THE COMPOSITION OF THE KETOGLUTARATE DEHYDROGENASE COMPLEX

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SUMMARY

Ketoglutarate dehydrogenase has been isolated from pig heart as a soluble complex of enzymes, containing protein bound thiamin pyrophosphate, lipoic acid and FAD in stoichiometric amounts. The flavin constituent of the complex has been shown to be lipoyl dehydrogenase. The complex was resolved into two fractions, one colourless, the other yellow, by fractionation in the presence of 2.5 *M* urea. The colourless fraction, which contains all of the protein-bound thiamin pyrophosphate and lipoic acid of the complex, was found to catalyse the oxidation of ketoglutarate when ferricyanide was used as electron acceptor. The yellow fraction was shown to be identical with lipoyl dehydrogenase (diaphorase). Neither fraction alone was capable of carrying out DPN^+-linked ketoglutarate oxidation, this activity was reconstituted on mixing the two fractions. From chemical analysis, and the stoichiometry of the reconstitution of DPN^+-linked ketoglutarate oxidation, the unit molecular weight of the ketoglutarate dehydrogenase complex was calculated to be about 260,000.

INTRODUCTION

The enzymic oxidation of α-ketoglutarate, like that of pyruvate, has been shown by work in the laboratories of GUNSALUS[1], REED[2] and SANADI[3] to be a complicated series of reactions involving the acylation of enzyme bound lipoic acid, transfer of the acyl group to CoASH, and reoxidation of the resulting reduced lipoic acid with DPN^+.

$$KG + \text{enz lip } S_2 \xrightarrow{TPP} CO_2 + S\text{—succinyl lipoyl enz.} \quad (I)$$

$$S\text{—succinyl lipoyl enz.} + CoASH \longrightarrow \text{enz lip } (SH)_2 + \text{Succinyl CoA.} \quad (II)$$

$$\text{enz lip } (SH)_2 + DPN^+ \rightleftharpoons \text{enz lip } S_2 + DPNH + H^+ \quad (III)$$

$$\text{overall: } KG + CoASH + DPN^+ \longrightarrow \text{succinyl CoA} + DPNH + CO_2 + H^+ \quad (IV)$$

The work of SANADI, LITTLEFIELD AND BOCK[4] demonstrated that the enzymes responsible for the preceding reactions are associated in a soluble complex in pig heart together with the protein-bound cofactors, lipoic acid and thiamin pyrophos-

The following abbreviations are used: DPN^+ and DPNH, the oxidized and reduced forms of diphosphopyridine nucleotide; FAD and $FADH_2$, oxidized and reduced flavin adenine dinucleotide; lip S_2 and lip $(SH)_2$, oxidized and reduced lipoic acid; CoASH, coenzyme A; TPP, thiamin pyrophosphate; DCPIP, 2,6-dichlorophenol indophenol; KG, α-ketoglutarate; SU, succinate.

phate. No separation of the constituent enzymes could be obtained during purification. In this respect pig heart ketoglutarate dehydrogenase appeared to differ from the pyruvate and ketoglutarate dehydrogenases of *Escherichia coli* which were easily separated into two fractions, fraction A, precipitating at relatively low $(NH_4)_2SO_4$ concentrations, and fraction B, precipitating at higher concentrations of $(NH_4)_2SO_4$[5,6]. While complete resolution was not obtained, fraction A was rich in the enzymes catalysing reactions (I) and (II) above, and fraction B was rich in lipoyl dehydrogenase (reaction (III)). Recent reports[7,8] show that the bacterial enzymes also exist in the form of soluble complexes. Following the demonstration that diaphorase was a powerful lipoyl dehydrogenase[9,10], it was of considerable interest to determine whether the lipoyl dehydrogenase associated with ketoglutarate oxidation was the same as diaphorase, as this would provide direct evidence for the physiological role of the enzyme. Accordingly ketoglutarate dehydrogenase was purified by the method of SANADI *et al.*[4]. The resultant enzyme was amber coloured, and could be purified considerably further by fractionation on a calcium phosphate gel column, to yield a bright yellow and highly fluorescent preparation. This preparation had 2–3 times higher specific activity than that reported by SANADI, and the flavoprotein present was shown to be diaphorase (lipoyl dehydrogenase) by several methods, including resolution of the complex into two fractions, similar to the fractions A and B of the bacterial enzyme systems[6].

MATERIALS AND METHODS

DPN^+, DPNH (92 %) and TPP (85 %) were obtained from the Sigma Chemical Co., CoASH from Pabst Laboratories and the Sigma Chemical Co. DL-lipoic acid was a gift from Dr. I. C. GUNSALUS and DL-lipoamide a gift from Dr. L. J. REED. Commercial α-ketoglutaric acid was recrystallized from ethyl propionate by the method of PRICE[11]. Other chemicals used were of analytical grade.

Lipoic acid analyses: Lipoic acid was assayed in biological materials after 6 *N* HCl hydrolysis by the method of GUNSALUS AND RAZZELL[12]. The lipoic acid-deficient cells of *Streptococcus faecalis*, strain 10 Cl, were freeze dried, and kindly grown by Dr. S. R. ELSDEN from a culture provided by Dr. I. C. GUNSALUS.

Thiamin assays: Thiamin pyrophosphate was assayed spectrofluorimetrically after conversion to thiamin by seminal acid phosphatase by the thiochrome method of BURCH *et al.*[13]. The fluorescence of the thiochrome was excited with light of 370 mμ (isolated by a grating monochromator from a xenon arc). The thiochrome fluorescence was isolated from a trace of flavin fluorescence present in some samples by a combination of filters; Corning UVIOL and Chance O.V. 1. The identity of the fluorescence with that obtained from authentic TPP (or thiamin) subjected to the same method was confirmed by the identity of the fluorescence excitation spectra as defined by WEBER AND TEALE[14].

KG dehydrogenase assays: The overall oxidation of KG shown in reaction (IV) was followed spectrophotometrically at 340 mμ, in a total volume of 1 ml at 25°. Each cell (1-cm light path) contained the following constituents; phosphate pH 7.4, 60 μmoles; CoASH, 0.045 μmole; cysteine HCl, 3 μmoles; DPN^+, 0.3 μmole; KG, 1 μmole. The reaction was begun by the addition of enzyme. Unit activity is defined as that amount of enzyme producing an O.D. change of 1.0/min.

$K_3Fe(CN)_6$-linked KG dehydrogenase assays: The reaction

$$KG + 2\ Fe(CN)_6^{\equiv} \longrightarrow SU + CO_2 + 2\ Fe(CN)_6^{\equiv} + 2\ H^+$$

was studied at 25° at a variety of pH values. Spectrophotometer cells contained the following constituents in a final volume of 3 ml, $K_3Fe(CN)_6$ 2 μmoles, phosphate buffer 150 μmoles, bovine serum albumin 2 mg, ketoglutarate 10 μmoles. Reaction was begun by the addition of enzyme and readings taken every 15 sec in order to determine the initial rate of reaction.

Lipoyl dehydrogenase assays: The oxidations of DPNH employing $K_3Fe(CN)_6$, lipoic acid and lipoamide as acceptors were carried out at 25° as described previously[10].

Preparation of pig heart particles: The particulate preparation from which the ketoglutarate dehydrogenase was extracted, was prepared by the method previously described[10].

Preparation of calcium phosphate gel – cellulose columns: The larger columns used in the purification of the KG dehydrogenase were prepared as described previously[10]. The smaller columns used in the urea resolution were prepared with 60 ml of the same mixture, to give a column of about 15 cm length, 1 cm diameter. The smaller columns were washed with 0.05 *M* phosphate pH 7.6 + 2.5 *M* urea and equilibrated in a cold room before use.

RESULTS

Purification of ketoglutarate dehydrogenase

A pig heart particulate preparation was extracted at pH 7–7.2 by freezing and thawing and fractionated with ammonium acetate according to the method of SANADI *et al.*[4]. The resultant amber-coloured enzyme had a specific activity of 4.35 μmoles KG oxidized/min/mg at 25° under the experimental conditions given in METHODS (variation 3.78 to 5.15 μmoles/min/mg in eight preparations). This activity is very similar to that given by SANADI *et al.*[4]. Considerable purification was then effected by fractionation on a calcium phosphate gel column. The enzyme as obtained above was dialysed for 2 h *vs* 0.1 *M* phosphate pH 7.6 and applied to a calcium phosphate gel column which had previously been washed with 0.1 *M* phosphate pH 7.6. After adsorption of the enzyme, washing with this buffer was continued, resulting in the elution of a reddish brown protein, as well as colourless protein, and leaving a bright yellow band at the top of the column. This band was then eluted with 0.1 *M* phosphate pH 7.6 + 4% (w/v) $(NH_4)_2SO_4$, and fractionated with $(NH_4)_2SO_4$ into 2 fractions; 0–0.36 saturation, and 0.36–0.70 saturation. The 0–0.36 fraction contains the KG dehydrogenase complex, the 0.36–0.70 fraction contains uncomplexed lipoyl dehydrogenase. (The complex precipitates sharply between 0.29–0.36 saturation; as no precipitate is obtained below 0.29 saturation this broader fraction is collected.) Table I summarises the results of a typical preparation.

Properties of the ketoglutarate dehydrogenase complex

The ketoglutarate dehydrogenase obtained is bright yellow and highly fluorescent, and has a specific activity of 14.9 μmoles KG oxidized/min/mg under the standard assay conditions given under METHODS. That the yellow colour and fluorescence are due to lipoyl dehydrogenase in a complex of enzymes is shown in several

TABLE I

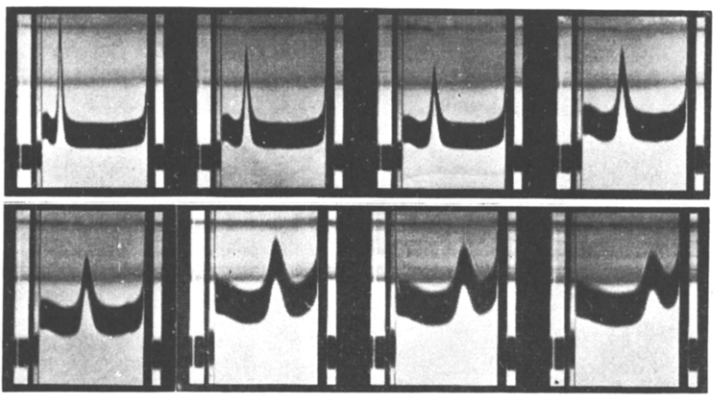
SUMMARY OF PURIFICATION PROCEDURE FOR THE ISOLATION OF KETOGLUTARATE DEHYDROGENASE

Procedure	*Volume (ml)*	*Protein mg/ml*	*Activity units/ml*	*Specific activity units/mg*	*Total units*
pH 7.2 extract of particle preparation from 1500 g washed pig heart mince	255	13.8	55	3.98	14,000
Precipitate obtained by fractionation with ammonium acetate between 37–77 g %	15.2	30	885	29.5	13,400
Fractionated on a column of calcium phosphate-cellulose (see text) Collected yellow fraction	40	5.3	282	53	11,300
Fractionated with $(NH_4)_2SO_4$ Fraction precipitating between 0–0.36 saturation	5	23.2	2160	93	10,800

ways, which will be detailed in this and the following sections. Evidence for association of the lipoyl dehydrogenase with other proteins comes first of all from a comparison of the salting out characteristics of ketoglutarate dehydrogenase and lipoyl dehydrogenase. Ketoglutarate dehydrogenase is precipitated out of solution between 0.29 and 0.36 saturation with $(NH_4)_2SO_4$; uncomplexed lipoyl dehydrogenase is precipitated between 0.55 and 0.70 saturation[10]. The yellow colour of the ketoglutarate dehydrogenase is not due to contamination with uncomplexed lipoyl dehydrogenase, since repeated fractionation with $(NH_4)_2SO_4$ between 0.29 and 0.36 saturation fails to remove the yellow colour. Secondly in agreement with the results of SANADI *et al.*[4] ketoglutarate dehydrogenase has been found to have a high sedimentation constant. (In several determinations with a Spinco Analytical Ultracentrifuge a value of $S_{20,w}$ (extrapolated to zero protein concentration) of 30 S was found.) In contrast lipoyl dehydrogenase has a low sedimentation constant, 5.4 S[10,15]. That the yellow colour of the ketoglutarate dehydrogenase is in the form of a complex is further supported by the observation that on ultracentrifugation the yellow colour is clearly associated with this fast sedimenting material. Fig. 1 shows the Schlieren patterns obtained on ultracentrifugation of the enzyme.

Spectral comparison of ketoglutarate dehydrogenase and lipoyl dehydrogenase

Fig. 2 shows for comparison the absorption spectra of the ketoglutarate dehydrogenase complex and isolated lipoyl dehydrogenase. The relatively higher absorption of the ketoglutarate dehydrogenase at lower wavelengths may be due to two causes. Firstly, solutions of the enzyme, while appearing perfectly clear when examined with direct light, appear to scatter light appreciably when viewed with indirect light. No such phenomenon is found with lipoyl dehydrogenase. Secondly, as shown in a later section, ketoglutarate dehydrogenase contains protein-bound lipoic acid wheras lipoyl dehydrogenase does not. This would be expected to lead to a greater absorption in the 300–400 mμ region, though not as great as the observed difference.



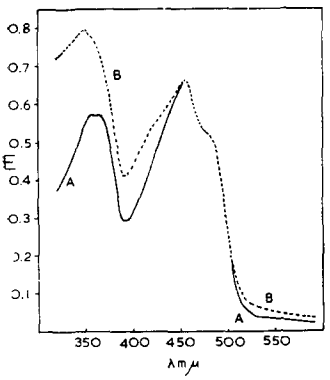


Fig. 1. Sedimentation patterns of ketoglutarate dehydrogenase (specific activity 93) obtained in the Spinco analytical ultracentrifuge. The enzyme was in 0.1 *M* phosphate pH 7.6, average temperature 12.1°. The total duration of the centrifugation (50,740 rev./min) was 40 min. Protein concentration, 7.7 mg/ml.

Fig. 2. Comparison of the absorption spectra of diaphorase (A) and ketoglutarate dehydrogenase (B) in 0.05 *M* phosphate pH 7.4. The absorptions were adjusted (by calculation) to give the same value at 455 mμ. Readings were taken at 5 mμ intervals with a Beckman model DU spectrophotometer.

Involvement of the ketoglutarate dehydrogenase flavin in ketoglutarate oxidation

The reversible reduction of the ketoglutarate dehydrogenase flavin in full accord with reactions (I–III) can be shown readily under anaerobic conditions. Fig. 3 summarises the results of several experiments of this sort. As expected from reactions I and II, the enzyme flavin is reduced only with a mixture of ketoglutarate and CoASH, neither alone causing reduction. Once reduced in this way, the flavin is reoxidized either by the addition of DPN^+ or lipoic acid (or lipoic acid derivatives). In accordance with the involvement of protein bound reduced lipoic acid in the reduction of the flavin by ketoglutarate and CoASH, this reduction is completely

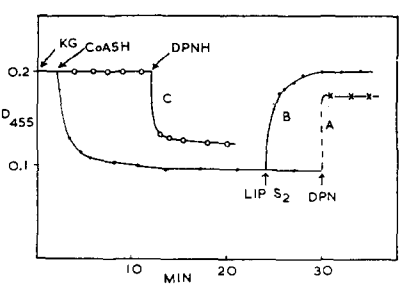


Fig. 3. The anaerobic reduction and reoxidation of flavin in the ketoglutarate dehydrogenase complex. Three anaerobic cells were used containing 0.06 *M* phosphate pH 7.4, and 10 mg KG dehydrogenase in a final volume of 3 ml. In each cell reduction was attempted by the addition of 0.15 μmole CoASH (reduced with cysteine) and 2 μmoles KG. Reoxidation was obtained in cell (A) on the addition of 2 μmoles DPN and in cell (B) 4 μmoles lip S_2. Cell (C) contained sodium arsenite to a final concentration of 10^{-3} *M*. This completely prevented reduction by KG and CoASH, but did not prevent reduction by the addition of 1 μmole DPNH. (Actually the incubation with KG and CoASH was continued for 90 min (without any change in O.D.) before the addition of DPNH; the time scale has been foreshortened here for the sake of convenience). Temperature, 4°.

inhibited by 10^{-3} *M* sodium arsenite. Also in accord with the flavin of ketoglutarate dehydrogenase being lipoyl dehydrogenase, reduction of the flavin is obtained by the addition of DPNH (reaction (III)); this reduction is not inhibited by arsenite.

Enzymic properties of the α-ketoglutarate dehydrogenase complex

Ketoglutarate oxidation coupled to DPN reduction: Under the assay conditions described in METHODS, the rate of ketoglutarate oxidation in the overall reaction:

$$KG + DPN^+ + CoASH \longrightarrow \text{Succinyl CoA} + DPNH + H^+ + CO_2$$

is 14.9 μmoles/min/mg. This rate is considerably higher than reported by SANADI *et al.* and is not due to the difference in assay conditions. When the above reaction is carried out under the assay conditions of SANADI *et al.*[8] the rate is 15.4 μmoles/min/mg. The increase in rate due to the higher temperature used in the assay of SANADI *et al.*[8] (30° instead of 25°) is apparently almost balanced by the slower rate due to the lower DPN concentration (10^{-4} *M* instead of $3 \cdot 10^{-4}$ *M*). Table II shows the Michaelis constants and extrapolated maximum velocities obtained by varying separately the concentrations of ketoglutarate, CoASH and DPN^+, while maintaining the other substrate concentrations constant as described in the table. The low K_m for CoASH permits the measurement of essentially maximum velocity at almost any concentration of CoASH, and a concentration of ketoglutarate of 0.01 *M* gives an observed velocity of 98 % of the extrapolated maximum velocity. However, a concentration of DPN of $1 \cdot 10^{-4}$ *M* (employed by SANADI *et al.*[8]) at 25° gives an observed velocity only 68 % the extrapolated maximum. (At $3 \cdot 10^{-4}$ *M* DPN, as used in the standard assay described in this paper, the observed velocity is 87 % of the maximum.) Thus the maximum velocity for infinite concentration of all substrates should approximate that found for DPN, *i.e.* 17.1 μmoles/min/mg.

TABLE II

MICHAELIS CONSTANTS AND MAXIMUM VELOCITIES OF THE REACTION

$KG + CoASH + DPN^+ \longrightarrow \text{Succinyl CoA} + CO_2 + DPNH + H^+$

*Variable**	*Extrapolated maximum velocity μmoles DPN^+ reduced/min/mg*	K_m
CoASH varied; DPN^+, $3 \cdot 10^{-4}$ *M*; KG, 10^{-2} *M*	14.9	$< 10^{-7}$ *M*
KG varied; CoASH, $4.5 \cdot 10^{-5}$ *M*; DPN^+, $6 \cdot 10^{-4}$ *M*	16.0	$1.3 \cdot 10^{-5}$ *M*
DPN^+ varied; CoASH, $4.5 \cdot 10^{-5}$ *M*; KG, 10^{-2} *M*	17.1	$4.5 \cdot 10^{-6}$ *M*

* Except for the concentrations shown in this column, the experimental conditions were the same as those detailed in METHODS.

Ketoglutarate oxidation linked to ferricyanide reduction: As reported by SANADI *et al.*[4], ketoglutarate dehydrogenase also oxidises ketoglutarate using $K_3Fe(CN)_6$ as acceptor. This reaction does not require CoASH or DPN, and is not inhibited by 10^{-3} *M* sodium arsenite, as is the DPN-linked reaction discussed previously. The reaction was shown to be:

$$KG + 2\ Fe(CN)_6^{\equiv} \longrightarrow Succinate + CO_2 + 2H^+ + 2\ Fe(CN)_6^{\equiv}$$

The experimental evidence for this stoichiometry is given in Table III. The pH optimum for this reaction was found to be pH 6.4. The K_m for ferricyanide, and the extrapolated maximum velocity, at 25° and pH 6.5 were respectively $2 \cdot 10^{-4}\ M$; 4.1 μmoles KG oxidized/min/mg.

TABLE III

STOICHIOMETRY OF $K_3Fe(CN)_6$-LINKED OXIDATION OF KETOGLUTARATE

The reduction of $K_3Fe(CN)_6$ was carried out at pH 6.8 as described in METHODS. In the second experiment, succinate was measured by the micromethod employing phenazine methosulphate-catalysed reduction of cytochrome c[20].

Experiment	*μmoles KG present*	*μmoles $K_3Fe(CN)_6$ reduced*	*μmoles succinate produced*	*Conclusion*
Enzyme reacted with limiting amounts KG	0.5	1.0	—	$KG + 2\ Fe(CN)_6^{\equiv} \rightarrow$
in presence of excess $K_3Fe(CN)_6$	1.0	2.0	—	
Enzyme reacted with excess KG and limiting amount of $K_3Fe(CN)_6$.	5	1.0	0.455	$KG + 2\ Fe(CN)_6^{\equiv}$ ↓
When $K_3Fe(CN)_6$ was fully reduced, enzyme was destroyed by heating at 100° for 1 min, and succinate estimated	5	2.0	0.995	$SU + 2\ Fe(CN)_6^{\equiv}$ $(+ CO_2 + 2H^+)$

Lipoyl dehydrogenase activity: As well as reacting with the protein bound lipoic acid, the lipoyl dehydrogenase of the ketoglutarate dehydrogenase complex also reacts with added lipoic acid and derivatives[8,16]. It also exhibits the ferricyanide linked DPNH dehydrogenase (diaphorase) activity of lipoyl dehydrogenase. The rates of reaction with these substrates, per mole of flavin, are identical with those of uncomplexed lipoyl dehydrogenase. Furthermore the Michaelis constants, at least under the conditions tested, are very similar. Thus it would appear that the complexing of lipoyl dehydrogenase in ketoglutarate dehydrogenase does not hinder its reaction with added lipoic acid derivatives or with pyridine nucleotides:

$$DPNH + H^+ + lip\ S_2 \rightleftharpoons DPN^+ + lip(SH)_2$$

A comparison of the experimental values with ketoglutarate dehydrogenase and isolated lipoyl dehydrogenase is given in Table IV.

Bound cofactors in ketoglutarate dehydrogenase

The early work of SANADI *et al.*[4] showed that firmly bound thiamin and lipoic acid were present in their preparations, and added more experimental support to the concept of ketoglutarate dehydrogenase as an organized complex of enzymes provided with some of the necessary cofactors for the catalytic oxidation of ketoglutarate. This finding is confirmed with the more purified preparation described in this paper. Table V shows the analysis of ketoglutarate dehydrogenase for bound thiamin pyrophosphate and lipoic acid, and gives as well the flavin content calculated from the yellow colour of the enzyme. It is seen that there are approximately equimolar amounts of thiamin pyrophosphate, lipoic acid and flavin, suggesting that the

TABLE IV

COMPARISON OF KINETIC CONSTANTS OF DIAPHORASE AND KETOGLUTARATE DEHYDROGENASE

All assays were at 25°, conditions as described previously[10], *i.e.* pH 5.9 with lipoic acid as acceptor, pH 6.5 with lipoamide and pH 4.8 with $K_3Fe(CN)_6$. V_{max} values are expressed as turnover numbers, *i.e.* as moles of DPNH oxidized/min/mole of enzyme flavin. The flavin concentration was calculated assuming a value of E_{455} of $1.13 \cdot 10^4$ cm²/mmole in both diaphorase and ketoglutarate dehydrogenase.

Enzyme \ *Acceptor*	DL-*lipoic acid*		DL-*lipoamide*		$K_3Fe(CN)_6$	
	V_{max}	Km mM	V_{max}	Km mM	V_{max}	Km mM
Diaphorase	1000	2	80,000	5	8000	0.27
Ketoglutarate dehydrogenase	1000	5	80,000	5.5	8400	0.22

TABLE V

BOUND COFACTORS OF KETOGLUTARATE DEHYDROGENASE

(+) lipoic acid and thiamin pyrophosphate were assayed as described in METHODS. Flavin was estimated from its O.D. at 455 mμ assuming E_{455} of $1.13 \cdot 10^4$ cm²/mmole.

Enzyme	(+) *Lipoic acid* μmoles/g	*Thiamin pyrophosphate* μmoles/g	*Flavin* μmoles/g
Ketoglutarate dehydrogenase complex	4.5	3.1	4.1
As II (colourless fraction obtained in urea resolution)	5.3	4.5	0
As III (yellow fraction obtained in urea resolution)	0.1	0	12.8
Diaphorase	0	0	13.3

constituent enzymes of the complex are present in stoichiometric amounts, presumably linked in a rather special way for the most efficient oxidation of ketoglutarate. The average value of 3.9 μmoles/g protein indicates a minimum molecular weight of 257,000 for the ketoglutarate dehydrogenase complex. This is considerably smaller than the value of 2,000,000 estimated by SANADI *et al.*[4] and confirmed here by the high sedimentation constant. Thus it would appear that ketoglutarate dehydrogenase as isolated is a polymer of a unit complex of enzymes; that about eight such unit complexes form one giant molecule. From the resolution of the complex with urea (detailed in the following section) it would appear that the forces holding the complex together are probably hydrogen bonds.

Resolution of the ketoglutarate dehydrogenase complex with urea

Ketoglutarate dehydrogenase can be resolved into two distinct protein fractions by treatment with urea. When the enzyme was incubated at 0° with different concentrations of urea, it was found even with the highest concentration tested (5.5 *M* urea) that the lipoyl dehydrogenase activity was not affected even after two days. This concentration of urea was sufficient, however, to destroy both DPN^+- and $K_3Fe(CN)_6$-linked ketoglutarate oxidations almost instantaneously. Examination in the analytical ultracentrifuge showed that such treatment had produced considerable polydispersity,

suggesting a dissociation of the ketoglutarate complex. Incubation with 1.1 *M* urea produced no inactivation of ketoglutarate oxidation even after 24 h; in 2.5 *M* urea full activity was maintained for at least 3 h and only half the activity was destroyed in 24 h; in 3.7 *M* urea 60 % of the activity was lost in 2 h and complete inactivation obtained before 24 h. This instability of the enzyme responsible for the initial stages of ketoglutarate oxidation places a fairly limited restriction on the concentration of urea which can be employed in attempting to separate the components of the complex. In practice fractionation in the presence of 2.5 *M* urea was found satisfactory; good resolution was obtained and comparatively little inactivation was obtained during the time taken to accomplish the fractionation. A typical resolution is described below.

A dialysed solution of ketoglutarate dehydrogenase was made 0.05 *M* with respect to phosphate pH 7.6 and 2.5 *M* with respect to urea and applied to a calcium phosphate gel column (see METHODS) which had previously been washed with 0.05 *M* phosphate pH 7.6 + 2.5 *M* urea. After adsorption of the enzyme the column was washed with the same buffer–urea solution. A colourless protein fraction was eluted in this way, leaving a yellow, fluorescent band still at the top of the column. This band was then eluted with 0.075 *M* phosphate pH 7.6 + 2.5 *M* urea + 4 % (w/v) $(NH_4)_2SO_4$. The elution pattern obtained is shown in Fig. 4. The colourless fraction, which contains the urea-unstable enzyme(s) was precipitated with $(NH_4)_2SO_4$ (to 0.45 saturation) as soon as possible after elution, the precipitate redissolved in 0.1 *M* phosphate pH 7.6 and then reprecipitated with $(NH_4)_2SO_4$ in order to dilute out the urea as rapidly as possible. The yellow fraction was concentrated by precipitation with $(NH_4)_2SO_4$ between 0.45 and 0.7 saturation. The $(NH_4)_2SO_4$ precipitates of both fractions were dissolved in 0.05 *M* phosphate pH 7.4. Neither fraction alone was able to catalyse the DPN^+-linked oxidation shown by the original enzyme; however on mixing the two fractions this activity was reconstituted (Fig. 5). As with the un-

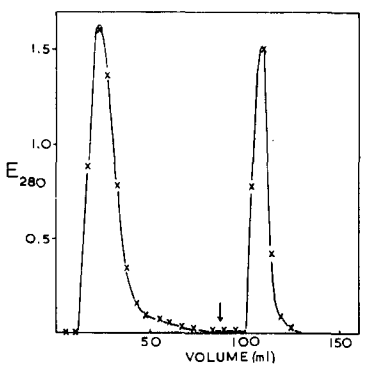


Fig. 4. The resolution of the KG dehydrogenase complex with 2.5 *M* urea employing a column of calcium phosphate gel suspended on cellulose. Elution of the first (colourless) fraction was obtained with 0.05 *M* phosphate pH 7.6 + 2.5 *M* urea. At the point indicated by the arrow the yellow band was still at the top of the column. It was then eluted with 0.075 *M* phosphate pH 7.6 + 2.5 *M* urea + 4 % (w/v) $(NH_4)_2SO_4$.

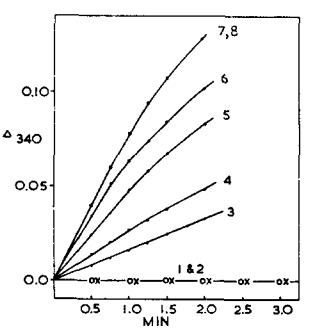


Fig. 5. The reconstitution of KG dehydrogenase activity (KG + DPN^+ + CoASH → succinyl CoA + CO_2 + DPNH + H^+) from the two fractions obtained by urea resolution. Assays were carried out at 25° as detailed in METHODS. Curve 1 shows the lack of effect of 2.12 μg of the colourless fraction in the absence of added yellow fraction. Curve 2 shows the lack of effect of 48 μg of the yellow fraction in the absence of added colourless fraction. Curves 3–8 show the effect of mixing 2.12 μg of the colourless fraction with varying amounts of the yellow fraction. The amounts of yellow fraction used were; Curve 3, 0.194 μg; curve 4, 0.292 μg; curve 5, 0.584 μg; curve 6, 1.94 μg; curves 7 and 8, 5.84 μg and 17.5 μg.

resolved enzyme, this reconstituted activity is inhibited completely by 10^{-3} *M* arsenite.

The colourless fraction contains all of the protein-bound thiamin pyrophosphate and lipoic acid of the ketoglutarate dehydrogenase complex (Table V). As would be expected from the flavoprotein nature of lipoyl dehydrogenase, the colourless fraction is completely devoid of lipoyl dehydrogenase activity. This fraction is however capable of ketoglutarate oxidation when ferricyanide is used as acceptor. This reaction is identical with the reaction catalysed by the intact complex; it has the same stoichiometry and the same pH optimum (pH 6.4). Also the specific activities are quite similar. The maximal rate with the intact ketoglutarate dehydrogenase is 4.1 μmoles ketoglutarate oxidized/min/mg at pH 6.5; the corresponding value with the colourless fraction is 6.0. This difference probably does not represent a real increase in activity on dissociation of the complex, but is merely a reflection of lower protein content/unit activity caused by the separation of the constituents of the complex (*cf.* Table VI).

TABLE VI

TURNOVER NUMBERS AT 25° OF REACTIONS CATALYSED BY THE KETOGLUTARATE DEHYDROGENASE COMPLEX AND ITS CONSTITUENT ENZYMES

The turnover numbers were calculated from the values previously quoted, assuming molecular weights of 260,000 for the whole complex, 180,000 for the colourless fraction (As II), and 80,000 for diaphorase (As III).

Reaction	*pH*	*Max turnover number*		
		Whole complex	*As II*	*As III*
KG + DPN^+ + CoASH → Succ CoA + DPNH + H^+ + CO_2	7.4	4,400	0	0
KG + 2 $Fe(CN)_6^{\equiv}$ → SU + CO_2 + $2H^+$ + 2 $Fe(CN)_6^{\equiv}$	6.5	1,060	1,080	0
H^+ + DPNH + lipoic ⇌ DPN^+ + reduced lipoic	5.9	1,000	0	1,000
H^+ + DPNH + lipoamide ⇌ DPN^+ + reduced lipoamide	6.5	80,000	0	80,000
DPNH + $2Fe(CN)_6^{\equiv}$ → DPN + $2Fe(CN)_6^{\equiv}$	4.8	8,400	0	8,000

Properties of the yellow fraction

The yellow fraction obtained by the urea resolution is clearly identical with lipoyl dehydrogenase (Straub diaphorase). Fig. 6 shows the very similar absorption spectrum of the yellow protein obtained from the ketoglutarate dehydrogenase compared with that of free lipoyl dehydrogenase[10]. The lipoyl dehydrogenase activity/mole of flavin is the same as that found either with free lipoyl dehydrogenase or with the enzyme in the ketoglutarate dehydrogenase complex (DPNH oxidation with lipoic acid, lipoamide, $K_3Fe(CN)_6$, DCPIP as acceptors). Further convincing proof that the yellow fraction is lipoyl dehydrogenase comes from ultra centrifugal analysis (Fig. 7). Only one protein component was observed, with a sedimentation constant of 5.4 S, the same as that previously reported for lipoyl dehydrogenase[15,10].

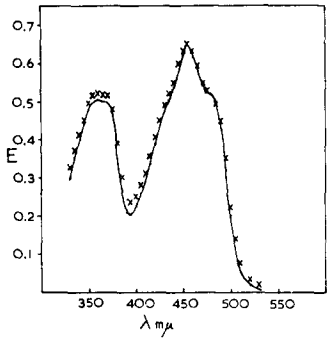


Fig. 6. Comparison of the visible spectrum of diaphorase[10] and the yellow fraction obtained by urea resolution of the KG dehydrogenase complex (see text). O.D. readings were taken at 5 mμ intervals with a Unicam SP 500 spectrophotometer. The solid line shows the spectrum of a solution of diaphorase in 0.05 *M* phosphate pH 7.4, protein concentration 4.3 mg/ml. The crosses show the O.D. under the same conditions of a 4.7 mg/ml solution of the yellow fraction obtained by urea resolution from ketoglutarate dehydrogenase.

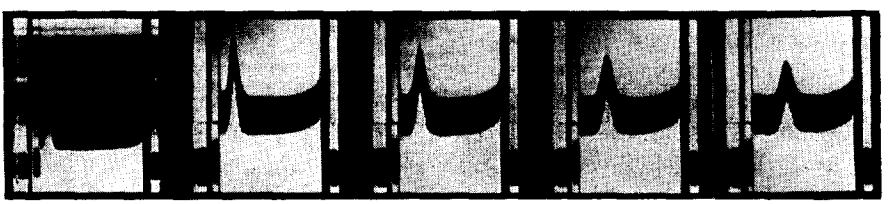


Fig. 7. The sedimentation of the yellow fraction from urea resolution in the Spinco analytical ultracentrifuge. The enzyme (4.7 mg/ml) was in 0.1 *M* phosphate pH 7.6; average temperature 17.5°; rotor speed 50,740 rev./min. Photographs were taken at 16 min intervals.

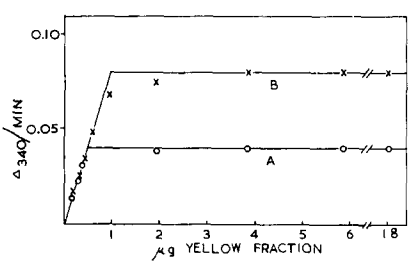


Fig. 8. The effect of titration of the colourless fraction from urea resolution with the yellow fraction on ketoglutarate dehydrogenase activity (KG + DPN^+ + CoASH → succinyl CoA + CO_2 + DPNH + H^+). Assays were carried out at 25° as detailed under METHODS. In curve A 1.06 μg of the colourless fraction was used; in curve B 2.12 μg of the colourless fraction was used.

Stoichiometry of reconstitution of ketoglutarate dehydrogenase activity

Fig. 8 shows that in the reconstitution of the overall ketoglutarate dehydrogenase activity obtained by mixing the two fractions, each fraction may be titrated against the other. Thus if the amount of colourless fraction is kept constant, the rate of ketoglutarate oxidation is directly proportional to the amount of the yellow fraction until saturation is obtained, when there is no further increase in activity even with a large increase in the yellow fraction.

From these results the specific activity of the reconstituted enzyme can be calculated. At the most favourable, *i.e.* when the one fraction is theoretically saturated

with the other the activity is 4.2 μmoles KG oxidized/min/mg. This is considerably less than the value before splitting of the complex (14.9 μmoles/min/mg). The lower value obtained may be due to slight changes in the structure of either or both fractions as a result of the separation in the presence of urea; the slightly modified enzymes, though unimpaired in their reaction with ferricyanide and externally added lipoyl derivatives, may not be able to arrange themselves in exactly the same way as in the natural complex. In support of this idea, lipoyl dehydrogenase prepared by the method previously described[10], even when added in great excess, functions in ketoglutarate oxidation with the colourless fraction at a rate only 0.1–0.25 that of the lipoyl dehydrogenase isolated from the complex. This is the only difference that has been found in the enzyme prepared by these two different procedures.

Estimates of unit molecular weight

From the sedimentation constant of 30 S a very high molecular weight is indicated. SANADI *et al.*[4] have given an estimate of 2,000,000 from their data. From two quite distinct sets of results reported here, estimates of minimum molecular weight can be made. Firstly from the cofactor composition shown in Table V, the average concentration of thiamin pyrophosphate, lipoic acid and FAD is 3.9 μmoles/g, corresponding to a minimum molecular weight of 257,000. An estimate of the molecular weight of the enzymically functional complex can also be made from the titration data of Fig. 8. Here it is seen that 2.12 μg colourless fraction combines with 0.97 μg lipoyl dehydrogenase. This means that the molecular weight of the colourless fraction is 2.20 times that of the lipoyl dehydrogenase, which is known to be about 80,000[15]. Thus the molecular weight of the colourless fraction would be about 180,000, and of the whole complex, about 260,000. The values obtained by these two different methods are in remarkable agreement, and give an average minimal molecular weight of about 260,000. Thus ketoglutarate dehydrogenase is not only a complex of enzymes linked in stoichiometric quantities together with tightly bound cofactors, but a polymer of this unit complex. From the molecular weight of SANADI *et al.*[4] calculated from sedimentation and diffusion data, there would be 8 such unit complexes/giant polymer complex.

The minimal molecular weights calculated above have been used to convert rates of reaction into turnover numbers, *i.e.* the number of molecules of substrate oxidized/min/molecule of enzyme. The results are given in Table VI.

DISCUSSION

The results show conclusively that the lipoyl dehydrogenase of the ketoglutarate dehydrogenase complex is a flavoprotein, and identical with the lipoyl dehydrogenase already described[10] (STRAUB diaphorase[15,17]). Thus there can be little doubt that the physiological function of diaphorase is as the lipoyl dehydrogenase of keto acid oxidation. Its function in ketoglutarate oxidation can be represented as in Fig. 9. It should be noted that while this enzyme can readily catalyse the reversible reaction,

$$DPNH + H^+ + \text{lip } S_2 \rightleftharpoons DPN^+ + \text{lip}(SH)_2$$

where lip S_2 applies to a number of lipoyl derivatives (including that bound in the ketoglutarate dehydrogenase complex), its normal function in oxidative metabolism

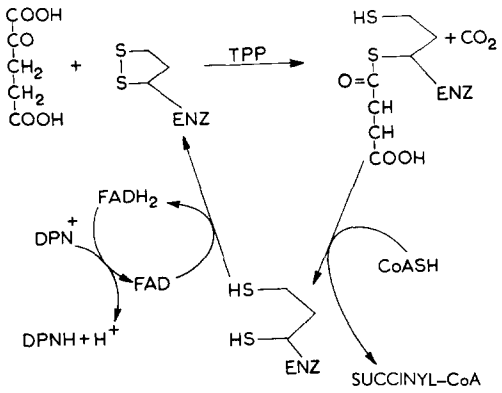


Fig. 9. Scheme of ketoglutarate oxidation by the KG dehydrogenase complex.

would be to reduce DPN^+, rather than to oxidize DPNH, the function previously ascribed to STRAUB diaphorase[18]. The DPNH formed is presumably reoxidized *in vivo* by way of the respiratory chain of enzymes. The pattern of electron and hydrogen transfer is thus much more complex with α-ketoglutarate than with any other of the tricarboxylic acid cycle intermediates:

$$KG \longrightarrow \underset{\text{(lipoyl dehydrogenase)}}{\text{Lip } S_2} \longrightarrow FAD \longrightarrow DPN^+ \longrightarrow \underset{\text{(DPNH dehydrogenase)}}{\text{flavin}} \longrightarrow \text{cytochrome system}$$

It is somewhat of a paradox that thiamin pyrophosphate, the first of the four vitamin cofactors recognized to be required in keto acid oxidation[19], is the only one whose function is not reasonably well understood. It is hoped that its presence in stoichiometric amounts in the ketoglutarate dehydrogenase complex, and its localization in the colourless fraction, will facilitate further studies on its function.

It is not yet possible to say anything about the number of different enzymes involved in the ketoglutarate dehydrogenase complex. The results of the urea resolution would suggest that only two enzymes are involved, but it must be pointed out that the colourless fraction may well be only a partly resolved complex, from which all the lipoyl dehydrogenase has been dissociated, but which may consist of any number of constituent enzymes.

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INFLUENCE OF INORGANIC PHOSPHATE IN THE FORMATION OF PHOSPHATASES BY *ESCHERICHIA COLI*

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SUMMARY

Both acid and alkaline phosphatase have been demonstrated in strains of *E. coli*. Bacterial acid phosphatase (pH optimum 4–5) displays a fairly high specificity for the hexosephosphates, while the alkaline enzyme (pH optimum 8.5–9.5) hydrolyzes all the phosphomonoesters tested.

The kinetics of formation of both enzymes have been studied and it has been shown that alkaline phosphatase in measurable amount is only formed when Pi becomes limiting in the medium, at which point the enzyme is formed in substantial amount at a maximum rate.

The implications of the findings with respect to mechanisms of controlling enzyme formation are discussed.

The following abbreviations are used: Pi (inorganic phosphate); Tris (trishydroxymethylaminomethane); NPP (*p*-nitrophenylphosphate); NP (*p*-nitrophenol); bis-NPP (bis-*p*-nitrophenylphosphate).

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