Pyridoxal-5-phosphate and Pyridoxamine-5-phosphate

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Principle

An apotransaminase can be isolated from brewer's yeast (see p. 609)[1] which can be reactivated with either pyridoxal-5-phosphate (PALP) or pyridoxamine-5-phosphate (PAMP)[2]. The extent of reactivation can be assayed by coupling the transamination reaction (1) with the malic dehydrogenase reaction (2) and spectrophotometric measurement of the reaction at 340 or 366 mμ[3-7].

(1) α-Oxoglutarate + aspartate $\rightleftharpoons$ (Trans-aminase) glutamate + oxaloacetate

(2) Oxaloacetate + DPNH + H^+ $\rightleftharpoons$ (Malic dehydrogenase) malate + DPN^+

If the malic dehydrogenase, DPNH, α-oxoglutarate, aspartate, and apotransaminase are present in excess, the amount of PALP or PAMP becomes rate-limiting. On plotting the enzymatic activity (ΔE/min. per 3 ml.) against the coenzyme concentration in the test mixture (mole/l), almost linear curves are obtained at low coenzyme concentrations. By comparison with standard curves obtained with authentic samples, the PALP or PAMP content of unknown preparations can be determined. PALP and PAMP can be differentiated by treatment with potassium borohydride which reduces PALP to an enzymatically inactive substance – probably pyridoxol-5-phosphate (POLP)[6,7].

Reagents

1. Triethanolamine
2. Reduced diphosphopyridine nucleotide, DPNH
   disodium salt, DPNH-Na_2; commercial preparations, see p. 1011.
3. Pyridoxal-5-phosphate *)
4. Pyridoxamine-5-phosphate *)
5. α-Oxoglutarate
   commercial preparations, see p. 1024.
6. Aspartic acid
7. Malic dehydrogenase, MDH
   from pig heart[8] or commercial preparations (see p. 989), suspension in 2.8 M ammonium sulphate solution.
8. Potassium borohydride, KBH_4
9. Apotransaminase
   preparation, see p. 609.

*) *e. g.* from Hoffman-La Roche.

1) *G. Schreiber, M. Eckstein, A. Oeser* and *H. Holzer*, Biochem. Z. *340*, 13 [1964].
2) *G. Schreiber, M. Eckstein, G. Maass* and *H. Holzer*, Biochem. Z. *340*, 21 [1964].
3) *J. S. LaDue, F. Wróblewski* and *A. Karmen*, Science [Washington] *120*, 497 [1954].
4) *A. Karmen, F. Wróblewski* and *J. S. LaDue*, J. Clin. Invest. *34*, 131 [1955].
5) *H. Holzer, U. Gerlach, G. Jacobi* and *M. Gnoth*, Biochem. Z. *329*, 529 [1958].
6) *H. Holzer* and *G. Schreiber* in: Proceedings of the Symposion on Chemical and Biological Aspects of Pyridoxal Catalysis, Rome 1962. Pergamon Press, London 1963, p. 523.
7) *G. Schreiber, M. Eckstein, A. Oeser* and *H. Holzer*, Biochem. Z. *340*, 35 [1964].
8) *S. Ochoa* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. 1, p. 735.

Preparation of Solutions

I. Triethanolamine buffer (0.2 M; pH 8.2):
Dissolve 5.97 g. triethanolamine in doubly distilled water and adjust pH to 8.2 with 2 N HCl, dilute with doubly distilled water to about 195 ml., readjust pH to 8.2, and add doubly distilled water to 200 ml.

II. Reduced diphosphopyridine nucleotide (1.2×10^{-2} M β-DPNH):
Dissolve 10 mg. DPNH-Na_2 (containing 78.5% β-DPNH) in 1 ml. doubly distilled water.

III. Pyridoxal-5-phosphate, PALP, standard solution (0.05 μg./ml.; 1.89×10^{-7} M):
Dissolve 5 mg. crystalline pyridoxal-5-phosphate ($C_8H_{10}O_6NP \cdot H_2O$) in 100 ml. doubly distilled water and dilute 1 ml. of this solution to 100 ml. with doubly distilled water; mix thoroughly.

IV. Pyridoxamine-5-phosphate, PAMP, standard solution (0.05 μg./ml.; 2.15×10^{-7} M):
Dissolve 5 mg. pyridoxamine-5-phosphate in 100 ml. doubly distilled water and dilute 1 ml. of this solution to 100 ml. with doubly distilled water; mix thoroughly.

V. α-Oxoglutarate (0.5 M):
Dissolve 730 mg. α-oxoglutaric acid (or 745 mg. of a 98% pure preparation) in a little doubly distilled water, neutralize with 2 N NaOH and dilute with doubly distilled water to 10 ml.

VI. Aspartate (0.5 M):
Dissolve 665.5 mg. aspartic acid in a little doubly distilled water, neutralize with 2 N NaOH and dilute with doubly distilled water to 10 ml.

VII. Potassium borohydride, KBH_4 (0.1 M KBH_4 in 0.02 N KOH):
Dissolve 270 mg. KBH_4 in 50 ml. 0.02 N KOH; prepare a fresh solution for each series of assays.

VIII. Malic dehydrogenase, MDH (*ca.* 40000 units[10]/ml.):
Suspend 0.5 mg. enzyme protein in 1 ml. of 2.8 M ammonium sulphate solution.

IX. Apotransaminase (*ca.* 30000 units*)/ml.):
Dilute preparation obtained according to[1] with 2.7 M ammonium sulphate solution if its activity is too high.

Procedure

Experimental material

Wash about 10 g. of baker's yeast twice with cold distilled water and suspend it in water by stirring. Make up the suspension to 100 ml. and add EDTA to a final concentration of 10^{-2} M in order to prevent metal-catalysed transaminations[9]. Boil the suspension for 5 min., and cool it immediately in an ice-bath. Centrifuge and use the clear supernatant for the assays.

Homogenize animal tissue samples with 5 times their weight of boiling water containing 10^{-2} M EDTA using an Ultra-Turrax (p. 51). Heat the homogenate at 100°C for 5 min., cool it in an ice-bath, and centrifuge. Use the clear supernatant for the assays.

*) One unit is the amount of enzyme which causes a change in the optical density of 0.001 in 1 min. at 366 mμ, in an assay mixture of 3 ml. and with a 1 cm. light path.

9) *E. E. Snell*, Fed. Proc. *20*, Suppl. No. 10, 81–83 [1961].

10) Units according to Bücher, see p. 33.

Standard curves

Prepare standard curves for PALP (0.63×10^{-9} to 6.3×10^{-9} M) and for PAMP (0.72×10^{-9} to 1.44×10^{-8} M). Use 0.01 ml. (= 0.0005 μg.) to 0.1 ml. (= 0.005 μg.) of standard solution III for PALP and 0.01 ml. (= 0.0005 μg.) to 0.2 ml. (= 0.01 μg.) of standard solution IV for PAMP. Plot the corrected reaction rates in ΔE/min. per 3 ml. (ordinate) against the concentration in moles/l of PALP or PAMP (abscissa). Check the assay system and reagents for every series of estimations by analysing samples containing known amounts of PALP and PAMP.

Reduction with potassium borohydride

Mix 0.1 and 0.2 ml. portions of the assay sample with 0.6 and 0.5 ml. respectively of solution VII in darkened test tubes. Stopper the test tubes and incubate them for 5 min. at 37°C. Cool the tubes in ice-water and add 0.3 ml. of 0.1 N H_2SO_4 to each. Restopper the darkened tubes and keep them at 90 – 100°C for 5 min. Cool in ice water. Use five to ten times as much of the reduced samples as of the non-reduced samples for the assays.

Spectrophotometric measurements

Wavelength: 366 mμ; light path: 1 cm.; final volume: 3 ml.; temperature: 25°C; read against water or air.

Dilute the sample until the reaction rate ΔE/min. lies on the linear portion of the standard curve after subtraction of the control value (without coenzyme). Pipette successively into the cuvettes:

	Experimental	*Control*
DPNH solution (II)	0.10 ml.	0.10 ml.
MDH suspension (VIII)	0.03 ml.	0.03 ml.
α-Oxoglutarate solution (V)	0.02 ml.	0.02 ml.
PALP or PAMP solution (III or IV) or unknown sample (unreduced and reduced)	0.003 – 0.2 ml.	equivalent amount H_2O
Apotransaminase suspension (IX)	0.01 ml.	0.01 ml.
Triethanolamine buffer (soln. I)	to 3.00 ml.	to 3.00 ml.

Mix, incubate for 5 min., read at 30 sec. intervals until there is no further change or until the small decrease in optical density becomes constant. Record the values for ΔE_1/30 sec. Start the transaminase reaction by mixing into both cuvettes

0.03 ml. aspartate solution (VI).

Measure the decrease in optical density at 30 sec. intervals for 5 to 10 min.: ΔE_2/30 sec.

Calculations

Calculate the mean values for ΔE_1/30 sec. and ΔE_2/30 sec. for each cuvette. Use these values to obtain the difference ΔE_2/30 sec. – ΔE_1/30 sec. This gives $\Delta E_{experimental}$/30 sec. and $\Delta E_{control}$/30 sec.

$\Delta E_{experimental}$/30 sec. – $\Delta E_{control}$/30 sec. = $\Delta E_{PALP + PAMP}$/30 sec.

The corrected ΔE/30 sec. values obtained for the standard solutions III and IV are used to construct the standard curves for PALP and PAMP respectively. The $\Delta E_{PALP + PAMP}$/30 sec. for the unreduced samples gives the value for PALP + PAMP, whereas for the reduced sample it gives PAMP only. Read off the PAMP concentration in the experimental cuvette from the PAMP standard curve. To obtain the PALP concentration in the experimental cuvette subtract the $\Delta E_{PALP + PAMP}$/30 sec. of the reduced sample from that of the unreduced sample and read off the concentration corresponding to this value from the PALP standard curve.

Specificity and Sources of Error

Of all the substances examined, only PALP and PAMP are active in this test[2,6]. The reaction is inhibited slightly by pyridoxal and orthophosphate, more strongly by pyridoxol-5-phosphate (POLP) and isonicotinic hydrazide. POLP may interfere when the sample contains a large amount of PALP[7], for the latter is probably reduced to POLP by KBH_4. Values for the PAMP content of such samples are too low [for a quantitative estimation of the inhibition, cf.[7]].

If the apoenzyme used is highly purified[1], as little as 10^{-4} μg. of PALP and PAMP can be determined.

Appendix

Apotransaminase from brewer's yeast[1]

Unless otherwise stated, all operations should be carried out at 0–4°C.

a) Maceration juice: Stir 200–300 g. dried brewer's yeast with three times its weight of distilled water for 3 hr. at 37°C, leave at 0°C. for several hours, and centrifuge. The protein content of the supernatant is 30–70 mg./ml.

b) Selective inactivation by heat: Heat the maceration juice gradually to 53–54°C within 4 min. and keep at this temperature for 8 min. Cool immediately in an ice-bath and centrifuge.

c) Fractionation with ammonium sulphate: Adjust the supernatant to a protein concentration of 40 mg./ml and add analytical grade ammonium sulphate slowly at 4°C until 1.5 M. Leave the solution for 20 min. in an ice-bath. Centrifuge and add ammonium sulphate to the supernatant to 2.1 M; keep in an ice-bath for 20 min. and centrifuge. The sediment contains the apotransaminase.

d) Gel filtration: Dissolve the sediment in a little 1 M tris buffer (pH 7.3) and add this solution to a column (40 cm. long, 6 cm. ∅) of Sephadex G-100 equilibrated with 0.01 M tris buffer. [Columns of up to 60 cm. height of gel still give fast enough flow rates; beyond this, lower yields are obtained as the enzyme is not very stable at this stage.] Elute the proteins from the column with 0.01 M tris buffer (pH 7.3) and measure the absorption of the eluate at 280 mμ. Several peaks are obtained; the enzyme appears in the second half of the first peak.

e) Chromatography: Transfer the eluate immediately (!) to a column of DEAE-cellulose (column volume 270 ml., height of packing after settling 44 cm.) equilibrated with 0.01 M tris buffer (pH 7.3). After the enzyme has been adsorbed, wash the column twice with its own volume of 0.01 M tris buffer (pH 7.3) and elute the enzyme with 0.05 M tris buffer (pH 7.3). Check the enzymatic activity and the optical density at 280 mμ of the eluate. Combine the fractions containing enzyme and bring with analytical grade ammonium sulphate to 3.1 M. Centrifuge, redissolve the precipitate in a little 1 M tris buffer (pH 7.3), add analytical grade ammonium sulphate to this solution to 3.1 M, and store it at −18°C.

This preparation contains less than 1% of holoenzyme saturated with PALP + PAMP. Its stability depends upon its purity. A preparation with 3000 units per mg. of protein had still half of the original activity after 106 days[2]. When apoenzyme solutions are incubated with PALP, the latter becomes firmly attached to the apoenzyme and cannot be removed by dialysis[2].