Crotonyl Coenzyme A

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Principle

The β-oxidation of even-numbered fatty acids results in the formation of crotonyl-CoA from butyryl-CoA[1,2]. The further conversion of the unsaturated compound proceeds by way of L-(+)-β-hydroxybutyryl-CoA to acetoacetyl-CoA[1,3]. Crotonyl-CoA can be determined by a combination of the hydration and oxidation reactions:

(1) $CH_3-CH=CH-CO\sim SCoA + H_2O \rightleftharpoons CH_3-CH(OH)-CH_2-CO\sim SCoA$

(2) $CH_3-CH(OH)-CH_2-CO\sim SCoA + DPN^+ \rightleftharpoons CH_3-CO-CH_2-CO\sim SCoA + DPNH + H^+$

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(3) $CH_3-CH=CH-CO\sim SCoA + H_2O + DPN^+ \rightleftharpoons CH_3-CO-CH_2-CO\sim SCoA + DPNH + H^+$

The enzymes crotonase and β-hydroxyacyl dehydrogenase (HOADH) have been crystallized and the methods for their isolation are easily reproducible. The equilibrium constant[4] K of the crotonase reaction (1) is 6.18×10^{-2} [l./mole] (for the equilibrium constant of reaction (2) and its dependence on pH, see p. 441). The increase in absorption at 340 mμ due to the formation of DPNH is measured.

Reagents

1. Potassium dihydrogen phosphate, A. R., KH_2PO_4
2. Disodium hydrogen phosphate, A. R., $Na_2HPO_4\cdot2H_2O$
3. Potassium hydrogen carbonate, A. R., $KHCO_3$
4. Potassium hydroxide, A. R.
5. Perchloric acid, A. R., sp. gr. 1.67, *ca.* 70% (w/w)
6. Hydrochloric acid, A. R., conc. (*ca.* 37% w/w)
7. Tris-hydroxymethyl-aminomethane, tris
8. Ethylene-diamine-tetra-acetic acid, EDTA
   disodium salt, EDTA-$Na_2H_2\cdot2H_2O$ (Titriplex III, Trilon B, Versene)
9. Diphosphopyridine nucleotide, DPN
   free acid, commercial preparation, see p. 1010.
10. β-Hydroxyacyl dehydrogenase, HOADH
    purified from sheep liver according to[5] or crystalline from pig heart according to[6] (see also p. 425); crystalline commercial preparation, see p. 984.
11. Crotonase
    crystalline from ox liver according to[7], suspended in 0.02 M potassium phosphate buffer (pH 7.4) containing 0.003 M EDTA. Isolation, see Appendix on p. 440.

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1) *W. Seubert* and *F. Lynen*, J. Amer. chem. Soc. *75*, 2787 [1953].
2) *D. E. Green, S. Mii, H. R. Mahler* and *R. M. Bock*, J. biol. Chemistry *206*, 1 [1954].
3) *J. R. Stern* and *A. Del Campillo*, J. Amer. chem. Soc. *75*, 2277 [1955].
4) *J. R. Stern* and *A. Del Campillo*, J. biol. Chemistry *218*, 985 [1956].
5) *F. Lynen* and *O. Wieland* in *S. P. Colowick* and *N. O. Kaplan:* Methods in Enzymology. Academic Press, New York 1955, Vol. I, p. 566.
6) *J. R. Stern*, Biochim. biophysica Acta *26*, 448 [1957].
7) *J. R. Stern, A. Del Campillo* and *I. Raw*, J. biol. Chemistry *218*, 971 [1956].

Preparation of Solutions

I. Potassium dihydrogen phosphate (0.2 M):
Dissolve 2.722 g. KH_2PO_4 in distilled water and make up to 100 ml.

II. Disodium hydrogen phosphate (0.2 M):
Dissolve 3.561 g. $Na_2HPO_4 \cdot 2H_2O$ in distilled water and make up to 100 ml.

III. Potassium hydroxide (*ca.* 8 N):
Dissolve 45 g. KOH in distilled water with cooling and make up to 100 ml.

IV. Potassium hydrogen carbonate (*ca.* 1 M):
Dissolve 10 g. $KHCO_3$ in distilled water and make up to 100 ml.

V. Tris buffer (*ca.* 0.5 M; pH 9.5):
Dissolve 12.1 g. tris in 150 ml. distilled water, add 0.35 ml. conc. HCl and dilute to 200 ml. with distilled water.

VI. Perchloric acid (*ca.* 4 M):
Dilute 35 ml. 70% $HClO_4$ to 100 ml. with distilled water.

VII. Ethylene-diamine-tetra-acetate (0.1 M):
Dissolve 1.86 g. EDTA-$Na_2H_2 \cdot 2H_2O$ in distilled water and make up to 50 ml.

VIII. Phosphate buffer (0.02 M; pH 7.4; 0.003 M EDTA):
Mix 4 ml. KH_2PO_4 solution (I), 16 ml. Na_2HPO_4 solution (II) and 0.6 ml. EDTA solution (VII) and dilute to 200 ml. with CO_2-free distilled water (boiled).

IX. Diphosphopyridine nucleotide (*ca.* 0.01 M β-DPN):
Dissolve 7.4 mg. DPN in about 0.5 ml. distilled water, neutralize with a few drops of $KHCO_3$ solution (IV) and dilute to 1 ml. with distilled water.

X. β-Hydroxyacyl dehydrogenase, HOADH (3 mg. protein/ml.):
Dilute the stock suspension with distilled water containing EDTA (0.05 ml. solution VII/ml.).

XI. Crotonase (5 μg. protein/ml.):
Dilute the crotonase suspension (5 mg. protein/ml.) with phosphate buffer (solution VIII).

Stability of the solutions

Concentrated or dilute enzyme solutions can be stored for a considerable period at 0°C or in the frozen state without appreciable loss of activity. Frequent freezing and thawing of the solutions causes deterioration. Crotonyl-CoA has a stability similar to that of other acyl-CoA derivatives (see p. 426), but the thiol esters of α,β-unsaturated acids are considerably more stable in alkali than other acyl mercaptans (refer to[8]). Neutral, aqueous solutions of DPN are stable for several weeks at 0°C or in the frozen state. All other solutions are stable indefinitely, providing that bacterial contamination is avoided by storage in a refrigerator. Use polyethylene containers for buffer and alkaline solutions.

Procedure

Extraction and deproteinization of the sample

See p. 420 and 426. Solutions III and VI are required.

[8] *K. Decker:* Die aktivierte Essigsäure. Ferd. Enke, Stuttgart 1959.

Spectrophotometric measurements

Preliminary remarks: Crotonyl-CoA should only be in contact with the alkaline assay medium for the minimum time possible, therefore it should be added just before the start of the reaction and a highly active enzyme should be used to shorten the reaction time.
Since the conversion of crotonyl-CoA is only quantitative at pH >9, it is necessary to check on completion of the reaction that the pH of the assay mixture has not fallen below 9. If this is the case, then the sample is too strongly buffered. The amount of 0.1 N KOH required to obtain the correct pH is determined and the assay is repeated with the inclusion of the requisite quantity of 0.1 N KOH just before the distilled water.

Method: Wavelength: 340, 334 or 366 mμ; light path: 1 cm.; final volume: 2 ml.; room temperature. Measure against a water blank or air.

Pipette successively into the cuvette:

tris buffer (solution V)	0.70 ml.
EDTA solution (VII)	0.03 ml.
DPN solution (IX)	0.05 ml.
HOADH solution (X)	0.01 ml.
sample	up to 1.20 ml.
distilled water	to 2.00 ml.

Observe the optical density until constant (at least 30 sec.) and then read the initial optical density E_1. Start the reaction by mixing in

0.005 ml. crotonase solution (XI; *ca.* 35 units according to [7]).

Read the final value E_2 when the optical density increase stops (constant readings for 1 minute).

Calculations

With measurements at 340 mμ, an assay volume of 2 ml. and a 1 cm. light path:

$$0.322 \times \Delta E \times \frac{V}{v} = \mu\text{moles crotonyl-CoA in the whole sample}$$

with measurements at 334 mμ: $0.341 \times \Delta E \times \frac{V}{v} = \mu$moles crotonyl-CoA in the whole sample

with measurements at 366 mμ: $0.607 \times \Delta E \times \frac{V}{v} = \mu$moles crotonyl-CoA in the whole sample

where V = volume of the whole sample in ml.
v = volume of the sample taken for assay in ml.
$\Delta E = E_2 - E_1$
ε = extinction coefficient for DPNH. The values are 6.22 cm.^{2}/μmole at 340 mμ; 5.87 at 334 mμ; 3.3 at 366 mμ.

Example

Coenzyme A (20 μmoles) was converted to crotonyl-CoA with crotonic anhydride [8]. The volume of the neutral solution was 5.4 ml. and 0.07 ml. was taken for the estimation. Wavelength: 366 mμ. $E_1 = 0.106$. After addition of the crotonase the optical density rose within 80 to 90 sec. to $E_2 = 0.398$ (constant for 1 min.). The final pH was 9.3. $\Delta E = 0.398 - 0.106 = 0.292$.

$$0.607 \times 0.292 \times \frac{5.4}{0.07} = 13.65\ \mu\text{moles crotonyl-CoA in the whole sample.}$$

When 0.05 ml. was taken $E_1 = 0.102$; $E_2 = 0.312$; $\Delta E = 0.210$; the amount of crotonyl-CoA in the whole sample: 13.73 μmoles. The reaction of coenzyme A with crotonic anhydride is therefore not quantitative.

Sources of Error

For the β-ketoacylthiolase content of the enzyme preparations, see p. 427. The presence of β-hydroxybutyryl-CoA in the sample, which would also lead to a reduction of DPN, is indicated by an increase in optical density before the addition of crotonase. If E_1 has reached a steady value before the start of the reaction then the accuracy of the crotonyl-CoA determination is not affected. The most important of the interfering compounds, which react with crotonase as well as with β-hydroxyacyl dehydrogenase (refer to "Specificity"), are the CoA derivatives of the α,β- (and β,γ-) unsaturated fatty acids. Mercaptans add to the double bond of α,β-enoylthiolesters, and the β-thioethers so formed do not react[8]. An excess of SH-compounds should therefore be avoided when working with crotonyl-CoA.

Specificity

β-Hydroxyacyl dehydrogenase and crotonase react with the CoA derivatives of all the α,β-unsaturated fatty acids[4,9]. The products and substrates of the reversible hydration are the L-(+)-β-hydroxyacyl-CoA derivatives. There are only quantitative differences in the activity of crotonase with positional isomers ($\Delta^{\alpha,\beta}$ and $\Delta^{\beta,\gamma}$) and *cis-trans* isomers (iso-crotonyl- and crotonyl-CoA)[4,9]. β-Methyl-crotonyl, tiglyl, sorbyl and β-methyl-glutaconyl-CoA are also hydrated[4].

Crotonase has a high degree of specificity with regard to the thiol component. Neither the *N*-acetylcysteamine, nor the glutathione derivative of crotonic acid are hydrated. Crotonylpantetheine reacts with the crystalline enzyme extremely slowly; however, in the presence of coenzyme A, crotonase can act on this substrate in the same manner as thioltranscrotonylase[4].

Other Methods for the Determination of Crotonyl-CoA

Crotonyl thiolester has a characteristic absorption spectrum with peaks at 225 and 263 mμ ($\varepsilon = 1.06 \times 10^7$ and 6.5×10^6 cm.²/mole)[1]. The decrease in absorption between 260 and 270 mμ *) on hydration can be used for the estimation of crotonyl-CoA, if the β-hydroxybutyryl-CoA formed is completely removed from the reaction mixture. This is accomplished by use of β-hydroxyacyl dehydrogenase, alcohol dehydrogenase, acetaldehyde and a catalytic amount of DPN. Another possibility for the determination of crotonyl-CoA is its reduction to butyryl-CoA by TPNH and an enzyme from liver microsomes[10,11]. None of these methods has any advantage over the procedure described here.

Appendix

Isolation of crotonase[7]

The starting material is deep-frozen bovine liver. The stages are: 1. Extraction with $KHCO_3$ and cysteine (pH 8.2). — 2. Heating to 55°C at pH 5.5. — 3. Acetone precipitation at −5°C, solution of the precipitate in phosphate buffer, pH 7.4 (0.003 M EDTA) and dialysis. — 4. Ammonium sulphate fractionation between 40 and 65% saturation; solution of the precipitate in 0.02 M potassium phosphate buffer, pH 7.4 (0.003 M EDTA and 0.001 M glutathione); dialysis. — 5. Crystallization by addition of 0.1 volumes ethanol to the dialysed solution. Recrystallize twice.

*) In most cases the nucleotide absorption in this range must be compensated by reading against a control cuvette containing the sample.

9) *S. J. Wakil* and *H. R. Mahler*, J. biol. Chemistry *207*, 125 [1954].
10) *R. G. Langdon*, J. Amer. chem. Soc. *77*, 5190 [1955].
11) *W. Seubert*, *G. Greull* and *F. Lynen*, Angew. Chem. *69*, 359 [1957].