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THE CHARACTERISATION OF INDUCIBLE DEHYDROGENASES SPECIFIC FOR THE OXIDATION OF D-ALANINE, ALLOHYDROXY-D-PROLINE, CHOLINE AND SARCOSINE AS PERIPHERAL MEMBRANE PROTEINS IN *PSEUDOMONAS AERUGINOSA*

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Summary

The interaction with the cytoplasmic membrane of the inducible, membrane-bound, cytochrome-linked dehydrogenases specific for the oxidation of D-alanine, allohydroxy-D-proline, choline and sarcosine in *Pseudomonas aeruginosa* was investigated. The susceptibility of D-alanine dehydrogenase to solubilisation by cation depletion or by washing with high ionic strength buffers indicated that it was a peripheral membrane protein. The effect of various divalent cations in reducing the amount of enzyme released by cation depletion suggests a requirement for Mg^{2+} in the binding of D-alanine dehydrogenase to the cytoplasmic membrane. The peripheral nature of all four dehydrogenases was confirmed by examination of the molecular properties and phospholipid content of preparations of the enzymes solubilised with 1 M phosphate buffer (pH 7.0). Additional confirmatory evidence was provided by Arrhenius plots of membrane-bound activity of D-alanine and allohydroxy-D-proline dehydrogenases which were monophasic and independent of the discontinuities attributable to membrane lipid phase separations which characterise such plots of the activity of integral membrane-bound enzymes. The shape of the Arrhenius plots obtained for the activities of known integral respiratory proteins of *P. aeruginosa* suggests that these enzymes may remain in a fluid environment throughout the course of the phase separation.

Introduction

The utilisation of any compound as a source of carbon and energy involves its sequential oxidation by enzymic catalysis. Although oxygenases, hydroxyl-

Abbreviations: EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid; DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulphate.

ases, and oxidases are frequently involved in this process, the majority of growth substrates are oxidised by dehydrogenases at one or more steps in their catabolism. This fact is reflected in the wide variety of dehydrogenases which have been detected in nutritionally versatile groups of bacteria. Many of the dehydrogenases characterised in members of the genus *Pseudomonas* have been shown to be associated closely with the cytoplasmic membrane and a large number are independent of pyridine nucleotide cofactors. One such group is the D-amino acid dehydrogenases found in *Escherichia coli*, *Salmonella typhimurium*, and various *Pseudomonas* species which are all inducible, membrane-bound, cytochrome-linked enzymes [1]. These dehydrogenases are all functionally similar and differ mainly in respect to substrate specificity.

In comparison with the constitutive, membrane-bound dehydrogenases of the respiratory chains of mitochondria and bacteria (e.g. succinate dehydrogenase, NADH dehydrogenase), the membrane binding properties of the inducible dehydrogenases of bacterial membranes have received little attention. Both succinate and NADH dehydrogenases fall within Singer's definition of integral proteins [2,3], the distinction between integral and peripheral proteins being deduced from differences in resistance to solubilisation by various processes and in molecular properties of the solubilised protein molecules. It is improbable that the membrane attachment of integral proteins would show the necessary specificity or saturability [3] to explain Kung and Henning's [4] observation that inducible dehydrogenases of *E. coli* compete for membrane binding sites and such enzymes are more likely to be peripheral membrane proteins.

This report deals with the membrane-binding properties of two D-amino acid dehydrogenases of *Pseudomonas aeruginosa* which are known to be inducible, membrane bound, and cytochrome linked: these are D-alanine dehydrogenase [5], and allohydroxy-D-proline dehydrogenase [6]. The effects of various solubilising agents upon the two enzymes are assessed and the properties of the solubilised molecules are discussed. The suggestions made concerning the nature of the enzyme-membrane interaction are supported by evidence based upon comparisons between Arrhenius plots of membrane-bound D-amino acid dehydrogenase activity and similar plots of the activity of succinate dehydrogenase. Also included are some observations on two other enzymes, choline and sarcosine dehydrogenases, originally described in *Pseudomonas reptilovora* [7]; these have subsequently been shown to be inducible, membrane-bound, and cytochrome-linked in *P. aeruginosa* (Bater, A.J., unpublished ohservations).

Some of these results were presented to the Society for General Microbiology in September 1975 [8].

Methods and Materials

Organism and culture conditions. The bacterium used was *P. aeruginosa* PAO 1 (ATCC 15692) [9]. The growth media used and the conditions for growth and harvesting were as previously described [4,5]. For cell preparations induced for D-alanine dehydrogenase, allohydroxy-D-proline dehydrogenase, choline dehydrogenase or sarcosine dehydrogenase, the cells were grown on minimal medium containing as sole source of carbon and energy DL-alanine,

L-hydroxyproline, choline chloride, or sarcosine, respectively, all at a final concentration of 10 mM. For uninduced cell preparations 10 mM sodium succinate was used as the sole carbon and energy source.

Cell breakage and fractionation. Cells harvested at the mid-exponential phase of growth were washed twice in cold 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM $MgSO_4$ (buffer M) and sedimented by centrifugation at 12000 × *g* for 15 min at 4° C. Cells were broken with a Hughes Press at —25° C and $2 \cdot 10^7$ kg/m^{-2}. The broken cell paste was homogenised with an equal volume of cold buffer M containing 1 mg/ml DNAase (Sigma Type 1) and freed of unbroken cells by centrifugation at 12000 × *g* for 15 min at 4° C. This cell-free extract was centrifuged at 256000 × *g* for 2 h at 4° C in an MSE 65 ultracentrifuge. The supernatant was discarded and the upper, red layer of the pellet resuspended in several volumes of cold buffer M and recentrifuged under identical conditions. This purification step was repeated once more before resuspending the final membrane preparation in a small volume of 50 mM potassium phosphate buffer (pH 7.0) (buffer P).

Enzyme assays. Dehydrogenase activities were measured by following the reduction of 2,6-dichlorophenolindophenol (DCIP) at 600 nm in a recording spectrophotometer. Test cuvettes contained 100 µmol phosphate buffer (pH 7.0), 30 µmol KCN, 0.3 µmol DCIP, 1.7 µmol phenazine methosulphate, 100 µmol substrate, and enzyme plus water to a total of 3.0 ml. Activities were calculated using an extinction coefficient for DCIP of $21 \cdot 10^3$ mol^{-1} · cm^{-1}, and expressed as nmol DCIP reduced · min^{-1} · ml^{-1}.

Oxidase assays were performed by following the decrease in oxygen concentration with a Clark type Pt/Ag electrode coupled to a Servoscribe potentiometric recorder. The incubation mixture, which was maintained at 30° C, contained 50 µmol phosphate buffer (pH 7.0), 100 µmol substrate and enzyme plus water to a total of 1.0 ml. Activities were calculated from the known oxygen content of air-saturated buffer at 30° C and expressed as nmol oxygen utilised · min^{-1} · ml^{-1}.

Catalase was estimated by a method described by Sigma U.K. Ltd. A substrate solution was prepared by adding 0.1 ml 30% H_2O_2 to 50 ml of buffer P and adjusting the absorbance at 240 nm to between 0.52 and 0.55 by the addition of extra buffer or H_2O_2 as appropriate. 0.1 ml enzyme preparation was added to 2.9 ml substrate solution and the time taken for the A_{240nm} to drop from 0.45 to 0.40 was noted; this corresponds to a decomposition of 3.45 µmol H_2O_2 in the 3 ml solution. Activity in the original enzyme preparation is given by (3.45/min required) 30 units/ml.

NADH oxidase was measured by following the decrease absorbance at 340 nm of a 33 µg/ml solution of NADH in buffer P. Activity was calculated using an extinction coefficient for NADH at 340 nm of $6.22 \cdot 10^3$ mol^{-1} · cm^{-1} and expressed as µmol NADH oxidised · min^{-1} · ml^{-1}.

Cytochrome oxidase was assayed by following the decrease in absorbance at 550 nm of a 333 µg/ml solution of cytochrome *c* in buffer P. The cytochrome solution was reduced before use by the addition of a small crystal of $NaBH_4$. Activity was calculated using an extinction coefficient for cytochrome *c* at 550 nm of $18.5 \cdot 10^3$ mol^{-1} · ml^{-1} and expressed as µmol cytochrome *c* oxidised · min^{-1} · ml^{-1}.

All assays requiring controlled temperatures were carried out in an SP1800 recording spectrophotometer (Pye-Unicam) equipped with a jacketed cuvette holder through which water was pumped from a controlled-temperature water bath. Where necessary the latter was cooled below room temperature by placing in it a stainless steel heat exchanger coil through which iced water was passed. The temperature in the sample cuvette was measured before and after each assay. Only assays which showed no temperature change throughout the recording period were used in the subsequent calculations.

Protein was estimated by the method of Lowry et al. [10] using bovine serum albumin as a standard.

Solubilisation treatment. Aliquots of membrane preparations were diluted to suitable protein concentration with either buffer P or buffer M as appropriate. Sufficient solubilising agent was added to give the required final concentration and the membrane suspension was then homogenised gently with a manual teflon-glass homogeniser. After standing at room temperature for 20 min the suspensions were centrifuged at 256000 × *g* for 2 h in an MSE 65. The supernatant was decanted and the pellet made up to the original volume with the appropriate buffer or with fresh solubilising agent if the extraction was to be repeated. The degree of solubilisation was assessed by assaying both supernatant and pellet fractions for enzyme activity and/or protein concentration.

Density gradient analysis of solubilised dehydrogenase. The method used was that of Martin and Ames [11] as adapted by Oltmann et al. [12]. 0.4 ml of a suitable membrane extract containing the appropriate dehydrogenase was mixed with 0.1 ml buffer P containing 10 units of bovine liver catalase. The mixed enzyme solution was layered onto a preformed 9.5 ml linear sucrose density gradient made up in buffer P. The gradients were centrifuged at 98000 × *g* for 20 h at 4°C in a 3 × 10 ml swing-out rotor in an MSE 50 ultracentrifuge. 0.4-ml fractions were collected automatically using an ISCO Density Gradient Fractionater. The fractions were assayed for dehydrogenase and catalase activities. The sucrose concentration in each fraction was determined with a 0—28% sugar refractometer (Bellingham and Stanley, London). Enzyme activities and sucrose concentration were plotted against fraction number.

Molecular weights were calculated by comparison of the sedimentation of the unknown (the dehydrogenases) with that of the internal standard (catalase) [11]. The distances sedimented from the sample: density gradient interface by catalase and by the dehydrogenase were determined from the graphs. Sedimentation coefficients were calculated from the formula.

$$\frac{S^0_{20,w}\text{unknown}}{S^0_{20,w}\text{standard}} = \frac{\text{distance sedimented by unknown}}{\text{distance sedimented by standard}}$$

Assuming bovine liver catalase to have an $S^\circ_{20,w}$ of 11.4 and a molecular weight of 240000 [12], the molecular weights of the dehydrogenases were calculated from the formula.

$$\frac{S^0_{20,w}\text{unknown}}{S^0_{20,w}\text{standard}} = \left[\frac{\text{molecular weight of unknown}}{\text{molecular weight of standard}}\right]^{2/3}$$

assuming similar partial specific volumes for both molecules.

Phospholipid analysis. Phospholipids were extracted from membranes or

enzyme solutions with chloroform/methanol (1 : 2, v/v) using the method of Johnson [13]. The extracts were evaporated to dryness under nitrogen and the residue dissolved in 0.25 ml of chloroform/methanol (1 : 2, v/v). 5-μl samples were applied to thin-layer chromatography plates of silica gel G and developed by ascending flow of chloroform/ethanol (2 : 1, v/v). Sample spots were detected by spraying with 2′,7′-dichlorofluorescein (2% ethanolic solution) and examined under ultraviolet light. Suitably diluted solutions of L-α-phosphatidylcholine and L-α-phosphatidylethanolamine in chloroform were used as phospholipid standards. Oleic acid was used as a fatty acid standard.

Chemicals. All enzymes, cofactors, enzyme substrates, detergents, phospholipids, and electron acceptors were obtained from Sigma U.K. Ltd. All other chemicals were reagent grade or better from B.D.H., Poole, Dorset.

Results

Effect of detergents on D-alanine dehydrogenase

Membrane preparations from cells of *P. aeruginosa* PAO 1 grown on minimal medium plus alanine (alanine-membranes) were treated with several detergents and the effects upon D-alanine dehydrogenase were assessed. Membrane preparations were diluted to 9 mg/ml with buffer M and 1-ml aliquots homogenised with sufficient detergent to give a final concentration of either 0.1 or 1.0 mg detergent/mg membrane protein. After incubation and centrifugation as described in Methods and Materials the supernatant and pellet fractions were both dialysed for 18 h against 50 volumes buffer M at 4°C. Both fractions were assayed for protein concentration and for D-alanine dehydrogenase activity. Table I lists the distribution and recovery of enzyme activity following treat-

TABLE I

SOLUBILISATION OF D-ALANINE DEHYDROGENASE BY VARIOUS DETERGENTS

Detergent concentration is expressed as mg detergent/mg membrane protein; membrane suspensions contained approx. 1 mg/ml protein during treatment. The enzyme activities are given as nmol DCIP reduced · min^{-1} · ml^{-1} and their distribution as a percentage of the total (particulate plus soluble) activity recovery was calculated as a percentage of the total activity of the control (no detergent).

Detergent	Concentration	Dehydrogenase activity		Distribution		Recovery (%)
		Particulate	Soluble	Particulate (%)	Soluble (%)	
None	—	2.76	0.11	96.2	3.8	100.0
Deoxycholate	0.1	2.79	0.11	96.2	3.8	101.0
	1.0	0.01	0.01	—	—	0.1
Cholate	0.1	2.64	0.08	97.1	2.9	94.7
	1.0	0.01	0.01	—	—	0.1
Dodecyl sulphate	0.1	2.61	0.11	96.0	4.0	94.8
	1.0	0.01	0.01	—	—	0.1
Triton X-100	0.1	2.77	0.20	93.3	6.7	103.5
	1.0	0.14	2.70	4.9	95.1	98.9
Brij 56	0.1	1.60	1.15	58.2	41.8	95.8
	1.0	0.36	1.82	16.5	83.5	75.9

ment with three anionic detergents and with two non-ionic detergents.

Whereas the enzyme was almost completely inactivated by the three ionic detergents, the activity was stable during treatment with non-ionic detergents; in particular, extraction of membranes with 1 mg Triton X-100/mg protein solubilised 95% of the dehydrogenase with a recovery of 98.9% of the activity. The results of a more detailed study of the effect of Triton X-100 are shown in Figs. 1—3. Alanine-membranes were treated as above with various concentrations of the detergent from 0.2 to 1.6 mg Triton X-100/mg protein and the ensuing fractions were assayed for total protein, for D-alanine dehydrogenase and D-alanine oxidase activities, and for the activities of succinate oxidase and succinate dehydrogenase (a known integral protein [14]). The excess of dehydrogenase activity over oxidase activity observed in Figs. 2 and 3 can be accounted for by the difference in stoichiometry of the assay reactions, and by the fact that the turnover numbers for dehydrogenases are often higher than those for other respiratory components [14]. The latter also explains why some decrease in dehydrogenase levels is possible without a concomitant drop in oxidase activity. The fall in particulate D-alanine dehydrogenase activity was accompanied by a corresponding rise in soluble activity; in contrast, no soluble succinate dehydrogenase activity was detectable.

Attempts were made to remove the Triton X-100 from the solubilised D-alanine dehydrogenase by several methods. Extensive dialysis or ultrafiltration were both ineffective in reducing the detergent concentration as measured by the method of Garewal [15], an assay specific for polyethoxy-type detergents.

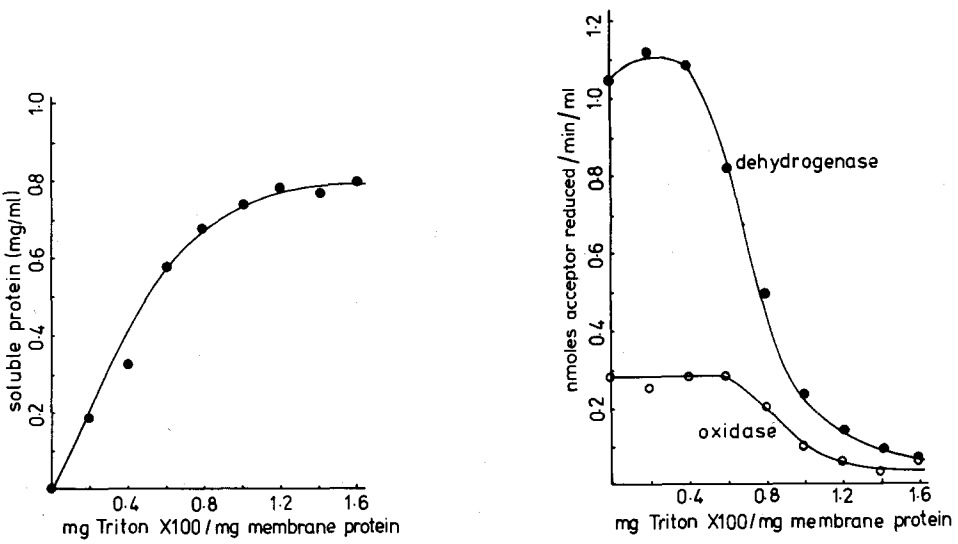

Fig. 1. Solubilisation of total membrane protein by treatment with Triton X-100. Conditions were as described in Methods and Materials. The protein concentration during treatment was 1 mg/ml.

Fig. 2. Solubilisation of succinate dehydrogenase by treatment with Triton X-100. Conditions were as described in Methods and Materials. The protein concentration during treatment was 1 mg/ml. Particulate succinate dehydrogenase (●———●) and succinate oxidase (○———○) were assayed with DCIP and molecular oxygen, respectively, as terminal electron acceptors. No soluble succinate dehydrogenase activity was detectable.

Although the ratio of detergent to protein could be reduced by approx. 20% by a single pass through a 15 × 1 cm column of Sephadex G-15, further treatment did not remove any extra Triton X-100.

Effects of cation depletion on D-alanine dehydrogenase

Using the general methods described for detergent treatment above, alanine-membranes were washed repeatedly with either 50 mM Tris · HCl buffer (pH 7.0) or 50 mM potassium phosphate buffer (pH 7.0; buffer P), both being free of inorganic, polyvalent cations. After five washes the particulate residue was resuspended to the original volume with the appropriate washing buffer. Each fraction was assayed for D-alanine dehydrogenase activity. Table II shows the results expressed as the percentage of the total recovered activity present in each wash or residue; at least 95% recovery was obtained in each case. With both buffers 20—30% of the initial activity was released into the third wash and 60—70% was solubilised overall. When the wash buffers contained 10 mM $MgSO_4$, no D-alanine dehydrogenase activity was detectable in any of successive washings, while approx. 100% of the initial activity was recovered in the final pellet. Lower concentrations of Mg^{2+} are effective in preventing the solubilisation of D-alanine dehydrogenase by repeated washing with dilute, neutral buffers as no measurable activity was present in any of three successive washes of a similar membrane suspension to that used above with buffer M, which contains only 1.0 mM $MgSO_4$.

The specificity of the requirement for the divalent cation involved in the enzyme-membrane interaction was investigated with the use of chelating agents; EDTA and EGTA, which have greatest affinity for Mg^{2+} and Ca^{2+},

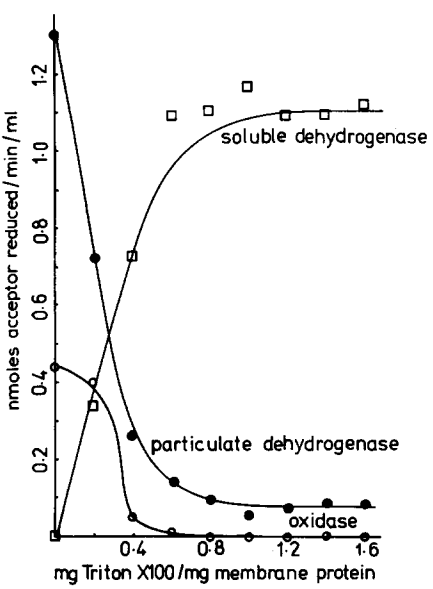

Fig. 3. Solubilisation of D-alanine dehydrogenase by treatment with Triton X-100. Conditions were as described in Methods and Materials. The protein concentration during treatment was 1 mg/ml. Soluble (□——□) and particulate (●——●) forms of D-alanine dehydrogenase and D-alanine oxidase (○——○) were assayed with DCIP and molecular oxygen, respectively, as terminal electron acceptors.

TABLE II

SOLUBILISATION OF D-ALANINE DEHYDROGENASE BY WASHING WITH AQUEOUS, NEUTRAL BUFFERS

Distributions of activity are expressed as percentage of the sum of the particulate activity plus the activity present in the five soluble fractions. Recoveries are the percentage of the activity present in the suspension prior to the first wash.

Buffer	Distribution of activity						Recovery (%)
	1st wash	2nd wash	3rd wash	4th wash	5th wash	Particulate residue	
50 mM phosphate, pH 7.0	6.2	11.2	28.7	13.1	10.3	30.3	98.7
50 mM phosphate, pH 7.0, plus 10 mM $MgSO_4$	0.0	0.0	0.0	0.0	0.0	100.0	100.4
1 M phosphate, pH 7.0	40.0	7.1	9.3	6.8	4.3	32.1	98.3
50 mM Tris · HCl, pH 7.0	4.0	16.5	29.3	6.8	5.0	38.1	97.2
50 mM Tris · HCl, pH 7.0, plus 10 mM $MgSO_4$	0.0	0.0	0.0	0.0	0.0	100.0	99.4
1 M Tris · HCl, pH 7.0	56.8	11.5	7.1	2.8	4.0	17.5	32.9

respectively, were utilised. Alanine-membranes were resuspended in 50 mM Tris · HCl at the required pH. Chelating agents were added to a final concentration of 10 mM with or without divalent cations also at 10 mM. A single extraction was carried out under the standard conditions, the pellet being resuspended in the appropriate extraction medium.

The results (Table III) demonstrate that Mg^{2+} are able to fulfill the requirement for a divalent cation. Although these results do not preclude the involvement of other common divalent cations, e.g. Zn^{2+}, Fe^{2+}, Mn^{2+}, Mo^{2+}, they show that Ca^{2+} is probably not active in this system.

Effects of high ionic strength on D-alanine dehydrogenase

When alanine-membranes were washed with 1 M Tris · HCl (pH 7.0) or with 1 M potassium phosphate buffer (pH 7.0), a significant proportion of the total recoverable activity was released in the first wash. Although a significant solubilisation of activity by 1 M Tris · HCl was observed (Table II) this was accompanied by a very poor (approx. 30%) recovery of activity. This loss of activity was irreversible as attempts to reduce the Tris · HCl concentration by dialysis or by ultrafiltration (Amicon 10 ml stirred ultrafiltration cell equipped with a UM20E membrane under $3.5 \cdot 10^4$ kg/m^2 nitrogen gas) both failed to yield enzyme preparations with increased activity.

As shown in Table II, 40% of the total D-alanine dehydrogenase activity was solubilised by a single wash with 1 M phosphate buffer (pH 7.0). In this case the recovery was 98.3%. The ionic strength of 1 M phosphate buffer is 6.0, which is much greater than that of the standard buffers used in this work, buffer P ($I = 0.3$) and buffer M ($I = 0.306$).

The effect of pH on extraction of D-alanine dehydrogenase by molar phosphate buffer was investigated. The pellet from the centrifugation step were resuspended in buffer P and the supernatants were dialysed overnight against buffer P before assay. Fig. 4 shows that the release of D-alanine dehydrogenase activity is more or less independent of pH. The progressive loss of recoverable

TABLE III

SOLUBILISATION OF D-ALANINE DEHYDROGENASE BY CHELATING AGENTS

Membrane suspensions contained approx. 1 mg/ml protein during treatment. Distribution of activity is expressed as percentage of total activity in each fraction. Recovery (percent of pH 7.0 control) was at least 95% in each case.

Chelator (10 mM)	Cation (10 mM)	pH (Tris · HCl, 50 mM)	Distribution of dehydrogenase activity	
			Particulate	Soluble
—	—	7.0	96.1	3.9
—	—	8.0	95.2	4.8
—	Mg^{2+}	8.0	96.9	3.1
—	Ca^{2+}	8.0	95.5	4.5
EDTA	—	7.0	95.0	5.0
EDTA	—	8.0	74.8	25.2
EDTA	Mg^{2+}	8.0	89.9	10.1
EDTA	Ca^{2+}	8.0	79.8	20.2
EGTA	—	7.0	96.4	3.6
EGTA	—	8.0	94.6	5.4

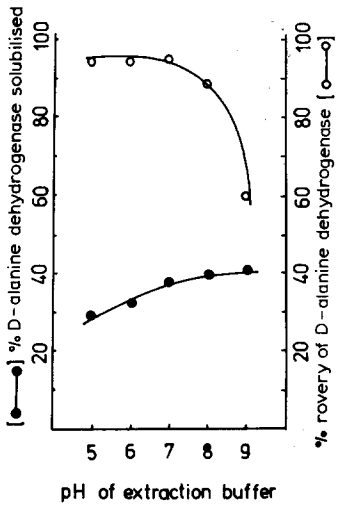

Fig. 4. Extraction of D-alanine dehydrogenase into a single wash with 1 M phosphate buffer at various pH values. Conditions were as described in Methods and Materials. Protein concentration during washing was 1 mg/ml. Pellets were resuspended in buffer P and supernatants were dialysed against buffer P before assay. Soluble D-alanine dehydrogenase (●———●) is expressed as a percentage of the total activity recovered after each extraction. Recovery (○———○) is quoted as the percentage of the activity recovered from a control sample washed once with buffer M.

activity, which becomes significant in the more alkaline buffers reflects the observation made by Pioli et al. [5] that D-alanine dehydrogenase activity is unstable at higher pH values.

Solubilisation of inducible dehydrogenases with 1 M phosphate (pH 7.0)

The extraction with 1 M phosphate buffer (pH 7.0) of other dehydrogenases and respiratory components of *P. aeruginosa* was compared. Membranes from cells grown on alanine, hydroxyproline, choline, sarcosine, or succinate were subjected to a single wash under the standard conditions and the resulting soluble and particulate fractions were assayed for various appropriate respiratory enzymes. Whereas between 20 and 45% of each inducible dehydrogenase was solubilised under these conditions, (as shown in Table IV) there was no decrease in the particulate levels of NADH oxidase or cytochrome oxidase and

TABLE IV

SOLUBILISATION OF INDUCIBLE DEHYDROGENASES BY 1 M PHOSPHATE BUFFER, pH 7.0

Membrane preparations of cells grown on the appropriate inducing carbon source were suspended to 1 mg/ml protein in 1 M phosphate buffer, pH 7.0. The percentage solubilised value represents that activity remaining in the supernatant after centrifugation; values quoted are the means of three separate determinations (range of individual values in parentheses). Recovery was at least 95% in each case.

Dehydrogenase	Percentage solubilised
D-Alanine	38.4 (29.4—45.6)
Allohydroxy-D-proline	34.6 (29.2—41.8)
Choline	27.8 (20.3—34.7)
Sarcosine	31.7 (26.1—38.9)

significant amounts of neither succinate dehydrogenase (less than 3%) nor cytochromes (undetectable) were released into solution.

Characterisation of the solubilised dehydrogenase

The fact that D-alanine dehydrogenase, allohydroxy-D-proline dehydrogenase, choline dehydrogenase, and sarcosine dehydrogenase can be solubilised in significant amounts by washing with a high ionic strength, neutral buffer supports the hypothesis that they might be peripheral membrane proteins as defined by Singer [3]. Experiments were accordingly designed to test solubilised preparations of the four inducible dehydrogenases listed above for possession of the two other properties of peripheral proteins discussed by Singer [3], viz. truly soluble or molecularly dispersed in neutral aqueous buffers, and essentially free of lipids in solution.

The solubility properties of the solubilised enzymes were investigated by sucrose density gradient centrifugation using a soluble marker enzyme as an internal standard. Before centrifugation, the 1 M phosphate buffer extracts were ultrafiltered under $3.5 \cdot 10^4$ kg/m^2 nitrogen through a PM-10 membrane in a stirred 10 ml ultrafiltration cell (Amicon); ultrafiltration was continued until the phosphate concentration was 50 mM and the enzyme had been concentrated 10-fold. Figs. 5 and 6 show the sedimentation profiles for 1 M phosphate-solubilised dehydrogenase preparations cocentrifuged with bovine heart catalase. Using the formulae quoted in Methods and Materials the distances sedimented from the buffer/sucrose interface by the inducible dehydrogenases were converted into values of $S^\circ_{20,w}$ and molecular weight; these values are listed in Table V.

Figs. 5a and 6a—6c demonstrate that the solubilised dehydrogenases sediment as more or less symmetrical bands, a mark of uniform molecular dispersal, and this clearly shows that the dehydrogenases are in true solution. Of five separate determinations of sedimentation behaviour performed on D-alanine dehydrogenases, three showed single, fairly symmetrical peaks with an $S^\circ_{20,w}$ of 5.7 (Fig. 5a), one showed a single, slightly negatively skewed peak with an $S^\circ_{20,w}$ of 8.5 (Fig. 5b), while the fifth showed a major peak with an $S^\circ_{20,w}$ of 8.5 (Fig. 5c). The corresponding molecular weights calculated from these sedimentation coefficients are 85000 and 156000 for the "light" and "heavy" forms, respectively. This suggests that the heavy peak represents a molecular species comprising twice as many monomeric units as the light peak.

In order to check for lipid association with the solubilised dehydrogenase molecules, an ultrafiltered preparation of 1 M phosphate buffer-solubilised D-alanine dehydrogenase containing catalase was preincubated with 1 mg/ml phospholipase C prior to layering onto sucrose density gradients; centrifugation and fractionation then proceeded as before. (The ability of the phospholipase C preparation to lyse PAO 1 sphaeroplasts indicates that the enzyme is able to degrade PAO 1 membrane lipids). The sedimentation profile of the phospholipase-treated sample was indistinguishable from that of the phospholipase-free control, and was similar to Fig. 5a.

The lack of an effect of phospholipase C suggests that solubilised D-alanine dehydrogenase is not associated with significant amounts of phospholipids. This conclusion was supported by the findings of thin-layer chromatographic

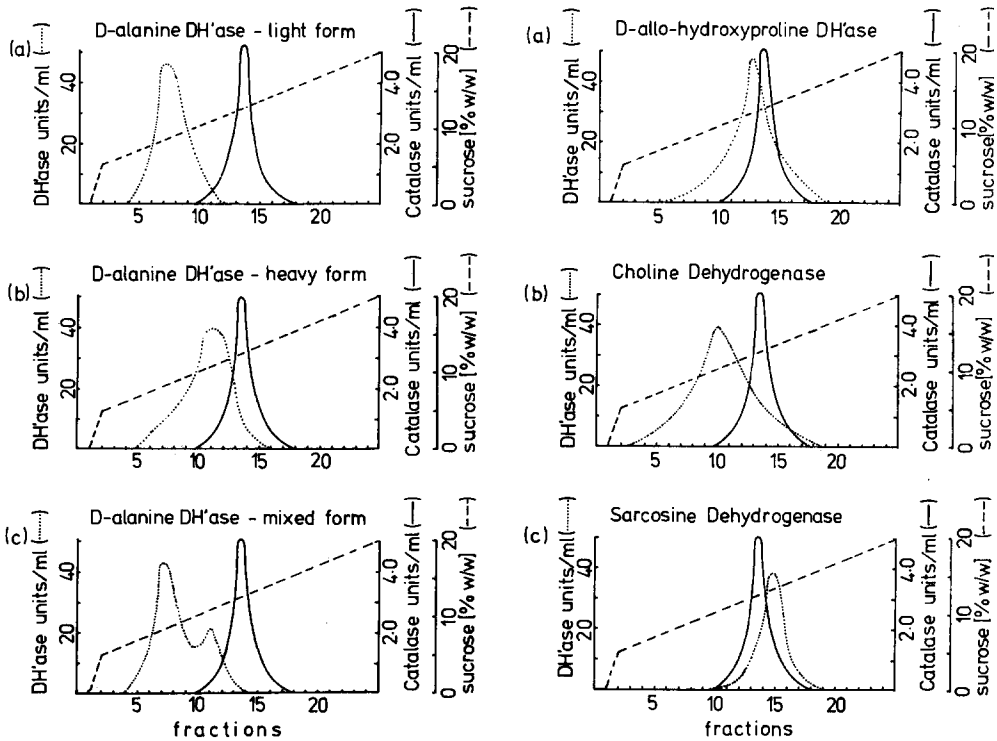

Fig. 5. Density gradient profile of solubilised D-alanine dehydrogenase with catalase as an internal standard. Dehydrogenase samples contained approx. 10 mg protein. The sucrose density gradients were centrifuged for 20 h at 98000 × *g* and 4°C. The labels light, heavy, and mixed forms appended to a, b and c, respectively, do not denote differences in the mode of sample preparation but are merely convenient descriptions of the observed behaviour patterns. Dehydrogenase units (DHase units) are nmol DCIP reduced/min.

Fig. 6. Density gradient profiles of solubilised preparations of (a) allohydroxy-D-proline dehydrogenase, (b) choline dehydrogenase, and (c) sarcosine dehydrogenase each with catalase as an internal standard. Each sample of solubilised dehydrogenase contained approx. 10 mg protein. The sucrose density gradients were centrifuged for 20 h at 98000 × *g* and 4°C.

TABLE V

SEDIMENTATION COEFFICIENTS AND MOLECULAR WEIGHTS OF MEMBRANE-BOUND DEHYDROGENASES

Sedimentation coefficients and molecular weights of inducible dehydrogenases solubilised with molar phosphate buffer were calculated from the data in Figs. 5 and 6 by comparison with catalase ($S^{\circ}_{20,w}$, 11.4; M_r = 240000 [12]). See text for discussion of the two apparent forms of solubilised D-alanine dehydrogenase.

Dehydrogenase	Sedimentation coefficient	Molecular weight
D-alanine (a)	5.7	85 000
(b)	8.5	156 000
Allohydroxy-D-proline	10.5	213 000
Choline	8.2	145 000
Sarcosine	12.3	266 000

analysis of solvent extracts of the solubilised dehydrogenase preparations. Preliminary tests with suitable dilutions of standard phospholipid solutions showed that the dichlorofluorescein reagent used could detect as little as 5 μg phosphatidylcholine or phosphatidylethanolamine, and that this figure could be reduced to 1—2 μg if the background fluorescence was quenched by spraying the plate with water prior to examination under ultraviolet light.

Active fractions from appropriate density gradients were pooled to give a solution of each enzyme containing a total of 5 mg protein. An aliquot containing 5 mg protein was also taken from a typical membrane suspension. Each sample was diluted with water to 2.0 ml. The samples were then extracted and chromatographed as described in Methods and Materials. Fig. 7 shows a tracing of a typical chromatogram. Samples 1 and 2 are a 10-fold dilution of the extract of a membrane suspension; a large spot corresponding to the position of phosphatidylethanolamine is visible, as is a fatty acid spot migrating with the solvent front. The other spot is probably phosphatidylglycerol, the other major phospholipid of *P. aeruginosa* membranes [18]. If the solutions of solubilised dehydrogenases had contained as little as 1 μg of any phospholipid species per 100 μg protein, a visible spot should have appeared on the chromatogram; however, no lipid spots were detected in the extract of any solubilised dehydrogenase. Although large quantities of lipids were detected in samples 1 and 2, inadequacies in the sample extraction procedure cannot be ruled out as the cause of the failure to detect lipids in the extracts of solubilised dehydrogenases.

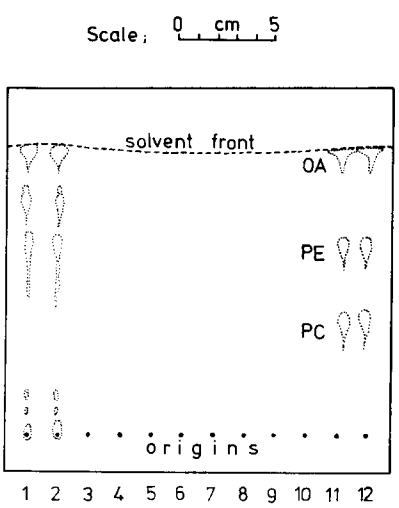

Fig. 7. Typical thin-layer chromatogram of chloroform/methanol (1 : 2, v/v) extracts of whole membranes (alanine-grown cells, 1, 2), extracts of solubilised dehydrogenases (D-alanine dehydrogenase, 3, 4; allohydroxy-D-proline dehydrogenase, 5, 6; choline dehydrogenase, 7, 8; sarcosine dehydrogenase, 9, 10), and lipid standards (OA, oleic acid; PE, phosphatidylethanolamine, PC, phosphatidylcholine, 11, 12). 5-μl samples were spotted onto the silica gel plate at the origins shown. The plate was developed in chloroform/ethanol (2 : 1, v/v), and the spots detected by examination under ultraviolet light after spraying with a 2% (w/v) ethanolic solution of 2',7'-dichlorofluorescein.

Effect of assay temperature on membrane-bound enzyme activity

The lateral phase separation which occurs when two or more components of a lipid bilayer have different temperatures of transition from the gel state to the sol state [19,20] are reflected as discontinuities in Arrhenius plots of various cellular phenomena in which membrane interactions are essential, e.g. growth [21,22], and transport [23]. Integral protein enzymes whose membranes association depends on strong protein-lipid interactions of a hydrophobic nature, should show polypasic Arrhenius kinetics, whereas peripheral protein enzymes, depending only on weak interactions for their membrane binding, should show monophasic Arrhenius plots. Suitable membrane preparations were assayed for the activities of a number of membrane-bound enzymes at various temperatures as described in Methods and Materials and the results presented as Arrhenius plots. Assays of cytochrome oxidase and NADH oxidase, were carried out at approx. 5°C intervals between 4 and 40°C. Arrhe-

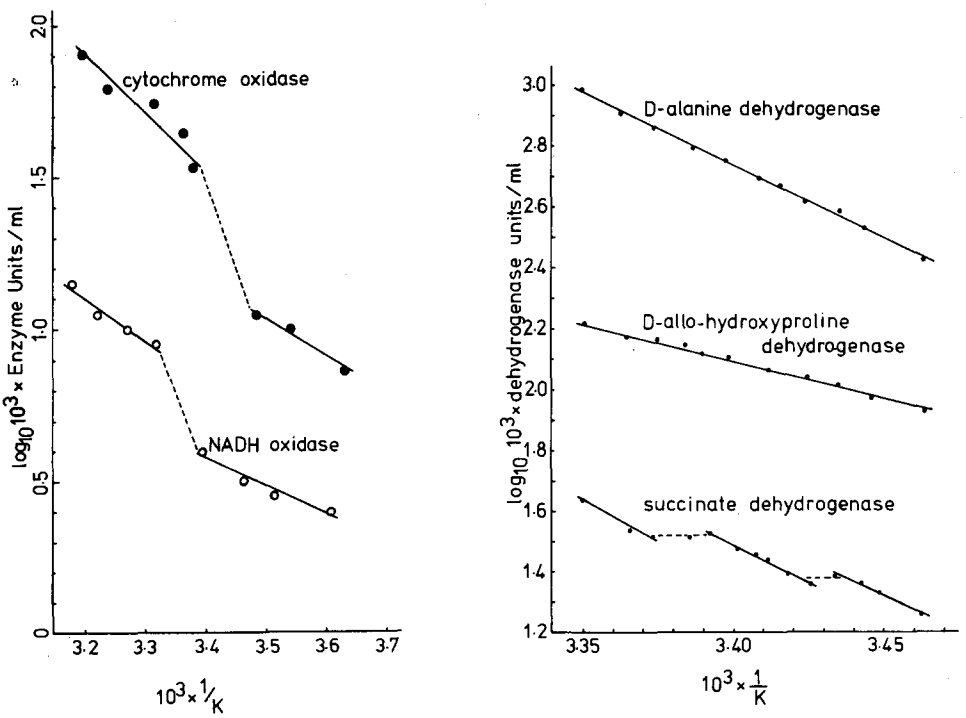

Fig. 8. Arrhenius plots of the activities of cytochrome oxidase (●———●) and NADH oxidase (○———○) in membrane preparations of succinate-grown cells. Enzyme units are as described in Methods and Materials. Assay mixture contained 0.71 mg membrane protein. Each point is the mean of two separate determinations. The straight lines are regression lines calculated from the individual (non-averaged) data.

Fig. 9. Arrhenius plots of the activity of (a) succinate dehydrogenase, (b) D-alanine dehydrogenase, and (c) allohydroxy-D-proline dehydrogenase in membrane preparations of cells grown on the appropriate carbon sources. Enzyme units are nmol DCIP reduced/min. Assay mixtures contained (a) 10.14, (b) 10.88, and (c) 9.81 mg protein. Each point is the mean of from two to five separate determinations. Straight lines are regression lines calculated from the individual (non-averaged) data.

nius plots of the data obtained are shown in Fig. 8; each point is the average of two separate determinations. The curves reflect the integral nature of cytochrome oxidase; as NADH oxidase is assayed via cytochrome oxidase, no conclusions can be drawn about the nature of NADH dehydrogenase, although other work suggests that this also is an integral protein [14]. Assays of succinate, D-alanine, and allohydroxy-D-proline dehydrogenases were carried out at approx. 1°C intervals between 15 and 25°C. Fig. 9 shows the Arrhenius plots of the resulting data; each point is the average of from two to five separate determinations. The straight lines in Figs. 8 and 9 are regression lines calculated from the individual (non-averaged) data using a Sumlock Compucorp 342 Statistician microcomputer, and all have a very good statistical fit to a straight line. In every case where discontinuities are apparent, a single regression line calculated from all the data has a much worse statistical fit. No test is available which would show whether the monophasic or the polyphasic plot is more probable, but the presentation chosen is in each case more consistent with the results of other workers.

Discussion

The solubilisation of D-alanine dehydrogenase by cation depletion (Table II) and of the D-alanine, allohydroxy-D-proline, choline and sarcosine dehydrogenases by high ionic strength buffer (Table IV) suggests that these enzymes are peripheral proteins in *P. aeruginosa* PAO 1. This hypothesis is substantiated by the lack of lipid associated with the solubilised enzymes (Fig. 7), and by the sedimentation of the latter through a density gradient as single molecular species (Figs. 5 and 6); as noted earlier, these sedimentation profiles do not necessarily indicate the solubilisation of the dehydrogenases as monomeric polypeptides.

During the intensive study of protein-lipid interaction in biological membranes one of the most common findings has been the involvement of divalent cations. This is true not only for the reattachment of solubilised membrane-bound enzymes, e.g. ATPases [24—27], hexokinase [28], malate and NADH dehydrogenases [29], but also for the reconstruction of totally solubilised membranes, e.g. *Acholeoplasma laidlawii* membranes dissociated with sodium dodecyl sulphate [30,31]. Membrane components carry a net negative charge at physiological pH values, and reconstitution experiments such as those with *A. laidlawii* membranes indicate that divalent cations are required to overcome the ensuing electrostatic repulsion and enable interacting molecules to move close enough together for hydrophobic bond formation. It is likely that the stabilisation of reconstituted membrane structure by formation of salt bridges between charged groups in adjacent membrane components also involves divalent cations [32].

Although calcium is as effective as magnesium in mediating the reconstitution of several bacterial ATPases [25,26], most peripheral enzymes which have been examined show a specific requirement for magnesium [24,28,29]. As demonstrated in Table III the relative effects of Mg^{2+} and Ca^{2+} in reducing the amount of D-alanine dehydrogenase solubilised from *P. aeruginosa* membranes by aqueous buffers or by chelating agents suggests that magnesium is the

divalent cation required in the protein-membrane interaction of this enzyme.

Work in progress in this laboratory on the reconstitution of solubilised D-alanine and allohydroxy-D-proline dehydrogenases with peripheral protein-depleted membrane preparations of induced and uninduced cells indicates that these peripheral enzymes bind to the membrane via interactions with a specific, inducible, integral protein(s), which may be common to both dehydrogenases (Bater, A.J. and Venables, W.A., unpublished observations). Singer [3] has suggested that such specific binding may require some degree of structural homology between the peripheral protein and the exposed hydrophilic portion of the integral "binding protein". If this were the case, the divalent cations would be required to neutralise excess negative charges so that the structurally homologous regions could approach sufficiently for molecular recognition to occur. Therefore Mg^{2+} could serve two functions; initiation of binding by charge neutralisation, and stabilisation of integral-peripheral protein complexes by salt-bridge formation.

The lack of stability of D-alanine dehydrogenase in the presence of ionic detergents (Table I) is in marked contrast to the stability of the mitochondrial respiratory components studied by Racker and his colleagues [33—35]. This is probably because most of the enzymes of the mitochondrial respiratory chain are integral proteins, and during their isolation from sub-mitochondrial particles detergent molecules replace the lipids with which the proteins would be associated with in vivo and hence help to stabilise the solubilised protein. As amphipathic proteins, such integral respiratory enzymes would have an asymmetric distribution of hydrophobic residues to which the detergent might be expected to bind preferentially [36], and since these hydrophobic ends would normally be buried deep in the bilayer where they can play only a minor role at most in catalysis, the detergent binding does not have a drastic effect on enzyme activity.

In contrast, peripheral proteins are not normally in close association with non-polar membrane lipids and have no mainly hydrophobic regions with which detergent molecules may interact preferentially. The binding of ionic detergents to the peripheral protein may therefore be expected to have a disruptive rather than a stabilising effect. Thus, the lack of stability of D-alanine dehydrogenase in the presence of ionic detergents supports the conclusion that it is a peripheral protein.

That the non-ionic detergent, Triton X-100, is bound very tightly to the D-alanine dehydrogenase is demonstrated by the difficulty encountered in trying to remove it from solubilised preparations of the enzyme. This tight binding is surprising in the light of the finding of Helenius and Simons [37] that naturally soluble (i.e. non-amphipathic) proteins bind little, if any, non-ionic detergents, an observation which should also apply to non-aphipathic peripheral proteins. It is possible that whereas molar phosphate buffer solubilises the dehydrogenase by breaking the ionic bonds between peripheral enzyme and integral binding protein, the Triton X-100 may exert its effect on the hydrophobic bonds between the integral binding protein and the bilayer phospholipids, and hence solubilise an intact dehydrogenase-binding protein complex. If this were so, the detergent molecules would form strong hydrophobic interactions with the non-polar end of the amphipathic binding protein,

leaving the associated dehydrogenase molecule unaffected. As Triton X-100 bears no charge, the ionic milieu of the solubilised complex would be no different from that of the membrane-integrated form, and there is therefore no reason why such non-ionic detergent treatment should dissociate the dehydrogenase from its integral binding protein. This assumption should be testable by attempting to dissociate the two components of the detergent-solubilised complex with molar phosphate buffer; the necessary experiments to demonstrate this are in preparation.

When presented as Arrhenius plots the data from temperature controlled assays of D-alanine and allohydroxy-D-proline dehydrogenases clearly demonstrate the independence of these enzymes of any effect of membrane lipid phase separations, reaffirming the conclusion that the enzyme-membrane interaction is of a peripheral nature. In contrast, the Arrhenius plots of the activity of integral respiratory enzymes of *P. aeruginosa* show irregularities attributable to the lipid phase changes (Figs. 8 and 9a); they are, however, different in shape from most published curves. The typical shape is shown, for example, by lactose transport in *E. coli* [23]; in this case the Arrhenius plots give two straight lines of different activation energy, with the change in slope occurring at the temperature of lipid phase transition or separation. The contrasting curves for the *P. aeruginosa* enzymes may be due to a different effect of the phase separation upon the enzymes involved. The lactose permease of *E. coli*, like other transport enzymes, would be expected to completely span the bilayer [3] and may be less mobile than other enzymes which interact with only the cytoplasmic face of the membrane. Lipid phase separations, in which the phospholipids with the highest transition temperature separates out as solid (less fluid) patches in an otherwise fluid matrix, are known to exclude fluorescent and electron spin resonance probes from the solid patches and may have a similar effect on membrane proteins [20]. In this case, the differential effect of the phase separation on mobile and less-mobile proteins may be that whereas the former are excluded from the solid patches during the phase change, the limited diffusion of the latter may preclude such an exclusion, i.e. the *E. coli* lactose permease would be "frozen" into a different lipid environment from the onset of phase separation whereas the *P. aeruginosa* respiratory enzymes would remain in a region of constant fluidity throughout the process. This would account for the more or less parallel slopes of the Arrhenius plots (i.e. similar activation energies) above and below the discontinuities which represent the phase separations.

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