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SYNTHESIS OF THE BIFUNCTIONAL DINUCLEOTIDE AMP–ATP AND ITS APPLICATION IN GENERAL LIGAND AFFINITY CHROMATOGRAPHY

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A new AMP derivative substituted with spacer arms both at position N^6 and C8 of the adenine moiety was synthesized and immobilized to Sepharose. To the immobilized ligand was subsequently coupled C8-substituted ATP in a solid-phase synthesis fashion yielding the bifunctional general ligand AMP–ATP. This affinity material was used in the separation of two major groups of enzymes, dehydrogenases and kinases. It was found that on passage of crude homogenates obtained from mouse kidney through the affinity column, several dehydrogenases and kinases were bound, which could be eluted separately using pulses of NADH and ATP, respectively. In the fractions obtained on NADH elution, lactate dehydrogenase, malate dehydrogenase, and α-glycerol phosphate dehydrogenase were found, whereas ATP eluted 3-phosphoglyceric acid kinase, pyruvate kinase, and aldolase.

INTRODUCTION

General ligand affinity chromatography is based on the principle that one single immobilized ligand is able to adsorb a family of enzymes such as dehydrogenases or kinases (1–9). The enzyme to be isolated can subsequently be eluted under conditions favoring biospecific elution. A vast number of enzymes have been purified in the last few years by this technique, usually by using a cofactor or a cofactor fragment as a general ligand. Immobilized N^6- or C8-substituted AMP and ATP derivatives have proved to be good general ligands for many dehydrogenases and kinases, respectively. In this communication, we describe the synthesis of a new N^6- and C8-disubstituted AMP derivative and a solid-phase synthesis of a Sepharose-bound AMP–ATP dinucleotide derivative. The usefulness of this bifunctional ligand in the separation and copurification of the two different classes of enzymes, dehydrogenases and kinases, was investigated.

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MATERIAL AND METHODS

AMP, ATP, idoacetic acid, and *N*-hydroxysuccinimide were obtained from Sigma Chemical Company (St. Louis, Missouri). Dicyclohexyl carbodiimide and 1,6-diaminohexane were purchased from Aldrich Chemical Company (Wisconsin). Thin-layer material was purchased from Merck (Darmstadt, West Germany) and Sepharose 4B from Pharmacia (Uppsala, Sweden).

Analytical Procedures

Thin-layer chromatography was performed on a silica gel using isobutyric acid–1 M ammonia (5 : 3 vol/vol) solvent saturated with disodium EDTA. Compounds were detected by viewing under an ultraviolet lamp (254 nm). AMP and AMP derivatives were also analyzed by high-resolution proton NMR (100 MHz) at a constant ambient probe temperature of 25°C. The samples were first lyophilized and then dissolved in D_2O at a concentration between 50 and 100 mM before the recording of the spectrum.

The enzyme activities of various dehydrogenases and kinases were assayed according to the methods given in the Boehringer catalog.

Preparation of Tissue Extracts

Kidney homogenates from a special strain of mice (C57BL/6J) were employed for the purification of several dehydrogenases and kinases by affinity chromatography. Five male mice (8–16 weeks old) were killed by cervical dislocation. The kidneys were homogenized in 5 vol of 10 mM phosphate buffer at pH 7.0 using a glass–Teflon homogenizer. After centrifugation at $16.000 \times g$ for 20 min, the supernatant was collected. All procedures were performed at 4°C. The supernatant was employed directly for enzyme purification without dialysis.

*N^6-Carboxymethyl-8-Br-AMP (**I**)*

The general course of the synthetic route employed is found in Fig. 1. One gram of AMP was dissolved in 10 ml of 1 M sodium acetate buffer at pH 4.5 and a threefold excess of bromine was added to the solution, which was stirred vigorously for 30 min. The unreacted bromine was extracted three times with 3 vol of carbon tetrachloride. The residual bromine was bleached by adding a slight excess of sodium bisulfite. 8-Br-AMP was precipitated from the solution by the addition of 3 vol of cold ethanol (−20°C). The yield of 8-Br-AMP was greater than 90%. One gram of 8-Br-AMP was dissolved

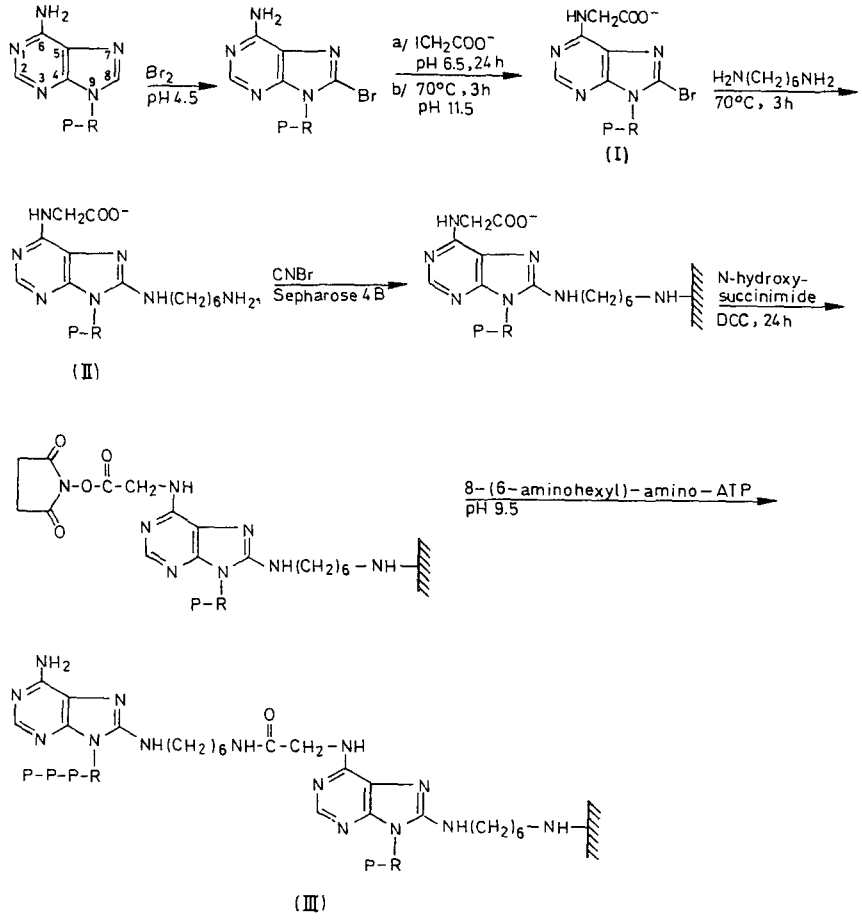

FIG. 1. Synthesis of N^6-carboxymethyl-8-Br-AMP (I), N^6-carboxymethyl-8-(6-aminohexyl)-amino-AMP (II), and Sepharose-bound AMP–ATP dinucleotide (III). R = ribose, P = phosphate, and DCC = dicyclohexylcarbodiimide.

in 10 ml of water containing 2 g of iodoacetic acid. The solution was kept in the dark at 37°C and the pH was adjusted to 6.5 with 1 M sodium hydroxide every hour. After 6 h, an additional 2 g of iodoacetic acid was added and the alkylation reaction was allowed to proceed for another 7 h. At this stage more than 90% of 8-Br-AMP was converted to N^1-carboxymethyl-8-Br-AMP as judged from thin-layer chromatography (Table 1) and the changes in UV absorbance. Subsequently 3 vol of cold ethanol (−20°C) were added to the solution to precipitate the nucleotides. The precipitate was dissolved

TABLE 1. **Thin-Layer Chromatography**

Compounds	R_f values with silica plates[a]
1. AMP	0.50
2. 8-Br-AMP	0.55
3. N^1-Carboxymethyl-8-Br-AMP	0.30
4. N^6-Carboxymethyl-8-Br-AMP	0.25
5. N^6-Carboxymethyl-8-(6-aminohexyl)-amino-AMP	0.13

[a] Solvent system: 1 M isobutyric acid—1 M aqueous ammonia (5 : 3 v/v), saturated with EDTA.

in 50 ml of water and the pH was adjusted to 11.5. The solution was then heated at 70°C and the rearrangement to N^6-carboxymethyl-8-Br-AMP was monitored by the shift of absorbance maximum from 263 to 272 nm as well as by thin-layer chromatography (Table 1). After 3 h the solution was passed through a Dowex 1×2 anion exchange column (100 ml resin/g nucleotides) which had been equilibrated with 2 M ammonium formate and water. The adsorbed N^6-carboxymethyl-8-Br-AMP was eluted with a 0–0.5 M formic acid gradient (0.5 liter × 0.5 liter). Proper fractions, i.e., those having an absorbance maximum at 272 nm and the expected thin-layer chromatographic characteristics, were pooled, concentrated on a rotary evaporator, and the nucleotide content precipitated with cold ethanol (−20°C).

N^6-carboxymethyl-8-Br-AMP could also be purified by a DEAE-cellulose column (300 ml resin/g nucleotides) which was preequilibrated with 2 M ammonium bicarbonate and water. Elution was made with a 0–1 M ammonium bicarbonate gradient (1 liter × 1 liter). N^6-Carboxymethyl-8-Br-AMP was eluted at a salt concentration of 0.3–0.5 M. Proper fractions were collected and lyophilized to dryness.

N^6-Carboxymethyl-8-(6-aminohexyl)-amino-AMP (II)

Purified N^6-carboxymethyl-8-Br-AMP, 0.5 g, was dissolved in 10 ml of water containing 1 g of 1,6-diaminohexane. The solution was heated to 70°C for 3 h. Conversion of N^6-carboxymethyl-8-Br-AMP to N^6-carboxymethyl-8-(6-aminohexyl)-amino-AMP was followed by the shift of UV absorbance maximum from 272 to 281 nm as well as by thin-layer chromatography (Table 1). After completion of the reaction, the solution was diluted 20-fold with water and passed through a Dowex 1×2 anion exchange column (100 ml resin/g nucleotides), which had been equilibrated with 2 M ammonium formate and water. The column was washed with 500 ml of

0.02 M ammonium chloride solution and the product was eluted with a linear gradient of 0–0.5 M formic acid (0.5 liter × 0.5 liter).

Proper fractions of N^6-carboxymethyl-8-(6-aminohexyl)-amino-AMP were pooled and concentrated on a rotary evaporator. The nucleotide was then precipitated with 3 vol of cold ethanol (−20°C) and the precipitate was dried under vacuum overnight. The overall yield of N^6-carboxymethyl-8-(6-aminohexyl)-amino-AMP from AMP was about 30%. The R_f values found on thin-layer chromatography of various intermediate AMP derivatives are given in Table 1 and the 100 MHz NMR proton spectra of N^6-carboxymethyl-8-Br-AMP and N^6-carboxymethyl-8-(6-aminohexyl)-amino-AMP are presented in Fig. 2.

Coupling of N^6-Carboxymethyl-8-(6-aminohexyl)-amino-AMP to Sepharose 4B

Sepharose 4B (4 g wet weight) was activated by the CNBr method (10) using 120 mg of CNBr in 6 ml of water. The activated gel was suspended in a solution of 0.1 M $NaHCO_3$ pH 9.5 containing 100 μmol of N^6-carboxymethyl-8-(6-aminohexyl)-amino-AMP and coupling was allowed to proceed for 14 h at 4°C. The gel was subjected to successive washings in water, 2 M NaCl, and water. The amount of bound nucleotide was approximately 1.2 μmol/g of wet gel as determined by the absorbance at 281 nm of a suspension of gel in 50% glycerol (vol/vol).

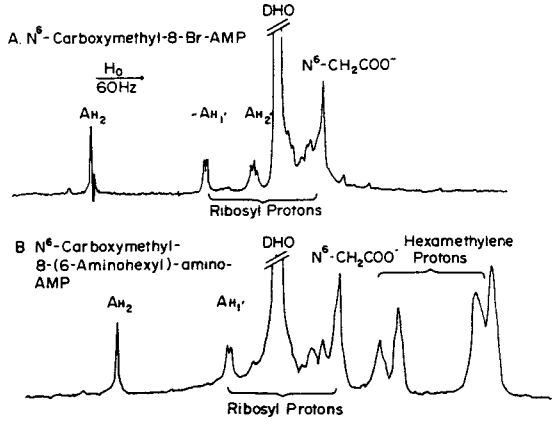

FIG. 2. 100 MHz proton spectra of (A) N^6-carboxymethyl-8-Br-AMP (I) and (B) N^6-carboxymethyl-8-(6-aminohexyl)-amino-AMP (II).

8-(6-aminohexyl)-amino-ATP

8-(6-Aminohexyl)-amino-ATP was prepared from ATP via 8-Br-ATP as described previously (4, 11).

Solid-Phase Synthesis of Sepharose-Bound AMP–ATP Dinucleotide (III)

The coupling of 8-(6-aminohexyl)-amino-ATP to the Sepharose-bound N^6-carboxymethyl-8-(6-aminohexyl)-amino-AMP proceeded over the hydroxysuccinimide intermediate of the latter (12): 10 g of N^6-carboxymethyl-8-(6-aminohexyl)-amino-AMP–Sepharose was suspended in 10 ml of dioxane–water solution (50% vol/vol) containing 0.1 M of both *N*-hydroxysuccinimide and dicyclohexylcarbodiimide. The mixture was shaken for 24 h at room temperature. The Sepharose was then filtered off and washed with dioxane and the water–dioxane mixture. It was then placed in 10 ml of 0.1 M $NaHCO_3$, pH 9.5, at 4°C containing 100 mM of 8-(6-aminohexyl)-amino-ATP. The replacement reaction was allowed to proceed for another 24 h. The resulting bifunctional AMP–ATP–Sepharose preparation was filtered and washed extensively with 2 M NaCl and water. Based on UV measurements, the ATP ligand derivative was determined as 0.6 μmol/g of AMP–Sepharose; i.e., every other AMP entity had been further substituted with an ATP entity.

RESULTS AND DISCUSSION

Synthesis of N^6-carboxymethyl-8-(6-aminohexyl)-amino-AMP followed essentially a combination of the reported procedures for N^6-substituted AMP (13) and C8-substituted AMP (11,14). Bromination of AMP followed by alkylation is the preferred route of synthesis (Fig. 1), since the inversed order of reaction often results in incomplete bromination of the N^6 alkylated nucleotide. In contrast to the previously reported procedure for alkylation of AMP, we found that the alkylation of 8-Br-AMP by the present procedure, i.e., elevated temperature and constant adjustment of pH, gave a much faster reaction rate. During the course of synthesis, the intermediate compounds such as N^6-carboxymethyl-8-Br-AMP and N^6-carboxymethyl-8-(6-aminohexyl)-amino-AMP were identified by either thin-layer chromatography (Table 1) or NMR spectra. The disappearance of H_8 proton and appearance of N^6-carboxymethyl protons in the NMR spectra indicate that both C8 and N^6 of AMP are substituted in these adenine nucleotide derivatives (Fig. 2).

Preliminary attempts to prepare a soluble AMP–ATP dinucleotide directly by a condensation reaction between N^6-carboxymethyl-8-Br-AMP

and 8-(6-aminohexyl)-amino-ATP in aqueous medium were not successful because of competing side reactions. Therefore a solid-phase synthesis was designed for the preparation of the AMP–ATP dinucleotide derivative on the Sepharose. By this procedure about 50% of the immobilized AMP ligands appears to be substituted with C8-hexyl-ATP.

Affinity Chromatography

The doubly substituted AMP analog, N^6-carboxymethyl-8-(6-aminohexyl)-amino-AMP, was first examined in terms of its properties as a general ligand. To this end a crude mouse kidney extract was passed through a N^6-carboxymethyl-8-(6-aminohexyl)-amino-AMP–Sepharose column. The ligand showed adsorption characteristics toward various dehydrogenases very similar to those of the corresponding monosubstituted AMP analog, Sepharose-bound 8-(6-aminohexyl)-amino-AMP.

Quantitative adsorption of both lactate dehydrogenase and α-glycerol phosphate dehydrogenase was observed whereas malate dehydrogenase showed only weak affinity to both affinity columns. The three dehydrogenases could be biospecifically eluted with a pulse of 1 mM NADH. The two affinity columns, on the other hand, did not exhibit any affinity for kinases present in the crude homogenate. These results strongly suggest that the dehydrogenases have a high "tolerance" toward various substitutions of the adenine moiety of the AMP inhibitor. This is in line with X-ray crystallographic data obtained with dogfish lactate dehydrogenase from which it can be seen that both N^6 and C8 positions of the adenine moiety in the binary complex (15) are exposed in an open cleft region and are available for substitution with, for instance, hydrocarbon spacers. Our present findings thus fit well with the X-ray structure of dehydrogenases in terms of the geometry of the coenzyme binding site. In this context it is worth mentioning that AMP coupled through its hydroxyl groups to a spacer matrix yields an affinity material which is useful for dehydrogenases (16), although it does not bind as strongly as the preparations just mentioned. This may indicate that dehydrogenases exhibit tolerance also to ribosyl substitutions.

The coupling of 8-(6-aminohexyl)-amino-ATP to the Sepharose-bound N^6-carboxymethyl-8-(6-aminohexyl)-amino-AMP did not appreciably alter the affinity behavior toward dehydrogenases, but did instead provide a second functionality, a relatively good affinity for kinases. Thus, when a mouse kidney homogenate was applied to this bifunctional AMP–ATP–Sepharose column, both dehydrogenases and kinases were adsorbed. Lactate dehydrogenase, α-glycerol phosphate dehydrogenase, and aldolase as well as pyruvate kinase were adsorbed quantitatively whereas malate dehydrogenase and 3-phosphoglyceric acid kinase were found to leak

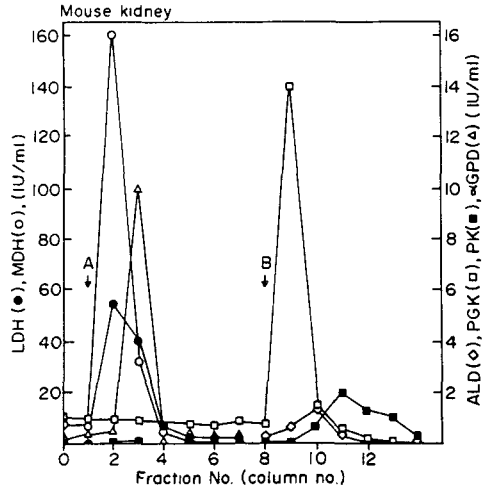

FIG. 3. Affinity chromatography on an AMP–ATP dinucleotide Sepharose column. Ten milliliters of centrifuged mouse kidney homogenate (protein concentration 7 mg/ml) in 10 mM phosphate buffer, pH 7.0, was applied to the column (0.5 × 15 cm). After washing with 10 mM phosphate buffer, pH 7.0, the enzymes were eluted with 10-ml pulses of nucleotides in phosphate buffer. At (A) 1 mM NADH was applied and at (B) 5 mM ATP. Enzyme activity is expressed in μmol/min/ml (IU) under standard assay conditions. One-milliliter fractions were collected.

considerably from the affinity column (≳50% adsorption). After washing with three column volumes of loading buffer, the three dehydrogenases were pulsed out with 1 mM NADH as seen in the elution pattern depicted in Fig. 3. In the dehydrogenase-enriched fractions, only small amounts of pyruvate kinase and aldolase activity were found. 3-Phosphoglyceric acid kinase, pyruvate kinase, and aldolase were subsequently pulsed out with 5 mM ATP in the same buffer. From the elution profile in Fig. 3 it is seen that 3-phosphoglyceric acid kinase was eluted in a relatively sharp peak in contrast to pyruvate kinase and aldolase. The fact that aldolase was adsorbed to the column and could subsequently be biospecifically eluted with ATP is in accord with the finding that 8-(6-aminohexyl)-amino-ATP is an inhibitor to the enzyme (4).

CONCLUSION

In the present investigation the synthesis of a bifunctional AMP–ATP ligand in a solid-phase fashion via Sepharose-bound disubstituted AMP is

described. The affinity material obtained was useful in the binding of the two groups of enzymes present in crude extracts, dehydrogenases and kinases, which could subsequently be eluted with NADH and ATP, respectively. The exact affinity-binding pattern of the two classes of enzymes to the gel material, containing both AMP–ATP ligand and unsubstituted immobilized AMP, remains to be established. The major object of the work described herein is to provide synthetic routes leading to bi or oligofunctional ligands which may be useful for affinity chromatography and other applications.

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