Purification and Properties of the $\alpha$-Ketoglutarate Dehydrogenase Complex of Cauliflower Mitochondria

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(Received for publication, April 16, 1970)

SUMMARY

A procedure is described by which the $\alpha$-ketoglutarate dehydrogenase-lipoyl transsuccinylase portion of the $\alpha$-ketoglutarate dehydrogenase complex of cauliflower mitochondria has been purified to a specific activity $>5$. The enzymatic activity is totally dependent on $\alpha$-ketoglutarate, DPN, reduced CoA, thiamine pyrophosphate, Mg$^{2+}$ or Ca$^{2+}$, and lipoyl dehydrogenase. Either cauliflower or pig heart lipoyl dehydrogenase will couple with the $\alpha$-ketoglutarate dehydrogenase-lipoyl transsuccinylase complex to produce DPNH and succinyl-CoA.

Because the cauliflower enzyme as prepared has a requirement for both thiamine-PPi, and Mg$^{2+}$, it was possible to show that the enzyme binds magnesium-thiamine-PPi rather than free Mg$^{2+}$ and free thiamine-PPi. The enzyme-magnesium-thiamine-PPi complex is relatively stable, with a half-life of about 2 min. The $K_m$ for magnesium-thiamine-PPi, determined on the basis of initial rates was $8.5 \times 10^{-4}$ M, while the $K_m$ determined by measurement of the length of the lag between the addition of magnesium-thiamine-PPi, and the initiation of the reaction was $6.1 \times 10^{-4}$ M. This close agreement supports the conclusion that the lipid phase found when the reaction is initiated with magnesium-thiamine-PPi, is due to the slow formation of the enzyme-magnesium-thiamine-PPi complex.

As usually isolated, the $\alpha$-ketoglutarate dehydrogenase-lipoyl transsuccinylase complex activity is apparently limited by an inadequate level of lipoyl dehydrogenase still complexed with the other two enzymes. When the $\alpha$-ketoglutarate dehydrogenase-lipoyl transsuccinylase was isolated completely free of lipoyl dehydrogenase, it was possible to saturate this complex by the addition of lipoyl dehydrogenase either from cauliflower or from pig heart. When the lipoyl dehydrogenase is present in excess it is possible accurately to determine the kinetic parameters for the substrates and cofactors of the complex.

The oxidation of $\alpha$-ketoglutarate is catalyzed by $\alpha$-ketoglutarate dehydrogenase (2-oxoglutarate : lipoate oxidoreductase (acceptor acylating), EC 1.2.4.2) which has been isolated as a complex with a molecular weight of about $2 \times 10^6$, from Escherichia coli, pig heart muscle, pigeon breast muscle, and bovine kidney mitochondria (1). This enzyme complex has not previously been obtained in an active soluble form from higher plant tissues.

All of the $\alpha$-ketoglutarate dehydrogenase complexes previously isolated have been shown to contain thiamine pyrophosphate, flavin adenine dinucleotide, covalently bound lipoic acid, and a divalent metal ion, all of which are required for the oxidation of $\alpha$-ketoglutarate by dihydrolipoyl dehydrogenase, resulting in the formation of succinyl-CoA as shown in the reaction

\[ \alpha \text{Ketoglutarate} + \text{DPN}^+ + \text{CoA-SH} + \text{Mg}^{2+} \rightarrow \text{succinyl-CoA} + \text{CO}_2 + \text{DPNH} + \text{H}^+ \]

This reaction proceeds successively via decarboxylation of $\alpha$-ketoglutarate, reductive succinylation of a protein-bound lipoic moiety, succinyl transfer, and electron transfer reactions as depicted in the sequence of reactions (2) at top of next page.

The complexes which catalyze this sequence of reactions in bacteria and mammals have been resolved into three separate enzyme fractions which can be reconstituted to give active complexes (1). The three enzymes which catalyze the reactions above are $\alpha$-ketoglutarate dehydrogenase (oxoglutarate dehydrogenase, EC 1.2.4.2) which catalyzes Reactions 1 and 2, lipoyl transsuccinylase (succinyl CoA : dihydrolipoyl S-succinyl transferase) which catalyzes Reaction 3, and lipoyl dehydrogenase (lipoamide dehydrogenase, EC 1.6.4.3) which catalyzes Reactions 4 and 5 (2).

The available evidence for the participation of cofactors in the bacterial and mammalian enzymes is shown in the reaction sequence above. The metal ion is presumed necessary for the binding of thiamine-PPi to the $\alpha$-ketoglutarate dehydrogenase, although clear evidence is lacking because of the presence of bound metal ions which are not removed by dialysis in the presence of 0.01 M EDTA (3).

The lipoic moiety is covalently bound to the lipoyl transsuccinylase portion of the complex where it is linked to the epsilon amino group of a lysine residue by an amide bond (4).

Highly purified preparations of KG-dehydrogenase complex from E. coli are completely dependent on added thiamine-PPi.

The abbreviation used is: KG-dehydrogenase, $\alpha$-ketoglutarate dehydrogenase.

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Preparation of Mitochondria—Preparations of the α-ketoglutarate dehydrogenase-lipoyl transsuccinylase complex and plant lipoyl dehydrogenase started with mitochondria isolated from the floral heads of cauliflower (Brassica oleracea var. botrytis) obtained from a local market. All leaves were removed, and the heads were grated with a conical stainless steel grater driven by a high torque motor, arranged so that cell disruption occurred under the surface of the isolation medium. All steps of mitochondrial and enzyme preparation were carried out between 0 and 4°C. A crude enzyme preparation were carried out between 0 and 4°C. A crude suspension of mitochondria was obtained by grating 1500 g of cauliflower tissue into 1500 ml of 0.6 M sucrose, 0.001 M MgCl₂, 0.005 M EDTA, and 0.05 M Tris at pH 7.0. The suspension was strained through a tightly woven nylon bag with the aid of a Pexton press and centrifuged at 10,000 X g for 30 min. This resuspended material was then centrifuged at 10,000 X g for 30 min. The sedimented material from this centrifugation was resuspended in 40 ml of 1 X 10⁻⁴ M EDTA, 0.01 M MgCl₂ and 0.05 M Tris, pH 7.0. A second preparation of the same size was routinely carried out in parallel with the first and the combination of the two preparations produced 80 ml of mitochondrial suspension containing about 8.5 mg of protein per ml. Further preparation of the enzyme from this mitochondrial suspension is described under “Results.”

Assay Procedures—Except as otherwise noted, the activity of the KG dehydrogenase lipoyl transsuccinylase complex was assayed by adding 0.005 to 0.01 unit of the complex to a reaction mixture already containing 10 μmoles of Tris-Cl (pH 6.9), 10 μmoles of MgCl₂, 5 μmoles of Tris DPN, 10 μmoles of dithiothreitol, 0.225 μ mole of thiamine-PPi, and 50 units of pig heart LipDH. This mixture was incubated for 90 sec in the cell compartment of the spectrophotometer (controlled at 25°C), after which the reaction was started by adding 5 μmoles of Tris-α-ketoglutarate and 0.1 μmole of CoA-SH in 25 μl of 0.01 M dithiothreitol. The final volume was 1.0 ml and the final pH was 6.9. The appearance of DPNH was followed at 340 nm. Since the reaction of this enzyme produces 1 mole of DPNH for each mole of α-ketoglutarate oxidized, 1 unit of KG-dehydrogenase-lipoxytranssuccinylase complex is defined as that amount of enzyme which catalyzes the oxidation of 1 μmole of α-ketoglutarate per min.

The activity of lipoyl dehydrogenase was determined in an assay mixture containing 50 μmoles of Tris-Cl, 2 μmoles of lipoamide, and 0.1 μmole of DPNH in a final volume of 1.0 ml at pH 7.0. The reaction was started with enzyme. The decrease in DPNH absorbance was followed at 340 nm and the rate is expressed as micromoles of DPNH consumed per min. One unit of lipoyl dehydrogenase is defined as that amount of enzyme which catalyzes the oxidation of 1 μmole of DPNH per min.

Protein concentrations were determined by the method (5) of Lowry et al. with crystalline bovine serum albumin as a standard or by calculation from the absorbance of the solution at 280 nm and 260 nm with the method of Warburg and Christian (5). Specific activity of enzymes is expressed as units per mg of protein.

Thiamine pyrophosphate was determined by conversion to thiochrome pyrophosphate by treatment with alkaline ferricyanide. Determinations were made in a volume of 5.0 ml containing 1.0 ml of 1 N NaOH and 0.1 ml of 0.01 M potassium ferricyanide. Fluorescence of the thiochrome was read in a Turner model 111 fluorometer with a primary filter with a peak transmission at 436 nm and secondary filters with a peak transmission at 436 nm. The fluorescence of known solutions of thiamine-PPi was found to be linear with concentration.

The dissociation constant (Kd) of magnesium-thiamine-PPi was determined at 25°C at pH 7.0 in a solution containing all of the components of the standard assay mixture except the KG-dehydrogenase complex, lipoyl dehydrogenase, and CoA. The free Mg²⁺ was determined as the magnesium complex of 8-hydroxyquinoline at 470 nm as a function of MgCl₂ concentration in the presence and absence of thiamine-PPi, with a cell with a 4-cm light path and a low (0.054 mm) concentration of 8-hydroxyquinoline. The Kd was estimated by the method of
O'Sullivan and Perrin (6) with the apparent dissociation constant determined at 0.75, 1.50, and 3.0 mM thiamine-PP1 and a plot of these values against thiamine-PP1 concentration extrapolated to zero thiamine-PP1 to give a $K_d$ of $4.07 \times 10^{-4}$ M for the magnesium-thiamine-PP1 complex.

Values of maximal velocity and $K_m$ were determined from plots according to the method of Lineweaver and Burk fitted by the procedure of Wilkinson (7) to provide appropriate weights. Plotted lines are those described by the equations generated by that fitting process.

Materials

Commercial Enzymes—Analytical reagent grade lipoamide dehydrogenase (NADH$_2$:lipoamide oxidoreductase, EC 1.6.4.3) from pig heart with a specific activity of 210 i.u. per mg was obtained from Boehringer Mannheim and dialyzed at 4°C against three changes of 0.005 M Tris (pH 7.0) containing 1 X $10^{-5}$ M EDTA. When assayed with lipoamide, the resulting pig heart lipoj dehydrogenase solution contained 1000 units of activity per ml at 25°C.

Highly purified bovine pancreatic ribonuclease A (type XA, solution in 0.2 M phosphate buffer, pH 6.4) and crystalline bovine pancreatic deoxyribonuclease I (type DN-C) were obtained from Sigma.

Reagents—Coenzyme A, α-ketoglutaric acid, and reduced diphosphopyridine nucleotide (disodium salt) were obtained from Boehringer Mannheim. Thiamine pyrophosphate chloro, oxidized diphosphopyridine nucleotide (free acid), and oxidized Nt-6,8-thiolydic acid amide (lipoamide) were obtained from Sigma. Dithiothreitol and crystalline bovine serum albumin were obtained from Calbiochem. The α-ketoglutaric acid, thiamine pyrophosphate, and oxidized pyridine nucleotide solutions were made to pH 7.0 with Tris. Enzyme grade Tris and enzyme grade (NH$_4$)$_2$SO$_4$ were supplied by Mann. All other chemicals used were reagent grade or better. All solutions were made in double distilled water, deionized before final glass distillation.

RESULTS

Early attempts to solubilize the α-ketoglutarate dehydrogenase from cauliflower were hampered by a high level of DPNH oxidase in mitochondria and preparations made from mitochondria. It was found that this activity was sensitive to repeated freezing and thawing cycles, and the use of this technique to eliminate DPNH oxidase made a reliable assay for α-ketoglutarate dehydrogenase possible.

In preliminary efforts at purification of the α-ketoglutarate dehydrogenase complex it was found that the lipoj dehydrogenase of the plant complex separates into two fractions during ammonium sulfate fractionation, one separating with the KG-dehydrogenase-lipoyl transuccinylase activity, and the remainder at a much higher ammonium sulfate saturation (see Fig. 1). Attempts to purify the entire α-ketoglutarate dehydrogenase complex by a procedure involving ultracentrifugation, pH precipitation, protamine precipitation according to Hirashima, Hayakawa, and Koike (8), gel filtration on Sephadex G-200 and Sepharose 2B, and solvent fractionation with ethyl alcohol resulted in partial or complete separation of the lipoyl dehydrogenase activity from the remainder of the complex. Procedures were therefore developed to purify the KG-dehydrogenase-lipoyl transuccinylase portion of the complex only.

![Fig. 1. Separation of cauliflower α-ketoglutarate dehydrogenase complex. The effect of ammonium sulfate fractionation on the separation of lipoyl dehydrogenase from the α-ketoglutarate dehydrogenase complex in an extract of 3 kg of cauliflower florets is shown. The complex was solubilized by sonic oscillation and freeze-thaw treatment of mitochondria from cauliflower heads (see text). ▲, lipoyl dehydrogenase activity; □, α-ketoglutarate dehydrogenase-lipoyl transuccinylase activity.](http://www.jbc.org/)

![Fig. 2. Saturation of α-ketoglutarate dehydrogenase-lipoyl transuccinylase by lipoyl dehydrogenase (LipDH). Rate of α-ketoglutarate dehydrogenase reaction (reported as micromoles of α-ketoglutarate oxidized per min) as a function of the addition of lipoyl dehydrogenase from cauliflower or pig heart to α-ketoglutarate dehydrogenase complex in an extract of 3 kg of cauliflower mitochondria. The assay mixture contained 1 mM DPN, 10 mM phosphate, 0.2 mM thiamine-PP1, 10 mM MgCl$_2$, 10 mg of bovine serum albumin, 2.5 mM α-ketoglutarate, and 0.1 mM CoA-SH in a total volume of 1 ml. The pH was adjusted to 6.9 with KOH. Each assay contained 2 µl of the enzyme from Step II of the purification procedure which included 0.001 unit of lipoyl dehydrogenase activity. The units of lipoyl dehydrogenase shown include both the additions of purified lipoj dehydrogenase and that added with the enzyme preparation. ▲, cauliflower lipoyl dehydrogenase; ●, pig heart lipoyl dehydrogenase.](http://www.jbc.org/)
TABLE I

Purification of cauliflower α-ketoglutarate dehydrogenase-lipoyl transsuccinylase complex

Procedures are as described in the text. Assays were performed as indicated under ‘‘Experimental Procedure.’’ The starting material for this preparation was 4.5 kg of grated cauliflower florets.

| Step | Volume | Total KG-dehydrogenase-lipoyl transsuccinylase | Protein | Specific activity | Purification Step | Recovery Overall |
|------|--------|-----------------------------------------------|---------|------------------|------------------|------------------|
|      | ml     | units                                        | mg      |                  |                  |                  |
| Step I: Sonically oscillated mitochondrial extract | 109.0 | 32.2 | 785.4 | 0.041 | 1 | 1 | (100) | (100) |
| Freeze-thaw cycle extract | 120.0 | 40.9 | 676.3 | 0.060 | 1.5 | 1.5 | 127 | 127 |
| Step II: 30-45% saturated ammonium sulfate precipitate | 9.0 | 45.3 | 261.5 | 0.173 | 2.8 | 4.2 | 111 | 141 |
| Freeze-thaw cycle of 30-45% saturated ammonium sulfate precipitate | 7.1 | 32.3 | 92.7 | 0.348 | 2.0 | 8.5 | 71 | 100 |
| Step III: Eluate from Sephadex G-200 after nuclease treatment | 13.6 | 21.9 | 18.7 | 1.17 | 3.4 | 28.5 | 68 | 68 |
| Step IV: 55-60% saturated ammonium sulfate precipitate | 1.0 | 13.8 | 4.5 | 3.07 | 2.6 | 73.9 | 63 | 43 |

In preparations such as the 30 to 40% saturation ammonium sulfate precipitate in Fig. 1, in which some lipoyl dehydrogenase activity separated with the KG-dehydrogenase-lipoyl transsuccinylase complex, the activity of the preparation as a function of enzyme concentration was highly nonlinear. This nonlinearity could be abolished by the addition of either cauliflower or pig heart lipoyl dehydrogenase, perhaps indicating a preferential binding of the plant enzyme, but the amount of lipoyl dehydrogenase required was considerably in excess of the levels of that activity which was assayed with increasing amounts of lipoyl dehydrogenase from either cauliflower or pig heart.

The amount of lipoyl dehydrogenase required to produce a maximum α-ketoglutarate dehydrogenase activity was much greater with the pig heart than with the cauliflower lipoyl dehydrogenase, perhaps indicating a preferential binding of the plant enzyme, but the amount of lipoyl dehydrogenase required was considerably in excess of the levels of that activity which could be isolated in combination with the KG-dehydrogenase-lipoyl transsuccinylase complex by any technique tried. Because of difficulties in the purification of the cauliflower lipoyl dehydrogenase, and the inhibition of the over-all reaction produced by impurities in this preparation, pig heart lipoyl dehydrogenase was routinely used for assay of the KG-dehydrogenase-lipoyl transsuccinylase complex in the work reported here.

Purification of α-Ketoglutarate Dehydrogenase-Lipoyl Transsuccinylase Complex

The purification scheme which produced enzyme with maximum specific activity and which was used for preparation of the KG-dehydrogenase-lipoyl transsuccinylase complex whose characteristics are reported here is as follows.

Step I: Extraction of Mitochondria—A mitochondrial suspension prepared as described under ‘‘Methods’’ was made 0.01 M in dithiothreitol and 40-ml aliquots were sonically disrupted in a 250-ml Rosett cooling cell immersed in an ice bath. The Branson model S-75 sonifier with a 0.5-inch step type horn was operated at 5 to 6 amp. The sonic oscillation continued for 2.5 min with 30 sec on and 30 sec off for cooling. The suspension was centrifuged at 20,000 × g for 30 min at −4°. The supernatant fractions were frozen at −15° for at least 4 hours and then thawed slowly at room temperature. The freezing-thawing cycle was repeated twice over a period of at least 24 hours. After the third thawing, precipitated protein was removed by centrifugation at 20,000 × g for 60 min.

Step II: Ammonium Sulfate Fractionation—To the suspension from Step I a solution of saturated ammonium sulfate in 0.001 M glycylglycine buffer, pH 7.0, was added dropwise with stirring. The concentration was first brought to 30% saturation (calculated as per cent of the saturated ammonium sulfate in the total volume), stirred for 1 hour, and centrifuged at 20,000 × g for 30 min. The supernatant liquid was brought to 45% saturation by addition of saturated ammonium sulfate, permitted to stir for 1 hour, and centrifuged as before. The precipitate was resuspended in a minimal volume (approximately 10 ml) of 0.05 M Tris (pH 7.0) containing 1 × 10−4 M EDTA. It was then frozen overnight at −15° and, after thawing, was centrifuged at 20,000 × g for 30 min to remove the precipitated protein.

Step III: Nuclease Treatment—Ribonuclease and deoxyribonuclease were added to the supernatant solution from Step II to give a final concentration of 25 μg of each nuclease per ml. The mixture was incubated at 25° for 60 min, after which it was placed on a Sephadex G-200 column (2.5 × 40 cm) and eluted with 0.05 M Tris buffer, pH 7.0, containing 1 × 10−4 M EDTA. Four of the 3.3-ml fractions containing the α-ketoglutarate dehydrogenase-lipoyl transsuccinylase activity were pooled and centrifuged for 20 min at 20,000 × g to remove a white colloidal material which forms in the column and elutes with the activity. Passage through the column separates the KG-dehydrogenase transsuccinylase complex from the nucleases, the nucleotides which they have produced from nucleic acid contaminants, and some other small proteins.
Fig. 3. Substrate requirements of \( \alpha \)-ketoglutarate dehydrogenase-lipooyl transsuccinylase complex. The reaction, which otherwise used standard conditions described in the text, was started by the addition of CoA-SH at Point A after a 90-set preliminary incubation with the other components of the assay. An equal aliquot of CoA-SH was added at Point B, resulting in the change in rate shown by the dashed line. The same experiment was repeated with the reaction started with DPN, \( \alpha \)-ketoglutarate, or lipooyl dehydrogenase. In each of these cases the rate was identical with that started with CoA-SH, except that the second addition at Point B had no effect on the rate (solid line).

Step IV: Second Ammonium Sulfate Fractionation—The supernatant solution from Step III was made to 33% ammonium sulfate saturation with saturated ammonium sulfate solution as before. After stirring for 1 hour, the suspension was centrifuged at 20,000 \( \times g \) for 30 min and the precipitate was discarded. The saturation with ammonium sulfate was then brought to 40% and the suspension was stirred for 1 hour and allowed to stand overnight. The precipitate was collected by centrifugation and resuspended in a minimal volume (1 to 5 ml) of 0.05 M Tris with 1 \( \times 10^{-4} \) M EDTA, pH 7.0.

A typical preparation by the steps outlined above is summarized in Table I. The enzyme prepared by this method was free of lipoyl dehydrogenase and DPNH oxidase activity, but contained 0.29 unit of pyruvate dehydrogenase per unit of \( \alpha \)-ketoglutarate dehydrogenase activity at the time of isolation. Attempts at further purification with sucrose density gradient techniques did achieve some separation of the \( \alpha \)-ketoglutarate dehydrogenase and pyruvate dehydrogenase activities with a further reduction of the pyruvate dehydrogenase contaminant, but the separation was not complete. The pyruvate dehydrogenase activity disappears during storage, and is not detectable after about 3 weeks of storage.

The KG-dehydrogenase-lipooyl transsuccinylase complex prepared in the manner described above is quite stable and can be stored at \(-15^\circ\)C for several months without significant loss of activity. Preparations stored for more than 1 year retained 80% of the original activity. The enzyme appears to be unaffected by freezing and thawing and loses no activity when stored at room temperature for several hours. When the preparation is assayed without the addition of dithiothreitol, there is an apparent loss of activity with time, but this is completely reversed by the addition of dithiothreitol to the assay medium. When assayed in the presence of saturating levels of pig heart lipooyl dehydrogenase, the activity is linear with respect to concentration of the KG-dehydrogenase-lipooyl transsuccinylase complex.

| Metal ion added | EDTA concentration | Rate \( \mu \)mols/min |
|-----------------|-------------------|---------------------|
| \( 0.01 \) Mg\( \text{II} \) | 0 | 0.0285 |
| 0 | 0 | 0.0251 |
| 0 | 3.3 | 0.0011 |
| 0.01 Mg\( \text{II} \) | 3.3 | 0.0089 |
| 0.01 Ca\( \text{II} \) | 3.3 | 0.0204 |

General Properties of \( \alpha \)-Ketoglutarate Dehydrogenase-Lipooyl Transsuccinylase Complex

The purified complex of \( \alpha \)-ketoglutarate dehydrogenase and lipooyl transsuccinylase isolated from cauliflower mitochondria is in many respects similar to the enzymes previously isolated from bacterial and mammalian sources. It is totally dependent on \( \alpha \)-ketoglutarate, CoA-SH, thiamine-PP\( \text{I} \), DPN, and lipooyl dehydrogenase. There were no detectable rates in the absence of any of these factors and starting the reaction with any of them resulted in identical rates. The course of a typical assay is shown in Fig. 3. The addition of either DPN, \( \alpha \)-ketoglutarate, CoA-SH, or lipooyl dehydrogenase at Point A after 90-set preliminary incubation of the enzyme in the presence of the other reaction components resulted in the initiation of the reaction, with the establishment of a rate which was linear for about 1 min. The rate of DPNH production then decreased to a slower second rate which was constant for 10 min or longer. The addition of a second aliquot of \( \alpha \)-ketoglutarate, DPN, or lipooyl dehydrogenase at Point B had no effect on the rate then established. However, a second addition of an equal amount of CoA-SH resulted in an increased rate as indicated by the dashed line of Fig. 3. The initial rate resulting from the second CoA-SH addition was always slower than that found when the reaction was first initiated and the total DPNH produced before the establishment of the second rate was less than after the first addition of CoA-SH. These observations suggest product inhibition by succinyl-CoA, and this compound has been found to inhibit the initial rate of the reaction.

The addition of either Mg\( \text{II} \) or Ca\( \text{II} \) was found to stimulate the activity of the cauliflower KG-dehydrogenase-lipooyl transsuccinylase complex in our assays, as has been reported for both bacterial and mammalian enzymes (3). Determination of calcium and magnesium in our preparations by atomic absorption revealed that the assay mixtures contained approximately 1 \( \times 10^{-5} \) M each of magnesium and calcium before the addition of any metal ions. Most of the ions present in the assay mixture were contributed by the Tris salt and dithiothreitol. In view of this significant contaminant of metal ions it was difficult to be certain that there was an absolute requirement for Mg\( \text{II} \) or Ca\( \text{II} \). This requirement was shown by the addition of 3.3 \( \times 10^{-3} \) M EDTA to the reaction mixture with the results shown in Table II. It may be seen that EDTA essentially abolishes the ac-
FIG. 4. Effect of pH on the activity of α-ketoglutarate dehydrogenase-lipooyl transsuccinylase complex. The standard assay procedure was used except for variations in buffer composition and pH described in the text. ●, mixed buffer (0.01 M each of Tris and potassium phosphate); ★, potassium phosphate buffer; ○, Tris buffer; □, mixed buffer with twice as much lipooyl dehydrogenase.

FIG. 5. Interaction of magnesium and thiamine pyrophosphate with α-ketoglutarate dehydrogenase-lipooyl transsuccinylase complex. Lines shown are tracings of recorder display of reactions carried out according to the standard assay procedure except that thiamine-PPi concentration was 2 × 10⁻⁵ M. In A the reaction was initiated by the addition of enzyme at the arrow. Identical reaction rates were observed when the reaction was initiated with Mg²⁺ or thiamine-PPi. The method of estimating τ (lag phase) is indicated. In B and C the reaction was initiated at the arrow by the addition of KG-dehydrogenase-lipooyl transsuccinylase complex which had been incubated for 24 hours with 0.01 M MgCl₂ and 0.1 mM thiamine-PPi. Curve B received Mg²⁺ and thiamine-PPi supplements in addition to that supplied with the enzyme; Curve C received only the Mg²⁺ and thiamine-PPi included in a 5-μl aliquot of the previously incubated enzyme.

Bound lipoylate was not determined, but the specificity of pig heart lipooyl dehydrogenase for lipoylate is strong evidence for the presence of bound lipoylate in the cauliflower complex.

The α-ketoglutarate dehydrogenase-lipooyl transsuccinylase complex was completely precipitated by centrifugation at 144,000 × g for 2.5 hours. This compares with the 2 hours required to sediment the entire α-ketoglutarate dehydrogenase complex isolated from pig heart by Sanadi (9) which has a molecular weight of 2 × 10⁷. In addition, the cauliflower KG-dehydrogenase-lipooyl transsuccinylase complex was eluted in the void volume of Sephadex G-200, which excludes all proteins with a molecular weight over 800,000 (10). Both of these observations suggest a high molecular weight, although no direct molecular weight determinations have been made.

The effect of pH on α-ketoglutarate dehydrogenase-lipooyl transsuccinylase activity was determined in a mixed buffer system containing both 0.01 M Tris-Cl and 0.01 M potassium phosphate. Determinations at the pH extremes were repeated with additional lipooyl dehydrogenase to ensure that the rate was not limited by an effect of pH on the lipooyl dehydrogenase. The mixed buffer gave the same rate as either buffer alone at the same pH. The effect of pH on the reaction rate is shown in Fig. 4, where the pH indicated for each point is that measured after completion of the assay. The pH optimum in 6.9, which is somewhat lower than that reported for the bacterial enzyme.

### Table III

Comparison of α-ketoglutarate dehydrogenase complexes

| pH optimum | Cauliflower | E. coli | Pig heart |
|------------|-------------|---------|-----------|
| 6.9        | 7.7 (8)     | 7.0 (8) | Urea on calcium phosphate gel (12) |
| Method of resolving lipooyl dehydrogenase | (NH₄)₂SO₄ | (NH₄)₂SO₄ (II) | |
| Bound thiamine-PPi | No | No (11) | Yes (12) |
| Stimulation by Mg²⁺ and Ca²⁺ | Yes | Yes (3) | Yes (3) |
| Bound Mg²⁺ and Ca²⁺ | No | Yes (3) | Yes (3) |
| Specific activity | 3.0 | 15 (2) | 3.5 (12) |
| Inhibition by DPNH | Yes | Yes (13) | |
| Kₘ for α-ketoglutarate | 1.2 × 10⁻⁴ M | 1.3 × 10⁻⁵ M (14) | (<10⁻⁷ M (14) |
| Kₘ for CoA | 6.1 × 10⁻¹ M | | |

activity in the absence of added metal ions, and that the addition of either Mg²⁺ or Ca²⁺ completely restores the activity.

The apparent complete dependence of the KG-dehydrogenase-lipooyl transsuccinylase complex on added thiamine-PPi for activity was an indication that the cauliflower complex as isolated differed from previous complexes (1) in not containing thiamine-PPi bound to the enzyme. Fluorometric analysis of this purified enzyme confirmed this by showing no detectable thiamine-PPi (<1 × 10⁻⁴ μmoles per mg of protein) bound to the KG-dehydrogenase-lipooyl transsuccinylase complex. The levels of magnesium and calcium in the enzyme suspension were not significantly different from those in the suspending medium and it is presumed that metal ions are not tightly bound to the complex. The absolute requirement of DPN, CoA-SH, and α-ketoglutarate suggests that none of these is present in a bound form, although no attempts have been made to confirm this.
Interaction of Metal Ions, Thiamine-PPi, and Enzyme Complex

Preliminary studies with nonsaturating thiamine-PPi concentrations showed a distinct lag phase when the reaction was initiated by thiamine-PPi, Mg"+, or enzyme which was not found when the enzyme was previously incubated with both thiamine-PPi and Mg"+. This lag phase is illustrated in Curve A of Fig. 5. If the enzyme is first incubated for 90 sec with thiamine-PPi and Mg"+, the reaction starts immediately on the addition of the previously incubated enzyme (Line B of Fig. 5). If the previously incubated enzyme is added to an assay mixture which contains no thiamine-PPi and Mg"+ other than that added with the enzyme, the reaction starts at the same time, but slows more rapidly than when thiamine-PPi and metal are provided (Line C of Fig. 5). This observation suggests the formation of a relatively stable enzyme-metal-thiamine-PPi complex.

The stability of this complex was studied by incubating the KG-dehydrogenase-lipoyl transsuccinylase complex for 24 hours at 0° in 0.05 M Tris (pH 7.0) containing 0.01 M Mg"+ and 1 x 10^{-4} M thiamine-PPi. Aliquots of 5 μl of this previously incubated mixture were then added to 1-ml assay mixtures lacking thiamine-PPi and incubated at 25° for periods up to 10 min. After various intervals of incubation the reaction was started by adding a mixture of CoA-SH and α-ketoglutarate as for the standard assay. A similar series of assays were previously incubated at 25° in the absence of both Mg"+ and thiamine-PPi. If a relatively stable complex were formed during the preliminary incubation at 0°, the decrease in rate found when it was subsequently diluted and incubated without additional Mg"+ or thiamine-PPi would reflect dissociation of that complex. As is shown in Fig. 6, when the natural logarithm of the initial rate of the reaction is plotted against the incubation time at 25°, the indicated pseudo-first order rate constant for decay of the complex (obtained from the slope of lines fitted by the method of least squares) is 0.0094 sec^{-1} in the absence of Mg"+ and 0.0025 sec^{-1} when Mg"+ is present during the 25° incubation. This result indicates that there is an enzyme-thiamine PPi magnesium complex formed which slowly dissociates, with a half-time of approximately 100 sec, when only very low concentrations of Mg"+ and thiamine-PPi are present. The presence of Mg"+ during the dissociation significantly retards the rate of breakdown, indicating the equilibrium nature of the reaction.

The delay in the initiation of the oxidation of α-ketoglutarate by the cauliflower complex which is found if the enzyme is not previously incubated with thiamine-PPi and Mg"+ offers an opportunity to evaluate the second order rate constant for the formation of the metal-thiamine-PPi-enzyme complex. Dixon and Webb (15) have derived equations for the relationship between the second order rate constant for the formation of an enzyme complex and the time elapsed before the establishment of the steady state for its reaction. They have defined τ, the length of the lag phase, as the time from initiation of the reaction to the point at which extrapolation of the linear steady state rate intersects the extrapolated zero product concentration line (see Fig. 5). In reciprocal form the equation of Dixon and Webb is

\[
\frac{1}{\tau} = k_{+1}S_0 + k_{+2}K_m
\]

where \(S_0\) is the initial concentration of the compound which binds to the enzyme. In this case, because of evidence presented below, this is considered to be magnesium-thiamine-PPi, the formation of which from Mg"+ and thiamine-PPi is assumed to be much more rapid than its joining in complex with the enzyme. In the data presented in Fig. 7, the length of the lag phase, \(\tau\), was measured by starting the standard assay with thiamine-PPi after a 90-sec preliminary incubation of the enzyme in the otherwise complete reaction mixture. A similar series of assays was run with 0.01 M CaCl2 substituted for 0.01 M MgCl2. As predicted by the equation above, the results with either ion give straight lines when \(1/\tau\) is plotted against thiamine-PP1 concentration. On the basis of the dissociation constant determined for magnesium-thiamine-PP1 (see below), the

![Graph](http://www.jbc.org/Downloaded_fromhttp://www.jbc.org/Issue_of_November_10_1970_L._L._Poulsen_and_R._T._Wedding)
thiamine-PP\(_1\) is presumed to be essentially completely in the form in complex under the conditions of these assays, and the same is probably true for the calcium thiamine-PP\(_1\) as well. With this assumption, the second order rate constants for the formation of enzyme-magnesium-thiamine-PP\(_1\) and enzyme-calcium-thiamine-PP\(_1\) complexes were found to be 687 m\(^{-1}\) sec\(^{-1}\) and 4469 m\(^{-1}\) sec\(^{-2}\), respectively, as indicated by the slopes of the fitted lines of Fig. 7. According to the equation, the slope of these lines is equal to \(k_{+1}\), the rate constant for the formation of enzyme-metal-thiamine-PP\(_1\) complex. These data also provide an opportunity for an independent evaluation of the \(K_m\) for the magnesium-thiamine-PP\(_1\) substrate of the reaction, which according to Dixon and Webb’s equation is equal to the intercept divided by the slope of the \(1/v\) against magnesium-thiamine-PP\(_1\) lines. The values obtained in this way for the \(K_m\) values were 6.1 \(\times\) 10\(^{-6}\) m for magnesium-thiamine-PP\(_1\) and 4.0 \(\times\) 10\(^{-5}\) m for calcium-thiamine-PP\(_1\). When \(K_m\) values are calculated from the linear rates measured in the same experiments by fitting weighted lines to the reciprocal of the rate against the reciprocal of the magnesium-thiamine-PP\(_1\) concentration in a conventional double reciprocal plot, the values were found to be 8.6 \(\times\) 10\(^{-6}\) m for magnesium-thiamine-PP\(_1\) and 1.0 \(\times\) 10\(^{-4}\) m for calcium-thiamine-PP\(_1\). When the \(K_m\) values derived in the two different ways are compared, this agreement lends support to the conclusion that the lag phase is the result of the slow formation of a complex of metal ion and thiamine-PP\(_1\) with the enzyme.

Because the cauliflower KG-dehydrogenase-lipoyl transsuccinylase complex is isolated without bound thiamine-PP\(_1\) or magnesium, it has been possible to investigate the question of whether Mg\(^{2+}\) and thiamine-PP\(_1\) bind independently to the enzyme either randomly or in an ordered sequence or whether a magnesium-thiamine-PP\(_1\) complex is first formed which then binds to the enzyme. For this purpose the apparent dissociation constant of magnesium-thiamine-PP\(_1\) was determined under the conditions used for the enzyme assay. This constant, \(K_d\) = 4.07 \(\times\) 10\(^{-4}\) m, is of the same order of magnitude as has been determined for magnesium-ADP (16).

This \(K_d\) has been used to calculate the apparent magnesium-thiamine-PP\(_1\) concentration at different levels of Mg\(^{2+}\) and thiamine-PP\(_1\). The \(\alpha\)-ketoglutarate oxidation rates found in the presence of Mg\(^{2+}\) concentrations from 10\(^{-4}\) to 10\(^{-3}\) M in combination with thiamine-PP\(_1\) concentrations ranging from 3.0 \(\times\) 10\(^{-6}\) m to 3.75 \(\times\) 10\(^{-5}\) m were plotted against the calculated magnesium-thiamine-PP\(_1\) concentration for each combination. In these experiments the standard assay procedure, including a 90-sec preliminary incubation of enzyme with magnesium and thiamine-PP\(_1\), was used, and the reactions started by adding \(\alpha\)-ketoglutarate and CoA-SH. Within the range indicated, the rates all fall essentially on the same line when \(1/v\) is plotted against \(1/[\text{magnesium-thiamine-PP}_1]\) concentration as shown in Fig. 8. This strongly suggests that magnesium-thiamine-PP\(_1\) is in fact the form which binds to the enzyme during the preliminary incubation, and the absence of significant inhibition when either free Mg\(^{2+}\) or free thiamine-PP\(_1\) is high relative to the concentration of magnesium-thiamine-PP\(_1\) indicates that the free forms do not bind significantly to the enzyme. In this experiment the apparent \(K_m\) for magnesium-thiamine-PP\(_1\) was found to be 1.1 \(\times\) 10\(^{-3}\) M.

**DISCUSSION**

The \(\alpha\)-ketoglutarate dehydrogenase complex of cauliflower mitochondria has proven to be impossible to isolate in its entirety by the methods attempted. Any complete complex which was purified contained levels of lipoyl dehydrogenase inadequate to saturate fully the potential activity of the preceding enzymes in the complex. This may be true of the complex isolated from other organisms, and may even represent the situation in vivo. The variability in the amount of lipoyl dehydrogenase which comes through various steps of purification still attached to the remainder of the complex, and the very high levels of lipoyl dehydrogenase activity which are found separately render any conclusions on this point uncertain. We have chosen to purify only the \(\alpha\)-ketoglutarate dehydrogenase-lipoyl transsuccinylase portion of the complex and to assay this under uniformly saturating levels of lipoyl dehydrogenase provided separately. We believe that these conditions provide a more reliable means for studying the activity of the KG-dehydrogenase-lipoyl transsuccinylase complex and accordingly give more reliable estimates of the properties of this complex.

The higher plant complex isolated in this way is obtained with a specific activity which approaches that of the enzyme previously purified from mammalian and bacterial sources. As with those complexes (16), there is some contamination of pyruvate dehydrogenase. However, since these activities can be separated to some degree by sucrose gradient centrifugation of the purified enzyme from Step IV of the procedure, and since the \(\alpha\)-ketoglutarate dehydrogenase activity is stable while the pyruvate dehydrogenase rapidly disappears in storage, there is little question that the pyruvate dehydrogenase activity is due to a different enzyme and is not a minor activity of the \(\alpha\)-ketoglutarate dehydrogenase complex.

The relative ease with which lipoyl dehydrogenase dissociates from the remainder of the KG-dehydrogenase complex and the high concentration of apparently unbound lipoyl dehydrogenase may indicate that the same situation obtains in the intact cell, with lipoyl dehydrogenase in loose association with the various \(\alpha\)-keto acid dehydrogenases, perhaps without specificity as to the preferred complex (12). However, the higher level of
activity obtained from the addition of cauliflower lipoyl dehydrogenase to the cauliflower KG-dehydrogenase-lipoyl transsuccinylase complex, as compared with that provided by the pig heart lipoyl dehydrogenase, indicates that the plant lipoyl dehydrogenase either binds better to the plant complex or for other reasons forms a better coupling system with it. The nature of the binding of the lipoyl transsuccinylase to lipoyl dehydrogenase is presently unclear. It may be that only the reduced lipoate of lipoyl transsuccinylase must bind to the lipoyl dehydrogenase, or it may be, as Reed (17) suggests, that the lipoyl dehydrogenase must itself be bound to the lipoyl transsuccinylase before it can accept the lipoate of that enzyme. One can speculate that either mechanism is possible, and that the reason for the lower activity with the pig heart LipDH is that it does not fit the binding site for lipoyl dehydrogenase on the lipoyl transsuccinylase and must accept the lipoate from an unfavorable configuration.

The absence of bound thiamine-PPi in the cauliflower complex has made it possible to show that the cofactor for this enzyme appears to be magnesium-thiamine-PPi rather than magnesium and thiamine PPi separately. The magnesium-thiamine-PPi dissociates readily, although rather slowly, from the enzyme. This dissociation of a required cofactor may represent one method of controlling the activity of KG-dehydrogenase in plants.

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J. Biol. Chem. 1970, 245:5709-5717.

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