D-2,3-Diphosphoglycerate

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Principle

2,3-Diphosphoglycerate is required as cofactor for the reaction:

(1) 3-phosphoglycerate $\rightleftharpoons$ 2-phosphoglycerate

Phosphoglycerate mutase (PGM) [1] catalyses this reaction. The initial rate of the reaction is proportional to the 2,3-diphosphoglycerate concentration, providing that this compound is present in limiting amounts. The 2,3-diphosphoglycerate content of the sample is determined by comparing its activating effect with that of a standard preparation. Reaction (1) is coupled with the reaction catalysed by enolase:

(2) Phosphoenolpyruvate $\rightleftharpoons$ 2-phosphoglycerate

Consequently the rate of reaction (1) is measured by the decrease in optical density at 240 mμ due to the removal of phosphoenolpyruvate [2] in reaction (2).

Reagents

1. Tris-hydroxymethyl-aminomethane, tris
2. Magnesium chloride, $MgCl_2 \cdot 6\,H_2O$
3. Hydrochloric acid, A. R., 5 N and 0.1 N
4. Sodium hydroxide, A. R., 0.1 N
5. Phosphoenolpyruvate, PEP
   crystalline cyclohexylammonium salt; commercial preparation, see p. 1024.
6. 2,3-Diphosphoglycerate
   prepared according to [3], converted to the brucine salt [4], recrystallized twice from water and then converted to the barium salt.
7. Enolase
   from yeast [5] or crystalline commercial preparation from muscle, see p. 973.
8. Phosphoglycerate mutase, PGM
   from yeast [6] or crystalline commercial preparation from muscle, see p. 995.

Purity of the enzyme preparations

The enzyme preparations need not be purified as far as the last step. It is sufficient to purify enolase as far as the eighth step and the phosphoglycerate mutase need not be crystallized. The preparations are stable for at least six months at 2°C.

Preparation of Solutions

I. Tris buffer (2.0 M; pH 7.4):
Dissolve 24.22 g. tris-hydroxymethyl-aminomethane in 50 ml. distilled water, adjust to pH 7.4 with *ca.* 35 ml. 5 N HCl and dilute to 100 ml. with distilled water.

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[1] *E. W. Sutherland, T. Posternak* and *C. F. Cori*, J. biol. Chemistry *181*, 153 [1949].
[2] *O. Warburg* and *W. Christian*, Biochem. Z. *310*, 384 [1941].
[3] *I. Greenwald* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1957, Vol. III, p. 221.
[4] *H. Jost*, Hoppe-Seylers Z. physiol. Chem. *165*, 171 [1927].
[5] *T. Bücher* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 427.
[6] *V. W. Rodwell, J. C. Towne* and *S. Grisolia*, Biochim. biophysica Acta *20*, 394 [1956].

II. Magnesium chloride (0.5 M):
Dissolve 10.2 g. $MgCl_2 \cdot 6H_2O$ in distilled water and make up to 100 ml.

III. Phosphoenolpyruvate (0.025 M PEP):
Dissolve 58.1 mg. PEP-tricyclohexylammonium salt in distilled water and make up to 5 ml.

IV. 2,3-Diphosphoglycerate standard solution (10^{-5} M):
Dissolve 10 mg. Ba salt in 10 ml. 0.1 N HCl. Remove the Ba^{2+} with Dowex 50 (H^+ form) and neutralize with 0.1 N NaOH. Determine the concentration of 2,3-diphosphoglycerate by measuring the bound organic phosphate and dilute to give 10^{-5} M 2,3-diphosphoglycerate.

V. Enolase:
Dissolve the preparation obtained according to[3] in distilled water to give 10 mg. protein/ml. Dilute the commercial preparation from muscle with distilled water to give 1 mg. protein/ml.

VI. Phosphoglycerate mutase, PGM:
Dissolve the preparation obtained according to[6] in distilled water to give 0.7 mg. protein/ml. Dilute the commercial preparation from muscle with distilled water to give 1 mg. protein/ml.

Stability of the solutions

The PEP and 2,3-diphosphoglycerate solutions keep for several weeks in the frozen state. Prepare the enzyme solutions freshly each day. The concentrated stock suspensions of the enzymes keep for several months at 0 to 4°C.

Procedure

Deproteinization

Heat samples for 5 min. at 100°C or add trichloroacetic acid (50% w/v) to give a final concentration of 5% (w/v). Centrifuge and neutralize the supernatant with 1 N KOH. If trichloroacetic acid is used, the concentration of 2,3-diphosphoglycerate in the sample must be sufficiently high so that only a small amount of the deproteinized sample need be taken for the assay, otherwise the trichloroacetate will interfere with the measurements at 240 mμ.

Spectrophotometric measurements

Wavelength: 240 mμ; silica cuvettes, light path: 1 cm.; final volume: 1 ml.; temperature: 25°C (constant for the sample and the standard curve). Measure against water.

Pipette into the cuvette:

0.02 ml. tris buffer (solution I)
0.01 ml. $MgCl_2$ solution (II)
0.03 ml. PEP solution (III)
0.01 ml. enolase solution (V)
distilled water to a final volume of 1 ml.

The optical density is constant within 1 min. and is about 1.5.
Add

0.01 ml. phosphoglycerate mutase solution (VI).

The optical density is constant within about 1 min. Mix in

0.01—0.06 ml. sample (containing 10^{-4} to 6×10^{-4} μmoles 2,3-diphosphoglycerate)

or for the standards

0.01 to 0.06 ml. 2,3-diphosphoglycerate standard solution (IV) (corresponding to 1×10^{-4} to 6×10^{-4} μmoles 2,3-diphosphoglycerate).

Start a stopwatch and read the optical density at minute intervals from 1 to 4 min. To obtain a standard curve plot the decrease in optical density ΔE/min. of the standards (ordinate) against the μmoles 2,3-diphosphoglycerate (abscissa).

Calculations

Obtain the 2,3-diphosphoglycerate content of the experimental cuvette by reading off from the standard curve the value corresponding to the ΔE/min.

Sources of Error

To check whether the sample contains interfering substances, analyse the sample and a standard together and separately. If no interfering substances are present, the value for the determination on the mixture should equal the sum of the two individual determinations.