Studies on the Use of Sepharose-*N*-(6-Aminohexanoyl)-2-Amino-2-Deoxy-D-Glucopyranose for the Large-Scale Purification of Hepatic Glucokinase

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The synthesis of *N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucose is described and it was shown to be a competitive inhibitor (K_i , 0.75 mM) with respect to glucose of rat hepatic glucokinase (EC 2.7.1.2). After attachment to CNBr-activated Sepharose 4B, this derivative was able to remove glucokinase quantitatively from crude liver extracts and release it when the columns were developed with glucose, glucosamine, *N*-acetylglucosamine or KCl. Repeated exposure of the columns to liver extracts led to rapid loss in their effectiveness as affinity matrices because proteins other than glucokinase are bound to the columns. The nature of such protein binding and methods for the rejuvenation of 'used' columns are discussed along with the effect of the mode of preparation of the Sepharose-ligand conjugate and the concentration of bound ligand on the purification of glucokinase. Glucose 6-phosphate dehydrogenase is cited as an example of both non-specific protein binding to the affinity column and of the importance of the control of ligand concentration in removing such non-specifically bound proteins. Some guidelines emerged that should be generally applicable to other systems, particularly those which involve affinity chromatography of enzymes that are present in tissue extracts in very low amounts and possess only a relatively low association constant for the immobilized ligand.

Despite the impact of affinity chromatography on protein-separation technology [see review by Cuatrecasas (1972) and references therein] most of the current literature on this subject has concerned itself with small-scale purifications, often using commercially available enzymes in model systems (e.g. Mosbach *et al.*, 1972; Brodelius & Mosbach, 1973; Lowe *et al.*, 1974a; Lowe & Mosbach, 1975). Only in a relatively few instances has the technology been applied to the large-scale purification of enzymes to homogeneity (e.g. Trayer & Hill, 1971; Barker *et al.*, 1972; Nicolas *et al.*, 1972). This is particularly true when either the ligand to be immobilized has only a relatively high dissociation constant (about 10^{-3} M) for the enzyme under study or the enzyme is present in the tissue source in only very small amounts.

In the large-scale purification of rat hepatic glucokinase (EC 2.7.1.2) (the following paper, Holroyde *et al.*, 1976), the key step was the affinity chromatography of the enzyme on a Sepharose-*N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose matrix. Our initial attempts (Chesher *et al.*, 1973) to utilize this immobilized glucosamine derivative, which in free solution is a competitive inhibitor with respect to glucose of the glucokinase reaction (K_i , 0.75 mM), in a chromatographic procedure gave variable results. A more detailed appraisal now

permits us to report the various parameters, such as the control of ligand concentration on the Sepharose matrix and the elution conditions used that can be varied to obtain optimum results. Correct washing procedures were found to be essential when processing crude extracts from up to 100 rat livers to maintain the effectiveness of the affinity matrix.

Although the results presented here are devoted to the affinity chromatography of glucokinase, some guidelines emerge that should be generally applicable to other systems. Some of this work has been reported in preliminary form (Holroyde & Trayer, 1974).

Materials and Methods

Materials

Chemicals. Coenzymes, nucleotides, Dowex ion-exchange resins and dithiothreitol were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Ethyl trifluorothiol acetate was from Pierce Chemical Co., Rockford, Ill., U.S.A., *N*-ethoxycarbonylthoxy-1,2-dihydroquinoline was obtained from Calbiochem, San Diego, Calif., U.S.A., and CNBr was from R. Emanuel, Wembley, Middx., U.K. Glucosamine hydrochloride, *N*-glycylglycine, *N*-(*N*-glycylglycyl)-glycine and 6-aminohexanoic acid were supplied by

BDH Chemicals, Poole, Dorset, U.K. Sepharose 4B was from Pharmacia (G.B.) Ltd., London W.5, U.K., and DEAE-cellulose (DE 52) was from Whatman Biochemicals, Maidstone, Kent, U.K. All other chemicals were AnalaR grade and used as supplied.

Proteins. Yeast hexokinase [ATP-D-hexose 6-phosphotransferase, EC 2.7.1.1; type C-302 (300units/mg)] and glucose 6-phosphate dehydrogenase [D-glucose 6-phosphate-NADP⁺ oxidoreductase, EC 1.1.1.49, from yeast, type VII (345 units/mg)] were purchased from Sigma Chemical Co. Pronase (B grade) was from Calbiochem.

Glucokinase preparations. The crude extracts of rat liver were prepared as described by Chesher *et al.* (1973). The partially purified enzyme preparations (post-DEAE-cellulose treatment) were obtained as described by Holroyde *et al.* (1975).

Methods

Preparation of *N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose. The synthesis of an amide linkage between the amino group of glucosamine and the carboxyl group of 6-aminohexanoic acid is described below. This involves prior blocking of the amino group of the amino acid before the coupling reaction and deblocking afterwards. This scheme has also been carried out with galactosamine (R. Barker, personal communication) and with a variety of other amino acids (A. S. Warsy & I. P. Trayer, unpublished work).

***N*-Trifluoroacetyl-6-aminohexanoic acid.** To 0.1 mol (13.2g) of 6-aminohexanoic acid, dissolved in 100ml of water and maintained at 4°C in an ice-bath, were added 18ml of ethyl trifluorothiol acetate in 1ml batches during a 3–4h period with vigorous stirring. The emulsion was maintained at pH9.5 by the occasional addition of 1M-NaOH. The reaction was judged complete (3–4h) when only a pale-pink (and not blue) colour was obtained after spotting a sample on paper, spraying with ninhydrin and heating at 100°C for 5 min. The reaction mixture was adjusted to pH5 with trifluoroacetic acid and concentrated to dryness at 40°C. The residue was redissolved in ethanol and reconcentrated from this (three times in all) and then three times from aq. 50% (v/v) ethanol to remove ethyl mercaptan. The residue was redissolved in 100ml of 50% (v/v) ethanol, adjusted to pH1.5 with trifluoroacetic acid, and passed through a column (30cm×3cm) of Dowex 50 (X8; H⁺ form; 20–50 mesh) equilibrated to 50% (v/v) ethanol to remove any unchanged amine. The acidic eluate was concentrated to dryness under vacuum at 45°C and then dried *in vacuo* over anhydrous CaSO₄ for 24h. This material (in aqueous solution) migrated as a single anionic component on high-voltage paper electrophoresis at pH6.5, was negative to ninhydrin but gave a yellow acidic spot when

stained with Bromophenol Blue. It was stored in a desiccator at room temperature and was stable over several years. The overall yield of pure compound was 20.3g (90%) and was used in the subsequent stages without further characterization.

***N*-(*N*-Trifluoroacetyl-6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose.** Glucosamine hydrochloride (2.15g, 10mmol) and *N*-trifluoroacetyl-6-aminohexanoic acid (2.27g, 10mmol) were dissolved in 400ml of water and adjusted to pH7.5 with 1M-NaOH. To this solution was added 15g (60mmol) of *N*-ethoxycarbonylethoxy-1,2-dihydroquinoline (Belleau & Malek, 1968) and the suspension was shaken vigorously at 37°C for 16h. During the first 2–4h of the reaction, three additions of 100ml of ethanol were made to bring the *N*-ethoxycarbonylethoxy-1,2-dihydroquinoline into solution slowly so that activation of the carboxyl groups occurred throughout the reaction. After 16h, the reaction mixture was evaporated to dryness at 40–50°C and the residue extracted twice with 200ml of water. The water-soluble extracts were combined, adjusted to pH1.5 with trifluoroacetic acid, and passed through a column (10cm×3cm) of Dowex 50 (X8; H⁺ form; 20–50 mesh) to remove unchanged glucosamine. The column was washed with 200ml of water. The eluate and washings were combined, adjusted to pH7 with 1M-NaOH, and evaporated to dryness. The product contained some unchanged *N*-trifluoroacetyl-6-aminohexanoic acid which was removed at a later stage.

The reaction was monitored by high-voltage paper electrophoresis at pH6.5. The disappearance of glucosamine (which is positive to both ninhydrin and the silver nitrate test for reducing sugars) correlates with the appearance of the product (ninhydrin-negative and silver nitrate-positive) at the origin. The yield was 70–80%. This material was chemically characterized in our early preparations (see below), but as a routine in later preparations was immediately deblocked as follows.

***N*-(6-Aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose.** The above product (about 3.5g) was dissolved in 12–13ml of 1.0M-piperidine at room temperature (20°C) and the solution was immediately chilled in an ice bath. After standing at 0°C for 2h, the reaction mixture was adjusted to pH6.0 with 0.5M-acetic acid and applied directly to a column (140cm×3.0cm) of Sephadex G-10 equilibrated in water. The product was located in the eluate by testing samples for reducing sugar with ferricyanide (Park & Johnson, 1949). Two ferricyanide-positive peaks emerged from the column. The first contained the final product which was further purified as described below. The second peak, which emerged together with the salt peak, located by conductivity measurements, contained some neutral material positive to silver nitrate together with some 6-aminohexanoate and piperidine.

Further deblocking of this unidentified material did not yield any product and so this second peak was discarded. After concentrating the appropriate fractions to dryness, the product was dissolved in 1 mM-HCl and applied to a column (25 cm $\times$ 0.9 cm) of Dowex 50 (X8; H⁺ form; 200–400 mesh) previously equilibrated with 1 mM-HCl. The column was washed with about 100 ml of 1 mM-HCl and a linear gradient (400 ml of 1 mM-HCl and 400 ml of 0.3 M-LiCl in 1 mM-HCl) was applied. The initial breakthrough peak was examined by high-voltage paper electrophoresis at pH 6.5 and contained 6-amino-hexanoic acid and neutral silver nitrate-positive material. This latter material could not be further deblocked to form additional product and appeared to be derived from the coupling agent *N*-ethoxycarbonyl-ethoxy-1,2-dihydroquinoline and was discarded. The single peak of material that emerged under the gradient and gave a positive reaction for reducing sugar was concentrated to dryness. It was desalted, if necessary, with Sephadex G-10 as described above, but it can be used for coupling to CNBr-activated agarose without further purification.

As judged by the ferricyanide procedure, the overall yield was 1.9 g (65%) and the product was stored, after adjusting it to pH 7.0 with 1 M-NaOH, in portions at -12°C .

The final product was not crystallized but was shown to be pure because it migrated as a single cationic species on high-voltage paper electrophoresis (mobility relative to glucosamine: glucosamine, 1.0; product 0.81) and on ascending paper chromatography in solvents 1 and 2 (see below) ($R_F1 = 0.65$; $R_F2 = 0.56$). The single component gave positive tests with both ninhydrin and silver nitrate. The product was further characterized by chromatographic analysis on an amino acid analyser (see below) after acid hydrolysis (6 M-HCl, 20 h at 105°C). The hydrolysate was shown to contain only 6-amino-hexanoic acid and glucosamine in a molar ratio of 1 : 1. On this basis, and the elementary analyses found for the *N*-trifluoroacetyl derivative (elementary analyses; found: C, 43.5, H, 6.1; N, 7.8; $\text{C}_{13}\text{H}_{23}\text{N}_2\text{O}_6\text{F}_3$ requires C, 43.3; H, 6.4; N, 7.8%), the structure shown in Fig. 1 was assigned to this product.

The equivalent *N*-glycylglycine and *N*-(*N*-glycylglycyl)glycine derivatives of glucosamine were prepared in a manner identical with that described above. These derivatives were shown to be pure by high-voltage paper electrophoresis at pH 6.5 and were characterized by chromatography on the amino acid analyser (see below) after acid hydrolysis. The expected molar ratios of glycine/glucosamine were found.

Preparation and characterization of Sepharose adsorbents. The ligands were attached to CNBr-activated Sepharose 4B (Axen *et al.*, 1967) under the conditions described by Trayer *et al.* (1974). These

procedures generally gave a coupling efficiency of 80–95% as judged by the analytical procedures described below.

As a routine, the ligands were left to couple to the activated gel at a concentration of 10–12 $\mu\text{mol/g}$ of gel on a rotary mixer overnight at 4°C . For the 'direct-linked' gels described in Fig. 6, the ligands were coupled at different concentrations, ranging from 2 to 10 $\mu\text{mol/g}$ of gel. (N.B. 1 g of gel in these experiments is defined as the weight of Sepharose 4B taken after packing it down in a Buchner funnel connected to a water suction pump until the Sepharose 4B began to 'crack'. This is not entirely satisfactory but does give reproducible results in our experience.)

After coupling, unchanged groups on the Sepharose 4B were treated by washing the gels with 1 M-ethanolamine adjusted to pH 8 with HCl. The gels (up to 100 g) were then washed on a Buchner funnel with 0.1 M- Na_2CO_3 , pH 9.5 (2 litres), water (1 litre), 2 M-KCl containing 1 mM-HCl (2 litres) and water (1 litre). The gels were characterized by taking a known weight of gel and washing by centrifugation in a bench centrifuge three times with acetone. The dried residue was hydrolysed *in vacuo* in a sealed tube with 2 ml of 6 M-HCl at 105°C for 24 h. The black precipitate that formed was removed by centrifugation, washed with 3×5 ml of water and the combined supernatants were concentrated to dryness from water. The residue was dissolved in a known volume of 0.2 M-sodium citrate adjusted to pH 2.2 with 1 M-HCl and an appropriate portion of it subjected to chromatography on a Beckman Spinco model 120B amino acid analyser. Losses due to mechanical transfer were eliminated by using norleucine as an internal standard. Known concentrations of glucosamine and 6-amino-hexanoic acid were similarly hydrolysed before their use as standards on the analyser to correct for any destruction occurring during hydrolysis.

Dilution of substituted gels to a required ligand concentration was performed by weighing out the substituted gels and fresh Sepharose 4B as described above and mixing in the appropriate proportions. When packing columns of diluted gels, the mixture was stirred continuously and small samples were withdrawn for introducing to the glass columns; this procedure decreased the possibility of local regions of either unsubstituted or substituted gel in the resulting column.

Operation of the affinity columns. These were always run at 4°C and were unpacked and washed by suction on a Buchner funnel with 2 M-KCl/6 M-urea after every use. Pronase treatment, when required, was carried out as follows: the gels (200–300 g) were first washed with the KCl/urea solution and then suspended in 20 mM-triethanolamine/HCl, pH 7.0, at room temperature. Pronase (20 mg) was added

and the gel suspension gently stirred. Two further additions of Pronase (20mg each) were made after 2h and 4h and the suspension was left stirring gently overnight. The treated gels were then washed carefully on a Buchner funnel with water (1litre), the KCl/urea solution (3litres), water (10litres) followed by the appropriate operating buffer (1 litre). The gels were stored in this buffer containing 0.2% sodium azide at 4°C.

The progress of glucose gradients was monitored by subjecting samples to the phenol/H₂SO₄ reaction for neutral sugars (Ashwell, 1966). Recoveries of glucokinase from these columns was always greater than 80% (usually nearer 100%) unless specifically stated to the contrary.

Enzyme assays. Glucokinase activity was measured at 30°C in a total volume of 0.75ml by the coupled assay of Parry & Walker (1966) as described by Storer & Cornish-Bowden (1974). Low-*K_m* hexokinase activities were estimated in the presence or absence of glucokinase as described by Holroyde *et al.* (1975). Any contaminating activities which may interfere with these assays and the method of correction for them are also described in that paper. Glucose 6-phosphate dehydrogenase activity was also measured by the increase in *E*₃₄₀ as NADP⁺ was reduced in a total volume of 0.75ml containing the same reagents as for the glucokinase assay except that the glucose, ATP and glucose 6-phosphate dehydrogenase were replaced by 20mM-glucose 6-phosphate.

One unit of enzyme activity is defined as that which catalyses the formation of 1μmol of either glucose 6-phosphate/min (glucokinase) or 6-phosphogluconate (glucose 6-phosphate dehydrogenase) at 30°C.

Analytical procedures. High-voltage paper electrophoresis and paper chromatography were carried out as described by Trayer *et al.* (1974). The solvents (referred to above) are: solvent 1, isobutyric acid/NH₃ (sp.gr. 0.88)/water (66:1:33, by vol.); solvent 2, propan-2-ol/aq. 0.25M-NH₄HCO₃ (13:7, v/v).

Chromatograms and electrophoretograms were developed with the ninhydrin/cadmium reagent for amines (Barrolier, 1957) and with Bromophenol Blue [a 0.1% solution in aq. 95% (v/v) ethanol titrated to its end point with 5M-NH₃] for acidic and basic groups. Reducing sugars on chromatograms were revealed by treatment with the silver nitrate reagent. A saturated solution of silver nitrate in water (0.1 ml) was added to 20ml of acetone and water added until the white precipitate disappeared. The dried chromatography paper was dipped in this solution and then immediately sprayed with 0.5M-NaOH in aq. 95% (v/v) ethanol. Reducing sugars appeared as dark-brown spots.

Salt peaks were detected in fractions after gel filtration by monitoring on a conductivity meter, type CDM (Radiometer, Copenhagen, Denmark).

Elementary analyses were performed by the Department of Chemistry (Analytical Section) of this University.

Results

Initial studies on the affinity chromatography of glucokinase on immobilized glucosamine derivatives

The structure of the glucosamine derivative prepared by synthesizing an amide link between the amino group of glucosamine and the carboxyl group of 6-aminohexanoic acid is shown in Fig. 1(a). The free ligand was a competitive inhibitor with respect to glucose in the glucokinase reaction, with a *K_i* (0.75mM) similar to that of free glucosamine (Fig. 1b), and was not phosphorylated. Another glucosamine derivative prepared by synthesizing a glycosidic bond between position 1 of *N*-acetylglucosamine and 6-aminohexan-1-ol (Barker *et al.*, 1972, 1974) did not inhibit the glucokinase reaction nor did this enzyme bind to this derivative linked to Sepharose. This result is consistent with the view that the position 1 of glucose is essential for the binding of glucose to a variety of hexokinase enzymes during their reaction (Crane, 1962).

When rat liver extracts were applied to conjugates of *N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose with Sepharose then all of the glucokinase activity was retained on these columns and could be released by the introduction of glucose to the

Fig. 1. Inhibition of glucokinase activity by *N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose

(a) Structure of *N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose. (b) Full details of the reaction mixture are given in the Materials and Methods section. The enzyme (0.02 unit/ml) was incubated at 30°C with 5, 10, 20, 30 and 50mM-glucose in the absence and presence of the glucosamine derivative. ○, Glucose only; ●, glucose + 0.66mM-glucosamine derivative; Δ, glucose + 1.33mM-glucosamine derivative.

Fig. 2. Chromatography of rat liver extracts on various Sepharose derivatives

To all columns (15cm×0.8cm) 2.5ml of liver extract (containing 0.8 unit of glucokinase activity/ml) was applied. The columns were operated in 20mM-triethanolamine/HCl, pH 7.0, containing 10mM-KCl, 4mM-EDTA, 7.5mM-MgCl₂, 1mM-dithiothreitol and 5% (v/v) glycerol at 20ml/h; 1.5ml fractions were collected. At A, 0.5M-glucose and at C, 0.5M-KCl, were included in the developing buffers. At B, the buffer was changed to 2M-KCl/6M-urea. (a) Sepharose-*N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose (ligand concentration: 2 μmol/g wet weight of packed gel); (b) Sepharose-glucosamine (6 μmol/g wet weight of packed gel); (c) Sepharose-6-aminohexanoate (6 μmol/g wet weight of packed gel); (d) Sepharose-*N*-(*N*-glycylglycyl)-2-amino-2-deoxy-D-glucopyranose (8 μmol/g wet weight of packed gel). ○, *E*₂₈₀; ●, glucokinase activity.

developing buffers (Fig. 2a). Under these conditions the liver low-*K_m* hexokinase activity appeared not to be retained on the columns but to pass through unretarded. Subsequent work has shown that mammalian hexokinases can be bound to these glucosamine derivatives under certain conditions (A. S. Warsy, M. J. Holroyde & I. P. Trayer, unpublished work). In separate experiments, yeast hexokinase did not exhibit any interaction with these glucosamine-containing matrices.

Glucokinase eluted from these derivatives (Fig. 2a) was found to be purified some 500- to 1000-fold. Rechromatography of the glucokinase fraction on the affinity columns under similar conditions resulted in additional purification but the final product was still heavily contaminated by other proteins and in

our best preparations the glucokinase only represented some 5% (w/w) of the total protein present. These columns lost much of their effectiveness as affinity matrices when repeatedly exposed to liver extracts. The apparent 'capacity' of the columns to bind glucokinase was gradually diminished with each subsequent usage. Their working life could be prolonged somewhat by exhaustive washing between runs with 6M-urea/2M-KCl but after four or five chromatography runs their effectiveness was much decreased. There was no evidence of any enzymic degradation of the ligand attached to the columns and subsequent experimentation suggested that the problem was a gradual build-up of apparently non-specific protein on the affinity matrix. As a consequence the interaction of proteins from the liver extracts with the component parts of the glucosamine derivative attached to Sepharose was investigated.

Although it was not possible to show any interaction between glucokinase and glucosamine attached directly to CNBr-activated Sepharose-4B without the intervening 6-aminohexanoate 'spacer' molecule (Fig. 2b), these derivatives did remove a considerable amount of protein when liver extracts were applied. A small proportion of this protein could be eluted by 0.5M-glucose and a large amount of the remaining protein could be released by eluting the columns with 6M-urea/2M-KCl (Fig. 2b). Even more protein was removed from liver extracts when these were applied to a Sepharose-6-aminohexanoate conjugate but all the glucokinase activity passed through unretarded. Again glucose caused some protein to be eluted and most of the remaining bound protein was released by the combined action of 6M-urea/2M-KCl (Fig. 2c).

Clearly, a series of combined interactions between the glucosamine derivative and the proteins found in the liver extract was occurring. Although the results in Fig. 2 support the view that the interaction between glucokinase and the Sepharose-glucosamine derivatives are truly specific, interactions of an ion-exchange and/or hydrophobic nature also occur between the other proteins in the crude extract and the CNBr-activated Sepharose matrix and the polymethylene backbone of the 'spacer' molecule. O'Carra *et al.* (1973) have also described hydrophobic interactions between the polymethylene 'spacer' groups most often used in affinity chromatographic matrices and proteins. In an effort to minimize this effect glucosamine derivatives containing more hydrophilic 'spacer' arms were synthesized by attaching either *N*-glycylglycine or *N*-(*N*-glycylglycyl)glycine in amide linkage to the glucosamine in place of the 6-aminohexanoate. Both the free diglycyl- and triglycyl- derivatives of glucosamine were competitive inhibitors of the glucokinase reaction with respect to glucose, having *K_i* values of 0.75 and 1.4mM respectively. When

attached to Sepharose 4B at ligand concentrations of up to $8\mu\text{mol/g}$, however, neither of these immobilized derivatives exhibited any strong affinity for the enzyme (Fig. 2d). Some interaction was observed since the glucokinase emerged from these columns after the main protein peak, but no conditions could be found which caused the glucokinase to bind without considerable leakage. The use of these immobilized glycol glucosamine derivatives was thus discontinued.

The problems encountered by applying crude liver extracts directly to the affinity columns thus appeared too great to be overcome easily and the subsequent experiments described below were therefore performed on extracts first purified some 200-fold by batchwise chromatography on DEAE-cellulose (Holroyde *et al.*, 1975) to increase the relative amounts of glucokinase to other proteins in the preparation.

Importance of adequate washing procedures between affinity-chromatography operations

Despite such partial purification of glucokinase before the affinity-chromatography step, the Sepharose-glucosamine derivatives still increasingly lost much of their effectiveness to bind the enzyme after several operations. This lowering in their apparent 'capacity' to bind the enzyme was now less than when liver extracts were applied directly but the problem remained even after exhaustive washing with the urea/KCl solution between operations. The physical appearance of the affinity matrix after six or so operations indicated clumping of the gel particles and the matrix had a pale-yellow colour. This contrasted with the clean white appearance of an unused matrix and suggested that the washing procedures were not preventing a gradual build-up of protein on the matrix. When the 'aged' matrices were left overnight with a non-specific proteinase preparation, Pronase, then the original 'capacity' of the columns could be virtually fully recovered (Fig. 3). Consequently, the working life of the columns was greatly prolonged for use in large-scale purification procedures by introducing the Pronase treatment after every second use of the columns. The urea/KCl wash was performed as a routine after every use and after the Pronase treatment.

Effect of ligand concentration

The concentration of ligand attached to the Sepharose matrix affects both the binding of glucokinase and non-specific protein-column interactions and hence the purification of the enzyme. Fig. 4 describes a series of column profiles where the same amount of a glucokinase preparation was

Fig. 3. Effect of Pronase treatment of the glucosamine derivative affinity columns

In each case glucokinase (70 units in 70 ml), purified by DEAE-cellulose chromatography, was applied to the columns ($10\text{cm} \times 3\text{cm}$) operated in the buffer described in Fig. 2. The arrows indicate where 0.5M -glucose was included in the developing buffer. The columns were operated at 30ml/h , and 7ml fractions were collected. (a) Sepharose-*N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose that had been used in previous experiments using liver extracts at least six times. Between operations this matrix had been washed with 2M -KCl/ 6M -urea. (b) The same column treated with Pronase overnight (full details in the Materials and Methods section). $\circ$, E_{280} ; $\bullet$, glucokinase activity.

applied to columns of the Sepharose matrix covalently substituted with different amounts of *N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose. After washing the columns, a linear gradient from 0 to 1.0M -glucose was applied. At low concentrations on the column (Fig. 4a), the enzyme activity appeared before the inclusion of glucose in the developing buffer. Increasing the ligand concentration caused the glucokinase to be bound quantitatively and necessitated increasingly higher concentrations of glucose to effect its elution (Figs. 4b and 4c). At very high ligand concentrations, no enzyme was eluted by up to 1M -glucose and 0.5M -KCl was needed to release the bound enzyme (Fig. 4d).

The size of the protein breakthrough peak before application of the glucose gradient also varied. This became noticeably smaller as the ligand concentration was increased and indicated that binding of non-specific protein was also increasing. A comparison of the specific enzymic activities

Fig. 4. Effect of ligand concentrations on the elution of glucokinase by glucose from Sepharose-*N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose

Each column (10cm×0.8cm) was equilibrated with the buffer described in Fig. 2 and operated at 20ml/h; 3.0ml fractions were collected. Glucokinase (2ml, 2units/ml) purified by DEAE-cellulose chromatography was applied to each column followed by 25ml of the equilibration buffer. At A, the columns were developed with a linear gradient formed from 75ml of the column buffer and 75ml of 1M-glucose dissolved in this buffer. At B, 0.5M-KCl was included in the buffer. (a) 1.2μmol of glucosamine derivative coupled per g wet weight of packed gel; (b) 3.75μmol/g; (c) 6.0μmol/g; (d) 10μmol/g. In this experiment the final ligand concentrations were achieved by diluting the 10μmol/g gel material with unsubstituted Sepharose (see the Materials and Methods section). ○, E_{280} ; ●, glucokinase activity; ----, glucose concentration.

of the eluting enzyme confirmed this; the enzyme fraction eluted with glucose shown in Fig. 4(a) had a higher specific activity (10units/mg) than the enzyme eluted in Fig. 4(c) (1.5units/mg). Thus for optimal use it was necessary to control the ligand concentration coupled to the Sepharose matrix to balance the degree of purification obtained against efficient binding of the enzyme. In practice, this meant working at as low a ligand concentration as would quantitatively remove the glucokinase from the protein applied to the column.

The above experiments were conducted with affinity columns that were linked initially at a concentration of about 10μmol of glucosamine ligand/g of Sepharose and then 'diluted' to the required concentration with unsubstituted Sepharose 4B (see the Materials and Methods section). Dilution would give rise to columns that contained local regions of high ligand concentration interspersed with unsubstituted Sepharose. The experiments were

repeated with affinity columns prepared at different ligand concentrations by direct coupling and the results compared (Fig. 5). The two lines converge at higher ligand concentrations, i.e. where dilution with unsubstituted Sepharose of the 'diluted' series is minimal. At lower ligand concentrations a higher concentration of glucose is required to elute the enzyme from a 'diluted' column containing a given ligand concentration than from the corresponding direct-linked gel. More non-specific protein bound to the direct-linked gels as judged by the amount of protein passing unretarded through the columns whereas the enzyme eluted from the 'diluted' columns, was of a consistently higher specific activity. This was particularly apparent when large amounts of enzyme were being processed. The presence of the non-specific protein may also have influenced the tightness of binding of glucokinase.

The results of these studies have been incorporated into a successful purification scheme for

Fig. 5. Comparison of the elution of glucokinase from affinity columns containing different concentrations of *N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose prepared by either dilution or direct-linking

The experiments were conducted exactly as described in Fig. 4. 'Binding' refers to the concentration of glucose in the fraction where the maximum glucokinase activity eluted from the column was found. Columns containing different ligand concentrations were prepared either by diluting a stock ligand-Sepharose conjugate (10 μmol/g) with unsubstituted Sepharose (○) or by coupling the ligand to the CNBr-activated Sepharose directly at different ligand/gel ratios (●). Full details are given in the Materials and Methods section.

glucokinase where, by using a ligand concentration of 2 μmol/g obtained by dilution after covalently coupling the ligand to the CNBr-activated Sepharose at about 8 μmol/g, the binding of non-specific proteins was lowered, thus facilitating a 20- to 50-fold purification step with a high yield in addition to that obtained on DEAE-cellulose (Holroyde *et al.*, 1975).

Other considerations

Glucokinase could also be displaced from the affinity column by including *N*-acetylglucosamine in the developing buffer. Elution by this compound, which is a competitive inhibitor with respect to glucose of the glucokinase reaction, did not offer any advantages over glucose elution since both enzyme preparations were of similar specific activity (results not shown in detail), but did substantiate the true affinity nature of the interactions between the glucokinase and the immobilized glucosamine ligand. Bound glucokinase could also be eluted by

Fig. 6. Non-specific binding of glucose 6-phosphate dehydrogenase to various Sepharose derivatives

Liver extract (3 ml) purified by DEAE-cellulose chromatography and containing 5 units of glucokinase and 2.5 units of glucose 6-phosphate dehydrogenase activity was applied to each column (5 cm × 0.8 cm). The column and samples were equilibrated with the buffer described in Fig. 2 and the various additions were made where indicated: A, 1.0 M-glucose; B, 0.5 M-KCl. The columns were operated at 25 ml/h, and 2 ml fractions were collected. (a) and (c) Sepharose-*N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose at coupled ligand concentrations of 4 μmol/g wet weight of packed gel (a) and 2 μmol/g of gel (c). (b) Sepharose-6-aminohexanoate (6 μmol/g of gel). ○, *E*₂₈₀; ●, glucokinase activity; Δ, glucose 6-phosphate dehydrogenase activity.

raising the KCl concentration, although in this case the eluted enzyme activity peak was of a much lower specific activity, because additional non-specific protein material had been removed from the column (Fig. 6a). For large-scale purifications, however, it seemed better to elute the enzyme from the column as a sharp peak by developing it with a high concentration of glucose (1 M) rather than eluting by a glucose gradient and spreading the enzyme activity over a greater number of fractions with a concomitant decrease in specific activity of the final preparation.

Glucose was, nevertheless, eluting proteins other than glucokinase from these columns. Whether or not these proteins were binding specifically to the immobilized ligand was not investigated. Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) has been identified (Storer, 1975) as one of the impurities and in this instance, at least, the interaction with the affinity matrix appeared to be non-specific. Although this dehydrogenase bound to the immobilized glucosamine derivative and could be displaced by either glucose or KCl (Fig. 6a), neither glucosamine, 6-aminohexanoic acid nor *N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose inhibited the enzymic activity in free solution.

In Fig. 6(a), 0.5M-KCl was used as the eluent; a similar elution profile could be obtained with glucose except that less E_{280} -absorbing material was then released. Further, glucose 6-phosphate dehydrogenase bound to a Sepharose-6-aminohexanoate derivative and could be eluted with glucose from this matrix (Fig. 6b). Control of ligand concentrations was found to be effective in separating the two enzymic activities, for the binding of the dehydrogenase to the immobilized glucosamine derivative was only noticed at ligand concentrations of $4\mu\text{mol/g}$ or higher. If the columns were operated at $2\mu\text{mol/g}$ (the value chosen for large-scale purification work from the earlier studies) then most of the dehydrogenase passed through the column before the application of the glucose step, albeit in a position slightly behind the main protein peak (Fig. 6c).

Discussion

The results presented here indicate the necessity for careful control of the various parameters involved in the operation and positioning of an affinity-chromatography step in a purification scheme. This is particularly important if the enzyme to be purified is only present as a minor component in the starting material. In our case glucokinase represents only some 0.005% (w/w) of the total protein in the rat liver extracts after centrifugation. A series of different interactions must occur between the applied proteins and the gel matrix that, unless controlled, can severely limit the working life of the columns. Fortunately, these problems can be virtually eliminated by (a) extensive column-washing procedures between successive utilizations, (b) the introduction of other purification step(s) before the affinity-column step to increase the relative concentration of required enzyme in the applied protein and (c) careful control of immobilized ligand concentration.

The yield of glucokinase in the glucose eluates from these columns was always in excess of 80% even in large-scale preparations suggesting that its interaction with the affinity column was essentially

of a specific nature. It was not determined whether the proteins other than glucokinase present in the glucose eluates from these affinity columns operated in the purification scheme were other glucosamine-binding proteins found in rat liver or whether they were the results of non-specific interactions. The latter seems most likely in view of the results obtained with glucose 6-phosphate dehydrogenase. The elution of this enzyme by glucose from immobilized ligands that do not affect its activity in free solution is difficult to explain. It only bound weakly to all the columns tested and may have been interacting, possibly through hydrogen bonds, with groups produced by the immobilization procedure (Ahrgren *et al.*, 1972) rather than any introduced ligands. Elution with glucose may have resulted from an effect on the hydration shell around the protein.

The results shown in Fig. 2 clearly indicate that most of the non-specific interactions are occurring either by an ion-exchange process [presumably due to the charged groups introduced by the CNBr-activation procedure (Ahrgren *et al.*, 1972)] or by hydrophobic interactions (presumably due to the presence of the pentamethylene 'spacer' group). These interactions are particularly troublesome and we have estimated that up to 25% (w/w) of the protein in a crude liver extract can be absorbed on a Sepharose-aminohexanoate matrix at a ligand concentration of $6\mu\text{mol/g}$. The use of immobilized polymethylene ligands, often containing charged groups, in hydrophobic chromatography is well documented (Er-el *et al.*, 1972; Yon, 1972; Shaltiel & Er-el, 1973). The 'disappearance' of protein from the breakthrough peak of the direct-linked gels compared with the 'diluted' gels (Fig. 4) points also to the effects of the modified gel matrix (by the CNBr-activation procedure) in contributing to this non-specific binding. We were surprised, however, that the combined action of urea and KCl could not completely remove all protein from these columns. Additional protein could be removed after the washing procedure if the gels were left overnight in 8M-urea, or by washing with an organic solvent [aq. 80% (v/v) dioxan] (Lowe & Mosbach, 1975). These treatments, however, were still not sufficient to completely renew an 'aged' column and it was more convenient and efficient to incubate with Pronase (Fig. 3).

All of the columns used in this study were prepared by linking the pre-synthesized and characterized glucosamine ligands directly to the CNBr-activated Sepharose. The columns could have been prepared more simply by the methods outlined by Cuatrecasas (1970), i.e. preparation of a Sepharose-aminohexanoate derivative and coupling the glucosamine directly to this. Since the coupling is unlikely to be 100% efficient and is chemically ambiguous, our experience suggests that the non-specific binding

encountered with the defined ligands would have been considerably enhanced in this situation. In addition careful control of ligand concentration coupled to the gel would not have been possible. This was essential in order to help minimize non-specific binding and to maximize the purification of the enzyme.

The relationship between immobilized ligand concentration and the concentration of glucose required for elution is linear up to about $6\mu\text{mol/g}$. Above this concentration increasingly higher concentrations of glucose are required to achieve elution, suggesting that additional factors, such as hydrophobic binding, may well play an increasing role in binding the enzyme to the column. Up to $6\mu\text{mol/g}$ it is most convenient to express the binding in terms of two competing equilibria, i.e., the competition of the immobilized ligand and the eluting molecule, glucose, for the same site on the enzyme. This is an oversimplified treatment since the presence of non-specifically bound protein may also indirectly influence these equilibria. The effect of ligand concentration on the binding of enzymes was first demonstrated by Harvey *et al.* (1974) in their small-scale model system studies. These workers (Lowe *et al.*, 1974b) have also shown that the total amount of immobilized ligand and even the column geometry must also be considered.

The dramatic effect of change in ligand concentration on the affinity chromatography of enzymes is probably only applicable in a situation as here where there is a relatively low association constant between the immobilized ligand and the enzyme. If the association constant is very high then it may not be possible to control the elution of the enzyme from the column so effectively by adjusting the immobilized ligand concentration.

The results obtained with the gels in which hydrophilic glycine arms were used were at first sight surprising. Both the diglycine and the 6-aminohexanoate derivatives of glucosamine were equally effective at inhibiting the glucokinase reaction in free solution ($K_i = 0.75\text{ mM}$). When attached to an agarose matrix, however, the glucosamine derivative with the hydrophobic 'spacer' moiety was much more effective as an affinity matrix. Barry & O'Carra (1973) and O'Carra *et al.* (1974) have suggested that the stronger binding at similar ligand concentrations found with the more hydrophobic derivative can be best explained by a hydrophobic interaction between the enzyme and the 'spacer' molecule reinforcing the specific enzyme-ligand interaction. This may well be the case in many systems (Lowe & Mosbach, 1975) but an alternative explanation is possible. The results with the glycyl derivative (Fig. 2) were exactly those found when the hexanoate-glucosamine derivative was operated at a ligand concentration of about $1\mu\text{mol/g}$.

The hydrophilic glucosamine derivative may not have been as physically available for interaction with the enzyme, perhaps owing to hydrogen-bonding along its whole length to the polysaccharide matrix, thus decreasing the effective ligand concentration.

Although our objective was to determine as precisely as possible the best operating conditions for this affinity step in the large-scale purification of hepatic glucokinase (Holroyde *et al.*, 1975), the results obtained should be applicable to many enzyme purification systems, particularly where the enzyme under study is present in the tissue extract in very low amounts and only possesses a relatively low association constant for the immobilized ligand. It is noteworthy that if our preliminary experiments with this system had been conducted under different conditions, e.g. at a lower ligand concentration, then the lack of binding that would have been observed might well have discouraged us from further use of this method.

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