Purification of the Hexokinases by Affinity Chromatography on Sepharose-*N*-Aminoacylglucosamine Derivatives

DESIGN OF AFFINITY MATRICES FROM FREE SOLUTION KINETICS

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The purification is described of rat hepatic hexokinase type III and kidney hexokinase type I on a large scale by using a combination of conventional and affinity techniques similar to those previously used for the purification of rat hepatic glucokinase [Holroyde, Allen, Storer, Warsy, Cheshier, Trayer, Cornish-Bowden & Walker (1976) *Biochem. J.* **153**, 363–373] and muscle hexokinase type II [Holroyde & Trayer (1976) *FEBS Lett.* **62**, 215–219]. The key to each purification was the use of a Sepharose-*N*-aminoacylglucosamine affinity matrix in which a high degree of specificity for a particular hexokinase isoenzyme could be introduced by either varying the length of the aminoacyl spacer and/or varying the ligand concentration coupled to the gel. This was predicted from a study of the free solution kinetic properties of the various *N*-aminoacylglucosamine derivatives used (*N*-aminopropionyl, *N*-aminobutyryl, *N*-aminohexanoyl and *N*-aminooctanoyl), synthesized as described by Holroyde, Cheshier, Trayer & Walker [(1976) *Biochem. J.* **153**, 351–361]. All derivatives were competitive inhibitors, with respect to glucose, of the hexokinase reaction, and there was a direct correlation between the K_i for a particular derivative and its ability to act as an affinity matrix when immobilized to CNBr-activated Sepharose 4B. Muscle hexokinase type II could be chromatographed on the Sepharose conjugates of all four *N*-aminoacylglucosamine derivatives, although the *N*-amino-hexanoylglucosamine derivative proved best. This same derivative was readily able to bind hepatic glucokinase and hexokinase type III, but Sepharose-*N*-amino-octanoylglucosamine was better for these enzymes and was the only derivative capable of binding kidney hexokinase type I efficiently. Separate studies with yeast hexokinase showed that again only the Sepharose-*N*-amino-octanoylglucosamine was capable of acting as an efficient affinity matrix for this enzyme. Implications of these studies in our understanding of affinity-chromatography operation are discussed.

Lack of efficient purification procedures for the mammalian hexokinases (ATP-D-hexose 6-phosphotransferases, EC 2.7.1.1) has hindered any in-depth investigations into their physiological control and mode of action. Until recently the only isoenzyme to be purified to homogeneity was type I from brain (Schwartz & Basford, 1967; Chou & Wilson, 1972) and pig heart (Easterby & O'Brien, 1973). The selective extraction of the mitochondria to which this isoenzyme is bound simplified the purification process, but the other three isoenzymes could not be obtained so easily. However, Holroyde & Trayer (1974) and Holroyde *et al.* (1976*a,b*) succeeded in purifying hepatic glucokinase (hexokinase type IV,

EC 2.7.1.2) to homogeneity by using, in conjunction with ion-exchange chromatography, a new affinity matrix of Sepharose-*N*-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose. Glucokinase differs from the other mammalian hexokinases in that it is only half their molecular weight (Holroyde *et al.*, 1976*b*), has a slightly narrower hexose specificity and a higher K_m for glucose (Parry & Walker, 1966) and displays a sigmoidal dependence of enzyme rate against glucose concentration (Storer & Cornish-Bowden, 1976).

Initial experiments with this glucosamine affinity matrix (Cheshier *et al.*, 1973) suggested that glucokinase bound selectively and reversibly, but that low- K_m hexokinase activity in liver extracts (presumed to be types I, II and III) and yeast hexokinase were unretarded. From the start this seemed puzzling,

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as it is well known that *N*-acetylglucosamine is a good competitive inhibitor of all four isoenzymes (Walker, 1966). It was eventually found, however, that, by adjusting the operating conditions slightly, hexokinase type II from rat muscle could be purified to homogeneity (Holroyde & Trayer, 1976). This prompted a more thorough investigation of the reactions between the hexokinases and this affinity matrix. Various glucosamine compounds were synthesized, giving a series of derivatives in which the length of the carbon chain attached to the amino group was varied. This had the effect of altering the length of the spacer arm attaching the glucosamine to the Sepharose. When the inhibition with respect to glucose of rat and yeast hexokinase isoenzymes by these compounds was investigated, a definite correlation was found to exist between the K_i of a compound in free solution and its effectiveness as an affinity matrix when immobilized to Sepharose. Thus, by varying either the length of the aminoacyl spacer molecule and/or the concentration of ligand attached to the gel, it has been possible to prepare a glucosamine affinity matrix capable of purifying each of the hexokinase isoenzymes to homogeneity on a large scale. Some of this work has been reported in preliminary form (Wright *et al.*, 1976).

Materials and Methods

Chemicals

Coenzymes, Dowex ion-exchange resins and dithiothreitol were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Ethyl trifluoromethanecarbothiolate was obtained from Pierce Chemical Co., Rockford, IL, U.S.A., and *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline was from Calbiochem, San Diego, CA, U.S.A. DEAE-cellulose (DE-52) was from Whatman Biochemicals, Maidstone, Kent, U.K., Sepharose 4B, DEAE-Sephadex A-50 and Sephadex G-25 were from Pharmacia (G.B.), London W.5, U.K., and CNBr was from Aldrich Chemical Co., Gillingham, Dorset, U.K. Hydroxyapatite was obtained from BioRad Laboratories, Richmond, CA, U.S.A. *N*-Acetylglucosamine hydrochloride, glucosamine hydrochloride, 3-aminopropionic acid, 4-aminobutyric acid, 6-aminohexanoic acid and 8-amino-octanoic acid were from BDH Chemicals, Poole, Dorset, U.K. All other chemicals were of analytical grade and used as supplied.

Proteins

Yeast hexokinase [ATP-D-hexose 6-phosphotransferase, EC 2.7.1.1; type C-302 (300 units/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. Gifts of purified yeast hexokinase types A and B and a crude yeast extract after acid precipitation (Ramel *et al.*, 1971) were generously given by Professor E. A. Barnard, Department of Biochemistry, Imperial College of Science and Technology, London S.W.7, U.K.

Hexokinase preparations

Rat hepatic glucokinase was prepared as described by Holroyde *et al.* (1976b) and rat skeletal-muscle hexokinase type II as described by Holroyde & Trayer (1976). Hexokinase types I and III were prepared from kidney and liver respectively. The initial stages of the preparation are described below and are based on our method of preparation of hepatic glucokinase and muscle hexokinase type II. Essentially the enzymes are extracted from the tissues, adsorbed batchwise on DEAE-cellulose and then chromatographed on either DEAE-Sephadex (hexokinase type III) or hydroxyapatite (hexokinase type I) before their final purification on the affinity columns described in the Results section.

Preparation of rat kidney hexokinase type I

Frozen rat kidneys (400g) (purchased from Olac (Northern), Bicester, Oxon., U.K.) were chopped finely with scissors and homogenized in 2 vol. of 20 mM-triethanolamine/HCl, pH 7.0, containing 0.2 M-MgCl₂, 10 mM-KCl, 50 mM-glucose, 1 mM-EDTA, 0.5 mM-dithiothreitol and 5% (v/v) glycerol in an Ato-Mix blender for 2 min. The homogenate was kept for 45 min at 4°C before centrifuging at 23 500g for 45 min. The supernatant was adjusted to pH 8 by the slow addition of 2 M-triethanolamine and then dialysed against several changes of the above buffer (at pH 8 and with the MgCl₂ omitted) until the conductivity of the non-diffusible material was approx. 2 mΩ⁻¹. The dialysed extract was recentrifuged as above and the supernatant adsorbed on DEAE-cellulose (approx. 35g of moist exchanger/100 ml of supernatant) previously equilibrated with the pH 8 buffer. The DEAE-cellulose was collected on a Buchner funnel, washed with the pH 8 buffer under suction and packed into a column (35 cm × 4.5 cm). The column was washed further until the A_{280} of the emerging eluate was less than 0.1 and it was then developed with a linear gradient to 0.4 M-KCl (in pH 8 buffer) over 2 litres. The active fractions, eluted between 0.04 M- and 0.08 M-KCl, were concentrated by ultrafiltration to between 50 and 100 ml and dialysed against three changes of 1.5 litres each of 10 mM-potassium phosphate buffer, pH 7.0, containing 50 mM-glucose, 1 mM-EDTA, 0.5 mM-dithiothreitol and 5% (v/v) glycerol on a rocking dialyser for about 3 h. The dialysis residue was then applied to a hydroxyapatite column (15 cm × 2.5 cm) equili-

brated in the above buffer. After a washing of the column with this buffer, a linear gradient to 0.6M-potassium phosphate (over 2 litres) was applied. The active fractions, eluted between 0.2M- and 0.25M-potassium phosphate, were pooled, concentrated by ultrafiltration and stored at 4°C. This isoenzyme proved to be very stable, retaining 98% activity over a period of 6 months. The enzyme was obtained in 75% yield with a specific activity of 11.6 units/mg (representing some 1400-fold purification at this stage) and was free of other hexokinase activity as judged by electrophoresis in 0.5% agarose (see below). This material was purified to homogeneity by chromatography on Sepharose-*N*-(8-amino-octan-oyl)-glucosamine as described in the Results section (Fig. 5).

Preparation of rat hepatic hexokinase type III

The 50 rats were starved for 24h and then fed with carbon tetrachloride (20%, v/v) in liquid paraffin (0.5ml/100g body wt.) as described by Ueda *et al.* (1975). (This treatment destroys the hepatic glucokinase and enhances the low- K_m hexokinase activity.) The rats were killed after an additional 48h without food, and their livers were removed and homogenized in 2vol. of 10mM-potassium phosphate buffer, pH7.0, containing 10mM-glucose, 5mM-EDTA, 0.5mM-dithiothreitol and 10% (v/v) glycerol for 2min in an Ato-Mix blender. After centrifugation (23 500g for 1h), the supernatant was adsorbed on DEAE-cellulose (about 10g of moist gel/100ml of supernatant) equilibrated with the extraction buffer, and the DEAE-cellulose was then collected on a Buchner funnel. It was washed with the above buffer containing 0.1M-KCl (1 litre) under suction and then packed into a column (20cm $\times$ 4.5cm), and the washing was continued until the A_{280} of the emerging fractions fell below 0.2. Hexokinase activity was step-eluted by raising the KCl concentration in this buffer to 0.4M. The active fractions were pooled and dialysed against two changes of 1.5 litres each of 50mM-potassium phosphate buffer, pH7.0, containing 10mM-glucose, 1mM-EDTA, 1mM-MgCl₂, 0.5mM-dithiothreitol and 10% glycerol (v/v) for about 3h until the conductivity of the non-diffusible material was down to 5m Ω^{-1} . This material was then applied to a DEAE-Sephadex column (20cm $\times$ 4.5cm) equilibrated with the above buffer but containing 0.1M-potassium phosphate, pH7.0. The column was washed further with this buffer until the A_{280} fell to 0.2 and was then eluted with a linear gradient to 0.5M-potassium phosphate, pH7.0, containing the above ingredients, over 2 litres. Some hexokinase activity appeared in the washings before application of the gradient and was shown to be hexokinase type II by electrophoresis (see below). This was occasionally saved and treated as described in the Results section (Fig. 6) should this isoenzyme be

required. The hexokinase activity eluted under the gradient contained both type II and type III isoenzymes, which could be most easily resolved by affinity chromatography (Fig. 6). The type III enzyme was not very stable at this stage of the preparation and the affinity-chromatography step was performed immediately. At this stage, the hexokinase activity was about 40% of that in the original liver extract. (Some of the 'loss' was hexokinase type I, which does not adsorb on the DEAE-cellulose at pH7.0, and type II, some of which is lost at each ion-exchange step, although there is still a certain amount in the preparation at this stage.) The specific activity was 12.1 units/mg, which represents a 2500-fold purification.

The kinetic experiments described in the early part of the Results section were performed with homogeneous samples of rat hepatic glucokinase, rat skeletal-muscle hexokinase type II and yeast hexokinase and with samples of hexokinase types I and III after the two preliminary purification steps described above. Rat kidney hexokinase type I is pure with respect to hexokinase type at this stage, but hepatic hexokinase type III was obtained by pooling fractions from the higher-ionic-strength side of the peak eluted from the DEAE-Sephadex column and checking for type-homogeneity by electrophoresis in 0.5% agarose (see below).

Assay of hexokinase activity

Hexokinase activity was measured at 30°C in a total volume of 0.75ml by the coupled assay method of Parry & Walker (1966) as described by Storer & Cornish-Bowden (1974). Glucokinase was assayed routinely at a glucose concentration of 100mM, and the low- K_m hexokinases were assayed at either 0.5mM-glucose or 0.1mM-glucose (type III). For the inhibition studies glucose was removed from the enzyme solutions by passage down a Sephadex G-25 column [usually a 1ml sample applied to a 20cm $\times$ 1cm column operated in 20mM-triethanolamine/HCl, pH7.0, containing 0.2M-KCl, 0.5mM-dithiothreitol, 1mM-EDTA, 1mM-MgCl₂ and 5% (v/v) glycerol] and the enzyme used straight away.

Glucose was removed from the glucose 6-phosphate dehydrogenase by overnight dialysis against 20mM-Tris/HCl buffer, pH7.5. The competitive inhibition constants (K_i values) were determined by the method of Dixon (1953) and the uncompetitive component (K'_i) by the method of Cornish-Bowden (1974, 1976).

One unit of hexokinase activity is defined as that which catalyses the formation of 1 μ mol of glucose 6-phosphate/min at 30°C.

Preparation of glucosamine derivatives

An amide linkage was synthesized between the amino group of glucosamine and the carboxy group

of the following amino acids: 3-aminopropionic acid, 4-aminobutyric acid, 6-aminohexanoic acid and 8-amino-octanoic acid (Holroyde *et al.*, 1976a). The final products were not crystallized, but gave single spots on high-voltage paper electrophoresis at pH 6.5 (mobilities relative to glucosamine: glucosamine, 1.0; products, 0.81–0.87) and on ascending paper chromatography in two solvent systems (Holroyde *et al.*, 1976a). Positive reactions were obtained with both ninhydrin and AgNO_3 (Holroyde *et al.*, 1976a). After acid hydrolysis (6M-HCl, 20h at 105°C) chromatographic analysis on an amino acid analyser showed only the appropriate amino acid and glucosamine in a molar ratio of 1:1. The structures shown in Fig. 1 were therefore assigned to the products.

Preparation of Sepharose adsorbents

The ligands were attached to CNBr-activated Sepharose 4B under the conditions described by Trayer *et al.* (1974). The ligands were coupled at a concentration of 10–12 $\mu\text{mol/g}$ of gel and characterized as described by Holroyde *et al.* (1976a). The gels were diluted to the required ligand concentration by dilution with unsubstituted Sepharose 4B (Holroyde *et al.*, 1976a). The adsorbents are designated as e.g., Sepharose- $\text{C}_3\text{-GlcN}$ for glucosamine linked to Sepharose by a C_3 spacer.

Electrophoretic methods

Enzyme purity and estimations of molecular weight were determined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969). To identify isoenzymes in the early part of this work, electrophoresis was carried out in the absence of sodium dodecyl sulphate in polyacrylamide gels and the gels were stained for enzymic activity (Grossman & Potter, 1974), but most frequently electrophoresis in 0.5% agarose was used (Allen & Walker, 1976). The latter procedure gave greatly improved separation.

Analytical methods

Conductivity measurements were made on a Radiometer type CDM conductivity meter (Radiometer, Copenhagen, Denmark). Protein determinations were made by the turbidimetric tannin micro method of Mejbaum-Katzenellenbogen & Dobryszcka (1959), with bovine serum albumin as standard. Amino acid analyses were performed with a Beckman model 120B amino acid analyser, with correction for losses as described by Holroyde *et al.* (1976a).

Results

Inhibitory properties of the N-aminoacylglucosamine derivatives

The general structure of the four glucosamine derivatives synthesized is shown in Fig. 1. The

Fig. 1. Structure of the *N*-aminoacylglucosamine derivatives

The synthesis of the various ligands [where $n = 2$, *N*-(3-aminopropionyl)-2-amino-2-deoxy- β -D-glucopyranose ($\text{C}_3\text{-GlcN}$), $n = 3$, *N*-(4-aminobutyryl)-2-amino-2-deoxy- β -D-glucopyranose ($\text{C}_4\text{-GlcN}$), $n = 5$, *N*-(6-aminohexanoyl)-2-amino-2-deoxy- β -D-glucopyranose ($\text{C}_6\text{-GlcN}$), and $n = 7$, *N*-(8-aminooctanoyl)-2-amino-2-deoxy- β -D-glucopyranose ($\text{C}_8\text{-GlcN}$)] is described in the Materials and Methods section.

competitive inhibition constant (K_i) of each isoenzyme for each ligand was determined first with impure enzyme fractions [but still homogeneous with respect to hexokinase type as judged by electrophoresis (see the Materials and Methods section)] and later, as the findings were extended, the experiments were repeated with pure isoenzymes. All the results were in good agreement, and in separate assays for ADP production (Storer & Cornish-Bowden, 1977) the glucosamine derivatives could not be shown to be substrates. Typical inhibition plots are shown in Fig. 2, where the K_i in free solution of hepatic hexokinase type III for the $\text{C}_3\text{-GlcN}$ derivative is shown to be 5.9 mM. The inhibition is purely competitive, as indicated by Fig. 2(b), where the parallel lines in the plot of $[S]/v$ against $[I]$ show that no uncompetitive inhibition component is detectable, i.e. K'_i approaches infinity. However, the inhibition of hexokinase type II by all four glucosamine derivatives, of hexokinase type III by the $\text{C}_6\text{-GlcN}$ and $\text{C}_8\text{-GlcN}$ derivatives, and of yeast hexokinase by the $\text{C}_8\text{-GlcN}$ derivative showed mixed inhibition (Cornish-Bowden, 1974); all other combinations of enzymes and inhibitors showed purely competitive inhibition, as illustrated in Fig. 2. In the cases where mixed inhibition occurred, this was demonstrated in the plot of $[S]/v$ against $[I]$, where the lines at different glucose concentrations intersected to the left of the ordinate.

The results of the determinations of competitive inhibition constant (K_i) obtained from the plot of $1/v$ against $[I]$ for all the enzymes are shown in Fig. 3 plotted against the number of carbon atoms in the aminoacyl chain of the glucosamine derivative. Results for *N*-acetylglucosamine, which is generally a better inhibitor of all the isoenzymes than the amino-

Fig. 2. Inhibition of rat liver hexokinase type III by *N*-(3-aminopropionyl)-2-amino-2-deoxy-D-glucopyranose (C_3 -GlcN) at various glucose concentrations

Initial rates (v) were measured as described in the Materials and Methods section at a constant concentration of $MgATP^{2-}$ (7.5 mM). (a) The competitive component of the inhibition was investigated by plotting $1/v$ against inhibitor concentration (Dixon, 1953). (b) The uncompetitive component of inhibition was investigated by plotting $[glucose]/v$ against inhibitor concentration (Cornish-Bowden, 1974). The parallel lines indicate that this component is not detectable, i.e. K'_i approaches infinity. Concentrations of glucose were 0.1 mM (Δ), 0.05 mM ($\bullet$) and 0.025 mM ($\circ$).

acyl derivatives, are shown for comparison. The introduction of an additional aminomethyl moiety to the *N*-acetylglucosamine side chain (i.e. the C_3 -GlcN derivative) considerably lessens its effectiveness as an inhibitor, but, as the number of carbon atoms in the chains was increased, so was the effectiveness of the various ligands as free solution inhibitors.

Small-scale affinity-chromatography experiments

A direct correlation was found to exist between the K_i of a particular derivative and its ability to act as an affinity matrix when immobilized on Sepharose. In a series of small-scale experiments, some of which are illustrated in Fig. 4, it became apparent that, provided

Fig. 3. Inhibition of the mammalian hexokinase isoenzymes by the various *N*-aminoacylglucosamine derivatives in free solution

The K_i values, estimated by plotting $1/v$ against inhibitor concentration as described in Fig. 2, are plotted against the number of C atoms in the *N*-aminoacyl side chain of the glucosamine derivatives depicted in Fig. 1. (Number of C atoms = $n + 1$ in Fig. 1.) $\circ$, Hexokinase type I (rat kidney); $\bullet$, hexokinase type II (rat muscle); $\square$, hexokinase type III (rat liver); $\blacksquare$, glucokinase (hexokinase type IV, rat liver). GlcNAc, *N*-acetylglucosamine, shown for comparison. The broken line at $K_i = 2$ mM represents the upper limit of K_i from which a successful affinity column can be made.

that the K_i in free solution (in this context it can be taken as the dissociation constant prevailing in the system) was ≤ 2 mM then an efficient affinity-chromatography system could be designed when the glucosamine derivatives were attached to Sepharose.

Thus the only really effective inhibitor of rat kidney hexokinase type I was the C_8 -GlcN derivative (K_i 1.3 mM) and only the Sepharose conjugate of this derivative proved a suitable affinity matrix (Fig. 5). If the C_6 -GlcN derivative was attached to Sepharose at very high ligand concentrations (12 μ mol/g of Sepharose) the hexokinase type I (K_i 2.75 mM) was partially retained on the column, but not sufficiently well to allow this to be used in a purification scheme. At lower ligand concentrations (Fig. 4b) the isoenzyme passes through unretarded. Similarly muscle hexokinase type II will bind efficiently to any of the immobilized glucosamine derivatives, but liver

Fig. 4. Chromatography of the mammalian hexokinase isoenzymes on various Sepharose-*N*-aminoacylglucosamine derivatives

All columns were the same dimensions (10cm $\times$ 0.8cm) and 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, and 2ml fractions were collected. Hexokinase (activity 2 units in 2–3 ml) was applied to each column. At the arrow, 0.5M-glucose was included in the developing buffer. (a) Composite of four individual column profiles obtained by applying each of the isoenzymes in turn to a Sepharose-C₄-GlcN matrix (ligand concentration: 8 μ mol/g wet wt. of packed gel). (b) Composite of four individual column profiles obtained by applying each of the isoenzymes in turn to a Sepharose-C₆-GlcN matrix (ligand concentration: 4 μ mol/g wet wt. of packed gel). (c) Glucokinase and hexokinase type II were applied to a Sepharose-C₆-GlcN matrix (6 μ mol/g) and eluted by a linear gradient formed from 20ml of the column buffer and 20ml of 1M-glucose dissolved in this buffer. $\circ$, Hexokinase type I (rat kidney); $\bullet$, hexokinase type II (rat muscle); $\square$, hexokinase type III (rat liver); $\blacksquare$, glucokinase (hexokinase type IV, rat liver); ----, glucose concentration.

hexokinase type III and glucokinase are best chromatographed on Sepharose conjugates of either C₆-GlcN or C₈-GlcN. Furthermore, in situations where the K_i values of two isoenzymes for a particular ligand differed, these enzymes could be separated chromatographically by either adjusting the ligand concentration attached to the gel or using a glucose (or sometimes even a KCl) gradient. For example,

hexokinase type II (K_i 0.4mM) and glucokinase (K_i 0.75mM) could be separated on Sepharose-C₆-GlcN in this way (Fig. 4c).

It should be emphasized that attachment of either glucosamine (Chesher *et al.*, 1973) or any of the various amino acids (or their aminoalkane analogues) directly to CNBr-activated Sepharose did not provide matrices that showed any significant interaction with

the isoenzymes. Nor did the amino acids and the aminoalkanes inhibit the hexokinase activities in free solution.

The importance of choosing the most effective ligand concentration attached to the gel (and how to adjust this) when designing a large-scale affinity-matrix operation has been reported earlier (Holroyde *et al.*, 1976a,b; Trayer *et al.*, 1978; Trayer, 1978; Harvey *et al.*, 1974). Furthermore, better purifications of these isoenzymes are obtained if a ligand concentration is chosen so that the enzyme is bound sufficiently tightly to allow the column to be thoroughly washed with as high a KCl concentration as possible, which, in addition to removing further unwanted protein, 'loosens' the enzyme-immobilized ligand interaction. Elution of the required enzyme can then be effected specifically in a small volume by including glucose in the developing buffer.

Large-scale preparation of hexokinase types I and III

The large-scale purifications of hexokinase type I from rat kidney (Fig. 5) and hepatic hexokinase type III (Fig. 6) were both carried out on a Sepharose-C₈-

GlcN affinity matrix; neither enzyme had been previously purified to homogeneity. After prior chromatography of the kidney extracts on DEAE-cellulose and hydroxyapatite (see the Materials and Methods section), the fractions containing hexokinase type I were applied to a Sepharose-C₈-GlcN column, which was operated as described in Fig. 5. Washing of the column with 70 mM-KCl removed unwanted protein, and the enzyme was specifically eluted by inclusion of 200 mM-glucose in the developing buffer. At this stage the hexokinase fraction gave one major and one minor band on polyacrylamide-gel electrophoresis in sodium dodecyl sulphate. A further passage down the affinity column removed the minor contaminant, leaving a band migrating in a position equivalent to a mol.wt. of 96000. Identical molecular weights have been recorded for hexokinase type I from heart (Easterby & O'Brien, 1973; Easterby, 1975) and brain (Chou & Wilson, 1972; Chakrabarti & Kenkare, 1974). The final product had a specific activity of about 180 units/mg, which represented a 21 500-fold purification. The two affinity-chromatography steps were performed in greater than 85% yield.

Fig. 5. Purification of rat kidney hexokinase type I on Sepharose-C₈-GlcN

The extracts of 400 g of rat kidneys were partially purified as described in the Materials and Methods section. The sample was dialysed against 20 mM-triethanolamine/HCl, pH 7.0, containing 10 mM-KCl, 4 mM-EDTA, 0.5 mM-dithiothreitol and 5% (v/v) glycerol and applied directly to the affinity column (25 cm × 3 cm, adjusted to 5 μmol of ligand per g wet wt. of packed gel as described under 'Preparation of Sepharose adsorbents') equilibrated in the same buffer. The column was operated at 40 ml/h, and 6 ml fractions were collected. At arrow A the KCl concentration in the buffer was increased to 70 mM, and at arrow B 200 mM-glucose was also included. ●, A₂₈₀; ○, hexokinase activity.

Fig. 6. Separation and purification of hexokinase types II and III from rat liver on Sepharose- C_8 -GlcN

The extracts of the livers of 50 carbon tetrachloride-treated rats were partially purified by ion-exchange chromatography as described in the Materials and Methods section. The sample, containing only hexokinase types II and III activities, was dialysed against the buffer described in Fig. 6 and applied directly to the affinity column (20 cm $\times$ 3 cm, adjusted to 3.85 μ mol of ligand/g of gel as described under 'Preparation of Sepharose adsorbents') equilibrated to this buffer. The column was washed with this buffer until the A_{280} of the emerging solution was ≤ 0.1 (not shown). At arrow A the KCl concentration of the buffer was increased to 140 mM, and at arrow B 50 mM-glucose was added to this. The column was operated at 40 ml/h, and 4 ml fractions were collected. $\bullet$, A_{280} ; $\circ$, hexokinase activity. Electrophoretic analysis (Allen & Walker, 1976) indicated that the protein eluted by the 140 mM-KCl alone contained only the hexokinase type II isoenzyme together with other protein material, whereas the KCl/glucose eluate contained homogeneous hexokinase type III.

Hepatic hexokinase type III could have been purified to homogeneity on either the C_6 -GlcN or the C_8 -GlcN derivatives attached to Sepharose; the C_8 -GlcN derivative was chosen since hexokinase type II is a major contaminant after the two ion-exchange steps (see the Materials and Methods section) and this glucosamine derivative best distinguishes between them (see Fig. 3). Fig. 6 shows the results of applying the partially purified hexokinase type III preparation to Sepharose- C_8 -GlcN (3.85 μ mol of ligand/g of gel). After washing of the column with the application buffer (containing 10 mM-KCl) (not shown), the column was developed with a buffer containing 140 mM-KCl. This eluted hexokinase type II quantitatively together with unwanted protein. The type III enzyme was then eluted specifically with 50 mM-glucose. If required, the hexokinase type II eluted with the 140 mM-salt could be purified by

passage directly down a Sepharose- C_6 -GlcN derivative (Holroyde & Trayer, 1976). The enzyme would bind in this buffer and could be subsequently eluted by glucose.

Hexokinase type III was found to be very unstable in our hands even on the addition to the buffers of glucose, dithiothreitol and glycerol, ingredients that help stabilize glucokinase (Holroyde *et al.*, 1976b). The freshly eluted enzyme had a specific activity of about 200 units/mg and gave a single migrating species on polyacrylamide gels in the presence of sodium dodecyl sulphate (mol.wt. 98000). The preparation appeared to contain trace amounts of proteinases, since the band migrating at a position equivalent to a mol.wt. of 98000 gradually disappeared on standing over a 2–3-week period to be replaced by faster-migrating bands with a concomitant loss of enzymic activity. We have not observed

Fig. 7. Affinity-chromatography studies on yeast hexokinase

(a) Inhibition of yeast hexokinase by the various *N*-aminoacylglucosamines in free solution. The experiments were performed as described in Figs. 2 and 3 with a pure sample of yeast hexokinase type B (see the Materials and Methods section). Virtually identical results were obtained with the type-A isoenzyme. (b) Crude (see the Materials and Methods section) yeast hexokinase (0.1 ml; $\Delta A_{340}/\text{min} = 7.0$) was applied to a column of Sepharose- C_6 -GlcN (5 cm $\times$ 0.9 cm, containing 5 μmol of glucosamine ligand/g of gel) operated in the buffer described in Fig. 6. At arrow A the KCl concentration was increased to 20 mM, and at arrow B 200 mM-glucose was added to this. The column was operated at 20 ml/h, and 2 ml fractions were collected. ●, A_{280} ; ○, hexokinase activity.

attempts to chromatograph this enzyme on Sepharose- C_6 -GlcN (Chesher *et al.*, 1973) were unsuccessful, an observation confirmed by others (E. A. Barnard, personal communication). The K_i values for the C_3 -GlcN, C_4 -GlcN and C_6 -GlcN derivatives increased rapidly as the length of the spacer molecule was increased (Fig. 7a). However, the addition of two methylene groups to the spacer molecule had a dramatic effect on the inhibitory properties of this glucosamine derivative (cf. K_i 24.2 mM for C_6 -GlcN and K_i 0.9 mM for C_8 -GlcN). It was therefore possible to chromatograph successfully a crude yeast extract on Sepharose- C_8 -GlcN (Fig. 7b) and obtain a 20–25-fold purification. Although this is only shown on a small scale here, our experience with the mammalian hexokinases suggests that it is also possible to scale up this method.

Discussion

For the first time it has been shown possible to purify to homogeneity all four mammalian hexokinases by a combination of conventional and affinity techniques. The key to each purification was the use of a glucosamine affinity matrix in which a high degree of specificity for a particular hexokinase isoenzyme could be introduced by either varying the length of the spacer molecule and/or varying the ligand concentration coupled to the gel. In principle the method should be applicable to the purification of any hexokinase inhibited by *N*-acetylglucosamine. It was particularly noteworthy that the choices of matrix, ligand concentration and operating conditions were largely predicted from free solution kinetic data. These results explain our earlier puzzling observations that, when liver extracts were applied to Sepharose- C_6 -GlcN, only glucokinase was bound and low- K_m hexokinase activity was unretarded (Chesher *et al.*, 1973). Under the conditions of these experiments, hexokinase type I, the major low- K_m hexokinase in liver, would not be expected to bind to this affinity matrix. In this respect the report by Rijkssen & Staal (1976) on the purification of human erythrocyte hexokinase type I is particularly noteworthy. These authors used an immobilized glucosamine derivative (Sepharose- C_6 -GlcN in our nomenclature) as the final step of their purification scheme. They showed that, although the enzyme did not bind very efficiently, it was sufficiently retarded to achieve the required purification. Although they did not report a ligand concentration attached to the gel, their mode of preparation suggests that it was at least 10 $\mu\text{mol/g}$ of gel, conditions where we observed a retardation (but not binding) of the rat kidney hexokinase type I. Our experience suggests that an immobilized C_8 -GlcN conjugate would provide a more efficient affinity matrix. There are considerable

this problem with any of our other hexokinase preparations.

Studies on yeast hexokinase

The usefulness of the kinetic data in helping to predict affinity-chromatography behaviour was very obvious in our studies on yeast hexokinase. Our early

differences, however, between the two type I enzymes; the rat hexokinase is more active (specific activity of 180 units/mg at 30°C compared with 25–50 units/mg at 37°C for the human enzyme) and smaller (mol.wt. 96000 as opposed to 132000).

The exact nature of the interaction of enzyme with immobilized ligand is difficult to assess. Increasing the length of the spacer molecule, in general terms, increases the strength of binding of the enzymes to the matrices, but it would be naive to suggest an increased steric availability of the glucosamine as being responsible for this. Indeed, the fact that hexokinase type II will bind to the immobilized conjugates of all the *N*-aminoacylglucosamine derivatives tried and, in more general terms, the strong correlation between the kinetic and chromatographic data argue for an altogether more specific explanation. Nevertheless it was somewhat unexpected that the K_i values that such a closely related family of enzymes exhibit towards the *N*-aminoacylglucosamine derivatives should differ so markedly as the carbon chain length is increased. This must reflect differences in their active-site conformations, and it is probable that the binding of the ligands to the enzymes involves a combination of steric, hydrophobic and electrostatic interactions. In this respect it should be emphasized that the positively charged amino function of the *N*-aminoacylglucosamine derivatives under the conditions of the kinetic studies would be retained in the isourea generated when these derivatives were coupled to CNBr-activated agarose (Wilchek, 1974).

It did not escape our notice that in most cases where the K_i value was less than 2 mM these values were obtained from mixed-inhibition systems (as opposed to purely competitive), although only the competitive component was used in the construction of Fig. 3. Since this increased inhibition generally occurs with the longer polymethylene chains, it could be interpreted as an increased hydrophobic component in the enzyme–ligand interaction that may help to reinforce the affinity effects (Barry & O'Carra, 1973). These affinity columns were always operated with a very high yield of hexokinase, usually 90–100%. If the total yield of applied protein was calculated, however, it was always much less than this and varied with the purity of the applied sample. These effects have been noticed in our previous studies (Holroyde *et al.*, 1976a,b). In addition, the 'capacity' of the affinity columns for these enzymes also depended on the purity of the applied sample: the purer the sample, the higher the 'capacity'. This phenomenon can be expected in low-affinity systems where non-specific adsorption of protein can readily interfere with specific interactions. Thus the more impure the fraction to be chromatographed the larger the column required (see also Paulson *et al.*, 1977; Schwyzler & Hill, 1977). The low-affinity systems

described here do have the one advantage that adjustment of ligand concentration can dramatically affect the subsequent chromatography. In very-high-affinity systems such an adjustment of ligand concentration is likely to run into steric/accessibility problems.

It has been suggested that the important quantitative differences in the behaviour of the hexokinase isoenzymes towards these various affinity matrices reflect (slight) differences in their active-site conformations. By this criterion, the active site of the yeast enzyme appears to differ most from the others, a prediction borne out by affinity-labelling experiments with *N*-bromoacetylglucosamine (cf. Otieno *et al.*, 1975, 1977; Connolly & Trayer, 1978).

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