Clostridium pasteurianum glutamine synthetase mechanism

Evidence for active site tyrosine residues

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Preliminary chemical modification studies indicated the presence of tyrosine, carboxyl, arginine, histidine and the absence of serine and sulfhydryl residues at or near the active site of *Clostridium pasteurianum* glutamine synthetase The conditions for tyrosine modification with tetranitromethane were optimized The inactivation kinetics follow pseudo-first-order kinetics with respect to enzyme and second order with respect to modifier per active site There was no inactivation at pH 6 5 suggesting the absence of thiol oxidation The synthetase and transferase reactions followed the same pattern of inactivation on enzyme modification and both were equally protected by glutamate plus ATP Thus tyrosine residues are present at the active site of the enzyme and are essential for both tranferase and synthetase activities

Clostridium pasteurianum *Glutamine synthetase* *Active site* *Tyrosine* *Tetranitromethane*

1. INTRODUCTION

Glutamine synthetase initiates ammonia assimilation by catalyzing its ATP-dependent incorporation into glutamate. It plays a central role in metabolism and is therefore a target for cellular control. As the enzyme catalyzes a multisubstrate biochemical transformation, studies of its reaction mechanism are of particular interest. Most such studies have been done kinetically. Only recently have limited investigations on active site residues with glutamine synthetase been reported, which implicate sulfhydryl [1–4], arginine [5] and lysine [6] at the active site of glutamine synthetase. Very recently, we reported the purification of this enzyme to homogeneity from *Clostridium pasteurianum*, a very active, anaerobic nitrogen fixer [7]. The enzyme has a comparatively high M_r (1.05×10^6) and 20 identical subunits vs 8–12 reported from other sources. It exhibits negative cooperativity in the binding of L-glutamate and its K_m for ADP is lowest among those reported from other sources. Thus, it is likely that the enzyme from anaerobes follows a reaction mechanism quite different from that suggested for sheep brain [8] or *E. coli* enzyme [9].

Preliminary chemical modification studies indicated that tyrosine, carboxyl, arginine and histidine and possibly lysine are near or at the active site of the enzyme

Modification of the enzyme with tetranitromethane, a tyrosine-specific reagent, under optimal conditions resulted in concomitant loss of enzymatic activity which suggests that the tyrosine residues are at or near the active site of *C. pasteurianum* glutamine synthetase.

2. MATERIALS AND METHODS

2.1. *Materials*

Bovine serum albumin, Coomassie brilliant blue R 250, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate, dimethylsuberimidate hydrochloride, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), *N*-ethylmaleimide, phenylmethanesulfonyl fluoride and Tris were purchased

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from Sigma (USA); DEAE-Sephadex A-50 and Sepharose 4B from Pharmacia, Uppsala; phenylglyoxal hydrate and 2,3-butanedione from Fluka and tetranitromethane (TNM) from K & K Laboratories, Jamaica, NY.

2 2. *Methods*

C. pasteurianum was grown, harvested and processed for purification of glutamine synthetase to homogeneity as in [7]. Polyacrylamide disc gel electrophoresis in the presence and absence of SDS showed the enzyme to be homogeneous. Purified enzyme had a specific activity of 65 units per mg protein as measured by the γ-glutamyltransferase assay, 1 unit being defined as μmol γ-glutamylhydroxamate formed per min. Protein was determined by the method of Lowry et al. [10] and enzyme activity by the synthetase and γ-glutamyltransferase activities [7].

Preliminary chemical modification studies were carried out under the conditions as given in table 1

3. RESULTS AND DISCUSSION

3.1. *Preliminary modification studies and optimization of modification of tyrosine with tetranitromethane*

Table 1 shows the loss in enzymatic activity on modification of glutamine synthetase with reagents specific for the likely active site amino acids These results implicate tyrosine, carboxyl, arginine and histidine and suggest the non-involvement of serine and sulfhydryl residues at or near the active site The loss in enzymatic activity in the presence of dimethylsuberimidate could be due to either cross-linking or modification of the lysyl residues. The retention of activity on reacting with sulfhydryl-specific reagents is in contrast to the findings in other organisms [1–4]

As the preliminary experiments indicated that among the active site amino acids tyrosine residue(s) play an essential role in glutamine synthetase catalysis, conditions were systematically optimized for its modification with TNM, which indicated that the modification proceeded optimally at· tetranitromethane, 0.21 mM; temperature, 30°C, buffer, 50 mM Tris-HCl (pH 7.7); incubation time, 30 min

TNM preferably nitrates tyrosine in the phenoxide form. Hence lowering the pH should slow down and at pH 6 0, practically stop the nitration of this residue [11] In the case of *C pasteurianum* enzyme, lowering the pH to 6 0 or raising it to 8.0 or above led to loss of activity in the enzyme itself, but modification at pH 6 5 led to only 10% loss vs 70% at pH 7.7.

Treatment of a protein with TNM at pH 6.5 can oxidize sulfhydryl groups with concomitant loss in enzymatic activity. Hence, modification of glutamine synthetase was carried out in the presence of DTNB (1 mM) at pH 7.0 and 6 5, but it did not affect the pattern of loss of activity at either pH. A high concentration of TNM can lead to cross-linking of protein with loss in enzymatic activity But in this case, determination of the M_r of the modified enzyme by gel permeation chromatography showed no change and hence loss of enzymatic activity was attributed to modification of tyrosine residue(s) and not due to either thiol oxidation or cross-linking of protein.

3.2. *Kinetics of inactivation of glutamine synthetase by tetranitromethane*

Glutamine synthetase was incubated with different concentrations of TNM at 30°C for 30 min at pH 7.7. Aliquots were withdrawn at different time intervals, dialyzed and assayed for γ-glutamyltransferase activity. Semilog plots of residual activity vs time were linear at all TNM concentrations employed, suggesting that the inactivation is pseudo-first order in enzyme (fig.1)

The number of molecules of modifier reacting per active unit of the enzyme was calculated by plotting $-\log (t_{0\,5})^{-1}$ vs log [TNM] (fig 1, inset a). A slope of 2.2 was obtained suggesting that 2 molecules of TNM reacted per active unit of the enzyme leading to its inactivation. When the observed pseudo-first-order rate constants (k_{obs}) were plotted vs [TNM] (fig 1, inset b) a linear plot was obtained, indicating a single-step reaction.

3.3. *Effect of tyrosine modification on synthetase activity*

Glutamine synthetase was incubated with 0 21 mM TNM in 50 mM Tris-HCl (pH 7 7) at 30°C for various time intervals. The loss in γ-glutamyltransferase and synthetase activities were determined as shown in fig 2. It was observed that the loss in the two activities on modification ran

Table 1

Inactivation of *C pasteurianum* glutamine synthetase on treatment with amino acid specific reagents

Specific reagents	Concentration of stock solution	Buffer and pH of the incubation mixture	Incubation temperature (°C)	Incubation time (min)	Concentration (mM)	Percent residual activity
Control						100
Sulfhydryl						
N-Ethylmaleimide	50 mM in 100 mM Tris-HCl (pH 7 2)	100 mM Tris-HCl (pH 7 2)	30	30	20	100
5,5′-Dithiobis-(2-nitrobenzoic acid)	20 mM in 100 mM Tris-HCl (pH 7 2)	100 mM Tris-HCl (pH 7 2)	30	30	10	100
Serine						
Phenylmethane-sulfonyl fluoride	25 mM in isopropanol	100 mM Tris-HCl (pH 7 2)	30	30	1	100
Histidine						
Rose Bengal dye	0 2% (w/v) in 50 mM Tris-HCl (pH 7 5)	50 mM Tris-HCl (pH 7 5)	4°C irradiated with 200 W bulb at a distance of 1 ft	10	0 04% (w/v)	51
Arginine						
Phenylglyoxal	160 mM in 50 mM borate buffer (pH 8 0)	50 mM borate buffer (pH 8 0)	30	60	20	26
2,3-Butanedione	91 mM in 50 mM borate buffer (pH 7 5), pH adjusted to pH 7 5 with 1 N NaOH	50 mM borate buffer (pH 7 5)	30	30	18 2	42
Tyrosine						
Iodine	10 mM in 500 mM KI	50 mM Tris-HCl (pH 7 5)	30	6	0 1	30
Tetranitromethane	21 mM in 95% ethanol	50 mM Tris-HCl (pH 7 5)	30	30	0 21 (ethanol concentration does not exceed 5% in incubation mixture)	38
Glutamate/aspartate						
1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate	125 mM in 40 mM Mes buffer (pH 6 5)	40 mM Mes buffer (pH 6 5)	4	30	25	20
Lysine						
Dimethylsuberimidate	150 mM in 50 mM Tris-HCl (pH 7 5), pH rapidly adjusted to 7 5 with 1 N NaOH	50 mM Tris-HCl (pH 7 5)	30	30	60	50

Aliquots (1 0 ml) of exhaustively dialyzed homogeneous glutamine synthetase (protein, 0 15 mg/ml) were incubated with indicated reagents under the stated conditions of pH, concentration, temperature and time in a total volume of 2 5 ml, dialyzed and assayed for γ-glutamyltransferase activity Controls were run simultaneously in the absence of the reagent, and their activity was taken as 100

more or less parallel implicating tyrosine in both activities

3.4 *Stability of modified glutamine synthetase*

Glutamine synthetase modified with 0.21 mM TNM at 30°C for 30 min and native enzyme were dialyzed against 50 mM Tris-HCl (pH 7 2) for 12 h with two changes and their residual activities were compared. The ratio of activities in the two samples remained unchanged. Thus the modified enzyme is stable

3 5 *Protection of glutamine synthetase against modification by substrates*

The presence of substrates, substrate analogs or competitive inhibitors protects only the active site amino acid residues against modification and thus establishes the site of modified amino acids.

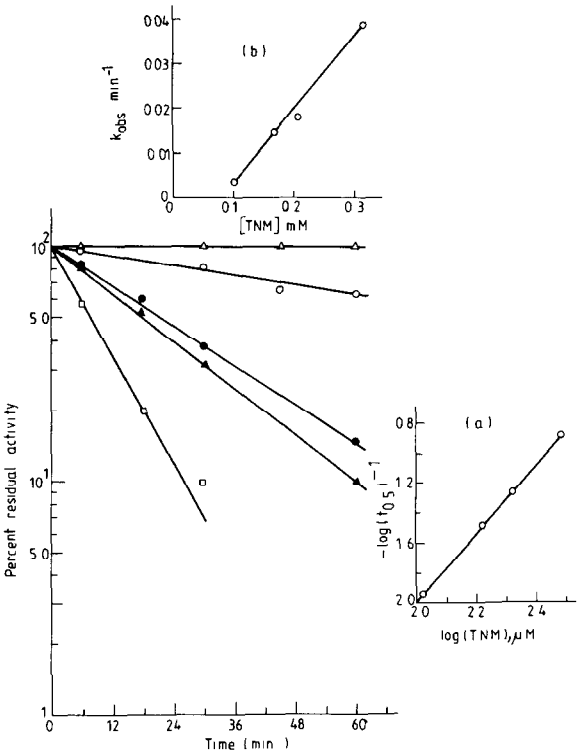

Fig 1 Semilogarithmic plot of glutamine synthetase inactivation by TNM. Aliquots (1.0 ml) of the enzyme solution (protein, 0 16 mg/ml) were incubated with 0 105 mM (○—○), 0 168 mM (●—●), 0 21 mM (▲—▲) and 0 315 mM (□—□) TNM in 50 mM Tris-HCl (pH 7 7) at 30°C in a total volume of 2 5 ml Aliquots were withdrawn at regular time intervals, dialyzed and assayed for γ-glutamyltransferase activity Controls (△—△) were incubated under similar conditions without TNM. The percent activity left in each sample was calculated by taking control value 100 (Inset a) Apparent order of the inactivation was determined by plotting the negative values of the logarithm of the half-time of inactivation $(t_{0.5})^{-1}$ vs log [TNM] (Inset b) Observed first-order rate constants were plotted vs [TNM] to determine the number of steps involved in the inactivation process

Tyrosine modification was therefore carried out in the presence and absence of substrates for 30 min, dialyzed and assayed for γ-glutamyltransferase activity (table 2). It was observed that neither Mg^{2+} nor NH_2OH alone provided any protection against modification. The presence of either ATP or glutamate alone protected the enzyme marginally The enzyme retained 50% activity in their presence

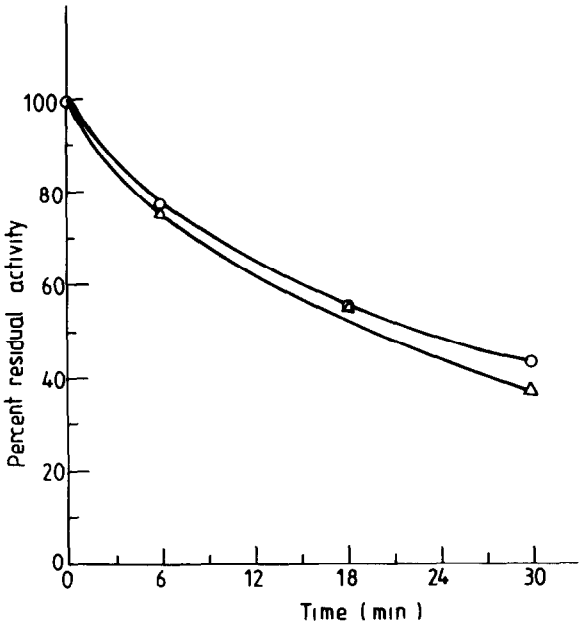

Fig 2 Effect of TNM on synthetase and transferase activities of glutamine synthetase Aliquots (1 0 ml) of the enzyme solution were incubated with 0 21 mM TNM in 50 mM Tris-HCl (pH 7 7) at 30°C in a total volume of 2 5 ml Aliquots were removed at different time intervals, dialyzed and assayed for synthetase (○—○) and γ-glutamyltransferase (△—△) activities Control was also run under the same conditions in the absence of TNM and percent activity left in each sample was calculated by taking control as 100

Table 2
Protection of glutamine synthetase by substrate(s) against modification on treatment with TNM

Modification in the presence of	Percent residual activity[a]
Nil	33 3
NH_2OH	22
$MgCl_2$	25
ATP	50
ATP + $MgCl_2$	60
Glutamate	48
Glutamate + ATP	95

[a] Activity of the unmodified enzyme was taken as 100 Glutamine synthetase (0 4 ml; protein. 0 16 mg/ml) was incubated with TNM (0 21 mM) in the presence and absence of L-glutamate (72 mM), ATP (28 mM), $MgCl_2$ (48 mM); NH_2OH (28 mM), for 30 min in 50 mM Tris-HCl (pH 7 7) at 30°C in a total volume of 1 0 ml, dialyzed and assayed for γ-glutamyltransferase activity

vs 33% in their absence. However, the presence of both glutamate and ATP gave nearly full protection to the enzyme against modification. A similar observation was made when synthetase activity was determined. Hence tyrosine residues are present at or near the active site of glutamine synthetase from *C. pasteurianum* and are essential for both its γ-glutamyltransferase and synthetase activities.

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