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Orthophosphite-Nicotinamide Adenine Dinucleotide Oxidoreductase from *Pseudomonas fluorescens*

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Information was obtained on the general properties and specificity of orthophosphite-nicotinamide adenine dinucleotide oxidoreductase. The enzyme was extracted from *Pseudomonas fluorescens* 195 grown in medium containing orthophosphite as the sole source of phosphorus. An enzyme preparation suitable for characterization was obtained from crude extracts by use of high-speed centrifugation, protamine sulfate precipitation, ammonium sulfate fractionation, and Sephadex gel filtration. The enzyme exhibited maximal activity at *p*H 7.0, and was inactivated within 6 min at 37 C. Arsenite, hypophosphite, nitrite, selenite, and tellurite were not oxidized by the enzyme. Sulfite inhibited the enzymatic oxidation of orthophosphite in an apparent competitive manner.

A variety of bacteria are capable of utilizing orthosphosphite as a sole source of phosphorus (1, 3). A study of the physiological aspects of phosphite metabolism of *Pseudomonas fluorescens* 195 and *Serratia marcescens* 24 revealed the presence of an inducible phosphite-oxidizing enzyme (7). Resting-cell suspensions and cell-free extracts prepared from bacteria grown in liquid medium containing orthophosphite as a sole source of phosphorus oxidized orthophosphite to orthophosphate. Dialysis of cell-free extracts resulted in loss of a cofactor which was subsequently identified as nicotinamide adenine dinucleotide (NAD).

The bacterial oxidation of orthophosphite has been proposed to be a step in a biological phosphorus cycle which involves the oxidation and reduction of phosphorus between oxidation states ranging from the −3 oxidation state of phosphine to the +3 and +5 oxidation states of orthophosphite and orthophosphate, respectively (2). The purpose of this investigation is to provide information concerning the general properties and specificity of orthophosphite-NAD oxidoreductase from *P. fluorescens* 195, which may aid in our understanding of the characteristics and importance of such a phosphorus cycle in nature.

Several enzymes have been demonstrated to be capable of oxidizing or reducing nitrogen or sulfur anions (8-10), and the microorganisms containing those enzymes have been postulated to be important in the nitrogen and sulfur mineral cycles (13). This report, however, represents the first description of an oxidoreductase which is specific for a phosphorus anion and which may be important in a biological phosphorus cycle.

MATERIALS AND METHODS

Chemicals. Reagents were obtained from the following sources: NAD, Sigma Chemical Co., St. Louis, Mo.; protamine sulfate, Calbiochem, Los Angeles, Calif.; ammonium sulfate (special enzyme grade), Mann Research Laboratories, New York, N.Y.; Sephadex G-100, Pharmacia, Uppsala, Sweden.

Sodium phosphite ($Na_2HPO_3 \cdot 5H_2O$), laboratory chemical grade, was obtained from Fisher Scientific Co., Pittsburgh, Pa. A sample was passed through an anion-exchange column of the type described by Pollard et al. (11), to determine whether this reagent contained a significant amount of contaminating phosphate. A single, symmetrical peak was obtained, which indicated that any orthophosphate present in phosphite reagent was below the 2% limit of resolution of the technique.

All other reagents were "certified reagent" grade (Fisher Scientific Co.).

Culture medium and growth conditions. The following basal medium was prepared with glass-redistilled water: glucose, 0.5%; NH_4Cl, 0.2%; KCl, 0.2%; Na_2SO_4, 0.002%; $MgCl_2 \cdot 6H_2O$, 0.05%; tris(hydroxymethyl)aminomethane (Tris) adjusted to *p*H 7.0 with HCl, 1.2%.

A 30-liter batch of basal medium containing approximately 10 ml of silicone antifoam (Dow Corning Corp., Midland, Mich.) was autoclaved in a 40-liter, stainless-steel fermentation tank. After the medium had cooled, a filter-sterilized phosphite solution was

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added to a concentration of 280 mg per liter, and the fermentor was inoculated with 5 liters of a 24-hr cultuer of *P. fluorescens* 195 grown in the same medium. Sterile air was passed into the medium, and the temperature was maintained at 30 C.

The culture was agitated continuously, and after 16 hr the cells were harvested in a Sharples supercentrifuge. The cell paste was suspended in an equal volume of acetate buffer (0.1 M, *p*H 6.0) and stored at −20 C.

Protein and phosphorus assays. Protein concentration was determined by the Folin-Ciocalteau reagent according to the procedure of Lowry et al. (5). Bovine serum albumin was used, arbitrarily, as a protein standard. The method of Chen, Toribara, and Warner (4) was employed in all phosphorus assays.

Enzyme assay. Phosphite-oxidizing activity in the first three steps of the purification procedure was assayed by measuring inorganic orthophosphate accumulation. A reaction mixture containing protein, 3×10^{-3} M NAD, 10^{-2} M HPO_3^{-2}, and 0.1 M acetate buffer (*p*H 6.0) was incubated at 25 C, and at appropriate intervals 0.4-ml samples were withdrawn and added to an equal volume of 10% trichloroacetic acid. After standing for 10 min, 1.2 ml of glass-redistilled water was added and the suspensions were centrifuged at 17,000 × *g*. The supernatant fractions were stored at 2 C until assayed for inorganic phosphate.

In step IV of the purification procedure and in all subsequent experiments, a spectrophotometric method of enzyme assay was employed. Spectrophotometric measurements were made at room temperature with a Beckman DU spectrophotometer equipped with a Gilford log converter and automatic cuvette changer attached to a recorder. Quartz microcuvettes (0.2-ml) containing 0.18 ml of extract in buffer (0.1 M Tris chloride, *p*H 7.0) and 0.02 ml of cofactors and substrates were employed. The blank contained 0.1 M Tris chloride buffer.

Specific activity was defined either as millimicromoles of NAD reduced or inorganic phosphate produced per minute per milligram of protein.

Purification and characterization of the enzyme. All steps in the purification procedure were carried out at 2 C. In steps I to III, 0.1 M acetate buffer (*p*H 6.0) was used. The enzyme was eluted from the Sephadex G-100 column with 0.01 M acetate buffer (*p*H 6.0).

The heat lability of the enzyme was determined by incubating samples of the purified enzyme preparation in Tris chloride buffer at 39 C and at intervals plunging portions of the heated sample into ice. After rewarming to room temperature, the samples were assayed for phosphite-oxidizing activity.

RESULTS

Enzyme purification. A procedure based on the finding that the enzyme is associated with the soluble portion of a cell-free extract was devised for the purification of the phosphite-oxidizing enzyme. It is summarized in Table 1 and described below.

Step I: high-speed centrifugation. A 12-ml amount of thawed cells suspended in 47 ml of buffer was disrupted in a French pressure cell and centrifuged at 17,000 × *g* for 20 min. The cell-free extract thus obtained was centrifuged at 144,000 × *g* for 1.5 hr, the pellet was discarded, and the supernatant fraction was adjusted to a protein concentration of 15 mg/ml by the addition of buffer. This fraction was designated "high-speed supernatant fraction."

Step II: protamine sulfate fractionation. Solid ammonium sulfate was added with stirring to the high-speed supernatant fraction to a final concentration of 0.25 M. One-fifth volume of 2% protamine sulfate was added dropwise, and the mixture was stirred for 20 min. The precipitate, which contained large quantities of nucleic acids, was removed by centrifugation. The total activity in enzyme units increased approximately 50% in this step.

Step III: ammonium sulfate fractionation. The $(NH_4)_2SO_4$ concentration in the protamine sulfate fraction was raised to 1.6 M, and, after 20 min of stirring, the resulting precipitate was removed by centrifugation. Ammonium sulfate was again added to the supernatant fraction, bringing the concentration to 2.4 M. After stirring for 20 min, the precipitate was collected by centrifugation and dissolved in buffer. The protein concentration of this fraction was 10.2 mg/ml.

Step IV: Sephadex gel filtration. Sephadex G-100 was hydrated in buffer and allowed to stand overnight. The fines were removed by repeatedly adding large quantities of buffer, stirring, and decanting the supernatant fluid. A glass column

TABLE 1. *Purification of orthophosphite-NAD oxidoreductase from Pseudomonas fluorescens 195 cells*

Step	Total activity (enzyme units)	Total protein	Specific activity (units/mg of protein)	Recovery	Purification factor
		mg		%	
I. High-speed supernatant fraction....	6,210	654	9.5	—	1
II. Protamine sulfate fractionation....	9,300	440	21.0	150	2
III. Ammonium sulfate fractionation (1.6 to 2.4 M)........................	4,210	112	37.5	70	4
IV. Sephadex G-100 chromatography..	2,900	11	265.0	50	28

(150 by 2.5 cm) was packed with the resulting gel, and the enzyme solution from step III was layered onto the top of the gel column. Buffer was passed through the column at a rate of 20 ml/hr, and 10-ml fractions were collected. The enzyme was eluted as a nearly symmetrical peak (Fig. 1).

This procedure, which removed membrane fragments and nucleic acids from the crude extract, enriched the phosphite-oxidizing protein 28-fold and provided a preparation suitable for characterization. The rate of oxidation of phosphite by the purified enzyme preparation was measured spectrophotometrically by following the increase in absorbance at 340 mμ. The initial reaction rate was linear for at least 15 min (Fig. 2). The influence of enzyme concentration on the rate of phosphite oxidation is illustrated in Fig. 3. The rate of reduced NAD ($NADH_2$) formation was directly proportional to the amount of enzyme in the reaction mixture over a wide range of concentrations.

Properties of the enzyme: required reaction components. The enzyme is specific for NAD, since nicotinamide adenine dinucleotide phosphate (NADP) did not serve as an electron acceptor (Fig. 2). There was essentially no oxidation of phosphite when flavin adenine nucleotide or flavin mononucleotide was added as the sole coenzyme to dialyzed crude extracts.

pH optimum. The effect of *p*H on the enzyme activity was determined in 0.1 M acetate, histidine-hydrochloride, and Tris chloride buffers by use of the spectrophotometric assay procedure. A broad, almost symmetrical bell-shaped curve of enzyme activity was obtained, with optimal activity at approximately *p*H 7.0 (Fig. 4). No detectable activity was observed below *p*H 4.0 and above *p*H 9.0.

Thermal inactivation. Heating the purified enzyme in Tris chloride buffer at 39 C for 6 min resulted in complete destruction of measurable orthophosphite-NAD oxidoreductase activity.

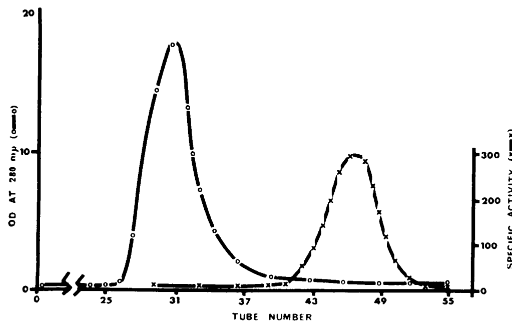

FIG. 1. *Elution pattern of orthophosphite-NAD oxidoreductase from Sephadex G-100 column.*

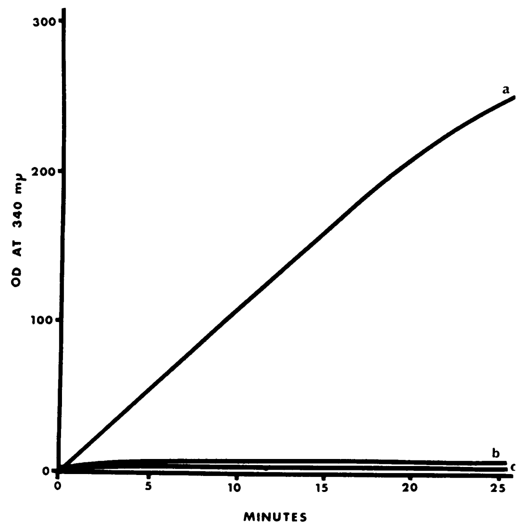

FIG. 2. *Oxidation of phosphite by the purified enzyme as a function of time. The reaction mixtures contained 10^{-3}M NAD, 2.0×10^{-3}M HPO_3^{-2}, and 0.1 mg of protein per ml. (a) Complete reaction mixture; (b) reaction mixture lacking either NAD or substrate; (c) reaction mixture containing 10^{-3}M NADP as a coenzyme. The curves are tracings of recorder plots.*

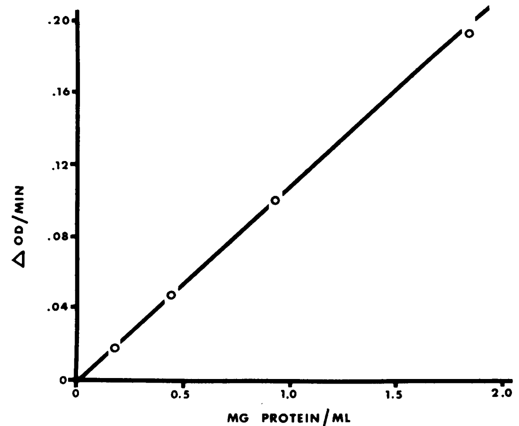

FIG. 3. *Reaction rate as a function of enzyme concentration. Reaction mixtures contained 3.0×10^{-3} M NAD and 10^{-2}M HPO_3^{-2}.*

Substrate affinity. The influence of phosphite concentration on the reaction rate is shown in Fig. 5. The amount of enzyme used in these experiments was saturated by 2.0×10^{-3} M orthophosphite. From these data, a Lineweaver-Burk plot was constructed and the value for the K_m was calculated to be 6.3×10^{-4} M.

Substrate specificity. The following anions were tested for activity as substrates for orthophos-

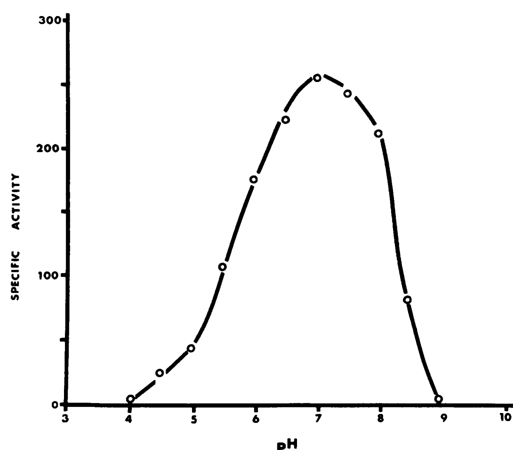

FIG. 4. *Effect of pH on the rate of phosphite oxidation by the purified enzyme preparation. The following buffers were employed: pH 4.0 to 6.0, 0.1 M acetate; pH 6.0 to 7.0, 0.1 M histidine hydrochloride; pH 7.0 to 9.0, 0.1 M Tris chloride.*

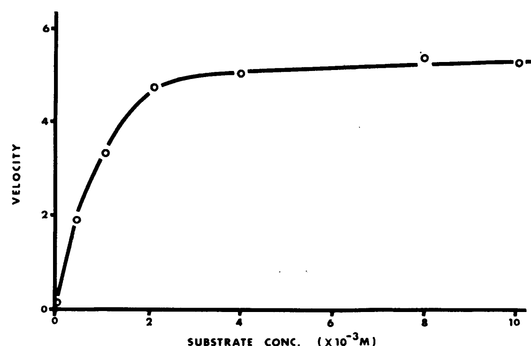

FIG. 5. *Effect of substrate concentration on the velocity of phosphite oxidation. The reaction mixtures contained 3.0 × 10^{-3} M NAD and 0.1 mg of protein per ml. Velocity given in millimicromoles of NAD reduced per minute.*

phite-NAD oxidoreductase: arsenite, hypophosphite, nitrite, orthophosphite, selenite, sulfite, and tellurite. To reaction mixtures containing protein and NAD at final concentrations of 0.4 mg/ml and 3.0 × 10^{-3} M, respectively, the anion to be tested for substrate activity was added to final concentrations of 10^{-2} and 10^{-3} M. During a 40-min period, NAD reduction was observed only in the reaction mixture containing orthophosphite. The other anions were inactive as substrates.

Inhibition by sulfite. One of the anions which was tested for substrate activity inhibited the enzymatic oxidation of phosphite. To reaction mixtures containing NAD, enzyme, and orthophosphite (10^{-3} M), each of the anions was added to final concentrations of 10^{-3} and 10^{-2} M. Sulfite alone inhibited the oxidation of phosphite in this system. The nature of this inhibition was investigated by adding various concentrations of sulfite to reaction mixtures containing protein, NAD, and orthophosphite. A double reciprocal plot of the data indicates that the inhibition is apparently competitive (Fig. 6).

DISCUSSION

Several anions are available commercially in which phosphorus is present in an oxidation state lower than the +5 oxidation state of the phosphorus in orthophosphate. These include hypophosphite and phosphine in addition to orthosphophosphite. Certain of these anions have been reported to occur in nature. Rudakow (12) observed the reduction of mineral phosphates in soil (to phosphite, hypophosphite, and phosphine) under anaerobic conditions. Different soil types were found to vary in their capacity to bring about the reduction of phosphate, and pure cultures of bacteria which were capable of reducing phosphate were isolated from the most active phosphate-reducing soil samples.

More recently, Tsubota (14) obtained evidence that phosphate reduction takes place in flooded paddy fields. *Clostridium butyricum* and *Escherichia coli* were demonstrated to reduce phosphate.

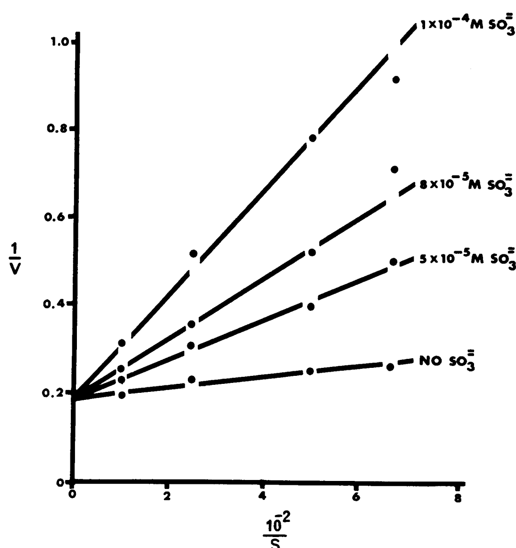

FIG. 6. *Apparent competitive inhibition of orthophosphite-NAD oxidoreductase by sulfite. Velocity given in terms of millimicromoles of NAD reduced per minute. Reaction mixtures contained protein and NAD at final concentrations of 0.1 mg/ml and 3.0 × 10^{-3} M, respectively.*

The products of these reduction reactions were identified by paper chromatography as phosphite and hypophosphite. These processes have been postulated to constitute a microbiological mineral cycle which is similar in its most general features to the nitrogen and sulfur mineral cycles (2). This paper contains the first analysis of the biochemical features of one step in the proposed cycle, the bacterial oxidation of orthophosphite.

A relatively simple, rapid, and efficient procedure was devised for the purification of the enzyme responsible for the oxidation of orthophosphite in *P. fluorescens* 195. A 28-fold purified preparation was considered sufficiently pure for the characterization studies, since all $NADH_2$ oxidase activity was eliminated during the purification procedure, and the assay system based on NAD reduction was demonstrated to be directly proportional to enzyme concentration.

The elution pattern obtained from chromatography on Sephadex G-100 indicates that the enzyme is probably of relatively small molecular size, since its flow through the column was greatly impeded as compared with the rate of flow of the major protein portion. The finding that the *p*H optimum of the reaction velocity was approximately 7.0 may indicate that the enzyme reacts preferentially with the divalent anion, HPO_3^{-2}, since the ionization of orthophosphite proceeds in the following manner:

$$H_3PO_3 \xrightarrow{pK\ 2.0} H_2PO_3^- \overset{pK\ 6.8}{\rightleftharpoons} HPO_3^{-2}$$

A variety of anions are known to be active in enzyme systems, and, in a few cases, enzymes catalyzing their oxidation or reduction have been described (8–10, 13). Several of these anions were tested for their ability to act as substrates in experiments in which NAD reduction was followed. It was discovered that only orthophosphite could function as a substrate. This finding, along with the determination of the high affinity of the enzyme for phosphite, leads to the conclusion that phosphite is indeed the natural substrate of the enzyme. The enzyme's primary function in cellular metabolism is, therefore, probably that of phosphite oxidation. The discovery that a wide variety of bacteria are capable of oxidizing phosphite (Malacinski, Ph.D. Thesis, Indiana Univ., Bloomington, 1966), presumably via an orthophosphite-NAD oxidoreductase, supports the hypothesis that microorganisms do play a role in the phosphorus cycle. The fact that bacteria retain the genetic capability to synthesize a phosphite-specific enzyme, although they utilize phosphate phosphorus preferentially, would suggest strongly that microorganisms are exposed continually to orthophosphite in nature and utilize it frequently as a source of phosphorus.

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LITERATURE CITED

1. ADAMS, F., AND J. P. CONRAD. 1953. Transition of phosphite to phosphate in soils. Soil Sci. **75:** 361–371.
2. ALEXANDER, M. 1961. Introduction to soil microbiology. John Wiley & Sons, Inc., New York.
3. CASIDA, L. E., JR. 1960. Microbial oxidation and utilization of orthophosphite during growth. J. Bacteriol. **80:**237–241.
4. CHEN, P. S., T. Y. TORIBARA, AND H. WARNER. 1956. Microdetermination of phosphorus. Anal. Chem. **28:**1756–1758.
5. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
6. MACLEOD, R. M., W. FARKAS, I. FRIDOVICH, AND P. HANDLER. 1961. Purification and properties of hepatic sulfite oxidase. J. Biol. Chem. **236:** 1841–1846.
7. MALACINSKI, G. M., AND W. A. KONETZKA. 1966. Bacterial oxidation of orthophosphite. J. Bacteriol. **91:**578–582.
8. NICHOLAS, J. D., AND P. J. WILSON. 1964. A dissimilatory nitrate reductase from *Neurospora crassa*. Biochim. Biophys. Acta **86:**466–476.
9. PECK, H. D., JR. 1962. The role of adenosine-5′-phosphosulfate in the reduction of sulfate to sulfite by *Desulfovibrio desulfuricans*. J. Biol. Chem. **237:**198–203.
10. PINCHINOTY, F. 1964. A propos des nitrate-reductase d'une bacterie denitrifiante. Biochim. Biophys. Acta **89:**378–381.
11. POLLARD, F. H., D. E. ROGERS, M. T. ROTHWELL, AND G. NICKLESS. 1962. Separation of hypophosphite, phosphite, and phosphate by anion-exchange chromatography. J. Chromatog. **9:** 227–230.
12. RUDAKOW, K. J. 1929. Die Reduktion der mineralischen Phosphate auf biologischem Wege. Zentr. Bakteriol. Parasitenk. Abt. II **79:** 229–245.
13. THIMANN, K. V. 1963. The life of bacteria. The Macmillan Co., New York.
14. TSUBOTA, G. 1959. Phosphate reduction in the paddy field. I. Soil Plant Food (Tokyo) **5:**10–15.
15. VAN GOOL, A., AND A. LAUDELOUT. 1966. The mechanism of nitrite oxidation by *Nitrobacter winogradskyi*. Biochim. Biophys. Acta **113:**41–50.