Identification of cysteine residues in carbamoyl-phosphate synthase I with reactivity enhanced by *N*-acetyl-L-glutamate

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Carbamoyl-phosphate synthase I (pig liver) is modified at the cysteine residues 1327 and 1337 (numbered according to the rat sequence) in the presence of 5 mM-*N*-acetyl-L-glutamate with enhanced rate. ATP/Mg²⁺ (≥ 5 mM) protects against alkylation of these two cysteines and loss of activity. According to the results obtained by limited proteolysis of monobromobimane-modified carbamoyl-phosphate synthase I, the accessible cysteines 1327 and 1337 are located in the C-terminal 20 kDa domain D of the enzyme. *N*-Bromoacetyl-L-glutamate is an allosteric activator and inactivates carbamoyl-phosphate synthase I in a slow reaction.

INTRODUCTION

Carbamoyl-phosphate synthase I (EC 6.3.4.16) (CPSI) has a nearly absolute requirement for *N*-acetyl-L-glutamate (AcGlu) [1] and shows a 1:1 binding stoichiometry with this allosteric effector [2]. When K⁺, Mg²⁺ and a mixture of either ADP and carbamoyl phosphate or ATP and HCO₃⁻ are present, AcGlu is bound with high affinity (*K*_d 1–6 μM) [2]. In the absence of one or more of the substrates and K⁺/Mg²⁺, the affinity of the CPSI for its allosteric effector is lowered drastically (*K*_d 0.365 mM) [3].

CPSI has two ATP-binding sites per molecule. Only one ATP (ATP_B), providing the phosphoryl group for carbamoyl phosphate, is bound with high affinity (*K*_d ~ 15 μM) [4,5]. Limited proteolysis and the comparison with carbamoyl-phosphate synthases from micro-organisms established a four-domain structure of the single peptide chain in CPSI [6,7]. Photolabelling of the CPSI (rat) by 8-azido-ATP disclosed the location of one ATP-binding site on each of the large internal domains B and C [8].

Although the effects of AcGlu on the catalytic and structural characteristics of CPSI are well studied [3,9,10], there has been no attempt to localize the AcGlu site of CPSI. The complex (CPSI–AcGlu) is rapidly inactivated by thiol-targeted reagents [10]. ATP/Mg²⁺/K⁺ provide complete protection. The influence of AcGlu on the reactivity of two, possibly proximate, thiol groups [10] and proteolytic sites [6] protected by ATP/Mg²⁺, can be considered as reasonable evidence for a spatial relationship between the AcGlu site and an ATP site in CPSI. In this paper, we identified the cysteines showing increased reactivity of thiol groups in the presence of AcGlu and which are protected against modification by Mg²⁺ATP.

MATERIALS AND METHODS

Materials

Sephadex media and Ultrogel AcA 34 were from

Pharmacia LKB. *N*-Bromoacetyl-L-glutamate was synthesized according to [11] and purified by chromatography on a Sephadex LH-20 column (2 cm × 96 cm) equilibrated with water. The substance was eluted by 18.5% acetonitrile from a Bakerbond wide-pore octadecylsilyl column (4.6 mm × 250 mm) using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid.

Purification and assay of CPSI

Pig liver CPSI was purified to homogeneity [12] and stored in 0.3 M-potassium phosphate (pH 7.6)/0.5 mM-dithiothreitol/0.1 mM-Na₂EDTA at –25 °C. Immediately before use, the stock enzyme (~ 80 μM) was incubated with dithiothreitol essentially as described in [10].

The activity of CPSI was determined at 25 °C using the pyruvate/lactate dehydrogenase system [12] with the following modifications: 1 unit of pyruvate kinase, 20 units of lactate dehydrogenase and 0.15 μmol of NADH in a total volume of 0.5 ml. Protein was determined according to [13] or from the *A*₂₈₀ assuming *A*₂₈₀^{1 cm} = 0.96 for a 1 mg/ml solution as calculated for purified CPSI [12].

Modification of CPSI with monobromobimane

CPSI pretreated with dithiothreitol was modified by monobromobimane (MBB; Calbiochem) in 50 mM-potassium Hepes/0.1 mM-Na₂EDTA, pH 7.6 (adjusted with KOH). The reaction was stopped by a 100-fold molar excess of dithiothreitol over MBB. Excess reagent was removed by gel chromatography on a Sephadex G-25 column (2 cm × 96 cm) with 0.1 M-NH₄HCO₃ at pH 8.2. The degree of modification of CPSI was determined according to [14].

Isolation and sequencing of CNBr peptides derived from CPSI

Native or modified CPSI was *S*-carboxymethylated in 6 M-guanidinium chloride (iodoacetate/CPSI molar ratio 500:1) and precipitated by dialysis against water. Cleavage of the *S*-carboxymethylated CPSI by CNBr (200-fold

Abbreviations used: AcGlu, *N*-acetyl-L-glutamate; bimane-labelled, labelled by the 4-methylene-3,6,7-trimethylbimane group; BrAcGlu, *N*-bromoacetyl-L-glutamate; CPSI, carbamoyl-phosphate synthase I (EC 6.3.4.16); MBB, monobromobimane; Tos-PheCH₂Cl, *N*-tosyl-L-phenylalanine chloromethane ('TPCK'); PAGE, polyacrylamide-gel electrophoresis.

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molar excess of CNBr over methionine) and the fractionation of the CNBr fragments on Sephadex G-75 were performed as described [14]. The peptides eluted from the Sephadex G-75 column with 10% (v/v) acetic acid were collected in nine pools. The peptides of the dried pool VII + VIII (Fig. 1) were dissolved in 1 ml of 70% (v/v) formic acid and separated by h.p.l.c. on a Hypersil C₄ wide pore column (Gynkotek) (4.6 mm × 250 mm) using an acetonitrile/0.1% trifluoroacetate gradient. Effluents were monitored by absorbance (220 nm), bimane fluorescence (excitation: 395 nm, emission: 475 nm) and tryptophanyl fluorescence (excitation: 295 nm, emission: 354 nm).

Homoserine lactone-containing peptides were coupled to CPG/3-aminopropyl(3-aminoethyl) glass (Pierce Chemicals) and subjected to automated Edman degradation in a Sequemat Mini 15. Phenylthiohydantoin amino acids were determined as reported by Lottspeich [15].

Tryptic digestion of bimane-labelled CPSI

Bimane-inactivated CPSI (46 nmol) was incubated with Tos-PheCH₂Cl-treated trypsin (28.4:1 molar ratio) in 76 mM-sodium Hepes/1 mM-AcGlu, pH 7.6, for 80 min at 37 °C in the dark. The digestion was stopped by adjusting the sample to a final concentration of 1 mM-phenylmethanesulphonyl fluoride. After subsequent drying, the residue was dissolved in 2 ml of 70% (v/v) formic acid, applied to a Sephadex G-75 column (1.7 cm × 130 cm) equilibrated with 10% (v/v) acetic acid and eluted with the same buffer.

RESULTS

Identification of the cysteine residues in CPSI with AcGlu-dependent thiol reactivity

In the presence of 5 mM-AcGlu, CPSI was completely inactivated by a 5- or 10-fold molar excess of the fluorogenic cysteine reagent MBB over CPSI, corresponding to a stoichiometry of ≤ 0.5 mol of cysteine reagent/mol of thiol group assuming 20 cysteine residues/molecule of porcine CPSI as in the rat enzyme [7]. Using a molar ratio MBB:CPSI = 1:1.2, inactivation of the CPSI did not exceed 70% of the original activity (5 mM-AcGlu, pH 7.6). Half-inactivation of CPSI (6.2–8.4 μ M) by a 10-fold molar excess of MBB was observed within 0.8 min in the presence of 5 mM-AcGlu and within 15 min in the absence of this activator. To identify the cysteines in CPSI protected by ATP/Mg²⁺ against AcGlu-enhanced alkylation, enzyme completely inactivated by MBB (degree of modification 1.9 ± 0.13 bimane groups/mol of CPSI) and CPSI modified with the same reagent in the presence of 10 mM-ATP/10 mM-Mg²⁺ (degree of modification 0.4–0.6 mol of bimane groups/mol of CPSI) were subjected to CNBr cleavage. Peptide maps obtained by chromatography of the CNBr digests on Sephadex G-75 disclosed that 10 mM-ATP/10 mM-Mg²⁺ prevents the labelling of pool VII + VIII peptides (Fig. 1). The distribution of the bimane fluorescence on the CNBr fragments of CPSI inactivated in the absence of 10 mM-ATP/10 mM-Mg²⁺ showed that about 35% of the reagent incorporated is not located in the cysteine peptides of the pool VII + VIII (Fig. 1a). Fractionation of the CNBr fragments in pool VII + VIII obtained from bimane-inactivated CPSI by h.p.l.c. resulted in the isolation of two main bimane-modified

Fig. 1. Gel chromatography of the CNBr peptides obtained from CPSI labelled by MBB (MBB/CPSI molar ratio 10:1; 5 mM-AcGlu) in the absence (a) and the presence (b) of 10 mM-ATP/10 mM-Mg²⁺.

The CNBr peptides were eluted from a Sephadex G-75 column (2.7 cm × 130 cm) by 10% (v/v) acetic acid [14]. —, A_{280} ; —, peptide pool VII + VIII collected; —, bimane fluorescence. The positions for the peak maxima of the standard proteins (bovine serum albumin, M_r 68 000; cytochrome c, M_r 12 500; insulin B chain disulphonate, M_r 3495; bacitracin, M_r 1450) are given.

peptides, CB2 and CB3 (Fig. 2). A third bimane-labelled peptide, CB1, was observed in smaller and varying amounts (Fig. 2). The structures of the fragments CB2 and CB3 established by Edman degradation are identical with the stretches Ala¹³³⁰–Met¹³⁵⁰ (with Cys-1337) and Phe¹³¹³–Met¹³²⁹ (with Cys-1327) respectively determined for the rat CPSI [7] (Fig. 3). CB3 contains an aspartyl-proline bond Asp¹³²²–Pro¹³²³ susceptible to cleavage by acid. During incubation of CB3 in 70% formic acid at 50 °C, the split peptides CB3A (N-terminus Phe) and the bimane-labelled CB1 (N-terminus Pro) showing no tryptophanyl fluorescence were formed. Gas phase sequencing confirmed the identity of CB1 with the fragment Pro¹³²³–Met¹³²⁹ arising from CB3.

N-Bromoacetyl-L-glutamate (BrAcGlu) is an allosteric activator of CPSI ($V_{AcGlu}/V_{BrAcGlu} = 1/0.97$ under the conditions of the enzymic assay) and inactivates the synthase slowly with a pseudo-first order constant of $3 \times 10^{-2} \text{ min}^{-1}$ (BrAcGlu 7.5 mM, CPSI 7.5 μ M). Evidence has been obtained by gel filtration of the CNBr peptides

Fig. 2. H.p.l.c. of the pool VII + VIII peptides obtained by gel chromatography of the CNBr digest from MBB-inactivated CPSI

Elution of the CNBr peptides from a Gynkochrom Hypersil C_4 wide-pore column (25 cm $\times$ 0.46 cm) was performed using a linear gradient (---) composed of 50% acetonitrile in 0.1% trifluoroacetate (solvent A) and 0.1% trifluoroacetate (solvent B). —, A_{220} ; ----, bimane fluorescence.

derived from CPSI that preincubation with BrAcGlu (CPSI/BrAcGlu molar ratio 1:1000 in 10 mM- $MgCl_2$ /0.1 mM-EDTA/50 mM-potassium Hepes, pH 7.6; activity loss > 95%) prevents the modification of cysteines 1327 and 1337 by MBB (CPSI/MB molar ratio 1:840) in 6 M-guanidinium chloride/1 M-Tris/HCl, pH 8.2.

Location of the cysteine residues protected by Mg^{2+} /ATP against modification in domain D of CPSI

Limited proteolysis of MBB-inactivated porcine CPSI by trypsin in the presence of 1 mM-AcGlu led to the formation of polypeptides previously described as 87 kDa and 62 kDa fragments corresponding to domains A + B and domain C of the CPSI respectively [6,16]. According to our results obtained by gel chromatography and SDS/PAGE (Figs. 4 and 5), the bimane label is for the

Fig. 3. Amino acid sequence of the CNBr peptides derived from CPSI containing the cysteine residues protected by ATP/ Mg^{2+} against alkylation by MBB

C*, cysteine residues labelled by MBB; ↓, peptide bond cleaved by acid. The numbering of the amino acid residues is based on the sequence of the CPSI from rat liver [7].

most part incorporated into the peptides of mass ≤ 4000 (pools III and IV in Fig. 4) assumed to originate from tryptic proteolysis of the domain D.

DISCUSSION

Differential labelling of the thiol groups using ATP/ Mg^{2+} as protective ligands allowed us to identify the cysteines in CPSI with enhanced thiol reactivity in the presence of AcGlu as residues 1327 and 1337, the modification of which causes inactivation of the enzyme.

Fig. 4. Isolation of the high- (pool I) and low- (pools II-IV) M_r fragments from a tryptic digest of bimane-inactivated CPSI (15 nmol) by gel chromatography on Sephadex G-75

—, A_{280} ; ----, bimane fluorescence. The experimental protocol is described in the Materials and methods section.

Fig. 5. SDS/PAGE under reducing conditions of the high- and low- M_r peptides obtained by gel chromatography of the tryptic digest from bimane-inactivated CPSI

(a) Lane a, bimane-modified CPSI (10 μ g); lane b, pool I (10 μ g) from Fig. 4; lane c, standard proteins (10 μ g) [glucose oxidase (M_r 93000), bovine serum albumin (M_r 68000), lactate dehydrogenase monomer (M_r 35000), myoglobin (M_r 17800)]; 7.5% acrylamide gel. (b) Lane a, pool II (10 μ g) from Fig. 4; lane b, pool III (10 μ g) from Fig. 4 (bimane fluorescence is marked by light dots in the gel); lane c, standard proteins (10 mg) [cytochrome *c* (M_r 12500), insulin B chain disulphonate (M_r 3495)]; 17.5% acrylamide gel.

Tryptic digestion of MBB-inactivated CPSI demonstrated the presence of both critical cysteines in the domain D of the enzyme. The mass of domain D was estimated to be 20 kDa [6] and it was assumed to comprise

the stretch "within about 160 residues of the COOH-end" [16]. As a cleavage site for trypsin a peptide bond was proposed about 14 residues upstream of the sequence Glu¹³²⁸-Gly¹³⁴¹ [16]. Our results localize the tryptic cleavage site between domains C and D at the Arg¹³²⁶-Cys¹³²⁷ bond or a peptide moiety within the arginine-rich and hydrophilic stretch Pro¹³¹¹-Arg¹³²⁶. The protection by ATP/Mg²⁺ could indicate that the critical cysteines and the cleavage site C are within or near to one of the ATP-binding regions of CPSI. The supposed proximity to an ATP site seems to be confirmed by the suggestion that ATP-binding segments in other adenine nucleotide-dependent enzymes are similar to the stretch 1259-1309 in CPSI (rat) [7]. However, the location of the ATP sites could not yet be established unequivocally by comparison of sequences, since according to Powers-Lee & Corina [8] more likely segments of an ATP site in domain C are the stretches 1070-1078 and 1168-1179, remote from the critical cysteines. The observation of a second trypsin cleavage site a few residues upstream of the C-terminal end within the sequence Ala¹⁴⁸⁰(?)-Ala¹⁵⁰⁰ [16] defines a second region in domain D interacting with ATP and AcGlu which is not similar to typical ATP-binding sequences. The molecular basis for an understanding of the allosteric effects caused by AcGlu is the localization of the AcGlu-binding centre in CPSI. Future studies using reactive analogues of AcGlu promise to provide a way for the labelling of the AcGlu site in CPSI.

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