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STUDIES ON THE ENZYMATIC HYDROLYSIS OF POLYGLUTAMYL FOLATES BY CHICKEN LIVER FOLYL POLY-γ-GLUTAMYL CARBOXYPEPTIDASE

II. STRUCTURAL STUDIES

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Summary

Further studies on the purified chicken hepatic folyl poly-γ-glutamyl carboxypeptidase (peptidyl-L-glutamate hydrolase, EC 3.4.12.10) have elucidated some of the structural characteristics of the enzyme. Various analytical studies described reveal 424 amino acid residues in the isolated native enzyme with molecular weight of around 57 900. β-Mercaptoethanol (14.3 mM) activated the enzyme 2.2-fold and induced reductive cleavage of an interchain disulfide linkage resulting in the splitting of the native enzyme into two active polypeptides (molecular weights 43 000 and 18 000). The constituent polypeptides have identical NH_2-terminal residues (valine) and exhibit a high degree of sequence homology as revealed by finger print analyses of their tryptic digests. The 10-fold greater sensitivity of the reductively cleaved enzyme to p-chloromercuribenzoate would imply that active site related sulfhydryl groups are not readily accessible in the native enzyme. Ionic strength effects in the presence of Mn^{2+} and Na^+ and the presence of low urea concentration (0.55 M) result in a further up to 5-fold stimulation of reductively cleaved native enzyme. Citrate inhibited and phosphate induced autolytic degradation of the enzyme. The physiological role of γ-glutamyl carboxypeptidase has been discussed.

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Abbreviations: SDS, sodium dodecyl sulfate; Nbs_2, 5,5'-dithiobis-2-nitrobenzoic acid; N_2Ph, *N*-2,4-dinitrophenyl; N_2Ph-F, 1-fluoro-2,4-dinitrobenzene.

Introduction

Enzymes capable of hydrolyzing pteroylpolyglutamates, the naturally occurring conjugated forms in which folic acid occurs, are widely distributed throughout the phylogenetic spectrum. Partial purification of folyl poly-γ-glutamyl carboxypeptidase (peptidyl-L-glutamate hydrolase, EC 3.4.12.10) from chicken pancreas [1], hog kidney [2] and human tissues [3] have been reported. An 80 000-dalton pteroyl-γ-oligoglutamyl endopeptidase which is part of a multienzyme complex hydrolyzing pteroylpolyglutamates [4] has recently been isolated from chick intestine [5]. A zinc-stabilized γ-glutamyl hydrolase purified from bovine liver with a molecular weight of 108 000 has also been reported [6]. We describe here some structural characteristics of the chick hepatic enzyme whose purification to homogeneity and some properties have been described in the previous article [7]. The native enzyme consists of two disulfide-linked polypeptides of unequal chain lengths, which are separable and independently active.

Materials and Methods

All the chemicals used were of analytical grade. Sephadex G-100 was a product of Pharmacia Fine Chemicals, Uppsala. Sucrose, urea (both "Ultra Pure") and the nonenzymatic marker proteins bacitracin, cytochrome *C*, myoglobin, chymotrypsinogen A, ovalbumin and albumin were purchased from Mann Research Laboratories, Orangeburg, New York. Nbs_2, sodium borohydride, N_2Ph amino acids, carboxypeptidase A, trypsin, Dextran Blue, aldolase and SDS were the products of Sigma Chemical Company, St. Louis, Missouri. Silica gel G and N_2Ph-F were the products of E. Merck AG, Darmstadt. Catalase was obtained from Calbiochem, Los Angeles whereas trypsin inhibitor was a product of Worthington Biochemical Corporation, Freehold, New Jersey.

Preparation of the purified enzyme and the enzyme assay

Folyl γ-glutamyl carboxypeptidase from chicken liver was purified and microbiologically assayed as described earlier [7]. Protein was measured according to the method of Lowry et al. [8].

Molecular weight determinations

Molecular weight determinations of the enzyme were carried out by (a) gel filtration on Sephadex G-100 columns according to the method of Andrews [9], (b) 5–15% sucrose density gradient analyses essentially according to the method of Martin and Ames [10] and (c) SDS polyacrylamide gel electrophoresis in 7.5% analytical gels essentially as described by Weber and Osborn [11]. Standard marker proteins of known molecular weight were run simultaneously under identical conditions. These included bacitracin, horse heart cytochrome *C*, sperm whale myoglobin, soybean trypsin inhibitor, beef pancreatic chymotrypsinogen A, rabbit muscle aldolase, ovalbumin, bovine liver catalase and bovine serum albumin. The purified enzyme protein was also run in the presence of indicated amounts of metal ions, anions and denaturants.

Amino acid analyses

Protein hydrolyzates of the enzyme were prepared as described by Crestfield [12] and the hydrolyzates were analyzed for the free amino acids as described by Spackman et al. [13] on a Beckman Unichrom automatic amino acid analyzer. Tryptophan content was determined spectrophotometrically by the method of Goodwin and Morton [14]. The nearest integral number of amino acid residues were averaged from three independent analyses with reference to the chromatogram obtained for a standard amino acid mixture.

End-group analyses

(a) NH_2-terminal end-group analysis. The amino-terminal end-group analysis of purified γ-glutamyl carboxypeptidase was carried out by the N_2pH-F method of Sanger [15], as described by Fraenkel-Conrat et al. [16]. The N_2Ph-amino acid derivatives of the protein (17 mg) were analyzed by thin (approximately 0.25 mm) layer chromatography on 20 × 20 cm Silica gel G plates. Two different solvent systems were used for developing the plates (a) chloroform/benzyl alcohol/acetic acid (70 : 30 : 3) and (b) chloroform/methanol/acetic acid (95 : 5 : 1) as described by Bailey [17]. The N_2Ph-amino acids were identified by running authentic samples (Sigma) under identical conditions. Quantitative NH_2-terminal amino acid analysis of the enzyme was carried out according to the method of Levy [18] and Fraenkel-Conrat et al. [16].

(b) COOH-terminal end-group analysis. The carboxy-terminal end-group analysis of purified chicken liver γ-glutamyl carboxypeptidase was carried out employing carboxypeptidase A essentially as described by Fraenkel-Conrat et al. [16]. The ratio of the enzyme protein to bovine carboxypeptidase A (Sigma) was 10 : 1 (w/w).

Peptide analysis

Tryptic hydrolyzates of the native enzyme and its dissociated polypeptides were prepared by digesting the protein with trypsin (100 : 1) for 24 h at 37°C in 0.05 M Tris · HCl buffer (pH 8.0). Hydrolysis was stopped by lowering the pH to 2.0 with 6 M HCl. The digests were analyzed for the cleaved tryptic peptide fragments by two-dimensional paper chromatography. The solvent systems employed were butanol/acetic acid/water (40 : 10 : 50) in the first dimension and phenol/water (80 : 20) in the second dimension.

Thiol assay

Total -SH, free -SH and disulfide (-S-S-) contents of the native enzyme and its dissociated polypeptides were determined essentially by the Nbs_2 method of Ellman [19] with some modifications as described by Butterworth et al. [20].

Effect of p-chloromercuribenzoic acid treatment

The purified enzyme (0.4 mg protein) either in absence or presence of β-mercaptoethanol (14.3 mM) in 0.4 ml 20 mM phosphate buffer (pH 6.0) was preincubated at 27°C for 25 min with from 25—250 μM *p*-chloromercuribenzoic acid and used to initiate the 2 ml γ-glutamyl carbopeptidase reaction as detailed earlier. In the concentrations used *p*-chloromercuribenzoic acid did not affect the microbiological assay.

Results

Molecular weight of the enzyme. An approximate molecular weight of 57 500 was determined for the purified folyl γ-glutamyl carboxypeptidase by Andrew's method [9] employing Sephadex G-100 exclusion chromatography. The protein eluted at an elution volume (V_e) of 50 ml and the distribution coefficient (K_{av}) calculated for the enzyme was found to be 0.386. On sucrose density gradient sedimentation analysis the enzyme migrated 1.8 ml starting from the upper mensiscus and a sedimentation coefficient ($S_{20,w}$) of 2.01 S was determined from the linear calibration curve obtained as described by Martin and Ames [10]. Accordingly an average molecular weight of 55 300 was calculated for the enzyme.

In earlier report [7] we have indicated that the native enzyme moves as a homogeneous band on polyacrylamide gel electrophoresis in the absence of mercaptoethanol. In the presence of mercaptoethanol however, it was found that activity was distributed in two bands. SDS gel electrophoresis resulted in further separation of two polypeptide components according to their molecular weights (Fig. 1(1)). The molecular weights of the constituent polypeptides were calculated to be 43 000 and 18 000 daltons which when added together is somewhat higher than the molecular weight of the undissociated native enzyme as determined by gel filtration (57 500) and the sucrose density gradient analyses (55 300).

The SDS-gel electrophoresis patterns of the enzyme obtained in the added presence of 0.1 M Na^+, 1.6 M Mn^{2+} and 0.55 M urea which caused 3—5-fold (optimal) stimulation of the enzyme [7] were exactly similar to that obtained in the presence of 14.3 mM β-mercaptoethanol alone (Fig. 1(2), 1(3), 1(4) and 1(1) respectively). In the presence of higher inhibitory concentrations of urea (8 M), SDS gel electrophoresis revealed a single species of molecular weight 57 500 suggesting a random coil denaturation of the enzyme without the reductive cleavage. The autolytic digestion promoted by 0.1 M phosphate [7] is evident in Fig. 1(6) where the smaller fragments formed (molecular weights 24 000, 21 000 and 17 000) are observed. At higher phosphate concentrations (0.5 M) even smaller peptides are observed which are completely inactive (untabulated results). The presence of inhibitory citrate levels however does not alter the separation pattern (Fig. 1(5)).

Amino acid content. Amino acid composition of the protein hydrolyzate of the purified chicken liver enzyme is presented in Table I. The enzyme is calculated to have 424 amino acid residues. It is evident that aspartic and glutamic acid contents of the hydrolyzate constitute the major part of the total amino acids (about 27% of the total amino acids). Other amino acid residues were leucine, glycine, lysine and proline (about 30% of the total amino acids). The enzyme seems to be relatively poor in methionine and histidine residues which represent less than 4% of the total amino acids of the protein.

NH_2-terminal end groups. Our results show that valine is the only NH_2-terminal amino acid detected by Sanger's method [15] for the purified enzyme. The N_2Ph-amino acid obtained in four experiments migrated as a single spot on Silica gel-G thin layer chromatography and its R_F value was identical with that of an authentic sample of N_2Ph-L-valine. Quantitative analysis of the N_2Ph-

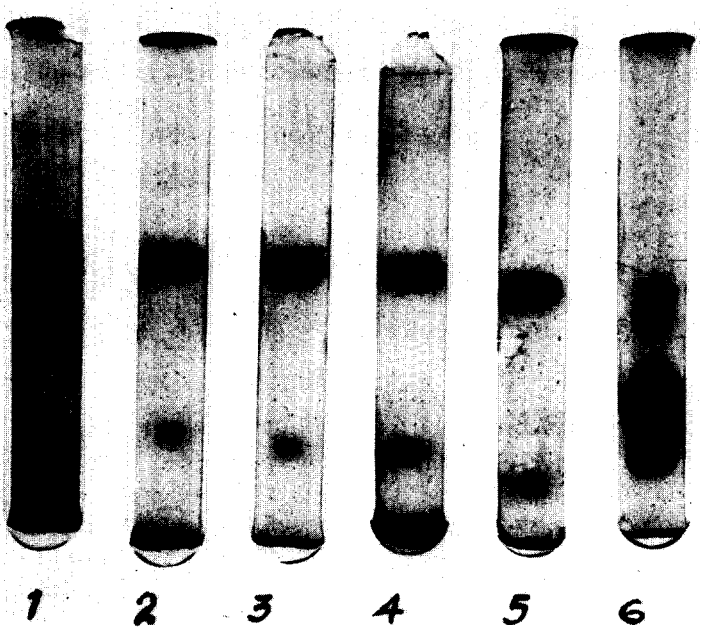

Fig. 1. SDS polyacrylamide gel electrophoresis of purified chicken liver γ-glutamyl carboxypeptidase. The enzyme preparations were treated and the gels run in presence of mercaptoethanol as described by Weber and Osborn [11]. Gel 1 represents the control SDS electrophoretogram of the enzyme. Gels 2, 3 and 4 represent the SDS electrophoretograms of the enzyme obtained in the presence of stimulatory concentrations of cations Na^+ (0.1 M) and Mn^{2+} (1.6 M) and 0.55 M urea respectively whereas the gels 5 and 6 represent the SDS electrophoretograms of the enzyme obtained in the presence of inhibitory concentrations of citrate (0.1 M) and phosphate (0.1 M) respectively. 7.5% Analytical polyacrylamide gels containing 0.1% SDS were employed using 0.05% Bromophenol Blue as the tracking dye. About 35—40 μg (40 μl) samples of the enzyme protein were applied over the gels and the electrophoresis was carried out with a net current of 8 mA/gel at 80 V for 3 h 10 min. The gels were stained in 1% Coomassie Brilliant Blue and destained in 7% acetic acid.

valine content (1.79 mol/mol) determined by the method of Levy [18] indicated the presence of two residues per molecule of the enzyme. This evidence confirms that the purified enzyme is comprised of two constituent polypeptide chains, each chain having valine as the NH_2-terminal amino acid.

COOH-terminal end groups. Hydrolysis of the purified chicken liver enzyme with carboxypeptidase A resulted in the release of four amino acids, viz. alanine, leucine, valine and isoleucine. Of these four amino acids, alanine and leucine were the first to be cleaved. Detectable amounts of alanine and leucine appeared as early as 30 min after carboxypeptidase A hydrolysis. About 0.6–0.7 residues each of these amino acids were detected after 3 h of digestion. A plateau value of 1 mol/mol of enzyme was reached after 6 h of hydrolysis. This value remained constant over a further period up to 48 h of hydrolysis. Valine was released at a much slower rate and only 0.5 residues per mol of enzyme was released after a 48-h digestion. The release of isoleucine (0.3 residue) was much slower in comparison with valine. The rate of release of various amino acids after carboxypeptidase A digestion is shown in Fig. 2. It is thus concluded that alanine and leucine are the carboxy terminal residues for two different polypeptide chains constituting the chicken liver γ-glutamyl carboxypeptidase

TABLE I

AMINO ACID COMPOSITION OF CHICKEN LIVER γ-GLUTAMYL CARBOXYPEPTIDASE

Amino acid	Nearest integral No. of amino acid residues/56 400 daltons *
Aspartic acid	55
Threonine	13
Serine	16
Glutamic acid	58
Proline	26
Glycine	28
Alanine	23
Valine	31
Methionine	10
Isoleucine	17
Leucine	45
Tyrosine	16
Phenylalanine	17
Lysine	28
Histidine	6
Arginine	15
Ammonia	13
Tryptophan	12
Total half-cystine **	8
Total	424

* Average molecular weight of the enzyme as determined by gel filtration and sedimentation analyses (see Results).

** As determined from the -SH content arrived at by the Nbs_2 method (see Results).

enzyme. These COOH-terminal residues were preceded by valine and isoleucine.

Peptide analyses. Peptide analyses of the tryptic digests of the native enzyme and its dissociated polypeptides by two-dimensional paper chromatography revealed 21 peptide fragments for the native enzyme. The larger polypeptide was resolved into 19 fragments while the smaller polypeptide contributed to 8 separable fragments. Six of these fragments from each of these two polypeptides have identical R_F values. Thus the evidence suggests a large degree of homology between the smaller polypeptide and portions of the larger polypeptide.

Sulfhydryl content. Total -SH, free -SH and the disulfide (-S-S-) contents of the chicken liver native enzyme were calculated to be 7.74, 2.84 and 2.45 mol/mol enzyme respectively by the procedure of Ellman [20]. Total -SH, free -SH and the disulfide contents of the larger constituent polypeptide component (polypeptide I) were measured to be 4.16, 1.94 and 1.11 mol/mol polypeptide whereas those of the smaller constituent polypeptide component (polypeptide II) were 1.77, 1.99 and 0.0 mol/mol polypeptide. Since the native enzyme has an additional disulfide linkage as compared to the total disulfide residues of the separated polypeptides, it could be inferred that in the native enzyme these two polypeptides are linked by a single interchain disulfide bridge. The larger polypeptide component alone has a single intrachain disulfide linkage and each polypeptide has in addition a free -SH group. Reductive cleavage in presence of β-mercaptoethanol probably occurs at the interchain disulfide linkage.

p-Chloromercuribenzoic acid inhibition. Pretreatment of the enzyme in the absence of mercaptoethanol with 50 μM *p*-chloromercuribenzoate resulted in

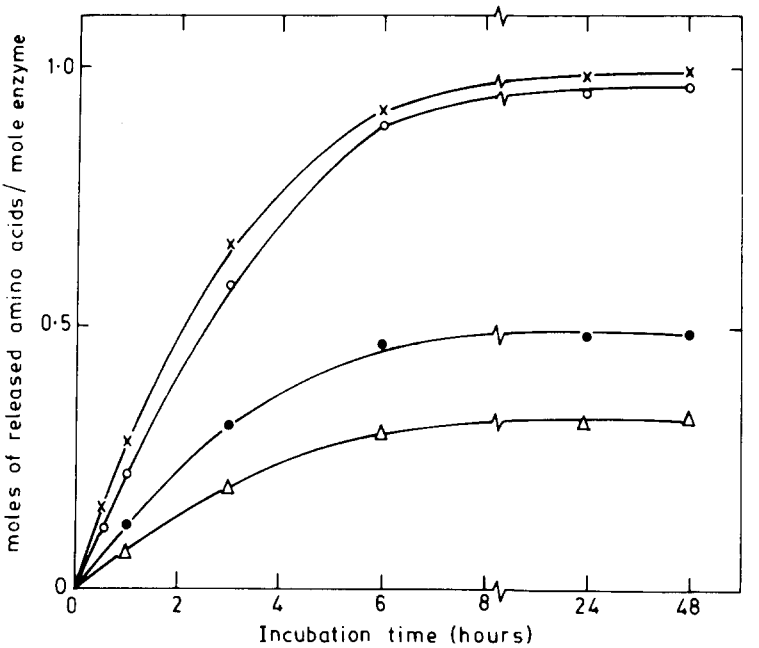

Fig. 2. Release of amino acids from purified chicken liver γ-glutamyl carboxypeptidase by the action of carboxypeptidase A. The enzyme protein (20 mg) was digested at 37°C by incubating with bovine pancreatic carboxypeptidase A (2 mg) in a final volume of 2 ml of 0.2 M $NaHCO_3$ buffer (pH 8.2) containing 3% Li Cl. Aliquots (0.1 ml) were withdrawn at various time intervals and the reactions terminated by precipitation with 0.2 M citrate buffer (pH 2.2) (0.9 ml). The precipitates were removed by centrifugation and the supernatants were analyzed for free amino acids on a Beckman Unichrom automatic amino acid analyzer [13]. Alanine X, Leucine ○, Valine ●, Isoleucine △.

50% inhibition of activity while 70% activity was lost with the 100 μM *p*-chloromercuribenzoate treatment. However approximately the same levels of enzymatic inhibition (48 and 68%) were observed when the enzyme was treated in the presence of 14.3 mM β-mercaptoethanol with 5 and 10 μM concentrations of *p*-chloromercuribenzoate respectively. These experiments clearly imply a role for reduced thiol groups in the catalytic activity of the enzyme and suggest a 10-fold sensitivity to *p*-chloromercuribenzoate of the reductively cleaved and activated enzyme.

Discussion

The experiments described in this paper have been aimed at characterizing a purified preparation of chicken liver folyl γ-glutamyl carboxypeptidase with respect to a number of its structural aspects. A mean molecular weight of 58 000 can be arrived at for the chicken liver enzyme from the values obtained on gel filtration (57 500), sucrose density gradient sedimentation (55 300), SDS gel electrophoresis (61 000), the average molecular weights of the constituant polypeptide chains obtained on gel filtration and gel electrophoresis in the presence of mercaptoethanol (42 000 and 17 500) and the molecular weight derived for the inactivated and undissociated enzyme in presence of 8.0 M urea (57 500).

There is ample evidence that the native enzyme consists of two enzymatically active and separable polypeptide chains of unequal lengths. The quantitative end group analyses data suggests the presence of two dissimilar chains each with valine at the NH_2-terminal end. Gel electrophoretic studies suggest that the native molecule is reductively split in the presence of mercaptoethanol to yield two unequal polypeptide fragments, with average molecular weights of 42 000 and 17 500. Although there is a 2.5-fold difference in the molecular sizes of the constituent polypeptides there is considerable homology in their amino acid composition as is evident from the peptide mapping of their tryptic digest fragments. Possibly this homology is confined to their NH_2-terminal end and extends to include sequences contributing to their catalytic activity. Autolytic deletions observed in the presence of higher phosphate concentrations could possibly proceed from the COOH-terminus and are of consequence after a crucial minimal chain length is reacted beyond which catalytic activity is adversely affected. The structure envisaged comprising of two linked unequal polypeptide chains with sequence homology could possibly have arisen as an artifact during isolation resulting from proteolytic degradation by lysosomal enzymes of an original homodimeric form. These peptides could possibly be transcribed from the same gene as is suggested for the native dimeric form of mouse liver β-glucuronidase [21] where also the enzymatic activity is attributed to components having different electrophoretic mobilities.

Both the constituent polypeptides have a free sulfhydryl group and are linked in the isolated native form by a single disulfide group. Maintenance of reduced sulfhydryl groups, augmented in the presence of mercaptoethanol is probably associated with the catalytic activity of the enzyme. Accordingly the enzyme is inhibited in the presence of organomercurials (see Results). The native enzyme is stimulated over two fold by mercaptoethanol, in the presence of which gel filtration and electrophoretic studies reveal that the polypeptide constituents exist independently after reductive cleavage of the interchain disulfide bridge. The further observation that β-mercaptoethanol sensitized the enzyme ten fold to organomercurials would imply that active site-related sulfhydryl groups were not as readily accessible in the isolated native enzyme. Stearic hinderance factors are probably relieved after cleavage of the native enzyme in the presence of mercaptoethanol activating the enzyme, but also making it more susceptible to sulfhydryl inhibitors.

Stimulatory ionic strength effects, observed in presence of Mn^{2+} and Na^{+} as well as the 5-fold stimulation induced by favourable secondary and tertiary structural alterations in presence of 0.55 M urea are also observed with the native enzyme in the reductively dissociated state. In the presence of inactivating urea concentrations random coil denaturation of the enzyme occurred without cleavage of the enzyme into its constituent polypeptides despite the presence of mercaptoethanol.

While polyglutamyl tetrahydrofolate derivatives are well recognized as the active folyl cofactors mediating most of the one-carbon transfer reactions known [22—24], certain folyl polyglutamates act as powerful inhibitors of thymidylate synthesis [25,23]. Monoglutamyl forms are preferred for storage, absorption and transport of the vitamin [26]. A key metabolic role could be played by folyl γ-glutamyl carboxypeptidase by altering the γ-glutamyl chain

lengths of various derivatives within the folate pool. In fact recently Krumdieck et al. have shown that cycles of uterine cell growth and involution could be mediated through hormonally induced changes in the levels of γ-glutamyl carboxypeptidase which act by controlling the conversion of folyl metabolic inhibitors to their active coenzyme derivatives and vice versa [27].

The enzyme presently described has the advantage of stability and simplicity of preparation in a highly pure form, making it probably the most extensively characterized folyl γ-glutamyl carboxypeptidase reported to date. Its properties including the multiple pH optima [7], its potent inhibition by citrate and its degradation induced by higher phosphate concentrations suggest mechanisms by which the enzyme activity could be sensitively modulated in vivo. The fairly large levels in which the enzyme occurs ubiquitously in natural material certainly calls for a more intensive search for its suggested, but little understood role in regulating cell growth and differentiation [27].

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