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Affinity Chromatography of the *Neurospora* NADP-Specific Glutamate Dehydrogenase, its Mutational Variants and Hybrid Hexamers

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The synthesis of an affinity adsorbent, 8-(6-aminohexyl)aminoadenosine 2′-phosphate–Sepharose 4B, is described. The assembly of the 2′-AMP ligand and the hexanediamine spacer arm was synthesized in free solution before its attachment to the Sepharose matrix. This adsorbent retarded the hexameric NADP-specific glutamate dehydrogenase of *Neurospora crassa*, showing a capacity for this enzyme similar to that of comparable coenzyme-analogue adsorbents for other dehydrogenases. The enzyme was eluted either at pH 6.8 in a concentration gradient of $NADP^+$, or at pH 8.5 in the presence of $NADP^+$ in concentration gradients of either dicarboxylates or NaCl. Anomalous effects of dicarboxylates in facilitating elution are discussed. 2′-AMP and its derivatives, 8-bromoadenosine 2′-phosphate and 8-(6-aminohexyl)aminoadenosine 2′-phosphate, which were used in the synthesis of the adsorbent, all acted as enzyme inhibitors competitive with $NADP^+$. The chromatographic properties of the wild-type enzyme were compared with those of mutationally modified variants containing defined amino acid substitutions. This approach was used to assess the biospecificity of adsorption and elution and the contribution of non-specific binding. The adsorbent showed a low capacity for the enzyme from mutant *am*[1] (Ser-336 replaced by Phe), a variant that has a localized defect in NADP binding, but an otherwise almost normal conformation, suggesting that non-specific interactions are at most weak. The enzyme from mutant *am*[3], a variant modified in a conformational equilibrium, was fully retarded by the adsorbent, but showed a significantly earlier elution position than the wild-type enzyme. This is consistent with measurements in free solution that showed the *am*[3] enzyme to have a higher K_i for 2′-AMP than the wild-type enzyme. The enzyme from mutant *am*[19] was eluted as two distinct peaks at both pH 6.8 and 8.5. The adsorbent was used to separate hybrid hexamers constructed *in vitro* by a freeze–thaw procedure from pairs of purified variants. Several chromatographically distinct peaks of differing enzymological properties were purified from each hybridization mixture in quantitites of up to a few milligrams, and represented distinct species of hybrid hexamers differing in subunit ratio.

There are several reports of the successful affinity chromatography of dehydrogenases with immobilized coenzyme analogues attached in a chemically defined way to a spacer arm (Mosbach *et al.*, 1972; Craven *et al.*, 1974; Trayer *et al.*, 1974). The use of such adsorbents as an analytical tool has also been investigated by several workers who have attempted quantitatively to relate ligand-specific chromatographic behaviour to ligand-binding parameters derived from studies of enzymes in free solution (Lowe *et al.*, 1974*a*,*b*; Trayer & Trayer, 1974; Harvey *et al.*, 1974*a*,*b*). The effectiveness of such analyses has been limited by the occurrence of non-specific binding of dehydrogenases to the adsorbents (Barry & O'Carra, 1973), possibly involving the spacer arm and the linkage of the spacer arm to the matrix. Despite this complication, biospecific binding has been established for several dehydrogenases and adsorbents.

We describe here studies of the affinity chromatography of the NADP-specific glutamate dehydrogenase (EC 1.4.1.4) from *Neurospora crassa*, by using immobilized 2′-AMP attached by a spacer arm to Sepharose 4B. This enzyme is a very well characterized hexamer of identical subunits, and the amino acid sequence of the chain of 452 residues has been determined (Holder *et al.*, 1975). Many mutationally modified variants of the enzyme exist, in some of which amino acid substitutions have been defined (Brett *et al.*, 1976). We report here the affinity-chromatographic behaviour of three of these variants that differ from the wild-type enzyme in coenzyme-binding and conformational properties as a result of single amino acid substitutions. The correlation between the altered properties in these variants and the differences in their chromatographic behaviour is used to assess the biospecificity of adsorption and elution and the contribution of non-specific binding.

Table 1. *Properties of wild-type and mutationally modified forms of NADP-specific glutamate dehydrogenase from Neurospora crassa*

References for the methods are: amino acid substitutions and interpretation of defects, Brett *et al.* (1976); conformational states, Ashby *et al.* (1974) and Ashby (1974); electrophoretic mobilities, Coddington *et al.* (1966). *A*, Active conformational state of high intrinsic tryptophan fluorescence; *I*, inactive conformational state of low intrinsic tryptophan fluorescence.

Strain	Conditions for activity	Conformational state		Electrophorectic mobility at pH8.3	Amino acid substitution	Interpretation of defect
		at pH6.8	at pH8.5			
Wild-type	Over pH range approx. 6.5–9.5	*I*	*A*	Normal	—	—
am[1]	Inactive under all conditions	Approx. 60% *A*, 40% *I*	*A*	Normal	Ser-336 to Phe	Defective NADP binding, slightly altered *A*/*I* equilibrium
am[3]	Above pH approx. 8 after activation by dicarboxylates	*I*	*I*	Normal	Glu-393 to Gly	Abnormally stabilized in *I* conformation
am[19]	Over pH range approx. 6.5–9.5 after prolonged activation by dicarboxylates	Predominantly an inactive state (distinct from *I*) at both pH values		Active state normal, inactive state fast	Lys-141 to Met	Abnormally stabilized in an inactive conformation. Very slow activation

Properties of the variants used are summarized in Table 1.

In this work we have used purified enzymes, prepared by established methods, which do not involve affinity chromatography, as the starting material for the affinity-chromatographic studies. One objective was to develop a system capable of separating hybrid heterohexamers of the enzyme which differ in subunit ratio. Such hybrids, composed of subunits derived from two different mutational variants or from a variant and the wild-type enzyme, are of considerable interest for studies of intersubunit interactions and the mechanism of interallelic genetic complementation [reviewed by Fincham (1966) and Zabin & Villarejo (1975)]. Previous work has established that the product of hybridization of subunits *in vitro* is a complex mixture of hexamers of different subunit ratio. No satisfactory separation of these different hybrids was achieved in attempts using ion-exchange chromatography, centrifugation methods, gel electrophoresis or isoelectric focusing (Fincham & Coddington, 1963; Coddington & Fincham, 1965; Coddington *et al.*, 1966; Sundaram & Fincham, 1967; D. H. Watson & J. C. Wootton, unpublished work). We describe in the present paper the separation of such mixtures of hybrids by affinity chromatography on the immobilized 2′-AMP adsorbent. There have been reports of the resolution or partial resolution of mixtures of isoenzyme hetero-oligomers by affinity chromatography (Brodelius & Mosbach, 1973; Andersson *et al.*, 1974; Visser & Strating, 1975).

Because of the desirability of minimizing non-specific interactions between the enzyme and adsorbent, we have used the strategy of synthesizing the ligand-spacer arm compound in free solution before its attachment to the Sepharose matrix, by using the type of approach developed in Mosbach's laboratory (Mosbach, 1974; Jergil *et al.*, 1974). This seemed preferable to the 'modular solid-state' strategy, in which the spacer arm is coupled to the matrix before attachment of the target ligand. There is evidence suggesting that adsorbents synthesized by the latter approach exhibit a significant amount of non-specific binding of enzyme to unsubstituted spacer arms (Barry & O'Carra, 1973).

Materials and Methods

Materials

2′-AMP was from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. $NADP^+$ and NADPH were from Boehringer (London) Corp., Lewes, Sussex, U.K. CNBr, anisole and hexanediamine were from Eastman–Kodak, Liverpool, U.K. DEAE-Sephadex, Sephadex G-200, Sepharose 4B and Sephadex G-10 were from Pharmacia Fine

Chemicals (G.B.), London W.5, U.K. Standard laboratory chemicals, of analytical grade, were from BDH Chemicals, Poole, Dorset, U.K., and were used without further purification.

Growth of Neurospora

Conidia and mycelium of the wild-type *N. crassa* STA4 were grown as described by Ashby *et al.* (1974). Conidia and mycelium of *am*^{1} (laboratory stock no. *am*^{1}-6-3*a*), *am*^{3} (stock no. *am*^{3}-6-1*a*) and *am*^{19} (stock no. *am*^{19}-6-1*a*) strains of *N. crassa* were grown by a similar method with the addition of 5mM-monosodium L-glutamate to the growth medium in each case. The wild-type and mutant strains yielded comparable amounts of mycelium.

Enzyme and protein assays

Three assay systems, A, C and S, were used (Coddington *et al.*, 1966). Wild-type enzyme was assayed in assay systems A and C. Assay system A measured the rate of reductive amination of 2-oxoglutarate and contained: 2.55ml of 0.1M-Tris/HCl buffer, pH8.5 (containing 1mM-EDTA), 0.10ml of 1M-NH_4Cl, 0.15ml of 0.2M-2-oxoglutaric acid (adjusted to pH8.5 with NaOH), 0.2ml of 0.15% (w/v) NADPH, and 1, 2 or 10μl of a suitably diluted solution of enzyme, all in the same buffer at 37°C. Assay system C measured the rate of oxidative deamination of L-glutamate and contained: 2.8ml of 0.16M-monosodium L-glutamate in 0.1M-Tris/HCl buffer, pH8.5 (containing 1mM-EDTA), 0.2ml of 0.2% (w/v) $NADP^+$ in the same buffer, and 1, 2 or 10μl of suitably diluted solution of enzyme, all at 37°C.

am^3 enzyme was assayed in systems A and C as described above and in certain cases (see the Results section) by a modified form of this assay, which contained: 2.8ml of 0.32M- or 0.08M-monosodium L-glutamate in 0.1M-Tris/HCl buffer, pH8.5 (containing 1mM-EDTA), 0.2ml of 0.2% $NADP^+$ in the same buffer, and 1, 2 or 10μl of a suitably diluted solution of enzyme, all at 37°C or in some cases other temperatures as indicated.

am^{19} enzyme was assayed in system C as described above, or in some cases (see the Results section) by a modified form of the assay, in which 1, 2 or 10μl of a suitably diluted solution of the enzyme were added to 1.4ml of 0.1M-Tris/HCl buffer, pH8.5 (containing 1mM-EDTA), at 37°C and preincubated at this temperature for 120min. Then 1.4ml of 0.32M-monosodium L-glutamate and 0.2ml of 0.2% $NADP^+$, both in the above buffer, were added and the initial rate was measured. *am*^{19} enzyme was also assayed in system S (Coddington *et al.*, 1966), which measures oxidative deamination of L-glutamate by succinate-activated enzyme; 1, 2 or 10μl of a suitably diluted solution of enzyme was added to 0.875ml of 0.17M-disodium succinate in 0.1M-Tris/HCl buffer, pH8.5 (containing 1mM-EDTA), and 0.1ml of 0.2% $NADP^+$ in the same buffer, both at 37°C. The mixture was incubated at this temperature for 120min; 0.45ml of 1.0M-L-glutamate, 0.1ml of 0.2% $NADP^+$, both in the above buffer, and 1.37ml of the buffer were then added.

Changes in A_{340} were measured on a Unicam SP.800 spectrophotometer attached to a Servoscribe chart recorder [Kelvin Electronics Co., (Smiths Industries), London, U.K.]. Substrate and inhibitor concentrations used for measurements of K_m and K_i values are indicated in the relevant sections.

Enzyme activity (initial rate) is expressed in units of μmol of $NADP^+$ reduced/min (assay systems C and S) or μmol of NADPH oxidized/min (assay system A).

Concentration of purified enzyme was determined spectrophotometrically by using the formula: mg/ml = $A_{280} \times 0.825$ (Ashby *et al.*, 1974).

Enzyme purification

NADP-dependent L-glutamate dehydrogenase was purified from freeze-dried mycelium of wild-type and amination-deficient strains of *N. crassa* as described by Ashby *et al.* (1974). Enzyme purified to constant specific activity was stored at 6°C as an $(NH_4)_2SO_4$ precipitate, being resuspended in and dialysed against the required buffer before use.

As reported by Ashby *et al.* (1974), no appreciable loss of specific activity was detectable during storage of the precipitated enzyme over several months. Similar yields of enzyme were obtained from the wild-type and from all the mutants, when assessed on the basis of the weight of purified protein obtained per weight of freeze-dried mycelium. The enzyme from mutants *am*^{3} and *am*^{19} was detected during purification by the appropriate assays described above. The enzyme from mutant *am*^{1}, which has no detectable glutamate dehydrogenase activity, was purified by the procedure used for the wild-type 'blind', and monitoring the later stages of purification by gel electrophoresis.

Enzyme was pure by the criteria used by Ashby *et al.* (1974) and contained no detectable bound coenzyme.

Enzyme hybridization

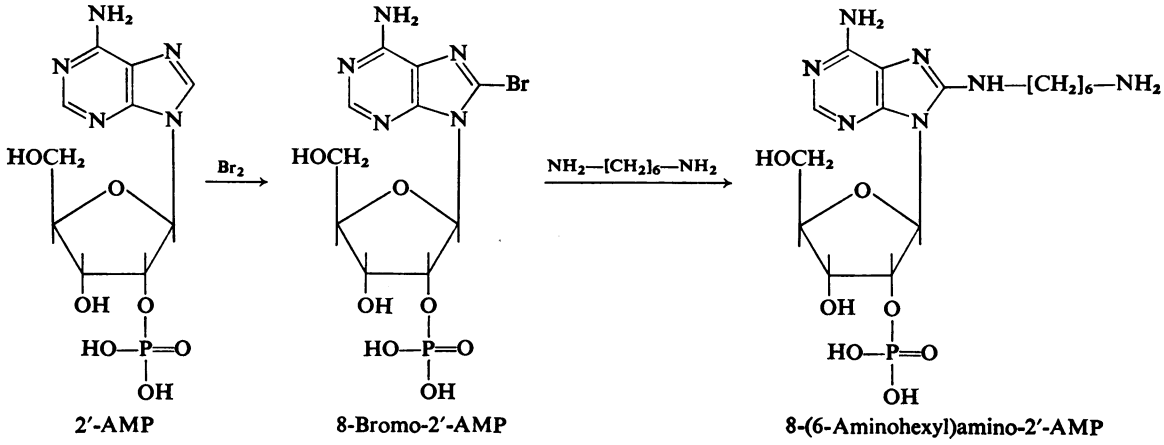
Hybrid mixtures of pairs of enzymes were formed by the method of Coddington *et al.* (1966) by mixing the two parent proteins (both in 50mM-sodium phosphate buffer, pH7.4, containing 1mM-EDTA)

and making this solution 0.10M with respect to NaCl by the addition of 1.0M-NaCl in the same buffer in 10μl portions. The mixture was then frozen at −20°C, left overnight at this temperature and then allowed to thaw at room temperature (approx. 25°C). The solution was then dialysed against the relevant affinity-chromatographic starting buffer before application to the column of adsorbent.

Synthesis of 8-(6-aminohexyl)amino-2'-AMP–Sepharose 4B

The synthesis of immobilized 2'-AMP described below is based closely on the comparable synthesis of 5'-AMP coupled to Sepharose 4B via C-8 of the adenine nucleus as described by Jergil *et al.* (1974). The synthesis is illustrated in Scheme 1.

8-Bromo-2'-AMP. This was synthesized by a method exactly analogous to that described by Jergil *et al.* (1974) for the synthesis of 8-bromo-5'-AMP. Thus bromine (7.5mmol) and the sodium salt of 2'-AMP (2.9mmol) were shaken in 50ml of 1M-sodium acetate buffer, pH3.9, at room temperature in the dark for 5 days. Anisole (10ml) was added and the shaking continued for a further 2 days. Resolution of the concentrated reaction mixture on a column (2cm×37cm) of Sephadex G-10 (100g), eluted with water, led to a pattern of overlapping peaks of bromine, bromine and nucleotides, and brominated nucleotides similar to that described by Jergil *et al.*



Scheme 1. *Synthesis of 8-(6-aminohexyl)amino-2'-AMP*
Details are given in the Materials and Methods section.

Table 2. *Comparative spectral and t.l.c. properties of C-8-substitution derivatives of 2'- and 5'-AMP*
U.v.-absorbance spectra of solutions, in 0.05M-sodium phosphate buffer, pH7.0, were measured on a Pye–Unicam SP.800 spectrophotometer. T.l.c. of 2'-AMP and its derivatives was carried out as described in the text on 0.025mm-thick cellulose plates, along with a control sample of 5'-AMP. R_F values for 5'-AMP and its derivatives (right-hand column) are taken from Jergil *et al.* (1974), who used 0.1mm-thick cellulose plates. N.M., not measured. First peak and second peak of brominated 2'-AMP refer to the peaks obtained from the second column of Sephadex G-10 (see the Materials and Methods section).

Compound	pH7.0 $\lambda_{max.}$ (nm)	pH7.0 $\lambda_{min.}$ (nm)	R_F values: 25μm Cellulose plates	R_F values: 100μm Cellulose plates
2'-AMP	259	229	0.57	N.M.
5'-AMP	259	227	0.55	0.27
Brominated 2'-AMP (first peak)	274–282	235	0.44	N.M.
Brominated 2'-AMP (second peak)	263	234	0.63	N.M.
8-Bromo-5'-AMP	263	N.M.	N.M.	0.36
8-(6-Aminohexyl)amino-2'-AMP	278	237	0.91	N.M.
8-(6-Aminohexyl)amino-5'-AMP	277	237	N.M.	0.48

(1974). T.l.c. of the pooled peak containing brominated nucleotides in system (a) described by Jergil *et al.* (1974) (2-methylbutan-2-ol/formic acid/water, 3:2:1, by vol.; chromatography on 0.025mm-thick cellulose plates) indicated two forms of bromo-2′-AMP (Table 2). Re-running of this pool on the above-described Sephadex G-10 column led to resolution of these two forms. One of these forms was closely similar to 8-bromo-5′-AMP as described by Jergil *et al.* (1974). The final yield of this form of bromo-2′-AMP was 0.96g (78%) from 1g of 2′-AMP. This product was homogeneous by t.l.c. and was used in the preparation of 8-(6-aminohexyl)-amino-2′-AMP.

8-(6-Aminohexyl)amino-2′-AMP. This was prepared by a method exactly analogous to that used by Jergil *et al.* (1974) for the preparation of the corresponding 5′-AMP derivative. The purified derivative had very similar spectral properties to the latter derivative and was homogeneous in the above t.l.c. system (Table 2). The final yield of purified derivative was 40mg (30%) from 118mg of bromo-2′-AMP.

Coupling of 8-(6-aminohexyl)amino-2′-AMP to Sepharose 4B. Coupling was carried out as described by Jergil *et al.* (1974) for the preparation of the corresponding 5′-AMP derivative. By using 5ml of CNBr-activated Sepharose 4B and 22mg of 8-(6-aminohexyl)amino-2′-AMP, approx. 9mg of nucleotide derivative were coupled to the gel as determined spectrophotometrically (i.e. 45μmol/g of dry Sepharose). Unbound nucleotide was removed by the washing procedure described by Jergil *et al.* (1974).

Chromatography on immobilized nucleotide

A column of adsorbent gel of dimensions 0.8cm × 8cm was used, and operated and maintained at 6°C unless otherwise stated. Between runs the column was washed with 2 bed vol. of freshly made 8M-urea in 0.05M-sodium phosphate buffer, pH7.4, to remove any remaining bound protein, then equilibrated with 6 bed vol. of the starting buffer for the next run. Adsorption and elution conditions for individual runs are described in the Results section. In all cases, after application of the enzyme to the column the flow was stopped for 1h to allow equilibration of the enzyme and adsorbent. Before elution with a gradient of substrate or salt, 3 bed vol. of starting buffer was run through the column to elute any unbound enzyme.

Operated in this way the column has been used reproducibly over a period of several months, with no loss of bound nucleotide detectable by scans of the u.v.-absorption spectra of washes of effluent. No change in elution properties of wild-type or any mutant variant of enzyme was found in replicate runs performed at intervals of several months.

Results and Discussion

Choice of immobilized ligand

The NADP-specificity of this glutamate dehydrogenase is reflected in the types of coenzyme analogues

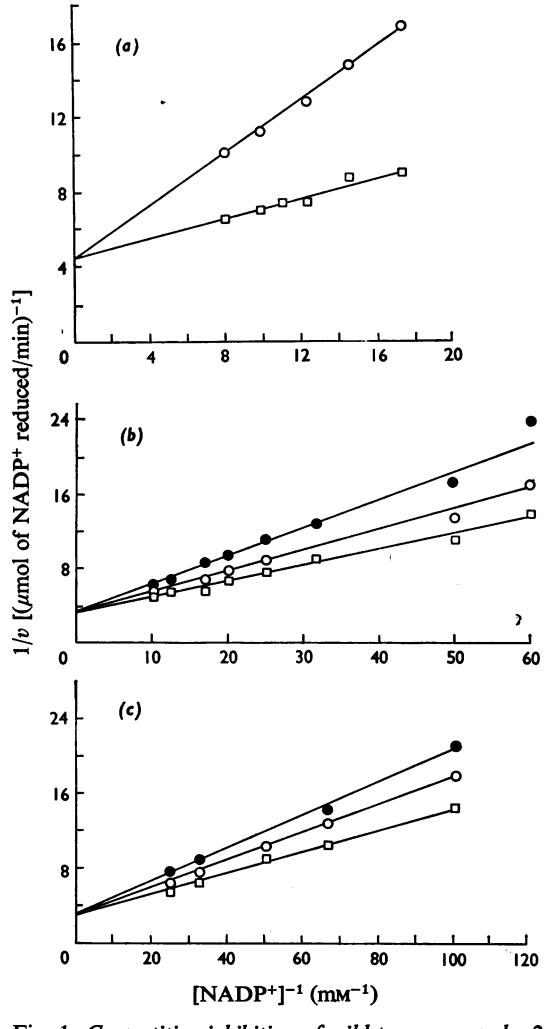


Fig. 1. *Competitive inhibition of wild-type enzyme by 2′-AMP, 8-bromo-2′-AMP and 8-(6-aminohexyl)amino-2′-AMP*

Enzyme was added last to assay system C (see the Materials and Methods section) except for variations in $NADP^+$ concentration as indicated. Initial velocities (v) are expressed in units of μmol of $NADP^+$ reduced/min. □, No inhibitor present. (*a*) ○, 2.7mM-2′-AMP present; (*b*) ○, ●, 2mM- and 4mM-8-bromo-2′-AMP present respectively. (*c*) ○, ●, 1.1mM- and 2.2mM-8-(6-aminohexyl)amino-2′-AMP present respectively. The lines represent linear unweighted least-squares regressions.

that show inhibition competitive with $NADP^+$. NADH binding is not detectable by fluorescence enhancement, and no competitive inhibition is observed with NAD^+, NADH or 5′-AMP (Gore *et al.*, 1972). However, 2′-AMP shows competitive inhibition [Gore *et al.* (1972), confirmed by us, Fig. 1] with an apparent K_i of 450 μM under conditions (Fig. 1) where the apparent K_m for $NADP^+$ is 30 μM. Also Blumenthal & Smith (1975) have reported competitive inhibition by the compound formed by removing nicotinamide from NADP, with an apparent K_i of 430 μM, compared with an apparent K_m for $NADP^+$ of 56 μM under their conditions. Taken together the above results imply that the primary interaction in coenzyme binding is with the 2′-phosphate group of the adenosine ribose moiety. Interactions with the adenine ring, the 5′,5″-pyrophosphate bridge and the ribose moiety of the NMN portion of the coenzyme appear to make at most a relatively minor contribution to binding. Interactions with the nicotinamide ring are implicated by the high affinity for coenzyme (K_D for NADPH = 12.5 μM at pH 8.2; Gore, 1972), but the mode of interaction with nicotinamide is uncertain because of the apparent lack of binding of NAD^+ and NADH.

This primary involvement of the 2′-phosphate may be a common feature of several NADP-dependent dehydrogenases. Brodelius *et al.* (1974) found that immobilized N^6-(6-aminohexyl)adenosine 2′,5′-bisphosphate was effective in the ligand-specific retardation of several yeast NADP-dependent dehydrogenases, including glutamate dehydrogenase, whereas this adsorbent did not retain NAD-dependent dehydrogenases. Before the publication of the report of Brodelius *et al.* (1974), one of us synthesized the immobilized 8-(6-aminohexyl)amino-2′-AMP described in the present paper and found it to be effective in retarding *Neurospora* NADP-specific glutamate dehydrogenase (D. H. Watson, unpublished work). It seemed desirable to avoid phosphates or other groups at the 5′-position of the 2′-AMP derivative, because of the possible introduction of additional non-specific interactions with the enzyme.

Both of the intermediates involved in the synthesis of the adsorbent, 8-bromo-2′-AMP and 8-(6-aminohexyl)amino-2′-AMP, showed competitive inhibition with respect to $NADP^+$ (Fig. 1), giving apparent K_i values of approx. 4.8 mM in both cases. Therefore we considered the adsorbent potentially suitable for the biospecific chromatography of NADP-specific glutamate dehydrogenase.

Choice of adsorption and elution conditions

Standard conditions of concentration of immobilized ligand, temperature, pH and column geometry and dynamics were established by preliminary experiments and on the basis of the experience of other workers who used comparable adsorbents (Harvey *et al.*, 1974*a*,*b*; Lowe *et al.*, 1974*a*,*b*; Cuatrecasas *et al.*, 1968). We have used a constant bound ligand concentration of 1.5 μmol/ml of wet gel, similar to published values for comparable 5′-AMP derivatives (Harvey *et al.*, 1974*b*; Brodelius *et al.*, 1974; Jergil *et al.*, 1974). This concentration proved adequate for the complete retardation of the wild-type and the potentially active mutant forms of the enzyme under appropriate conditions of pH and temperature.

The capacity of the adsorbent for the enzyme was greater at lower pH values in the range pH 8.5–6.8 (Table 3). This parallels the increasing affinity of the enzyme for NADPH with decreasing pH in this range (Gore, 1972). A similar effect of pH has been

Table 3. *Effect of pH and temperature on the capacity of* 8-(6-*aminohexyl*)*amino*-2′-*AMP–Sepharose* 4*B for wild-type and* am^3 *enzymes*

Enzyme (0.44 mg in 0.2 ml) was applied in the stated buffer, which in each case contained 1 mM-EDTA, at the stated temperature to a 4 ml bed volume column of adsorbent (see the text) equilibrated in the same buffer and at the same temperature. Unretarded enzyme was eluted by the same buffer. Retarded enzyme was eluted from the pH 8.5, 7.9 and 7.5 runs with 50 mM-Tris/HCl buffer, pH 8.5, containing 1 mM-EDTA, 0.2 M-L-glutamate and 1.25 mM-$NADP^+$, and from the pH 6.8 runs with 10 mM-sodium phosphate buffer, pH 6.8, containing 1 mM-EDTA and 2.5 mM-$NADP^+$. In all cases between 90 and 100% of applied enzyme activity was recovered in total. N.M., not measured.

Temperature (°C)	Buffer	pH	Wild-type enzyme retarded (mg/ml bed volume of adsorbent)	am^3 enzyme retarded (mg/ml bed volume of adsorbent)
6	50 mM-Tris/HCl	8.5	0.029	N.M.
6	50 mM-Sodium phosphate	7.9	0.075	N.M.
6	50 mM-Sodium phosphate	7.5	0.10	0.10
6	50 mM-Sodium phosphate	6.8	0.11	0.11
6	10 mM-Sodium phosphate	6.8	0.11	0.11
23	50 mM-Sodium phosphate	7.5	0.025	N.M.

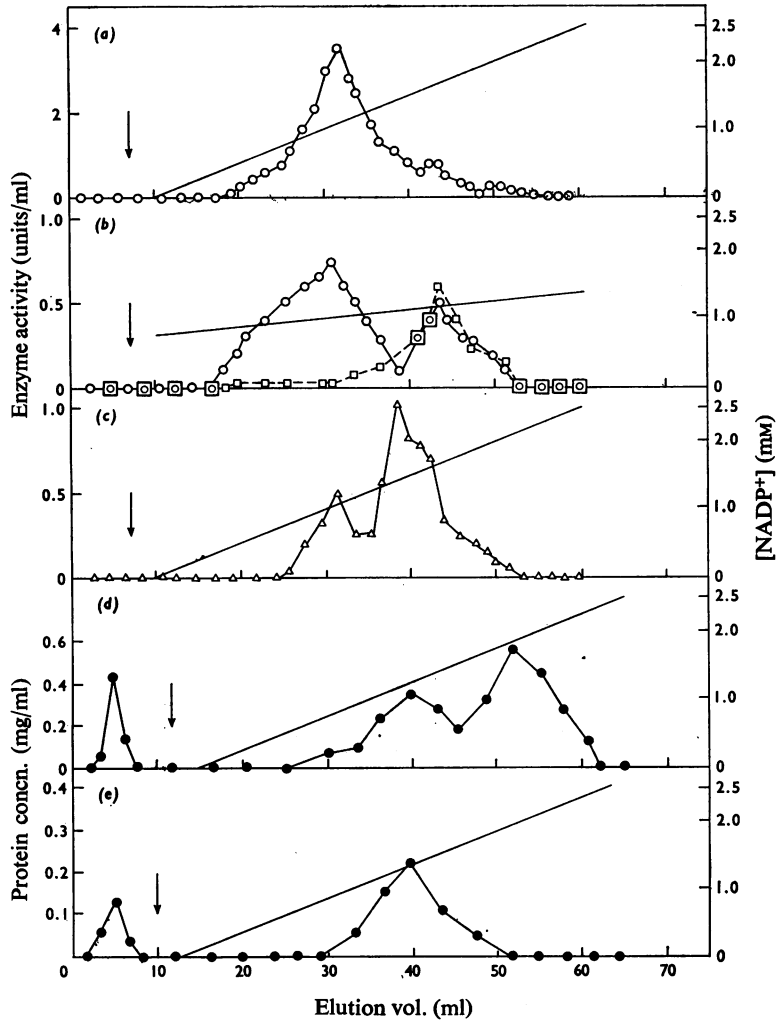


Fig. 2. *Chromatography of wild-type and mutant variant forms of glutamate dehydrogenase on* 8-(6-*aminohexyl*)*amino*-2′-*AMP–Sepharose* 4*B at pH* 6.8

Enzyme was applied in 10mM-sodium phosphate buffer, pH6.8, containing 1mM-EDTA to a column of adsorbent equilibrated as described in the Materials and Methods section. Unbound protein was eluted with the same buffer. Retarded enzyme was then eluted with a linear gradient of 0–2.5mM-$NADP^+$ (*a*, *c*, *d* and *e*) or 0.8–1.1mM-$NADP^+$ (*b*). The gradient was started at the arrow; ——, concentration of $NADP^+$ in the eluted fractions, calculated from the point of application of the gradient and the column bed volume. The linearity of this gradient was confirmed in many cases by measurement. Enzyme activity was assayed in system C (○), system A (values divided by 3 are plotted) (□) or system S (△). ●, Protein concentration. For assays by system A and protein measurements, fractions were dialysed to remove $NADP^+$, which would otherwise interfere. (*a*) Wild-type enzyme: 16 system-C activity units applied in 0.8ml of buffer; 14.5 units were recovered in eluate. (*b*) Mixture of am^3 and wild-type enzymes: 9 system-A units (3.3 system-C units) of wild-type enzyme applied, mixed with 4.1 system-C units of am^3 enzyme (which has negligible system-A activity) in final volume of 0.1ml; 8.9 system-A units and 7.35 system-C units were recovered in total (*c*) am^{19} enzyme: 7 system-S units applied in 0.1ml; 6.9 units recovered. (*d*) am^1 enzyme: 2.77mg of protein applied in 0.27ml; 2.60mg of protein recovered. (*e*) Rechromatography of the first retarded peak of am^1 protein. Fractions containing the elution volume from 26 to 45ml shown in (*d*) were pooled, precipitated with 60%-satd. $(NH_4)_2SO_4$, redissolved in application buffer and dialysed against the same buffer: 1.72mg of protein was applied in 0.27ml; 1.60mg of protein was recovered.

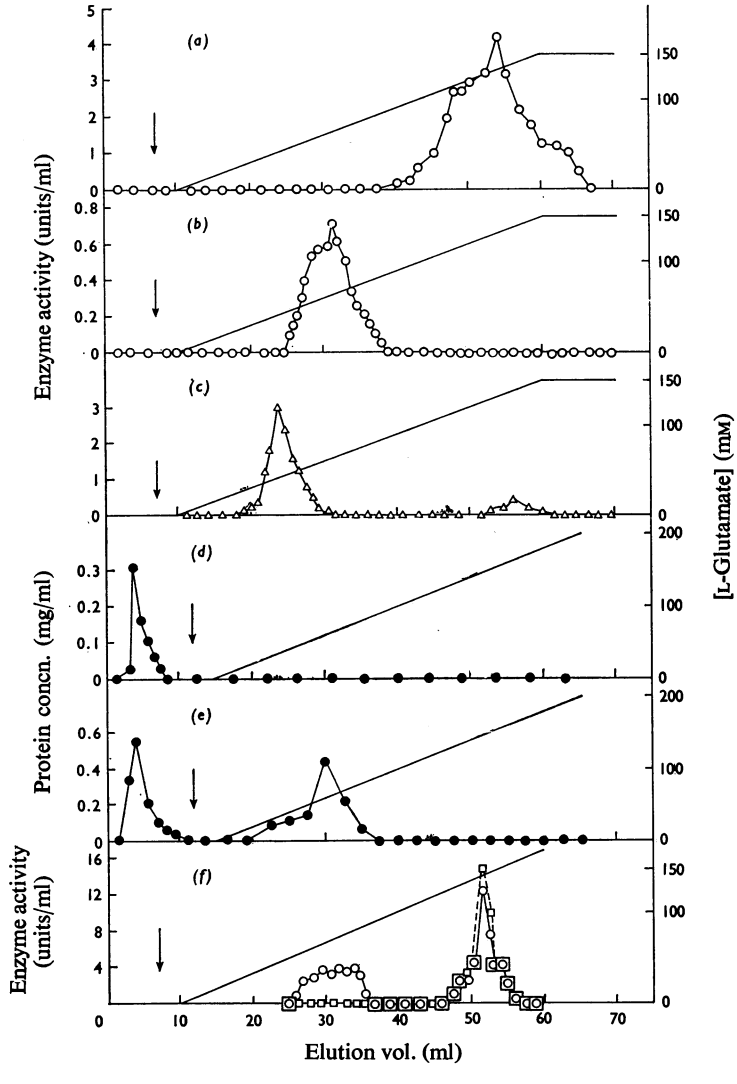


Fig. 3. *Chromatography of wild-type and mutant variant forms of glutamate dehydrogenase on* 8-(6-*aminohexyl*)*amino*-2′-*AMP–Sepharose* 4*B at pH* 8.5

Enzyme was applied in 50mM-Tris/HCl buffer, pH8.5, containing 1mM-EDTA to a column of adsorbent equilibrated as described in the Materials and Methods section. Retarded protein was eluted with a linear gradient of L-glutamate as indicated in the above buffer containing 1.25mM-$NADP^+$. The start of the gradient was applied as indicated by the arrow; ——, concentration of L-glutamate in the eluted fractions, calculated from the point of application of the gradient and the column bed volume. The linearity of this gradient was confirmed in many cases by measurement. Enzyme activity was assayed in system C (○), system A (values divided by 3 are plotted) (□) or system S (△). ●, Protein concentration. For system-A assays and protein measurements fractions were dialysed to remove L-glutamate and $NADP^+$, which would otherwise interfere. (*a*) Wild-type enzyme: 30 system-C activity units applied in 0.3ml of buffer; 28.5 units recovered in eluate. (*b*) am^3 enzyme. 6.5 system-C activity units applied in 0.1ml of buffer; 6.2 units recovered. (*c*) am^{19} enzyme: 15 system-S activity units applied in 0.2ml of buffer; 14.65 units recovered. (*d*) am^1 enzyme: 1.0mg of protein applied in 1.0ml; 0.98mg of protein recovered. (*e*) am^1 enzyme: 2.15mg of protein applied in 0.3ml; 2.16mg of protein recovered. (*f*) Mixture of am^3 and wild-type enzymes: 36 system-C units of am^3 enzyme mixed with 47 system-C units (150 system-A units) of wild-type enzyme were applied in a final volume of 1.0ml; 81.3 system-C units and 146 system-A units were recovered in total.

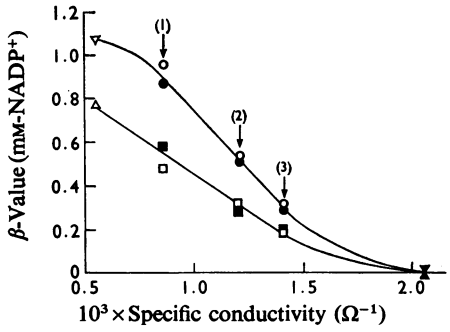


Fig. 4. *Effect of* L-*glutamate and NaCl on the elution of wild-type and am*^{3} *enzymes from* 8-(6-*aminohexyl*)*amino*-2′-*AMP–Sepharose* 4*B by a concentration gradient of NADP*$^+$ *at pH*6.6

Enzyme was applied in 10mM-sodium phosphate buffer, pH6.8, containing 1mM-EDTA as for Fig. 2. For both *am*^{3} and wild-type enzymes 0.5mg was applied in 0.5ml, and was totally retarded on the column in all cases. Elution was by a linear gradient of 0–2.5mM-NADP$^+$ (except for points ▲ and ▼ defined below) with either L-glutamate (●, ■) or NaCl (○, □) added at the following respective concentrations: (1), 15mM, 8.5mM; (2), 30mM, 17mM; (3), 40nM, 22mM. These concentrations are expressed on a common axis of specific conductivity, measured with a Radiometer CDM2d conducting meter (cell constant 1cm). β-Value indicates the concentration of NADP$^+$ that eluted the peak fraction of enzyme activity in the NADP$^+$ gradient (Lowe *et al.*, 1974*c*). ▽, △, Enzyme eluted by the NADP$^+$ gradient alone in the absence of L-glutamate and NaCl. ▼, ▲, Enzyme eluted by a concentration gradient of 70mM-L-glutamate in the absence of NADP$^+$; the points indicate the concentration (and specific conductivity) of L-glutamate that eluted the peak fraction of activity. ○, ●, ▽, ▼, Wild-type enzyme; □, ■, △, ▲, *am*^{3} enzyme.

reported for pig heart lactate dehydrogenase binding to N^6-(6-aminohexyl)-5′-AMP–Sepharose (Lowe *et al.*, 1974*b*). The capacity of our adsorbent for glutamate dehydrogenase was considerably higher at 6°C than 23°C, in accordance with the effects of temperature reported by Harvey *et al.* (1974*a*).

The column dimensions and conditions for equilibration of protein with adsorbent were as described in the Materials and Methods section. The elution flow rate, 8ml/h, was sufficiently low to avoid possible undesirable effects of high flow rates, which according to Lowe *et al.* (1974*c*) decrease column capacity.

Chromatographic behaviour of the wild-type enzyme

Because the wild-type enzyme shows a pH-dependent reversible conformational change, its chromatographic properties were studied in detail at both pH6.8 and 8.5. At pH8.5 the enzyme is essentially completely in the active conformation, characterized by a high intrinsic tryptophan fluorescence, and at pH6.8 the enzyme is completely in an inactive conformation with a tryptophan fluorescence approx. 25% lower (Ashby *et al.*, 1974).

Enzyme retarded at pH6.8 was eluted more readily than enzyme retarded at pH8.5. At pH6.8 complete elution occurred in an NADP$^+$ gradient at a concentration of approx. 1mM (Fig. 2*a*). At pH8.5, NADP$^+$ alone at concentrations up to 1.27mM (approx. 40 times the apparent K_m for NADP$^+$ at 37°C and pH8.5) failed to elute the enzyme. However, elution was achieved reproducibly in the presence of a constant concentration of 1.25mM-NADP$^+$ with a concentration gradient of L-glutamate (Fig. 3*a*), the peak of enzyme being eluted, in almost 100% yield as shown by activity measurements, at approx. 140mM-L-glutamate.

Table 4. *Effect of dicarboxylic acids and NaCl on the elution of wild-type and am*^{3} *enzyme forms at pH*8.5

Enzyme (1mg) was applied in 1ml of 50mM-Tris/HCl buffer, pH8.5, containing 1mM-EDTA to a column of adsorbent equilibrated as described in the text. In all cases the protein was totally retarded, and was eluted with a linear gradient of dicarboxylic acid or NaCl, as indicated, in the above buffer containing 1.25mM-NADP$^+$. The β-values indicate the concentrations of dicarboxylic acid or NaCl which eluted the peak fractions of enzyme activity in the concentration gradients. These concentrations are also expressed in terms of specific conductivity, measured with a Radiometer CDM2d conductivity meter (cell constant 1cm) on standard solutions of the same compounds in the same buffer at 6°C. In each case enzyme was eluted as a single peak of activity, and between 90 and 100% of applied activity was recovered in total in all cases.

	β-values				
Ligand	L-Glutamate	NaCl	D-Glutamate	2-Oxoglutarate	Succinate
Wild-type enzyme					
Ligand concentration (mM)	140	28.5	130	9	19
10^3 × Ligand specific conductivity (Ω^{-1})	5.6	2.8	3.4	0.9	1.6
am^3 enzyme					
Ligand concentration (mM)	65	14	50	12	11
10^3 × Ligand specific conductivity (Ω^{-1})	3.1	1.6	1.6	1.45	1.4

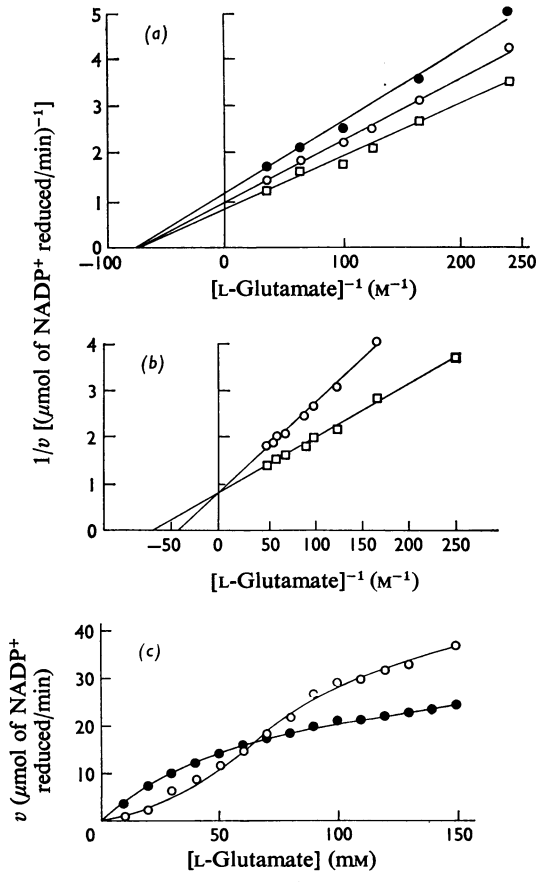


Fig. 5. *Effect of succinate and* D-*glutamate on activity of wild-type enzyme and of* D-*glutamate on activity of* am^3 *enzyme*

Enzyme was added last to assay system C (see the Materials and Methods section), except for variations in L-glutamate concentration as indicated. Initial velocities (v) are expressed in units of μmol of $NADP^+$ reduced/min. (*a*) Wild-type enzyme: □, no inhibitor present; ○, 10mM- and ●, 20mM-succinate (disodium salt adjusted to pH8.5) present. (*b*) Wild-type enzyme: □, no inhibitor present; ○, 5mM-D-glutamate present. (*c*) am^3 enzyme: ○, no inhibitor present; ●, 40mM-D-glutamate present. In (*a*) and (*b*) the lines represent linear unweighted least-squares regressions.

The roles of L-glutamate and other dicarboxylic acids in facilitating or retarding elution at pH8.5 were studied further in comparative experiments (Table 4), but the results reveal a complex situation. In the simpler case of elution at pH6.8, the effects of L-glutamate in facilitating elution are very similar to those of NaCl. The data in Fig. 4, where concentrations of L-glutamate and NaCl are expressed on a common axis of specific conductivity, suggest that at pH6.8 L-glutamate is acting mainly non-specifically as an ion. However, in elution at pH8.5, in addition to non-specific effects such as ion exchange, the following specific interactions of dicarboxylic acids could possibly be involved and have not been distinguished: ternary complex formation of the enzyme with 2-oxoglutarate and $NADP^+$ or with L-glutamate and $NADP^+$ (with the possible additional complication of product formation in the latter case); action of dicarboxylic acids as allosteric activators of enzyme activity in the direction of reductive amination (West *et al.*, 1967; Ashby *et al.*, 1974); action of succinate as a non-competitive inhibitor (Fig. 5*a*) and D-glutamate as a competitive inhibitor (Fig. 5*b*) with respect to L-glutamate.

Chromatography of mutant variant forms of glutamate dehydrogenase

The biospecificity of adsorption and elution in affinity chromatography can in principle be studied by exploiting mutational variants known from studies in free solution to be distinct from the wild-type enzyme in specific ligand-binding properties. Of the variants of NADP-specific glutamate dehydrogenase for which single amino acid substitutions have been determined in this laboratory (Brett *et al.*, 1976), am^1 (Ser-336 replaced by Phe), am^3 (Glu-393 replaced by Gly) and am^{19} (Lys-141 replaced by Met) have been chromatographed on the 2′-AMP–Sepharose column.

Interestingly, the am^1 enzyme, which is totally inactive in all assay systems because of a defect in NADP binding (see below), showed a considerably lower affinity for this adsorbent than did the wild-type enzyme. At pH8.5, 1mg of am^1 protein applied in 1ml was not retarded at all (Fig. 3*d*), virtually 100% of the protein being eluted before the application of $NADP^+$ and the L-glutamate gradient. At a higher concentration of am^1 protein (2.15mg applied in 0.3ml), partial retardation occurred at pH8.5 (Fig. 3*e*), the retarded fraction of enzyme being completely eluted in an earlier part of the L-glutamate gradient than is wild-type enzyme. At pH6.8, 2.77mg of am^1 protein applied in 0.4ml was retarded by approx. 80% (Fig. 2*d*), and the retarded fraction was eluted, in two peaks, later in the $NADP^+$ gradient than is the wild-type enzyme.

Two peaks have been reproducibly obtained from the retarded portion of am^1 enzyme at pH6.8. This phenomenon is not understood, but may indicate some structural heterogeneity in this mutant variant protein. After pooling, concentration and rechromatography under the same conditions, the first of the two peaks ran as an unretarded fraction and a single retarded peak in the original position in the gradient, showing that there is no rapid interconversion of the two retarded fractions (Fig. 2*e*).

The low affinity of am^1 enzyme for the 2′-AMP adsorbent is interesting in view of our current picture of the effect of amino acid substitution (Ser-336 replaced by Phe) in this mutant enzyme (discussed in Brett *et al.*, 1976). This variant completely fails to bind at least the nicotinamide portion of NADPH, as indicated by the absence of enhancement of nicotinamide fluorescence under all conditions tried (Gore, 1972; Ashby, 1974; confirmed by us at pH 6.52, 6.8, 7.4 and 8.54 and concentrations of NADPH of up to 70 μM). This is probably a localized defect in coenzyme binding, because the following two lines of evidence suggest that the conformational properties of the am^1 protein resemble those of the wild-type enzyme. First, am^1 enzyme is similar to (but not identical with) the wild-type enzyme in both the kinetics and equilibrium state of the pH-dependent conformational transition (Ashby *et al.*, 1974; Ashby, 1974), and secondly in hybrid oligomers am^1 subunits can constrain subunits of conformationally defective mutants (e.g. am^3 and am^{19}) in an active conformation (Coddington *et al.*, 1966). A stereochemical model of a localized alteration in the nicotinamide-binding site caused by the Ser-336 to Phe substitution has been proposed (Brett *et al.*, 1976), based on sequence homology between *Neurospora* NADP-specific glutamate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase in this region (Wootton, 1974). The decreased affinity of the am^1 enzyme for the 2′-AMP adsorbent, compared with the wild-type enzyme, suggests that this variant is defective in binding the 2′-AMP portion of the NADP molecule in addition to the nicotinamide portion. Without further experimental evidence on the binding of free 2′-AMP by am^1 enzyme it is not possible to say whether the partial retardation of this enzyme is caused by specific but low affinity for 2′-AMP or by non-specific binding to the spacer arm and its link to the Sepharose. However, it is clear that, even if retardation of am^1 protein is due entirely to non-specific binding, such binding is weak. Since the am^1 protein is probably conformationally normal apart from its localized defect in coenzyme binding, we can conclude that with the wild-type enzyme also non-specific binding is at most weak.

In contrast with am^1 enzyme, the potentially active am^3 variant is fully retarded by the adsorbent at pH 8.5 (Fig. 3*b*), pH 7.5 (Table 3) and pH 6.8 (Fig. 2*b*). However, at both pH 8.5 and 6.8 this variant is eluted earlier in the respective gradients than is the wild-type enzyme, and co-chromatography of the am^3 and wild-type enzymes shows that they are well separated at both pH values (Figs. 2*b* and 3*f*).

This earlier elution of the am^3 enzyme is consistent with the effects of $NADP^+$ and 2′-AMP on its kinetics in free solution. Fig. 6 shows that inhibition of am^3 enzyme activity by 2′-AMP is competitive with respect to $NADP^+$, as for the wild-type

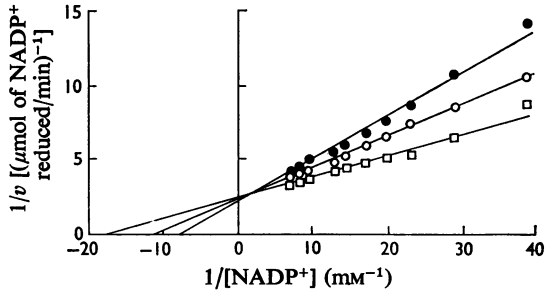


Fig. 6. *Competitive inhibition of am^3 enzyme by 2′-AMP*
am^3 enzyme was added last to a modified system-C assay mixture in which the final concentration of L-glutamate was 0.3 M, and $NADP^+$ was varied as indicated. Initial velocities (v) are expressed in units of μmol of $NADP^+$ reduced/min. □, No inhibitor present; ○, 1.25 mM-2′-AMP; ●, 2.5 mM-2′-AMP. The lines represent linear unweighted least-squares regressions.

enzyme. However, both the apparent K_m for $NADP^+$ (60 μM, compared with 30 μM for the wild-type enzyme) and the apparent K_i for 2′-AMP (1.8 mM, compared with 450 μM for the wild-type enzyme) are higher for the am^3 enzyme. Although values of K_i cannot be equated with inhibitor dissociation constants for this enzyme, the simplest interpretation is that the am^3 enzyme has a lower affinity for 2′-AMP than the wild-type enzyme. If, as the relative K_m and K_i values possibly suggest, the affinity for $NADP^+$ is lowered in the am^3 enzyme to a lesser extent than the affinity for 2′-AMP, this explains the earlier elution of the am^3 enzyme.

Apart from its earlier elution the behaviour of the am^3 enzyme resembles that of the wild-type enzyme in chromatography at both pH values. The effects of L-glutamate, other dicarboxylic acids and NaCl in facilitating elution are similar for both enzymes (Table 4; Fig. 4). The am^3 variant shows a sigmoid dependence of enzyme activity on L-glutamate concentration (Fig. 5*c*), which adds further difficulty to the interpretation of effects of L-glutamate on elution.

The major effect of the am^3 substitution (Glu-393 replaced by Gly) is an abnormal stabilization of the inactive conformation of low intrinsic tryptophan fluorescence (Ashby *et al.*, 1974; Ashby, 1974; Brett *et al.*, 1976). The general similarity of the elution behaviour of the am^3 and wild-type enzymes contrasts with their considerable difference in the position of the conformational equilibrium between active and inactive states. This suggests that the active and inactive conformations differ little in their chromatographic properties on this adsorbent.

The enzyme from mutant am^{19} (substitution of Lys-141 by Met) differs from the wild-type and am^3

enzymes in being eluted as two retarded peaks at both pH 6.8 and 8.5 (Figs. 2*c* and 3*c*). This is perhaps a consequence of the known conformational heterogeneity of the am^{19} enzyme (Coddington *et al.*, 1966; Ashby, 1974), but this possibility has not been tested further.

Separation of hybrid hexamers

Since the wild-type, am^1, am^3 and am^{19} species of glutamate dehydrogenase show distinct differences in adsorption to and elution from immobilized 2′-AMP, this adsorbent might be expected to separate heterohexamers of different subunit ratio made from these species. Because the best separation observed between homohexamers was that shown by the wild-type, am^1 and am^3 enzymes in the pH 8.5 system (Fig. 3), initial attempts to resolve the products of the freeze–thaw hybridization procedure (see the Materials and Methods section) were made at pH 8.5, by using equimolar hybridization mixtures of wild-type–am^3 and wild-type–am^1 enzyme pairs. The results were disappointing, showing a single broad peak in each case. Variations in enzymological properties across these peaks suggested that several unresolved hybrid species were present, but the system showed little improvement as a means of separating hybrid hexamers compared with the ion-exchange chromatography of Coddington & Fincham (1965).

In contrast, very encouraging results were obtained from the pH 6.8 system. Fig. 7(*a*) shows the set of several resolved or partially resolved peaks, as measured by activity in assay system C, obtained from chromatographing the products of a hybridized equimolar mixture of am^1 and am^3 enzymes. Protein measurements on the early fractions showed that no detectable am^1 homohexamer, which would be eluted as unretarded inactive protein, was present. The pattern of peaks of enzyme activity is very reproducible in both the relative heights and widths of the successive peaks and in their elution concentrations in the $NADP^+$ gradient: profiles very similar to Fig. 7(*a*) have been obtained from seven different equimolar hybridization mixtures of am^1 and am^3 enzymes chromatographed under the same conditions. These partially resolved peaks appear to represent chromatographically distinct species of enzyme that are stable to precipitation, redissolving and dialysis, as shown by rechromatography (Figs. 7*b*–7*e*). Repetition of chromatography and rechromatography as described in Fig. 7 enabled us to purify milligram quantities of these chromatographically distinct species for further study.

Simple physicochemical techniques for the determination of subunit ratio, comparable with the electrophoretic methods commonly used to analyse hybrid isoenzymes, are not applicable to hybrid hexamers of *Neurospora* NADP-specific glutamate dehydrogenase because the homohexameric variants are of identical mobility in gel electrophoresis and isoelectric focusing. However, several lines of evidence demonstrate that the chromatographically discrete peaks of Fig. 7(*a*) represent distinct hybrid enzymes which differ in subunit ratio. First, the peaks differ in enzymological properties, as shown, for example, by the ratio of activity given by 300 mM- and 75 mM-L-glutamate (Fig. 7*a*). Most of the peaks show, to different extents, evidence of complementation activity (defined as enzyme activity shown by hybrid oligomers under conditions in which the original homo-oligomers are significantly less active or completely inactive), since the activity that they give with 75 mM-L-glutamate is significantly greater than that given by the am^3 homohexamer. Similar complementation activity of unresolved am^1–am^3 hybridization mixture was observed by Fincham & Coddington (1963). Secondly, in experiments using different peaks purified by rechromatography as in Figs. 7(*b*)–7(*e*), the distinct chromatographic species differed in the pH-dependence of enzyme activity, showing complementation activity to different extents at pH below 7.9. These species differed also in the pH-dependence of a conformational change, corresponding to that described for the wild-type enzyme by Ashby *et al.* (1974), detectable by a change in the intrinsic tryptophan fluorescence of the protein. Thirdly, an experiment was performed in which the homohexamers of am^1 and am^3 enzymes were labelled respectively with ^{14}C and ^3H by partial carboxymethylation with labelled iodoacetate before hybridization. Controls showed that this modification did not detectably alter the chromatographic properties or activity of the enzymes. With this doubly-labelled hybridization mixture the chromatographically distinct peaks from a run similar to that of Fig. 7(*a*) showed clear differences in the ratio of ^{14}C to ^3H. These ratios were very close to the integral values expected for hybrid hexamers of subunit ratios 5:1, 4:2, 3:3, 2:4 and 1:5. The latter experiments, although of interest for studies of hybrid oligomers, are not discussed in detail here because the purpose of the present paper is to describe the chromatographic system and its potentialities.

Chromatographic profiles showing similar resolution to that of Fig. 7(*a*) were obtained in other experiments by using the pH 6.8 system, from hybridized mixtures of the am^1–am^{19}, wild-type–am^1 and wild-type–am^3 pairs of enzymes. In each of these experiments differences in enzymological properties suggested that the partially-resolved peaks represented different hybrid species. Therefore this chromatographic system opens up the possibility of studies of subunit interactions and complementation in several interesting species of isolated hybrid hexamers of this glutamate dehydrogenase.

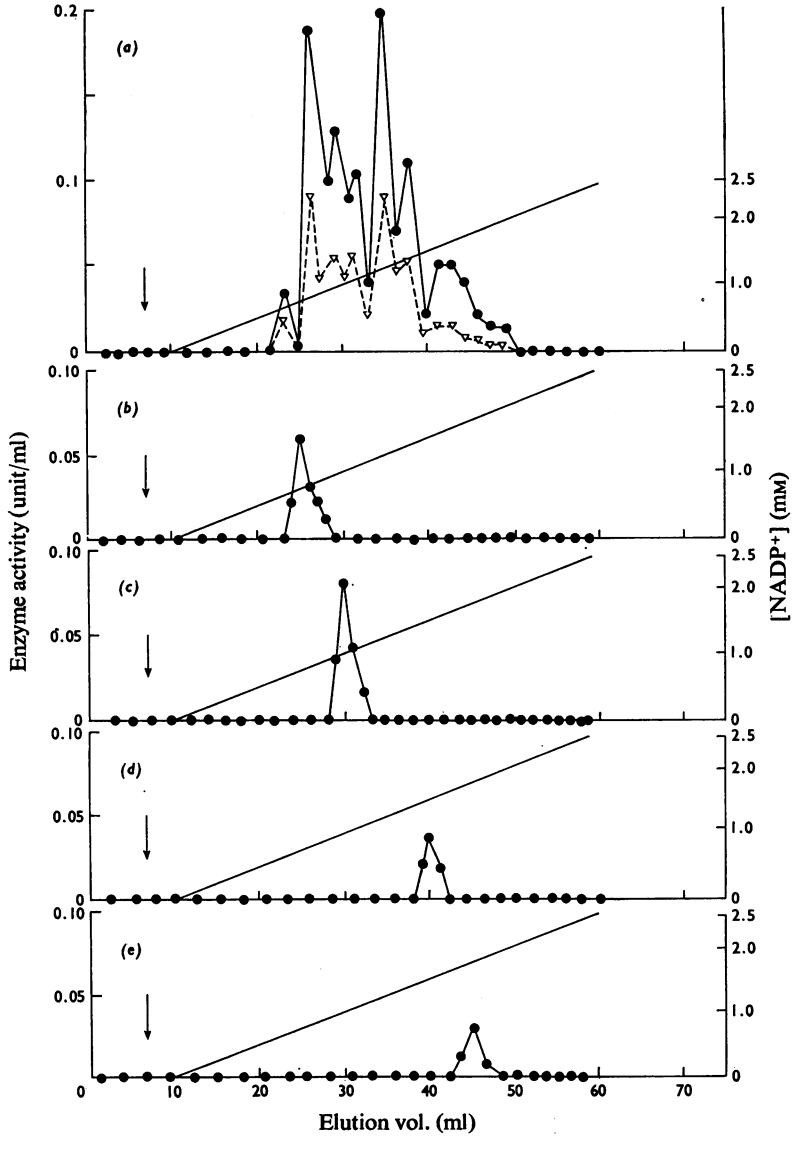


Fig. 7. *Chromatography on* 8-(6-*aminohexyl*)*amino*-2′-*AMP*–*Sepharose* 4*B at pH*6.8 *of the mixture of hybrid hexamers obtained by freezing and thawing an equimolar mixture of* am^1 *and* am^3 *enzymes*

After the hybridization procedure at pH7.4 (see the Materials and Methods section), the enzyme mixture was dialysed against 10mM-sodium phosphate buffer, pH6.8, containing 1mM-EDTA, and 0.1ml was applied to the column as for Fig. 2. All enzyme was retarded. (*a*) Elution profile of this mixture of hybrids obtained with a linear gradient of 0–2.5mM-$NADP^+$. Enzyme was assayed in modified system-C assays in which the final concentrations of L-glutamate were 0.3M (●) and 0.075M (▽). (*b*) to (*e*): rechromatography of peak fractions of profile (*a*). In each case enzyme was precipitated with 60%-satd. $(NH_4)_2SO_4$, redissolved in approx. 0.05ml of application buffer, dialysed against this buffer and applied to the column. Fractions corresponding to the following elution volumes of profile (*a*) were rechromatographed as shown: in (*b*) 25.0–26.25ml; (*c*) 31.0–32.25ml; (*d*) 36.5–37.75ml; (*e*) 41.5–42.75ml. ——, $NADP^+$ concentration as described for Fig. 2. Units of enzyme activity (0.3M-L-glutamate assay) applied and recovered were, respectively: in (*a*) 2.01 and 1.99 units; in (*b*) 0.24 and 0.23 unit; in (*c*) 0.18 and 0.18 unit; in (*d*) 0.14 and 0.11 unit; in (*e*) 0.07 and 0.08 unit.

General Discussion

Our results illustrate the value of using mutationally modified variants of an enzyme, which differ only in single amino acid substitutions, in assessing the biospecificity of elution and adsorption and the contribution of non-specific interactions. Effects of such substitutions on specific ligand-binding properties may be estimated from measurements in free solution. A limitation of this approach is the possibility that the substitutions may also affect non-specific interactions in an unknown way, possibly through indirect conformational effects. Nevertheless, taking together the results from the wild-type, am^1 and am^3 enzymes, it is reasonable to conclude that retardation of *Neurospora* NADP-specific glutamate dehydrogenase by this adsorbent is predominantly ligand-specific, that non-specific interactions are at most weak, and that the conformational difference between the active high-fluorescence and inactive low-fluorescence states of the enzyme has no significant effect on chromatographic behaviour.

Further work is required to provide an understanding of the apparent heterogeneity in affinity chromatography of purified am^1 and am^{19} enzyme variants. Purified wild-type and am^3 enzymes may also be exhibiting chromatographic heterogeneity in giving single peaks (Figs. 2*a*, 2*b*, 3*a*, 3*b* and 3*f*) which are possibly broader than would be expected from a single chromatographic species. Considerably narrower peaks were obtained from similar runs of hybridized mixtures in the pH6.8 system (Fig. 7).

We have not used 8-(6-aminohexyl)amino-2′-AMP–Sepharose 4B in large-scale purifications of this enzyme or of other NADP-specific dehydrogenases. Ox liver glutamate dehydrogenase has been purified by affinity chromatography on immobilized GTP (Godinet *et al.*, 1974), but this method would not be applicable to microbial NADP-specific glutamate dehydrogenases because they do not show interactions with GTP. An adsorbent synthesized by the modular solid-state approach, *N*-carboxymethyl-L-glutamate–Sepharose 4B, has been used by Blumenthal and Smith (1973) in the purification of *Neurospora crassa* NADP-specific glutamate dehydrogenase. A disadvantage of this adsorbent is that low-pH buffers, which normally cause partial inactivation of the enzyme, are required for elution. We have confirmed the results of Blumenthal & Smith (1973), which showed considerable losses of enzyme activity during chromatography on this adsorbent. In comparison with these alternatives, 8-(6-aminohexyl)amino-2′-AMP–Sepharose 4B appears to be a suitable adsorbent for the purification of glutamate dehydrogenases that utilize NADP.

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