The role of diaphorase in ketoglutarate oxidation

Ketoglutaric oxidase has been purified from pig heart as an apparent complex of enzymes by SANADI *et al.*[1]. It was shown[2] that the complex exhibited lipoic dehydrogenase activity; hence by analogy with the similar pyruvate oxidase system from *Escherichia coli*[3,4] the following mechanism could be written.

$$KG + \text{lip } S_2 \xrightarrow{DPT?} \text{S-succinyl lipoate} + CO_2 \quad (1)$$

$$\text{S-succinyl lipoate} + CoA \rightleftharpoons \text{succinyl CoA} + \text{lip}(SH)_2 \quad (2)$$

$$\text{lip}(SH)_2 + DPN^+ \rightleftharpoons \text{lip } S_2 + DPNH + H^+ \quad (3)$$

$$KG + CoA + DPN^+ \longrightarrow \text{Succinyl CoA} + CO_2 + DPNH + H^+ \quad (4)$$

SANADI and collaborators[1,2] reported the presence of diaphorase as an impurity in their preparations. The recent finding that STRAUB[5] diaphorase is a powerful lipoic dehydrogenase[6] made it likely that the diaphorase reported by the previous workers[1,2] was not an impurity, but being identical with lipoic dehydrogenase, an integral part of the KG oxidase system. This conclusion is confirmed by the partial resolution of the KG oxidase complex into fractions inactive except in the presence of added diaphorase and by the demonstration that the flavoprotein of the complex is reduced by CoA + KG and reoxidized by DPN or lip S_2.

KG oxidase was obtained by the method of SANADI *et al.* and further purified on a column of cellulose and $Ca_3(PO_4)_2$ gel. After elution of considerable impurities with 0.1 *M* phosphate, pH 7.6, the yellow band containing diaphorase (both free and in the KG oxidase complex) was eluted with 0.1 *M* phosphate, pH 7.6, + 4% (w/v) $(NH_4)_2 SO_4$. The fractions eluting just in front of the yellow, fluorescent band had no KG oxidase activity; however on the addition of purified diaphorase[6], KG oxidation occurred (Fig. 1).

While the separation achieved in this way clearly shows that diaphorase is required for KG oxidation, the amount of resolution obtained is quite small (1–5% total KG oxidase). The bulk of the complex could not be resolved and was further purified by fractionation with $(NH_4)_2SO_4$ between 0.3 and 0.35 satn., followed by sedimentation for 100 min in the No. 40 rotor of the Spinco ultracentrifuge. The pellet obtained was bright yellow and highly fluorescent, and appeared to be 80–85% pure in the Spinco analytical ultracentrifuge, with a sedimentation coefficient of 30 S. Its absorption spectrum in the visible was very similar to that of diaphorase, with maxima at 455 mμ and 360 mμ. As well as KG oxidase activity (12.2 μmoles KG oxidized/min/mg protein under the assay conditions of Fig. 1) it showed very similar lipoic dehydrogenase activity (with both DL-lipoic acid and DL-lipoamide) to that of free diaphorase. That the flavoprotein of this complex is definitely associated with KG oxidation and lip S_2 reduction was shown by the bleaching of the yellow colour of the enzyme (and loss of fluorescence) on reaction with both CoA and KG and the reoxidation of the flavin by the addition of DPN (Fig. 2A) or DL-lipoic acid (Fig. 2B).

The involvement of flavoprotein in the pyruvate oxidase complex of *E. coli* has also been demonstrated by REED AND KOIKE[7]. This and the present demonstration of

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Abbreviations: KG, α-ketoglutarate; DPT, diphosphothiamin; DPN^+ and DPNH, oxidized and reduced diphosphopyridine nucleotide; lip S_2 and $\text{lip}(SH)_2$, oxidized and reduced lipoic acid; CoA, coenzyme A.

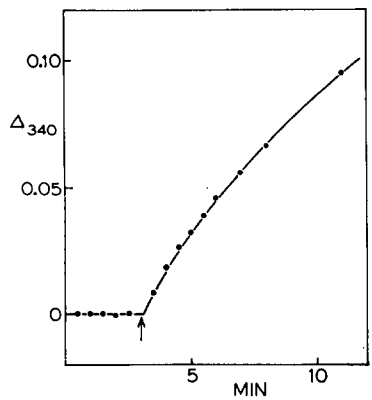

Fig. 1. Partial resolution of KG oxidase. Microcells (1-cm light path) contained in a total vol. of 1.0 ml: 0.06 *M* phosphate, pH 7.4; $4.5 \cdot 10^{-5}$ *M* CoA; $3 \cdot 10^{-3}$ *M* cysteine; $3 \cdot 10^{-4}$ *M* DPN and 3 μg of a fraction eluted from a column in front of the KG oxidase (see text). At the time shown by the arrow 8 μg purified diaphorase was added. An identical result was obtained by reversing the order of addition of the two protein solutions. Temp., 25°.

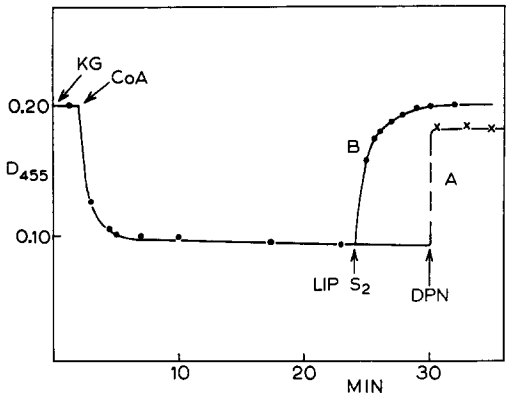

Fig. 2. The anaerobic reduction and re-oxidation of flavin in the KG oxidase complex. Two anaerobic cells were used containing 0.06 *M* phosphate, pH 7.4, and 10 mg KG oxidase in a final volume of 3.0 ml. Reduction was obtained by the addition of 0.15 μmole CoA (reduced with cysteine) and 2 μmoles KG. Reoxidation was obtained on the addition in cell (A) of 2 μmoles DPN or (B) 4 μmoles lip S_2. Temp., 4°.

diaphorase in the KG oxidase system of pig heart suggest very strongly that the physiological role of diaphorase is as the lipoic dehydrogenase of keto acid oxidation rather than as a component of the electron-transport chain in DPNH oxidation.

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Labeling of the pH-5 enzyme with adenosine triphosphate

In a previous communication from this laboratory[1] we have shown that the exchange between ATP and ^{32}P-labeled inorganic pyrophosphate by the pH-5 enzyme from rabbit liver is sensitive to ribonuclease. To obtain more direct evidence concerning the role of RNA in this reaction, ^{32}P labeling of the pH-5 enzyme with [$\beta\gamma$-$^{32}P_2$]ATP was studied.

[$\beta\gamma$-$^{32}P_2$]ATP was prepared by oxidative phosphorylation with rat-liver mitochondria in the presence of $H_3{}^{32}PO_4$, and purified twice by chromatography on

Abbreviations: ATP, adenosine triphosphate; PP, inorganic pyrophosphate; RNA, ribonucleic acid.