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PROPERTIES OF *PSEUDOMONAS* AM1 PRIMARY-AMINE DEHYDROGENASE IMMOBILIZED ON AGAROSE

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Summary

1. The primary-amine dehydrogenase of *Pseudomonas* AM1 (primary-amine:(acceptor) oxidoreductase (deaminating), EC 1.4.99.-) was purified by an improved method and covalently attached to cyanogen bromide-activated Sepharose 4B. The immobilized enzyme showed very little change in its sensitivity to heat and to inhibition by semicarbazide as compared with the soluble enzyme, but had enhanced stability at 0°C. The pH optimum of the immobilized enzyme remained unchanged at pH 7.4.

2. A new type of spectrophotometric assay is described in which sedimentation of the immobilized enzyme in the cuvette is prevented by increasing the viscosity by the presence of 10% (w/w) polyethylene glycol (M_r 20 000). Detailed kinetic analysis using this assay showed only insignificant differences in the K_m values for *n*-butylamine and phenazine methosulphate between the soluble and Agarose-bound enzymes. The results are compared with those for other oxidoreductase enzymes immobilized on Sepharose.

Introduction

Although much work has been done on the chemical linkage of enzymes to insoluble matrices [1], much of the work in the literature has been on hydrolytic enzymes (e.g. Zaborsky, [2]). The primary-amine dehydrogenase of *Pseudomonas* AM1 (primary-amine:(acceptor) oxidoreductase (deaminating), EC 1.4.99.-) [3,4] has a number of properties which make it suitable for immobilization studies. It is unusually stable to extremes of temperature [3] and pH [5]. Its kinetics have been throughly examined [4]. We present here a report

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on the properties of the enzyme after it has been insolubilized by direct covalent coupling to cyanogen bromide-activated Sepharose 4B [6]. We find, in agreement with the work of Lowe [7—9] with lipoamide dehydrogenase, that there are very few significant detectable changes in the properties of the enzyme when it has been immobilized.

Materials and Methods

Enzyme purification

The primary-amine dehydrogenase of *Pseudomonas* AM1 [4] was partially purified by an improved method.

1. Crude extract. 25 g of methylamine-grown *Pseudomonas* AM1 [3] were suspended in 50 ml ice-cold 20 mM sodium-potassium phosphate buffer, pH 7.0, and passed once through a French pressure cell at 30 MPa. The resulting material was centrifuged for 40 min at 4°C and 50 000 × *g*. The pellet was resuspended in 30 ml ice-cold buffer and again passed through the French press and centrifuged. The two supernatants were combined.

2. Heat treatment. The combined supernatants were stirred in a stainless steel beaker at exactly 75°C for 20 min and cooled. Heat-denatured protein was removed by centrifuging for 20 min as above. This material was then dialysed for 2 h against 2 mM sodium-potassium phosphate, pH 7.0.

3. 5-Aminopentyl-Sepharose chromatography. The dialysed enzyme was applied to a column (2.5 × 10 cm) of 5-aminopentyl-Sepharose 4B (prepared as described in Ref. 10) equilibrated with 2 mM phosphate pH 7.0. The column was then washed with 10 mM phosphate, pH 7.0, and the enzyme was then quantitatively eluted with 20 mM sodium-potassium phosphate, pH 7.0.

4. Concentration. The combined active fractions from step 3 were concentrated 10-fold in a rotary evaporator under reduced pressure at 45°C. This material was 19-fold purified in 66% yield (Table I), and is suitable for immobilization. During purification, the enzyme was assayed as in [3].

Estimation of dry weight of immobilized enzyme

A measured volume of gel suspension (usually 3 ml) was filtered through a 0.45 μm pore size Sartorius membrane filter. The retained material was washed with 100 ml distilled water and dried to constant weight at 110°C and compared with a control membrane used to filter 100 ml of water only. Dry weights of gel could also be approximately estimated by turbidity measurement

TABLE I

PURIFICATION OF PRIMARY-AMINE DEHYDROGENASE FROM *PSEUDOMONAS* AM1

Step	Volume (ml)	Total protein	Total units	Specific activity	Purification	Yield (%)
Crude extract	84	1260	233.5	0.185	1	100
Heat treatment	73	394	227.3	0.576	3.1	97.3
Combined fractions after hydrophobic chromatography	143	44.3	166.2	3.74	20.1	71.1
Residue after concentration	14.2	44.3	154.8	3.50	18.9	66.2

at 570 nm when the gel was suspended in 10% w/w polyethylene glycol P-2263.

Immobilization of amine dehydrogenase

Activation of Sepharose 4B with CNBr in acetonitrile solution was carried out by the method of March et al. [11]. After activation, the slurry of activated Sepharose was washed as described in Ref. 11. The amine dehydrogenase was dialysed for 4 h at 4°C against 20 mM $NaHCO_3$, pH 9.0. The enzyme is known to be stable at this pH [5]. The dialysed enzyme (about 20 mg protein) was then added to 20 ml of activated Sepharose slurry (containing 10 ml of Sepharose 4B and 10 ml 0.1 M $NaHCO_3$, pH 9.5) and stirred at 4°C for 20 h. The slurry was then washed by filtration with a small amount of 0.1 M $NaHCO_3$ and the amount of uncoupled protein in this washing was estimated. Thus the amount covalently linked to the gel could be calculated by difference [12]. Usually 97% of the protein was bound. Protein was estimated by the method of Lowry et al. [13]. Any unreacted imidocarbonate groups in the Sepharose were then destroyed by addition of 5 ml of 1 M ethanolamine, followed by stirring for 2 h at 25°C [14]. The coupled Sepharose beads were then washed as described in [14] and finally resuspended in 10 mM sodium-potassium phosphate buffer, pH 7.0.

Control gel

For comparison with soluble enzyme, a sample of CNBr-activated Sepharose was reacted immediately with 1 M ethanolamine instead of coupling with enzyme. After washing as before, it was suspended in 10 mM phosphate buffer, pH 7.0. This material was added to soluble amine dehydrogenase to provide a control with the same turbidity and tendency to settle out as the immobilized enzyme and with an equivalent level of enzyme activity. No attempt at equivalence on a specific activity basis was made.

Enzyme assay

The insoluble enzyme has to be dispensed most carefully into the assay mixture if reproducible rates of reaction are to be measured. This was done by keeping the chilled enzyme preparation continuously stirred with a magnetic stirrer and removing and dispensing samples for assay with an automatic pipette using a fairly wide-mouthed disposable tip.

(a) Oxygen electrode method. The thermostatically controlled reaction vessel contained in a total volume of 3.4 ml, 200 μmol sodium phosphate buffer, pH 7.4, 1 μmol phenazine methosulphate, 50 mg catalase and about 2.5 mg (dry weight) immobilized enzyme at 30°C. The reaction was started by addition of 20 μmol of methylamine hydrochloride into the magnetically stirred vessel from a syringe. The oxygen uptake measured with the Clark electrode of a Beckman model 777 oxygen monitor was recorded on a Servoscribe recorder. The rate of oxygen uptake was linear with enzyme concentration at the stirring rate used up to at least 3 mg (dry weight) of gel in the reaction vessel. One unit of enzyme is the amount catalysing the uptake of 1 μmol O_2/min per ml reaction mixture.

(b) Spectrophotometric method. Polystyrene cuvettes (1 cm light path) con-

tained, in a total volume of 3.1 ml: 266 μmol sodium-potassium phosphate buffer, pH 7.5, 1.33 μmol phenazine methosulphate, 1.1 ml 28.6% w/w polyethylene glycol (P-2263) solution, 0.19 μmol 2,6-dichlorophenol-indophenol, and about 2.5 mg (dry weight) immobilized amine dehydrogenase. The reaction was started by addition of amine substrate (27 μmol methylamine-HCl or *n*-butylamine-HCl) and the change in absorbance followed at 25° C and 600 nm against a blank containing all components except 2,6-dichlorophenol-indophenol and amine substrate in a Unicam SP1800 spectrophotometer with AR 25 linear recorder. A cuvette with no gel or no amine gave no dye reduction over a period of 3 min, and the recorded rates were linear. The rates were proportional to enzyme concentration up to at least 3 mg (dry weight) of gel in the cuvette. One unit of enzyme is the amount required to catalyse the reduction of 1 μmol of 2,6-dichlorophenol-indophenol/min at 25° C.

Materials

Sepharose 4B was obtained from Pharmacia (London), cyanogen bromide from Aldrich (Gillingham, Dorset, U.K.), cadaverine (1,5-diaminopentane), phenazine methosulphate, 2,6-dichlorophenol-indophenol, catalase, polyethylene glycol (product No. P-2263) (molecular weight 20 000) from Sigma, London (Poole, Dorset). *Pseudomonas* AM1 (NCIB 9133) was grown on methylamine as described previously [3].

Results

Development of assay methods

(a) Oxygen-electrode assay. This was based on measurement of oxygen uptake [15] when the electron acceptor phenazine methosulphate (PMS) was reoxidized by oxygen in the presence of catalase

$$CH_3NH_3^+ + PMS + H_2O \rightarrow HCHO + NH_4^+ + PMSH_2 \quad (1)$$

$$PMSH_2 + O_2 \rightarrow PMS + H_2O_2 \quad (2)$$

$$H_2O_2 \rightarrow H_2O + \tfrac{1}{2} O_2 \quad (3)$$

Sum:

$$CH_3NH_3^+ + \tfrac{1}{2} O_2 \rightarrow HCHO + NH_4^+ \quad (4)$$

This method worked satisfactorily but it was slow and rather insensitive compared with the spectrophotometric method.

(b) Spectrophotometric assay. Assay methods have been described in the literature involving pumping part of the reaction mixture through a spectrophotometric flow cuvette [16] or involving stirring of the cuvette [17]. These are unsatisfactory for a number of reasons. The flow cell method requires only a small volume of the reaction mixture to be away from the reaction vessel at any given instant, and filtration devices to exclude the insoluble enzyme are necessary. There can be problems of creating a vortex in the stirred cuvette methods. It seemed to us that instead of stirring to keep the insolubilized enzyme in suspension, the same result might be achieved by increasing the viscosity of the mixture in the assay cuvette by the introduction of a suitable

inert viscous nonelectrolyte. Sucrose and glycerol were found to be unsuitable, but polyethylene glycol ('Carbowax') (M_r 20 000) introduced into the assay cuvette at a final concentration of 10% (w/w) produced a suspension in which there was no detectable sedimentation of the enzyme over the period of the assay, no interference with the reaction catalysed and no alteration in the molar extinction coefficient of 2,6-dichlorophenol-indophenol. This allowed the accurate measurement of small differences in rate.

Effect of immobilization on the pH optimum of the enzyme

There was no effect on the position of the pH optimum on immobilization. With methylamine as substrate it remained at pH 7.4 (Fig. 1).

Temperature stability

Since the soluble enzyme is extremely stable to heat [3], relatively high temperatures were necessary before an effect was observed, but it is clear that the immobilized enzyme is somewhat more stable at 84 and 86° C (Fig. 2). At 0° C in ice, the immobilized enzyme had a half-life of 130 days. No activity was released from the gel during this period. The soluble enzyme at concentrations showing comparable activity lost all its activity in 50 days.

Sensitivity to semicarbazide inhibition

Primary-amine dehydrogenase is extremely sensitive to semicarbazide [3]. Low concentrations of semicarbazide hydrochloride had an identical effect on both soluble and immobilized enzymes (Fig. 3), but at higher concentrations the immobilized enzyme was slightly less rapidly inactivated.

Kinetic experiments

n-Butylamine hydrochloride and phenazine methosulphate were used in

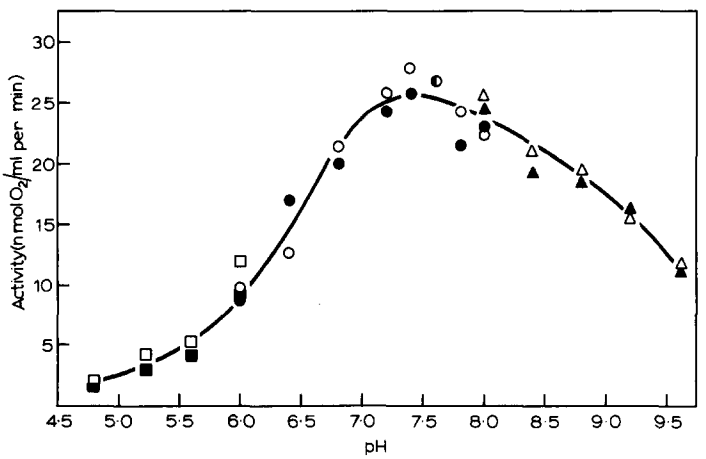

Fig. 1. Effect of pH on the oxidation of methylamine by soluble and Agarose-bound primary-amine dehydrogenase. Methylamine concentration, 5.9 mM. Phenazine methosulphate concentration, 0.29 mM. Enzyme concentration was 75 μg protein for purified enzyme or 0.15 mg dry weight for Agarose-bound enzyme. Solid symbols, soluble enzyme; open symbols, immobilized enzyme. Oxygen uptake was measured in an oxygen electrode at 30° C. Buffers used were: □, acetate; ○, phosphate; △, glycine-NaOH (each 60 mM).

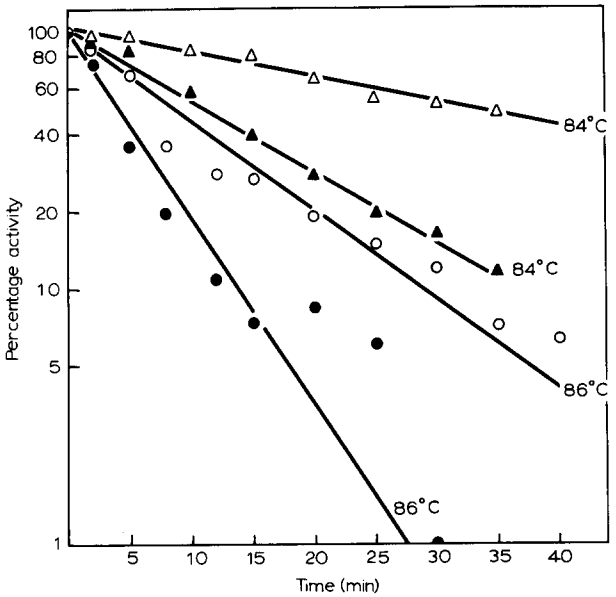

Fig. 2. Effect of heat on the activity of Agarose-bound primary-amine dehydrogenase and soluble enzyme (in the presence of control gel). Samples (0.2 ml) of both enzymes were incubated in small tubes at the temperatures indicated. At the times shown, a tube was removed, cooled in ice and then assayed by the spectrophotometric method. Solid symbols, soluble enzyme, open symbols, immobilized enzyme.

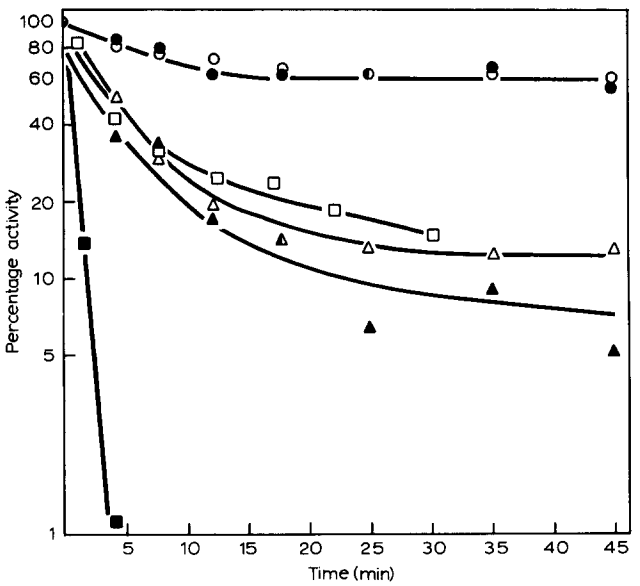

Fig. 3. Time-course of the inhibition of soluble and Agarose-bound primary-amine dehydrogenase by various concentrations of semicarbazide. Enzyme (1.2 mg dry weight of immobilized enzyme or 600 μg of soluble enzyme) was incubated with semicarbazide-HCl in 50 mM phosphate buffer pH 7.0 at 21°C, and samples removed at intervals and assayed by oxygen electrode assay at 30°C. Solid symbols, soluble enzyme; open symbols, immobilized enzyme. Semicarbazide concentrations: ○, 2 μM; △, 20 μM; □, 200 μM.

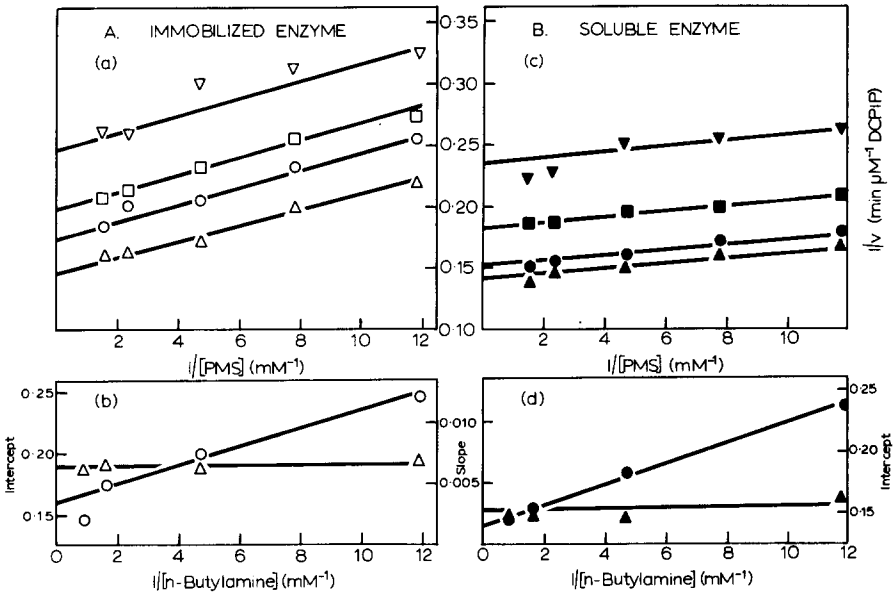

Fig. 4. Primary and secondary double-reciprocal plots of activity of soluble and immobilized primary-amine dehydrogenase as a function of substrate concentration. In the primary plots phenazine methosulphate (PMS) concentration was varied at a series of fixed concentrations of *n*-butylamine. In the secondary plots, the slopes (△) and intercepts (○) of the primary plots are plotted as functions of reciprocal *n*-butylamine concentration. The spectrophotometric assay at 25° C was used.

kinetic studies using the spectrophotometric enzyme assay with either gel-bound enzyme or a mixture of soluble enzyme with control gel. In both systems ping-pong kinetics [18] were observed (Fig. 4). From the replot (Figs. 4b and 4d) of the slopes and intercepts of the primary plots [19], it was shown that immobilization changed the K_m value for phenazine methosulphate from 27 to 41 μM and for *n*-butylamine from 61 to 46 μM. These slight changes are probably not significant.

Discussion

We found that the direct coupling of primary-amine dehydrogenase to CNBr-activated Sepharose yields highly efficient binding of enzyme to the matrix, as also observed by others [14,20], and the resultant material is highly active catalytically, but it is extremely difficult to make comparisons on an activity/mg protein basis between immobilized and soluble enzyme. It seems likely that the creation of covalent linkages (presumably between ε-amino groups of lysyl side chains and the Sepharose matrix [21]) will decrease the specific activity of the enzyme. Moreover, uncertainty of the nature and number of these covalent linkages prevents any kind of prediction of the stability or other properties of the gel-bound enzyme. In this connection it is noteworthy that recent work [5,22—24] suggests that the primary-amine dehydrogenase consists of two kinds of subunit, and which of these is linked to the matrix is not known.

The original intention of this work was to attempt, by use of a column of immobilized amine dehydrogenase, to detect the in vivo natural electron

acceptor of the enzyme, which it was thought might bind to the immobilized enzyme if crude cell extracts were passed at low ionic strength down the column. However, because such binding would be stoicheiometric, it was found to be impossible to immobilize a sufficient quantity of enzyme for detectable amounts of material to bind to it.

The sensitivity of the enzyme to heat and to inhibition by semicarbazide and the position of the pH optimum seem to be little affected by immobilization, though such changes as there are agree with the concept of the immobilized enzyme having greater stability. Only insignificant effects were apparent also on the affinity of the enzyme for its substrate as measured by 'apparent' K_m. The term 'apparent' K_m is used here as defined by Mosbach [1] in connection with immobilized enzymes. The parameter we have determined is not an apparent K_m in the sense of K_m at a noninfinite concentration of the second substrate [25], our values reported here are calculated for infinite concentration of the fixed varying substrate. The observed mechanism is in reasonable agreement with our previous work on the soluble enzyme [4]. In the spectrophotometric assay in the increase in the viscosity of the medium introduced by the presence of polyethylene glycol is certain to influence the diffusion rates and hence accessibility of small molecules to the enzyme active centre. For this reason we have found it desirable to compare reaction rates for the immobilized enzyme with those for the soluble enzyme in the presence of an equivalent amount of gel without bound enzyme.

The approach to assaying insolubilized enzymes spectrophotometrically by increasing the viscosity of the assay mixture to minimize sedimentation has not, so far as we are aware, been previously attempted. The only practical difficulty in using it is the slowness with which the polyethylene glycol solution is dispensed into the cuvette. We have found it important to measure the volume actually dispensed, which is always less than expected, due to retention of the viscous solution in the pipette.

The insignificant change in properties observed when the enzyme was covalently attached to Sepharose has also been observed with lipoamide dehydrogenase [7—9]. D-Amino acid oxidase however, changed its pH optimum but not its affinity for substrate [20]. In contrast, considerable changes in properties were observed when lactate dehydrogenase was immobilized on agarose [26] or on porous glass beads [27]. At the moment our limited knowledge of the molecular structure of the primary-amine dehydrogenase (see Refs. 23, 24) prevents a more sophisticated approached to the chemistry of the immobilization process being made (cf. the work of Lowe [7—9] with lipoamide dehydrogenase). The requirement of the enzyme for coloured dyes as electron acceptors makes possible technological use of the gel-bound enzyme, for example in the removal of amines from aqueous solutions, difficult to envisage, but one possible method might be to co-immobilize the electron acceptor with the enzyme on to the Sepharose. If this were to be attempted, it would be better to test 8-dimethylamino-2,3-benzophenoxazine (Meldola Blue, Boehringer) as a possible acceptor for the enzyme, since it is not photo-labile as phenazine methosulphate is. An electron acceptor used in this way would need to be an autoxidizable one, so that the passage of aerated amine through a column or stirred reactor would continuously regenerate the

reduced immobilized dye. It would also need to have reactive groups suitable for covalent linkage to Sepharose.

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