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Plasmodium knowlesi : Partial Purification and Characterization of NADP-Glutamate Dehydrogenase

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ABSTRACT

An NADP-linked glutamate dehydrogenase (L-Glutamate dehydrogenase NADP-oxidoreductase, EC 1.4.1.4.) was purified 181 folds from Plasmodium knowlesi (simian malaria parasite)by ammonium sulfate fractionation, gel filtration and hydroxylapatite column chromatography. The molecular weight of the enzyme was found to be 295,000 as determined by gel filtration. The enzyme appeared to be heat stable (4 h at 56°C) and activated about 39% and 14% by KCl and NaCl respectively. It catalysed the amination of α-Ketoglutarate and the deamination of glutamate with optimum activity at pH 7.4 and 8.6 respectively. Hyperbolic kinetics were observed for the substrates and cofactors yielding Km values of 0.25±0.02mM for α-Ketoglutarate, 1.3±0.2mM for ammonium acetate, 0.011±0.001mM for NADPH, 1.8±0.1mM for glutamate and 0.050±0.002 mM for NADP. The amination reaction was about 10 times more active as compared to the deamination reaction. Purine nucleotides did not show any effect on enzyme activity. These results suggest that the amination reaction may predominate in P.knowlesi parasites.

INTRODUCTION

Glutamate dehydrogenase (L-glutamate dehydrogenase NADP-oxidoreductase, EC 1.4.1.4, GDH) catalysing the interconversion of glutamate and α-ketoglutarate is of considerable importance due to its pivotal role in linking of protein and carbohydrate metabolism as well as in providing auxillary energy source in many prokaryotic and eukaryotic organisms including certain protozoan and helminth parasites (Cazzulo et al., 1977; Mustafa et al.,1978; Sherman,

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1979; Smith et al., 1975; Turner and Lushbaugh, 1988). The enzyme has been demonstrated in many species of malarial parasites and has been shown to be different from that of vertebrate tissues as it requires NADP instead of NAD as cofactor (Sherman,1979). The NADP-specific glutamate dehydrogenase appears to be useful marker enzyme for malaria parasites as it is not present in red cells. A parasite specific single isoenzymic form of NADP-GDH has been shown in rodent, simian as well as human malarial parasites (Carter, 1978;Carter and Walliker, 1977; Watts et al., 1988a). Though a considerable amount of work has been done on the characterization of NADP-specific glutamate dehydrogenase from a number of malaria parasites, there is no conclusive evidence regarding the exact role it is playing in the parasite metabolism. In some species of malaria parasites, the NADP-GDH has been shown to be a well documented source of endogenous NADPH production (Sherman et al., 1971; Walter et al., 1974) while in others it has been suggested to have a predominant role in the amination reaction i.e. glutamate production (Langer et al., 1970; VanderJagt et al., 1989). In the present communication we describe the partial purification and characterization of NADP-linked glutamate dehydrogenase from *P.knowlesi* and its possible significance in parasite metabolism.

MATERIALS AND METHODS

Parasites: *Plasmodium knowlesi* infection was maintained in rhesus monkeys (*Macaca mulatta*) of either sex weighing 3-6 kg by intravenous infusion of $1X10^6$ schizont infected red blood cells from an infected donor monkey to a healthy monkey. Parasitaemia was monitored by microscopic examination of giemsa stained thin blood smears. Blood was collected at about 40% parasitaemia(mainly schizonts)in acid citrate dextrose and washed with phosphate buffered saline (PBS, pH 7.2) by centrifuging at 800Xg. The platelets and leucocytes from the infected blood were removed following the procedure of Scheibel and Miller (1969), the schizonts were purified using one step percoll gradient as described elsewhere (Kaushal et al., 1988).

Preparation of homogenate: A twenty percent homogenate of the schizonts was prepared in 25mM triethanolamine buffer (pH 7.4) containing 1 mM β-mercaptoethanol (βME) and 0.1 mM ethylene diamine tetraacetic acid (EDTA) in a potter Elvehjem homogeniser under ice cold conditions. The homogenate was centrifuged at 10,000Xg for 30 min (4^oC) and the supernatant obtained was used as the enzyme source. βME and EDTA were found to be useful in stabilizing the enzyme activity and were therefore added to all buffers.

Enzyme assay: The activity of glutamate dehydrogenase(GDH) was estimated by the method of Schmidt and Schmidt (1983) with slight modifications. The reaction mixture in a total volume of 3.0 ml contained 25mM triethanolamine buffer (pH 7.4); 3.3 mMα-Ketoglutarate(α-KG), 3.3 mM ammonium acetate and 0.2mM NADPH. The reverse reaction with glutamate as substrate and NADP as cofactor was estimated according to the method of Strecker (1955) as modified by Sherman et al.(1971). One unit of enzyme is defined as the amount of enzyme that catalyzes the conversion of 1 nmoles of coenzyme min^{-1}. Specific activity is expressed as enzyme units

mg^{-1} protein. The protein contents of all the samples, after TCA precipitation, were estimated by the method of Lowry et al. (1951).

Enzyme purification: Glutamate dehydrogenase of *P.knowlesi* was purified by ammonium sulphate fractionation followed by molecular sieving and adsorption chromatography. All steps performed at 4-8°C unless otherwise stated. The crude parasite extract was brought upto 40% saturation with respect to ammonium sulphate and was allowed to stand for 3 h in cold with gentle stirring. The precipitated proteins were removed by centrifugation at 10,000Xg for 30 min(4°C). The supernatant was taken to 75% saturation of ammonium sulphate and again stirred for 3 h in cold. The precipitate containing most of the GDH activity was recovered by centrifugation at 10,000Xg (30 min) and dissolved in minimum volume of 10mM phosphate buffer (pH 7.0) containing 1 mM βME and 0.1 mM EDTA. The 40-75% ammonium sulphate fraction was applied to Sephadex G-200 column (1.6 X 30.5cm) pre-equilibrated with 10mM phosphate buffer (pH 7.0) containing 1 mM βME and 0.1 mM EDTA. The column was washed with the same buffer and 2.0 ml fractions were collected at a flow rate of 12 ml/hr until the GDH was eluted. Fractions from Sephadex G-200 column having GDH activity were pooled and passed through a small hydroxylapatite column (1.0 X 1.2 cm) which had been pre-equilibrated with 10mM phosphate buffer (pH 7.0) containing 1mM βME and 0.1 mM EDTA. After washing the column with 20 mM and 50 mM phosphate buffer (pH 7.0) the enzyme was eluted with 150 mM phosphate buffer (pH 7.0). The fractions containing high enzyme activity were pooled and used for studying the properties of GDH.

Molecular Weight Determination: The molecular weight of *P.knowlesi* GDH was determined by gel filtration through Sephadex G-200 column according to the method of Andrews (1965) using standard proteins of known molecular weights viz. bovine liver glutamate dehydrogenase (335,000) bovine liver catalase (232,000), rabbit muscle lactate dehydrogenase (135,000), yeast hexokinase (110,000) and ovalbumin (45,000).

RESULTS

Purification of NADP-GDH of P.knowlesi: GDH of *P.knowlesi* was purified by combination of ammonium sulphate precipitation, gel filtration on Sephadex G-200 and adsorption chromatography on hydroxylapatite column. The summary of purification is given in Table-1. The fraction sedimenting between 40-75% saturation of ammonium sulphate contained 96% of GDH activity. Fig.1a represents a typical elution profile of NADP-GDH of *P.knowlesi* from Sephadex G-200 column. Out of the three protein peaks the enzyme activity was localized in the fractions (26 ml-40 ml) obtained just after the first protein peak. No activity was detected in other column fractions. The fractions having GDH activity (55% recovery of enzyme activity) were pooled and applied to hydroxylapatite column. The elution profile of protein separated by the hydroxylapatite and their activities with respect to GDH are given in Fig. 1b. After this step the enzyme was purified about 181 folds with an overall recovery of 52%.

Table-1 Purification and recovery of NADP-glutamate dehydrogenase of P.knowlesi

Steps	Total volume (ml)	Total protein (mg)	Total enzyme activity (units)	Specific activity	%Recovery	Fold purification
1. crude extract	10.0	215.2	1114	5.2	100	0
2. Ammonium sulphate precipitation (40-75%)	1.0	38.8	1075	27.7	96	5
3. Gel filtration (Sephadex G-200)	16.0	6.5	615	94.6	55	18
4. Hydroxylapatite	1.5	0.62	584	942	52	181

The 10,000X g supernatant of a 20% parasite homogenate was prepared in 25mM triethanolamine buffer (pH 7.4) and used as the enzyme source. Total activity is expressed as units in the total volume of extract.

Unit is defined as the amount of enzyme that catalysed the conversion of 1 nmole of coenzyme ml^{-1} under the assay conditions stated. Specific activity is units mg^{-1} protein.

Properties of purified NADP-GDH: The enzyme activity using α - Kg/NADPH for the amination reaction and glutamate/NADP for deamination reaction was estimated using buffers of different pH. The pH optima for the amination reaction with α-Kg/NADPH and the deamination reaction with glutamate/NADP were found to be 7.4 and 8.6 respectively (data not shown). The highest activity of GDH(236 units/ml) was observed in the amination reaction at pH 7.4 (optimum pH for amination). The GDH activity in the deamination reaction was found to be 79 units/ml at pH 8.6 (optimum pH for deamination)and 23.58 units/ml at pH 7.4 (optimum pH for amination). The kinetic studies for amination reaction using α-Kg , ammonium acetate and NADPH were done at pH 7.4 while pH 8.6 was used for studying the kinetics of glutamate and NADP in the deamination reaction. The NADP- GDH of P.knowlesi exhibited hyperbolic type of kinetics with respect to the substrate and cofactors. The double reciprocal plots of α - ketoglutarate, ammonium acetate, NADPH, glutamate and NADP are shown in Fig. 2. The Km values calculated from the Lineweaver Burk plots

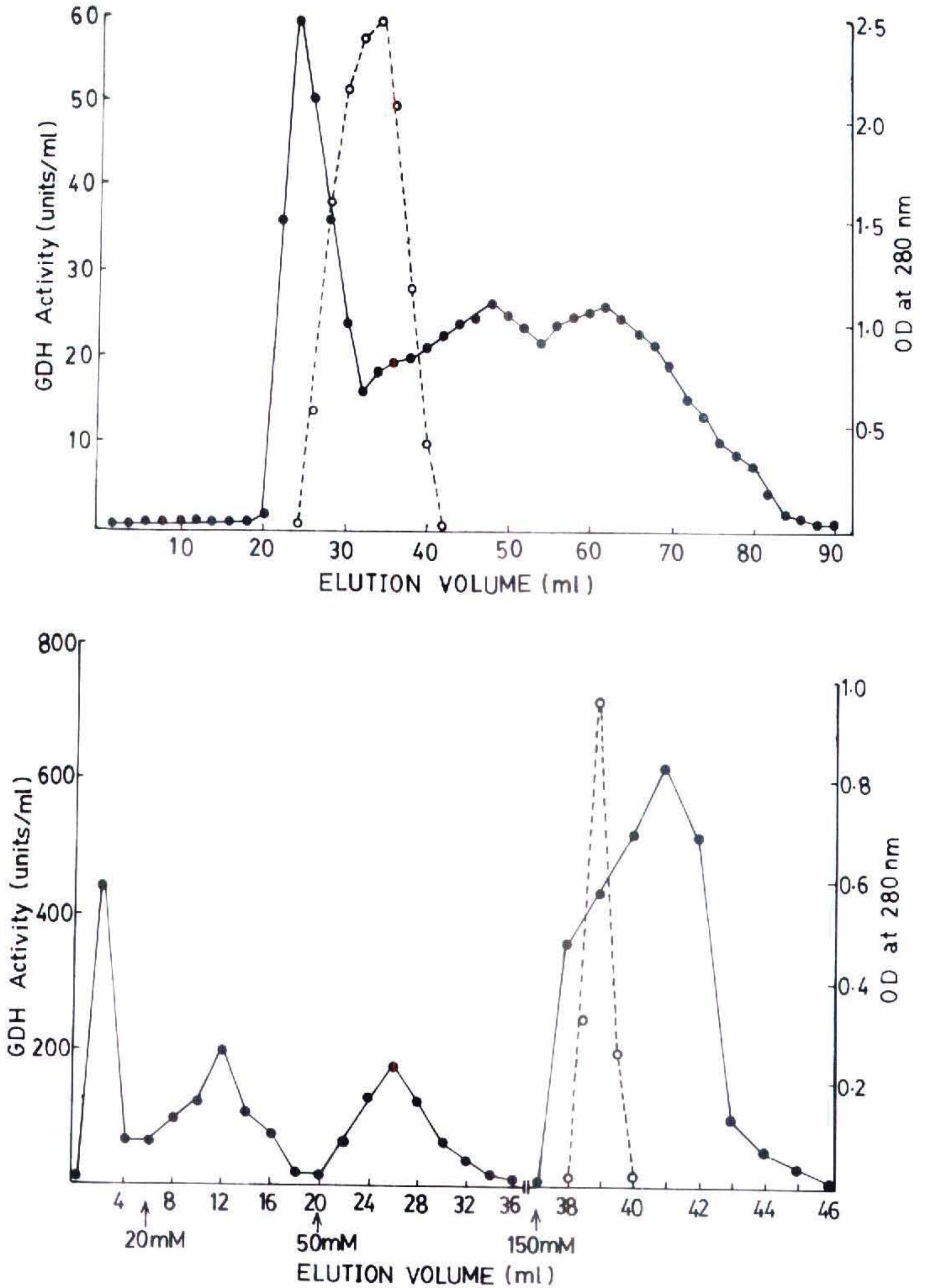

Fig.1: Elution profile of P.knowlesi NADP-glutamate dehydrogenase on sephadex G-200 column(a) and hydroxylapatite column(b)

(O--O) GDH activity; (●—●) Absorbance at 280 nm

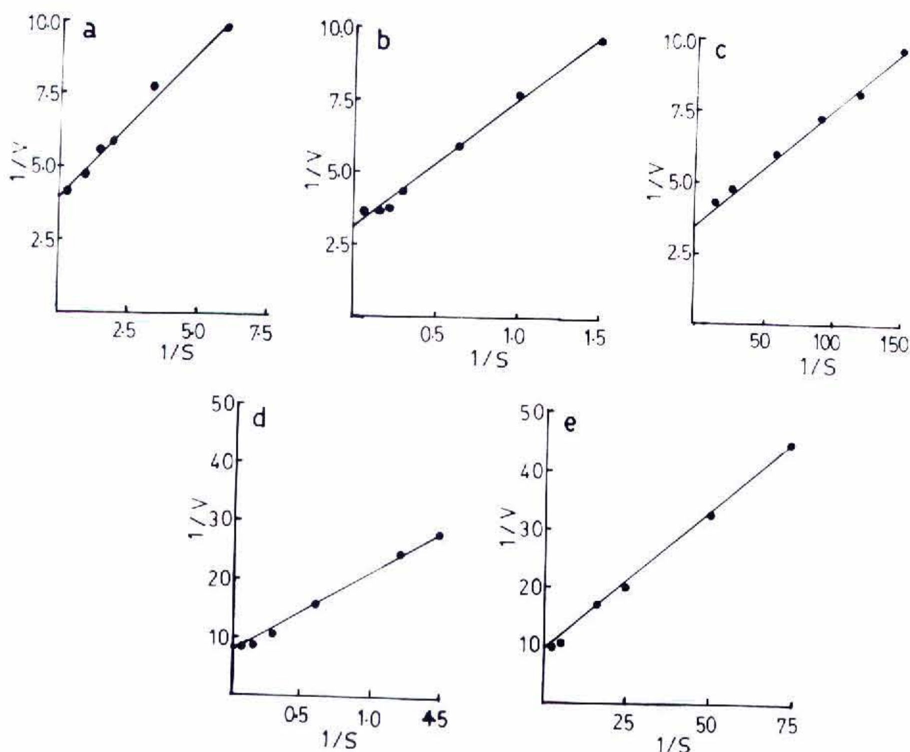

Fig.2: Lineweaver-Burk plots of α-Ketoglutarate(a), ammonium acetate (b), NADPH (c), glutamic acid (d), NADP(e) for NADP-GDH of P.knowlesi.

were found to be 0.25±0.02, 1.3±0.2, 0.011±0.001 mM for α-Ketoglutarate,ammonium acetate and NADPH respectively. Correspondingly the Km values for glutamate and NADP were 1.8±0.1 and 0.50±0.002 mM.

Studies on sensitivity to temperature indicated that P.knowlesi NADP-GDH was stable upto 56°C following 10 min incubation and only about 7% activity was lost when the enzyme was incubated at 60°C. On increasing the incubation time to 1 h the enzyme did not show any loss of activity till 56°C, whereas about 11% and 18% loss in enzyme activity was observed at 60°C and 70°C respectively (Fig.3). Even after incubating the enzyme for 4h a 56°C no loss in enzyme activity was observed (Data not shown).

The effect of different concentration of KCl and NaCl on the activity of P.knowlesi GDH is shown in Fig.4. The addition of KCl to the enzyme showed more activation as compared to NaCl. About 39% and 14% activation was observed at 100mM KCl and 50 mM NaCl respectively . On increasing the concentrations of these salts decrease in activationof the enzyme was observed. The activity reached to the control value at about 100 mM NaCl and

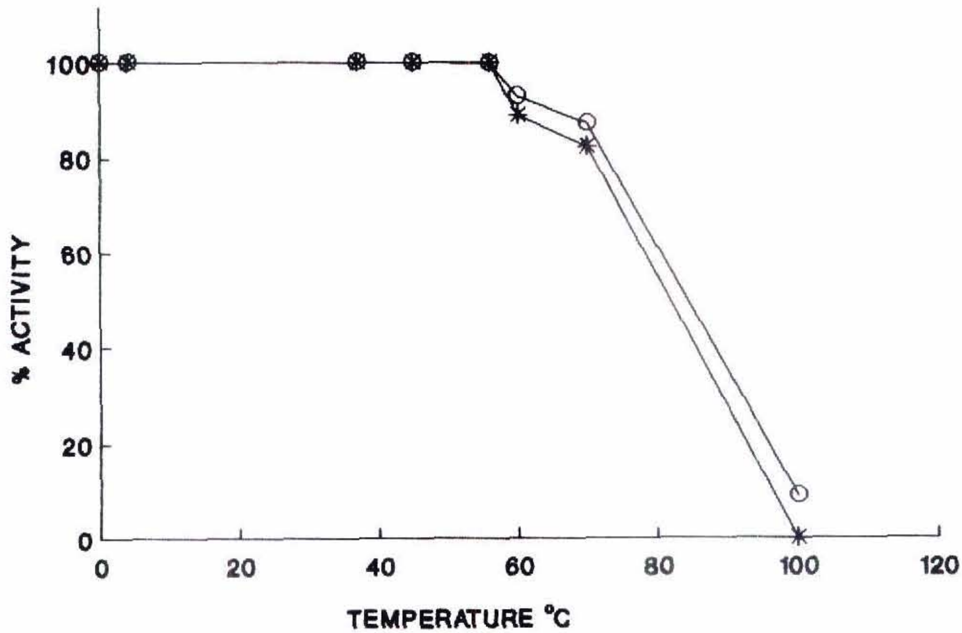

Fig.3: Effect of different temperatures on the activity of NADP-glutamate dehydrogenase of P.knowlesi after 10 (-⊖-) min and 60 (-✳-) min incubation.

Percent activity was calculated as remaining GDH activity after incubation at different temperature with respect to control activity (enzyme kept at 4°C).

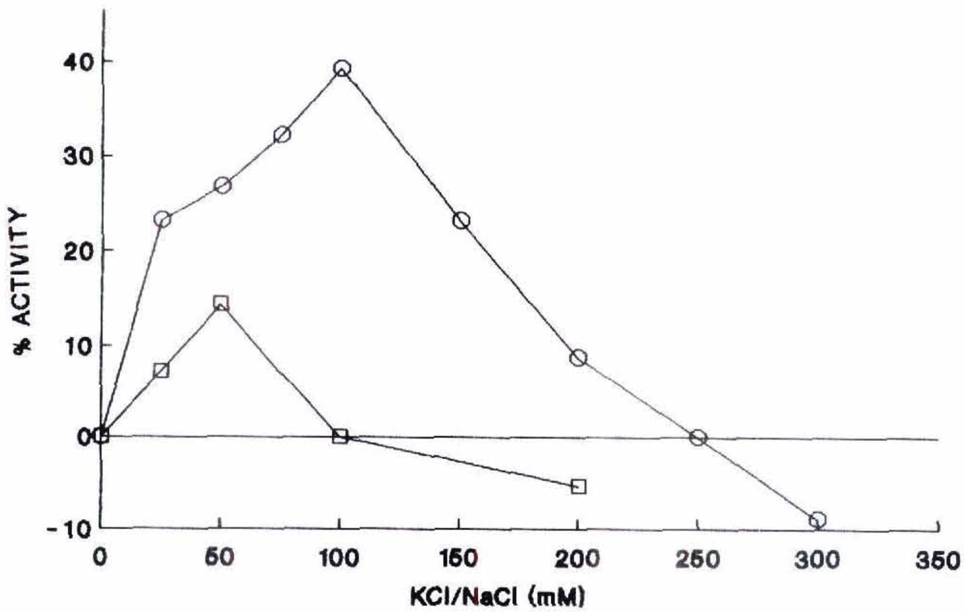

Fig.4: Effect of different concentrations of KCl(-⊖-) and NaCl (-⊟-) on NADP-glutamate dehydrogenase of <u>P.knowlesi</u>.

and 250 mM KCl. Further increase in concentration of both the salts resulted in inhibition of the enzyme activity.

The effect of purine nucleotides viz. ADP, ATP, GDP, GTP, IMP and ITP was studied on NADP-GDH of P.knowlesi. These purine nucleotides had no effect on the enzyme activity at concentrations of 0.1 and 1 mM (Data not shown). The molecular weight of NADP-GDH of P.knowlesi as determined by molecular sieving through Sephadex G-200 column was found to be 295,000.

DISCUSSION

Three types of glutamate dehydrogenase with different coenzyme specificities (NAD specific, NADP specific and with dual pyridine nucleotide specificity) have been reported from various sources (Smith et al., 1975). The present study demonstrates the existence of a parasite specific NADP-linked glutamate dehydrogenase in P.knowlesi as has been shown in other species of malarial parasites (Sherman, 1979; Vander Jagt et al., 1989). Since this enzyme is not present in red blood cells, and also because of the loss of GDH activity (10%) during the isolation of parasites free of red cell contaminants (Watts et al., 1988b), the schizont infected erythrocytes of P.knowlesi were used as starting material for enzyme purification. The procedures employed for the purification of P.knowlesi GDH, in the present study, resulted in about 181 folds enrichment of enzyme activity with an overall recovery of 52 percent. The P.knowlesi enzyme had a molecular mass of about 295,000, as determined by molecular sieving, which is accordance with the molecular mass reported for NADP-GDH of P.falciparum(Ling et al., 1986). The molecular weight of GDH isolated from other malarial and trypanosome parasites varies from 245,000-280,000 (Juan et al, 1978; Walter and Ebert, 1979; Walter et al., 1974). The mammalian enzyme from bovine liver and heart has been reported to have slightly higher molecular mass of about 330,000 (Mc Daniel et al., 1986; Smith et al., 1975). The enzyme activity of P.knowlesi NADP-GDH was found to be affected by sats like KCl and NaCl as has been reported for NADP-linked enzyme from Trypanosoma cruzi (Juan et al., 1978), yeast (Camardella et al., 1976) and Bacteroides fragilis (Yamamoto et al., 1987). The NADP-dependent GDHs from various sources have been shown to be thermostable in nature (Schmidt and Schmidt, 1983). Similarly the NADP-GDH of P.knowlesi can also withstand heat upto 56°C for 4 h. The biochemical properties and the physiological role of glutamate dehydrogenase vary depending on the enzyme source. The enzyme from animal sources (mammalian GDH) has dual coenzyme specificity, utilising both NAD(H) and NADP(H), and is affected by the purine nucleotides (Smith et al., 1975). However, the glutamate dehydrogenase from non-animal sources has been shown to be specific for either NAD(H) or NADP(H) and its activity is not affected by the purine nucleotides except for few NAD-specific GDHs(Smith et al., 1975). In the present study, the glutamate dehydrogenase isolated from P.knowlesi was found to be specific for NADP(H), not influenced by the purine nucleotides (ADP, ATP, GDP, GTP, IMP, ITP) and therefore appears to belong to the group of GDH from non-animal sources. The GDH from other species of malaria parasites has also been shown to

be NADP dependent (Langer et al., 1970; Sherman et al., 1971; Vander Jagt et al., 1989; Walter et al., 1974).

The NADP-specific glutamate dehydrogenase of malarial parasites appears to play different roles in different malarial parasites. The enzyme has been shown to be the major source of endogenous NADPH production in some species of malarial parasites (Langer et al., 1970; Sherman et al., 1971; Walter et al., 1974) while P.falciparum GDH mainly participates in glutamate formation (VanderJagt et al., 1989). Similarly, the NADP-linked glutamate dehydrogenase of P.knowlesi appears to have a predominant role in glutamate synthesis as shown by high levels (10 folds) of NADPH dependent amination activity in comparison to deamination activity at pH 7.4 . These findings are supported further by the studies on enzyme kinetics of amination and deamination reactions of P.knowlesi GDH. The NADP-GDH of P.knowlesi showed lower Km values for the substrate (α-Ketoglutarate) and cofactor(NADPH) of the amination reaction and these are quite comparable to the Km values reported for the P.falciparum NADP-GDH which also showed high amination activity (Vander Jagt et al., 1989). The GDHs from P.chabaudi (Walter et al., 1974) and T.cruzi (Juan et al., 1978) showed higher Km values for α- Ketoglutarate and the GDH in these parasites has been shown to catalyse more efficiently the deamination reaction that is towards the production of reduced NADP. The presence of an NADP-dependent isocitratee dehydrogenase (NADP-IDH) has been demonstrated in P.falciparum and NADP-IDH instead of NADP-GDH has been suggested to be a preferred source of NADPH production in this parasite (Vander Jagt et al., 1989). Recently, NADP-dependent isocitrate dehydrogenase has also been reported in P.knowlesi (Sahni et al., 1992).

The present investigation reveals that the properties of NADP-specific glutamate dehydrogenase of P.knowlesi are quite similar to that of P.falciparum NADP-GDH. These findings also suggest that P.knowlesi GDH may probably be having a predominant biosynthetic role (glutamate synthesis) in parasite metabolism.

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