

Purification of M-MLVH- RT on a 9-Aminoethyladenine-(1,6-diamine-hexane)-triazine Selected from a Combinatorial Library of dNTP-Mimetic Ligands

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Abstract

Reverse transcriptase (RT) catalyzes the formation of dsDNA from single-stranded retroviral RNA genome. This enzyme is unique among DNA polymerases in its ability to use either RNA or DNA as a template. Moloney Murine Leukemia virus reverse transcriptase lacking RNase H activity (M-MLVH- RT) especially holds particular interest because of its ability to eliminate the deleterious effect of RNase H, which results in more efficient synthesis of full-length cDNA from mRNA. Therefore, the development of a simple purification method attracts the attention of retroviral drug and enzyme researchers and manufacturers. The present work is the first purification example of a non-tagged (native) RT by affinity chromatography using synthetic affinity ligands. In this study, the ligand was selected from a structure-biased combinatorial library of dNTP-mimetic ligands, and it was evaluated for its ability to bind and purify M-MLVH- RT from inclusion bodies of recombinant *E. coli*. The selected ligand (AEAd), bearing 9-aminoethyladenine and 1,6-diamine-hexane both linked on the same triazine scaffold, displayed the highest enzyme purifying ability after applying mild desorption conditions (6 mM MnCl₂ in 20 mM Tris-HCl buffer, pH 7.5). The binding capacity of immobilized AEAd with M-MLVH- RT was determined to be equal to approximately 1 mg enzyme/g moist weight gel. Adsorption studies with immobilized AEAd and soluble M-MLVH- RT demonstrated that the formation of the respective complex was perturbed by ATP. Quality control tests of the purified M-MLVH- RT essentially showed a single band (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and absence of nucleic acids and contaminating nuclease activities.

Introduction

Recombinant proteins are used throughout biomedical and biological sciences. However, their widespread application was made possible thanks to the development of simple and effective protein purification processes that are able to produce proteins of certain purity and quantity, as they may be required. In this endeavor, affinity chromatography (1–3) is the most reliable and

indispensable partner for preparing pure proteins because it explores the ability of biologically active macromolecules to form specific and reversible complexes with appropriate affinity ligands.

The selection and design of affinity ligands has progressed significantly over the years mainly because of knowledge derived from the scientific interaction between X-ray crystallography and bioinformatics as well as defined and combinatorial chemical synthesis and high throughput screening (1–5). We have reported the design and chemical synthesis of a combinatorial library of novel nucleotide-mimetic synthetic ligands (6,7). These ligands were generated following the so-called “structure-guided” or “directed” combinatorial method (2), for which one should have enough in-hand structural data for the target protein. The “winner” ligand is selected from a limited library built around a rationally designed “lead” ligand, a notion that is reminiscent of drug design. In the present study, we explore the dNTP-mimetic ligand library for selecting a ligand suitable for affinity chromatography purification of Moloney Murine Leukemia virus reverse transcriptase lacking RNase H activity (M-MLVH- RT) derived from recombinant *Escherichia coli* cells. Possible success of this task would lead to the first purification method for a non-tagged (native) reverse transcriptase by synthetic ligand affinity chromatography.

Reverse transcriptase (RT) is an essential retroviral enzyme that catalyzes the formation of dsDNA copy from the single-stranded retroviral RNA genome (8). RT is unique among related DNA polymerases in its ability to use either RNA or DNA as a template. The molecular architecture of the enzyme includes fingers, palm, thumb, connection, and RNase H domain and is based on an anthropomorphic resemblance to a right hand (9). M-MLVH- RT is a monomeric 75 kDa enzyme and belongs to the family of RTs that share significant structural and functional similarities with other DNA polymerases, which suggests a common catalytic mechanism for these enzymes (10–14). Moreover, it is a multifunctional enzyme as it comprises on the same polypeptide chain the following activities: RNA-dependant DNA polymerase activity, DNA-dependant DNA polymerase activity, and RNase H activity (15). The polymerases and RNase H activities reside in physically separable domains of the enzyme

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Table IA. The Structures of the Ligands of the Combinatorial Library (6)

Entry	Ligand Designation	R1	R2	
1	AMS			
2	AES			
3	APP			
4	ABP			
5	<i>o</i> ABS			
6	<i>m</i> ABS			
7	<i>p</i> ABS			
8	AEAd			
9	AEGu			
10	AMSAd			
11	AESAd			
12	APPAd			
13	ABPAD			

Table IB. The Structures of the Ligands of the Combinatorial Library (6)

Entry	Ligand Designation	R1	R2
14	<i>o</i> ABSAd		
15	<i>m</i> ABSAd		
16	<i>p</i> ABSAd		
17	AMSGu		
18	AESGu		
19	APPGu		
20	ABPGu		
21	<i>o</i> ABSGu		
22	<i>m</i> ABSGu		
23	<i>p</i> ABSGu		
24	AEAd-AEAd		
25	AEGu-AEAd		
26	AEGu-AEGu		

and can function independently of each other (16). In addition to polymerase activity, RNase H degrades the RNA to an RNA/DNA hybrid, which sets up a competition between the two activities. The extent to which the RNase H activity destroys the hybrid prior to the initiation of polymerization determines the maximum number of priming events that can actually occur (17). This process reduces the yield of cDNA by removing a portion of the mRNA from the reaction, which results in the production of truncated cDNA molecules with mass yields not exceeding 50% (18,19). Removal of RNase H activity with the use of recombinant DNA technology leads to the production of M-MLVH⁻ RT, thus improving the efficiency of cDNA synthesis from mRNA catalyzed by RT (19–21).

Purified reverse transcriptases attract the attention of retroviral drug researchers and manufacturers (e.g., HIV-RT). Especially M-MLVH⁻ RT is an irreplaceable tool of the recombinant DNA technology (18) because of its ability to eliminate the deleterious effect of RNase H, which results in more efficient synthesis of full-length cDNA. A facile and successful purification method ideally would involve a single chromatography step and make M-MLVH⁻ RT suitable for molecular biology applications.

Experimental

Materials

The pQE-70 vector and *Escherichia coli* M15 (pREP4) strain was purchased from Qiagen (Cologne, Germany). *Escherichia coli* XL-1 Blue strain was purchased from Stratagene (La Jolla, CA). Taq Pol, Pfu Pol, and dNTPs were purchased from Promega (Southampton, U.K.). The photometric enzyme immunoassay kit for the quantification of reverse transcriptase activity through incorporation of digoxigenin-labeled dUTP into DNA was obtained from Roche Applied Science (Indianapolis, IN). The pRT30-2 vector carrying the MMLV pol coding region was a thankful offer from Dr. Alice Telesnitsky of the Department of Biochemistry and Molecular Biophysics at the Columbia University of Physicians and Surgeons.

Construction of the ligand library

The design and the chemical synthesis of the ligand library were described previously (6). The chemical formulae of the ligands are shown in Table I.

Construction of the expression plasmid pQERTH⁻

Based on the DNA sequence of M-MLV pol (Gene Bank Accession No. NC001501), two primers were synthesized: (A) the N-terminal sense primer 5'-TTTTGCATGCTAAATATAGAA GATGAG-3' carrying a unique SphI site (underlined), which includes an ATG starting site of translation, and (B) the antisense primer 5'-TTAAGCTTTTAAATCAAGGCAGTTGTGTTG-3' carrying a unique underlined HindIII restriction site, followed by the stop codon. DNA amplification was performed using 2.5 U of Pfu Pol in a 50 μ L reaction mixture of PCR reaction buffer (supplied by vendor), 10 pmol of each primer, 0.2 mM of each dNTP, and 50 ng of pRT30-2 plasmid DNA. A standard PCR protocol was applied to amplify the approximately 1500-bp fragment of the M-MLVH⁻ RT

gene by using 30 amplification cycles (1 min at 94°C, 2 min at 52°C, 3.5 min at 72°C) and ending with incubation at 72°C for 10 min. The resulting amplification product was digested with SphI and HindIII and cloned into pQE-70, which was previously digested with SphI and HindIII.

Recombinant plasmids were transformed in *E. coli* XL-1 Blue strain and plated on LB media with ampicillin. Positive clones, carrying the M-MLVH⁻ RT coding region, were verified by PCR screening and designated as pQERTH⁻. To test for protein expression, pQERTH⁻ was transformed into *E. coli* M15 (pREP4). One colony was picked up and was grown on LB media with 100 μ g/mL ampicillin and 25 μ g/mL 1 kanamycin at 30°C. An overnight culture (50 mL) was used to inoculate 1 L (500 mL 2×2) LB with ampicillin and kanamycin. The cultures were grown at 30°C until the optical density (595 nm) was equal to 0.3, induced with 0.1 mM isopropyl β -D-thiogalactopyranoside (IPTG) and allowed to grow for 4 h. Cells were harvested (10,000 $\times g$, 10 min) and suspended in Tris-HCl buffer (50 mM, pH 8.0, 1 mM DTT, 2 mM PMSF). The cell suspension was disrupted by sonication on ice (5 min, 10 s pulse \times 10 s pause intervals) and centrifuged (13,000 $\times g$, 15 min, 4°C). The supernatant was discarded, whereas the pellet with inclusion bodies was collected, washed twice by resuspending it in 50 mM Tris-HCl buffer (pH 8.0) containing 2.5% (v/v) Triton-X 100, 1 mM EDTA, and 20% (w/v) sucrose (5 mL/g wet weight pellet), centrifuged (13,000 $\times g$, 15 min, 4°C), and finally stored at -20°C until use.

Refolding of M-MLVH⁻ RT

The washed pellet from the previous step was solubilized in 50 mM Tris-HCl (pH 8.0) containing 8 M urea (5 mL/g inclusion body pellet) at 4°C for 5 h with gentle stirring. Insoluble material was separated by centrifugation (13,000 $\times g$, 15 min, 4°C), and the supernatant was added dropwise into cold "refolding" buffer (100 mM Tris-HCl, pH 8.0, 10 mM DTT, 20% glycerol) to a final protein concentration of less than 0.2 mg/mL and stirred for 16 h at 4°C. After centrifugation (13,000 $\times g$, 15 min, 4°C) to remove aggregates, the supernatant was kept at 4°C for several days.

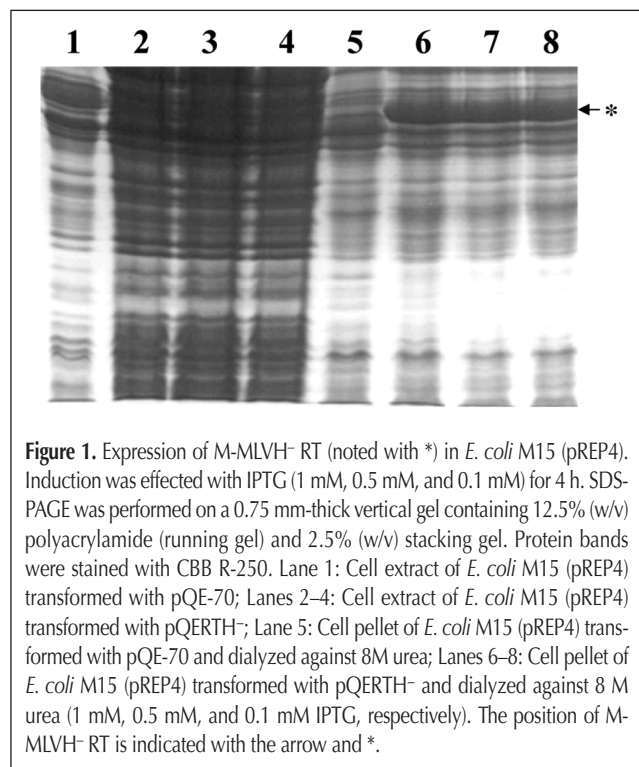
Screening of the library of immobilized ligands

Chromatographic procedures were performed at 4°C using refolded M-MLVH⁻ RT extract dialyzed in 20 mM Tris-HCl buffer (pH 7.5). The adsorbents bearing the formulae of Table I (1 mL, 0.9 g moist weight gel) were equilibrated in the same buffer and loaded with refolded M-MLVH⁻ RT extract (10 mL, 1 mg protein) before being washed with equilibration buffer until effluent absorbance (280 nm) was less than 0.01. Bound proteins were eluted with 2 M KCl (4 mL) in the same equilibration buffer. The collected fraction (4 mL) was dialyzed against water, lyophilized and the purity of the recovered M-MLVH⁻ RT was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (22).

Effect of pH on the purification of M-MLVH⁻ RT from the adsorbent AEAd

Chromatographic procedures were performed at 4°C. A column containing adsorbent No. 8 (AEAd) (1 mL, 0.9 g moist weight gel) was equilibrated, in four separate experiments with 20 mM Tris-HCl (pH 7.0, 7.5, 8.0, and 8.5). A sample of refolded

M-MLVH⁻ RT extract (10 mL, 1 mg protein) previously dialyzed against the same equilibration buffer was applied to the adsorbent. The column was washed with equilibration buffer until the effluent absorbance (280 nm) was less than 0.01. Adsorbed protein was eluted with equilibration buffer (4 mL) containing 60 mM KCl. Collected fraction (4 mL) was dialyzed against water, lyophilized, and analyzed by SDS-PAGE (22).



Purification of M-MLVH⁻ RT on the adsorbent AEAd

Chromatographic procedures were performed at 4°C. A column containing adsorbent No. 8 (AEAd) (1 mL, 0.9 g moist weight gel) was equilibrated with 20 mM Tris-HCl buffer (pH 7.5). A sample of refolded M-MLVH⁻ RT extract (10 mL, 1 mg protein) previously dialyzed against the same equilibration buffer was applied to the adsorbent. The column was washed with the equilibration buffer until effluent absorbance (280 nm) was less than 0.01. M-MLVH⁻ RT was eluted with the addition of 6 mM MnCl₂ (in equilibration buffer, 20 mM Tris-HCl buffer, pH 7.5, 4 mL). Collected fraction (4 mL) was dialyzed against water, lyophilized, and analyzed by SDS-PAGE (22).

Adsorption studies of M-MLVH⁻ RT with the adsorbent AEAd

In a total volume of 1 mL tris-HCl buffer (20 mM, pH 7.5), varying amounts of purified M-MLVH⁻ RT (2.5–70 µg), previously dialyzed in the same equilibration buffer, were mixed with 5 mg wet weight of adsorbent AEAd in the presence or in the absence of ATP (15 mM). The suspensions were shaken for 120 min in order for the system to reach equilibrium. The mixture was then centrifuged (5,000 × *g*, 2 min), and the amount of unbound protein in the supernatant was determined by the method of Bradford (23). Bound protein was calculated by subtracting the amount of unbound protein from the total amount of protein added.

Determination of the apparent capacity of adsorbent AEAd for M-MLVH⁻ RT

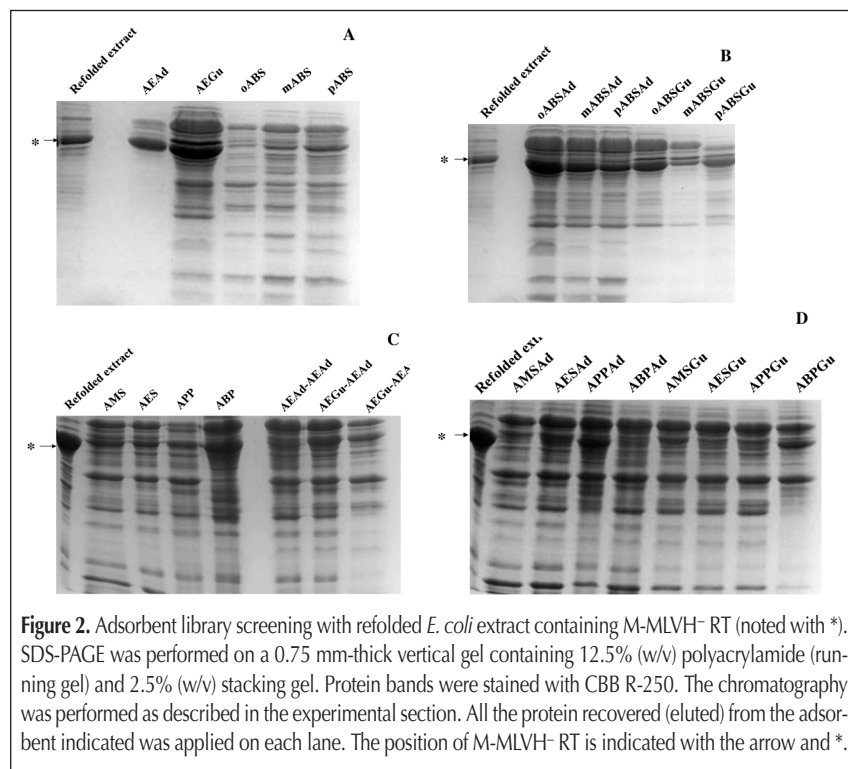
Chromatographic procedures were performed at 4°C. A column containing adsorbent No. 8 (AEAd) (0.12 g moist weight gel) was equilibrated with Tris-HCl buffer (20 mM, pH 7.5), and a solution of in-laboratory purified M-MLVH⁻ RT in the same buffer (0.07 mg/mL, 4 mL) was continuously applied on the column until effluent absorbance (280 nm) was constant. After washing the adsorbent with equilibration buffer, bound M-MLVH⁻ RT was eluted with 1 M KCl in the same equilibration buffer (1 mL).

Determination of RT activity and protein concentration

Quantification of reverse transcriptase activity was carried out using the photometric enzyme immunoassay kit (Roche Applied Science) via incorporation of digoxigenin-labeled dUTP into DNA according to manufactures' instructions. The protein concentration was determined by the method of Bradford (23).

Molecular docking

Docking studies were carried out by the CDocker module (24) of Discovery Studio (Accelrys, San Diego, CA) using the crystal structure of M-MLVH⁻ RT determined at 1.80 Å resolution (PDB code 1MML) (25) as the binding enzyme (receptor molecule). Water molecules from the M-MLVH⁻ RT structure were removed prior to the docking. Binding



sites were identified by using the binding site identification tool in Discovery Studio. The ligand was generated with ChemSketch 11.0 (Advanced Chemistry Development, Toronto, Ontario, Canada) and exported as a *.mol file to the Discovery Studio. Hydrogen atoms were added to the protein and the ligand. According to the CDOCKER protocol, random conformations of the ligand were first translated into the binding site using a CHARMM-based molecular dynamics simulation scheme. The ligand poses were then searched using rigid-body rotations followed by simulated annealing with a grid potential. Final refinement of the ligand poses was carried out by a full-force field potential. Docking was carried out with the ligand bearing the spacer 1,6-diamino-hexane moiety.

Results

Expression of M-MLVH⁻ RT in *E. coli* M15 (pREP4)

Expression experiments of *E. coli* M15 (pREP4) transformed with pQERTH revealed that M-MLVH⁻ RT was overexpressed in the form of inclusion bodies, while a small percentage was observed in the supernatant. Induction experiments with varied IPTG concentrations (1 mM, 0.5 mM, and 0.1 mM) showed that the expression level was remained unaffected (Figure 1). The enzyme was solubilized in 50 mM Tris-HCl (pH 8.0) containing 8 M urea and refolded in 100 mM Tris-HCl (pH 8.0) containing 10 mM DTT and 20% glycerol.

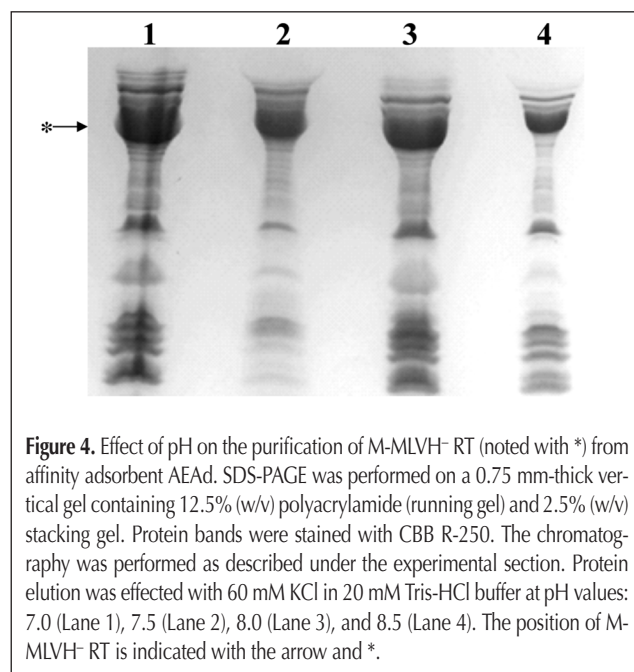
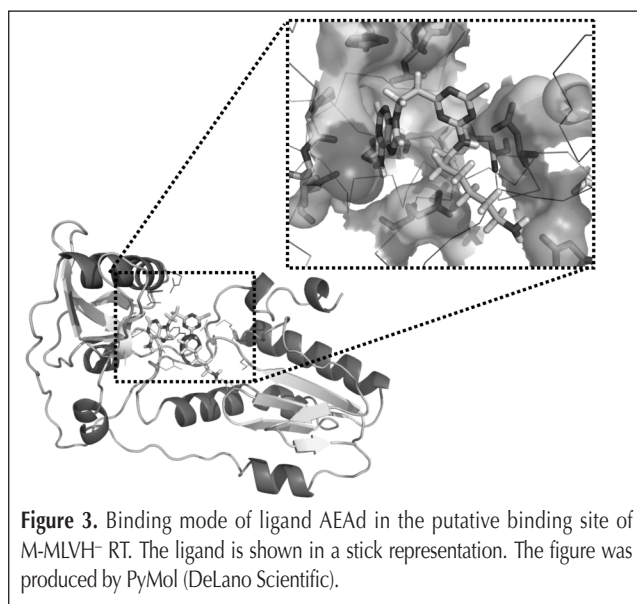
Screening of the adsorbent library and determination of binding capacity with M-MLVH⁻ RT

All adsorbents (Table I) were evaluated for their ability to bind and purify M-MLVH⁻ RT from refolded inclusion bodies extract. Assessment of the purifying effectiveness of the adsorbents was based on SDS-PAGE analysis, using all the amount of protein eluted from each adsorbent, thus ensuring direct comparability of the adsorbents. Examination of the gels (Figure 2) revealed that all adsorbents exhibited a significant variation in their

enzyme-purifying ability and recovery (elution). Specifically, adsorbents having no adenine or guanine moiety or having two purines (Table I, entries 1–7 and 24–26) bound the enzyme but presented either low purifying ability (Figure 2C: adsorbents ABP, AEAd-AEAd, and AEGu-AEAd) or low purifying ability and recovery (Figure 2A: adsorbents *o*ABS, *m*ABS, *p*ABS; Figure 2C: adsorbents AMS, AES, APP, and AEGu-AEGu). Furthermore, adsorbents bearing a purine and an anionic substituent on the triazine scaffold either failed to bind the enzyme (Figure 2D: adsorbents ABPad, AESGu, and APPGu) or bound the enzyme but presented low purifying ability and recovery (Figure 2B: adsorbents *o*ABSAd, *m*ABSAd, *p*ABSAd, *o*ABSGu, *m*ABSGu, and *p*ABSGu; Figure 2D: adsorbents AMSAd, AESAd, APPAd, AMSGu, and ABPGu). Finally, adsorbents bearing only adenine or guanine moiety (Table I, entries 8 and 9) bound the enzyme and presented good enzyme recovery; however, only adsorbent AEAd bearing the 9-aminoethyladenine-(1,6-diamine-hexane)-triazine ligand (Figure 2A) displayed also high purifying ability, which makes a promising tool for M-MLVH⁻ RT purification.

Molecular docking

Molecular docking studies were carried out to provide in silico structural information and help to locate the putative AEAd binding site on the M-MLVH⁻ RT molecule. The model for AEAd binding to the enzyme (Figure 3) was constructed based on the proposed dNTP binding site (25). The best results were obtained in a conformation in which the adenine substituent and the 1,6-diamine hexane spacer is transferred to the chloro-triazine ring (Figure 3). With this setting, the following binding mode of AEAd is obtained: The chloro-triazine ring is positioned close to Asp153 residue towards the solvent. Its secondary amine group makes a strong hydrogen bond with Asp153. The adenine ring points towards a small, comparatively hydrophobic pocket formed by residues Leu87, Pro111, Val112, His77, Trp185, and Phe156. The 1,6-diamino-hexane spacer is oriented towards the bulk solvent and makes no important interaction with other



amino acid residues or the peptide backbone except its terminal amino group which, in its free form, is hydrogen bonded with Asp224. However, after coupling of the NH₂-spacer to the agarose matrix, it is likely that this H-bond is no longer present.

Capacity of the AEA_d adsorbent for recombinant M-MLVH⁻ RT and the effect of ATP

Adsorption studies between solid adsorbent AEA_d and soluble recombinant M-MLVH⁻ RT, in the presence and absence of ATP, have shown that the adenine-nucleotide perturbs the equilibrium of the ligand-enzyme complex by approximately 30%, which indicates some degree of specificity between ligand and enzyme. The same adsorbent has shown a binding capacity of approximately 1 mg enzyme/g moist weight gel.

Purification of recombinant M-MLVH⁻ RT on the AEA_d adsorbent bearing 9-aminoethyladenine-(1,6-diamine-hexane)-triazine ligand

Prior to developing the purification protocol, the influence of pH on the binding and desorption conditions were investigated. At pH 7.5, affinity adsorbent AEA_d (Figure 4) displayed optimum behavior with regards to purifying ability and enzyme recovery. At higher pH values, either the purifying ability (pH 8.0) or the recovery (pH 8.5) of M-MLVH⁻ RT declined. Furthermore, at a lower pH of 7.0, the purifying ability declined. Consequently, pH 7.5 was chosen for the enzyme purification protocol.

With regard to the desorption conditions, the agents glycerol (30%), MgSO₄ (6 mM), MgCl₂ (6 mM), EDTA (10 mM), MnCl₂ (6 mM), and adenosine triphosphate (15 mM) were tested in 20 mM Tris-HCl buffer (pH 7.5). MnCl₂ led to the highest purification and recovery and, therefore, was adopted for the purification of M-MLVH⁻ RT. All other agents either failed to desorb the enzyme or to lead to acceptable purification (results not shown). SDS-PAGE analysis of the purified enzyme showed a major protein band corresponding to 56 kDa (Figure 5).

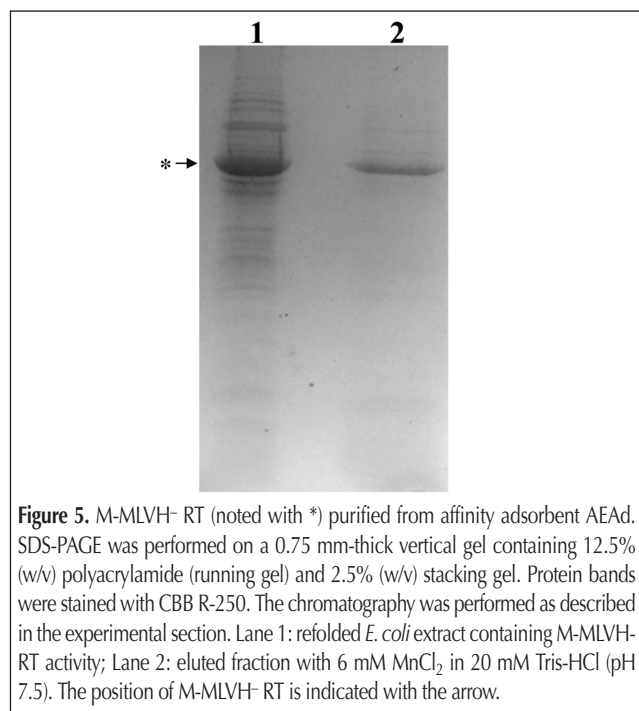


Figure 5. M-MLVH⁻ RT (noted with *) purified from affinity adsorbent AEA_d. SDS-PAGE was performed on a 0.75 mm-thick vertical gel containing 12.5% (w/v) polyacrylamide (running gel) and 2.5% (w/v) stacking gel. Protein bands were stained with CBB R-250. The chromatography was performed as described in the experimental section. Lane 1: refolded *E. coli* extract containing M-MLVH⁻ RT activity; Lane 2: eluted fraction with 6 mM MnCl₂ in 20 mM Tris-HCl (pH 7.5). The position of M-MLVH⁻ RT is indicated with the arrow.

Discussion

The comparison of the electrophoretic patterns obtained for all adsorbents (Figure 2) clearly reveals that adsorbent AEA_d presents the higher purifying ability. The encouraging behavior of the AEA_d ligand is supported by adsorption studies, which suggests the existence of some degree of specificity between immobilized AEA_d and soluble M-MLVH⁻ RT, after perturbation of the respective complex by ATP. The lower enzyme binding capacity of the AEA_d adsorbent, compared to the acclaimed higher (by up to 10-fold) for immobilized triazine dyes (26,27), may be counterbalanced by its effective enzyme purifying ability (Figure 2A and Figure 5). Therefore, adsorbent AEA_d was justifiably chosen for M-MLVH⁻ RT purification.

The predicted mode of interaction of the AEA_d with M-MLVH⁻ RT is shown in Figure 3. The binding may primarily be achieved by hydrophobic and electrostatic interactions that provide the driving force for ligand positioning and recognition. The bulk of interactions with the enzyme involve hydrophobic (Leu87, Pro111, Val112, Trp185, and Phe156) and hydrophilic residues (His77, Asp153). Apparently, this part of the enzyme structure is rather flexible and becomes ordered only upon complex formation (25). Because the docking approach does not take protein flexibility into account, it should be kept in mind that upon complexation with the triazinyl ligand certain amino acid side-chains may adopt a different conformation compare (25). In addition, this flexibility may also be important for ligand binding because it may require some additional space in this region, should the binding mode suggested by docking for the free ligands be adopted. The binding modes observed by molecular modelling, however, do not provide a clear clue of the relative ligand-binding affinities as they have been observed from the screening procedure (Figure 2).

However, one should consider that the presence of a large negatively charged substituent on the triazine ring may promote non-favorable interactions with the negatively charged Asp153, which is positioned close to the triazine ring. It is worthwhile noting that entropic and/or solvation/desolvation contributions also play an important role in determining binding affinities but are not adequately accounted for by the docking procedure.

A classical purification protocol for M-MLVH⁻ RT (20) combines ammonium sulfate precipitation, followed by three chromatographic steps (phosphocellulose, Heparin-Sepharose, and Mono-S chromatography). An alternative purification protocol employs metal chelate chromatography (known also as immobilized metal affinity chromatography, IMAC) followed by Mono-S ion exchange chromatography (34). However, IMAC adsorbents are restricted to His-tagged enzymes, which is an inherent drawback if considered for the purification for native RTs. Furthermore, the IMAC-based purification method for M-MLVH⁻ RT incorporates the protease thrombin for hydrolyzing the His-tag from the purified enzyme (34,35). We believe that the method presented in this report is simpler, as it employs a single chromatography and a low-cost elution agent (6 mM MnCl₂ in 20 mM Tris-HCl buffer, pH 7.5) and potentially can be applied to RTs from various sources. The purified M-MLVH⁻ RT was kept at -20°C for more than one year in 20 mM Tris-HCl (pH 7.5) containing 200 mM NaCl, 0.1 mM EDTA, 1 mM DTT,

0.01% Nonidet P-40, and 50% glycerol without appreciable loss of its activity, and it is used in-house for routine molecular biology applications.

Conclusions

M-MLVH⁻ RT from inclusion bodies of recombinant *E. coli* cells can be purified by affinity chromatography on agarose-immobilized 9-aminoethyladenine-(1,6-diamine-hexane)-triazine ligand (AEAd). The enzyme is desorbed from the adsorbent in the presence of 6 mM MnCl₂. The binding capacity of immobilized AEAd for M-MLVH⁻ RT was approximately 1 mg enzyme/g moist weight gel. Adsorption studies indicated some selectivity between immobilized AEAd and soluble M-MLVH⁻ RT because the respective complex was perturbed by ATP. The purified enzyme is suitable for routine molecular biology applications. The proposed purification method may be useful for the purification of HIV-RT necessary for *in vitro* evaluation of anti-AIDS drugs.

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