EVIDENCE FOR PYRROLIDONE CARBOXYLIC ACID IN β-GALACTOSIDASE FROM E. COLI

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SUMMARY. Acidic peptides isolated from pronase digests of β-galactosidase by chromatography on Dowex 50-X2 contain N-terminal pyrrolidone carboxylic acid. This was not due to cyclization of glutamine or glutamic acid to pyrrolidone carboxylic acid during the procedure as addition of excess fluorodinitrobenzene immediately following digestion and prior to the chromatographic step did not alter the results. Acetylation of β-galactosidase prior to the procedure resulted in recovery of additional amino acids which were compatible with the previously established N-terminal threonine. These data indicate the presence of multiple polypeptide chains in the β-galactosidase monomers of 135,000 molecular weight, with pyrrolidone carboxylic acid and threonine as N-terminal residues.

The enzyme β-galactosidase from Escherichia coli has been characterized as a tetramer consisting of four identical monomers of 135,000 molecular weight each (1-6). Previous studies have shown that the monomer represents a unique sequence of amino acids (4). The possibility that these 1100 amino acids which do not have major repeating sequences represent a single or several polypeptide chains has been the subject of much discussion. Wallenfels et al. (7) and Steers et al. (4) concluded from physico-chemical studies that the monomer represents three or four sequentially different polypeptide chains. Fluorodinitrobenzene has revealed less than one mole of amino-terminal threonine per 135,000 (8-10) although Wallenfels reported glutamic acid in amounts as high as 0.7 moles per monomer in addition to threonine (8). Brown et al. showed a single carboxy-terminal lysine per 135,000 molecular weight leading them to conclude that the monomer represented a single polypeptide chain (10).

Pyrrolidone carboxylic acid is the amino-terminal residue of a variety of peptides and proteins (11). By pronase digestion and ion-exchange chromatography of the resulting peptides, we have identified pyrrolidone

carboxylic acid in β-galactosidase in amounts greater than 1 mole per mole of 135,000 M.W. These peptides are acidic because of the absence of a free Dowex-50 (H^+ form) on which they will not be retained.

EXPERIMENTAL. Purification. The β-galactosidase employed in this study was prepared from the regulator constitutive strain of E. coli K_{12}, 3300, by a minor modification of the procedure used previously (12, modification published in detail in 13). The purified protein was stored under ammonium sulfate at 4° or carboxymethylated following reduction with mercaptoethanol and stored in the inactive form.

Isolation of Acidic Peptides. Carboxymethylation of β-galactosidase was performed as previously described (9,12) and the protein dialyzed against 0.2 M NH_4HCO_3. Pronase (Calbiochem) was added (2% w/w) and proteolytic digestion was carried out at 37° for 3 hours. The resulting hydrolysate was lyophilized, taken up in de-ionized water, and passed at 4°C over a column (1 x 10-12 cm) of Dowex-50 (X-2, H^+ form, Bio-Rad) previously washed with water. The acidic peptides were eluted with water, and detected spectrophotometrically at 230 mμ. In some cases the β-galactosidase was acetylated prior to the pronase digestion. The β-galactosidase was dissolved in 7 M guanidine-HCl (Aldrich) half saturated with sodium acetate and 1/25 vol. acetic anhydride (Allied Chemicals) was added three times at 5 minute intervals at 0°C. It was then dialyzed against 0.2 M NH_4HCO_3, reduced and carboxymethylated, and handled as above. Alternately, fluorodinitrobenzene (Aldrich) was added (0.2 mg/100 mg) at the end of the pronase digestion and incubation at 37° continued for four hours. At the end of two hours, a second aliquot of the reagent was added. The excess fluorodinitrobenzene was largely removed by multiple extractions with ether. The solution was then acidified and most of the DNP-peptides removed by extraction with ethylacetate. The extracted solution was neutralized and chromatographed on Dowex-50 as before.

Characterization of Peptides. N-terminal analyses were performed by a modification of the fluorodinitrobenzene procedure (14) utilizing one

molar trimethylamine acetate (Eastman) for the reaction buffer, and by the dansyl (1-dimethylaminonapthalene-5-sulfonyl) chloride (Pierce) procedure (15) Pyrrolidone carboxylic acid was opened to glutamic acid by acid hydrolysis in 1N HCl for 10 minutes at 100°C or by alkaline hydrolysis in 1N NaOH for 20 minutes at 100°C or 12 days at 25°. Performic acid oxidation of peptides (16) was also performed so as to detect any possible methionine by the dansyl procedure. Amino acid analyses were performed with the Beckman Model 120B Amino Acid Analyzer following hydrolysis in 6N HCl.

RESULTS. The peptides isolated on Dowex-50 from a pronase digest of β-galactosidase contained several amino acids with glutamic acid predominating (Table I). The peptides were unreactive with ninhydrin, dansyl chloride, or fluorodinitrobenzene indicating the absence of free amino groups (Table II). When treated by mild acid hydrolysis (1N HCl at 100° for 10 min.) glutamic acid, with trace amounts of aspartic acid, was found by both the dansyl and FDNB methods. The small amount of aspartic acid suggests some peptide bond cleavage. Fluorodinitrobenzene treatment of mild base-hydrolyzed peptides (1N NaOH at 25° for 12 days or at 100° for 20 min.) gave glutamic acid as the N-terminal amino acid. (The dansyl method could not readily be used with the products of alkaline hydrolysis because of the large amounts of salt.) Dansylation after performic acid oxidation did not reveal methionine. Tryptophan would not be detected by the dansyl procedure as we have performed it. These results are only compatible with the presence of N-terminal pyrrolidone carboxylic acid in the acidic peptide fraction eluted from the Dowex columns. Highly acidic peptides with a free α-amino group might be eluted from the Dowex-50 but the dansyl and dinitrofluorobenzene analyses of the intact peptides show that this has not occurred with β-galactosidase. However, we have found acidic peptides with free α-amino groups following the pronase digestion of thyroglobulin, bovine serum albumin and pronase itself (auto-digestion) while treatment of these peptides with 1N HCl for ten minutes at 100°C does not result in large amounts of N-terminal glutamic acid.

TABLE I

Amino Acid Composition of Hydrolyzed Acidic Peptides Isolated by Chromatography on Dowex-50-X2 from β-galactosidase Digested with Pronase.

Amino acid	β-galactosidase		β-galactosidase FDNB added after digestion with pronase		Acetylated β-galactosidase	
	mole/mole 135,000	mole %	mole/mole 135,000	mole %	mole/mole 135,000	mole %
Lys					.316	4.2
SCMC*	.6	17.5	.104	2.7	.989	13.2
Asp	.373	10.9	.594	15.1	.575	7.7
Thr	.211	6.2	.446	11.3	.624	8.3
Ser	.219	6.4	.311	7.9	1.159	15.5
Glu	1.232	36.2	1.108	28.2	1.313	17.5
Pro	.105	3.1	.203	5.2	.340	4.5
Gly	.438	12.8	.527	13.4	.803	10.7
Ala	.235	6.9	.345	8.8	.470	6.3
Val			< .1	7.4	.203	2.7
Met			< .1		.105	1.4
Isoleu			< .1		.154	2.1
Leu			< .1		.195	2.6
Tyr			< .1		.113	1.5
Phe			< .1		.138	1.8

* Carboxymethylcysteine

Equally, peptides containing a high proportion of basic amino acids could be missed although the chromatographic behavior of the small peptides presumably resulting from pronase digestion should be dominated by the blocked α-amino group. In this regard, aminoethylation of β-galactosidase somewhat decreases the yield of acidic peptides.

The cyclization of glutamine or glutamic acid to pyrrolidone carboxylic

TABLE II

N-terminal Amino Acid Determinations of Acidic Peptides Isolated by Chromatography on Dowex 50-X2 from β-galactosidase Digested with Pronase

Source of peptides, treatment	Dansyl	FDNB
β-galactosidase		
no treatment	0	0
1N HCl, 100°, 10'	glu > asp	glu, asp
1N NaOH, 100°, 20'	---	glu
1N NaOH, 25°, 12 days	---	glu
performic acid oxidation	glu	---
β-galactosidase, FDNB added after digestion with pronase		
no treatment	glu (trace)	---
1N HCl, 100°, 10'	glu	---
Acetylated β-galactosidase		
no treatment	0	---
1N HCl, 100°, 10'	glu > asp ≃ ser > thr	---

acid is weak acid or base catalyzed while strong acid or base hydrolyzes the ring structure to glutamic acid (17). This occurs with glutamine or glutamic acid in N-terminal positions of peptides and proteins and may be catalyzed by the acidic sulfonic acid groups of the Dowex-50 under certain conditions (see 11 for review). Such cyclization has been shown not to occur at 4° with one synthetic, glutamine N-terminal tripeptide (18). To further test this point we added a large excess of fluorodinitrobenzene to the pronase digestion mixture to block all free N-terminals and prevent their possible cyclization as a result of subsequent handling. We see in Table I that the yield of glutamic acid per monomer remains the same although there is less carboxymethyl cysteine. It is possible that the first yield represents the relative

acidity of the carboxymethylcysteine-reduced, carboxymethylated bovine serum albumin yielded acidic peptides with a large amount of carboxymethylcysteine while aminoethylated BSA yielded much smaller amounts. It is not clear why the fluorodinitrobenzene treatment would reduce the yield of such cysteic peptides from β-galactosidase and why the end-group analyses did not reveal free α-amino groups corresponding to them.

It was of interest to see whether amino acids corresponding to the previously described threonylmethionine N-terminal sequence (19) could be obtained in the acidic peptide fraction by blocking the free amino group with acetic anhydride. The aceidic peptide mixture isolated from the acetylated β-galactosidase showed larger amounts of peptides eluted, presumably due to acetylation of the basic lysines which are now present in significant amounts (Table I). The acetylated fraction contained more threonine and methionine than the acidic peptides from the unacetylated β-galactosidase (the 0.6 M of threonine per mole 135,000 M.W. should be compared with .2 M and .4 M for unacetylated and unacetylated with FDNB-treatment, respectively). More significantly, following mild acid hydrolysis, dansyl-reactive threonine was detected along with serine and aspartic acid which were perhaps adjacent to labile peptide bonds. This is compatible with the hydrolysis of acetyl-threonine to threonine.

DISCUSSION. One of the difficulties with theories of multiple polypeptide chains determined by the *z* gene has been the failure to find the appropriate quantities of N- and C-terminal residues in β-galactosidase. Brown *et al.* (10) have also isolated acidic peptides by chromatography on Dowex-50, with an even higher yield of glutamic acid than we find. They concluded, however, that the result was due to post-digestion cyclization of glutamine residues. This does not appear to be the explanation as evidenced by the results of fluorodinitro-benzene treatment. We have not excluded the possibility that cyclization occurs during the pronase digestion. Such cyclization within the short period of time employed in this study is highly unlikely and has not been found with a number

of proteins where pronase digestion was utilized: α_1-glycoprotein (18), heavy chains of immunoglobulins (20,21), and light chains of γ-globulin (22).

The 1.1-1.3 mole of glutamic acid per mole of monomer that we find following acid hydrolysis of the acidic peptides certainly represents a minimum value when losses are taken into consideration. We conclude that there are more likely two pyrrolidone carboxylic acid residues per monomer of β-galactosidase as determined by the above procedure. Preliminary characterization of the composition and partial sequences of several ninhydrin negative - NaClO-KI positive peptides isolated from peptide maps are compatible with two different PCA N-terminal sequences (23).

The question remains whether there is a DNA code word for pyrrolidone carboxylic acid, perhaps as a minor initiator, or whether glutamine or glutamic acid are cyclized in the cell or during protein purification when N-terminal. It has been recently shown that pyrrolidone carboxylate-tRNA may be formed from glutaminyl-tRNA (24) but the product has not been shown to participate in protein synthesis *in vitro* or *in vivo*.

To our knowledge, this is the first time that pyrrolidone carboxylic acid has been demonstrated in a bacterial protein. The existence of pyrrolidone carboxylyl peptidase, an enzyme which removes N-terminal pyrrolidone carboxylic acid, in *Pseudomonas fluorescens* (25) and *Aerobacter cloacae* (26) suggests a more wide-spread distribution of N-terminal pyrrolidone glutamic acid in bacterial species.

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