## The identity of TPN-linked isocitric dehydrogenase and oxalosuccinic carboxylase

The oxidative decarboxylation of *iso*citric acid to α-ketoglutaric acid can occur by two mechanisms. One is catalysed by an *iso*citric dehydrogenase which requires DPN as co-enzyme.

$$d\text{-}iso\text{citric acid} + \text{DPN} \rightarrow \alpha\text{-ketoglutaric acid} + \text{DPN}\cdot 2\text{H} + CO_2$$

There is no evidence that oxalosuccinic acid is an intermediate and the reaction is not reversible[1,2].

The other mechanism occurs in two steps, the first being catalysed by a TPN-linked *iso*citric dehydrogenase[3,4].

$$d\text{-}iso\text{citric acid} + \text{TPN} \rightleftarrows \text{oxalosuccinic acid} + \text{TPN}\cdot 2\text{H}$$

Oxalosuccinic acid is then decarboxylated by oxalosuccinic carboxylase[5,6]

$$\text{oxalosuccinic acid} \rightleftharpoons \alpha\text{-ketoglutaric acid} + CO_2$$

Both these steps are reversible[7], but it was not certain whether one enzyme or two are responsible. The question is of particular interest in view of certain similarities between the *iso*citrate-α-ketoglutarate system and the malate-pyruvate system[8]. In the case of malate, a DPN-specific dehydrogenase causes the oxidation to oxaloacetate which is then decarboxylated by oxaloacetic carboxylase, these two reactions being catalysed by two distinct and separable enzymes: nevertheless a TPN-linked "malic" enzyme has been shown to bring about the whole process, possibly with enzyme-bound oxaloacetate as an intermediate. Six-fold purification of the TPN-linked *iso*citric dehydrogenase and oxalosuccinic carboxylase from pig heart by GRAFFLIN AND OCHOA[9] did not separate the two activities but it was possible that further purification might do so.

The enzymes have now been obtained in a high state of purity and the results of the present work show that one protein is responsible for both enzymic activities.

*Preparation of enzyme.* An acetone powder of pig hearts was prepared according to the method of STRAUB[10] as used by GRAFFLIN AND OCHOA[9]. The dry powder was extracted at room temperature with 0.05 *M* phosphate buffer at pH 7.3 containing 0.15 *M* NaCl. The extract was fractionated by precipitation with ammonium sulphate followed by adsorption on calcium phosphate gel at pH 7.3. A fraction of high specific activity was obtained which represented 15–25% of the original activity and which behaved largely as a single component in several physical tests. Details of purification of the enzyme and its properties will be presented elsewhere.

*Enzyme activity tests.* (a) *Isocitric dehydrogenase.* Reduction of TPN in the presence of excess *iso*citrate at 24° was measured in the Beckman model DU spectrophotometer at a wavelength of 340 mμ using cuvettes of 1 cm light path. The reaction mixture (3 ml) was the same as that of GRAFFLIN AND OCHOA[9] except that "tris" (tris(hydroxy methyl)amino methane) buffer pH 7.3 at a final concentration of 0.012 *M* was used in place of glycyl-glycine buffer. One dehydrogenase unit was defined as the amount of enzyme causing an increase in optical density of 0.010 in 1 min. Protein was estimated by optical density at 280 mμ[11]. The specific activity ($SA_D$) was defined as the number of units per mg protein.

(b) *Oxalosuccinic carboxylase.* Production of carbon dioxide from oxalosuccinate at pH 5.6 in citrate buffer at 18° was measured in Warburg manometers. Simultaneous determinations of the spontaneous decarboxylation of oxalosuccinate were made, and these values substracted from total carbon dioxide production in presence of enzyme. The reaction mixture was identical with that of OCHOA AND WEISZ-TABORI[12]. One carboxylase unit was defined as the amount of enzyme producing 1 μl $CO_2$/5 min, and specific activity ($SA_C$) as the number of units/mg protein.

*Results.* A comparison of *iso*citric dehydrogenase and oxalosuccinic carboxylase activity of the different fractions showed that no separation of the two enzymes took place during the purification (Table I). A similar comparison of activity in fractions undergoing inactivation gave the same result, that is to say any inactivation of one enzyme was accompanied by inactivation of the other to the same degree. Fractions E1 and E2 (Table I) were samples of the original extract which had been allowed to autolyse. Over a 200-fold range of specific activity the ratio of the two enzymic activities remained constant within experimental error.

The purest fractions (corresponding in specific activity to K1 of Table I) were subjected to several physical tests. Sedimentation in the Spinco Model E Ultracentrifuge showed a single component representing 90–95% of the total protein (Fig. 1). The sedimentation constant was calculated assuming a partial specific volume for the solute of 0.73, and found to be

$$S^{W}_{20} = 4.80 \cdot 10^{-13}\ \text{cm}^2/\text{sec}\ \text{(extrapolated to zero protein concentration)}$$

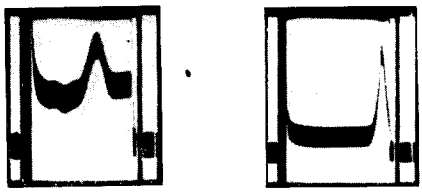
A value of 64,000 was obtained for the molecular weight of the enzyme, using a diffusion constant calculated from the spread of the sedimenting boundary[14]. Electrophoresis was carried out at pH 5.6, 7.3 and 8.6 and showed one major component (see Fig. 2) of very low mobility. Some difficulty was experienced because of the instability of the enzyme after prolonged dialysis, and a certain amount of inactivation and precipitation occurred.

TABLE I

| *Fraction* | *Yield activity* % | $SA_D$ | $SA_C$ | $\frac{SA_D}{SA_C}$ |
|---|---|---|---|---|
| Extract | 100 | 190 | 58 | 3.3 |
| Purification | | | | |
| F 2 | 58 | 690 | 183 | 3.8 |
| H 2 | 41 | 1000 | 300 | 3.3 |
| K 1 | 20 | 1500 | 480 | 3.1 |
| Inactivation | | | | |
| E 1 | 12 | 60 | 23 | 2.6 |
| E 2 | 10 | 8.6 | 2.4 | 3.5 |

TABLE II

| *Fraction* | $SA_D$ | $SA_C$ | $\frac{SA_D}{SA_C}$ |
|---|---|---|---|
| A 1 | 0 | 0 | |
| A 2 | 700 | 220 | 3.2 |
| A 3 | 1000 | 344 | 2.9 |
| D 1 | 0 | 0 | |
| D 2 | 500 | 150 | 3.3 |
| D 3 | 420 | 197 | 2.2 |
| D 4 | 293 | 132 | 2.2 |
| D 5 | 1120 | 363 | 3.1 |



a b

Fig. 1. Sedimentation in 0.05 *M* "Tris" buffer pH 7.3 at 56,100 r.p.m. Exposures at 16 and 96 mins.

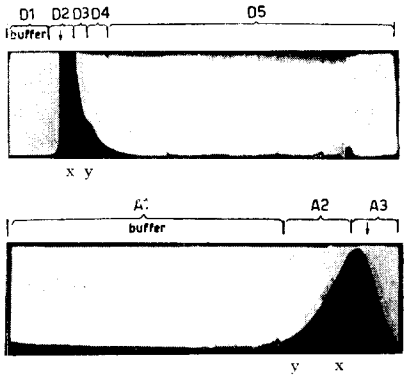



Fig. 2. Electrophoresis in 0.05 *M* "Tris" buffer pH 7.3 containing 0.002 *M* $MnCl_2$. After 5 h. (a) descending boundary; (b) ascending boundary. Initial position of boundaries marked ;.

By making use of the elegant apparatus of CROOK *et al.*[13] it was possible to fractionate the purest material after prolonged electrophoresis and separate the major component (*x*) from the faster moving, small component (*y*). Fractions were collected as indicated in Fig. 2. The ratio of the two enzymic activities remained constant over the whole boundary, and it was the major component (*x*) which was active and not the small component (*y*) (Table II). It is clear that electrophoresis causes no separation whatsoever of the two activities. It must therefore be concluded that both *iso*citric dehydrogenase activity and oxalosuccinic carboxylase activity are possessed by a single protein which has been obtained, on the evidence so far available, 90–95% pure.

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