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Identification of amino acid residues essential for enzyme activity of sheep liver 5,10-methylenetetrahydrofolate reductase

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Sheep liver 5,10-methylenetetrahydrofolate reductase was subjected to specific chemical modification with phenylglyoxal, diethyl pyrocarbonate and *N*-bromosuccinimide. The second-order rate constants for inactivation were calculated to be 54 $M^{-1} \cdot min^{-1}$, 103 $M^{-1} \cdot min^{-1}$ and 154 $M^{-1} \cdot min^{-1}$ respectively. This inactivation could be prevented by incubation with substrates or products, suggesting that the residues modified, namely arginine, histidine and tryptophan, are essential for enzyme activity.

INTRODUCTION

The enzyme 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4folate) reductase (EC 1.1.99.5), which catalyses the nicotinamide nucleotide-dependent reduction of 5,10-CH_2-H_4folate to 5-methyltetrahydrofolate (5-CH_3-H_4folate), was isolated from sheep liver in a homogeneous form, and the kinetic mechanism of the reaction was established to be Bi Bi Ping Pong (Varalakshmi *et al.*, 1983). A similar mechanism was earlier proposed for this enzyme from pig liver (Matthews, 1982). There is, however, no information on amino acid residues at the active site of the sheep liver reductase. We therefore decided to identify the amino acid residues essential for enzyme activity by using inactivation of the enzyme by specific reagents acting as a probe. The present paper reports the requirement of arginine, histidine and tryptophan for the activity of sheep liver 5,10-CH_2-H_4folate reductase.

EXPERIMENTAL

Materials

All biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. 5-[^{14}C]CH_3-H_4folate was from Amersham International, Amersham, Bucks., U.K.

Methods

Enzyme preparation. Enzyme was purified and assayed as described previously (Varalakshmi *et al.*, 1983). Protein was estimated by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Modification of the enzyme by phenylglyoxal, diethyl pyrocarbonate (DEPC) and *N*-bromosuccinimide (NBS). (*a*) The sheep liver reductase (70 μg) in 50 mM-potassium phosphate buffer, pH 7.2, containing 0.3 mM-EDTA, was preincubated with different fixed concentrations (25, 30, 40 and 50 mM) of phenylglyoxal, and after various time intervals portions were withdrawn, diluted into enzyme reaction mixtures and assayed for the residual enzyme activity. Inactivation by DEPC was carried out essentially as described for phenylglyoxal, except that L-histidine (4 mM) was included in the assay mixture after inactivation but before assay. Modification by NBS was carried out at pH 5.6 in a similar manner to the above two procedures. For determining the protective effects of different ligands, the enzyme was first preincubated with the ligands for 10 min at 37 °C at the concentrations indicated (Table 1) and the modification of the enzyme was carried out as described above.

(*b*) The modification of the enzyme by DEPC was reversed by hydroxylamine as described below. The enzyme modified with DEPC (1.5 mM) for 2 min was incubated overnight at 4 °C with 0.2 M-hydroxylamine, pH 7.0. The mixture was dialysed at 4 °C overnight and assayed for residual enzyme activity.

RESULTS AND DISCUSSION

Inactivation of the enzyme by phenylglyoxal

The inactivation of the enzyme with different fixed concentrations (25–50 mM) of phenylglyoxal followed pseudo-first-order kinetics, and the pseudo-first-order rate constants ($k_{app.}$) were obtained from the slopes (Fig. 1). The replot of $\ln k_{app.}$ against ln[phenylglyoxal] gave a slope value of 2, indicating that at least two molecules of phenylglyoxal were essential to inactivate the enzyme (Fig. 1 inset). Similar 2:1 stoichiometry in the reaction of phenylglyoxal was observed in other enzymes also (Daemen & Riordan, 1974; Berghauser, 1975; Lobb *et al.*, 1976). A second-order rate constant of 54 $M^{-1} \cdot min^{-1}$ was calculated (Fig. 1 inset). When the enzyme was preincubated with 5-CH_3-H_4folate (12 mM) before modification by phenylglyoxal, the enzyme was partially protected against inactivation, as indicated by a decrease in the pseudo-first-order rate constant in the presence of the substrate (Table 1). The second substrate, NADPH (0.9 mM), was without effect, suggesting that an arginine residue was probably essential for interaction with 5-CH_3-H_4folate and not NADPH. It was observed in many enzymes that the guanidinium groups of arginine residues are the possible binding sites for the anionic substrates containing phosphate or carboxy groups

Abbreviations used: 5,10-CH_2-H_4folate, 5,10-methylenetetrahydrofolate; 5-CH_3-H_4folate, 5-methyltetrahydrofolate; DEPC, diethyl pyrocarbonate; NBS, *N*-bromosuccinimide.

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Table 1. $k_{app.}$ values for inactivation of the enzyme in the presence of ligands

The concentrations at which the ligands were added are indicated in parentheses. —, Not determined.

Ligand	$k_{app.}$ (min^{-1})		
	Phenylglyoxal (25 mM)	DEPC (1.5 mM)	NBS (2 mM)
None	0.036	0.71	0.012
5-CH_3-H_4folate	0.015 (12 mM)	0.71 (6 mM)	Complete protection (3 mM)
NAD^+	—	0.53 (20 mM)	—
$NADP^+$	—	0.43 (20 mM)	0.018 (20 mM)
Nicotinamide	—	0.04 (20 mM)	—
NADPH	0.059 (0.9 mM)	0.71 (5 mM)	0.023 (0.3 mM)

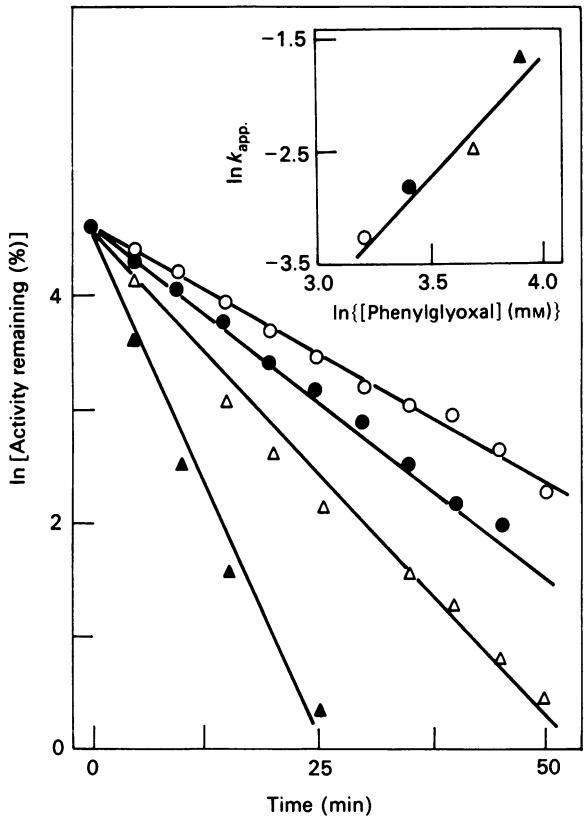

Fig. 1. First-order plots for the inactivation of the enzyme by phenylglyoxal

Enzyme (60–70 μg) in 50 mM-potassium phosphate buffer, pH 7.2, containing 0.3 mM-EDTA and 5% (v/v) glycerol, was placed in a set of tubes and different concentrations (○, 25 mM; ●, 30 mM; △, 40 mM; ▲, 50 mM) of phenylglyoxal were added. The reaction mixture (80 μl) was incubated at 37 °C, and 6 μl portions were withdrawn at different time intervals (5–50 min) into the reaction mixture and assayed for the 5-CH_3-H_4folate menadione oxidoreductase activity (Varalakshmi *et al.*, 1983). The inset shows the replot of the slopes against ln[phenylglyoxal].

(Riordan *et al.*, 1977; Riordan, 1979; Wong & Wong, 1981). The observed protection by 5-CH_3-H_4folate in the case of the sheep liver reductase suggested that the carboxy groups of glutamic acid residues of 5-CH_3-H_4folate might be interacting with the guanidinium groups of arginine residues. The involvement of arginine residues of sheep liver serine hydroxymethyltransferase in the binding of the carboxy groups of H_4folate was demonstrated previously (Manohar & Appaji Rao, 1984).

Inactivation by DEPC

From the time course of inactivation at different concentrations of DEPC (0.5–2 mM) the $k_{app.}$ values were calculated and were replotted as ln $k_{app.}$ versus ln[DEPC]. A second-order rate constant of 103 $M^{-1} \cdot min^{-1}$ and a slope value of 0.7 were obtained from this plot, indicating that a histidine residue might be essential for the activity of the reductase (Fig. 2).

The products $NADP^+$ (20 mM) and NAD^+ (20 mM) partially protected the enzyme against inactivation, whereas nicotinamide (20 mM) gave complete protection (Table 1). However, protection was not afforded by 5-CH_3-H_4folate (6 mM), NADPH (5 mM) or AMP (20 mM). The $k_{app.}$ values for the inactivation of the enzyme in presence of various ligands are given in Table 1.

Histidine is implicated as a proton donor in several enzymes (Millar & Schwert, 1963; Peterson & Martinez-Carrion, 1970; Harrigan & Trentham, 1973). It is possible that the histidine residue is playing a similar role in the reduction of 5,10-CH_2-H_4folate catalysed by the sheep liver reductase. Further evidence in support of this suggestion was the significant regain of the enzyme activity (80%) upon treatment of the DEPC-modified enzyme with hydroxylamine. This observation also suggested that an amino group or thiol group was probably not responsible for the inactivation by DEPC. The partial protection of the enzyme by $NADP^+$ and NAD^+ and the significant protection by nicotinamide, as well as the absence of protection by 5-CH_3-H_4folate, suggested that histidine is probably required for the binding of NADPH.

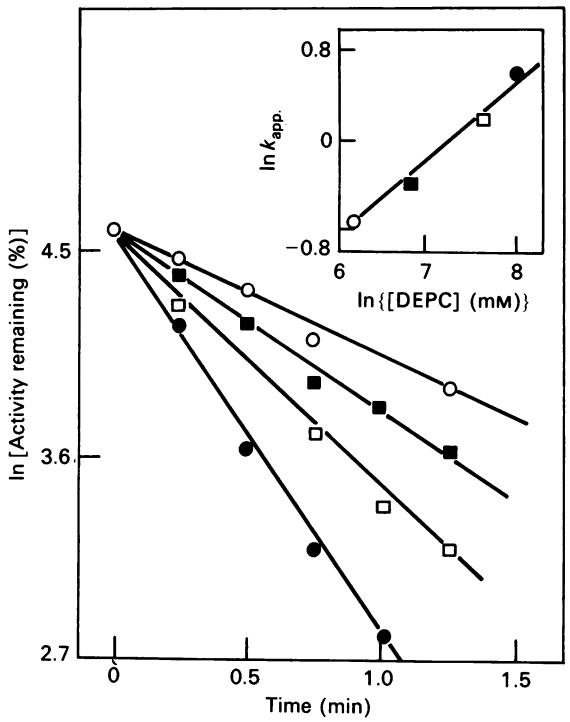

Fig. 2. Modification of the reductase by DEPC

To a set of tubes containing the enzyme (50 μg) in 50 mM-potassium phosphate buffer, pH 7.2, containing 0.3 mM-EDTA, different concentrations (○, 0.5 mM; ■, 1 mM; □, 2 mM; ●, 3 mM) of DEPC were added from a stock solution of DEPC in ethanol to start the inactivation. At various times (0.25–2 min), 20 μl portions were withdrawn from the reaction mixture (200 μl) and added to the assay mixture containing 4 mM-histidine. From the slopes of the first-order plots, $k_{app.}$ values were calculated and plotted as ln$k_{app.}$ against ln[DEPC] (inset).

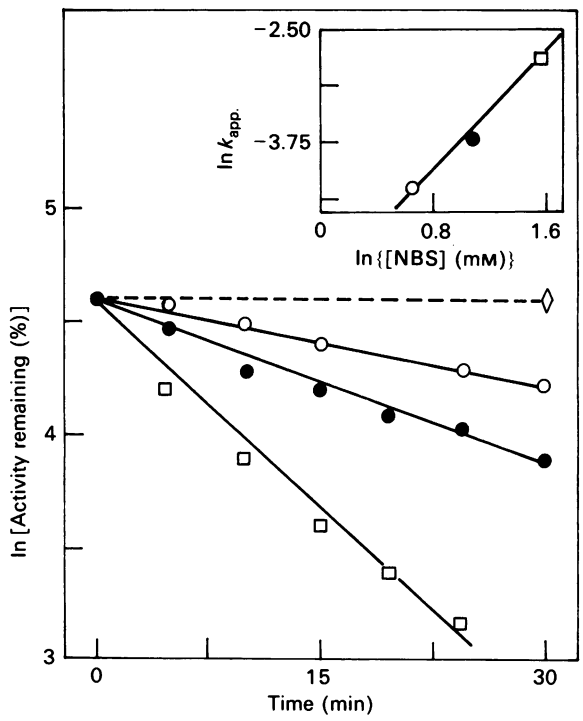

Fig. 3. Time courses of inactivation for the modification of the enzyme by NBS

The enzyme (70 μg) in 0.45 M-acetate buffer, pH 5.6, was placed in a set of tubes and was inactivated by different concentrations of NBS (◇, 0 mM, ○, 2 mM; ●, 3 mM; □, 5 mM). At various times (5–30 min), 10 μl portions were removed from the reaction mixture (100 μl), diluted into the assay mixture and the residual enzyme activity was determined. ln(Percentage of activity remaining) was plotted against time. The inset shows the slope replot as ln$k_{app.}$ against ln[NBS].

Identification of a tryptophan residue at the active site of the reductase

NBS was used for inactivating the reductase by the modification of tryptophan and the $k_{app.}$ values were calculated from the time course of inactivation (Fig. 3). The slope value (1.6) obtained from a replot of ln$k_{app.}$ versus ln[NBS] suggested the presence of one or two tryptophan residues essential for enzyme activity. A second-order rate constant of 154 $M^{-1} \cdot min^{-1}$ was calculated. The requirement of tryptophan residues for activity was confirmed by the complete protection afforded by 5-CH_3-H_4folate (3 mM) against inactivation. However, $NADP^+$ (20 mM) or NADPH (0.3 mM) did not protect the enzyme against inactivation (Table 1).

Tryptophan has been shown to be present at the active site of some flavoproteins and is known to form charge-transfer complexes with flavins (Nishikimi & Kyogoku, 1973; Ishida & Inone, 1979). The pseudo-first-order rates of inactivation by NBS suggested that tryptophan residue might be important for the activity of 5,10-CH_2-H_4folate reductase. The protection by 5-CH_3-H_4folate indicated that the folate substrate could be interacting with the tryptophan, whereas the lack of protection by NADPH strengthened the postulation that the two substrates may be interacting with different residues at the active site.

The results presented here implicate the involvement of an arginine, a histidine and a tryptophan residue in the reaction catalysed by sheep liver 5,10-CH_2-H_4folate reductase.

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