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HEXOKINASES AND MYOSIN: A PROBLEM OF ISOENZYME SEPARATION

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CONTENTS

1. INTRODUCTION

From its inception in 1968[1], affinity chromatography for protein purification has utilized immobilized substrates or substrate analogues primarily for the adsorption and elution of a single enzyme. "Group specific" or "general ligand" affinity chromatography, where a ligand capable of interacting with a wide variety of enzymes is immobilized, was a more recent development of this procedure and has been used extensively for the separation of nicotinamide nucleotide-dependent dehydrogenases on columns containing covalently-bound AMP or $NAD(P)^+$ derivatives[2,3]. Further developments of the technique have made possible a study of a variety of protein–ligand interactions, *e.g.* an investigation of enzyme mechanistic processes[3,4], separation of the lactate dehydrogenase isoenzymes[5] and the study of complex formation between enzymes and other ligands[6]. Indeed, it has been possible to use this technique to obtain accurate values for the binding constants between the protein and the immobilized ligand and eluting ligand[7–9].

By definition, affinity chromatography relies on the existence of a simple equilibrium between the immobilized and free interacting components. Thus, it is advisable to adapt any aspect of the free solution kinetic behaviour of the various enzyme–ligand interactions to the chromatography system under study. In this way, the specificity of both the adsorption and desorption phases can often be greatly increased with concomitant benefit to the experiment. The purpose of this report is to illustrate how this relationship between the free solution and chromatographic behaviour has been developed in our studies on the purification of the mammalian hexokinase isoenzymes and the fractionation of vertebrate skeletal muscle myosin isoenzymes. In the former case, the problem was essentially one of protein purification whereas although myosin isoenzymes have been suspected of existing for the last decade[10] it is only very recently that this has been established unequivocally[11].

2. STUDIES ON THE HEXOKINASE ISOENZYMES

There are four hexokinase isoenzymes found in mammals, numbered I to IV in order of increasing acidity on electrophoresis, whose distribution is largely tissue-specific; type I is found principally in the brain, heart and kidney, type II in skeletal muscle and adipose tissue, type III is found in all tissues in very small amounts but principally in the liver and spleen, whereas type IV, also known as glucokinase, is restricted to the liver and is the main glucose phosphorylating enzyme of that tissue. Although the glucose phosphorylation step is a prime candidate for regulation in metabolism, lack of efficient purification procedures for these enzymes 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 the type I from brain[12,13] and pig heart[14]. 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 and Trayer[15] and Holroyde *et al.*[16,17] succeeded in purifying glucokinase to homogeneity using, in conjunction with ion-exchange chromatography, affinity chromatography on a Sepharose-bound glucosamine derivative [N-(6-aminohexanoyl)-2-amino-2-deoxy-D-glucopyranose].

Initial experiments with this matrix[18] suggested that the glucokinase bound selectively and reversibly but that the low K_m hexokinase activity (presumed to be a mixture of hexokinase types I, II and III) in liver extracts and yeast hexokinase were unretarded. At first this was a puzzling observation since it is well known that both glucosamine and N-acetylglucosamine are good inhibitors of all four isoenzymes[19]. Eventually, it was found that hexokinase type II from skeletal muscle could be purified to homogeneity on this matrix[20]. This prompted a more thorough investigation of the reactions between the hexokinases and the glucosamine derivatives. Various glucosamine derivatives were synthesized giving a series of compounds in which the length of the polymethylene chain between the amide linkage to glucosamine and the primary amino group for attachment to the Sepharose matrix was varied. These glucosamine derivatives [N-aminopropionyl-(C3), N-aminobutyryl-(C4), N-aminohexanoyl-(C6) and N-aminooctanoyl-(C8)] had the effect of altering the length of the spacer molecule attaching the glucosamine to the Sepharose.

All of these N-aminoacyl glucosamine derivatives in free solution proved to be competitive inhibitors with respect to glucose of the rat and yeast hexokinases but with differing K_i values[21]. K_i can be taken as a direct measure of the dissociation constant in these systems. In fact, a direct correlation was found to exist between these kinetic data and the effectiveness of a particular derivative as an affinity chromatographic medium when immobilized to Sepharose. Thus the only really effective inhibitor for rat kidney hexokinase type I was the C-8 glucosamine derivative (K_i = 1.3 mM) and only the Sepharose conjugate of this derivative proved a suitable affinity matrix. This isoenzyme was probably responsible for our initial observations of low K_m hexokinase activity appearing in the wash-through fraction when liver extracts were applied directly to the Sepharose-C6 glucosamine matrix[18]. Subsequent chromatography experiments showed that providing the K_i for the ligand was $\leqslant$2 mM then a suitable affinity matrix could be constructed. For example, hexokinase type II could be chromatographed successfully on any of the immobilized derivatives since in free solution its K_i for all four glucosamine derivatives was less than 2 mM. Similarly,

glucokinase and hexokinase type III could be adsorbed most effectively to the immobilized C-6 and C-8 glucosamine derivatives. Furthermore, in situations where the K_i of two isoenzymes for a particular ligand differed (but were both less than 2 mM) these enzymes could be separated by either adjusting the ligand concentration attached to the gel or employing a glucose gradient for elution. Hexokinase type II ($K_i = 0.4$ mM) and glucokinase (0.75 mM) could be separated on a Sepharose-N-aminohexanoylglucosamine conjugate in this way. It should be pointed out that none of the hexokinases showed any interaction with Sepharose derivatives formed by linking either glucosamine or the various amino acid spacer molecules directly to the support. Although these initial experiments demonstrating the relationship between the free solution behaviour and the subsequent chromatographic behaviour were carried out on a small scale, it has been possible to handle large quantities of tissue extracts in this way. Consequently, in conjunction with conventional methods, it was possible to purify to homogeneity hexokinase type I from 500 g of rat kidneys, and hexokinase types II and III from the livers of 50 rats on Sepharose-N-(8-aminooctanoyl)glucosamine matrices.

These experiments illustrate the usefulness of adapting the kinetic behaviour to the affinity adsorbent operation. Thus, by varying either the length of the spacer molecule and/or the concentration of ligand attached to the gel[16] a glucosamine affinity matrix can be designed specifically for the purification of each hexokinase isoenzyme. These data also suggest that, at least with this hexokinase system, the spacer molecule appears to be making a specific contribution to the enzyme–immobilized ligand interaction. This was very apparent during our comparative investigations with yeast hexokinase. Our initial experiments indicated that this hexokinase would not bind to our C6-glucosamine matrix. Subsequent kinetic studies revealed that as the length of the spacer molecule was increased up to six C atoms, the K_i had increased to about 24 mM. It was a surprise, however, that when an additional two methylene groups were added, *i.e.* the C8-glucosamine, the K_i decreased to 1 mM and an efficient chromatographic adsorbent has been prepared from this derivative.

There have been reports in the literature that yeast hexokinase will not bind efficiently to an ADP-affinity adsorbent[22]. Studies on the kinetics of this enzyme (for review see ref. 23) show that although the enzyme exhibits a random ordered addition of substrates at high $Mg \cdot ATP^{2-}$ concentrations, a mechanism by which glucose binds before $Mg \cdot ATP^{2-}$ is the preferred pathway. The affinity chromatographic behaviour of the enzyme substantiates this behaviour. If yeast hexokinase is applied to an N^6-(6-aminohexyl)-ADP-Sepharose matrix (N^6-ADP) in the absence of glucose the enzyme is partially retarded but efficient binding to the column is only observed when glucose is included in the application buffer.

3. STUDIES ON THE MYOSIN ISOENZYMES

Myosin from the fast twitch muscles of rabbit is hexameric and comprises two polypeptide chains of molecular weight approximately 200,000 (heavy chains, HC) and four moles (per mole of myosin) of light chains: two moles of identical phosphorylatable polypeptides of molecular weight 18,000 (the P-LC) and two moles (total) of two further polypeptides of molecular weight 22,000 and 16,000 (the so-called alkali light chains, A1 and A2 respectively, named after their method of removal

from the native myosin). In overall design the myosin molecule is rod-shaped and divided at one end into two globular head regions (for review see ref. 24). The HC run throughout its length, whereas the light chains are associated exclusively with the head regions; the P-LC are possibly located near the junction between the rod and head regions[25,26] and the alkali light chains reside within the globular head regions, which possess both the actin-binding and ATPase activities of the myosin.

We have been using affinity chromatography techniques in our studies on the structure and function of myosin to answer two problems: the role of the light chains in the interactions of this protein with actin and ATP and whether or not myosin from vertebrate skeletal muscle exists as isoenzymes. Myosin from heart muscle, from many non-vertebrate muscles and from non-muscle sources invariably contains only 2 moles each of two different light chains per 2 moles of HC and with these proteins the subunit structure of the hexameric molecule is easy to envisage. Densiometric and radiochemical studies on rabbit fast twitch muscles have shown that the A1 and A2 light chains exist in a molar ratio of 1.35:0.65 respectively[27,28]. The presence of different heavy chain populations in myosin isolated from these species is also indicated by the observation of amino acid substitutions in certain peptide sequences[29]. Thus, the existence of myosin isoenzymes has frequently been invoked to explain these differences, but until recently[11] these had not been separated. With respect to light chain composition, each myosin head contains one P-LC and three myosin isoenzymes are suggested based upon the alkali light chain distribution: the symmetrical homodimers A1/A1 and A2/A2 and an A1/A2 heterodimer. Since myosins isolated from many vertebrate skeletal muscles contain a P-LC and two other electrophoretically distinct light chains, the existence of isoenzymes is likely to be a widespread phenomenon. The difficulties in separating any isoenzymes is compounded by the fact that myosin is insoluble at low ionic strength and so cannot be easily subjected to normal electrophoretic techniques. Fragments of myosin that are soluble at low ionic strength and that contain the biological activities of the parent molecule have been prepared for a number of years by proteolysis with different proteases. Much of the work on the functioning of myosin has thus been carried out using the water-soluble proteolytic fragments, heavy meromyosin (HMM), which contains both heads and half the rod portion of the molecule, and subfragment-1 (S-1), which is a preparation of the separated, single globular heads. Until recently the large number and variety of polypeptide components found on SDS-gel electrophoresis of these digests made it difficult to identify their origin unambiguously. By controlled digestion with chymotrypsin, however, Weeds and his co-workers[26,30] were able to obtain S-1 preparations that contained a single HC species (of molecular weight approximately 90,000) and the alkali light chains (with total loss of the P-LC) and HMM preparations that also contained a homogeneous HC species (of molecular weight about 140,000) and a full complement of light chains.

Chymotryptic S-1 from rabbit skeletal muscle myosin could be fractionated on columns of either Sepharose-bound G-actin[31] or F-actin (stabilized by glutaraldehyde cross-linking) into species containing only the A1 light chain [S-1 (A1)] or the A2 light chain [S-1 (A2)] by elution with gradients of ATP, ADP or PP_i (either as their free acids or Mg salts) or even KCl (ref. 32). In all cases the S-1 (A1) species required a higher concentration of nucleotide to dissociate it from the immobilized actin than the S-1 (A2) species. This observation agrees with the free solution kinetic analyses where

the apparent K_m for actin of the S-1 (A1) is less than that for the S-1 (A2)[30,33]. These results are not unique to S-1 populations isolated from the myosin of fast-twitch rabbit muscle. Corresponding experiments with S-1 populations isolated from myosin from rabbit slow-twitch muscle and chicken breast muscle also revealed two light chain species of S-1 with distinct binding properties[32]. Again the S-1 species containing the larger light chain showed the greater affinity for actin, but both species bound more tightly to the actin matrix, as judged by the nucleotide concentration required for elution, than the corresponding S-1's from the rabbit fast-twitch muscle. Indeed, a careful comparison of the elution profiles of these S-1's and bovine cardiac muscle S-1 (whose myosin contains only one type of alkali light chain) revealed a spectrum of actin-binding affinities which are in accord with their apparent K_m values for actin[33]. Furthermore, S-1 species isolated from slower-twitch muscle myosins bind to the actin matrices more tightly than those from fast-twitch muscles.

The experiments described here do not distinguish between the relative roles played by the heavy and light chains in actin binding. However, a role for the alkali light chains in determining the strength of binding to actin is indicated when these results are taken in conjunction with the observations of Wagner and Weeds[34]. The authors showed that the differences observed in the actin-activated ATPase activities of isolated native and hybridized rabbit S-1 populations were solely due to the particular alkali light chain present. It is noteworthy that the larger A1 light chains of myosin from chicken breast and rabbit white skeletal muscle and the cardiac light chain all contain proline and alanine-rich N-terminal regions[35,36] and it is tempting to speculate that these segments are responsible for the enhanced actin binding, especially of the S-1 (A1) types over the S-1 (A2) species from the same muscle.

S-1 populations from rabbit fast-twitch and slow-twitch muscle and from chicken breast muscle could also be reversibly bound to columns of Sepharose-bound ADP derivatives [N^6-ADP and 8-(6-aminohexyl)amino-ADP; ref. 37]. In each case, the two populations of S-1 could be fractionated when the columns were developed with ADP^{3-} or ATP^{4-} but not when either their Mg^{2+} or Ca^{2+} salts were used[38]. This is very interesting since no differences in either the Mg or Ca-ATPase activities of the separated S-1 species could be found.

Experiments with S-1 populations do not, however, help to decide about the existence of myosin isoenzymes since the A1- and A2-containing species could have originated from the same or separate myosin molecules. Thus, similar experiments to the above were carried out with chymotryptic HMM prepared from myosins isolated from both rabbit fast-twitch and chicken breast muscles. These HMM preparations could be fractionated into their homodimers on columns of matrix-bound actin, ADP and pyrophosphate using gradient elution techniques but this was much more difficult than fractionation of the S-1 populations. It was necessary to employ long thin columns (up to 30 × 0.9 cm) with long gradients to achieve the best separations and even so these were incomplete. The protein eluting at the lowest nucleotide concentration contained only the A2/A2 homodimer whereas that eluting at the highest nucleotide concentration contained only the A1/A1 homodimer. In between, HMM containing all three light chains was found. Similar fractionations were obtained from each affinity matrix using a variety of eluants: ATP, ADP, PP_i (free and as their Mg salts) and KCl. These studies clearly demonstrate the existence of homodimers but do not entirely eliminate the possibility of heterodimers being present. As yet we have been

unable to obtain two clearly separated peaks of homodimers. However, when the central portion of such a profile, containing all three light chains, is taken and re-applied to a second affinity column, then the material re-fractionated as above. A third re-run of the central portion again fractionated as well as the first application. These data suggest that homodimers predominate, at least, and any heterodimers must be present in only very small amounts.

Thus, careful affinity chromatographic studies have helped to elucidate the basic structure of the myosin molecule and the probable role of the alkali light chains. Each myosin molecule contains two moles of identical (alkali) light chains situated within the head region, which control the strength of binding of myosin to actin, and two moles of metal-binding (phosphorylatable in vertebrates) light chains[25], possibly arranged at the head–tail junction[25,26], which play a role in the regulation of the actomyosin ATPase. A regulatory role for these light chains in myosins from molluscan[39] and smooth[40] muscle systems and in some non-muscle myosins[41] is well-documented. Their exact role in vertebrate skeletal muscle myosins is as yet unknown although their phosphorylation and dephosphorylation by a specific kinase and phosphatase is established[42,43]. It seems that only vertebrate skeletal muscle myosin populations possess two types of actin-binding (alkali) light chains, suggesting that isoenzymes are invoked in cases where both maintained muscle tone and variation in contractile speed are required, and that this is achieved by the presence of myosin species which differ in their affinity for actin and therefore in their ability to generate force.

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5. SUMMARY

A direct correlation exists between the ability of various N-aminoacyl-glucosamine derivatives (propionyl, butyryl, hexanoyl and octanoyl) to inhibit the four mammalian hexokinase isoenzymes and yeast hexokinase in free solution and their effectiveness as affinity chromatographic media for these enzymes when immobilized to Sepharose. Thus, by using Sepharose conjugates with different spacer molecules and/or adjusting the final ligand concentration attached to the gel, glucosamine affinity matrices have been designed to purify specifically each isoenzyme on a large scale. Chromatography of vertebrate skeletal myosin on columns of immobilized actin or ADP has shown that these myosin preparations contain mixtures of symmetrical homodimer isoenzymes with respect to the light chains, *i.e.* each head of the myosin molecule is identical and contains two different light chains. Functionally these homodimers differ in their ability to bind to actin.

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