrate reduction proceeds 3 to 4 times as fast under anaerobic conditions than in air; and the appreciable endogenous rate of DPNH oxidation in the absence of nitrate is considerably reduced under anaerobic conditions.

Prosthetic flavin group. Unlike that of Neurospora nitrate reductase the flavin of the *E. coli* enzyme is not easily dissociated. Three to 4 ammonium sulfate precipitations of the enzyme at pH 4.0 failed to show a flavin requirement. However, when the enzyme was subjected at 0 C to 3 to 5 minutes of ultraviolet light illumination (McElroy *et al.*, 1954) with a Keese lamp, its activity was decreased by 90 per cent,

and it was restored almost completely by adding either FAD, FMN, or boiled pig heart extract. Subsequently, it was found that 7 to 10 ammonium sulfate precipitations at pH 4.0 gave some dissociation of the flavin group although not as effectively as the ultraviolet light treatment. Both FAD and FMN are equally effective in restoring enzyme activity (figure 2), half maximal activation occurring at about 0.5×10^{-6} moles per liter. Evidence for the identity of nitrate reductase flavin as FAD was provided by its activity with the apoenzyme of the FAD-specific D-amino acid oxidase as well as by fluorometric analysis as used previously (Nason and Evans, 1953). The latter method showed that more than 90 per cent of the flavin can be accounted for as FAD, the boiled enzyme preparation containing 7×10^{-4} μM FAD per ml of enzyme.

Inhibitors and metal component. The sulfhydryl nature of the enzyme was indicated by the complete reversal of *p*-chloromercuribenzoate inhibition (60 per cent inhibition at 5×10^{-4} M) by glutathione at 10^{-3} M final concentration.

Cyanide, azide, and 8-hydroxyquinoline at 10^{-3} M final concentration resulted in an appreciable inhibition of enzyme activity (80, 90, and 40 per cent, respectively) indicating a metal component. Carbon monoxide, however, did not inhibit the enzyme. The addition to the reaction mixture of various metal ions (Mn^{++}, Fe^{++}, Fe^{+++}, Cu^{++}, Co^{++}, $BO_3^{=}$, MoO_4^{-}, or Zn^{++} at 10^{-4} M final concentration) failed to enhance the activity of the enzyme. Extracts of molybdenum-deficient *E. coli* showed a one-third reduction in specific activity of nitrate reductase whereas iron-deficient extracts showed no change. Removal of trace metals from the basal culture solutions was achieved by purification methods described previously (Nicholas, 1952).

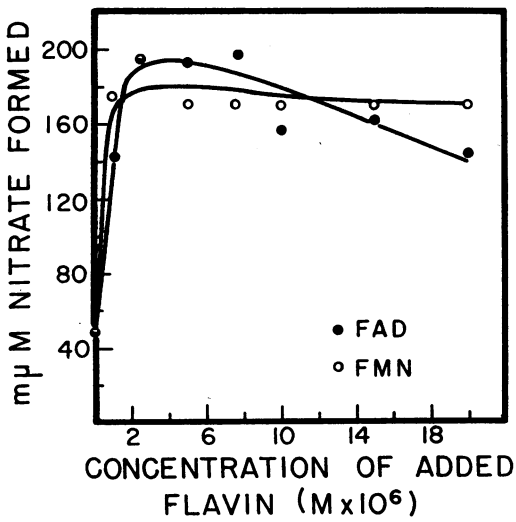

Figure 2. Effect of flavin concentration on nitrate reductase activity. The flavin concentrations are the final concentrations in the test.

In order to remove the metal constituent of nitrate reductase the enzyme was dialyzed successively against cyanide and phosphate-glutathione solutions using glutathione soaked membranes as described elsewhere (Nicholas and Nason, 1954). Of the various metals tried (Fe^{++}, Fe^{+++}, Cu^{++}, Mn^{++}, Zn^{++}, Mg^{++}, Cr^{+++}, W^{++}, V^{++}) only molybdate (1 μg of metal per 0.1 ml of enzyme) was effective in reactivating the dialyzed enzyme—to the extent of 65 per cent of its original activity.

DISCUSSION

The preceding experiments have shown that *E. coli* has a DPN-linked nitrate reductase with FAD and probably molybdenum as cofactors, thus resembling the corresponding molybdoflavoproteins in Neurospora and soybean leaves. It is likely that the mechanism of electron transport is similar to that established for the enzyme in Neurospora and in soybean leaves, namely that the sequence of electron transport is as follows:

$$\text{DPNH} + \text{FAD (or FMN)} \rightarrow \text{Mo} \rightarrow NO_3^-$$

Evans (1954) recently has reported the presence of a DPN-specific nitrate reductase with a metal component and a probable flavin requirement from a *Rhizobium* species from soybean root nodules.

The nitrate reducing system in *E. coli* described by Egami and co-workers allegedly containing iron as the metal component can probably be ascribed to an entirely different and independent system from that presented here. A particulate DPN-cytochrome c requiring system with a strong nitrate reducing activity has been observed by McElroy (*personal communication*) in *Achromobacter fischeri*.

SUMMARY

The purification and properties are described of a soluble nitrate reductase from *Escherichia*

coli which catalyzes the reduction of nitrate to nitrite by reduced diphosphopyridine nucleotide and not reduced triphosphopyridine nucleotide. The enzyme which has been purified 15 times is a flavoprotein with flavoadenine dinucleotide as the prosthetic group as shown by reactivation studies, fluorometric analysis, and the D-amino acid oxidase test. The presence of —SH groups on the enzyme was indicated by glutathione reversal of *p*-chloromercuribenzoate inhibition. Further similarity to the enzyme in Neurospora and soybean leaves was shown by the presence of a metal constituent tentatively identified as molybdenum; this was indicated by inhibition, by metal binding agents, a decrease in activity under molybdenum-deficient conditions, and restoration specifically by molybdate of cyanide dialyzed enzyme.

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