Experimental Parasitology 129 (2011) 368–374

Contents lists available at SciVerse ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Purification and characterization of *Plasmodium yoelii* adenosine deaminase

Sarika Yadav [a], Jitendra Kumar Saxena [a,*], U.N. Dwivedi [b]

[a] Division of Biochemistry, Central Drug Research Institute (Council of Scientific and Industrial Research), Post Box No. 173, Chatter Manzil Palace, Lucknow 226001, India
[b] Department of Biochemistry, Lucknow University, Lucknow 226001, India

ARTICLE INFO

Article history:
Received 18 February 2011
Received in revised form 26 August 2011
Accepted 31 August 2011
Available online 16 September 2011

Keywords:
Adenosine deaminase
Drug resistance
Inhibitor
Malaria
Plasmodium yoelii
Protozoa
Purine salvage pathway

ABSTRACT

Plasmodium lacks the de novo pathway for purine biosynthesis and relies exclusively on the salvage pathway. Adenosine deaminase (ADA), first enzyme of the pathway, was purified and characterized from *Plasmodium yoelii*, a rodent malarial species, using ion exchange and gel exclusion chromatography. The purified enzyme is a 41 kDa monomer. The enzyme showed K_m values of 41 μM and 34 μM for adenosine and 2′-deoxyadenosine, respectively. Erythro-9-(2-hydroxy-3-nonyl) adenine competitively inhibited *P. yoelii* ADA with K_i value of 0.5 μM. The enzyme was inhibited by DEPC and protein denaturing agents, urea and GdmCl. Purine analogues significantly inhibited ADA activity. Inhibition by *p*-chloromercuribenzoate (*p*CMB) and *N*-ethylmaleimide (NEM) indicated the presence of functional –SH groups. Tryptophan fluorescence maxima of ADA shifted from 339 nm to 357 nm in presence of GdmCl. Refolding studies showed that higher GdmCl concentration irreversibly denatured the purified ADA. Fluorescence quenchers (KI and acrylamide) quenched the ADA fluorescence intensity to the varied degree. The observed differences in kinetic properties of *P. yoelii* ADA as compared to the erythrocyte enzyme may facilitate in designing specific inhibitors against ADA.

1. Introduction

Malaria caused by the protozoan parasites *Plasmodium falciparum*, *Plasmodium ovale*, *Plasmodium vivax* and *Plasmodium malariae* is the major cause of mortality worldwide. It affects more than 300 million people causing about 2 million deaths annually (Fidock et al., 2004; WHO, 2008). Due to the emergence of drug-resistant strains of *Plasmodium*, it is getting difficult to control the disease with existing anti-malarials (Blair et al., 2006; Restrepo-Pineda et al., 2008). Thus, there is an urgent need to identify new drug targets and develop new pharmacophores with unique structures and mode of action. Targeting the parasite metabolic pathways that lead to the formation of functional and structural components of the parasite, can be a good strategy. The de novo pathway of purine biosynthesis is absent in most parasitic protozoa and the entire requirement of purine nucleotides for RNA and DNA synthesis is met through the salvage of preformed purine bases from host (Carter et al., 2008; Downie et al., 2008). Therefore, enzymes involved in this pathway can be utilized as potential targets to develop new anti-malarial compounds (Berg et al., 2010). Adenosine deaminase (ADA; EC 3.5.4.4), the first enzyme of the salvage pathway, catalyses the conversion of adenosine into inosine which, upon subsequent conversion, yields hypoxanthine, the major precursor for purine salvage pathway (Kicska et al., 2002).

The present paper reports the purification and characterization of *Plasmodium yoelii* ADA.

2. Material and methods

2.1. Chemicals

Adenosine, 2′-deoxyadenosine, EHNA, inosine, 2′-deoxyinosine, 6-mercaptoguanosine, adenine, 5′-amino-4-imidazole-carboxamide, DEPC, NEM, *p*CMB, acrylamide, potassium iodide (KI), urea, GdmCl, EDTA, Freund's adjuvants were obtained from Sigma (St. Louis, USA). Pre-stained SDS–PAGE protein markers were obtained from MBI, Fermentas. DEAE-Sephadex, Sephadex G-100, protein markers were obtained from Amersham Biosciences.

2.2. Infection maintenance

Swiss mice (20–25 g) were intra-peritoneally injected with *P. yoelii* parasitized RBCs in 3% tri-sodium citrate (pH 7.4) containing 0.85% NaCl.

Abbreviations: EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; *p*CMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide; EDTA, ethylenediamine tetraacetic acid; GdmCl, guanidinium hydrochloride; DEPC, di-ethyl pyrocarbonate.

* Corresponding author. Fax: +91 522 2623405.
E-mail address: jkscdri@yahoo.com (J.K. Saxena).

0014-4894/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2011.08.021

Table 1
Purification of *P. yoelii* adenosine deaminase.

Steps	Total protein (mg)	Activity (Units)	Sp. activity (U/mg protein)	Yield (%)	Purification (fold)
(a) Crude	28.0	2.5310	0.0903	100	1.0
(b) Ammonium sulfate (35–70%)	4.98	0.9722	0.1952	38.4	2.2
(c) DEAE-Sephadex	0.710	0.7253	1.0215	29.0	11.3
(d) Sephadex G-100	0.230	0.5185	2.25	20.5	25.0

One unit is defined as the amount of enzyme which deaminates one micromole of adenosine to inosine per min.

2.3. Isolation of parasites from infected blood

Blood collected from infected mice at 40–50% parasitaemia by cardiac puncture was passed through the CF-11 column to remove leukocytes (Kapoor and Banyal, 2011). The resultant infected erythrocytes were collected by centrifugation at 800*g* for 5 min and parasites were liberated by adding an equal volume of 0.15% (w/v) saponin in 1 × phosphate-buffered saline (PBS, pH 7.4). Parasites were sedimented by centrifugation at 1300*g* for 5 min. The parasite pellet was washed 5 times with ice cold PBS and stored at −80 °C until used.

2.4. Purification of ADA

Parasites were sonicated by Ultrasonic processor (Heat system Inc., Model-XL-2020) with pulse-rest cycle (5 cycles, 10 s. pulse at 25 W with 1 min interval) and supernatant was collected by centrifugation at 1300*g* for 30 min at 4 °C. The ADA precipitated by ammonium sulfate saturation between 35% and 70%, was re-suspended in Buffer A [50 mM potassium phosphate buffer (pH 7.4) containing 1 mM β-mercaptoethanol] and dialyzed against the same buffer. The dialyzed protein (2.0 ml) was loaded on the DEAE-Sephadex column (21 cm × 1.5 cm) pre-equilibrated with Buffer A and was eluted with continuous gradient of NaCl (0.1–1.0 M). The fractions containing ADA activity were pooled, concentrated to 2.0 ml and applied onto the Sephadex G-100 column (30 cm × 1.5 cm). Protein was eluted by Buffer A and fractions showing ADA activity were pooled. The purity of ADA was checked on 10% SDS–PAGE according to Laemmli (1970).

2.5. Enzyme assay

ADA activity was estimated spectrophotometrically at 265 nm by the modified method of Kalckar (1947). The 1.0 ml reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.4) and 30 μM adenosine. The protein concentration was measured by the method of Bradford (1976).

2.6. Kinetic investigations

K_m values for adenosine and 2′-deoxyadenosine were determined by Michaelis–Menten equation. pH–activity profile was determined between pH 6.5–9.0 using potassium phosphate buffer, pH 6.5–8.0 and Tris–Cl buffer, pH 8.0–9.0. The effect of temperature was determined by incubating the enzyme at different temperatures for 10 min before measuring activity.

Effect of various inhibitors *viz*. EHNA, *p*CMB, NEM, DEPC, EDTA, purine analogues, and protein denaturing agents (Urea and GdmCl) was studied by incubating the enzyme with inhibitors for 10 min at room temperature before measuring activity. The effect of known anti-malarials was also studied in similar manner.

2.7. Generation of polyclonal antibodies

Purified ADA (250 μg) was emulsified with equal volume of Freund's complete adjuvant and injected subcutaneously in a 6 months old rabbit. Two booster doses of 200 μg ADA were given after 3rd and 5th weeks, in Freund's incomplete adjuvant. Seven days after the second booster, the rabbit was bled to collect serum. Anti-ADA antibody titer was measured by ELISA and specificity was confirmed by Western blotting.

2.8. Determination of subunit and native molecular weight

The subunit molecular weight of ADA was determined on SDS–PAGE (10% w/v) according to Laemmli (1970). Native molecular weight was determined using a Superdex 200HR 10/300 column, pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.5) with AKTA-FPLC (Amersham) at flow rate of 0.35 ml/min. Standard protein markers *viz*. Ferritin (440 KDa), alcohol dehydrogenase (150 KDa), albumin (67 KDa) and carbonic anhydrase (29 KDa) were used to calibrate the column. The molecular weight of ADA was determined by plotting the curve between

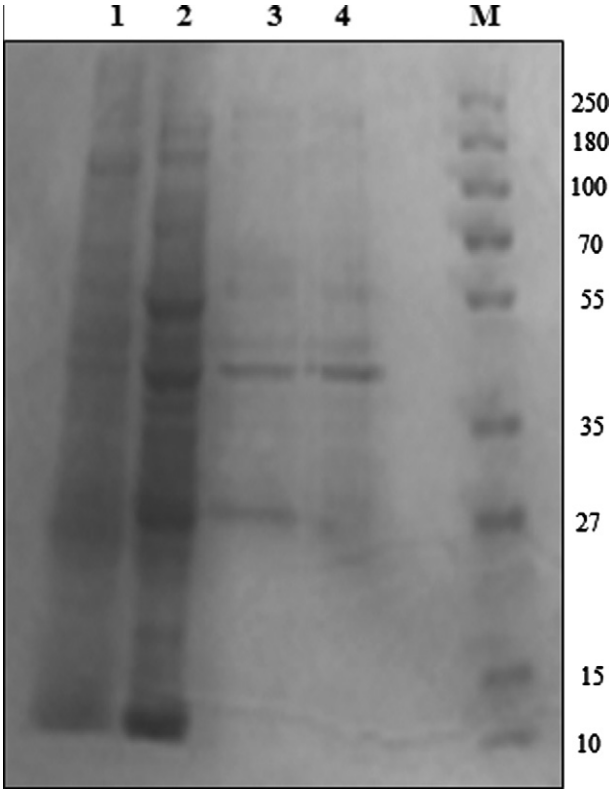

Fig. 1. Analysis of purified *P. yoelii* ADA on 10% SDS–PAGE. *Lane 1*: Parasite lysate; *Lane 2*: ammonium sulfate fraction (35–70%); *Lane 3*: DEAE-Sephadex fraction (eluted with 0.2 M NaCl); *Lane 4*: Sephadex G-100 fraction; and *Lane M*: pre-stained protein markers (kDa).