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GENERAL LIGAND AFFINITY CHROMATOGRAPHY IN ENZYME PURIFICATION

Ligands, Affinity Chromatography, Enzyme Purification

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INTRODUCTION

Recent developments in enzyme purification have revolutionized isolation techniques and at the same time provided a more profitable manner in which to obtain them. These developments are primarily concerned with the preparation and usability of immobilized ligands for the selective isolation and purification of biologically active substances[1-5], particularly enzymes. This technique is based on the unique principle that the biological macromolecule is able to be adsorbed to the immobilized ligand, specifically and reversibly, unlike the traditional methods by which proteins are separated by their differences in physicochemical properties. Numerous proteins have been purified since this technique was first applied by Campbell et al.[6] for the purification and isolation of antibodies. From these preliminary efforts it is apparent that further development of this method would greatly facilitate our understanding and utilization of enzymes.

Purification of proteins based on biospecific adsorption is generally termed "affinity chromatography"[1-3]. There are two basic procedures that one may use to purify enzymes via affinity column techniques. One is to prepare a ligand which is specific to the enzyme under study[1]. The ligands of high specificity are usually inhibitors, substrates, or perhaps antibodies which can bind a

particular enzyme[1, 6, 7] to the column. The second method is the use of a general ligand which would have the capacity to adsorb a large number of enzymes or family of enzymes[8, 9]. Ligands exhibiting general specificity are able to bind a family of enzymes which have cofactors or inhibitors in common; compounds immobilized on Sepharose are usually derivatives of these.

We have applied the technique of affinity chromatography primarily to two classes of enzymes, namely, dehydrogenases and kinases. Dehydrogenases are pyridine nucleotide-dependent enzymes which require either DPN^+ (NAD^+) or TPN^+ ($NADP^+$) as cofactors. These enzymes which utilize nucleotide cofactors are usually involved in the enzymatic oxidation-reduction of alcohols, aldehydes, ketones, or acids. Kinases, on the other hand, catalyze phosphate transfers, where the cofactors ATP and ADP are phosphate donors and acceptors, respectively.

Since there are a large number of dehydrogenases and kinases, the question arises then as to whether a particular type of general ligand column can be employed to initially bind them and then specifically elute a given enzyme from the column. Specific elution would use either an inhibitor, substrate, or dead-end ternary complex which is specific only for this particular enzyme.

Adenine nucleotide derivatives, such as 5'-AMP, 5'-ADP, 5'-ATP, DPN^+, or TPN^+, are known either as inhibitors or cofactors of dehydrogenases as well as kinases[8-12]. Various adenine nucleotide derivatives immobilized on Sepharose are found to bind numerous dehydrogenases and kinases [10, 12-14]. Thus the success of using general ligand affinity columns for protein purification may very well depend on whether the bound enzyme can be eluted specifically from the column under the proper experimental condition. In this article we present a brief review on the purification of these enzymes using general ligand affinity column techniques.

VARIOUS ADENINE NUCLEOTIDE DERIVATIVES PREPARED FOR GENERAL LIGAND AFFINITY CHROMATOGRAPHY

We have mentioned that the ligands used as general ligands in affinity chromatography are able to retain a family of enzymes from crude extracts of various sources. Most of the general ligands prepared exhibit relatively high affinity either for dehydrogenases or kinases. However, some of these ligands have the capacity to adsorb more than one class of enzymes.

The Sepharose-bound ligands are usually derivatives of adenine mononucleotides as well as diphosphopyridine or triphosphopyridine coenzymes which are covalently attached to Sepharose through long carbon chain spacers. As shown in Table 1, at least twenty different types of derivatives have been prepared as general ligands for affinity columns. Based on the positions of the attachment of spacers to the ligand, five major groups of affinity adsorbants can be discerned. These general ligands are denoted by Roman numerals in Table 1 and in the following descriptions within the text of the paper.

Attachment Through C^8 Position of Adenine Mononucleotides or Dinucleotides

There are several affinity ligands in which the spacer 1,6-diaminohexane was attached to the C^8 position of adenine nucleotides[12, 13, 15, 16]. These adsorbants are listed as follows: 8-(6-aminohexyl)-amino-5'-AMP-Sepharose (I), 8-(6-aminohexyl)-amino-TPN^+-Sepharose (IV)[13], and corresponding 8-substituted ADP- and ATP-Sepharose (V and VI)[13, 15]. Ligands (I) and (II) exhibit good affinity to DPN^+-dependent dehydrogenases; ligands (III) and (IV) exhibit good affinity to TPN^+-dependent enzymes. 8-(6-Aminohexyl)-amino-5'-AMP-Sepharose exhibited high affinity not only to DPN^+-dependent dehydrogenases but also to other classes of enzymes such as nucleases, phosphorylase a, DPN^+-dependent isocitrate dehydrogenase from yeast, and other enzymes which are either activated or inhibited by 5'-AMP. Ligands (V) and (VI) exhibit good affinity to kinases.

TABLE 1. Nucleotide Derivatives as General Ligands for Affinity Columns

	Name of ligand	Position of space attachment	General specificity of enzymes
I.	8-(6-Aminohexyl)-amino-5'-AMP[12,15]	C^8 of adenine base	DPN^+-dependent dehydrogenases; nucleases, enzymes which are activated or inhibited by 5'-AMP
II.	8-(6-Aminohexyl)-amino-DPN^+ [12]	C^8 of adenine base	DPN^+-dependent dehydrogenases
III.	8-(6-Aminohexyl)-amino-2'-AMP[12]	C^8 of adenine base	TPN^+-dependent dehydrogenases from; transhydrogenase from Pseudomonas aeruginosa
IV.	8-(6-Aminohexyl)-amino-TPN^+ [12]	C^8 of adenine base	TPN^+-dependent dehydrogenases
V.	8-(6-Aminohexyl)-amino-ADP[15]	C^8 of adenine base	Dehydrogenases and kinases
VI.	8-(6-Aminohexyl)-amino-ATP[15]	C^8 of adenine base	Kinases
VIA.	P-8-Diazo-DPN^+[N-(6-aminohexyl)-benzamide][21, 22]	C^8 of adenine base	DPN^+-dependent dehydrogenases
VIB.	P-8-Diazo-ADP[N-(6-aminohexyl)-benzamide][24]	C^8 of adenine base	Kinases and a few dehydrogenases
VIC.	P-8-Diazo-ATP[N-(6-aminohexyl)-benzamide][24]	C^8 of adenine base	Kinases
VII.	N^6-(6-Aminohexyl)-5'-AMP[14]	N^6 of adenine bases	DPN^+-dependent dehydrogenases; enzymes activated or inhibited by 5'-AMP

VIII.	DPN^+-[N^6-N-(6-aminohexyl)-acetamide][22]	N^6 of adenine base	DPN^+-dependent dehydrogenases
IX.	6-Mercapto-purine riboside-5'-phosphate S^6-[N-(6-aminohexyl)-acetamide][22]	S^6 of 6-mercapto purine	DPN^+-dependent dehydrogenase; adenosine deaminase
X.	6-Mercapto-purine riboside-5'-triphosphate[22]	S^6 of 6-mercapto purine	Kinases
XI.	6-Mercapto-purine-DPN^+ [22]	S^6 of 6-mercapto purine	DPN^+-dependent dehydrogenases
XII.	5'-AMP (6-amino-caproiate)[9, 29]	Ribose of nucleotides	DPN^+-dependent dehydrogenases
XIII.	DPN^+-(6-amino-caproiate)[9, 29]	Ribose of dinucleotides	DPN^+-dependent dehydrogenases
XIV.	TPN^+-(6-amino-caproiate)[9, 29]	Ribose of dinucleotides	TPN^+-dependent dehydrogenases
XV.	DPN^+-(6-amino-adipic hydrazide)[30, 31]	Ribose of nucleotides	DPN^+-dependent dehydrogenases
XVI.	TPN^+-(6-amino-adipic hydrazide)[30, 31]	Ribose of nucleotides	TPN^+-dependent dehydrogenases
XVII.	ATP-(6-amino-adipic hydrazide)[30, 31]	Ribose of nucleotides	Kinases; myosine
XVIII.	3-Amino-pyridine-DPN^+-N^3-[N-(6-amino-caproamide)]	N^3 of 3-amino-pyridine DPN^+	DPN^+-dependent dehydrogenases; kinases

TABLE 1 (Continued)

XIX.	3-N-(6-Aminohexyl)-nicotinamide-DPN^+	Nicotinamide of DPN^+	DPN^+-dependent dehydrogenases; kinases
XX.	3'-N-(6-Aminohexyl)-nicotinamide-deamino-DPN^+ [13]	Nicotinamide of deamino-DPN^+	DPN^+-dependent dehydrogenases; kinases
XXI.	3-[4-(2-Aminoethyl)-amino naphthyl]-azo-pyridine-deamino-DPN^+	C^3 of pyridine of pyridine-deamino-DPN^+	Dehydrogenases
XXII.	P^1-(6-Aminohexyl)-P^2-(5-adenosine)-pyrophate[15]	Terminal phosphate of 5'-ADP	DPN^+-dependent dehydrogenases; kinases
XXIII.	8-(6-Aminohexyl)-amino-desulfo-CoA[17, 20]	C^8 of adenine base	CoA-dependent enzymes
XXIV.	8-(6-Aminohexyl)-amino-2',5'-ADP[17]	C^8 of adenine base	TPN^+-dependent enzymes
XXV.	8-(6-Aminohexyl)-amino-3',5'-ADP[17]	C^8 of adenine base	CoA-dependent enzymes
XXVI.	TPN^+-N^6-[N-(6-aminohexyl)-acetamide][9]	N^6 of adenine base	TPN^+-dependent enzymes
XXVII.	N^6-(6-Aminohexyl)-2',5'-ADP[26]	N^6 of adenine base	TPN^+-dependent enzymes
XXVIII.	N^6-(6-Aminohexyl)-3',5'-ADP[26]	N^6 of adenine base	CoA-dependent enzymes
XXIX.	ATP-N^6-[N-(6-aminohexyl)-acetamide][28]	N^6 of adenine base	ATP-dependent enzymes; citrate synthase

Preparations of these ligands follow a relatively simple and straightforward procedure (Fig. 1). This usually involves a two-step synthesis, i.e., an initial direct bromination at the C^8 position of nucleotides and then substitution with 1,6-diaminohexane. However, some modifications are required for the preparations of 8-substituted DPN^+ or TPN^+ derivatives. Since the aqueous alkaline conditions are not favorable for oxidized coenzymes, 8-Br-DPN^+ or 8-Br-TPN^+ should be reduced enzymatically before the substitution reactions can proceed in an organic solvent such as DMSO. The yield for the preparations of these 8-substituted adenine nucleotide derivatives ranged from 50 to 95%[13].

Recently the direct synthesis of 8-(6-aminohexyl)-amino-ATP (compound III) has been developed in our laboratory[13]. This involves a direct bromination of ATP to 8-Br-ATP[12] and then a subsequent replacement reaction by 1,6-diaminohexane under alkaline conditions to form 8-(6-aminohexyl)-amino-ATP. Unlike the indirect synthesis reported by Trayer et al.[15], this improved procedure allows a relatively large-scale preparation of the general ligand for many ATP-dependent enzymes[17-19].

Based on a similar two-step procedure, 8-(6-aminohexyl)-amino-desulfo-CoA-Sepharose (XXIII) was also prepared in our laboratory[20]. This affinity gel has been shown to exhibit good affinity to many CoA-dependent enzymes including citrate synthase and succinate thiokinase from pig heart mitochondria[17] and phosphotransacetylase from Clostritium kluyveri[20]. 8-(6-Aminohexyl)-amino-2', 5'-ADP- and 8-(6-aminohexyl)-amino-3',5'-ADP-Sepharose (XXIV and XXV) were also prepared from 5'-AMP by a four-step procedure starting with 5'-AMP. This involves first the bromination of 5'-AMP, phosphorylation of 8-Br-5'-AMP in the presence of $POCl_3$ and triethylphosphate, subsequent reaction with 1,6-diaminohexane to prepare mixtures of 8-(6-aminohexyl)-amino-2',5'-ADP and -3',5'-ADP and finally separation of these two derivatives by ion exchange resin. These two affinity gels have been shown to exhibit relatively good affinity to TPN^+-dependent dehydrogenases and CoA-dependent enzymes, respectively[17].

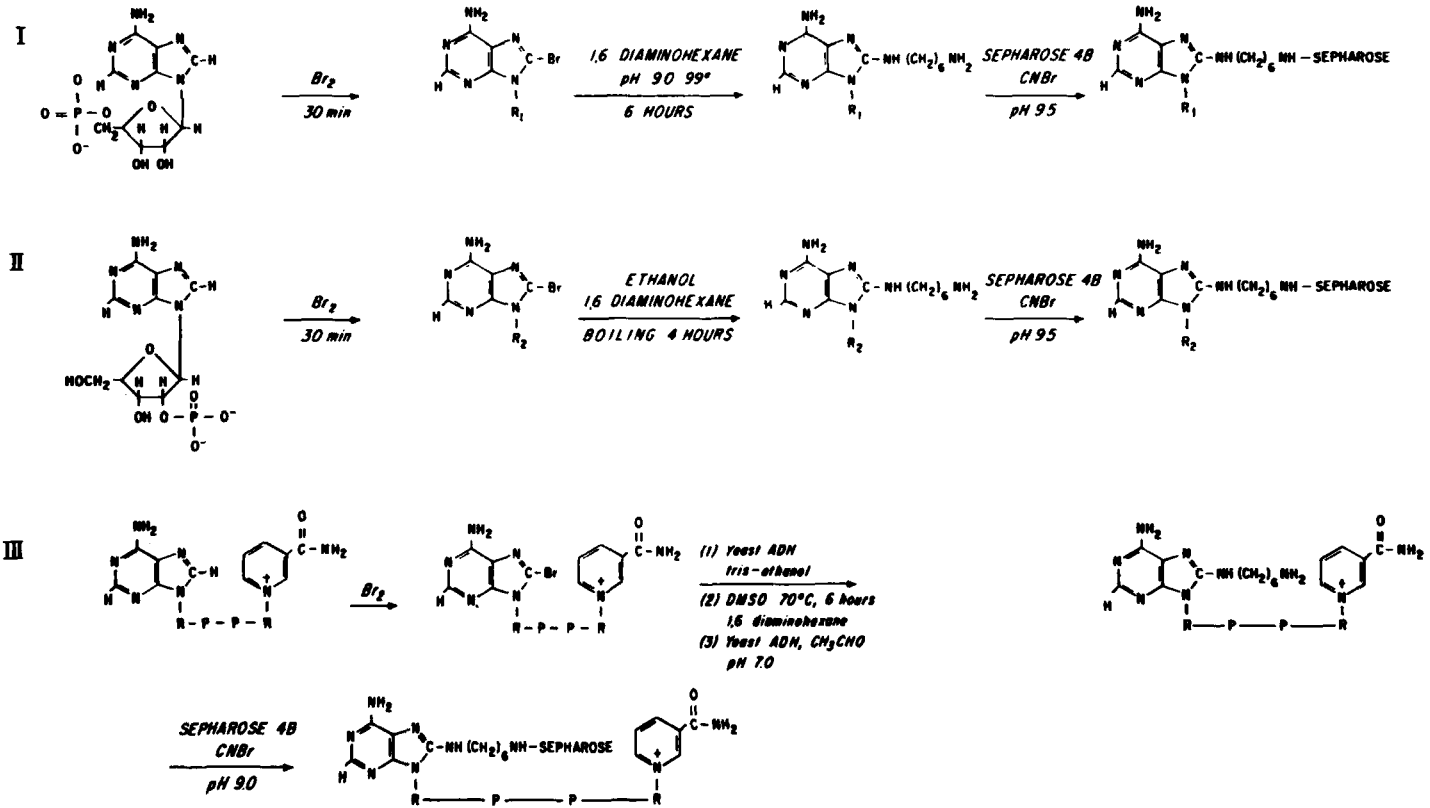

FIG. 1
A general scheme for the synthesis of (I) 8-(6-aminohexyl)-amino-5'-AMP-Sepharose, (II) 8-(6-aminohexyl)-amino-2'-AMP-Sepharose, and (III) 8-(6-aminohexyl)-amino-DPN$^+$-Sepharose[13].

The 8-substituted AMP-Sepharose is relatively stable over a wide pH range. However, DPN^+ and TPN^+ Sepharose are stable only under neutral and acidic conditions. These columns can be regenerated by washing the Sepharose with 8 M urea and high salt solutions. All these biospecific Sepharoses can be used repeatedly without any significant loss of affinity toward enzymes. An 8-substituted DPN^+ (VIA) coupled by means of an azo bond through the spacer to Sepharose was also reported[21, 22]. However, the detailed structure of the bound DPN^+ was not well characterized. This ligand showed good affinity to a number of dehydrogenases, such as lactate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, but not malate dehydrogenase[22]. The limited application of this Sepharose-bound DPN^+ may be due to the fact that the bulky benzoyl substitution of the ligand may allow the binding of only a few enzymes. Preparation of 8-azo-ADP- and 8-azo-ATP-Sepharose columns was also reported by Lee and Kaplan[13] for the purification of kinases (Table 1, VIB and VIC) (Fig. 2).

Attachment Through N^6 Position of Adenine Mononucleotides and Dinucleotides

This type of ligand was first prepared by Guilford et al.[14] and Craven et al.[23] using N^6-(6-aminohexyl) group as a spacer for 5'-AMP (VII) and N^6-[N-(6-aminohexyl)-acetamide] group as a spacer for DPN^+ (VIII)[24]. Other types of nucleotide derivatives using 6-mercapto-purine riboside 5'-phosphate (IX) and 5'-triphosphate (X) and 6-mercapto-purine DPN (XI) were also reported[25]. N^6-(6-aminohexyl)-5'-AMP was prepared either from (1) 6-chloropurine riboside via a two-step synthesis involving phosphorylation and substitution with 1,6-diaminohexane, or (2) direct substitution of 6-mercapto-5'-AMP with 1,6-diaminohexane[23] (Fig. 3). The starting material is usually expensive when obtained from commercial sources. A large-scale preparation of 6-substituted AMP usually starts from adenosine via several steps of synthesis. This usually involves diazotization of adenosine to inosine and protection of the ribose hydroxyl group followed by chlorination with $POCl_3$ to prepare 6-chloropurine riboside.

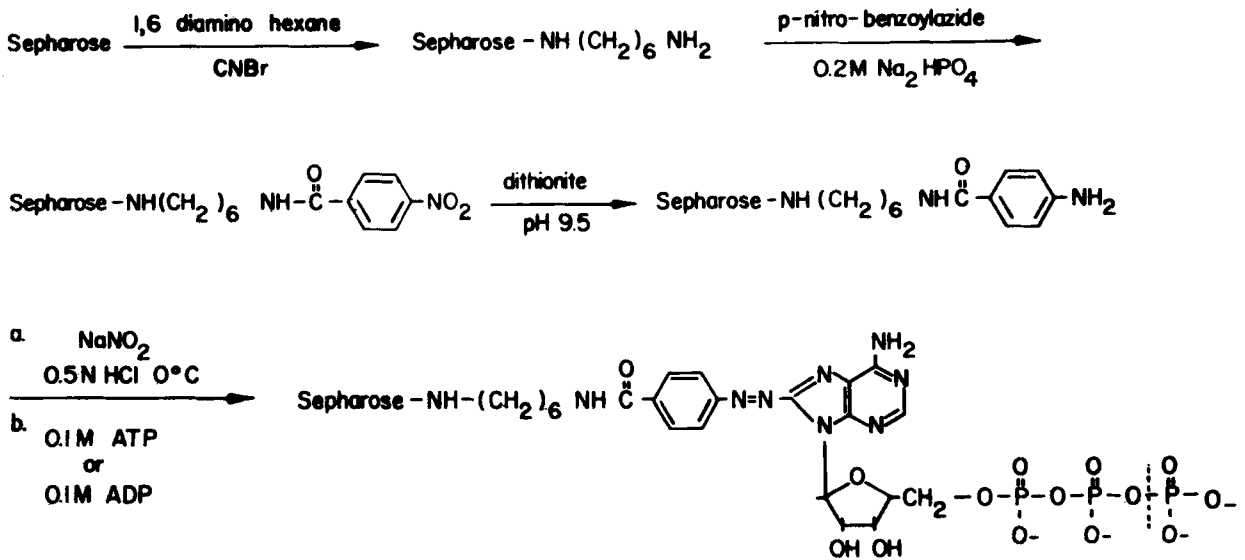

FIG. 2
A general scheme for the synthesis of 8-substituted ADP or 8-substituted ATP-Sepharose[13].

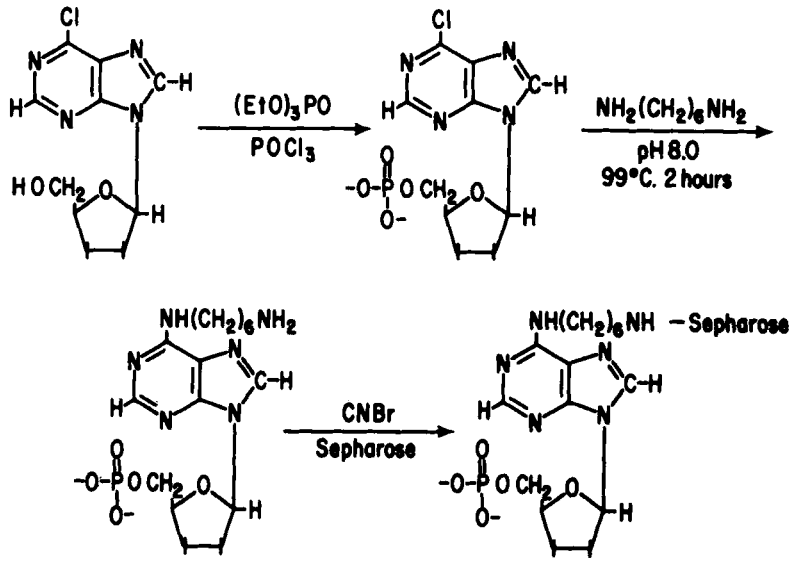

FIG. 3
A general scheme for the synthesis of N^6-(6-aminohexyl)-5'-AMP-Sepharose[14].

DPN^+-N^6-[N-(6-aminohexyl)-acetamide] and TPN^+-N^6[N-(6-aminohexyl)-acetamide] (XXVI) were prepared by a three-step procedure. This involves the coupling and the rearrangement of iodoacetate to the N^6 position of DPN^+ and TPN^+ and then the covalent attachment of 1,6-diaminohexane by DCC coupling. The yield of this compound was reported to be low. These two N^6-substituted ligands exhibited relatively good affinity for various DPN^+-dependent dehydrogenases. The stability of these biospecific gels is similar to that reported for the 8-substituted nucleotide Sepharose[12, 13]. Attachment of 6-mercapto-purine nomonucleotide or dinucleotide derivatives was achieved by the condensation reaction between the ligand and Sepharose bound bromoacetyl group at alkaline pH[25]. However, no detailed studies regarding the nature of these ligands were reported. Preparation of N^6-(6-amionhexyl)-2',5'-ADP- and N^6-(6-aminohexyl)-3',5'-ADP-Sepharose (XXVII and XXVIII) were independently reported

by Brodelius et al.[26] and Morelli and Benatti[27]. This involves a three-step procedure starting with 6-chloropurine riboside. N^6-(6-aminohexyl)-2',5'-ADP-Sepharose was shown to exhibit relatively good affinity to many TPN^+-dependent enzymes from yeast extracts. ATP-N^6-[N-(6-aminohexyl)-acetamide]-Sepharose (XXIX) was prepared by Lindberg and Mosbach[28]. This 6-substituted ATP-Sepharose has been shown to exhibit relatively good affinity to citrate synthase and could be used for a single step purification of this enzyme[28].

Attachment Through Ribosyl Position of Nucleotides

Ligands bound through the ribosyl moiety were first prepared by Larsson and Mosbach[29]. They are prepared by coupling the appropriate nucleotides such as 5'-AMP (XII), DPN^+ (XIII), or TPN^+ (XIV) to ε-aminocaproyl-Sepharose by carbodiimide reaction in 80% pyridine. The formation of the ester linkage between the ribose hydroxyl group and Sepharose-bound spacers was suggested[29]. Since the attachment was relatively simple, the method has been widely adopted for enzyme purification. However, the ester linkage between the cofactor and the spacer was reported to be unstable[27].

Another type of ligand involves the periodate oxidation of ribose hydroxyl group by periodate oxidation followed by the covalent attachment with Sepharose-bound adipic hydrazide derivatives of AMP, ADP, ATP, DPN^+, and TPN^+ as well as their derivatives (XV, XVI, XVII). These were first prepared by Lamed et al.[30, 31]. Since the ribosyl groups of the ligands are modified by oxidation, only a limited number of dehydrogenases or kinases show good affinity to these Sepharose-bound liganda.

Attachment Through Pyridine Moiety of Coenzymes

Because the adenine moiety of pyridine coenzymes is normally essential for the binding of coenzymes to dehydrogenases, modification by various substitutions on the adenine ring may effect the binding of some dehydrogenases to the Sepharose-bound liganda[32]. Therefore the attachment of coenzyme derivatives to Sepharose through pyridine moiety showed good affinity to the binding of some dehydrogenases such as transhydrogenase from chicken heart mitochondria and aldehyde dehydrogenases from Clostritium kluyveri. The preparation of this type of ligand is relatively simple (Fig.

4). Normally 3-substituted pyridine analogs of DPN^+ or deamino-DPN^+ are used for coupling to Sepharose. Examples of these ligands are 3-aminopyridine-DPN^+ (XVIII) (Fig. 4), 3-nicotinic acid-DPN^+ (XIX), 3-nicotinic acid-deamino-DPN^+ (XX), and 3-azopyridine deamino-DPN^+ (XXI).

These ligands not only exhibit good affinity for some dehydrogenases but also for some kinases such as adenylate kinase as well as creatine kinase, since the free ADP moiety may be essential for the binding of kinases.

Attachment Through the Phosphate Group of the Adenine Nucleotides

Harvey et al.[33] and Trayer et al.[15] reported the preparation

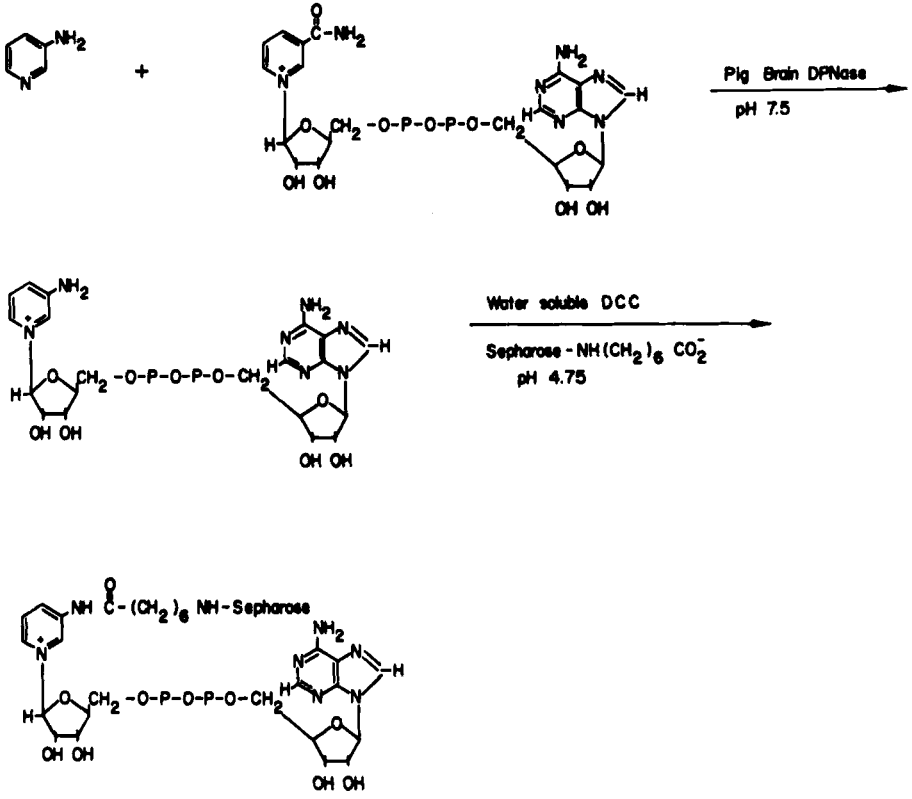

FIG. 4
Preparation of 3-amino pyridine-DPN^+-N^3-[N-(6-amino-caproamide)-Sepharose (see Table 1).

of P^1-(6-aminohexyl)-P^2-(5'-adenosine)-pyrophosphate Sepharose (XXII). The Sepharose-bound ligand was made through the covalent attachment to the pyrophosphate groups, and showed good affinity to both dehydrogenases and a few kinases[15, 33]. The preparation of this ligand is similar to that of UDP derivatives reported earlier by Barker et al.[34].

PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES OF FREE AND SEPHAROSE-BOUND LIGANDS

Any Sepharose-bound ligand which exhibits high affinity for enzymes must be either an inhibitor, a substrate, or a coenzyme of the respective enzyme. This can be shown by steady-state kinetic studies using these modified ligands in various enzymatic systems[13]. In the case of 8-(6-aminohexyl)-amino-DPN^+ and $-TPN^+$, these derivatives usually exhibited a higher apparent K_m than the natural coenzymes with most of the enzymes (Table 2). However, the V_{max}'s are less than those using DPN^+ or TPN^+. This indicates that the modified coenzymes may be bound to the enzymes similar to the natural coenzymes. However, in contrast there is one exceptional case: N^6-(6-aminohexyl)-5'-AMP exhibits a K_i (1×10^{-4} M) for lactate dehydrogenase which can be one order of magnitude lower than 5'-AMP itself. The activity of Sepharose-bound DPN^+-N^6-[N-(6-aminohexyl)-acetamide] in an enzymatic process has been demonstrated by Lindberg et al.[24] using a recycling multiple enzyme complex (malate dehydrogenase-citrate synthase-lactate dehydrogenase).

Effect of Chemical Modifications on the Structure of Adenine Nucleotide Derivatives

Significant changes in the interproton coupling constants of ribosyl protons were observed in the C^8-alkyl substitutions of adenine nucleotides[13]. In the case of 8-(6-aminohexyl)-amino-5'-AMP, the coupling constant between the 1'- and 2'-ribosyl protons, $J_{1'-2'}$, was observed to be 7.9 Hz in contrast to 5.5 Hz observed for 5'-AMP. This indicates that a conformational change in the ribose and/or in the glycosydic bond between the base and sugar ring occurs in the modified nucleotide derivatives. In addition to the steric hindrance caused by alkyl substitution, this may be the

reason that some enzymes exhibit relatively low affinity to these modified ligands.

Physicochemical Studies of General Ligands

Physicochemical characteristics of the Sepharose-bound ligand, especially N^6-(6-aminohexyl)-5'-AMP, were studied in detail by Harvey et al.[33, 35-37]. The concentration of the Sepharose-bound ligand has been shown to be a critical factor in determining the nature and strength of the enzyme-ligand interaction, and the capacity of the adsorbants was related to column geometry, total amount of ligand, time required for the equilibration, as well as pH of the loading buffer[35, 36]. Biospecific absorption of enzymes on Sepharose-bound ligands is also strongly temperature dependent[37]; i.e., the binding decreases drastically with increasing temperatures. The dissociation constants of the complex between Sepharose-bound ligand and several enzymes were also determined by Lowe et al.[38]. In the case of the binding of lactate dehydrogenase and glycerokinase to Sepharose-bound N^6-(6-aminohexyl)-5'-AMP, the dissociation constants were measured to be as low as 1.1 μM under batchwise conditions. This is significantly lower than that reported for the complex between N^6-(6-aminohexyl)-5'-AMP and lactate dehydrogenase (K_d = 100 μM)[14]. The apparent high affinity of the enzymes to Sepharose-bound ligands may reflect a hydrophobic effect superimposed on the intrinsic affinity of the enzyme for this ligand[38].

In short, these physicochemical studies demonstrate the fact that Sepharose-bound ligands could exhibit very different properties from those of the free ligand in terms of binding to enzymes. The structural characteristics of Sepharose could also allow the immobilized ligands to bind enzymes more effectively during its application for affinity chromatography.

Since the Sepharose-bound adenine nucleotide derivatives usually carry a negatively charged phosphate or pyrophosphate group in addition to the long hydrocarbon spacer, they could also nonspecifically adsorb a number of proteins due to electrostatic

TABLE 2. Biochemical Studies of Various Substituted Adenine Nucleotide Derivatives

Enzyme	Natural or modified ligand	Rate relative to the natural compound	K_m or K_i (M)	Relative V_{max}
Adenylate kinase (rabbit muscle)	5'-AMP[13]	1.00		
	8-(6-Aminohexyl)-amino-5'-AMP	$\leq 1 \times 10^{-3}$		
Transhydrogenase (Pseudomonas aeruginosa)	2'-AMP[13]	1.00		
	8-(6-Aminohexyl)-amino-2'-AMP[13]	$\sim$1.00		
Lactate dehydrogenase (chicken muscle)	DPNH[13]		1×10^{-5}	1.00
	8-(6-Aminohexyl)-amino-DPNH[13]		8×10^{-6}	0.60
Glucose-6-phosphate dehydrogenase yeast)	TPN^+[13]		3.6×10^{-5}	1.00
	8-(6-Aminohexyl)-amino-TPN^+		4.4×10^{-5}	0.42
Transhydrogenase (Pseudomonas aeruginosa)	TPNH[13]	1.00		
	8-(6-Aminohexyl)-amino-TPNH[13]	$\sim$1.00		

Alcohol dehydrogenase (liver)	DPN^+ [24]	1.00	
Lactate dehydrogenase (pig heart)	DPN^+-N^6-[N-(6-aminohexyl)-acetamide]	~1.00 (ADH)	
Malate dehydrogenase (pig heart)		0.50 (LDH)	
		0.75 (MDH)	
Lactate dehydrogenase	5'-AMP		1×10^{-3}
	N^6-(6-Aminohexyl)-5'-AMP [a]		1×10^{-4}

[a] K. Mosbach, personal communication.

and/or hydrophobic interactions. The elimination of these nonspecific proteins from an affinity column is an important factor in selective protein purification. Usually the nonspecific charge interaction between proteins and immobilized ligands are related to the surface charge distributions on the protein which are strongly pH and ionic strength dependent. However, these nonspecific charge interactions can be minimized by the proper adjustments of the pH as well as the ionic strength of the solution during the specific elution of proteins from the affinity columns. The length of spacer arms between Sepharose and the ligand was also found to be an important factor in determining the relative affinity of the Sepharose-bound ligand to enzymes[39]. A comparative study was performed with the binding of chicken H_4LDH to Sepharose-bound 8-substituted 5'-AMP using various lengths of carbon chain spacers[40]. It was found that the affinity of the Sepharose-bound ligand toward the enzyme increases with increasing spacer length over the range from a two- to a five-carbon chain. However, high affinity was first obtained when the spacer was longer than five carbons. Although 1,6-diaminohexane was usually employed as the optimum spacer arm for the affinity ligands in most of the binding studies carried out, the proper length of spacer arm between the Sepharose and the ligand could vary with the enzymes studied. This result indicates that Sepharose and the surface of the active site of the enzyme should be at least 10 to 12 Å away from each other in order to achieve maximum affinity. Spacer arms longer than a six-carbon chain usually do not increase the affinity between the enzyme and the Sepharose-bound ligand and, in some cases, the affinity is decreased.

APPLICATIONS OF GENERAL LIGAND AFFINITY CHROMATOGRAPHY FOR SELECTIVE PROTEIN PURIFICATION

The essential approach to the development of general ligand affinity chromatography was based upon the premise that a single, immobilized derivative might be useful in the selective purification of a number of enzymes only if the proper conditions for

specific elution could be achieved[14, 24]. Based upon such principles and criteria, about 60 enzymes have indeed been highly purified from crude extracts of various sources. The results of this purification are presented in Table 3. Brief descriptions of the purification of each enzyme are given in this section.

Since the Sepharose-bound general ligand is able to retain a family of enzymes based on their general binding characteristics, selective purifications of enzymes can be achieved only when the elution utilizes an inhibitor, substrate or dead-end ternary complex which is specific for only one of the enzymes. This possibility has been demonstrated by a model study by Kaplan and his coworkers[11]. Three different pure dehydrogenases, lactate dehydrogenase (LDH), malate dehydrogenase (MDH), and alcohol dehydrogenase (ADH), were mixed and diluted in 0.1 M phosphate buffer at pH 7.5. The mixture was then passed through an N^6-(6-aminohexyl)-5'-AMP column. After all three enzymes were adsorbed on the column, MDH, LDH, and ADH were specifically eluted from the column as separate enzymes with reduced oxalacetate-DPN adduct, pyruvate-DPN adduct, and a mixture of DPN^+ and hydroxylamine, respectively. Excellent separations of these three dehydrogenases were obtained since each element is known to be a specific inhibitor of the respective enzyme. The recovery of all three enzymes, after the appropriate dialysis to remove inhibitors, was almost quantitative (90%). Hence a mixture of at least three purified enzymes could be effectively resolved using the techniques and principles of specific elution from an AMP-Sepharose column (Fig. 5).

Selective purification of two enzymes from one crude extract was demonstrated in our laboratory[41]. An extract of chicken heart was passed through an 8-(6-aminohexyl)-amino-DPN^+-Sepharose column in 0.01 M maleate buffer, pH 6.0. After the enzymes were adsorbed on the column, it was extensively washed with buffer to eliminate all nonspecifically bound proteins. Specific elution of MDH was carried out with 1.2×10^{-4} M reduced oxalacetate-DPN adduct and LDH was then eluted with 1×10^{-4} M reduced pyruvate-DPN adduct.

TABLE 3. Enzyme Purifications Using Derivatives of Adenine Nucleotides as General Ligands in Affinity Chromatography

Enzyme	Source of crude extracts	Ligand in affinity chromatography	Fold of purification	Purity
Lactate dehydrogenase[12]	Dogfish muscle	8-(6-Aminohexyl)-amino-5'-AMP	37	~90%
Lactate dehydrogenase[12]	Dogfish muscle	8-(6-Aminohexyl)-amino-DPN^+	44	~95%
Lactate dehydrogenase[11]	Dogfish muscle	N^6-(6-Aminohexyl)-5'-AMP	34	~90%
Lactate dehydrogenase[a]	Rabbit muscle	8-(6-Aminohexyl)-amino-5'-AMP	271	$\geq$90%
Lactate dehydrogenase[a]	Beef heart	8-(6-Aminohexyl)-amino-5'-AMP	147	$\gtrsim$80%
Lactate dehydrogenase[a]	Beef liver	8-(6-Aminohexyl)-amino-5'-AMP	167	$\gtrsim$90%
Lactate dehydrogenase[a]	Lobster tail	8-(6-Aminohexyl)-amino-5'-AMP	370	$\gtrsim$90%
Lactate dehydrogenase[12]	Losbter tail	8-(6-Aminohexyl)-amino-DPN^+	86[i]	$\gtrsim$80%
D-Lactate dehydrogenase[b]	Abalone	8-(6-Aminohexyl)-amino-5'-AMP	17[i]	~90%
D-Lactate dehydrogenase	Limulus polyphemus	8-(6-Aminohexyl)-amino-DPN^+	10[i]	~15%
Lactate dehydrogenase[12]	Chicken heart	8-(6-Aminohexyl)-amino-DPN^+	33	~80%
Lactate dehydrogenase[41]	Human heart	8-(6-Aminohexyl)-amino-5'-AMP	90	~80%
Lactate dehydrogenase[41]	Human liver	8-(6-Aminohexyl)-amino-5'-AMP	145	~90%

Lactate dehydrogenase[41]	Human erythrocytes	8-(6-Aminohexyl)-amino-5'-AMP	19,000	~99%
Lactate dehydrogenase[41]	Human heart	8-(6-Aminohexyl)-amino-DPN^+	11	~10%
Lactate dehydrogenase[41]	Human liver	8-(6-Aminohexyl)-amino-DPN^+	65	~50%
Lactate dehydrogenase[41]	Human erythrocytes	8-(6-Aminohexyl)-amino-DPN^+	2,430	~15%
Lactate dehydrogenase[11]	Human serum	8-(6-Aminohexyl)-amino-DPN^+	17,000	5%
Transhydrogenase[12]	Pseudomonas aeruginosa	8-(6-Aminohexyl)-amino-2-AMP	110[j]	25%
Transhydrogenase[11]	Pseudomonas aeruginosa	N^6-(6-Aminohexyl)-amino-5'-AMP	314	25%
Transhydrogenase[c]	Chicken heart mitochondria	8-(6-Aminohexyl)-amino-5'-AMP	15	
Transhydrogenase[c]	Chicken heart mitochondria	3-Azo-pyridine-deamino-DPN^+	20	
Glutamate dehydrogenase[d]	Chicken liver	8-(6-Aminohexyl)-amino-5'-AMP	700	90%
Glucose-6-phosphate dehydrogenase[13]	Yeast	8-(6-Aminohexyl)-amino-TPN^+	4,000	98%
Glucose-6-phosphate dehydrogenase[13]	Human erythrocytes	8-(6-Aminohexyl)-amino-TPN^+	3,900	10%

TABLE 3 (Continued)

Glucose-6-phosphate dehydrogenase[11]	Human erythrocytes	TPN^+-(6-amino-caproiate)	4,600	10%
Glucose-6-phosphate dehydrogenase	Rat liver	8-(6-Aminohexyl)-amino-TPN^+	200	5%
Malate dehydrogenase[e]	Chicken heart	8-(6-Aminohexyl)-amino-5'-AMP	110[i]	99%
Alcohol dehydrogenase[f]	Yeast	8-(6-Aminohexyl)-amino-DPN^+	21	95%
Creatine kinase[18]	Chicken heart	3-Amino pyridine DPN^+ (compound XVIII)	80	80%
D-Mannitol-1-phosphate dehydrogenase[g]	Escherichia coli	8-(6-Aminohexyl)-amino-fructose-6-phosphate-DPN^+ adduct	100	50%
Aldehyde dehydrogenase[h]	Clostridium kluyveri	8-(6-Aminohexyl)-amino-DPN^+	40	30%
Adenylate kinase[13, 18]	Rabbit muscle	8-Azo-ATP (compound VIC)	100	~40%
Adenylate kinase[13, 18]	Beef liver	8-Azo-ATP (compound VIC)	20	~10%
Pyruvate kinase[18]	Rabbit muscle	8-Azo-ADP (compound VIB)	10	~30%
Hexokinase[18]	Yeast	3-Amino pyridine-DPN^+ (compound XVIII)	30	~20%
Creatine kinase[18]	Human brain	8-Hexyl-ATP (compound VI)	200	~60%

Creatine kinase[18]	Chicken muscle	8-Azo-ADP (compound VIB)	20	30%
Creatine kinase[18]	Beef heart mitochondria	8-Azo-ATP (compound VIC)	80	90%

[a]This work was carried out in collaboration with Dr. S. S. Taylor.

[b]This work was carried out in collaboration with Dr. G. Long.

[c]This work was carried out in collaboration with Drs. L. Lazarus and Y. Levin.

[d]This work was carried out in collaboration with Mr. F. Esch.

[e]This work was carried out in collaboration with Mr. B. Bachman and Mr. F. E. Stolzenbach.

[f]This work was carried out in collaboration with Dr. C. Wills.

[g]C.-L. T. Lee and N. O. Kaplan, manuscript in preparation.

[h]This work was carried out in collaboration with Mrs. L. T. Smith.

[i]After ammonium sulfate fractionation.

[j]After DEAE cellulose column.

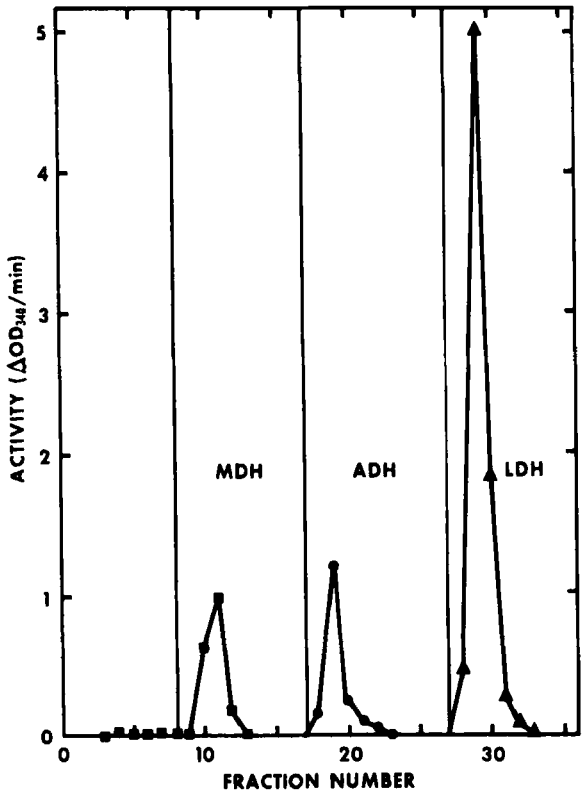

FIG. 5.
Elution pattern of MDH, ADH, and LDH from the N^6-(6-aminohexyl)-5'-AMP-Sepharose column. One-half a milliliter of a solution containing the three enzymes in 0.1 M phosphate buffer, pH 7.5, was applied to the column and the column was subsequently washed with buffer. The MDH was eluted using 0.12 mM reduced DPN-oxaloacetate adduct in 0.1 M phosphate buffer, pH 7.5; the ADH was eluted with a mixture of 0.5 mM DPN^+ and 3 mM hydroxylamine in buffer. The fractions at which the elution buffers were changed are indicated by vertical lines. Recoveries of each enzyme were greater than 90%. The activities obtained were after removal of the adduct of hydroxylamine by dialysis. Column size: 6.0 x 2.5 cm^{11}.

Little overlap of these two enzyme fractions was observed in this preparation. The purity of LDH and MDH obtained from this affinity column was greater than 50%. However, similar experiments using 6-substituted of 8-substituted AMP-Sepharose columns did not yield

satisfactory results for the crude extracts from chicken heart. Both MDH and LDH fractions overlap even in the presence of eluents specific for one of the enzymes[42].

Purification of Dehydrogenases from Crude Extracts

Using either an N^6-(6-aminohexyl)-5'-AMP or 8-(6-aminohexyl)-amino-5'-AMP-Sepharose column, M_4 lactate dehydrogenase was first purified from the muscle extract of dogfish (Fig. 6). The enzyme was readily eluted from the column with 1 x 10^{-4} M reduced pyruvate DPN^+ adduct[11, 12]. The specific activity of the purified enzyme appears to be equivalent to that obtained from the lengthy multi-step procedures. Subsequently, LDH's from other sources, such as chicken heart, beef liver, rabbit muscle, and lobster tail, were also purified using either 8-(6-aminohexyl)-amino-5'-AMP or -DPN^+-Sepharose columns. The purity of the enzymes eluted from these

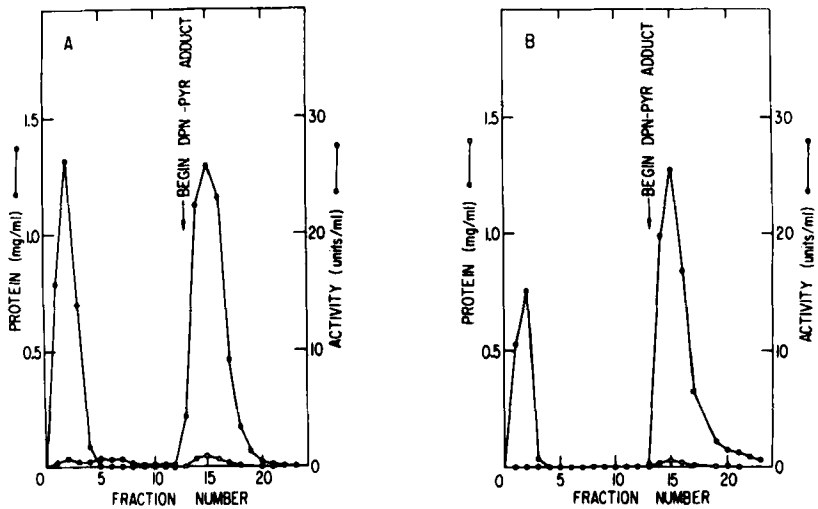

FIG. 6

Illustration of chromatographic separation of lactate dehydrogenase from an extract of dogfish muscle. Specific activities and gel electrophoresis of peak tubes revealed the enzymes to greater than 90% pure. (A) 8-(6-Aminohexyl)-amino-DPN^+-Sepharose column. (B) 8-(6-Aminohexyl)-amino-5'-AMP-Sepharose column. Elution of enzyme was made with DPN pyruvate adduct at a concentration of 2 x 10^{-4} M[13].

columns was greater than 90%. It is worthwhile to mention that 8-(6-aminohexyl)-amino-5'-AMP-Sepharose exhibits much better affinity for LDH from lobster tail or D-LDH from abalone than N^6-(6-aminohexyl)-5'-AMP-Sepharose. Selective purification of these two enzymes from crude extract could be demonstrated by use of the 8-substituted 5'-AMP or -DPN^+-Sepharose columns[12]. Purification of LDH from various human tissues was first carried out by Bachman and Lee in our laboratory (Table 3)[41]. Both 8-(6-aminohexyl)-amino-5'-AMP and -DPN^+-Sepharose columns were used for comparison. Under the identical conditions for elution, the 8-(6-aminohexyl)-amino-5'-AMP-Sepharose column yielded a much higher fold of purification for LDH from heart tissue and erythrocytes than 8-DPN^+-Sepharose column. However, similar purification of human liver LDH was obtained for both affinity columns. In the case of LDH from human heart and erythrocytes, 8-(6-aminohexyl)-amino-DPN^+-Sepharose exhibited a greater affinity for the enzyme than 8-AMP-Sepharose. Therefore the enzyme could not be specifically eluted from the 8-DPN^+-Sepharose column using relatively low concentrations of reduced pyruvate-DPN^+ adduct. As a result, the enzyme was eluted from the 8-AMP column in a relatively sharp peak, whereas a broad peak was obtained from the elution of the 8-DPN^+ column. This observation indicates that LDH isoenzymes from human heart (mostly H-type isozyme) and liver (mostly M-type isozymes) show different affinities for 8-DPN^+-Sepharose. Thus, using 8-(6-aminohexyl)-amino-5'-AMP-Sepharose, LDH can be purified in one step from three different human tissues. This study demonstrated that affinity chromatography techniques can be applied to clinical and medical research in the near future. Subsequently, lactate dehydrogenases from at least ten other different sources were also purified by this one-step affinity column procedure (Table 3).

Malate dehydrogenase from chicken heart was also successfully purified with an AMP-Sepharose column (Table 3). Because of the difficulties in the specific elution of MDH and LDH from this general ligand column, these two enzymes were first separated by

ammonium sulfate precipitation before being applied to the column. Malate dehydrogenase was eluted readily from the affinity column with 0.2 mM of reduced oxalacetate-DPN^+ adduct at pH 6.0, 1 mM phosphate buffer. The specific activity of the eluted enzyme appeared to be equivalent to that obtained from the conventional ten-step procedures.

The purification of glutamate dehydrogenase from chicken liver using an 8-(6-aminohexyl)-amino-5'-AMP-Sepharose column has been studied in our laboratory (Table 3). It was found that the purity of the eluted enzyme strongly depended upon the conditions of elution. This requires the proper choice of pH and ionic strength. The use of reduced-α-ketoglutarate-DPN adduct alone could not elute the enzyme even at high pH (∿7.5). The column was first washed with 0.06 M phosphate at pH ∿6.0 to remove contaminating proteins, and the enzyme was then eluted with 1 mM adduct in 0.03 M potassium phosphate buffer, pH 7.5. The specific activity of the purified enzyme was as high as 42 units/mg (∿90% purity) and the overall recovery was greater than 90%.

Glucose-6-phosphate dehydrogenase (G-6-PDH) from crude extracts of various sources was also purified using the approach of general ligand affinity chromatography. This enzyme was purified from human erythrocytes using either 8-(6-aminohexyl)-amino-TPN^+-Sepharose[13] or TPN^+-[5-aminocaproiate]-Sepharose[11]. Approximately 4000-fold purification was achieved via one-step elution by a 0 to 1 mM TPN^+ gradient. However, since this elution scheme is rather non-specific, the purity of the enzyme was only 10%. Subsequently, the enzyme was further purified over a DEAE-Sephadex column and, as a result, a homogeneous enzyme was obtained in the second step[11]. Glucose-6-phosphate dehydrogenase from Neurospora crassa was also purified using TPN^+-(6-aminocaproiate)-Sepharose (Table 3). After removing the nonspecifically bound protein with high salt (0.1 M NaCl) at high pH (7.5), the enzyme was readily eluted with 0.1 M potassium phosphate at pH 7.5. The purity of the eluted enzyme ranged from 50 to 100%. Elution with phosphate buffer seems to be

rather specific since the phosphate ion was shown to be an inhibitor of this enzyme[43].

Glucose-6-phosphate dehydrogenase from yeast extract was purified by using an 8-(6-aminohexyl)-amino-TPN^+-Sepharose column[13] (Table 3). Purity of the eluted enzyme was very sensitive to the conditions during the washing of the column. If the crude extract was loaded on the column and washed with 0.1 M phosphate buffer at pH 6.0 in the presence of 10% glycerol, the purity of the enzyme obtained by elution with a 0 to 1 mM TPN^+ gradient was only approximately 10%. However, if the column was thoroughly washed with 0.1 M phosphate buffer at pH 6.0 in the presence of 20% glycerol, most of the weakly bound TPN^+-dependent enzymes were eliminated with about 70% retention of glucose-6-phosphate dehydrogenase. It could then be readily eluted with a 0 to 1 mM TPN^+ gradient from the column with a purity of approximately 95%. Multi-enzyme purification of TPN^+-dependent enzymes was demonstrated by Brodelius et al.[26] and by Lee and Kaplan[13]. Upon the adsorption of yeast extract onto either an N^6-(6-aminohexyl)-amino-2',5'-ADP-[26] or an 8-(6-aminohexyl)-amino-TPN^+-Sepharose column[13], four to five different TPN^+-dependent enzymes were subsequently eluted from the affinity column by a proper TPN^+ gradient[13, 26].

An attempt was made to purify glucose-6-phosphate dehydrogenase and 6-phospho-gluconic acid dehydrogenase from rat liver extracts. A 200-fold purification was obtained when the enzymes were eluted with 1 mM TPN^+ from the column[13], although the enzymes were not homogeneous after this procedure.

The pyridine nucleotide transhydrogenase from Pseudomonas aeruginosa was purified by either 8-(6-aminohexyl)-amino-2'-AMP-Sepharose[12] or N^6-(aminohexyl)-5'-AMP-Sepharose columns[11]. In the case of the 6-substituted-5'-AMP-Sepharose column, the enzyme was readily eluted with 1 mM DPNH at neutral pH with an observed purity of only 10% due to the nonspecific elution of this enzyme. However, a homogeneous enzyme could be obtained by passing this preparation through a G-200 Sephadex column[11]. This enzyme is

known to form an aggregated species and can be eluted from the column in the void volume, while the contaminating proteins were excluded by the Sephadex.

Transhydrogenase from chicken heart mitochondria was also purified by about 20-fold using affinity chromatographic techniques. This enzyme can only be properly purified by using various 3-substituted pyridine-DPN^+-Sepharose. The extreme lability of this enzyme makes affinity chromatographic techniques useful methods for which this enzyme can be purified.

Yeast alcohol dehydrogenase was purified in cooperation with Dr. C. Wills at UCSD, using 8-(6-aminohexyl)-amino-DPN^+-Sepharose. Neither an 8-substituted nor 6-substituted 5'-AMP-Sepharose column exhibits good binding for this enzyme from crude extracts and therefore does not yield satisfactory results in terms of purifications. After the crude extract was loaded on an 8-substituted-DPN^+-Sepharose column, extensive washing with salt at low pH (6.0) eliminated the nonspecifically bound protein. The enzyme was readily eluted with 0.1 mM reduced acetaldehyde-DPN adduct. Purity was greater than 90% with a recovery of almost 100%. The application of affinity chromatography techniques for purification is likely to make possible the rapid isolation of isoenzymes of alcohol dehydrogenase from various mutants of yeast. Multi-enzyme purification from a single crude extract was first demonstrated by using an 8-(6-aminohexyl)-amino-5'-AMP-Sepharose column[19]. After passing the rabbit muscle extracts through the affinity column, at least four different dehydrogenases (MDH, α-GPD, LDH and TPD) were biospecifically eluted from the column by a 0 to 1 mM DPNH gradient. The purity of the isolated enzymes ranges from 50 to 90%. It is worthwhile mentioning that only dehydrogenases were adsorbed on the 8-substituted-AMP-Sepharose column whereas most kinases were recovered in the eluent during the loading of extracts. They could be purified subsequently by an ATP-Sepharose column[19].

There is one interesting observation which deserves further enumeration here. Saturation of the affinity column with respect

to a given enzyme often results in the exclusion of other weakly bound enzymes. Readsorption of the unbound enzymes on a second affinity column usually gives a higher degree of purification. An example of this procedure is the purification of three different dehydrogenases from chicken muscle extract. LDH, α-GPD and MDH were purified separately by two 8-substituted-AMP and one NAD-Sepharose columns, respectively, based on this saturation and readsorption technique. This concept was also successfully applied to the purification of several kinases[18, 19].

Isoenzyme purification of horse liver alcohol dehydrogenase was first reported by Andersson et al.[44]. EE, SS and ES isoenzymes were eluted specifically and separately from an N^6-(6-aminohexyl)-5'-AMP-Sepharose column. The homogeneous isoenzymes could be obtained by this affinity column procedure.

Purification of Kinases from Crude Extracts

Recently it was found in our laboratory that the Sepharose-bound ligands which possess a free 5'-ADP moiety exhibit relatively high affinity for various kinases. In the case of a 3-amino-pyridine-DPN^+-N^3-[N-(6-amino-caproamide)]-Sepharose column, it exhibits low affinity for most dehydrogenases, but good affinity for several kinases, such as hexokinase from yeast, creatine kinase from chicken heart[18], and protein kinase from pig muscle[40]. The adsorbed enzymes can be readily eluted from the column with proper concentrations of ATP. For example, using a linear ATP gradient for elution, creatine kinase from chicken heart extracts can be purified from this column with a purity greater than 80% (Table 3).

Hexokinase was also purified by using a 3-amino-pyridine-DPN^+-Sepharose column (Table 1, Compounds X and VIII). The adsorbed enzyme was readily eluted either with 0.10 M phosphate buffer at pH 7.0 or with a 0 to 10 mM ATP gradient at pH 6.0. The purity of the enzyme obtained in this preparation is about 20%, which corresponds to a 30-fold purification of the protein (Table 3).

3-Amino-pyridine-DPN^+-Sepharose column does not exhibit good affinity to adenylate kinase, pyruvate kinase from rabbit muscle, and creatine kinase from chicken muscle. Purification of these enzymes can be achieved only with other types of general ligand affinity columns.

With the use of 8-azo-ATP-Sepharose column (Table 1, Compounds VIB and VIC), adenylate kinase from rabbit muscle can be purified 100-fold by an elution of 0 to 10 mM ATP linear gradient. The specific activity of the enzyme obtained from this affinity column can be as high as 700 units/mg which corresponds to 40% in purity. With the use of an 8-azo-ADP-Sepharose column, pyruvate kinase from rabbit muscle can be purified by using an elution with an ATP gradient. As a result, the purity of the enzyme can be as high as 30% after this affinity column. The summary of purification of several kinases is presented in Table 3.

Multi-enzyme purification of kinases from a single extract has been demonstrated by using either an 8-azo-ATP- or 8-(6-aminohexyl)-amino-ATP-Sepharose column[18, 19]. At least three different kinases plus aldolase were eluted biospecifically by 0 to 10 mM ATP gradient. Adenylate kinase and creatine kinase were coeluted at an ATP concentration of 0.5 mM, whereas pyruvate kinase and aldolase were eluted together at 2 to 3 mM ATP. After the single-step elution, a sole mixture of two enzymes was obtained in the peak fraction of each enzyme[18]. These four enzymes can be further purified to homogeneity by one more step of protein purification with conventional procedures[18].

A similar approach has been successfully applied for the purification of creatine kinase and adenylate kinase from many different sources. These include human heart, brain, liver, beef heart mitochondria and fish muscle (Table 3). Generally speaking, a unified two-step procedure, affinity chromatography and isoelectric focusing were required to obtain homogeneous enzymes from all these tissue extracts.

Recently Dr. S.Taylor has purified the holoenzyme from of 3', 5'-cyclic AMP-dependent protein kinase by employing 8-(6-amino-hexyl)-amino-ATP-Sepharose[40]. The enzyme activity can be eluted either with a salt gradient or with ATP and recoveries range from 60 to 90%. The eluted enzyme is fully dependent on cyclic AMP for enzymatic activity and has a molecular weight indistinguishable from that of the holoenzyme[40].

Special Applications of General Ligand Affinity Chromatography

It is known that most dehydrogenases are strongly and specifically inhibited by the adduct prepared from the alkaline condensations of an oxidized coenzyme (DPN^+) and their respective substrates[45]. A Sepharose column prepared from the adduct of 8-(6-aminohexyl)-amino-DPN^+ and the oxidized substrate of a given dehydrogenase becomes a "specific ligand" affinity column for this particular enzyme. This method is extremely useful when the enzyme does not bind well to the "general ligand" column. This is demonstrated by the purification of D-mannitol-1-phosphate dehydrogenase using a DPN^+-fructose-6-phosphate adduct Sepharose column. This column exhibits an extremely high capacity with respect to the binding of this enzyme from crude extracts of E. coli (Table 3). The enzyme, which was purified 100-fold after this affinity column, could be further purified to a homogeneous state after passage through Sephadex G-100. This work was carried out in our laboratory by Mrs. C.-L. Lee.

Purification of Other Classes of Enzymes by General Ligand Affinity Chromatography

Recently attempts have been made to purify CoA-dependent enzymes and amino acyl-t-RNA synthetases by the approach of general ligand affinity chromatograhy. With the use of either N^6-substituted ATP-Sepharose[28] or 8-(6-aminohexyl)-amino-ATP-Sepharose column[17], citrate synthase was purified by a single-step procedure. In the case of 8-substituted-ATP-Sepharose column, a crude extract of pig heart mitochondria was passed through affinity column with a capacity of 3 mg of enzyme per ml of affinity gel. Biospecific

elution was made with a 0.5 mM CoASH. Since very few CoA-dependent enzymes were inhibited by ATP and were adsorbed on the ATP-Sepharose, citrate synthase was obtained with greater than 95% in purity in the peak fractions. On the other hand, elution with ATP did not result in a satisfactory purification of this enzyme, since the obtained citrate synthase was contaminated with many other ATP-dependent enzymes[17]. Purification of citrate synthase with an 8-(6-aminohexyl)-amino-desulfo-CoA-Sepharose column was also reported[17]. However, other CoA-dependent enzymes such as succinate thiokinase were co-eluted with citrate synthase by an elution with 0.5 mM CoASH. As a result, the obtained citrate synthase was not homogeneous by this affinity column procedure.

Recently general ligand affinity chromatography was applied to the purification of amino-acyl-t-RNA synthetases[46]. With the use of 8-(6-aminohexyl)-amino-ATP-Sepharose, the valine enzyme from E. coli (partially purified by DEAE column) was adsorbed on the affinity column. Valyl-t-RNA-synthetase was eluted specifically with a 0 to 5 mM ATP gradient in the presence of 5 mM Mg^{++} and 0.1 mM valine. As a result, the obtained enzyme is greater than 85% pure[46].

COMMENTS ON ENZYME PURIFICATION USING GENERAL LIGAND AFFINITY CHROMATOGRAPHY TECHNIQUES

Although the basic principles of using "general ligand" for protein purification are known, successful purification of a given enzyme from the affinity column strongly relies upon the following factors: (1) proper choice of a general ligand and carrier[39], (2) conditions of washing, and (3) specific elution. The proper combinations of these conditions will permit an efficient purification from crude homogenates.

It is apparent that the proper choice of an affinity ligand must be made for the successful application of affinity column techniques. The binding affinity between the ligand and the enzyme of interest must be great enough to prevent its elution with nonspecific eluents[39]. However, if the binding is too strong, the

tightly bound enzyme cannot be readily eluted under mild conditions. Therefore successful application of affinity chromatographic techniques cannot be achieved in either case.

The strength of binding of a given immobilized ligand to the enzyme is also influenced by several factors. These include: (1) the distance of the ligand from the surface of the solid support as discussed previously[39], (2) proper choice of the solid-support carrier, and (3) the coupling methods.

Sepharose is generally accepted as one of the best carriers for affinity columns[1, 2, 47-49]. There are several advantages for using this carrier in the synthesis of affinity columns: (1) weak nonspecific interactions of its surface with proteins, (2) good hydrophilic and flow properties, (3) a high degree of porosity and high concentration of functional groups which can easily be activated usually by means of cyanogen bromide for the attachment with the desired ligand[50-54], and (4) a high degree of physical and chemical stability.

In addition to the selection of proper ligands and solid-support matrix, there are several important steps which have to be taken into account before proceeding with purification of enzymes from crude extracts.

(1) Before passing crude extracts through the Sepharose columns, they should be dialyzed against a low ionic strength and low pH buffer (0.01 M salt and pH $\leq$ 7.0) in order to prevent interference from the contaminating nucleotides in the original extracts. Some tissue extracts should also be diluted in order to decrease the ionic strength effects arising from high protein concentrations.

Experimentally, it is generally observed that the binding of enzymes to the nucleotide Sepharose columns is better at a low pH and low ionic strength buffer. Since the surface charge distribution and the conformation of protein strongly depend on these two parameters, solutions of low pH and low ionic strength in general decrease the nonspecific repulsion force between the bound ligand and proteins; binding is therefore more readily facilitated.

(2) After the crude extract is loaded on the column, it is important that nonspecifically bound proteins be extensively removed before the specific elution of an enzyme commences. This is usually accomplished by extensive washing of the column with buffer. A buffer of high ionic strength and high pH is preferred provided that the enzyme to be purified can be preferentially retained on the column.

(3) Since an entire family of enzymes are usually retarded by the general ligand on the Sepharose columns, purification of the enzyme of interest can be achieved only when a specific inhibitor or substrate is applied to elute the enzyme from the column. In the case of dehydrogenases, it was reported by Everse et al.[45] that some dehydrogenases are specifically inhibited by an addition product of DPN^+ with their respective substrate, such as reduced DPN-pyruvate adduct for lactate dehydrogenases and reduced DPN-α-ketoglutarate adduct for glutamate dehydrogenase. Under certain circumstances the enzyme can be eluted specifically with DPN^+ and its oxidized substrate or inhibitor. Since the formation of a ternary complex often prevails, resulting in a strong inhibition, the enzyme can then be eluted. In case a specific eluent is not available for a given enzyme, a proper coenzyme or substrate gradient can be used for selective elution[55, 56]. The enzyme can frequently be purified with a high degree of purity in this manner. This is exemplified by the purification of glucose-6-phosphate dehydrogenase from yeast extracts as we have previously described[13].

Although not all the enzymes we have studied can be purified in one step by these affinity columns, homogeneous enzyme preparations can usually be obtained by an additional step of conventional procedures, such as ammonium sulfate fractionation, DEAE cellulose, or Sephadex chromatography. General ligand affinity chromatographic techniques are now extensively applied to the large-scale preparation of lactate dehydrogenase from various sources. With a half liter column of 8-(6-aminohexyl)-amino-5'-AMP-Sepharose, one can process and crystallize 1 gram of purified

LDH from crude extracts in a day or so, unlike the traditional procedures for which a week or a month are required to achieve the same goal. Large-scale purification of other dehydrogenases and kinases using the developed affinity column technique is expected in the near future. The idea that a single general ligand affinity column can be used to purify a number of enzymes has now become a reality. There is no doubt that this method appears to be simple and inexpensive; in many cases the purification involves a single adsorption and elution.

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