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Bi-substrate analogue ligands for affinity chromatography of protein kinases

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Abstract Novel affinity ligands, consisting of ATP-resembling part coupled with specificity determining peptide fragment, were proposed for purification of protein kinases. Following this approach affinity sorbents based on two closely similar ligands AdoC–Aoc–Arg_4–Lys and AdoC–Aoc–Arg_4–$NH(CH_2)_6NH_2$, where AdoC stands for adenosine-5′-carboxylic acid and Aoc for amino-octanoic acid, were synthesized and tested for purification of recombinant protein kinase A catalytic subunit directly from crude cell extract. Elution of the enzyme with MgATP as well as L-arginine yielded homogeneous protein kinase A preparation in a single purification step. Also protein kinase A from pig heart homogenate was selectively isolated using MgATP as eluting agent. Protein kinase with acidic specificity determinant (CK2) as well as other proteins possessing nucleotide binding site (L-type pyruvate kinase) or sites for wide variety of different ligands (bovine serum albumin) did not bind to the column, pointing to high selectivity of the bi-functional binding mode of the affinity ligand.

Key words: Protein kinase; Affinity chromatography; Bi-substrate inhibitor

1. Introduction

Protein kinases transfer the phosphoryl group from ATP to acceptor amino acids in proteins involved in regulatory phosphorylation phenomena and they form the largest family of enzymes in eukaryotic cells [1]. The predicted number of protein kinases in human genome has risen above 1000 [2], while the physiological role of most of these enzymes has remained unknown. Only a few hundred of the protein kinases have been characterized on protein level and substrate specificity of these enzymes has been investigated even in fewer cases. Slow progress in these areas can be related to complexity of the enzyme isolation protocols, based on conventional chromatography methods [3,4]. We expect that introduction of the novel type of affinity sorbents proposed in this report will simplify the purification procedure and dramatically change the situation.

An attempt has been made to use peptides as affinity chromatography ligands for isolation of glycogen synthase kinase and casein kinase II [5]. However, these sorbents had too low affinity as the peptides alone possess relatively low binding effectiveness to protein kinases in general [6]. Further, an attempt was made to use a potent peptide inhibitor for affinity chromatography of protein kinase A [7]. Although this inhibitor is potent, the enzyme binding with the column required too long incubation time and the purification yield was low. Alternatively, the protein kinase ligands directed towards the ATP binding pocket on these enzymes have been used as affinity ligands. For example, the γ-ATP-Sepharose has been tested for isolation of several kinases [8,9]. This affinity sorbent, however, cannot be specific for particular protein kinases and even for these enzymes in general, as many different proteins bind effectively ATP and other adenosine nucleotides.

In the present report novel bi-substrate inhibitors were introduced to design affinity sorbent for protein kinases. These inhibitors consist of a nucleotide-resembling part (AdoC) coupled with a peptide fragment *via* a linker group [10]. By using structural elements of both substrates involved in the phosphorylation reaction, affinity and selectivity of the inhibitors can be increased and even modulated by selecting appropriate peptide motifs. Therefore it would be possible to design affinity columns for group-selective isolation of basophilic, acidophilic or proline-oriented protein kinases, but also for isoenzyme separation, if their primary structure specificity motifs are known. In this report two affinity ligands AdoC–Aoc–Arg_4–Lys (ligand A) and AdoC–Aoc–Arg_4–$NH(CH_2)_6NH_2$ (ligand B) were synthesized for protein kinases of basophilic substrate specificity and tested for isolation of protein kinase A.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP and epoxy-activated Sepharose were from Amersham Pharmacia Biotech. Kemptide (LRRASLG) was obtained from Bachem. Peptide RRRADDSDDDDD (substrate for protein kinase CK2) and KRAKRKTAKKR (substrate for protein kinase C) were synthesized at the Department of Medical Biochemistry and Microbiology, Uppsala University, Sweden. All other chemicals were of the highest purity commercially available.

2.2. Synthesis of AdoC–Aoc–Arg_4–Lys (ligand A)

Wang-type peptide synthesis resin, Fmoc–Lys(Boc)–resin (200 mg, 0.6 mmol/g; Advanced ChemTech) was swollen in DMF. Fmoc protection was removed by treatment of the resin with 20% piperidine in DMF. The following arginines were attached to the resin in the form of Fmoc–Arg(Pmc) with Bop/Hobt activation. After removal of the N-terminal Fmoc protection the following 8-Fmoc–Aoc and 2′,3′-isopropylideneadenosine-5′-carboxylic acid were attached to the peptide

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Abbreviations: AdoC, adenosine-5′-carboxylic acid; Aoc, amino-octanoic acid; DMF, dimethylformamide; IPTG, isopropyl β-D-thiogalactopyranoside; PMSF, phenyl methyl sulfonyl fluoride; SDS, sodium dodecylsulfate; TFA, trifluoroacetic acid

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chain with Bop/Hobt activation. Cleavage of the ligand from the resin and removal of the protection groups were achieved by treatment of the resin with a cocktail containing triisopropylsilane (100 μl), ethanedithiol (100 μl), water (100 μl) and TFA (2 ml) for 2 h. AdoC–Aoc–Arg_4–Lys was purified by an acetonitrile–water (0.1% TFA) gradient on a preparative C18 HPLC column. Lyophilization of the solution gave the pure ligand in the form of trifluoroacetate salt.

2.3. Synthesis of AdoC–Aoc–Arg_4–$NH(CH_2)_6NH_2$ (ligand B)

The ligand was synthesized and characterized as described for A with the modification that diaminohexane trityl resin (Novabiochem, 0.56 mmol/g) was used as starting resin.

2.4. Coupling of ligand A to epoxy-activated Sepharose

0.4 g of freeze-dried epoxy-activated Sepharose 6B was washed with 50 ml of water. The ligand AdoC–Aoc–Arg_4–Lys (10 mg) was dissolved in 2.0 ml of 0.1 M carbonate buffer (pH 10.5) and added to the gel suspension. After coupling of the ligand on a shaker (60 h at room temperature) remaining epoxide groups were blocked with 0.5 M 2-mercaptoethanol in the same carbonate buffer. The gel was washed with carbonate (0.1 M, pH 9.5) and acetate (0.1 M, pH 4.5) buffers and water.

2.5. Coupling of ligand B to NHS-activated Sepharose™ 4 fast flow

1.0 ml of the NHS-activated gel was washed with 50 ml of 0.1 mM HCl. The ligand AdoC–Aoc–Arg_4–$NH(CH_2)_6NH_2$ (B, 5.0 mg) was dissolved in 2.0 ml of 0.1 M carbonate buffer (pH 8.5) and added to the gel suspension. After coupling of the ligand on a shaker (15 h, room temperature) excess active groups were blocked with 1.0 M ethanolamine (pH 8, 1 h). The gel was washed with carbonate (0.1 M, pH 8.5) and acetate (0.1 M, pH 4.5) buffers and finally with water.

2.6. Enzymes

The expression construct vector Cat-pRSETb for expression of protein kinase A catalytic subunit was a generous gift from Dr. S.S. Taylor (La Jolla, CA, USA) and the expression was performed as described in [11]. Shortly, the vector was transformed into *Escherichia coli* strain BL21(DE5) and the expression was induced at OD_{600} between 0.55 and 0.80 by adding 0.5 mM final concentration of IPTG. The cells where grown additionally for 6 h, harvested and the pellets were stored in portions at −70°C. The thawed cells were disrupted by sonication. For this procedure 20 ml of 30 mM Tris–HCl, 50 mM KCl, 1 mM EDTA, 5 mM β-mercaptoethanol (pH 7.5) was added to pellet obtained from 1 l of culture media. The suspension was centrifuged at $12\,000\times g$ for 30 min and the supernatant was pooled for affinity chromatography experiments. For comparison the classical protocol was used to purify protein kinase A [11]. Protein kinase CK2 and L-type pyruvate kinase were purified from rat liver as described in [12] and [13], respectively.

2.7. Affinity chromatography of recombinant protein kinase A catalytic subunit from E. coli extract

Affinity columns (0.5 ml bed volume) were equilibrated with 20 mM Tris–HCl (pH 7.5). The supernatant of *E. coli* cell homogenate was diluted to a protein concentration of 15 mg/ml and 200 μl of this mixture was applied to the column. Thereafter the column was washed with 10 volumes of the equilibration buffer containing 200 mM NaCl. The enzyme was eluted with the same buffer containing 20 mM $MgCl_2$ and ATP at different concentrations. The capacity was at least 1 mg of protein kinase A per ml of resin. Fractions (250 μl) were collected and enzyme activity was measured. Finally the column was washed with the equilibration buffer containing 1.2 M NaCl and regenerated by washing with 10 column volumes of 0.1 M Tris–HCl, 0.5 M NaCl (pH 8.5) and 10 column volumes of 0.1 M sodium acetate, 0.5 M NaCl (pH 4.5). The flow rate in all steps was 0.25 ml/min. In other experiments the elution buffer was complemented with L-arginine (50–200 mM) or NaCl (100–1000 mM).

2.8. Affinity chromatography of protein kinase A from pig heart

Pieces of freshly frozen pig heart were homogenized using Polytron Aggregate® homogenizer in four volumes (w/v) of homogenization buffer (20 mM Tris–HCl, pH 7.5, 10 mM EDTA, 10 mM EGTA, 15 mM β-mercaptoethanol, 1 mM PMSF). The homogenate was centrifuged for 30 min at $4000\times g$. The supernatant was filtered through glass wool, cAMP was added up to concentration of 10 μM and 1 ml of the obtained solution (protein concentration 16 mg/ml) was applied to a 0.5 ml affinity column equilibrated with 20 mM Tris–HCl, pH 7.5, 100 mM NaCl, 10 μM cAMP. The column was washed with ten volumes of 20 mM Tris–HCl, pH 7.5, 400 mM NaCl and with two column volumes of 20 mM Tris–HCl, pH 7.5, 20 mM $MgCl_2$. The protein kinase was eluted with a 0–2.5 mM gradient of ATP in buffer 20 mM Tris–HCl, pH 7.5, 20 mM $MgCl_2$ including 10% glycerol. In experiments where a preceding ion exchange step was performed, the pH and the ionic strength of the supernatant were adjusted according to that of 20 mM Tris–HCl, pH 7.5. The obtained material was loaded onto 10 ml of DE-52 column (Whatman) equilibrated with the same buffer. The column was washed with ten volumes of equilibration buffer and the proteins were eluted with 40 ml 20 mM Tris–HCl, pH 7.5, 400 mM NaCl. The ionic strength of the eluted protein solution was adjusted to that of the equilibration buffer of the affinity columns and the affinity chromatography was performed as described above except that the total protein amount loaded to the column was 40 mg. The obtained protein was concentrated using Filtron 10K cutoff concentration tubes.

2.9. SDS–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel (12%) was performed using a discontinuous buffer system according to Laemmli [14] as modified by O'Farrell [15]. After electrophoresis the proteins were visualized by colloidal staining in 1.6% ortho-phosphoric acid, 8% ammonium sulphate, 0.08% of Coomassie brilliant blue G-250 and 20% methanol overnight. Water was used for destaining.

2.10. Protein kinase assay

Activity of protein kinase A was assayed as described before [10] by using 50 μM of Kemptide (LRRASLG) and 100 μM [γ-^{32}P]ATP as substrates. The final dilution factor of the tested fractions in incubation mixture was 1000–5000. The activity of cGMP-dependent protein kinase was assayed analogously using Kemptide by including 2 μM cGMP into the incubation mixture. The assay of protein kinase C was performed as described in [10]. Protein kinase CK2 activity was measured as described in [10] except that the peptide RRRADDSDDDDD was used as substrate and the assay mixture contained 200 mM NaCl. The inhibition constants of the ligands were estimated using Lineweaver–Burk analysis as described in [16] assuming the sequential pathway of the phosphorylation reaction with ATP binding first [17].

3. Results and discussion

The affinity ligands AdoC–Aoc–Arg_4–Lys (ligand A) and AdoC–Aoc–Arg_4–$NH(CH_2)_6NH_2$ (ligand B) were effective reversible inhibitors of protein kinase A (Table 1). Both of the constituent parts of the ligands, adenosine and the oligo-arginine peptide, inhibited the enzyme and the appropriate inhibition constants together with the dissociation constant for ATP are listed in Table 1. It can be seen that coupling of the adenosine and peptide blocks via the linker group significantly increased the affinity of the ligand. The bi-substrate ligand AdoC–Aoc–Arg_4–Lys was coupled to the epoxy-activated Sepharose via the side chain amino group of its terminal lysine (Fig. 1A). Ligand AdoC–Aoc–Arg_4–$NH(CH_2)_6NH_2$ was coupled to NHS-Sepharose (Fig. 1B) yielding a resin

Table 1
Interaction of the affinity ligand AdoC–Aoc–Arg_4–Lys with protein kinase A compared to oligo-arginine Arg_6, adenosine and ATP

Ligand molecule	K_i (μM)
AdoC–Aoc–Arg_4–Lys	0.33
AdoC–Aoc–Arg_4–$NH(CH_2)_6NH_2$	0.083
Arg_6	29.5
Adenosine	84.5
ATP	25.0[a]

[a]Dissociation constant for ATP from [18].

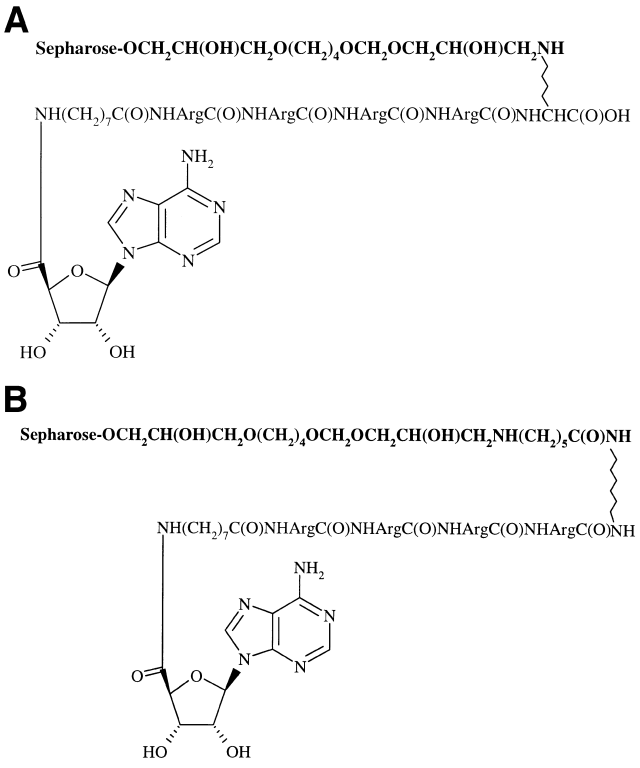

Fig. 1. A: Affinity ligand AdoC–Aoc–Arg_4–Lys coupled to epoxy-Sepharose 6B. B: Affinity ligand AdoC–Aoc–Arg_4–$NH(CH_2)_6NH_2$ coupled to NHS-Sepharose.

with longer spacer and lacking the carboxyl group of the terminal lysine of the ligand. The coupling efficiency for ligand B was approximately two-fold better than for A yielding 0.5 and 1.4 micromoles of ligand per ml of swollen gel, respectively, as calculated from amino acid analysis. The enzyme binding properties of the two resins were close to identical in respect to the characteristics given below.

The supernatant of the *E. coli* cell homogenate (Fig. 2, lane 1) was directly loaded to the ligand A affinity column. The breakthrough fractions (Fig. 2, lane 2) showed the same protein pattern as the loaded supernatant with a substantial decrease of the 40 kDa band of protein kinase A and it was sufficient to use 20 mM Tris–HCl containing 200 mM NaCl (pH 7.5) as washing buffer to remove non-specifically bound proteins from the column. The protein kinase A was eluted with 1 mM ATP in 20 mM $MgCl_2$. The product appeared as a homogeneous protein on SDS–polyacrylamide gel electrophoresis (Fig. 2, lane 3) and the yield of this one-step purification procedure was over 90%. In separate experiments it was shown that the specific activity of the eluted enzyme was similar to the specific activity of protein kinase A purified by the conventional protocol.

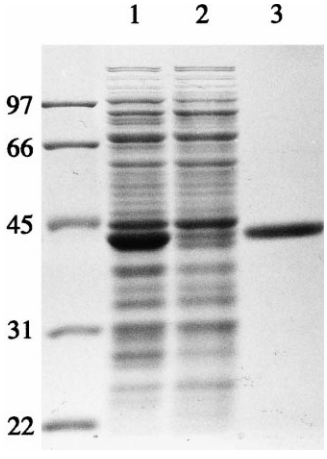

Fig. 2. SDS–polyacrylamide gel showing the affinity purification of the recombinant catalytic subunit of protein kinase A from *E. coli* extract. The left lane shows the molecular weight markers in kDa units. Lane 1, loaded supernatant of *E. coli* extract containing overexpressed protein kinase A; lane 2, breakthrough fraction; lane 3, elution of protein kinase A with the equilibration buffer containing 1 mM ATP and 20 mM $MgCl_2$.

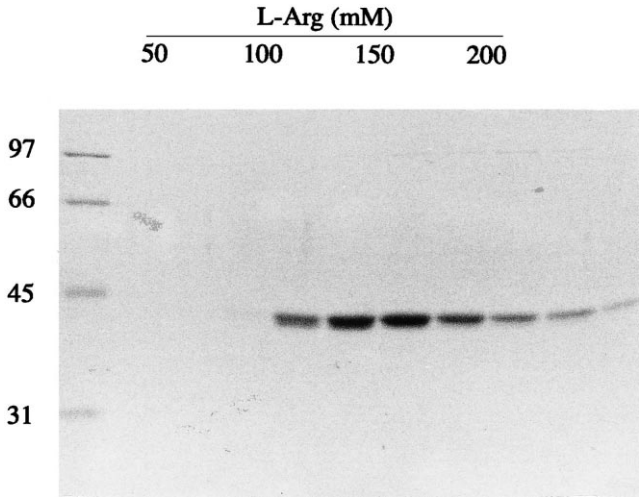

Fig. 3. SDS–polyacrylamide gel showing the elution of protein kinase A using increasing concentrations of L-arginine (pH 7.5) as indicated. The left lane shows the molecular weight markers in kDa units.

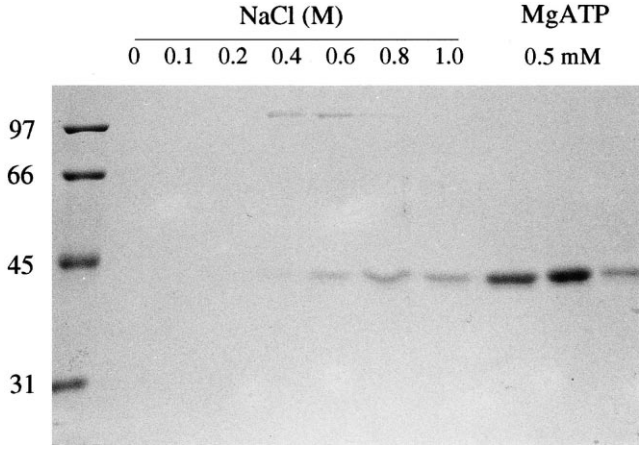

Fig. 4. SDS–polyacrylamide gel showing the influence of NaCl concentration on the binding of protein kinase A to the affinity column. The left lane shows the molecular weight markers in kDa units. Further lanes from left to right show the stepwise gradient of NaCl, which was applied after the protein kinase was bound and the column washed with the equilibration buffer. The last three lanes in the right side show the elution of protein kinase after subsequent application of the equilibration buffer containing 0.5 mM ATP and 20 mM $MgCl_2$.

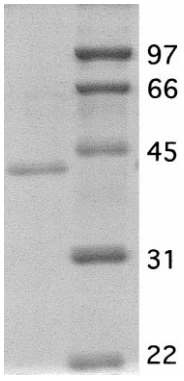

Fig. 5. SDS–polyacrylamide gel showing affinity purified protein kinase A from pig heart. The pig heart homogenate was first bulk purified on DE-52 followed by affinity chromatography as described in Section 2. The left lane represents the eluted protein kinase A from the affinity column and right lane shows the molecular weight markers in kDa units.

As the bi-substrate ligand AdoC–Aoc–Arg_4–Lys contained several arginines, a solution of L-arginine was also effective for elution of the enzyme. If stepwise gradient of this eluent was used, the kinase started to elute at 100–150 mM L-arginine concentrations (Fig. 3) and the enzyme obtained was a pure protein following the Coomassie stained SDS–polyacrylamide gel electrophoresis.

On the other hand, control experiments were performed with β-mercaptoethanol blocked epoxy-activated Sepharose lacking the ligand. In this case no binding of protein kinase A to the column was observed. These data, as well as the fact that only trace amounts of the enzyme were eluted from the affinity column at 0.6 M NaCl, if stepwise gradient of the salt was used (Fig. 4), gave additional evidence for high specificity of protein kinase A binding with the affinity sorbent.

Further, it was found that protein kinase CK2 did not bind to the affinity sorbent and all of its activity appeared in the flow-through fractions. This finding was in good agreement with the fact that this enzyme is characterized by clearly acidic substrate specificity determinants.

The binding of other adenosine nucleotide binding enzymes with the affinity sorbent was tested by using L-type pyruvate kinase as a model enzyme. A small fraction of this enzyme was bound to the affinity column if there was no salt added to the washing buffer. However, in the presence of 200 mM NaCl this binding was completely abolished pointing to a relatively weak interaction between this protein and the affinity ligand. Similar results were obtained if bovine serum albumin was loaded onto the column.

Finally, protein kinase A was isolated from pig heart using both ligand A and B resins. Due to the low amount of protein kinase A relative to the bulk proteins in the heart extract, resin B was used in the majority of experiments in order to improve the visualization of the isolated protein kinase, as the ligand density and ligand affinity of this resin were higher than that of resin A. It was possible to purify protein kinase A from crude cell extract by one-step procedure by eluting with ATP analogously as described above for recombinant kinase. In some experiments, in order to produce higher amounts of kinase without much destroying the affinity gel by excess amounts of loaded proteins, a preceding DEAE-cellulose step, frequently used before affinity chromatography, was used. Protein kinase A was separated from other protein kinases known to be present in the DE-52 pool as only the protein kinase A band was detectable in the SDS–PAGE of the elution (Fig. 5). All the loaded protein kinase A activity was bound by the column. Under the conditions used no activity of two basophilic protein kinases – cGMP-dependent protein kinase and PKC – closely related to protein kinase A was detected. Interestingly, protein kinase A bound equally well both when cAMP was absent or present in the loaded protein solution. As the bi-substrate type ligands presumably occupy both the adenosine binding pocket and the autoinhibitory pseudosubstrate site, it means that during the loading of the column the ligand successfully competed with the regulatory subunit for the pseudosubstrate site on the catalytic subunit. This fact is supported by the finding that the protein kinase A activity in the eluate, determined by a highly specific substrate peptide Kemptide, was not stimulated by cAMP showing that the enzyme was not bound and eluted as a holoenzyme but as a free catalytic subunit.

In summary, the affinity sorbent, based on the bi-substrate protein kinase inhibitor, provided an efficient one-step purification of protein kinase A directly from crude cell extract. The yield of this simple one-step purification process was almost quantitative and selectivity of the column was sufficient to separate the protein kinase from other adenosine nucleotide binding proteins. The affinity gel was chemically and biologically stable and could be used repeatedly without decrease of its efficiency.

It is noteworthy that the approach described may be applied, in combination with peptide phosphorylation assay, for quantitative detection of protein kinases in biological extracts. In this case the peptide phosphorylation assay should be preceded by the step of affinity chromatography to remove ATPases, phosphatases, peptidases, endogenous substrates of protein kinases under investigation or other contaminating proteins possibly interfering with the assay.

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