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On the Mechanism of α -Oxoglutarate Oxidation in *Escherichia coli*

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The enzymic formation of succinyl-coenzyme A from α -oxoglutarate is known to require diphosphopyridine nucleotide (DPN⁺), thiamine pyrophosphate (TPP), lipoic acid (lip-S_{ox} or lip-S_{red}) and coenzyme A (CoA). It has been postulated (Gunsalus, 1953; Kaufman, 1955; Sanadi, Langley & White, 1959) that the sequence of reactions involved in the overall reaction is:

- (1) α -oxoglutarate + TPP $\rightleftharpoons$ [succinic semialdehyde]-TPP + CO₂;
- (2) [succinic semialdehyde]-TPP + lip-S_{ox} $\rightleftharpoons$ succinyl-S-lip + TPP;
- (3) succinyl-S-lip + CoA-SH $\rightleftharpoons$ succinyl-S-CoA + lip-S_{red};
- (4) lip-S_{red} + DPN⁺ $\rightleftharpoons$ lip-S_{ox} + reduced DPN + H⁺.

Sum:

Attempts to isolate the enzymes catalysing these individual steps have been largely unsuccessful, although lipoic dehydrogenase (reaction 4) has been separated and shown to be a component of the α -oxoglutarate-dehydrogenase system of *Escherichia coli* (Hager & Gunsalus, 1953). More recently the mammalian α -oxoglutarate-dehydrogenase system has also been resolved with respect to lipoic dehydrogenase (Massey, 1960) and it has been shown to be a flavoprotein (Massey, 1958; Searls & Sanadi,

1959) identical with Straub's (1939) diaphorase (Massey, 1960).

It is the purpose of this paper to characterize the biochemical lesion in a succinate-requiring mutant of *E. coli*. The experiments reported indicate that the mutant lacks the enzyme catalysing reaction 1, α -oxoglutarate carboxylase, or reactions 1 and 2. It therefore provides a source for the α -oxoglutar-

ate-dehydrogenase complex which is deficient in this enzyme or these enzymes.

METHODS AND MATERIALS

Maintenance of stock culture. *E. coli* W (wild type) and the succinate-requiring mutant (*E. coli* 309-1 R) were gifts from Professor B. D. Davis (Harvard University Medical School, Boston, Mass., U.S.A.). The organisms were maintained in stock culture on agar slopes containing (per 100 ml. of medium) 1 g. of tryptone, 1 g. of Difco yeast extract, 0.5 g. of dipotassium hydrogen phosphate, 0.3 g. of glucose, 0.2 g. of sodium succinate and 2 g. of agar agar (Hopkin and Williams Ltd., Chadwell Heath, Essex).

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Preparation of inoculum and growth of cells. Both wild-type and mutant cells were grown in an identical manner. The inoculum was prepared by transferring cells from the stock-culture agar slopes to 50 ml. of liquid medium containing the same ingredients (minus agar) as the agar slopes. These cultures were incubated aerobically with shaking overnight at 30° and served as inocula for growth carboys (vol. 10 l.). The growth medium contained (per 100 ml.) 0.1 g. of Difco yeast extract, 0.1 g. of tryptone, 0.7 g. of dipotassium hydrogen phosphate, 0.3 g. of potassium dihydrogen phosphate, 0.1 g. of ammonium sulphate, 0.01 g. of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g. of glucose and 0.05 g. of sodium succinate. The 10 l. cultures were grown overnight at 30° with forced aeration aided by sintered-glass spargers. The cells were harvested by centrifuging in a Sharples centrifuge and the cell paste was stored at -20°. The yield of wild-type cells was 65 g. of cell paste/10 l. of culture. The yield of mutant cells was 40 g. of cell paste/10 l. of culture.

Preparation of cell-free extracts. Cell-free extracts of both wild-type and mutant cells were prepared by suspending 2 g. of cell paste in 10 ml. of 0.02M-2-amino-2-hydroxy-methylpropane-1:3-diol(tris) buffer, pH 7.0, and subjecting the resultant slurry to the output of a 600 w Mullard magnetostrictor oscillator (operating at 3.5 A, 25 kcyc./sec.) for 3 min. Unbroken cells and cell debris were removed by centrifuging at 20 000 g for 15 min. at 4° in a PR-2 International refrigerated centrifuge. The pellet obtained from the centrifuging was discarded. The supernatant fraction was dialysed overnight against 100 vol. of 0.02M-tris buffer, pH 7.0, and subsequently stored at -20°.

Substrates and cofactors. α -Oxoglutarate was a gift from Professor Sir Hans Krebs, F.R.S.; DPN and CoA were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.; TPP and oxidized lipoic acid were purchased from the California Research Foundation; the last-named compound was reduced according to the method of Gunsalus, Barton & Gruber (1956). Sodium [^{14}C]bicarbonate was prepared from $\text{Ba}^{14}\text{CO}_3$, obtained from The Radiochemical Centre, Amersham, Bucks., as previously described (Kornberg, Davies & Wood, 1952).

RESULTS

Oxidation of α -oxoglutarate with diphosphopyridine nucleotide as electron acceptor and coenzyme A as acyl acceptor. Crude extracts of wild-type *E. coli* catalyse the overall oxidation of α -oxoglutarate to form reduced diphosphopyridine nucleotide (DPNH) and succinyl-CoA (reaction 5). Fig. 1, curve 1, shows the formation of DPNH from α -oxoglutarate, in the presence of substrate amounts of CoA, catalysed by the crude extract prepared from wild-type cells. The extract prepared from the succinate-requiring mutant failed to catalyse this reaction (Fig. 1, curve 3), as would be expected, since growth experiments (Davis, Kornberg, Nagler, Miller & Mingioli, 1959) predict that the mutant lacks one of the enzymes involved in the oxidation of α -oxoglutarate to succinate. A mixture of the mutant extract plus the wild-type extract formed DPNH at the same rate as did the

wild-type extract alone (Fig. 1, curve 2). This finding indicates that the mutant extract lacks an enzyme of the α -oxoglutarate-dehydrogenase system, and that its failure to catalyse the overall

Fig. 1. Oxidation of α -oxoglutarate with DPN as electron acceptor and CoA as acyl acceptor. The complete system contained, in 3 ml.: 300 μ moles of potassium phosphate buffer, pH 7.0, 200 μ moles of magnesium chloride, 0.5 μ mole of thiamin pyrophosphate, 100 μ moles of cysteine, 1 μ mole of coenzyme A, 0.3 μ mole of diphosphopyridine nucleotide, 100 μ moles of α -oxoglutarate, and cell-free extracts. 1 (○), Wild-type extract (6 mg. of protein); 2 (●), wild-type extract plus mutant extract (6 mg. of protein of each); 3 (▲), mutant extract (6 mg. of protein). Control cuvettes contained the above mixtures with DPN omitted. All reactions were run anaerobically at room temperature. The oxidation of DPN was measured in a Beckman spectrophotometer as the increase in extinction at 340 μ .

Fig. 2. Oxidation of α -oxoglutarate with ferricyanide as electron acceptor. The complete system contained (in a total volume of 1 ml.): 100 μ moles of potassium phosphate buffer, pH 6.0, 20 μ moles of sodium α -oxoglutarate, 0.5 μ mole of TPP, 25 μ moles of potassium ferricyanide plus wild-type extract (12 mg. of protein: curve 1), wild-type extract (6 mg. of protein: curve 2) or mutant extract (12 mg. of protein: curve 3). The reaction mixtures were incubated at 30° for 30 min. under N_2 and the rate of CO_2 evolution was measured in Warburg manometers.

reaction is not due to an inhibitor of the enzyme complex in the mutant extract.

Oxidation of α -oxoglutarate with ferricyanide as electron acceptor. A comparison of the mutant and wild-type extracts for the ability to effect the oxidative decarboxylation of α -oxoglutarate with ferricyanide as electron acceptor is shown in Fig. 2. It is evident that, whereas the wild-type extract catalysed the oxidative decarboxylation of α -oxoglutarate at rates proportional to the amounts of extract added, the extract prepared from the mutant did not catalyse this reaction. Since TPP is the only cofactor required for its occurrence, it has been postulated (Gunsalus, 1953; Schweet, Fuld, Cheslock & Paul, 1953) that this reaction is a measure of α -oxoglutarate-carboxylase activity (reaction 1). The finding that the mutant did not catalyse the ferricyanide-linked reaction therefore offers circumstantial evidence about the metabolic lesion in the mutant.

Comparison of the wild-type and mutant extracts for their ability to catalyse $^{14}\text{CO}_2$ exchange with α -oxoglutarate. The exchange of $^{14}\text{CO}_2$ with α -oxoglutarate provides a more reliable assay for α -oxoglutarate-carboxylase activity. It has been previously established that TPP is the only cofactor requirement for this exchange reaction (Goldberg & Sanadi, 1952). Furthermore, it has been shown that $^{14}\text{CO}_2$ exchange with pyruvate is a measure of the carboxylase activity of the pyruvate-dehydrogenase complex (A. D. Gounaris & L. P. Hager, unpublished experiments). The ability of extracts

paper with 5% sodium bicarbonate. The sodium bicarbonate solution was acidified with HCl and the hydrazone extracted into ethyl acetate as described above; the ethyl acetate solution was then concentrated and plated on aluminium planchets for radioassay.

The 2:4-dinitrophenylhydrazone of α -oxoglutarate isolated after incubation with the wild-type extract gave 200 counts/min., but that isolated after incubation with the mutant extract was devoid of radioactivity. Hence, whereas extracts of the wild type catalyse $^{14}\text{CO}_2$ exchange with α -oxoglutarate, extracts of the mutant failed to catalyse this exchange reaction.

Other enzymes associated with the α -oxoglutarate-dehydrogenase complex. Both the wild-type extract and the mutant extract contained lipoid dehydrogenase (Table 1). The lipoid-dehydrogenase content of the extracts was estimated by following the disappearance of reduced lipoic acid in the presence of catalytic amounts of DPN plus pyruvate and lactic dehydrogenase (Hager & Gunsalus, 1953).

In addition, both the wild-type and mutant extracts contained succinic thiokinase, as assayed by the hydroxamic acid-trapping method (Kaufman, 1955). The mutant extract contained approximately 75% of the succinic-thiokinase activity of the wild-type extract (Table 1). Advantage was taken of the fact that both extracts contained succinic thiokinase in developing an assay for lipoic trans-succinylase. Succinic thiokinase was employed to generate catalytic amounts of succinyl-CoA according to equation 6:

of the wild type and the mutant organisms to catalyse the exchange of $^{14}\text{CO}_2$ with α -oxoglutarate was tested as described below.

The complete system contained 200 μ moles of magnesium chloride, 0.5 μ mole of TPP, 10 μ moles of sodium α -oxoglutarate, 2 μ moles of $\text{NaH}^{14}\text{CO}_3$ (containing 2×10^6 counts/min.) and wild-type or mutant extract (20 mg. of protein of each) in a total volume of 1.8 ml. The reaction mixtures were incubated at 30° for 1 hr. under nitrogen. After incubation, the reactions were stopped by the addition of 0.6 ml. of 6N-HCl, and denatured protein was removed by centrifuging. A saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl (1 ml.) was added and the solution was allowed to stand at room temperature for 2 hr. The 2:4-dinitrophenylhydrazone of α -oxoglutarate together with unreacted 2:4-dinitrophenylhydrazine were extracted from the acid solution by four extractions of 2 ml. each of ethyl acetate. The ethyl acetate extract was concentrated to a small volume, approx. 0.5 ml., under nitrogen at 60°. The concentrated solution was applied as a streak to Whatman no. 3 filter paper and chromatographed in the solvent of El Hawary & Thompson (1953). The α -oxoglutarate 2:4-dinitrophenylhydrazone was eluted from the

The transfer of the succinyl moiety from CoA to reduced lipoic acid, to yield succinyl lipoic acid (reaction 7),

was then detected by the lipoic acid-dependent formation of succinyl hydroxamic acid. In this case, hydroxylamine was added at the end of the

Table 1. *Lipoic-dehydrogenase and succinic-thiokinase activity of wild-type and mutant extracts*

Lipoic-dehydrogenase activity was assayed by the system developed by Hager & Gunsalus (1953); specific activity is defined as μ mole of reduced lipoic acid oxidized/hr./mg. of protein. Succinic-thiokinase activity was assayed by the hydroxamic acid method (Kaufman, 1955); specific activity refers to μ mole of succinic hydroxamate formed/hr./mg. of protein.

Extract	Specific activity	
	Lipoic dehydrogenase	Succinic thiokinase
Wild type	0.80	0.19
Mutant	0.88	0.14

incubation period. Some succinyl hydroxamate formation took place in the absence of lipoic acid since catalytic amounts of succinyl-CoA were generated by succinic thiokinase. In addition, both the wild-type and mutant extracts gave a colour in the ferric chloride test for succinyl hydroxamate. These two factors combine to yield the apparent 'succinyl hydroxamate' at zero time shown in Fig. 3. However, the intensity of this colour did not increase with time in the absence of lipoic acid: lipoic trans-succinylase action is shown as the formation as a function of time of succinyl hydroxamate, which depended on the presence of succinate and lipoic acid (Fig. 3). The two extracts have essentially equal, but low, catalytic activities in this assay system.

Succinate formation from malate under anaerobic conditions. Growth studies (Davis *et al.* 1959) have shown that the succinate-requiring mutant will grow anaerobically and utilize glucose in the absence of succinate. This finding implies that the mutant is capable of forming succinate under anaerobic conditions, presumably by the reduction of oxaloacetate, malate and fumarate. Undialysed cell-free extracts of the mutant were tested for their

ability to convert [14 C]malate (Dixon, Kornberg & Lund, 1960) into succinate under anaerobic conditions. The crude, undialysed extract, incubated for 1 hr. under nitrogen with tris buffer and magnesium chloride but without added electron donors, catalysed the conversion of approximately 80 % of added [14 C]malate into succinate: cell-free extracts of the mutant therefore reflect the ability of the intact cell to effect the anaerobic synthesis of succinate from malate.

DISCUSSION

Experimental verification of the proposed four-step reaction for the oxidation of pyruvate and α -oxoglutarate linked to DPN, CoA and lipoic acid has been hampered by the lack of success in separating the individual components. Both the pyruvate- and α -oxoglutarate-dehydrogenase systems fractionate as complexes from both bacterial and mammalian sources. At this time it is not known whether the complex formation primarily involves covalent bonding or merely electrostatic interaction, although with the lipoic-dehydrogenase component of the α -oxoglutarate complex it appears that electrostatic interaction is involved (Massey, 1960).

The isolation of mutants of *E. coli* requiring acetate or succinate offers a unique approach to the resolution and study of the pyruvate- and α -oxoglutarate-dehydrogenase complexes. Genetic considerations render it probable that these mutant organisms specifically lack only one of the sequential enzymes; they therefore offer a source of the enzyme complex resolved with respect to one of its components.

The experiments reported in this paper indicate that the succinate-requiring mutant (309-1R) of *E. coli* specifically lacks the ' α -oxoglutarate carboxylase' portion of the α -oxoglutarate-dehydrogenase complex. Extracts of the mutant, in contrast with those of the wild-type organism, failed to catalyse the exchange of $^{14}\text{CO}_2$ into α -oxoglutarate and the ferricyanide-linked oxidation of α -oxoglutarate as well as the overall formation of DPNH and succinyl-CoA from α -oxoglutarate. Since both the wild-type and the mutant extracts contain lipoic dehydrogenase, lipoic trans-succinylase and succinic thiokinase, it is probable that the primary decarboxylative step in the oxidation of α -oxoglutarate is ascribable to a discrete enzyme in the complex of enzymes not normally resolvable.

SUMMARY

1. Cell-free extracts of the succinate-requiring mutant of *E. coli*, 309-1R, and of its parent wild-type *E. coli* W, were tested for their ability to catalyse reactions involved in the overall oxidation of α -oxoglutarate to succinate.

Fig. 3. Formation of succinyl hydroxamate dependent upon the presence of ATP, CoA, succinate and reduced lipoic acid. The complete system contained (in 1 ml.): 100 μ moles of potassium phosphate, pH 7.5, 100 μ moles of ATP, 0.2 μ mole of CoA, 20 μ moles of sodium borohydride, 10 μ moles of reduced DL-lipoic acid and 8 mg. of wild-type extract (○) or 8 mg. of mutant extract (●). Reaction mixtures were incubated at 30°. At the time intervals indicated 800 μ moles of neutralized hydroxylamine were added and the mixture was allowed to stand at room temperature for 5 min. Succinyl hydroxamate colour was then developed by the addition of FeCl_3 reagent (Lipmann & Tuttle, 1945). The reading at zero time indicates the colour formed in an incubation mixture containing all the components of the complete system but with either succinate or reduced lipoic acid omitted: the intensity of this colour did not increase with time.

2. Whereas extracts of the wild type catalysed the reduction of diphosphopyridine nucleotide in the presence of α -oxoglutarate, thiamin pyrophosphate and coenzyme A, extracts of the mutant did not. Addition of mutant extract to that of the wild type did not affect the latter's ability to oxidize α -oxoglutarate; this shows that the inability of the mutant extract to effect this reaction was due not to the presence of an enzymic inhibitor, but to the lack of an enzymic component of the α -oxoglutarate-dehydrogenase system.

3. Extracts of the wild-type organism catalysed the anaerobic evolution of carbon dioxide from α -oxoglutarate in the presence of thiamin pyrophosphate and ferricyanide as electron acceptor. The rate of gas evolution was proportional to the amounts of extract added. Extracts of the mutant failed to effect this reaction.

4. Extracts of the wild-type organism in the presence of thiamin pyrophosphate catalysed the incorporation of isotope from [^{14}C]bicarbonate into α -oxoglutarate, by isotopic exchange. Extracts of the mutant did not catalyse this reaction.

5. Extracts of both types of organism contained succinic thiokinase, lipoic dehydrogenase and lipoic trans-succinylase: a new assay procedure for the last-named enzyme is reported.

6. These results show that the mutant lacked the enzymes catalysing the first step or first two steps in the overall oxidation of α -oxoglutarate. They also indicate that the formation of the succinic semialdehyde-thiamin pyrophosphate complex and carbon dioxide from α -oxoglutarate is effected by a discrete enzymic component of the α -oxoglutarate-dehydrogenase system.

7. Undialysed cell-free extracts of the mutant, in the absence of added electron donors, catalysed the anaerobic reduction of [^{14}C]malate to [^{14}C]succinate: this reflects the ability of the mutant to grow anaerobically even in the absence of succinate.

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The Measurement of Aconitase Activity in the Leaves of Various Normal and Variegated Plants

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Aconitase, an enzyme widely distributed in living organisms, was first found in higher plants (kidney beans, cucumber seeds) by Martius (1939), and was later shown to occur in leaves of cabbage (Jacobssohn & Soares, 1940) and of rhubarb (Morrison & Still, 1947).

Studies by Dickman & Cloutier (1951) and by Morrison (1954) have shown that during its purification from animal tissues the enzyme loses activity, which can be restored by the addition of ferrous iron together with cysteine, thioglycollate, ascorbate or glutathione. It has therefore been