STUDIES ON THE FATTY ACID OXIDIZING SYSTEM OF ANIMAL TISSUES

VIII. RECONSTRUCTION OF FATTY ACID OXIDIZING SYSTEM WITH TRIPHENYLTETRAZOLIUM AS ELECTRON ACCEPTOR

by

S. MII* AND D. E. GREEN

Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin (U.S.A.)

INTRODUCTION

The isolation and properties of each of the five enzymes which are involved in β-oxidation of fatty acids have been described in previous communications of this series[1-7]. The methods of study which led to the discovery of the three enzymes engaged in the conversion of the fatty acyl-CoA to the corresponding β-ketoacyl CoA are quite different in character from those employed once the individual enzymes were recognized. The present communication deals with an assay system which requires the presence of all the enzymes of the fatty acid oxidizing system and which can be used for the assay of any one enzyme. It was indeed this assay system which led step by step to the recognition and identification of the three hitherto unknown enzymes.

The assay system is based on the overall balanced reaction:

$$\text{ATP}^{**} + \text{Butyrate} + 4\ \text{TTZ} + 2\ \text{Malate} \longrightarrow \text{AMP} + \text{P—P}_1 + 4\ \text{Fz} + 2\ \text{Citrate} \quad (1)$$

The balanced reaction is the sum of the following component reactions where the letters over the arrows refer to the specific enzymic catalysts:

$$\text{ATP} + \text{CoA} + \text{Butyrate} \xrightarrow{\text{A}^{***}} \text{AMP} + \text{P—P}_1 + \text{Butyryl CoA} \quad (2)$$

$$\text{Butyryl CoA} + \text{MB} \xrightarrow{\text{B}} \text{Butenoyl CoA} + \text{MBH}_2 \quad (3)$$

$$\text{Butenoyl CoA} + \text{H}_2\text{O} \xrightarrow{\text{C}} \beta\text{-Hydroxybutyryl CoA} \quad (4)$$

$$\beta\text{-Hydroxybutyryl CoA} + \text{DPN}^+ \xrightarrow{\text{D}} \text{Acetoacetyl CoA} + \text{DPNH} + \text{H}^+ \quad (5)$$

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* Post doctoral trainee of the National Heart Institute of the National Institutes of Health.

** The following abbreviations will be used in the text: ATP, adenosinetriphosphate; AMP, adenosine-5-phosphate; TTZ, 2,3,5-triphenyltetrazolium; P–P_1, inorganic pyrophosphate; CoA, coenzyme A; MB, methylene blue; MBH_2, leuco methylene blue; Fz, formazan; DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide.

*** The following letters designate the various enzymes of the fatty acid oxidizing system: A, fatty acid activating enzyme; B, butyryl CoA dehydrogenase; C, hydrase; D, β-hydroxy acyl CoA dehydrogenase; E, β-ketoacyl CoA cleavage enzyme; F, malic dehydrogenase; G, condensing enzyme; and H, diaphorase.

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$$\text{Acetoacetyl CoA} + \text{CoA} \xrightarrow{E} 2\ \text{Acetyl CoA} \quad (6)$$

$$2\ \text{Malate} + 2\ \text{DPN}^+ \xrightarrow{F} 2\ \text{Oxalacetate} + 2\ \text{DPNH} + 2\ \text{H}^+ \quad (7)$$

$$2\ \text{Acetyl CoA} + 2\ \text{Oxalacetate} + 2\ \text{H}_2\text{O} \xrightarrow{G} 2\ \text{Citrate} + 2\ \text{CoA} \quad (8)$$

$$3\ \text{DPNH} + 3\ \text{H}^+ + 3\ \text{MB} \xrightarrow{H} 3\ \text{DPN}^+ + 3\ \text{MBH}_2 \quad (9)$$

$$4\ \text{MBH}_2 + 4\ \text{TTZ} \longrightarrow 4\ \text{MB} + 4\ \text{Fz} \quad (10)$$

The complexity of the assay system is of course far too great to recommend it in place of a one step assay for any one of the enzymes. But as a point of departure for reconstructing fatty acid oxidation with unidentified soluble enzyme fractions its virtue lay in this very completeness. Another point of interest is that the test conditions simulate physiological conditions. In reactions (2) and (6) the presence of free CoA in the reduced or SH form is mandatory. However five of the nine reactions are oxidative in nature. The problem thus arises of maintaining CoA in its reduced form in presence of oxidizing agents such as methylene blue. Under the special assay conditions described below with methylene blue acting in a shuttling, catalytic capacity between the substrates and TTZ, CoASH can coexist with oxidizing agents, and thus it becomes possible to study fatty acid oxidation in an overall sense with combinations of the five basic enzymes (A–E) and the three supplementary enzymes *viz.* malic dehydrogenase (F), condensing enzyme (G) and diaphorase (H).

METHODS

TTZ assay system

The complete system has a final volume of 0.2 ml and contains in addition to the requisite enzymes 0.5 μM of L-malate, 0.27 μM of ATP, 2.4 μM of TTZ, 3.3 μM of histidine (adjusted to pH 7), 0.06 μM of DPN, 3.3 μM of butyrate, 0.03 μM of reduced CoA and 0.66 μM of MB. The experiments are carried out anaerobically in narrow test tubes. Satisfactory anaerobic conditions are obtained by evacuating with an oil pump the desiccator in which the tubes are kept. To maintain temperature control the tubes are placed in a beaker partly filled with paraffin oil at 38°. In turn the beaker rests in paraffin oil (38°) covering the bottom of the vacuum desiccator. The desiccator is rapidly evacuated (3 to 5 min) and then kept for one hour in an air thermostat kept at 38°. At the end of the experiment the content of each test tube is immediately acidified with 0.05 ml of 6 NHCl, and then mixed with 1.5 ml of acetone and 4.5 ml of carbon tetrachloride. After thorough mixing the emulsion is centrifuged and the blue top layer is discarded. The absorption at 485 mμ provides a measure of formazan production. An optical density of 1.00 at 485 mμ indicates a concentration of 25 μg of formazan per ml or 0.0833 μM per ml. Each experimental tube has its own separate control from which the fatty acid is omitted. All experimental values given in the text are corrected for their corresponding blank which is usually small or negligible.

DPN assay system

This assay was devised originally for following the enzyme which catalyzed reaction 5, the second oxidative step. The complete system has a final volume of 0.2 ml and contains in addition to the requisite enzymes 1 μM of L-malate, 1 μM of magnesium chloride, 1 μM of ATP, 28 μM of glycylglycine (adjusted to pH 8.2), 1 μM of DPN and 0.024 μM of reduced CoA. The experiment is carried out in narrow test tubes which are immersed in a water bath at 38° for 15 min. The contents are then mixed with four volumes of 0.5 M phosphate buffer. The absorption at 340 mμ is used as a measure of DPNH formation. The molar extinction coefficient of DPNH was taken to be $6.22 \cdot 10^6$ sq. cm $\times$ mole^{-1} [8].

Preparation of enzymes

Initially three enzyme fractions were used in conjunction to supply the eight enzymes required for the TTZ assay system. Fraction I was prepared from beef liver mitochondria according to GREEN *et al.*[3]; fraction II from the same source according to MAHLER, WAKIL AND BOCK[1]; and fraction III from beef heart mitochondria essentially according to the procedure described by GOLDMAN[7] for the first stages in the purification of the cleavage enzyme from beef kidney mitochondria. Fraction I

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is a source of fatty acyl CoA dehydrogenase (B), fraction II of the activation enzyme (A), and fraction III is a composite fraction containing enzymes C–H.

In later studies fraction III was replaced by fractions IV, V and VI which are sources, respectively, of the β-hydroxyacyl CoA dehydrogenase and hydrase, of acetoacetyl CoA cleavage enzyme and of condensing enzyme. The source and methods of preparation of these fractions are as follows: IV, beef liver mitochondria, WAKIL *et al.*[6]; V, beef liver mitochondria, GOLDMAN[7] and VI, pig heart OCHOA *et al.*[9].

Chemical determinations. The following assay procedures were used: citric acid[10]; acetone[11]; and formazan[12] with the modifications mentioned in the text.

Materials. CoA was prepared by the method of BEINERT *et al.*[13] and used in its reduced form after treatment with amalgam[13]. DPN and ATP were obtained from the Schwartz and Pabst laboratories and TTZ from the Montclair Research Corporation.

RESULTS

Table I contains a component study in which the complete requirement for ATP, CoA, DPN, MB and malate is demonstrated. Fig. 1 shows activity as a function of the concentration respectively of ATP, CoA, DPN, MB and TTZ. The reaction is linear with time except for an initial 10 to 15 minute lag period which is referable to the time required to attain a saturating level of butyryl CoA generated according to reaction 2.

TABLE I

COMPONENT STUDY OF THE COFACTORS NEEDED FOR THE TTZ ASSAY SYSTEM

	μM Formazan/60 min
Complete system	0.22
No malate	0.02
No ATP	0.01
No DPN	0.00
No MB	0.00
No CoA	0.00

Standard conditions as described in text under heading of TTZ assay system. The following amounts of the 3 enzyme fractions were used: I, 10 γ; II, 85 γ; III, 22 γ.

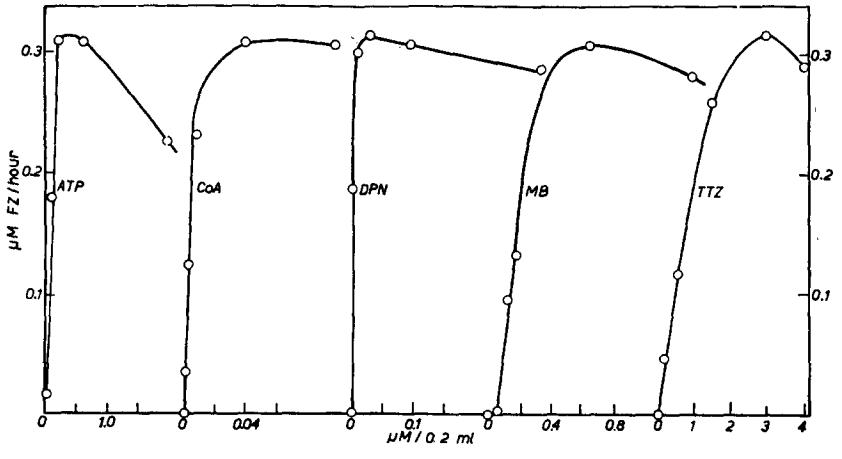

Fig. 1. Reaction rate as a function of the concentration of ATP, CoA, DPN, MB, and triphenyltetrazolium. Details as in legend for Table I. The three enzyme fractions were used in the following amounts: I, 10 γ; II, 85 γ; III, 22 γ.

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The requirement for each of the three enzyme fractions is shown in Table II. When any two fractions are present in excess the test system can be used for assay of the third fraction. For example Fig. 2 shows how the rate of the reaction depends upon the concentration of fraction I. The linear part of the curve falls within a rather narrow range of enzyme concentration.

TABLE II

COMPONENT STUDY OF THE ENZYME FRACTIONS NEEDED FOR THE TTZ ASSAY SYSTEM

	μM Formazan/60 *min*
Complete system	0.31
Without fraction I	0.03
Without fraction II	0.02
Without fraction III	0.00

Fractions I, II and III were used in the following amounts per 0.2 ml; 10, 16 and 20 γ respectively.

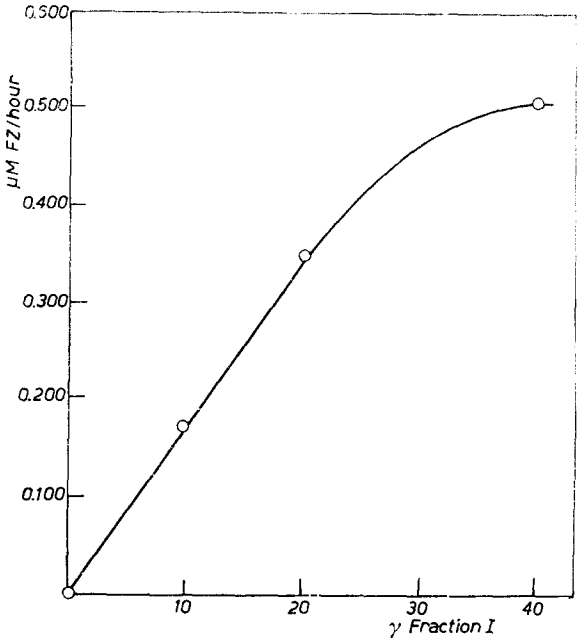

Fig. 2. Reaction rate as a function of the concentration of Fraction I. Details as for legend of Table I. The following amounts of fractions II and III respectively were used: 70 γ and 240 γ.

According to equation (1) the ratio, moles TTZ reduced per mole of citrate formed, should be 2. The observed stoichiometry (*cf.* Table III) is in agreement with theory. No correction has been applied for the equilibrium established by aconitase between citric acid on the one hand and *iso*citric acid and *cis*-aconitic acid on the other. When crude preparations of fraction III were used in the assay it was found that the yield of citrate was far below theory. This discrepancy was eventually traced to the presence in the preparation of a DPN *iso*citric dehydrogenase which catalyzed the further oxidation of citrate by way of *iso*citrate. However addition of fluorocitrate[14] prepared enzymically prevented this oxidative loss of citrate.

TABLE III

FORMAZAN—CITRATE RATIO

	Fz μM	*Citrate* μM	*Fz/Citrate*	
			Observed	*Theory*
(1)	0.29	0.12	2.4	2.00
(2)	0.21	0.11	1.9	2.00

The optimum pH for the assay system has been found to be about 6.5. This is not to be confused with the maximal pH for any of the participating enzymes. Formazan production in the controls without fatty acid increases in the more alkaline pH range —a fact which suggests oxidation of reduced CoA by TTZ mediated by MB.

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The reduction of TTZ to Fz involves the liberation of one equivalent of acid per mole of TTZ reduced. This property has been made the basis of a manometric assay. The assay system is made up to 15 times usual scale *i.e.* to a final volume of 3.0 ml. Bicarbonate (60 μM) is used instead of histidine. White phosphorus sticks are inserted in the center well of the manometer cups and the gas space is filled with a mixture of 95% N_2 and 5% CO_2. Fig. 3 shows a time curve in presence and absence of malate. In this particular experiment crude enzyme fractions were used. Later some comment will be made about the reaction in absence of added malate. According to theory the ratio, moles CO_2 formed per mole of TTZ reduced should be 1.0. In two experiments ratios of 0.74 and 0.94 were observed.

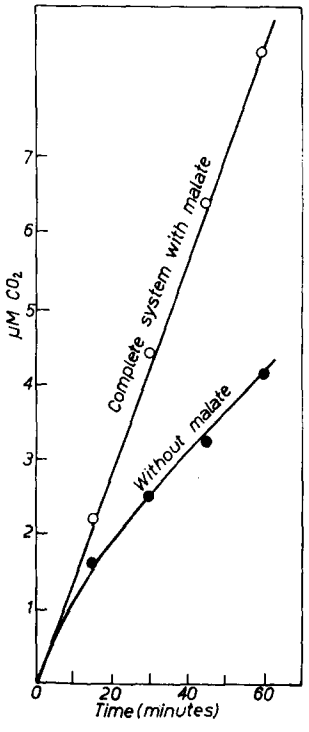

Fig. 3. Time curve for the manometric TTZ assay system. The details of the manometric assay are described in the text. The following amounts of crude enzyme fractions were used: I, 2.45 mg; II, 1.5 mg and III, 1.5 mg.

When fractions I and II were used at a lower level of purity it was observed that the reaction proceeded in complete absence of added malate except that the rate was reduced to one half (*cf.* Fig. 3). Under these conditions acetoacetyl CoA is disposed of according to the reaction:

$$\text{Acetoacetyl CoA} + H_2O \longrightarrow \text{Acetoacetate} + \text{CoA} \quad (11)$$

Crude preparations of fractions I and II contain a deacylase which liberates CoA from acetoacetyl CoA. Thus deacylation can replace citrate formation as a device for shifting the equilibrium of reactions 3 and 5 from left to right. The formation of acetoacetate determined as acetone after decarboxylation[11] could indeed be demonstrated when malate was omitted. The observed ratio, moles TTZ reduced per mole of acetone formed was 1.74 while theory is 2.0.

It followed from the complete requirement of the assay system for DPN that reactions 3 or 5 or both required DPN. The decision between these alternatives was arrived at in the following way. Fraction I contains the enzyme which catalyzes the first oxidation shown in equation 3 and fraction II the activating enzyme. If fractions I and II together can catalyze the oxidation of butyrate by TTZ in absence of DPN then DPN is not needed for the first oxidation. This was indeed found to be the case. Thus the participation of DPN in the second oxidative step was established. Furthermore Fraction III was localized as the fraction which among other enzymes contained the dehydrogenase for catalyzing reaction 5.

There were two possibilities as to which was the substrate for the second dehydrogenation—either the β-hydroxyacyl CoA derivative or the α-β-unsaturated derivative. Since both derivatives were acted upon by the enzyme fractions which were used in the TTZ assay clearly these two derivatives were in equilibrium with one another. Among the various substances tried as substrate for the second dehydrogenase sorbic acid which is $\Delta_{2,3}\,\Delta_{4,5}$ hexene-dioic acid proved to be the most satisfactory under the conditions of the assay. It was now possible to devise a simple assay for the second dehydrogenase. Fraction I could be eliminated, and TTZ + MB could be replaced by DPN

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which now served as the terminal electron acceptor. The overall reaction equation is:

$$ATP + Sorbate + 5\ DPN^+ + 3\ Malate \longrightarrow AMP + P\text{—}P_1 + 5\ DPNH + 3\ Citrate. \quad (12)$$

A typical component study is shown in Table IV. The oxidation of sorbate by DPN required ATP, CoA, magnesium ions and fractions II and III. According to theory 1 mole of citrate should be formed for each 1.67 moles of DPNH formed. The observed ratio was 0.43 while theory is 0.60. Again the observed value for citrate formation has not been corrected for the equilibrium with *iso*citric and *cis*-aconitic acids established by aconitase.

TABLE IV

COMPONENT STUDY FOR DPN ASSAY SYSTEM WITH SORBATE AS SUBSTRATE

	μM DPNH/15 min
Complete system	0.27
Without fraction II	0.01
Without fraction III	0.02
No CoA	0.01
No ATP	0.00
No magnesium ions	0.19

The complete system was set up as described in the text under the heading of DPN assay system. Fractions II and III were used in the following amounts: 10 γ and 20 γ respectively.

The next simplification introduced was the elimination of the coupling between the dehydrogenation step and citrate formation. To accomplish this CoA had to be used in stoichiometric rather than catalytic amount (*cf.* Table V). Under those conditions the overall reaction sequence conforms to the equation:

$$ATP + CoA + Sorbate + 2\ DPN^+ \longrightarrow AMP + P\text{–}P_1 + 2\ DPNH + 1.5\ Acetoacetyl\ CoA. \quad (13)$$

TABLE V

DPNH FORMATION AS A FUNCTION OF CoA CONCENTRATION IN ABSENCE OF MALATE

	μM CoA added	*μM DPN added*	*μM DPNH formed*
(1)	0.024	1.0	0.025
(2)	0.072	1.0	0.052
(3)	0.216	1.0	0.100
(4)	0.216	5.0	0.122

Details as for Table IV. Time 1 hour at 38°.

Thus in effect a one step assay for the dehydrogenase (reaction 5) became available since the appropriate acyl CoA derivate could be supplied directly rather than be generated *in situ* by reaction 2.

This assay led to rapid progress in the purification of the dehydrogenase and finally to the demonstration by GREEN *et al.*[3] and WAKIL *et al.*[6] that the proper substrate of the enzyme was the *d*-β-hydroxyacyl CoA derivative and not the unsaturated acyl derivative.

At this point it was possible to reconstruct the fatty acid oxidation system using

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the TTZ assay with five separate enzyme fractions (*cf.* Table VI). These included fractions I and II as usual. However, fraction III was replaced by fractions IV, V and VI which are sources respectively of the β-hydroxyacyl CoA dehydrogenase cum hydrase, acetoacetyl CoA cleavage enzyme and condensing enzyme. Thus only two enzymes remained which had to be added as separate fractions *viz.* malic dehydrogenase and diaphorase. These enzymes were present as contaminants of the five fractions used in the experiment shown in Table VI.

TABLE VI

RECONSTRUCTION OF FATTY ACID OXIDIZING SYSTEM WITH FIVE ENZYME FRACTIONS

	μM Fz/60 min
Complete system	0.31
Without I	0.00
II	0.00
IV	0.00
V	0.01
VI	0.01

Details as for Table I. The following amounts of enzyme were used: Fraction I, 10 γ; Fraction II, 7 γ; Fraction IV, 10 γ; Fraction V, 2 γ; Fraction VI, 5 γ.

There was one final point which the TTZ assay system was instrumental in resolving. When the assay was carried out with crude preparations of the green flavoprotein (fraction I) the enzymic system was found to be equally active on butyrate and octanoate. With purification of the green flavoprotein activity towards octanoate disappeared. This suggested a second acyl CoA dehydrogenase active on the higher acyl CoA's. Elsewhere BEINERT, CRANE, GREEN AND MII[15] have reported on the isolation of a second enzyme from beef liver mitochondria which is active on acyl CoA's from C_4–C_{18} in chain length.

DISCUSSION

The discovery of the oxidative enzymes involved in β-oxidation has also been made independently in the laboratory of F. LYNEN in Munich[16,17]. A very different approach was used by this group. A synthetic substrate S-acetoacetyl N-acetyl thioethanolamine was used as a guide to the purification of the β-hydroxyacyl CoA dehydrogenase by studying reaction 5 in reverse. Similarly S-crotonyl N-acetyl thioethanolamine was used as oxidant for the acyl CoA dehydrogenase with leuco safranine as electron donor. Two requirements had to be met to make possible our studies of the oxidative processes from a left to right direction: (1) Coenzyme A of high purity had to be available in considerable amount. (2) The activation enzyme had to be available for preparing any of the desired coenzyme A derivatives.

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SUMMARY

An assay system is described in which butyrate is oxidatively converted to citrate with 2,3,5-triphenyl tetrazolium as terminal electron acceptor. The role of this assay system in the recognition, one by one, of all the enzymes involved in the β-oxidation of fatty acids has been described.

RÉSUMÉ

Les auteurs décrivent un système expérimental qui réalise l'oxydation du butyrate en citrate, en utilisant le 2,3,5-triphényl-tétrazolium comme accepteur final d'électrons. L'emploi de ce système pour la mise en évidence, un par un, de tous les enzymes qui participent à la β-oxydation des acides gras est également décrit.

ZUSAMMENFASSUNG

Ein Versuchssystem wird beschrieben das Butyrat oxydativ in Citrat mit 2,3,5-Triphenyltetrazolium als Endelektronenacceptor umwandelt. Die Rolle dieses Versuchssystems bei der Erkennung aller an der β-Oxydation der Fettsäuren beteiligten Enzyme wurde beschrieben.

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