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PRINCIPLES OF MULTIENZYME PURIFICATIONS BY AFFINITY CHROMATOGRAPHY

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Recently in our laboratory, up to 20 different enzymes and their genetic variants have been purified from mouse and *Drosophila* by affinity chromatography. By virtue of the specific coenzyme requirements, up to ten different enzymes could be copurified from a single tissue extract either by biospecific elutions with different coenzymes or inhibitors, or by sequential passages of the extract through several cofactor-related affinity columns. Important principles were developed to purify enzymes exhibiting low affinity to the affinity columns. By "affinity filtration" of the extract through the affinity column, enzymes of low affinity can be retarded and separated effectively from strongly bound and nonadsorbed proteins. By the "saturation readsorption" procedure, enzymes of low affinity could be effectively separated from those of high affinity by overloading of the extracts on the affinity columns. Readsorption of the leaked low affinity enzymes to a second affinity column often results in better enzyme purification because of the elimination of competitive high affinity enzymes. With the application of these principles, the following enzymes and their genetic variants were highly purified via a single- or two-step affinity column procedure: lactate dehydrogenase-A, lactate dehydrogenase-B, lactate dehydrogenase-X, phosphoglycerate kinase-A, phosphoglycerate kinase-B, cytoplasmic and mitochondrial isocitrate dehydrogenase, malate dehydrogenase, malic enzyme, glucose-6-phosphate dehydrogenase, glutathione reductase, phosphoglucose isomerase and pyruvate kinase from mouse tissues; alcohol dehydrogenase, malate dehydrogenase, α-glycerol-phosphate dehydrogenase, malic enzyme, and glucose-6-phosphate dehydrogenase from *Drosophila*.

INTRODUCTION

Recent developments in affinity chromatography have revolutionized the methods involved in enzyme purification either in biomedical research or in the industrial production of enzymes (1–3). More specifically, general ligand affinity chromatography allows the purification of coenzyme-requiring enzymes based on their common binding properties to the nucleotide cofactors (2). During the last few years, more than 100 different enzymes from numerous sources have been purified by employing this methodology (1–5).

We are currently interested in the comparison of genetic variations in many enzymes from both mice and *Drosophila*. The simultaneous purification of many enzymes from a single sample is of considerable value for studies on these species. General ligand affinity chromatography fits this goal, since it allows the fractionation of many enzymes from a crude homogenate according to their respective coenzyme requirements.

In this communication, we would like to present our results on the facile purifications of dozens of mouse and *Drosophila* enzymes by employing some newly developed principles in affinity chromatography.

MATERIALS AND METHODS

Chemicals

The following chemicals were purchased from Sigma Chemical Company: AMP, ATP, NAD^+, $NADP^+$, NADP, NADPH, pyruvate, oxalacetate, α-glycerol-phosphate, isocitrate, glucose-6-phosphate, 1-malate, fructose-6-phosphate, and 3-phosphoglycerate.

Affinity Columns

In this study, several 8-(6-aminohexyl)-amino-adenine nucleotide derivatives immobilized on Sepharose columns were employed as affinity ligands for enzyme purifications. These affinity ligands and their general specificity are listed in Table 1. The preparation of these affinity ligands has been described previously (6–11).

Protein Determinations

The purity of enzymes from affinity columns was routinely analyzed by polyacrylamide gel electrophoresis in the presence and in the absence of SDS. Gels of 5% and 10% were used for native and SDS gel electrophoresis, respectively. The protein concentration was determined by the procedure of Böhlen et al. (12). The specific activity of enzymes purified from affinity columns was also employed as criteria for purity of the enzymes listed in Table 2.

Enzyme Assays

Assays of the various enzymes reported in this work were performed according to the standard procedures reported in the Boerhinger catalog (13).

TABLE 1. **8-Substituted Adenine Nucleotide Derivatives as General Ligands for Affinity Columns**[a]

	Ligand	Enzyme specificity
I.	8-(6-Aminohexyl)-amino-5'-AMP (8-hexyl-AMP)	NAD^+-dependent dehydrogenases, ribonucleases, deoxyribonucleases, enzymes which are activated or inhibited by 5'-AMP, tRNA synthetases
II.	8-(6-Aminohexyl)-amino-2'-AMP	$NADP^+$-dependent dehydrogenases; (ribonucleases)
III.	8-(6-Aminohexyl)-amino-5'-ADP (8-hexyl-ADP)	Kinases, NAD^+-dependent dehydrogenases
IV.	8-(6-Aminohexyl)-amino-5'-ATP (8-hexyl-ATP)	Kinases, NAD^+-dependent dehydrogenases, tRNA synthetases
V.	8-(6-Aminohexyl)-amino-NAD^+ (8-hexyl-NAD^+)	NAD^+-dependent dehydrogenases
VI.	8-(6-Aminohexyl)-amino-$NADP^+$ (8-hexyl-$NADP^+$)	$NADP^+$-dependent dehydrogenases
VII.	8-(6-Aminohexyl)-amino-2', 5'-ADP (8-hexyl-2', 5'-ADP)	$NADP^+$-dependent dehydrogenases
VIII.	8-(6-Aminohexyl)-amino-3', 5'-ADP (8-hexyl-3', 5'-ADP)	Coenzyme A-dependent enzymes
IX.	8-(6-Aminohexyl)-amino-desulfo-COA	Coenzyme A-dependent enzymes

[a]See References 6–11.

Preparation of Tissue Homogenates

The inbred strains of mice (DBA/2J) were obtained from Jackson Laboratory. Tissues, including muscle, testes, kidneys, and hearts, were removed by surgery and frozen to −70°C until the enzyme purification. The homogenates of frozen tissues were prepared by a Virtis homogenizer in 10 mM phosphate buffer at pH 6.5 with a 1–2 vol/wt ratio. The homogenates were centrifuged at $27{,}000 \times g$ for 20 min. The supernatant was then applied to affinity columns for enzyme purification. The supernatant of the mouse muscle extract was first fractionated with ammonium sulfate (40–80%). The pellet was dialyzed overnight against 5 mM phosphate buffer before being applied to affinity columns for enzyme purification. The homogenate of *Drosophila melanogaster* was also prepared with a similar procedure. After homogenization, the supernatant was subjected to a 40–80% ammonium sulfate fractionation. The resulting pellet was redissolved in buffer and dialyzed overnight against 10 mM phosphate buffer at pH 6.5 before being applied to affinity column for enzyme purification.

RESULTS AND DISCUSSION

We have previously reported that 8-substituted adenine nucleotide derivatives immobilized on Sepharose could be effective general ligands for the purification of many coenzyme-dependent enzymes, especially dehydrogenases and kinases (5). Several important new principles have been developed regarding multienzyme purification from crude extracts by general ligand affinity chromatography. These principles are (a) the use of multifunctional affinity columns for simultaneous purification of more than two different classes of enzymes (9), (b) affinity filtration, and (c) saturation and readsorption.

Multifunctional Affinity Column

Among the general ligands shown in Table 1, 8-(6-aminohexyl)-amino-ATP-Sepharose column exhibits multifunctional affinity properties in enzyme purification. ATP is not only coenzyme for ATP-dependent enzymes such as kinases and synthetases, but also is a common inhibitor to many NAD^+-dependent enzymes or sugar-phosphate-related enzymes. Besides, ATP-Sepharose also acts as an ion exchange resin, similar to phospho-Sepharose. Several enzymes and their genetic variants have been copurified from mice and *Drosophila* with this affinity column, because of these multifunctional properties.

As shown in Fig. 1, when a kidney homogenate from DBA/2J mice was passed through an ATP-Sepharose column in 10 mM phosphate buffer at pH 6.5, most of the dehydrogenases and kinases were quantitatively adsorbed. Dehydrogenases, such as lactate dehydrogenase, malate dehydrogenase and α-glycerol-phosphate dehydrogenase, could be eluted biospecifically with 0.5 mM NADH, whereas kinases and other ATP-dependent enzymes, such as 3-phosphoglycerate kinase, aldolase, and

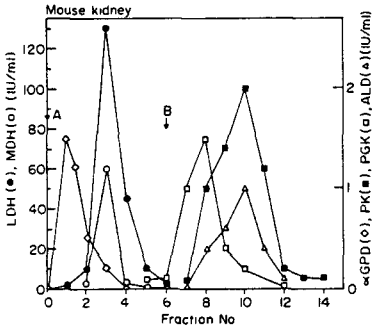

FIG. 1. Profiles for the elution of dehydrogenases and kinases in mouse kidney extract for an 8-(6-aminohexyl)-amino-ATP-Sepharose column (1.5 × 5 cm). A, Elution with 0.5 mM NADH in 10 mM phosphate buffer at pH 6.5; B, elution with 5 mM ATP in the same buffer. Fractions of 0.6 ml were collected.

pyruvate kinase, were eluted subsequently from the column with 5 mM ATP.

The same affinity column was also employed for the purification of three muscle enzymes from DBA/2J mice, malate dehydrogenase, pyruvate kinase, and phosphoglucose isomerase. Phosphoglucose isomerase was adsorbed onto this affinity column mainly because of the ion exchange properties of ATP column and possibly because 8-hexyl-ATP is a weak inhibitor of this enzyme. When some of the muscle extract (2 g total proteins) was loaded on the affinity column (2.5×20 cm), these three enzymes were preferentially adsorbed. After extensive washing with the same buffer, phosphoglucose isomerase was eluted biospecifically with 2 mM glucose-6-phosphate. Malate dehydrogenase was eluted subsequently with 0.2 mM NADH. Pyruvate kinase was finally eluted with a 0–2 mM ATP gradient (Fig. 2).

In this sequential elution procedure, no overlaps in enzyme activities among these three enzymes were observed, and the purity of the enzymes eluted from this affinity column ranged from 40 to 80%. The results of these enzyme purifications are summarized in Table 2. The same principle was employed in the purification of two sperm-specific enzymes, 3-phosphoglycerate kinase and lactate dehydrogenase-X, from testes of DBA/2J mice. As shown in Fig. 3, after adsorption and buffer wash, these two enzymes were eluted sequentially from ATP-Sepharose column with 2 mM ATP and 0.2 mM reduced NAD^+-pyruvate adduct, respectively. Both

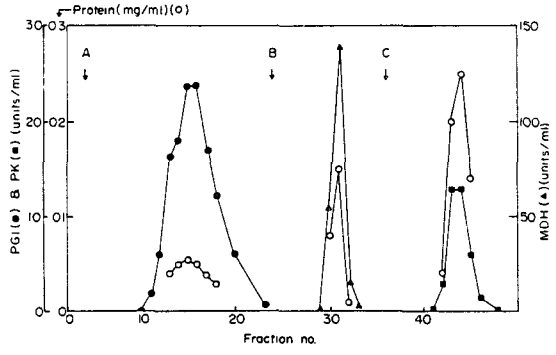

FIG. 2. Elutions of phosphoglucose isomerase (●), malate dehydrogenase (▲), and pyruvate kinase (■) in mouse muscle extract from an 8-(6-aminohexyl)-amino-ATP-Sepharose column (2.5×20 cm). A, B, and C denote, respectively, the elutions with 2 mM glucose-6-phosphate, 0.2 mM NADH, and 2 mM ATP in the 10 mM phosphate buffer at pH 6.5.

TABLE 2. **Enzymes Purified from Mice and *Drosophila* by Affinity Chromatography**

Enzyme (Source)	Affinity ligands	Fold of purification[a] (purity%)	Methods[b] used
Lactate dehydrogenase (Mouse kidney)	8-Hexyl-ATP	80	I
Malate dehydrogenase (Mouse kidney)	8-Hexyl-ATP	60	I
α-Glycerol-phosphate dehydrogenase (Mouse kidney)	8-Hexyl-ATP	23	II
Pyruvate kinase (Mouse kidney)	8-Hexyl-ATP	40	I
Aldolase (Mouse kidney)	8-Hexyl-ATP	20	I
3-Phosphoglycerate kinase-A (Mouse kidney)	8-Hexyl-ATP	25	I
Phosphoglucose isomerase (Mouse muscle)	8-Hexyl-ATP	500 (40)	I
Malate dehydrogenase (Mouse muscle)	8-Hexyl-ATP	100 (80)	I
Pyruvate kinase (Mouse muscle)	8-Hexyl-ATP	30 (40)	I
3-phosphoglycerate kinase-A (Mouse muscle)	8-Hexyl-ATP	26 (20)	I
3-Phosphoglycerate kinase-B (Mouse testes)	8-Hexyl-ATP	130 (10)	I
Lactate dehydrogenase-X (Mouse testes)	8-Hexyl-ATP	500 (80)	I
Isocitrate dehydrogenase (Mitochondria, mouse hearts)	8-Hexyl-AMP	22 (40)	I
Lactate dehydrogenase (Mouse hearts)	8-Hexyl-AMP	150 (90)	I
Glucose-6-phosphate dehydrogenase (Mouse testes)	8-Hexyl-AMP	700 (20)	I
Isocitrate dehydrogenase (Mitochondria, mouse hearts)	8-Hexyl-$NADP^+$	2.5 (100)	II
Isocitrate dehydrogenase (Cytoplasmic mouse kidneys)	8-Hexyl-$NADP^+$	5 (95)	II
α-Glycerol-phosphate dehydrogenase (*Drosophila*)	8-Hexyl-AMP	80 (20)	II
Alcohol dehydrogenase (*Drosophila*)	8-Hexyl-AMP	50 (50)	II

TABLE 2—*contd.*

Enzyme (Source)	Affinity ligands	Fold of purification[a] (purity%)	Methods[b] used
Malate dehydrogenase (*Drosophila*)	8-Hexyl-AMP	100 (80)	II
α-Glycerol-phosphate dehydrogenase (Chicken muscle)	8-Hexyl-AMP	180 (60)	III
Glutathione reductase (Mouse kidneys)	8-Hexyl-2',5'-ADP	400 (10)	III
Glucose-6-phosphate dehydrogenase (Mouse kidneys)	8-Hexyl-2',5'-ADP	356 (10)	IV
Malic enzymes (Mouse kidneys)	8-Hexyl-2',5'-ADP	200 (30)	IV
Glucose-6-phosphate dehydrogenase (*Drosophila*)	8-Hexyl-2',5'-ADP	210 (15)	IV
Malic enzyme (*Drosophila*)	8-Hexyl-2',5'-ADP	150 (20)	150N

[a] The recoveries of enzymes purified from these affinity columns ranged from 20 to 80%, depending on enzymes.

[b] I refers to the use of conventional affinity chromatography; II to the use of multifunctional columns; III to the application of the saturation readsorption procedure; and IV to the use of the affinity filtration procedure.

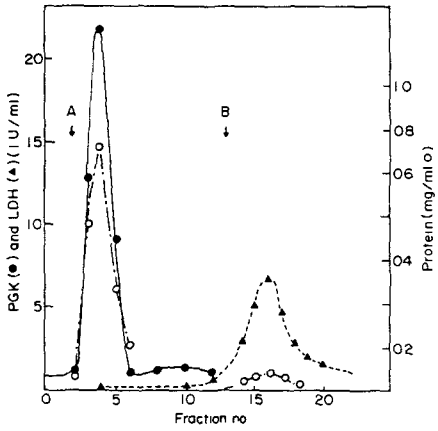

FIG. 3. Elution of 3-phosphoglycerate kinase (●) and lactate dehydrogenase (▲) from DBA/2J mouse testes from an 8-(6-amino-hexyl)-amino-ATP-Sepharose column (2× 10 cm). A and B denote the elution by 2 mM ATP and 0.2 mM reduced NAD^+-pyruvate adduct in 10 mM phosphate buffer at pH 6.5, respectively. (○) The protein concentration. Fractions of 5 ml were collected for 3-phosphoglycerate kinase and 10 ml lactate dehydrogenase.

enzymes were enriched about 100-fold from the crude extract by this single-step affinity chromatographic procedure (14).

8-(6-Aminohexyl)-amino-AMP-Sepharose is known to be a good affinity gel for many NAD^+-dependent enzymes. However, it also exhibits good affinity to several $NADP^+$-dependent enzymes from mice, such as mitochondrial $NADP^+$-dependent isocitrate dehydrogenase from heart and glucose-6-phosphate dehydrogenase from testes. Thus, from the heart extract, one could copurify lactate dehydrogenase and isocitrate dehydrogenase by sequential elutions with 0.2 mM reduced NAD^+-pyruvate adduct and 0.5 mM $NADP^+$, respectively. From the testicular extract, one could also use 8-hexyl-AMP-Sepharose column to purify both lactate dehydrogenase-X and glucose-6-phosphate dehydrogenase by separate elutions with 0.2 mM reduced NAD^+-pyruvate adduct and 0.5 mM $NADP^+$, respectively. The fold of purification and the purity of these mouse enzymes after the single-step affinity column procedure are presented in Table 2.

Affinity Filtration

Low affinity enzymes from the crude homogenate could be highly purified from the affinity columns by an affinity filtration method which combines the principles of gel filtration and affinity chromatography. The low affinity enzymes in the homogenate could be separated effectively from the strongly bound enzymes and nonadsorbed proteins in the affinity column by a gel filtration of the homogenate through the affinity columns because of the retardation of low affinity enzymes by weak enzyme–ligand interactions.

Two isozymes of isocitrate dehydrogenase from DBA/2J mice were purified to homogeneity based on this methodology. It was generally observed that isocitrate dehydrogenases from mice and many other species show little affinity to 8-(6-aminohexyl)-amino-$NADP^+$-Sepharose column. These enzymes could not, therefore, be purified by conventional affinity chromatography since the enzymes could not be permanently adsorbed on this affinity column. However, they are slightly retarded by the affinity column. Therefore, when a partially purified enzyme preparation in a relatively small volume (about 5% of the column volume) was allowed to filter through the affinity column, the enzyme was preferentially retarded such that the nonadsorbed proteins could be effectively separated from isocitrate dehydrogenase which could later be eluted biospecifically with $NADP^+$ from the affinity column. With this affinity filtration procedure, two isozymes of isocitrate dehydrogenases were purified to homogeneity from mouse hearts and kidneys, respectively.

Three dehydrogenases in *Drosophila*, alcohol dehydrogenase, malate dehydrogenase, and α-glycerol-phosphate dehydrogenase, are known to be coded by three linked genes located on the second chromosome. Numerous genetic variants and mutants have been identified from natural and mutagen-treated *Drosophila* populations. Current work in this laboratory is directed toward understanding the biochemical genetics of naturally occurring and mutationally induced enzyme variants. To achieve this goal, it is essential to develop facile purification procedures. These three dehydrogenases were found to be only retarded by 8-(6-aminohexyl)-amino-AMP-Sepharose column. Leakage of the enzymes occurred upon continuous washing of the affinity column with buffer alone. Therefore, the technique of affinity filtration was employed to purify these three enzymes simultaneously, to a high degree of purity in each case.

When the crude *Drosophila* extract (50 g of frozen flies), after ammonium sulfate fractionation, was concentrated to about 5% of the column volume and loaded on the affinity column (2.5 × 70 cm), one could observe that most of the other proteins appeared within the first column volume, whereas alcohol dehydrogenase, malate dehydrogenase, and α-glycerol-phosphate dehydrogenase were substantially retarded and separated from the main protein peak. α-Glycerol-phosphate dehydrogenase appeared within the second column volume. Alcohol dehydrogenase was subsequently eluted with the application of a solution containing 0.2 mM NAD^+ and 5 mM pyrazole, and malate dehydrogenase was finally eluted with a 0–0.5 mM NADH gradient. The profiles for the elution of these three enzymes from the affinity column are shown in Fig. 4. The purity of these three enzymes could be greater than 50% by this single-step affinity filtration procedure. Further details regarding the purification of these three *Drosophila* enzymes to homogeneity are presented elsewhere (15).

Saturation and Readsorption

Another principle that can be employed to purify enzymes that have low affinity to affinity columns is saturation-readsorption. Owing to the mutual competition of enzymes in the extract for the immobilized ligand, one would expect that enzymes of high affinity would eventually displace the enzymes of low affinity. Reloading of the weakly bound enzymes on a separate affinity column often results in better binding of these enzymes and a higher degree of purity. Several examples regarding the application of this technique for enzyme purifications have been reported (6, 14). One of the examples to be presented here is the purification of α-glycerol-phosphate dehydrogenase from chicken muscle. In comparison to lactate

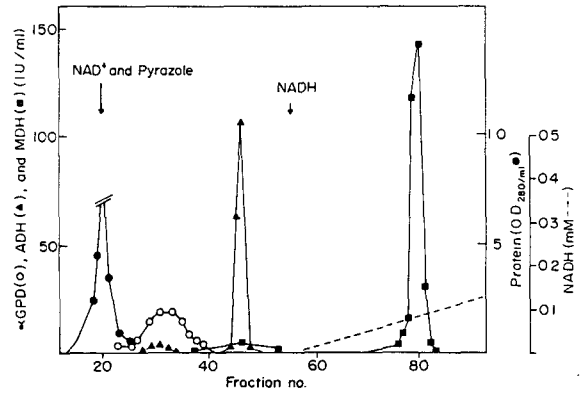

FIG. 4. Elution of three *Drosophila* dehydrogenases from an 8-(6-aminohexyl)-amino-AMP-Sepharose column by means of the affinity filtration procedure. Column size 2.5 × 70 cm, flow rate 2 ml/min. Elutions by NAD^+ plus pyrazole and by NADH gradient in 10 mM phosphate buffer at pH 6.5 are indicated.

dehydrogenase from chicken muscle, α-glycerol-phosphate dehydrogenase binds much more weakly to 8-(6-aminohexyl)-amino-AMP-Sepharose column (5 × 25 cm). During the loading of crude homogenate, leakage of α-glycerol-phosphate dehydrogenase appeared to increase with increasing loading. When approximately 1 mg of lactate dehydrogenase was adsorbed per milliliter of affinity gel, greater than 95% of α-glycerol-phosphate dehydrogenase was desorbed from the column. The desorbed enzyme in the eluent was free of lactate dehydrogenase after the first passage. The eluent containing α-glycerol-phosphate dehydrogenase was readsorbed on the second equilibrated affinity column of a similar size. α-Glycerol-phosphate dehydrogenase was found to be adsorbed much better on the second passage than on the first one. As a result, about 100 mg of α-glycerol-phosphate dehydrogenase could be obtained from the second affinity column upon the biospecific elution with a 0–0.5 mM NADH gradient. The eluted enzyme was shown to be greater than 50% in purity.

We also applied the same principle to purify three $NADP^+$-dependent dehydrogenases from mouse kidneys. By saturation of the first 8-(6-aminohexyl)-amino-2′, 5′-ADP-Sepharose column, glutathione reductase was effectively separated from the high affinity enzymes, glucose-6-phosphate dehydrogenase and malic enzymes. In the second passage, glutathione reductase was readsorbed on the same affinity column and a threefold increase in capacity was observed. The adsorbed glutathione

reductase was then eluted with a 0–0.5 mM $NADP^+$ gradient. Glucose-6-phosphate dehydrogenase and malic enzyme were eluted in the first column with a 0.2 mM $NADP^+$ in the washing buffer. With the manipulation of this technique, three $NADP^+$-dependent enzymes were copurified from a single homogenate.

CONCLUSION

Enzymes in crude homogenates could be effectively fractionated according to their coenzyme specificities as indicated in Table 1 by virtue of functional differences between various immobilized adenine nucleotide derivatives. Because of the broad specificity and ion exchange characteristics of 8-hexyl-ATP-Sepharose, as many as ten different enzymes could be copurified with this affinity gel. When two enzymes exhibit low affinity to a given affinity column, one could employ affinity filtration to separate the nonretarded proteins in the extract and to obtain highly purified enzymes. On the other hand, when two enzymes with the same coenzyme specificity have distinctly different affinities for the same ligand on the affinity column, a saturation readsorption technique could be applied to separate effectively, as well as purify, them at the same time. The techniques introduced here have been routinely employed for the purification of at least a dozen isozymes and variants from mice and *Drosophila* (Table 2). It is expected that the principles described here could also be useful in the purification of other enzymes from other sources.

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