Biochimica et Biophysica Acta 965 (1988) 22–28
Elsevier

BBA 22900

Purification of bovine liver *S*-adenosylhomocysteine hydrolase by affinity chromatography on blue dextran-agarose

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(Received 30 July 1987)
(Revised manuscript received 21 December 1987)

Key words: Adenosyl homocysteine hydrolase; Affinity chromatography; Blue dextran; Adenosine; NAD; Enzyme stability

_S_-Adenosylhomocysteine (AdoHcy) hydrolase (adenosylhomocysteinase, EC 3.3.1.1) was purified from bovine liver by conventional protein purification procedures (differential centrifugation, ammonium sulfate fractionation and DEAE-cellulose chromatography) followed by affinity chromatography on blue dextran coupled to agarose. The enzyme was eluted from the blue dextran-agarose column with adenosine and the adenosine was removed by chromatography on Sephadex G-75. The affinity chromatography step resulted in a substantial increase in total AdoHcy hydrolase activity (about 600%) suggesting either removal of some inhibitory substance or a change in the structure of the protein producing a more catalytically efficient enzyme. The isolation procedure afforded over 3400-fold purification of the enzyme, which was shown to be homogeneous by polyacrylamide gel electrophoresis. Using high pressure liquid chromatography, the nucleotide content of the freshly purified enzyme was determined to be 2 mol of nicotinamide adenine nucleotide per mol of enzyme tetramer. The ratio of the reduced to the oxidized form of the nucleotide was correlated to the activity of the enzyme preparation.

Introduction

S-Adenosylhomocysteine (AdoHcy) is the product of biological transmethylation reactions that utilize *S*-adenosylmethionine (AdoMet) as the methyl donor. AdoHcy is a potent inhibitor of AdoMet-dependent methyltransferases in vitro, but this inhibition is normally minimized in vivo by the conversion of AdoHcy to adenosine and homocysteine by *S*-adenosylhomocysteine hydrolase (AdoHcy hydrolase, EC 3.3.1.1) [1,2]. This AdoHcy hydrolase-dependent catalyzed reaction is reversible and the equilibrium when studied in vitro is strongly in the direction of synthesis of AdoHcy [3]. However, under physiological conditions, the rapid enzymatic removal of adenosine and homocysteine by other metabolic pathways increases the net flux in the hydrolytic direction [3]. Because of its key role in regulating the intracellular concentration of AdoHcy and the activity of AdoMet-dependent methyltransferases, AdoHcy hydrolase has become an important target

Abbreviations: AdoHcy, *S*-adenosylhomocysteine; AdoHcy hydrolase, *S*-adenosylhomocysteine hydrolase; AdoMet, *S*-adenosylmethionine; CM-Sephadex, carboxymethyl Sephadex: DEAE-cellulose, diethylaminoethyl cellulose; DTT, dithiothreitol; SP-Sephadex, sulfopropyl Sephadex; DMSO, dimethyl sulfoxide.

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for the design of potential therapeutic agents (e.g., antiviral, antitumor, etc.) [4–8]. In order to elucidate the mechanism of action of potential inhibitors of AdoHcy hydrolase (e.g., nephanocin A) and rationally design more effective inhibitors, sources of homogeneous AdoHcy hydrolase are required.

There have been several reports of partial and complete purification of AdoHcy hydrolase from various sources including leaves of spinach beet [9], yellow lupin seeds [10], rat liver [11–13], calf liver [14], beef liver [2], *Dictyostelium discoideum* [15] and *Alcalegenes faecalis* [16]. These purifications have generally involved convenient protein purification procedures which are lengthy, afford incomplete separation and low recovery of enzyme activity. Affinity chromatography has recently been employed to purify the enzyme from human placenta [17]. Recently, our laboratory has encountered instability problems with the prokaryotic AdoHcy hydrolase, which could be correlated with the instability of the enzyme-bound NAD^+ involved in the catalytic process [18]. These data prompted us to identify a more rapid and efficient means of purifying the bovine liver AdoHcy hydrolase and study the relationship of its catalytic activity to its content of NAD^+. The results of our research are described in this paper.

Materials and Methods

All chemicals were of reagent grade. [2,8-^{3}H]Adenosine (40 mCi/mmol) was purchased from ICN Radiochemicals, Irvine, CA. Blue dextran-agarose, adenosine, aprotinin, DTT, phenylmethylsulfonyl fluoride, SP-Sephadex C-25, NAD^+, NADH and calf intestinal adenosine deaminase were obtained from Sigma Chemical Co, St. Louis, MO. [2,8-^{3}H]AdoHcy (specific activity, 1.8 mCi/mmol) was synthesized enzymatically from [2,8-^{3}H]adenosine and DL-homocysteine using partially purified bovine liver AdoHcy hydrolase, according to the method of Chabannes et al. [19].

Purification of AdoHcy hydrolase from bovine liver

All manipulations were conducted at 4° C. The initial steps in the purification (through DEAE-cellulose chromatography) were carried out according to Hershfield et al. [17] after certain modifications.

Bovine liver (1 lb) was minced in 100–200 ml of a cold solution of 1 mM EDTA and 1 mM DTT, containing 3 ml of aprotinin and 100 mg of phenylmethylsulfonyl fluoride (dissolved in 2 ml DMSO) to inhibit proteolysis and homogenized in a Waring blender for 10 min. The tissue homogenate was centrifuged at $10\,000 \times g$ for 60 min. The supernatant obtained from the above step was made 75 mM with respect to KCl and mixed with an excess volume of DEAE-cellulose (Cellex D, Biorad) that had been equilibrated with 25 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 1 mM DTT. KCl (75 mM) was also added to this equilibration buffer. The suspension was left undisturbed for 15 min and filtered into a flask containing 100 mg of phenylmethylsulfonyl fluoride. The matrix was rinsed with about 50 ml of the equilibration buffer. Solid $(NH_4)_2SO_4$ was slowly added to the combined filtrate to achieve 70% saturation. The solution was stirred gently and then left undisturbed. After 60 min, the precipitate was collected by centrifuging at $10\,000 \times g$ for 30 min. The precipitate was dissolved in 50–70 ml of the same Tris buffer used for equilibration and dialyzed for 48 h against this buffer with four to six changes.

The dialyzed material was loaded onto a column (5×28 cm) of DE-52 (Whatman) cellulose that had been equilibrated with 25 mM Tris-HCl, pH 7.4 containing 1 mM EDTA, 1 mM DTT and 25 mM KCl. Materials bound to the column were eluted with a linear gradient formed from 1 liter of the buffer, containing 50 mM KCl and 1 liter of buffer containing 175 mM KCl. Fractions containing AdoHcy hydrolase activity were pooled, adjusted to pH 6.0 with acetic acid and mixed with an excess volume of CM-Sephadex (Sigma) that had been equilibrated with 10 mM Tris-acetate buffer, pH 6.0. After 5 min, the slurry was filtered into a flask containing 10 ml of 1 M Tris-HCl, pH 7.4. The gel was washed with 50 ml of Tris-acetate buffer, pH 6.0 and filtered into the same flask. The enzyme was concentrated by pressure ultrafiltration to a volume of 100 ml.

The concentrated enzyme was loaded batchwise onto a blue dextran-agarose (Sigma) column (1.5×20 cm) which had been equilibrated with 10

mM Tris-HCl, pH 6.8, containing 1 mM EDTA and 1 mM DTT. The column was washed with 60 ml of the equilibration buffer, followed by 60 ml of buffer containing 0.5 mM adenosine. AdoHcy hydrolase which bound to blue dextran-agarose was preferentially eluted by adenosine. All fractions containing enzyme activity were pooled and passed through an Amicon protein concentrator. The membrane (PM 30) fitted on the concentrator had a 30000 molecular weight cut-off. This process permitted the removal of adenosine and the enzyme sample was concentrated to 1–2 ml.

The concentrated enzyme was loaded onto a Sephadex G-75 column (1 × 30 cm) equilibrated with 10 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 1 mM DTT. AdoHcy hydrolase was eluted in the void volume by the same buffer. Fractions containing AdoHcy hydrolase activity were pooled and stored at −20 °C in the presence of 5% glycerol.

Assay of AdoHcy hydrolase activity

AdoHcy hydrolase was assayed in the hydrolytic direction using a modified procedure of Richards et al. [14]. The reaction mixture (total volume 500 μl) contained 150 mM potassium phosphate buffer, pH 7.6, 1 mM EDTA, 740 μM [2,8-^{3}H]AdoHcy (specific activity, 1.8 mCi/mmol) and 4 units of adenosine deaminase. The reaction was initiated by the addition of AdoHcy hydrolase and incubated for 5 min at 37 °C. The reaction was terminated by the addition of 100 μl of 5 N formic acid. The mixture was then layered onto an SP-Sephadex C-25 column (1.2 × 2.4 cm) equilibrated with 0.1 N formic acid. The first 0.5 ml wash was discarded and the column was eluted with 8 ml of 0.1 N formic acid. The eluate containing [2,8-^{3}H]inosine was counted by a liquid scintillation spectrophotometer.

Determination of protein

Protein was measured by the method of Bradford [20] using bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis

The homogeneity of purified AdoHcy hydrolase was verified by SDS gel electrophoresis in 7.5% polyacrylamide gel, pH 8–9, according to King and Laemmli [21]. The gel, stained with Coomassie blue, was scanned using a Shimadzu dual wavelength scanner (Model CS 930), fitted with a data recorder.

Determination of AdoHcy hydrolase-associated NAD^+ and NADH by HPLC

Lyophilized AdoHcy hydrolase (64 μg) was dissolved in 0.1 M potassium phosphate buffer, pH 7.0 and treated with 50 μl of 5% SDS (the final concentration of SDS being about 1%) for 10 min at 4 °C to release the bound nucleotides. The reaction mixture was filtered through an Amicon micro-partition system at 4 °C. A sample of the filtrate was analyzed for the presence of the nucleotides (NAD^+/NADH) by HPLC on a C_{18} reverse phase column (ODS Hypersil, 150 × 4.6 mm), eluted with 0.1 M sodium phosphate buffer, pH 7.0, containing 2.5% methanol at a flow rate of 1 ml per min. Analyses were performed using an HPLC system equipped with a Beckman 112 solvent delivery module and a programmable integrator from Perkin Elmer (model LC 100). The chromatograms were monitored at 254 and/or 340 nm using a Spectroflow 783 programmable detector.

Results and Discussion

Dye ligand chromatography has gained widespread popularity in recent years as an affinity medium for a plethora of proteins [22]. Recent literature on nucleotide-dependent enzymes deals with their purification by affinity elution chromatography on immobilized blue dextran. Blue dextran is a semi-specific high molecular weight compound substituted with the monochlorotriazinyl dye Cibacron blue F3GA, which can mimic a polynucleotide, presumably due to an attraction of the blue chromophore moiety to a nucleotide-binding site of the enzyme [22]. It was based on this information and the fact that AdoHcy hydrolase contains NAD^+, NADH and adenosine-binding sites that we attempted to purify AdoHcy hydrolase from the bovine liver using affinity chromatography on blue dextran-agarose.

The various steps involved in the purification procedure are listed in Table 1. The initial steps in the purification methodology are basically those

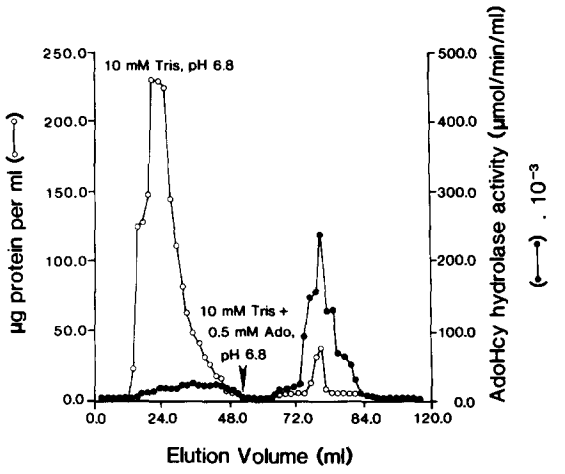

Fig. 1. Elution profile of bovine liver AdoHcy hydrolase on blue dextran-agarose. A column of blue dextran-agarose (1.5 × 20 cm), equilibrated with 10 mM Tris-HCl, pH 6.8, containing 1 mM EDTA and 1 mM DTT was loaded with an aliquot of bovine liver AdoHcy hydrolase (10.6 mg). The column was eluted with the same buffer (60 ml), followed by 60 ml of buffer containing 0.5 mM adenosine. Fractions were assayed for enzyme activity and protein concentration was determined as described in the Materials and Methods section.

described by Hershfield et al. [17]. Fig. 1 shows a representative elution profile of AdoHcy hydrolase from blue dextran-agarose. AdoHcy hydrolase which is specifically bound to the affinity column is eluted by the addition of 0.5 mM adenosine in the equilibration buffer. Adenosine, which has a very high affinity for bovine liver AdoHcy hydrolase, was found to be the most effective eluant. As shown in Table I, we were able to recover approximately 600% of the initial activity with about 3000-fold purification. The significant increase in the total activity occurred during the blue dextran-agarose chromatography step. This increase in the activity of the enzyme may result from the nucleotide-mimicing dye chromophore of blue dextran inducing a conformational change in the enzyme. Enhancement of enzyme activity was earlier observed in the case of purification of isocitrate dehydrogenase [23] and NADH-dependent nitrate dehydrogenase [24] by dye affinity chromatography. The authors attributed the increase in the enzyme activity to the removal of inactive enzymes or a change in the stereospecificity of the purified sample. The purified sample of NADH-dependent nitrate dehydrogenase displayed a new stereospecificity for the 'A' form of NADH. In earlier studies, our laboratory showed that the activity of bovine liver AdoHcy hydrolase could be increased upon incubation with exogenous NAD^+ [26]. In addition, Hohman et al. [27] have reported that native AdoHcy hydrolase from *D. discoideum* can bind an additional mol of NAD^+ per tetramer. This NAD^+ was suggested to serve a regulatory function and was thought to bind to a site distinct from the catalytic site. Since the chromophore of blue dextran has been reported to mimic nucleotides like NAD^+, it is not unreasonable to suggest that blue dextran-agarose chromatography may induce changes in the structure of the enzyme and thus alter its catalytic activity.

TABLE I

PURIFICATION OF *S*-ADENOSYLHOMOCYSTEINE HYDROLASE FROM BOVINE LIVER

See the Materials and Methods section for experimental details.

Steps in purification	Total activity (μmol/min)	Total protein (mg)	Spec. act. (μmol/min per mg)	Fold
10000 × *g* supernatant	24	55760	0.0004	1
$(NH_4)_2SO_4$ precipitate	34	31900	0.0011	2.5
DEAE-cellulose	70	5000	0.026	60
Blue dextran-agarose + Sephadex G-75	126	97	1.3	3250

This method of purification of AdoHcy hydrolase is evidently superior to methods reported which obtained recoveries in the range of 8–34% [9–18]. We have found that one of the major contaminants of AdoHcy hydrolase preparations is adenosine deaminase. The blue dextran-agarose chromatography step followed by gel filtration on Sephadex G-75 eliminates the residual adenosine deaminase from our preparations. This was confirmed by assaying for adenosine deaminase by the method of Bissbort et al. [25] (data not shown). In this case, the relative affinity of blue dextran and adenosine, presumably for the same site on the hydrolase enzyme, facilitated the selective de-

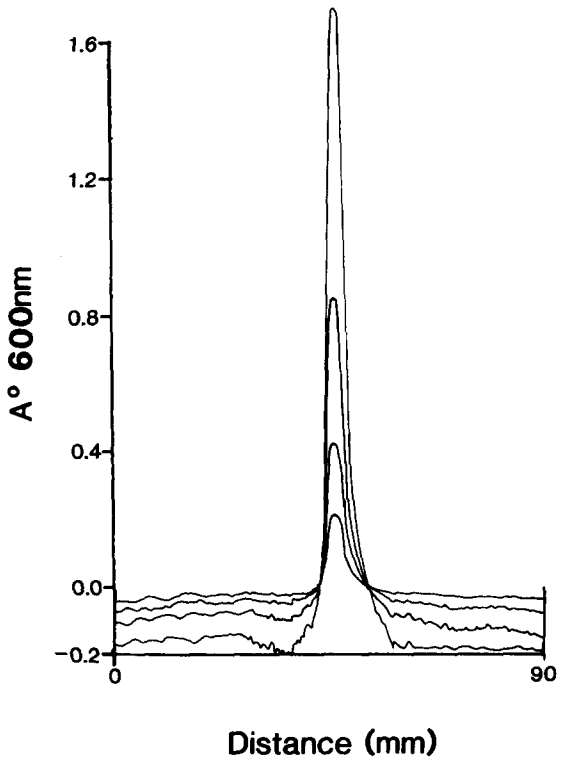

Fig. 2. The gel scan of purified bovine liver AdoHcy hydrolase after polyacrylamide gel electrophoresis. Purified samples (5, 7.5, 12.5 and 17 μg) of bovine liver AdoHcy hydrolase were electrophoresed on 7.5% polyacrylamide gel as described in Materials and Methods. The gel was stained with Coomassie blue and scanned at 600 nm using a Shimadzu dual wavelength scanner with the photomode adjusted for absorbance reflection as described in the Materials and Methods section.

sorption of AdoHcy hydrolase from the affinity matrix.

We established the homogeneity of our purified enzyme by SDS gel electrophoresis. The gel scan showed one sharp peak and this is depicted in Fig. 2. However, some preparations of AdoHcy hydrolase exhibited two close peaks during polyacrylamide gel electrophoresis, suggesting non-equivalency in the subunits. Similar observations were reported earlier when AdoHcy hydrolase was electrophoresed in the presence of SDS and during isoelectric focusing [10,1415].

It was recently shown that the catalytic activity of AdoHcy hydrolase is directly related to its NAD^+ content [18]. The nucleotide content of AdoHcy hydrolase purified by the method of Palmer and Abeles [2], followed by affinity chromatography on AdoHcy-agarose was reported to be 2 mol of NAD^+/NADH per enzyme tetramer, the NAD^+ content being 0.85 mol per enzyme tetramer [18]. In this study we compared the NAD^+ content of the AdoHcy hydrolase prepared by chromatography on blue dextran with the data of NAD^+ content reported by others [17,26,27].

In earlier studies, the nucleotide content of AdoHcy hydrolase was measured by a two-step process [26,27]. In the first step, NAD^+ was enzymically converted to NADH. In the following step NADH was quantified either by a spectrophotometric method or by measuring its intrinsic fluorescence [26,27]. Recently, Hershfield et al. reported an HPLC procedure to determine the nucleotide content of AdoHcy hydrolase from the human placenta [17]. Although this method detected the NAD^+ content of the enzyme directly, the NADH content was quantified indirectly by its acid degraded product. We have developed an HPLC method to directly quantify the amounts of NAD^+, NADH, adenine, adenosine and other ligands bound to microgram quantities of AdoHcy hydrolase. Both NAD^+ and NADH were quantified at pH 7.0, their optimum pH for stability. At acidic pH, decomposition of NADH occurs at the labile nicotinamide ring. In basic solution, oxidation of NADH can occur as well as cleavage at the sugar or phosphate groups [28]. However, NADH can be distinguished from NAD^+ as well as from most of its breakdown products by the characteristic absorbance of NADH at 340 nm. The major breakdown products not having a maximum at 340 nm, have ultraviolet spectra similar to each other and to the spectrum of NAD^+, with maxima between 254 and 290 nm. Hence, we could easily monitor any degradation of NAD^+ and/or NADH with a programmable detector. A typical HPLC profile is shown in Fig. 3. A fresh preparation of AdoHcy hydrolase obtained by blue dextran chromatography contained about 2 mol of NAD^+/NADH per enzyme tetramer (Table II). This finding is in agreement with the earlier work reported from our laboratory using a fluorescent method of detection [26] and with that reported by Hohman et al. for the hydrolase from *D. discoideum* [27]. Variable amounts of adenosine were also detected in our enzyme preparations. Ueland and Saebo [29] had observed that a frac-

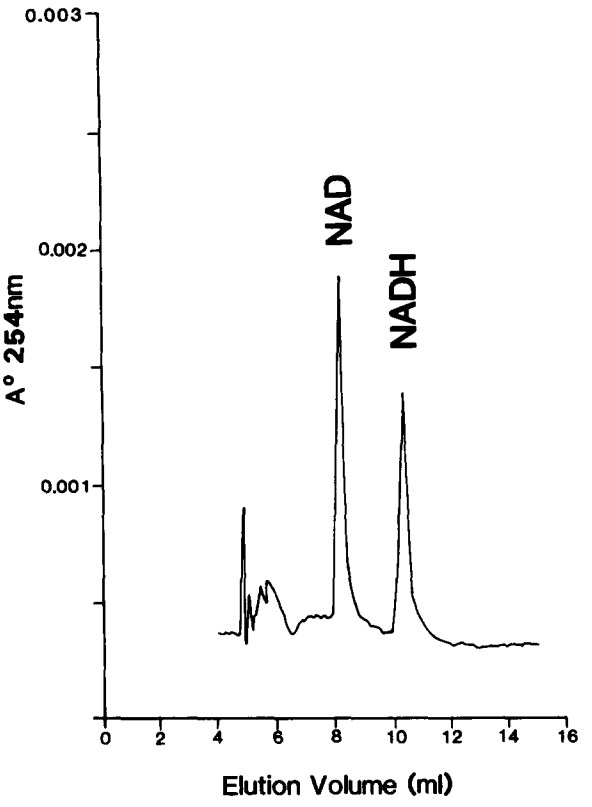

Fig. 3. HPLC analysis of ligands bound to bovine liver hydrolase. Purified bovine liver AdoHcy hydrolase was treated with 5% SDS and analyzed on a C_{18} reverse phase column as described in the Materials and Methods section. The injected sample (equivalent to 85 pmol of protein) contained 96 pmol of NAD^+ and 72 pmol of NADH.

TABLE II

NUCLEOTIDE CONTENT OF NATIVE AND DENATURED *S*-ADENOSYLHOMOCYSTEINE HYDROLASE

Lyophilized bovine liver AdoHcy hydrolase (64 μg) was dissolved in 0.1 M potassium phosphate buffer, pH 7.0 and treated with 5% SDS at 4°C. A sample of the above extract was analyzed for its nucleotide content and assayed for its catalytic activity as described in the Materials and Methods section.

Residual enzyme activity (%)	mol/mol of enzyme tetramer		
	NAD^+	NADH	adenine
100	1.1	0.8	0
40 [a]	0.36	1.1	0
0 [a]	0	n.d. [b]	0.047

[a] The enzyme preparation was stored at room temperature.
[b] n.d., not determined.

tion of adenosine bound to hydrolase is converted to adenine or a substance liberating adenine and that a significant amount of this is dissociated from the enzyme. Accordingly, we checked for the presence of adenine in our enzyme preparations, since our eluting buffer contained 0.5 mM adenosine. No adenine was released from fresh preparations of AdoHcy; however, traces of adenine were detected when the enzyme was denatured at room temperature (Table II), We also report the presence of NADH in fresh preparations of AdoHcy hydrolase. As in the case of the bacterial enzyme, over a course of time, the bovine liver AdoHcy hydrolase showed a direct correlation between the NAD^+ content and the residual activity of the enzyme sample [18]. It is interesting to note that *A. faecalis* AdoHcy hydrolase does not have affinity for blue dextran-agarose and thus this method cannot be employed for purification of the bacterial enzyme (data not shown). This difference between the bovine liver enzyme and the bacterial enzyme may relate to the fact that the bovine liver enzyme has two nucleotides (NAD^+/NADH) bound per tetramer [26] whereas the bacterial enzyme has six nucleotides (NAD^+) bound per hexamer [16,18]. All of the nucleotide-binding sites on the bacterial enzyme are occupied, thus the enzyme is unable to bind the chromophore on the blue dextran-agarose. In contrast, the bovine liver enzyme may have unoccupied nucleotide-binding sites, thus allowing for interaction with the chromophore on blue dextran.

NADH was detected in bovine liver AdoHcy hydrolase when the enzyme was fresh [26] and when it was inactivated by 2′-deoxyadenosine [30] and neplanocin A [26]. NADH was also found on freshly prepared AdoHcy hydrolase from *D. discoideum* [27] and *A. faecalis* [18]. However, the function of the enzyme-bound NADH remains unclear. A possible explanation is that this NADH is the result of in vivo inactivation involving reduction of NAD^+. It could also be the result of inactivation during the isolation procedure. The amino acid sequence of AdoHcy hydrolase from rat liver was recently derived from the cDNA sequence [31]. Although the region in the protein featuring a NAD^+-binding domain was identified, there is no conclusive evidence as to the number of moles of nucleotides bound per mole of enzyme

tetramer. The authors also suggested the existence of a dinucleotide fold in AdoHcy hydrolase from rat liver. Although blue dextran does not serve as a selective probe for the dinucleotide fold in proteins, several researchers in the field of dye–protein interaction have suggested and provided data that support the theory that blue dextran will bind to proteins possessing the dinucleotide fold [32].

Affinity chromatography has been employed before to purify AdoHcy hydrolase. However, synthetic media like immobilized dyes can prove useful because of their low cost, high binding capacity, regenerability and durability. In summary, it can be stated that this is a more efficient and less time-consuming method for the purification of bovine liver AdoHcy hydrolase. The method yields a substantial increase in the amount of activity recovered. AdoHcy hydrolase purified by this method contains both NAD^+ and NADH. The loss of enzymatic activity in the course of time can be correlated with the disappearance of NAD^+ and appearance of NADH.

Acknowledgement

This work was supported by a research grant from the National Institute of General Medical Sciences (GM 22357).

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