Thiamine Pyrophosphate

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Yeast pyruvic decarboxylase which was discovered by *Neuberg* and *Karczag*[1] in 1911, was shown by *Auhagen*[2] in 1932 to require a coenzyme, whose structure was elucidated by *Lohmann* and *Schuster*[3] in 1937 as being that of thiamine pyrophosphate (TPP). By alkali treatment TPP can be dissociated from the pyruvic decarboxylase. The pyruvic apodecarboxylase exhibits no enzymatic activity unless TPP is added.

Principle

The activity of pyruvic decarboxylase can be measured spectrophotometrically by coupling the pyruvic decarboxylase reaction:

(1) Pyruvate ⟶ acetaldehyde + CO_2

with the acetaldehyde reducing system (whose equilibrium lies to the right):

(2) Acetaldehyde + DPNH + H^+ ⇌ ethanol + DPN^+

since with a suitable excess of alcohol dehydrogenase (ADH) reaction (1) is rate limiting[4,5]. If the apoenzyme obtained by alkaline cleavage is used instead of the pyruvic holodecarboxylase then the rate of the two reactions (when saturated with Mg^{2+} ions) is dependent on the TPP concentration. The plot of activity versus TPP concentration is linear with low TPP concentrations and can be used as a standard curve for the determination of TPP. The Michaelis constant of the apodecarboxylase for TPP is 2.4×10^{-5} [moles/l.] (calculated according to[6]).

Reagents

1. Sodium hydroxide, 2 N
2. Disodium hydrogen phosphate, $Na_2HPO_4 \cdot 12H_2O$, A. R.
3. Glycine, A. R.
4. Maleic acid, pure, anhydrous
5. Magnesium sulphate, $MgSO_4 \cdot 7H_2O$, A. R.
6. Sodium pyruvate, A. R.
   commercial preparation, see p. 1027.
7. Ammonium sulphate, A. R.
8. Ammonia solution (*ca.* 25% w/v)
9. Reduced diphosphopyridine nucleotide, DPNH
   sodium salt, DPNH-Na_2; commercial preparation, see p. 1011.
10. Alcohol dehydrogenase, ADH
    from yeast, crystalline suspension in 2.4 M ammonium sulphate solution containing 3% $Na_4P_2O_7$ and 1% glycine, pH 8. Commercial preparation, see p. 969.
11. Thiamine pyrophosphate*), TPP

*) *e.g.* from E. Merck, Darmstadt, Germany.

1) *C. Neuberg* and *L. Karczag*, Biochem. Z. *37*, 170 [1911].
2) *E. Auhagen*, Hoppe-Seylers Z. physiol. Chem. *204*, 149 [1932]; *209*, 20 [1932].
3) *K. Lohmann* and *Ph. Schuster*, Biochem. Z. *294*, 188 [1937].
4) *H. Holzer:* 4. Colloquium dtsch. Ges. physiol. Chem., Springer-Verlag, Heidelberg 1953, p. 89.
5) *H. Holzer, G. Schultz, C. Villar-Palasi* and *J. Jüntgen-Sell*, Biochem. Z. *327*, 331 [1956].
6) *H. Lineweaver* and *D. Burk*, J. Amer. chem. Soc. *56*, 658 [1934].

12. Pyruvic decarboxylase
    prepared from yeast according to[5]. See Appendix, p. 605.
13. Pyruvic apodecarboxylase
    preparation, see p. 605.
14. Ethylene-diamine-tetra-acetic acid, EDTA
    disodium salt, EDTA-$Na_2H_2 \cdot 2H_2O$*).

Purity of the enzyme preparations

The pyruvic decarboxylase used for the preparation of the apoenzyme should have a specific activity of 0.8×10^3 to 1.0×10^3 units (according to *Bücher* et al.[7]) per mg.
The ADH preparation should have a specific activity of not less than 10^4 units (according to *Bücher* et al.[7]) per mg.

Preparation of Solutions

I. Disodium hydrogen phosphate (0.2 M):
Dissolve 7.16 g. $Na_2HPO_4 \cdot 12H_2O$ in doubly distilled water and make up to 100 ml.

II. Glycine (10% w/v):
Dissolve 10 g. glycine in doubly distilled water and make up to 100 ml.

III. Magnesium sulphate (0.5 M):
Dissolve 12.3 g. $MgSO_4 \cdot 7H_2O$ in doubly distilled water and make up to 100 ml.

IV. Maleate buffer (0.2 M; pH 6.6):
Dissolve 2.32 g. maleic acid in 70 ml. doubly distilled water, adjust pH to 6.6 with *ca.* 18 ml. 2 N NaOH and make up to 100 ml.

V. Sodium pyruvate (1 M):
Dissolve 11.0 g. sodium pyruvate in doubly distilled water and make up to 100 ml.

VI. Ammonium sulphate (2.7 M):
Dissolve 35.7 g. ammonium sulphate in doubly distilled water and make up to 100 ml.

VII. Reduced diphosphopyridine nucleotide (*ca.* 1.4×10^{-2} M β-DPNH):
Dissolve 10 mg. DPNH-Na_2 in 1 ml. doubly distilled water.

VIII. Alcohol dehydrogenase, ADH (1 mg. protein/ml.):
Dilute the commercially available enzyme suspension with 2.7 M ammonium sulphate solution (VI)

IX. Thiamine pyrophosphate (1 μg. TPP/ml.):
Dissolve 1 mg. thiamine pyrophosphate in doubly distilled water and make up to 1000 ml.

X. Pyruvic apodecarboxylase (*ca.* 1 mg. protein/ml.):
Dissolve several mg. of the ammonium sulphate paste of the apoenzyme in 1.0 ml. glycine-phosphate buffer [90 ml. 0.2 M Na_2HPO_4 solution (I) + 6 ml. glycine solution (II)] which contains a final concentration of 10^{-3} M EDTA. Determine the optical density change with this solution in the assay system and with 1 μg. thiamine pyrophosphate per cuvette. The absorption change should lie between 0.010/min. and

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*) *e.g.* Titriplex III from E. Merck, Darmstadt, Germany.
[7] *G. Beisenherz, H. J. Boltze, Th. Bücher, R. Czok, K. H. Garbade, E. Meyer-Arendt* and *G. Pfleiderer*, Z. Naturforsch. *8b*, 555 [1953]; cf. p. 33.

0.040/min. If it is higher dilute the apoenzyme solution with doubly distilled water; if lower add correspondingly more ammonium sulphate paste. An apoenzyme solution containing *ca.* 2 mg. protein/ml. is usually suitable.

XI. Ammonia solution (*ca.* 10%).

Procedure

Preliminary remarks: It is important that the temperature remains constant for a series of estimations (refer also to p. 9), since the rate of the decarboxylation reaction (1) which is used for the determination of the amount of TPP is very dependent on temperature. With each series a control without TPP is included and the rate given by this control is subtracted from all values with added TPP.

Standard curve

Use 0.1, 0.2, 0.4 and 0.8 μg. TPP [0.1, 0.2, 0.4 and 0.8 ml. of the TPP solution (IX)] instead of the sample in the assay system. Plot the rates ΔE/min. Against μg. TPP after subtraction of the ΔE/min. for the control. The optical density changes after subtraction of the control value should lie between 0.005/min. and 0.100/min. If the solution to be analysed contains an inhibitor, a constant volume of the sample should be added to the standards.

Spectrophotometric measurements

Wavelength: 366 mμ; light path: 1 cm.; final volume: 3.0 ml.; temperature: constant *ca.* 25°C. Read against air or a cuvette containing water.

Pipette successively into the cuvette (to the control cuvette add distilled water instead of TPP or sample):

1.5 ml. maleate buffer (solution IV)
0.10 – 1.18 ml. sample [for standard curve: TPP solution (IX)]
0.05 ml. Mg^{2+} solution (III)
0.06 ml. DPNH solution (VII)
0.06 ml. ADH suspension (VIII)
0.05 ml. apoenzyme solution (X)
distilled water to 2.90 ml.

Mix, allow to stand for 30 min. at temperature of measurements, then start the reaction by mixing in

0.10 ml. pyruvate solution (V).

Read the optical density change at 30 sec. intervals.

By increasing the amount of apodecarboxylase in the assay the sensitivity can be increased. The amount which can be added is limited by the TPP content of the enzyme due to incomplete cleavage of the holodecarboxylase.

Calculations

The amount of thiamine pyrophosphate corresponding to $\Delta E_{sample}/min. - \Delta E_{control}/min.$ is read from the standard curve.

Specificity

According to the work of *Lohmann* and *Schuster*[3] thiamine and thiamine monophosphate are not active as coenzymes. We found that in the above assay thiamine monophosphate gave less than 1% of the optical density change given by equimolar amounts of TPP. *Viscontini* et al.[8] have reported that thiamine triphosphate is just as active as thiamine pyrophosphate. According to *Velluz* et al.[9] thiamine pyrophosphate can be replaced by thiamine triphosphate if the apoenzyme is incubated with larger amounts of the latter. Their experiments showed that the triphosphate had only 80% of the activity of the true coenzyme.

Appendix

Pyruvic decarboxylase[5]

Yeast maceration juice: Stir 1 part of dried brewer's yeast with 3 parts distilled water, and allow to stand for 3 hours at 37° C. Centrifuge for 1 hour at 2000 g.

Acetone precipitation: Cool the juice to 0° C, add 59 ml. acetone per 100 ml. juice and allow to stand for 1 hour. Centrifuge and for every 100 ml. of the supernatant add 15 ml. acetone at 0° C. Centrifuge, finely suspend the sediment in 5% glycerol-water and centrifuge for 1 hour at 2000 g. Dilute the supernatant with 5% glycerol-water to give 40 mg. protein/ml.

Alcohol precipitation: To every 100 ml. of the solution add 50 ml. ethanol dropwise at 0° C, centrifuge for 15 min. at 2000 g. To every 100 ml. of the supernatant drop in a further 28 ml. ethanol and centrifuge for 10 min. at 2000 g. Dissolve the sediment in 5% glycerol-water to give 40 mg. protein/ml.

Ammonium sulphate precipitation: To every 100 ml. of the solution add 30.6 g. $(NH_4)_2SO_4$, centrifuge at 15000 g, wash the precipitate with a little 2.5 M $(NH_4)_2SO_4$ solution and suspend in 1.9 M $(NH_4)_2SO_4$ solution to give 40 mg. protein/ml. Mix thoroughly for 30 min. at 0°C and centrifuge for 20 min. at 6000 g. To every 100 ml. of the supernatant add 7 g. $(NH_4)_2SO_4$, centrifuge off the precipitate at high speed and store as a paste at −15 to −20°C.

Preparation of the apodecarboxylase from pyruvic decarboxylase

The apodecarboxylase is prepared by dissolving the holodecarboxylase in alkaline buffer (cf.[3,10]) This splits the coenzyme from the apoenzyme. The apoenzyme is precipitated by ammonium sulphate, while the coenzyme remains in solution and can be separated. By repeated washing of the precipitated apoenzyme a virtually complete separation is obtained.

Procedure: Mix 90.0 ml. 0.2 M Na_2HPO_4 solution (I) with 6.0 ml. 10% glycine solution (II). Adjust pH to 8.9 with 2 N NaOH and dilute to 100 ml. with doubly distilled water. Then dissolve *ca.* 2 g. of the holoenzyme (ammonium sulphate paste) in about 3 ml. ice-cold doubly distilled water and add this solution to the buffer. Allow to stand for 30 min. in an ice bath, and while stirring, add 45 g. ammonium sulphate over a period of 20 min. By addition of ammonia solution (XI) ensure that the pH does not fall below 8.5 during the addition of ammonium sulphate. Centrifuge for 30 min. at 20000 g (Spinco centrifuge) and discard the supernatant. Suspend the precipitate in 2.7 M ammonium sulphate solution (VI) and re-centrifuge for 30 min. at 20000 g. Discard the supernatant and repeat the washing with 2.7 M ammonium sulphate solution (VI). Store the precipitated apoenzyme in the centrifuge tube at −18° C as an ammonium sulphate paste. The apodecarboxylase is practically free of TPP. Stability of the ammonium sulphate paste: *ca.* 3 weeks.

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8) *M. Viscontini, G. Bonetti,* and *P. Karrer,* Helv. chim. Acta *32,* 1478 [1949].
9) *L. Velluz, G. Amiard* and *J. Bartos,* Bull. Soc. chim. France *1948,* 871.
10) *H. Holzer* and *H. W. Goedde,* Biochem. Z. *329,* 192 [1957].