# Effects of solubilisation on some properties of the membrane-bound respiratory enzyme D-amino acid dehydrogenase of *Escherichia coli*

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Solubilisation, delipidation and partial purification of the membrane-bound enzyme D-amino acid dehydrogenase of *Escherichia coli* K12 produced significant changes in several of its properties. Solubilised enzyme showed a broader substrate specificity, increased affinity for at least three substrates, and a lower pH optimum with D-alanine as substrate. Solubilised enzyme was more heat-labile than native enzyme, particularly at 37°C, and re-binding to envelope preparations restored protection against heat denaturation. Activity of delipidated enzyme could be increased by addition of pure phospholipids. Native enzyme showed biphasic Arrhenius kinetics associated with phase changes of membrane lipids.

*D-Amino acid* *Solubilisation* *Dehydrogenase* *Oxidase* *Membrane* *Integral*

## 1. INTRODUCTION

Several membrane-bound, respiration-linked flavoprotein dehydrogenases have been solubilised from *Escherichia coli* and characterised [1–5], but data on the effects of solubilisation on the catalytic and other properties of this class of enzymes is still fragmentary. Such information is of great potential interest as it should enable the evaluation of the contribution made by membrane integration to enzyme properties. In [6] we reported the solubilisation of the integral membrane enzyme D-amino acid dehydrogenase from envelopes of *E. coli* K 12; here, some properties of the membrane-integrated (native) and solubilised forms of the enzyme are compared, and the influence of phospholipids on enzyme activity is examined.

## 2. MATERIALS AND METHODS

### 2.1. *Organism and culture method*

*E. coli* K 12, strain AB259, was grown in 11 litre-batch cultures in a Microferm Laboratory Fermenter (New Brunswick) with forced aeration to $A_{550}$ 0.8–1.0, and harvested in a continuous flow separation unit (Alfa-Laval, Middlesex). The medium comprised (per litre): $(NH_4)_2SO_4$, 1 g; 1 M potassium phosphate buffer (pH 7.2), 20 ml; thiamine, 25 mg; yeast extract (Difco) 1 g; D,L-alanine, 3 g; and 5 ml of a salts solution as in [7]. Harvested cells were washed in 50 mM phosphate buffer (pH 7.2) and stored at −20°C.

### 2.2. *Preparation of envelope fractions and enzyme solubilisation*

Cells were broken in a Hughes press and envelopes prepared by differential centrifugation as in [6]. D-amino acid dehydrogenase in this envelope fraction is referred to subsequently as 'native enzyme'. D-amino acid dehydrogenase was solubilised by treatment of envelopes with Triton X-100 (0.5 mg/mg envelope protein). Removal of Triton X-100 from the enzyme solution and partial purification of the enzyme was effected by precipitation with $(NH_4)_2SO_4$, and chromatography with Sephadex G-200 and Biobeads (Bio-Rad) as in [6]. This preparation is lipid-free and is subsequently referred to as 'solubilised enzyme'.

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2.3. *Assays*

D-amino acid dehydrogenase was measured by following the reduction of 2,6-dichlorophenol-indophenol with phenazine methosulphate as intermediate electron carrier as in [7]. Protein estimation was as in [8].

## 3. RESULTS

### 3.1. *Substrate specificity*

Native, membrane-bound D-amino acid dehydrogenase is most active with D-alanine as substrate. Solubilisation, and partial purification increased its specific activity with D-alanine by a factor of 9.8. Table 1 shows the effect of enzyme solubilisation on the relative maximum velocities (relative to D-alanine, and henceforth to be referred to as relative activities) obtained with a selection of 11 substrates. The general effect of solubilisation of D-amino acid dehydrogenase was an increase in the relative activities of all substrates. For most substrates the increase was between 1.5- and 6-fold, though in the cases of arginine and allohydroxy-D-proline it was about 10-fold, and D-α-amino-*iso*-butyrate had no detectable activity with the native enzyme, but 10% relative activity with solubilised enzyme. D-Methionine replaced D-alanine as the most active substrate. $K_m$-Values of the native and solubilised enzymes for 4 of the most active substrates were determined from Lineweaver Burk plot (table 1). Affinity of the enzyme for D-alanine, D-asparagine and D-α-amino-*n*-butyrate was significantly increased by solubilisation, particularly the latter substrate where the increase was about 25-fold.

Table 1

Effect of solubilisation on substrate specificity of D-amino acid dehydrogenase

| Substrate | Relative activity (%) with: Native enzyme | Solubilised enzyme |
|---|---|---|
| D-Alanine | 100 (30 mM) | 100 ( 6.25 mM) |
| L-Alanine | 1.2 | 7 |
| D-α-Alanine methyl ester | 35 | 50 |
| D-α-Amino-*n*-butyrate | 38 (25 mM) | 80 ( 0.86 mM) |
| D-Asparagine | 26 (24 mM) | 60 (12.2 mM) |
| D-Valine | 6 | 18 |
| D-Serine | 20 | 50 |
| D-Methionine | 35 (50 mM) | 130 (50 mM) |
| Allohydroxy-D-proline | 2.1 | 26 |
| D-α-Amino-isobutyrate | 0 | 10 |
| D,L-Arginine | 3.8 | 36 |
| D-Histidine | 8.5 | 32 |

Actual specific activities of native and solubilised enzymes with D-alanine as substrate, were 36 and 354 nmol.min$^{-1}$.mg protein$^{-1}$, respectively, ($K_m$)

### 3.2. *pH optimum*

With D-alanine as substrate, pH optimum was 8.9 for native enzyme, and 7.9 for solubilised enzyme. The pH/activity profiles were similar, both showing a broad peak and rapid fall off of activity on the alkaline side of optimum.

### 3.3. *Effect of temperature on enzyme stability*

To ascertain the contribution made by membrane association to the thermal stability of D-amino acid dehydrogenase, native and solubilised preparations were held at 25, 37 and 45°C, and time samples assayed for enzyme activity (fig.1). Solubilisation was found to increase the thermolability of the enzyme significantly. Maximum effect was seen at 37°C where, after 10 min, the solubilised enzyme had lost 90% of initial activity whereas the native enzyme had lost only 20%. The effect of solubilisation was less marked at 25 and 45°C.

Solubilised D-amino acid dehydrogenase has been shown to bind to envelope preparations of the type used here with concomitant restoration of D-alanine-stimulated cytochrome reduction and oxidase activity. It has been argued that about 50% of the newly-bound dehydrogenase occupies its physiological position in the membrane bilayer [6]. Fig.2 compares the decay kinetics at 37°C of such newly-bound enzyme with those of native and solubilised preparations. The newly-bound enzyme is clearly less heat-labile than the solubilised enzyme, and the curved decay plot is more similar to that of the native enzyme. This indicates that integration of the enzyme within the membrane bilayer confers direct protection against thermal denaturation at 37°C.

### 3.4. *Effect of lipids on enzyme activity*

As the above results indicate that membrane in-

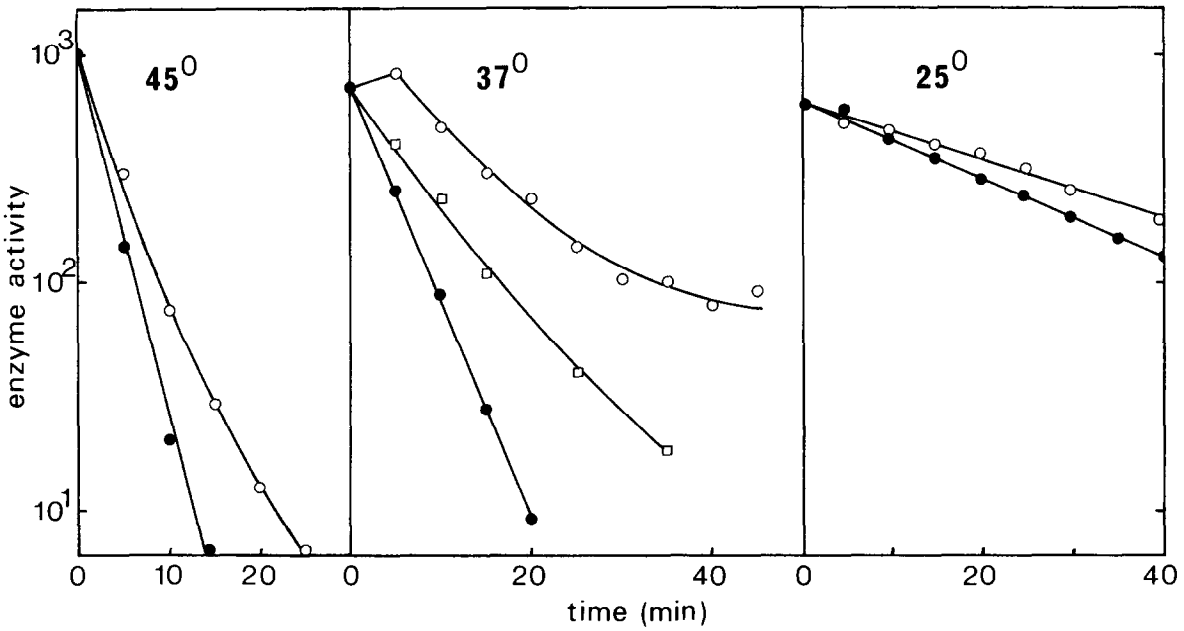
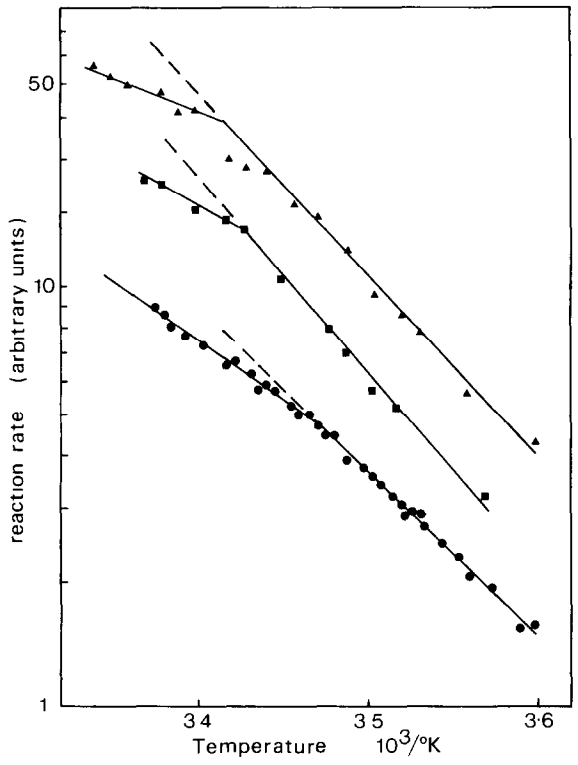



Fig.1. Temperature inactivation of D-amino acid dehydrogenase. Enzyme preparations were held at the temperatures indicated and time samples were assayed at 20°C: (○—○) membrane-bound (native) enzyme; (●—●) solubilised enzyme; (□—□) solubilised enzyme bound to membrane preparation. Enzyme activity is expressed in arbitrary units.




tegration influences enzyme properties, and as this influence is likely to be lipid-mediated, it seemed essential to demonstrate directly an interaction between D-amino acid dehydrogenase and membrane lipids. In some membrane-bound enzymes, association with the lipids of the bilayer can be detected as a biphasic Arrhenius plot in which a 'break' occurs at the temperature of the lipid–phase transition. In fig.2, the Arrhenius kinetics of native D-amino acid dehydrogenase from cells grown at 19°C and 37°C, and of solubilised enzyme (without removal of Triton X-100 or any purification) from cells grown at 37°C are shown. Native enzyme (37°C growth) showed a break in the plot at 20°C whereas in native enzyme (19°C growth) it was at 15°C, reflecting the greater degree of unsaturation and hence lower melting point of membrane lipids in cells grown at lower temperatures.

Fig.2. Arrhenius plots of D-amino acid dehydrogenase: (▲—▲) membrane-bound (native) enzyme; (■—■) solubilised enzyme, both from cells grown at 37°C; (●—●) membrane-bound (native) enzyme from cells grown at 19°C.

Table 2

Effect of addition of phospholipids to solubilised, delipidated D-amino acid dehydrogenase

| Lipid added | Relative activity (%) |
|---|---|
| None | 100 |
| Phosphatidyl glycerol (1.0) | 140 |
| Diphosphatidyl glycerol (1.0) | 150 |
| Phosphatidyl choline (1.0) | 180 |
| Phosphoglyceric acid (8.0) | 129 |
| Cardiolipin (10.0) | 189 |

Dry phospholipids were suspended in phosphate buffer (50 mM, pH 7.2) by sonication and added to enzyme solution + $MgCl_2$ (10 mM) to give concentrations (mg lipid/mg protein) shown in parentheses. Activities were determined after incubation of 5 min. Each cuvette contained 0.075 mg protein. Enzyme without phospholipid addition had spec. act. 38 $nmol.min^{-1}.mg\ protein^{-1}$

As with L-phenylalanine dehydrogenase [9] a break in the plot also occurred with solubilised enzyme, indicating a continued association between enzyme and lipids in Triton X-100 micelles. These results are consistent with the existence of a strong hydrophobic protein–lipid interaction between D-amino acid dehydrogenase and the *E. coli* cytoplasmic membrane.

The importance of lipids in D-amino acid dehydrogenase activity was directly demonstrated by addition of pure lipids to solubilised enzyme which had been delipidated and partially purified as described in section 2. Results (table 2) show that phosphatidyl glycerol, diphosphatidyl glycerol, phosphatidylcholine, phosphoglyceric acid and cardiolipin, produced increases of 40–90% in dehydrogenase activity in the presence of 10 mM $Mg^{2+}$. The effect of the first 3 lipids was reduced by a factor of about 3, if $Mg^{2+}$ was omitted. The effect of $Mg^{2+}$-omission was not tested with the other two lipids. Magnesium on its own did not increase enzyme activity. Presumably, $Mg^{2+}$ play a role in the neutralisation of negative charges on the phospholipids which may facilitate association with the enzyme.

## 4. DISCUSSION

We have shown that association with phospholipids stimulate the activity of D-amino acid dehydrogenase and this interaction is reflected in biphasic Arrhenius plots obtained with native, membrane-bound enzyme preparations. Presumably it is the removal of phospholipid interaction which results in changes of substrate specificity and affinity, pH optima and thermolability when the enzyme is solubilised. We suggest, therefore, that although the primary function of membrane integration may be to link the enzyme to the respiratory chain, it also has an essential role in the physiological optimisation of other catalytic properties of the enzyme and stabilisation of its optimum conformation. Solubilisation of D-lactate dehydrogenase produces changes in $K_m$ and pH optima, and a small relaxation of substrate specificity [1], but some other flavoprotein dehydrogenases do not show this behaviour [2–4]. Limitation of its activity by the influence of the membrane bilayer may be particularly important for an enzyme of such potentially broad substrate specificity as D-amino acid dehydrogenase.

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