A Comparative Study of Dihydrolipoyl Dehydrogenase and Diaphorase*

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The purification and some of the properties of a flavoprotein component of α-ketoglutaric dehydrogenase complex have been described in a previous communication (1). In several of its properties, this flavoprotein shows a remarkable resemblance to the flavoprotein first isolated by Straub (2) and later studied by Savage (3). They have the same prosthetic group (flavin adenine dinucleotide) and are both highly fluorescent. They catalyze the reduction of 2,6-dichlorophenolindophenol by reduced diphosphoryridine nucleotide at comparable rates. They have the same electrophoretic mobility at pH 7.2 and at pH 6.5. Finally, the spectrum of the flavoprotein component of α-ketoglutaric dehydrogenase complex and the spectrum obtained after its reduction with excess DPNH are very similar to the spectra obtained under similar conditions by Savage.

While the above work on the components and reaction sequence of the α-ketoglutaric dehydrogenase complex was under investigation and partially completed in our laboratory, the physiological role of diaphorase was being investigated by Massey. He reported that diaphorase prepared by a new and more gentle procedure had dihydrolipoyl dehydrogenase activity (4). He later showed that α-ketoglutaric dehydrogenase complex can be obtained in a resolved form consisting of a flavoprotein and a colorless component, and that the flavoprotein can be replaced by diaphorase in reconstituting the full activity of the complex. From these results, he proposed that the true role of the classic diaphorase is that of a component of the α-ketoglutaric dehydrogenase complex (5).

In this paper we wish to compare further the spectral and enzymatic properties of the flavoprotein isolated by our method (1) from α-ketoglutaric dehydrogenase complex and the flavoprotein isolated by Straub. These enzymes have previously been referred to in terms of their original assay, dihydrolipoyl dehydrogenase and the second as Straub flavoprotein in this paper.

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EXPERIMENTAL PROCEDURE

The preparation of the KGDH1 flavoprotein has been described (1). Some preparations which did not reach the desired level of purity were further purified by adsorption on calcium phosphate gel (3 to 6 mg of gel by dry weight per mg of protein in 0.01 M phosphate buffer pH 7.2), washing twice with 0.1 M phosphate pH 7.6, and finally elution with 0.1 M phosphate containing 4% ammonium sulfate. The Straub flavoprotein was purified by the procedure of Straub (2). DPNH-cytochrome c reductase was prepared and assayed as described by Mahler et al. (6). The preparation was not carried through the complete procedure and had only 70% of the activity expected of the final fraction. The dihydrolipoyl dehydrogenase assay (LS2 reduction assay) has been described (1).

The diaphorase assay was modified several times. Initially the conditions were those of Savage (3) except that the dye was added 2 minutes after the enzyme, in order to reduce an initial lag (1). Under these conditions, the reaction rate was reproducible and proportional to enzyme concentration but did not seem to be proportional to flavin content from one preparation to another. It was later found that KGDH flavoprotein, which had aged at -10°C, lost activity when it was diluted extensively for the LS2 reduction assay unless it was stabilized with albumin or EDTA. If the enzyme was diluted for the diaphorase assay in the presence of these compounds, no initial lag was observed, but the activity was one-third to one-fifth that which is shown in their absence (see also Reference (7)), and it still was not proportional to the flavin content from one preparation to another. Finally, it was found that addition of cadmium chloride to the enzyme would give very high rates for the diaphorase assay, and that these rates were proportional to flavin content (8). Similar effects have been obtained with Cu++ (7). The reaction mixture for the assay in the presence of Cd++ contained 150 μmoles of phosphate pH 7.3, 0.2 μmole of DPNH, 0.03 ml of 0.1% 2,6-dichlorophenolindophenol, and 0.2 μmole of cadmium chloride in 3.0 ml. Other experimental conditions and procedures have been described previously (1).

RESULTS

Massey has reported that a flavoprotein which he has isolated from the Keilen-Hartree type particles by a new procedure had both diaphorase and dihydrolipoyl dehydrogenase activity (4).

1 The following abbreviations will be used: KGDH, α-ketoglutaric dehydrogenase; EDTA, ethylenediaminetetraacetate.
This flavoprotein was believed to be identical with the Straub flavoprotein on the basis of the spectrum of the oxidized enzyme, the sedimentation constant, and activity in the diaphorase assay. The flavoprotein isolated, by a method which followed closely the original procedure of Straub, also showed a high level of dihydrolipoil dehydrogenase activity (Table I). The diaphorase activity found was less than that reported by Savage, presumably because of the aforementioned assay difficulties, but the activity in the dihydrolipoil dehydrogenase assay was the same as the activity of KGDH flavoprotein of the same flavin content (1).

The DPNH-cytochrome c reductase of Mahler, in contrast, had very little dihydrolipoil dehydrogenase activity (Table II). It contained some dihydrolipoil dehydrogenase activity at all stages, but the ratio of DPNH-cytochrome c reductase activity to dihydrolipoil dehydrogenase activity increased with purification. It would appear that if this enzyme does, in fact, have any activity in the dihydrolipoil dehydrogenase reaction, it cannot be more than 1% of the activity of Straub or KGDH flavoprotein.

The similarity of the spectrum of KGDH flavoprotein before and after reduction with DPNH to the spectra of Straub flavoprotein obtained under the same conditions (3) was one of the observations which suggested early in our studies (9) that these two flavoproteins might be identical. When KGDH flavoprotein was reduced with dihydrolipoate, a spectrum was obtained which has no counterpart in the literature. The difference spectrum showed peaks at 485 m\u00b4, 460 m\u00b4, and 375 m\u00b4, and negative peaks at 520 m\u00b4, 420 m\u00b4, and 320 m\u00b4 (1). Fig. 1 shows the spectrum of Straub flavoprotein reduced under the same conditions. Superimposed on this is the difference spectrum obtained upon reduction of KGDH flavoprotein with dihydrolipoate (Spectrum D). Spectrum C (the difference spectrum for Straub flavoprotein) and Spectrum D (the difference spectrum for KGDH flavoprotein) are seen to be nearly identical.

Straub flavoprotein and KGDH flavoprotein were also compared on the basis of their enzymatic properties. Fig. 2 shows the variation with pH of the activity of each enzyme for the reduction of lipoamide by DPNH. The maximal activity for both enzymes was obtained at pH 7.1. It is of interest to note that the original a-ketoglutaric dehydrogenase complex has a lower pH optimum (Curve C) which is indicative either of a change in the disengaged flavoprotein or of the effect of binding of the flavoprotein to the rest of the a-ketoglutaric dehydrogenase complex. Fig. 3 shows the pH activity curve for each enzyme for the reduction of lipoic acid by DPNH. The rates increased from pH 7.0 to pH 5.6 and were constant from pH 5.6 to pH 5.2. There was no detectable destruction of DPNH down to pH 5.2 under these conditions; the absorbancy at 340 m\u00b4u and the total decrease in absorbancy upon completion of enzyme reaction remained constant. There was an extremely rapid decrease in activity between pH 5.2 and pH 4.8. The rate was no longer linear with time below pH 4.9 (Fig. 4). The initial absorbancy at 540 m\u00b4u did not change, but the net change caused by the enzymatic reaction became less. It is not known whether this was due to destruction of the DPNH, to denaturation of the enzyme, or to the existence of an important ionization at this pH. A somewhat different effect was obtained with the lipoamide as the acceptor (Fig. 5). In this case, the reaction rate

### Table I

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<th>Dihydrolipoil dehydrogenase activity of Straub flavoprotein</th>
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<tr>
<td>Alcohol-(NH(<em>{4}))(</em>{2})SO(_{4}) solution</td>
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<td>Alumina eluate</td>
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<tr>
<td>First (NH(<em>{4}))(</em>{2})SO(_{4}) fractionation</td>
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<td>Second (NH(<em>{4}))(</em>{2})SO(_{4}) fractionation</td>
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* Assayed as described by Savage (3).

### Table II

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<tr>
<th>Dihydrolipoil dehydrogenase activity of DPNH-cytochrome c reductase</th>
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<td>First (NH(<em>{4}))(</em>{2})SO(_{4}) fractionation</td>
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<tr>
<td>Second (NH(<em>{4}))(</em>{2})SO(_{4}) fractionation</td>
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Fig. 1. Reduction of Straub flavoprotein by dihydrolipoate. Curve A is the spectrum of 3.3 mg of Straub flavoprotein in 0.70 ml of phosphate buffer pH 7.3. Curve B shows the spectrum after addition of 1.7 amoles of dihydrolipoate in 0.1 ml. Curve C is the difference spectrum. Curve D is the difference spectrum of KGDH flavoprotein which has been reduced under identical conditions. All spectra have been corrected for dilution. The reduction was carried out in an atmosphere of nitrogen (1).

Fig. 2. The pH-activity curve for the reduction of \(a\)-lipoamide by DPNH. These assays were carried out under the standard conditions (1). The pH was adjusted by varying the ratio of potassium phosphate to monopotassium phosphate, keeping the total phosphate concentration constant. The pH was measured at the end of each experiment. Curve A represents the data with KGDH flavoprotein and Curve B, those with Straub flavoprotein. Curve C was obtained with intact \(a\)-ketoglutaric dehydrogenase complex.
The pH-activity curve for the reduction of di-1ipoate by DPNH. The reaction mixture contained 0.25 μmole of DPNH, 1.5 μmoles of lipoic acid, 0.2 μmole of EDTA, and varying ratios of sodium acetate and histidine hydrochloride. The total reaction volume was 1.0 ml containing 50 μmoles of buffer. The pH was measured at the end of each experiment. Curve A represents the data with KGDH flavoprotein and Curve B, those with Straub flavoprotein. Curve C was obtained with intact α-ketoglutaric dehydrogenase complex in phosphate buffer.

The effect of lipoamide concentration on reaction rate. The reaction mixture contained 50 μmoles of phosphate pH 7.0, 0.24 μmole of DPNH, 1 μmole of EDTA, and 0.075 to 0.75 μmole of di-1ipoamide in 0.05 ml of ethanol in a total volume of 1.0 ml. The rate of DPNH oxidation after the addition of enzyme was measured at 30°. S = μmoles of lipoamide; v = change in absorbancy per minute at 340 nm. ○—○, 0.40 μg of KGDH flavoprotein (1 mole of flavin per 70,000 g) in 0.002 M EDTA-0.01 M phosphate; □—□, 0.44 μg of Straub flavoprotein (1 mole of flavin per 100,000 g) in 0.002 M EDTA-0.01 M phosphate.

Table III

Effect of heat on enzyme activities

The diaphorase activity of Straub flavoprotein was measured by the procedure of Savage (3). The diaphorase activity of KGDH flavoprotein was carried out in the presence of cadmium ion. Before each experiment, the flavoprotein was exhaustively dialyzed against glass-distilled water. The pH was then adjusted as indicated with 0.1% NH₄OH and heated for 5 minutes in a water bath. The activity is expressed in μmoles of DPNH oxidized per minute per mg of protein.

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<th>0°</th>
<th>20°</th>
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<td>25.1</td>
<td>21.4</td>
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<tr>
<td>pH 6.2</td>
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accelerated at pH 6.0, but was linear at pH 6.3 or above. Surprisingly, the final rate at pH values below 6.0 approaches the initial rate at pH 7.1.

The Michaelis constants and turnover numbers for these two enzymes with lipoamide as substrate were determined by the double reciprocal plot procedure (10) (Fig. 6). The Michaelis constant for the KGDH flavoprotein was 1.3 × 10⁻⁴ M, and for the Straub flavoprotein, 1.5 × 10⁻⁴ M. These values may be high because of the presence of polymerized lipoamide (1). The maximal rates were determined from these plots, and the turnover numbers were calculated as the maximal number of μmoles of DPNH oxidized per minute per μmole of enzyme-flavin adenine dinucleotide at 30°. The values obtained under our experimental conditions were 19,000 and 18,000 for the two flavoproteins, respectively. These values are approximately one-fourth of the values reported by Massey (5), although the ex-
Experimental conditions are not far different. We have no explanation for this discrepancy.

Heat Stability—The heat stability of Straub flavoprotein was one of its unusual qualities (2). Heating for 5 minutes at 70° destroyed only 10% of the activity of the purified enzyme. The KGDH flavoprotein was found to have equal heat stability (Table III). Heating for 5 minutes at 80° at pH 6.2 or pH 8.0 did not destroy the activity of either flavoprotein in either assay. Even after heating for 8 minutes at 90°, significant activity in both assays with either flavoprotein was retained.

Discussion

In the course of his elegant investigation of the physiological role of diaphorase, Massey found that DPNH-cytochrome c reductase and diaphorase could be isolated separately from the same particulate preparation of hog heart. He found further that diaphorase isolated by a more gentle method than that of Straub could transfer electrons from DPNH to lipoate. With the discovery that α-ketoglutaric dehydrogenase complex contains a flavoprotein, he proposed that the true role of diaphorase is as a member of the α-ketoglutaric dehydrogenase complex.

Simultaneous with this work, an investigation of the components and mechanism of α-ketoglutaric dehydrogenase complex was being carried on in our laboratory. Independently, it was found that digestion of the complex with trypsin would split it into two fractions, one of which was a flavoprotein with high diaphorase activity. The properties of this flavoprotein were so similar to the known properties of Straub diaphorase that further comparison of the two enzymes was undertaken. This has been of considerable value because of the conflicting views of Ziegler et al. (11). The properties of the two enzymes which have so far been documented are: (a) both have the same initial source; (b) both have the same prosthetic group (flavin adenine dinucleotide); (c) both catalyse the same reactions; (d) neither can be resolved with respect to flavin adenine dinucleotide with acid ammonium sulfate; (e) both precipitate between 50 and 60% of saturation with ammonium sulfate and both are soluble in the absence of salt; (f) both are extremely heat stable; (g) both are fluorescent; (h) they have identical spectra oxidized, reduced with DPNH, or reduced with dihydrolipoic acid; (i) they have the same Michaelis constant with lipoamide and the same turnover number in this reaction; (j) they have the same mobility on electrophoresis. The properties which are unique in this list are fluorescence and the spectrum obtained upon reduction with dihydrolipoic acid. We have yet to find a property by which the two flavoproteins may be distinguished.

The single discordant fact was the variation of the ratio of dihydrolipoyl dehydrogenase activity to diaphorase activity. Whether the flavoprotein was purified according to Straub or from α-ketoglutaric dehydrogenase complex, the dihydrolipoyl dehydrogenase activity was purified to a greater extent, particularly in the final stages. More recently it has been found that if the diaphorase activity is measured in the presence of cadmium ion, the results are more reproducible; and this ratio remains constant throughout purification (8, 10).

Summary

The flavoprotein component of α-ketoglutaric dehydrogenase has been compared with Straub diaphorase on the basis of its enzymatic, catalytic, and physical properties. To this time, we have been unable to find a property in which these two flavoproteins differ.

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References

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