Methods in Enzymology

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Vitamins and Coenzymes

Part B

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table shows that all the tetrahydroderivatives are very unstable under aerobic conditions. They are degraded to a series of compounds for the most part unspecifically and by nonenzymatic reactions. The deamination, i.e., the transition from the pterin to the lumazine series, unequivocally is an enzymatic step. The participation of xanthine oxidase in the introduction of the oxygen function into position 6 of the pteridine ring (dihydroxanthopterin, xanthopterin, and 6-hydroxylumazine) has been clearly established.[11,12] But as is shown by the table, the same products are obtained by nonenzymatic reactions, although with lower yields.

[201] Prosthetic Group of an Alcohol Dehydrogenase of a Pseudomonad[1]

By C. Anthony

The prosthetic group of this dehydrogenase is included here as present evidence suggests that it may be a pteridine derivative. This evidence consists primarily of fluorescence spectra[2] and of data on cochromatography of the purified prosthetic group with known pteridines.[3] A definite identification has not been possible. Methods are given below for preparation and purification of the enzyme, together with procedures for preparation of the prosthetic group from the purified enzyme.

The Alcohol Dehydrogenase

The enzyme, which has been found only in bacteria capable of growth on methanol as sole source of carbon and energy, oxidizes methanol to the oxidation level of formaldehyde. When isolated, ammonia is required as activator and phenazine methosulfate as primary hydrogen acceptor; the natural hydrogen acceptor is unknown.[4,5]

Assay Method

The more rapid spectrophotometric assay using 2,6-dichlorophenolindophenol as final hydrogen acceptor is given below. An alternative, more accurate, manometric assay,[5] using oxygen as final acceptor, may be used,

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[1] The bulk of the work on this enzyme and its prosthetic group has been published elsewhere in collaboration with Dr. L. J. Zatman.

[2] C. Anthony and L. J. Zatman, *Biochem. J.* **104,** 960 (1967).

[3] C. Anthony, unpublished results.

[4] C. Anthony and L. J. Zatman, *Biochem. J.* **92,** 609 (1964).

[5] C. Anthony and L. J. Zatman, *Biochem. J.* **92,** 614 (1964).

but the spectrophotometric assay is sufficiently accurate for use during purification of the enzyme.

Reagents

Tris-HCl buffer, 0.6 *M*, pH 9.0
Methanol, 0.15 *M*
Ammonium chloride, 0.45 *M*
N-Methylphenazonium methosulfate (phenazine methosulfate), 0.033 *M*
2,6-Dichlorophenolindophenol, 0.0013 *M*

Procedure. The assay system in a 1-cm light path cuvette contains 0.5 ml of Tris buffer, 0.1 ml of methanol, 0.1 ml of ammonium chloride, 0.1 ml of phenazine methosulfate, and 0.1 ml of 2,6-dichlorophenolindophenol. The final volume in the cuvette is made to 3 ml with water, and the reference cuvette contains deionized water. Enzyme solution is blown in with a pipette, which may be used then for rapid mixing. The initial rate of dye reduction is taken as twice the change in E_{600} occurring between 15 and 45 seconds after addition of enzyme. The amount of enzyme is adjusted to give a rate of change of E_{600} of less than 0.6/minute.

Definition of Unit and Specific Activity. One unit of enzyme activity is defined as the amount of enzyme that produces a change in E_{600} of 0.01/minute between 15 and 45 seconds after addition of the enzyme; 570 of these units are equivalent to 1 standard unit as defined in Enzyme Nomenclature (1965).[6] A high rate of dye reduction is sometimes observed in the absence of substrate when using the spectrophotometric assay.[5] This is ignored and is not subtracted from the reduction measured in the presence of substrate. The specific activity is defined as the number of units per milligram of protein as determined by the method of Lowry *et al.*[7]

Purification Procedure

Growth of Bacteria[8] *and Preparation of Crude Extract. Pseudomonas* sp. M27[4] or *Pseudomonas* AMI[9,10] may be used, but *Pseudomonas* sp. M27 is

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[6] Enzyme Nomenclature. "Recommendations (1964) of the International Union of Biochemistry on the Nomenclature and Classification of Enzymes." Elsevier, Amsterdam, 1965.

[7] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.* **193,** 265 (1951).

[8] The bacterial strains may be obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, U.K. *Pseudomonas,* sp. M27 is catalog No. NC1B 9686. *Pseudomonas* AMI is catalog No. NC1B 9133; it is also available as ATCC 14718.

[9] D. Peel and J. R. Quayle, *Biochem. J.* **81,** 465 (1961).

[10] P. A. Johnson and J. R. Quayle, *Biochem. J.* **93,** 281 (1964).

preferable for large-scale preparations as the enzyme is constitutive in this organism and gives more consistently a high specific activity. Stock cultures are maintained on methylamine-agar slopes.[9] The growth medium is a defined medium containing methanol + sodium lactate as carbon source. The bacteria are grown at 30° in well-aerated cultures and are harvested at the end of the exponential growth phase, which usually occurs 36–48 hours after inoculation. The inoculum is 5% of a late exponential phase culture. The yield is usually 2–3 g wet weight per liter. Harvested organisms may be stored at −20° for any length of time, and extracts may be prepared by means of Braun homogenizer, Hughes press, French pressure cell, or ultrasonic disintegrator. After cell breakage, crude extracts are prepared by centrifugation at 40,000 *g* for 1 hour at 2°. There are a number of procedures for purification of the enzyme,[5,11] but the best method for dealing with large quantities of material and for obtaining the purest product is given below.

Preparation of Pure Enzyme. The pH of crude extract is lowered very carefully with 1 *N* HCl to pH 4.0 at room temperature, and the heavy precipitate is removed by centrifugation at 40,000 *g* for 20 minutes. The pH of the supernatant liquid is raised to 6.0 with 1 *N* NaOH, and solid ammonium sulfate is added to give 65% saturation; after removal of the precipitate by centrifugation, ammonium sulfate is added to give 85% saturation. The precipitated enzyme is dissolved in 20 m*M* Tris-HCl buffer, pH 8.0, and passed through a column of DEAE-cellulose equilibrated with the same buffer. The enzyme is not adsorbed, but is eluted in the solvent front with 20 m*M*-Tris HCl buffer, pH 8.0. The pH of the pooled active fractions is lowered to 6.0, and solid ammonium sulfate is slowly added. Any precipitate that forms at less than 65% ammonium sulfate saturation is removed, and the 65–80% saturated fraction is dissolved in the minimum volume of 0.1 *M* Tris-HCl buffer, pH 8.0. At this stage the enzyme is dark brown. The enzyme solution is passed through a large column of Sephadex G-150 equilibrated with the same buffer; the total volume of the column should be about twenty times that of the sample. The main protein peak (as indicated by E_{280}) is collected and pooled. It should be golden yellow and completely separated from the red cytochrome *c*. If the absorption spectrum of the pooled fractions shows any absorption at 550 nm due to cytochrome *c*, they are concentrated with ammonium sulfate and passed through a similar column of Sephadex G-150. The pooled active fractions are dialyzed against 1000 volumes of deionized water at 2° before freeze-drying.

A summary of the purification procedure is given in the table.

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[11] C. Anthony and L. J. Zatman, *Biochem. J.* **104,** 953 (1967).

Purification of Alcohol Dehydrogenase from *Pseudomonas* sp. M27

Stage of purification	Volume (ml)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Crude extract	415	1,050,000	43	100	—
pH 4.0 Supernatant	365	893,000	220	85	4.9
Ammonium sulfate fraction	34	650,000	326	62	7.6
Pooled fractions from DEAE-cellulose	34	495,000	384	47	8.9
Pooled fractions from Sephadex G-150	60	347,000	420	33	9.8

Purity and Stability of the Enzyme

Enzyme prepared as described above is at least 95% pure and is stable for at least 4 months in the freeze-dried state. Solutions of the enzyme in 20 m*M* Tris buffer, pH 8.0, lose about 15% activity after storage for 2 months at −22° while the same solutions are stable at 2° for about a week.

Properties of the Enzyme

A full description of the enzyme including molecular weight, amino acid analysis, absorption spectrum, substrate specificity, and sensitivity to inhibitors has been published elsewhere.[2,5,11,12]

The Prosthetic Group

The prosthetic group is defined as the low molecular weight, green-fluorescent material produced from the nonfluorescent enzyme by boiling, or by lowering the pH to about 3, or by raising the pH to about 12. The prosthetic group has not been reversibly resolved from the enzyme; all attempts to do so either have failed to release the prosthetic group or have resulted in concomitant irreversible denaturation of the enzyme. A description of the methods used to prepare, purify, and assay this prosthetic group is given below.

Assay[2,3]

Because no assay that depends on reactivation of a resolved enzyme is possible, a method that involves measurement of fluorescence is used. The best ultraviolet source for detection of fluorescence is a lamp giving radiation at predominantly 366 nm; this is suitable for detection of fluorescent solutions or of fluorescent spots on chromatography or electrophoresis paper. Measurement of the prosthetic group is by means of a spectrophoto-

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[12] C. Anthony and L. J. Zatman, *Biochem. J.* **96**, 808 (1965).

fluorimeter; the maximum excitation wavelength is at about 365 nm, and maximum fluorescence is at about 470 nm. These values are uncorrected for the instrument and vary with the pH value of the solution. The wavelengths for maximum excitation and fluorescence should be determined on the particular instrument to be used, and all solutions should be adjusted to the same pH value before measurement. There is relatively little fluorescence in alkaline solution.

Preparation[2,3]

Although the prosthetic group may be released from the enzyme by a variety of methods, the most straightforward is to boil the enzyme preparation (about 10 mg/ml) for 2 minutes. Details of the kinetics of liberation of prosthetic group from the enzyme have been published.[2] The supernatant liquid, after a brief centrifugation to remove denatured protein, contains all the fluorescent material. Ten milligrams of pure enzyme dissolved in 1 ml of 0.05 M phosphate buffer, pH 6.0, and treated in this way, yields a fluorescent supernatant solution which gives a reading of about 60% full-scale deflection using an Aminco-Bowman spectrophotofluorimeter with a xenon lamp and a type IP21 photomultiplier (sensitivity at 25; slit arrangement No. 4; meter multiplier at 0.3).

Purification[2,3]

The procedure described below removes from the supernatant liquid about half of the material which absorbs in the ultraviolet range of the spectrum (240–320 nm).

After boiling the enzyme, the supernatant liquid is passed through a small column of DEAE- or TEAE-cellulose equilibrated with 50 mM ammonium acetate buffer, pH 6.0. A column of about 1-cm diameter is suitable, and about 1 cm in height should be allowed for every 20 mg of pure enzyme originally used. The column is washed with the same buffer until no more UV-absorbing material is eluted and then with 0.5 M ammonium acetate buffer, pH 6.0, to remove any trace of blue-fluorescent material that may be present. The adsorbed green-fluorescent material, visible as a red-brown band, is eluted with a linear gradient of 0.7–1.2 M ammonium acetate buffer, pH 6.0. All the green-fluorescent fractions, which should have the same excitation/fluorescence characteristics, are pooled, diluted five times with water, and adsorbed on a small pad of DEAE- or TEAE-cellulose. This pad should be as small as possible, but large enough to adsorb all the fluorescent material, which is then eluted with the minimum volume (usually less than 1 ml) of 10 M ammonium acetate. This concentrated material is then freeze-dried to remove the ammonium acetate. The yield, as indicated by total (arbitrary) fluorescence units, is about 90%.

The procedure described here may also be used for preparation of prosthetic group from enzyme protein that has not been completely purified. The last and most difficult stage of the enzyme purification procedure is the removal of cytochrome *c* by gel filtration. When this last stage has been omitted, the preparation obtained after purification of the prosthetic group has not been seen to be different from that obtained when starting with pure enzyme. It should be noted, however, that this technique has not been used extensively, and although it saves considerable time and labor, it should be used with caution.

Properties[2,3]

At room temperature, the purified prosthetic group is freely soluble in water but not in methanol, ethanol, acetone, diethyl ether, light petroleum (b.p. 40°–60°), hexane, cyclohexane, or chloroform. The prosthetic group, prepared as described here, often appears to have two or more fluorescent species; the relative proportions of these depend on time of storage. Minor fluorescent species may be removed from the major green-fluorescent species by paper chromatography in 0.1 *M* phosphate buffer, pH 7.0, or by high-voltage electrophoresis in the same buffer; at pH 7.0 the prosthetic group is negatively charged. Excitation and fluorescence spectra are given elsewhere; the major component has a fluorescence maximum at about 470 nm with excitation maximum at about 365 nm at pH 7.0 (uncorrected values).

Addendum to Section VII

A Bioautographic Procedure for Detecting TPN, DPN, NMN, and NR[1]

By Bernard Witholt

Bioassays are usually considerably more sensitive than chemical assays. Accordingly, a bioautographic technique based on the V factor requirement of *Hemophilus parainfluenza*, has[1a] been developed for several nicotinamide derivatives.

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[1] This work was supported by grants from P-L Biochemicals Inc. and the American Cancer Society (P-77M) to Dr. N. O. Kaplan.

[1a] A. Lwoff and S. Lwoff, *Proc. Roy. Soc.* (London), *B*, **122**, 352 (1937).