β-Hydroxy-β-methylglutaryl Coenzyme A

Joachim Knappe

Principle

The cleavage of β-hydroxy-β-methylglutaryl coenzyme A (HMG-CoA) to acetoacetate and acetyl-CoA by the HMG-CoA cleavage enzyme[1] can be used for the determination of HMG-CoA:

(1) HMG-CoA $\rightleftharpoons$ acetoacetate + acetyl-CoA

The equilibrium lies practically completely on the side of the cleavage products.

Combination with one of the spectrophotometric methods for the determination of acetyl-CoA (refer to p. 419) allows the measurement of HMG-CoA in a coupled optical assay[2]. Acetyl-CoA is determined according to[3] with malic dehydrogenase (MDH) and citrate condensing enzyme (CE):

(2) Acetyl-CoA + L-malate + H_2O + DPN^+ $\rightleftharpoons$ citrate + CoA + DPNH + $2\,H^+$

The balance equation is:

(3) HMG-CoA + L-malate + H_2O + DPN^+ $\rightleftharpoons$
citrate + acetoacetate + CoA + DPNH + $2\,H^+$

The measure of the reaction is the increase of optical density at 340, 366 or 334 mμ due to the formation of DPNH.

Reagents

1. Tris-hydroxymethyl-aminomethane, tris
2. Hydrochloric acid, A. R., 0.5 N
3. Magnesium chloride, $MgCl_2 \cdot 6\,H_2O$, A. R.
4. Thioglycollic acid
5. Potassium hydroxide, A. R., 1 N
6. DL-Malic acid
7. Diphosphopyridine nucleotide, DPN
   free acid; commercial preparation, see p. 1010.
8. Malic dehydrogenase, MDH
   from pig heart[4]; suspension of *ca.* 10 mg. protein/ml. in 2.8 M ammonium sulphate solution; specific activity 10000—50000 units*)/mg.; commercial preparation, see p. 988.
9. Citrate condensing enzyme, CE
   crystalline, from pig heart[5]; suspension of *ca.* 10 mg. protein/ml. in 2.5 M ammonium sulphate solution; specific activity *ca.* 4800 units**)/mg.; isolation, see Appendix, p. 448.

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*) Units according to[4] measured in tris-buffer. A unit is the amount of enzyme which changes the optical density at 340 mμ by 0.010 in 1 min., in a 3 ml. assay mixture. For the different activity of MDH in several buffer systems, see p. 988.

**) A unit is the amount of enzyme which changes the optical density at 340 mμ by 0.010 in 1 min., in a 1.5 ml. assay mixture.

1) *B. K. Bachhawat, W. G. Robinson* and *M. J. Coon*, J. biol. Chemistry *216*, 727 [1955].
2) *J. Knappe*, Ph. D. Thesis, Universität Munich (1957).
3) *J. R. Stern, S. Ochoa* and *F. Lynen*, J. biol. Chemistry *198*, 313 [1952].
4) *S. Ochoa* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 735.
5) *S. Ochoa* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 685.

10. HMG-CoA cleavage enzyme
from ox liver, *ca.* 2 mg. protein/ml. Suitable preparations are those with a specific activity of *ca.* 2000 units *)/mg. according to *Lynen* [6]. Isolation, see Appendix, p. 448. The dialysed solutions are stable for several months at −18°C but repeated freezing and thawing leads to a rapid loss of activity.

Preparation of Solutions

I. Tris buffer (*ca.* 0.5 M; pH 8.0):
Dissolve 60 g. tris-hydroxymethyl-aminomethane in distilled water and make up to 500 ml., then dilute to 1000 ml. with 0.5 N HCl.

II. Magnesium chloride (0.1 M):
Dissolve 2.03 g. $MgCl_2 \cdot 6\ H_2O$ in distilled water and make up to 100 ml.

III. Thioglycollate (0.35 M):
Dissolve 0.24 ml. 80% thioglycollic acid in *ca.* 4 ml. distilled water, neutralize with 1 N KOH and dilute to 10 ml. with distilled water. Prepare freshly each day.

IV. DL-Malate (0.1 M):
Dissolve 1.341 g. DL-malic acid in distilled water, neutralize with 1 N KOH and dilute to 100 ml. with distilled water.

V. Diphosphopyridine nucleotide (*ca.* 0.02 M β-DPN):
Dissolve 30 mg. DPN in 1 ml. distilled water, neutralize with 0.1 N KOH and dilute to 2 ml. with distilled water.

VI. Malic dehydrogenase, MDH (*ca.* 10 mg. protein/ml.):
Use the commercially available suspension undiluted.

VII. Citrate condensing enzyme, CE (*ca.* 10 mg. protein/ml.):
Use the suspension undiluted.

VIII. HMG-CoA cleavage enzyme (*ca.* 2 mg. protein/ml.):
If necessary, dilute the solution obtained according to p. 448 with 0.05 M tris buffer (pH 7.5).

Procedure

Experimental material

The assay mixture should not contain more than *ca.* 0.2 μmoles HMG-CoA. A high salt concentration in the sample is to be avoided. Trichloroacetic acid or perchloric acid (final concentration 3%) can be used to deproteinize the samples, which should be cooled in an ice bath. Remove trichloroacetic acid by repeated extraction with peroxide-free ether; blow off the residual ether with N_2 and adjust the solution to pH 5 with KOH. Carefully adjust solutions deproteinized with perchloric acid to pH 5 with KOH, allow to stand for 10 min. in an ice bath and decant off from the precipitated $KClO_4$.

Like all CoA thiolesters, HMG-CoA is sensitive to oxidizing agents (*e.g.* ether peroxides, H_2O_2) and is easily hydrolysed by alkaline pH (half-life time for the hydrolysis of HMG-CoA at 15°C in 0.1 N NaOH is 8 min.). At pH 4 to 5 (optimum) HMG-CoA is stable for several days at 0°C, while at −18°C it is virtually stable indefinitely.

*) A unit is the amount of enzyme which changes the optical density at 405 mμ by 0.001 in 5 min., in a 1.8 ml. assay mixture.

6) *F. Lynen, U. Henning, C. Bublitz, B. Sörbo* and *L. Kröplin-Rueff*, Biochem. Z. *330*, 269 [1958].

Spectrophotometric measurements

Wavelength: 340, 366 or 334 mμ; light path: 1 cm.; final volume: 1.80 ml.; temperature: 20–25°C. Measure against air or water.

Pipette successively into the cuvette:

0.40 ml. tris-buffer (solution I)
0.20 ml. $MgCl_2$ solution (II)
0.02 ml. thioglycollate solution (III)
0.05 ml. malate solution (IV)
0.05 ml. DPN solution (V)
1.00 ml. sample (if necessary, make up to 1 ml. with distilled water)
0.001 ml. MDH suspension (VI)
0.001 ml. CE suspension (VII).

After 20 sec. read the initial optical density E_1 *) and start the reaction by addition of

0.08 ml. HMG-CoA cleavage enzyme solution (VIII) (*ca.* 300 units).

The reaction is complete after 10–20 min. Read the optical density E_2. $E_2 - E_1 = \Delta E$ is used for the calculations.

If the HMG-CoA cleavage enzyme preparation is strongly coloured, then its optical density must by determined separately in a cuvette which contains distilled water instead of the sample; E_1 should be corrected for this value. With the enzyme preparations used here the reaction occurring in this blank is usually insignificant, but it should be checked before each series of measurements.

Calculations

According to the general formula for the calculations (p. 37), with an assay volume of 1.80 ml. and a light path of 1 cm., the amount of HMG-CoA in the sample taken for assay is
for measurements at 340 mμ: $\Delta E \times 0.289$ = μmoles HMG-CoA
for measurements at 366 mμ: $\Delta E \times 0.545$ = μmoles HMG-CoA
for measurements at 334 mμ: $\Delta E \times 0.300$ = μmoles HMG-CoA.

To convert to μg. it is necessary to multiply by the molecular weight of HMG-CoA (911.1).

Other Determinations

Acetyl-CoA can be determined in the same assay mixture: the optical density before and after the addition of CE is followed until constant (also refer to p. 419). The HMG-CoA can then be determined by the addition of the HMG-CoA cleavage enzyme.

Specificity

The HMG-CoA cleavage enzyme only reacts with the naturally occurring HMG-CoA isomer whose configuration corresponds to that of (+)-mevalonic acid[7].

Other Methods for the Determination of HMG-CoA

HMG-CoA can be determined by the action of HMG-CoA cleavage enzyme (equation 1) and arylamine transacetylase[8] as indicator enzyme (refer to acetyl-CoA, p. 419). For the determination in the presence of acetyl-CoA see[9].

*) The malate oxidation reaction reaches equilibrium in a few seconds.

7) *J. Knappe, E. Ringelmann* and *F. Lynen*, Biochem. Z. *332*, 195 [1959].
8) *H. Tabor, A. H. Mehler* and *E. R. Stadtman*, J. biol. Chemistry *204*, 127 [1953].
9) *O. Wieland, G. Löffler, L. Weiß* and *I. Neufeld*, Biochem. Z. *333*, 10 [1960].

There is a significant inhibition of arylamine transacetylase by free CoASH ($\geqq$ 0.1 μmole per assay mixture) and long-chain acyl-CoA derivatives, such as palmityl-CoA ($\geqq$ 0.02 μmoles)[8]. This can result in a considerable increase in the time taken for the reaction.

Appendix

Isolation of citrate condensing enzyme[5]

The following steps are employed:

1. Two extractions of minced pig hearts with phosphate buffer.
2. Precipitation with acetic acid at pH 5.5.
3. Adsorption on calcium phosphate gel and elution.
4. Alcohol precipitation.
5. Fractionation with ammonium sulphate between 50 and 60% or 60 and 70% saturation.
6. Refractionation with ammonium sulphate between 40 and 60% saturation.
7. Crystallization and recrystallization.

Isolation of HMG-CoA cleavage enzyme[6]

The following steps are employed:

1. Preparation of an acetone-dried powder of ox liver.
2. Extraction of the acetone powder with phosphate buffer.
3. Heating the extract to 50° C (20 min.).
4. Fractionation with acetone at −8° C.
5. Precipitation of inactive protein with zinc acetate solution.
6. Fractionation with ammonium sulphate between 0 and 50% saturation.