PARTIAL PURIFICATION OF A 2-OXO-GLUTARATE:GLYOXYLATE CARBOLIGASE FROM RAT LIVER MITOCHONDRIA

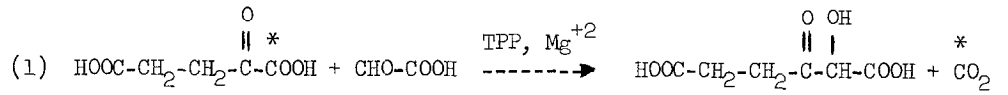
Jürgen Koch and E. L. R. Stokstad

Dept. Nutritional Sciences
University of California
Berkeley, California, USA

Received March 14, 1966

Glyoxylic acid is rapidly metabolized by mitochondria (Nakada & Sund, 1958). These authors reported the oxidative decarboxylation of glyoxylic acid with formation of N-formyl-glutamate by extracts of rat liver mitochondria. Crawhall & Watts (1962) showed that glyoxylate and 2-oxo-glutarate are decarboxylated synergistically by intact mitochondria. Recently Okuyama et al. (1965) reported the occurrence of a 2-oxo-glutarate dependent oxidation of glyoxylate in Rhodopseudomonas spheroides.

This communication describes the partial purification of a new carboligase type enzyme from rat liver mitochondria catalyzing the following reaction:

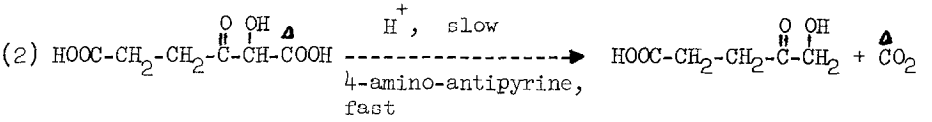


(1) $HOOC-CH_2-CH_2-C(=O)^{*}-COOH + CHO-COOH \xrightarrow{TPP,\ Mg^{+2}} HOOC-CH_2-CH_2-C(=O)-CH(OH)-COOH + {}^{*}CO_2$

METHODS

Preparation of mitochondria. Rat livers were homogenized (2-4°C) in a Waring blendor at low speed in an isotonic medium (0.163 M NaCl, 0.155 M $MgSO_4$, 0.15 M K-Na-phosphate, pH 7.4, 70:2.5:15). The precipitate (10 min., between 500 and 10,000 xg) was resuspended and recentrifuged twice (20 min., 6,000 xg). The final precipitate was suspended in water and homogenized with 20 vol. acetone (-20°C) in a Waring blendor. The acetone powder was collected by filtration and dried in vacuo.

Assay. When U-^{14}C-glyoxylate was the labeled substrate, the enzyme was assayed by following the decarboxylation of the reaction product 2-hydroxy-3-oxo-adipate, either the slow decarboxylation in acid medium, or the fast decarboxylation in the presence of 4-amino-antipyrine, which is a well known catalyst for the decarboxylation of β-ketoacids (MacDonald and Stanier, 1957). The latter system was used as the standard assay.



The recovery of $^{14}CO_2$ was also followed, using as substrates unlabeled glyoxylate and 2-oxo-(1-^{14}C) glutarate, generated from 1-^{14}C-glutamate with glutamate dehydrogenase and NAD (equ. 1).

Incubation. Incubations were performed for 1 hour at 30°C in a H_2-atmosphere in Warburg vessels. The main compartment contained: enzyme, 100 µmoles K-Na-phosphate pH 7.0, 5 µmoles $MgCl_2$, 0.2 µmoles TPP and 2.5 µmoles 2-oxo-glutarate. When 1-^{14}C-glutamate was used as a source of 2-oxo-(1-^{14}C)glutarate, unlabeled 2-oxo-glutarate was omitted, and 2.5 µmoles 1-^{14}C-glutamate (120,000 dpm), 2.5 µmoles NAD and 0.1 units glutamate dehydrogenase (bovine liver, NH_4^+-free, CalBiochem) was added. The reaction was started by tipping from one side arm either 2 µmoles U-^{14}C-glyoxylate (100,000 dpm) or 2 µmoles unlabeled glyoxylate when labeled 2-oxo-glutarate was the other substrate. Final volume was 2.5 ml. The reaction was stopped by adding either 0.2 ml 25% trichloroacetic acid or a mixture of 0.05 ml TCA and 0.1 ml 0.2 M 4-amino-antipyrine from the other side arm. $^{14}CO_2$ was equilibrated with alkali-soaked filter paper in the center well for another hour (unless otherwise indicated). The contents of the center well were counted in a scintillation counter (Bray solution, Packard 314-DC) with internal standardization.

Protein was determined according to Lowry et al. (1951), with crystalline bovine serum albumin as standard.

Paper chromatography of 2,4-dinitrophenylhydrazones was carried out

ascending on Whatman #1. Solvent: butanol-2N NH_3-ethanol (7:2:1), v/v.

Radioactivity of dinitrophenylhydrazones was determined on planchets in a gas-flow counter (Nuclear-Chicago 161A). Chromatograms were scanned in a Packard 385/7200. Absorption spectra were taken from a Cary 11.

Hydroxylapatite was prepared according to Levin (1962).

RESULTS

Purification of the enzyme. All procedures were carried out at 2-4°C. All buffer solutions were pH 7.0 and contained mercaptoethanol, 10^{-3}M. The light yellow mitochondrial acetone powder was extracted with 0.05M phosphate for 30 min. (100 ml per 4 g). Table 1 gives the purification procedure starting with 600 g rat liver (38 g acetone powder). The extract was centrifuged (1 hr, 14,000 xg) and to the supernatant ammonium sulfate was added to 60% saturation. The precipitate was collected by centrifugation (30 min., 14,000 xg), dissolved in 100 ml 0.05M phosphate and dialysed overnight against 10 l.0.05 phosphate. The dialysed solution was passed through

Fraction	Protein mg	Spec. Activity units/mg protein	Total Activity units	Yield %
Extract	5250	0.06	315	100
0-60% ammonium sulfate	2500	0.11	275	87
DEAE-cellulose	190	1.04	198	63
Hydroxylapatite	20	5.15	103	33

Table 1. Activity was determined by the standard assay system with U-^{14}C-glyoxylate and unlabeled 2-oxo-glutarate. The reaction was stopped with the mixture of TCA and 4-amino-antipyrine. 1 unit is equal to 1 μmole $^{14}CO_2$/hr.

a column (4 x 25 cm) of DEAE-cellulose equilibrated with 0.05M phosphate. The column was washed with this buffer until the eluate was free of protein. The active protein was than eluted with 0.05M phosphate containing 0.1M NaCl, and this solution applied to a hydroxylapatite column (4 x 10 cm). After

washing the column with 0.15M phosphate, the enzyme was eluted with 0.2M phosphate.

Properties of the enzyme. Solutions of the 85-fold purified enzyme are colourless at concentrations of 2 mg protein/ml. The requirements of the enzyme are demonstrated in Table 2. Metal ions as Mg^{+2} or Mn^{+2} and TPP are required for optimal activity. Pyruvate is not able to substitute for 2-oxo-glutarate and there is little decarboxylation of glyoxylate when enzyme or 2-oxo-glutarate are omitted. Hydroxomercuribenzoate (10^{-3}M) inhibits the reaction almost completely, but 10^{-3}M iodoacetamide increases the activity to a small extent. The enzyme has a sharp pH-optimum of 6.2 (Fig. 1).

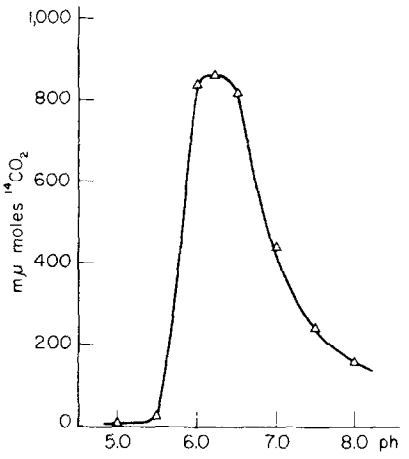


Fig. 1

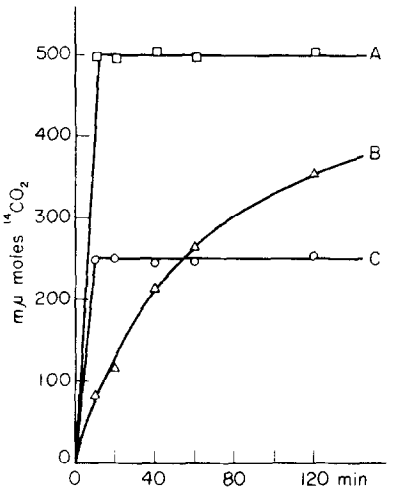


Fig. 2

Fig. 1. Standard assay with 0.09 mg enzyme and 150 μmoles phosphate of indicated pH.

Fig. 2. $^{14}CO_2$-release from the reaction mixtures as a function of time afte stopping the reaction. A: labeling in glyoxylate, stopped with the mixture of TCA and 4-amino-antipyrine. B: labeling in glyoxylate, stopped with TCA alone. C: labeling in 2-oxo-glutarate, stopped with TCA alone. 0.1 mg enzyme.

At pH 7.0 the reaction rate remains linear with time for 1 hour under the assay conditions. The rate is directly proportional to the enzyme concentration until 70% of the substrate has been used in the assay system. Storage of

the enzyme in 0.2M phosphate pH 7.0, 10^{-3}M mercaptoethanol at 0°C causes 5% loss of activity in 24 hours.

Assay System	mμmoles CO_2	Assay System	mμmoles CO_2
Complete	860	Mg^{+2} replaced by Mn^{+2}	930
2-oxo-glutarate omitted	41	2-oxo-glutarate replaced by pyruvate	39
Mg^{+2} "	450	Iodoacetamide added (10^{-3}M)	940
TPP "	500	Hydroxomercuribenzoate added (10^{-3}M)	30
Enzyme "	9		

Table 2. Standard assay with 0.166 mg purified enzyme and indicated changes.

Kinetics of the $^{14}CO_2$-release in the different assay systems. The slow release of $^{14}CO_2$ from the system with labeled glyoxylate, stopped with acid alone (curve B) shows that $^{14}CO_2$ evolution here is due to a slow decarboxylation of the reaction product in the acid medium and not to the preceding enzymatic reaction. In the presence of 4-amino-antipyrine the $^{14}CO_2$-release in the assay system is completed within 10 min. (curve A) and as rapid as the rate of the $^{14}CO_2$-release when the $^{14}CO_2$ is generated by the enzymatic reaction in the case of labeled 2-oxo-glutarate (curve C). The yield of $^{14}CO_2$ in this assay is lower due to the unfavorable equilibrium of the glutamate dehydrogenase reaction by which the 2-oxo-glutarate is generated.

Isolation of the reaction product as 2,4-dinitrophenylhydrazone. One mg of enzyme was incubated for 1 hour at 25°C in 1 ml 0.2M phosphate pH 7.0 with 1 μmole U-^{14}C-glyoxylate (24,000 cpm, gas-flow counter), 1.5 μmoles 2-oxo-glutarate, 0.2 μmoles TPP and 5 μmoles $MgCl_2$. The reaction was stopped by adding 2.5 ml 2,4-dinitrophenylhydrazine solution (10^{-2}M in 2N HCl). After 12 hours at 25°C, the mixture was extracted with ether in a Kutscher-Steudel extractor. The ether was evaporated and the residue dissolved in ethyl acetate and subjected to paper chromatography. About 19% (4,500 cpm) of the radioactivity was recovered

in a brown dinitrophenylhydrazone, Rf 0.1 (glyoxylate, Rf 0.52, 0.37; 2-oxo-glutarate, Rf 0.14), which turned blue after spraying with a saturated solution of KOH in ethanol, indicating a bis-2,4-dinitrophenylhydrazone. The spectrum of the compound in 0.05N NaOH with a maximum at 555 nm is practically identical to that of the dinitrophenylhydrazone obtained from 2-hydroxy-3-oxo-propionic acid (maximum at 560 nm), but distinct from the spectrum of the hydroxy-pyruvate-dinitrophenylhydrazone which lacks the band in the 560 nm region. Assuming the same molecular extinction coefficient as the dinitrophenylhydrazone from 2-hydroxy-3-oxo-propionic acid, $\varepsilon = 2.8 \times 10^4$ [1 x mole^{-1}x cm^{-1}], (Koch, 1964), the specific activity of the compound was calculated to be 23,800 cpm x μmole^{-1}. This fact indicates that both C-atoms of the glyoxylate are present in the reaction product. These results are consistent with the kinetics illustrated in Fig. 2 and contribute strong evidence that the reaction product is a 2-hydroxy-3-oxo-acid.

A glyoxylate carboligase reaction has been detected in microorganisms (Krakow and Barkulis, 1956), and the mechanism of this reaction has been studied by Jaenicke and Koch (1962) and Kohlhaw, et al. (1965). The carboligase reaction described in this communication might have a very similar mechanism. In this case, the reaction would proceed by a successive decarboxylation of 2-oxo-glutarate and condensation of the remaining fragment, an "activated succinic aldehyde", with glyoxylate to yield 2-hydroxy-3-oxo-adipate.

Acknowledgment. This work was supported in part by NIH Grant AM 08171-03.

REFERENCES

Crawhall, J. C., and Watts, R. W. E., Biochem. J. 85, 163 (1962).

Jaenicke, L., and Koch, J., Biochem. Z. 336, 432 (1962).

Koch, J., Thesis University of Cologne (1964).

Kohlhaw, G., Deus, B., and Holzer, H., J. Biol. Chem. 240, 2135 (1965).

Krakow, G., and Barkulis, S. S., Biochim. Biophys. Acta 21, 593 (1956).

Levin, O., in Colowick, Kaplan: Methods in Enzymology V, 27 (1962).

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).

MacDonald, D. L., Stanier, R. Y., in Colowick, Kaplan: Methods in Enzymology III, 614 (1957).

Nakada, H. J., and Sund, L. B., J. Biol. Chem. 233, 8 (1958).

Okuyama, M., Tsuiki, S., and Kikuchi, G., Biochim. Biophys. Acta 110, 66 (1965).