OLIGOMERIZATION OF α-THIOGLUTAMIC ACID

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Abstract. A decaglutamic acid primer is extended very slowly by α-thioglutamic acid. The addition of bicarbonate to the reaction mixture greatly accelerates the reaction. We believe the N-carboxy-anhydride of glutamic acid is an intermediate in the accelerated reaction. When $K_3Fe(CN)_6$ and bicarbonate are both present in the reaction mixture, oligoglutamic acids up to at least the 15-mer are formed rapidly. The acylating agent is the oxidation product of thioglutamic acid, a diacyldisulfide.

Keywords: bicarbonate, ferricyanide, oligomerization, thioglutamic acid

1. Introduction

Thioesters have been used extensively in the synthesis and ligation of peptides (Dawson *et al*., 1994), but there have been few reports of peptide synthesis from free α-amino thioacids. Wieland showed that the thioacid of alanine yields significant amounts of peptides when incubated in aqueous solution if bicarbonate is present, but not in the absence of bicarbonate (Wieland, 1988). He postulated that the N-carboxyanhydride is an intermediate in the productive reaction. More recently there has been renewed interest in thiol activation of amino acids in the context of prebiotic chemistry. It has been suggested that life originated in the deep-sea vents and that peptide synthesis may have occurred in the vents on the surface of transition-metal sulfides (Wächtershäuser, 1988, 1992). Thioacids or possibly thioesters are thought to be likely intermediates.

We have recently become interested in the oxidative acylation using thioacids. We found that while the thiocarboxylic acids themselves are not effective acylating reagents in aqueous solution, the dithio-diacids, that are formed when thioacids are oxidized, are extremely effective (Liu and Orgel, 1997). Phenylalanine, for example, is converted very rapidly and completely to N-acetylphenylalanine in the presence of thioacetic acid and an oxidizing agent. Here we discuss the effect of oxidation on the formation of peptides from α-thioglutamic acid (GluSH).

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2. Experimental Section

2.1. Materials and methods

Nα-t-Boc-L-Glutamic acid γ-t-butyl ester N-hydroxysuccinimide ester (Boc-glu-(OtBu)-OSu) was purchased from Novabiochem, lithium sulfide from Aldrich Chemical Company. The decamer $(L\text{-glu})_{10}$ was synthesized in the Peptide Biological Laboratory at the Salk Institute or purchased from Cybergene laboratory.

Paper chromatography was carried out on Whatman 3MM paper using the descending method. The solvent system was n-butanol-acetic acid-water (4/2/2). Bands corresponding to molecules containing GluSH were visualized under U.V. irradiation. Elution from paper was carried out using water. Column chromatography was performed on DEAE cellulose.

HPLC analysis was performed on an RPC-5 analytical column (Joyce *et al.*, 1984) using a Waters automated gradient controller in combination with a Waters model 510 solvent delivery system and a sample processor model 715 WISP. A linear gradient of $NaClO_4$ (0 to 0.03 M in 40 min at a flow rate of 1 mL min_{-1}) was used to elute the oligomers formed in the reaction. The eluate was monitored at 220 nm using a Waters 490E programmable multiwavelength detector.

2.2. Preparation of α-thioglutamic acid from a Nα (-Cγ) protected N-hydroxy succinimide ester of glutamic acid and Li_2S

N-protected thio-α-aminoacids can be synthesized by the interaction of the corresponding N-hydroxysuccinimide esters with Li_2S (Mitin and Zapevalova, 1990; Yamashiro and Blake, 1981; Liu, 1996). Boc-glu(Otbu)-COSH obtained in this way is soluble in organic solvents.

A solution of Li_2S (0.460 g) in 5 mL DMF was added dropwise to a solution of Boc-Glu(OtBu)-OSu (1 g; 2.5 mmol) in DMF (10 mL) cooled to 0 °C under argon. The mixture was stirred at 0 °C for 1 hr, then water was added (5 mL) and the mixture was stirred for a further 1 hr at 0 °C. After adition of aqueous HCl (1 N, about 18 mL) to adjust the pH to 3, the solution was extracted with ethylacetate (2×50 mL). The organic layer was washed with water (3×25 mL), then with aqueous saturated NaCl (>6 N, 25 mL) and finally dried over anhydrous $MgSO_4$. After filtration and evaporation under reduced pressure, the residue was deprotected.

The residue was dissolved at 0 °C in TFA (25 mL) under argon and the solution was stirred at 0 °C for 1 hr, then for 1 hr at room temperature. Solvent was removed under reduced pressure and the cream-colored oil obtained was triturated with anhydrous diethylether. The solid thioglutamic acid was filtered and washed with diethylether (overall yield: 70%). After drying, the solid was dissolved in aqueous KOH to reach a pH > 5.5, aliquoted, and then stored at –90 °C.

GluSH was purified by chromatography either on paper or on DEAE cellulose. Paper chromatography was carried out on Whatman 3 MM paper using the descending method. The solvent system was n-butanol-acetic acid-water (4/2/2, bubbled with argon) and elution from paper was carried out using water. Column chromatography was carried out on DEAE cellulose by stepwise elution with acetic acid (0.01 M – 0.07 M – 0.08 M – 0.09 M). After evaporation of the acetic acid, the potassium salt of GluSH was obtained by adding KOH. Then the solution was lyophilized to give the potassium salt of thioglutamic acid (GluSK) as a solid. The overall yield is 65–70%, λmax 250 nm, εM = 7415 M^{-1} cm^{-1}. Aqueous GluSK solutions at pH 5.5 are stable for at least 40 hr either at room temperature or at –20 °C.

Electrospray mass spectra of the product in the negative mode gave major peaks at 162 corresponding to Glu-S^- and at 128, corresponding to a fragmentation product that had lost H_2S. Small peaks at 146 and 59 probably correspond to impurities of glutamic and acetic acids. Spectroscopy in the positive mode gave the major peak a 239.9 corresponding to (GluSK)K^+ with a smaller peak at 201.9 (GluSK)H^+. A minor peak at 224.0 may correspond to (GluSK)Na^+. The data for positive and negative modes, taken together indicate that the GluSH is at least 90% pure (data not shown).

Microanalysis was performed by The Centre National de la Recherche Scientifique, service central d'analyse (Vernaison). The observed molar ratios are: N = 1.009 (theoretical value : 1), C:N = 5.36, (theoretical value 5), H:N = 9.3 (theoretical value : 8), K:N = 1.14 (theoretical value : 1). The observed molar excess of C, H and K can be attributed to small quantities of potassium acetate and potassium glutamate that are known from mass spectroscopy to be present in the sample.

2.3. OLIGOMERIZATION REACTIONS

Oligomer formation from GluSH (200 mM) or elongation of decaglutamic acid $(L\text{-}Glu)_{10}$ (0.4 mM) by GluSH (200 mM) in the presence of various combinations of $NaHCO_3$ (250 mM), $K_3Fe(CN)_6$ (100 mM) and $MgCl_2$ (50 mM) was carried out in 200 μL tubes at 50 °C. The total volume of the reaction mixture was always 150 μL. In each case, the pH of the reaction was adjusted to 8.2. When $K_3Fe(CN)_6$ was present in the reaction mixture, a precipitate formed. To desorb the products from the precipitate we added 100 μL $K_4P_2O_7$ (20 mM) and tumbled the suspension for 30 min. A similar procedure was used with the homogeneous samples to facilitate direct comparison of the results obtained by chromatography. The 'supernatants' were analyzed by HPLC on RPC-5 using a perchlorate gradient 0 to 0.03 M in 40 min. Control reactions containing glutamic acid instead of thioglutamic acid, were carried out under the same conditions.

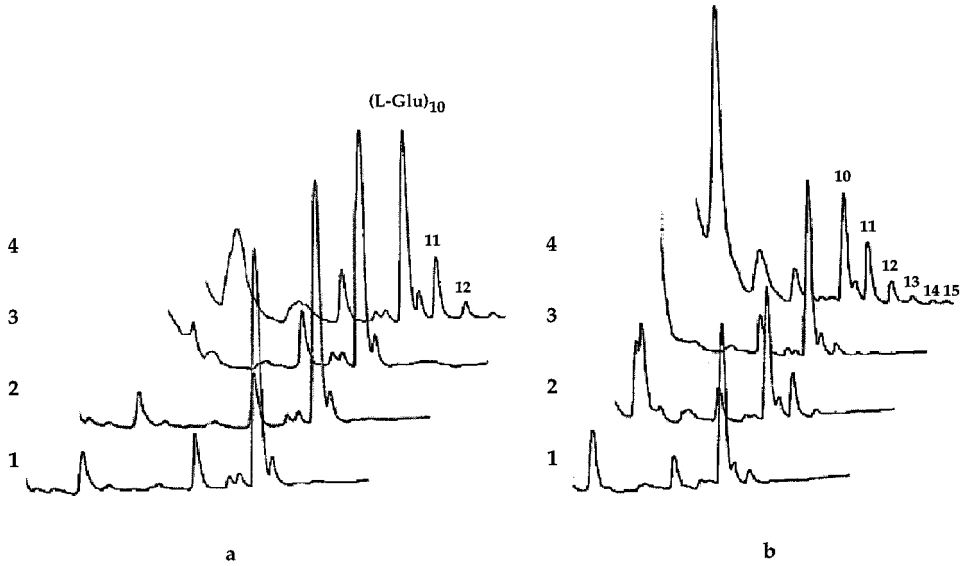


Figure 1. HPLC elution profiles of the products of elongation of (L-glu)$_{10}$ after (a) 0 time (b) 24 hr; Profile 1 with GluSH, Profile 2 with GluSH + bicarbonate; Profile 3 with GluSH + ferricyanide; Profile 4 with GluSH + bicarbonate + ferricyanide. GluSH (200 mM); (L-Glu)$_{10}$ (0.4 mM); $NaHCO_3$ (250 mM); $K_3Fe(CN)_6$ (100 mM) and $MgCl_2$ (50 mM). The HPLC run time is 20 min.

3. Results

3.1. PRODUCTS FROM THE ELONGATION OF (glu)$_{10}$

The elution profiles of products from the elongation of (glu)$_{10}$ are illustrated in Figure 1. In the absence of bicarbonate, the extension of (glu)$_{10}$ is slow, and after 24 hr only small amounts of (glu)$_{11}$ can be detected (Figure 1b, Profile 1). Little further reaction occurs after 7 days (data not shown). In the presence of bicarbonate alone, a significant yield of (glu)$_{11}$ and a trace of (glu)$_{12}$ are visible after 24 h (Figure 1b, Profile 2). Traces of some higher oligomers are formed after 7 days (data not shown). No extension of the primer can be observed on treatment with ferricyanide alone. When both bicarbonate and ferricyanide are present a substantial yield of (glu)$_{11}$ and some higher oligimers are found immediately after mixing (0 – time) (Figure 1a, Profile 4). Subsequently, a slower reaction leads to the formation of oligomers up to at least the 15mer (Figure 1b, Profile 4). In a parallel set of control experiments in which thioglutamic acid was replaced by glutamic acid, no extension of (glu)$_{10}$ was observed.

Samples of oligomers from (glu)$_{11}$ to (glu)$_{14}$ were recovered from an RPC5 column, purified and identified by electrospray mass spectrometry. (glu)$_{11}$: 1437.84±0.21 (theoretical value: 1438.27), (glu)$_{12}$: 1566.92±0.08 (theoretical value: 1567.4), (glu)$_{13}$: 1696.23±0.63 (theoretical value: 1696.51), and (glu)$_{14}$: 1824.99± 0.1 (theoretical value: 1825.62).

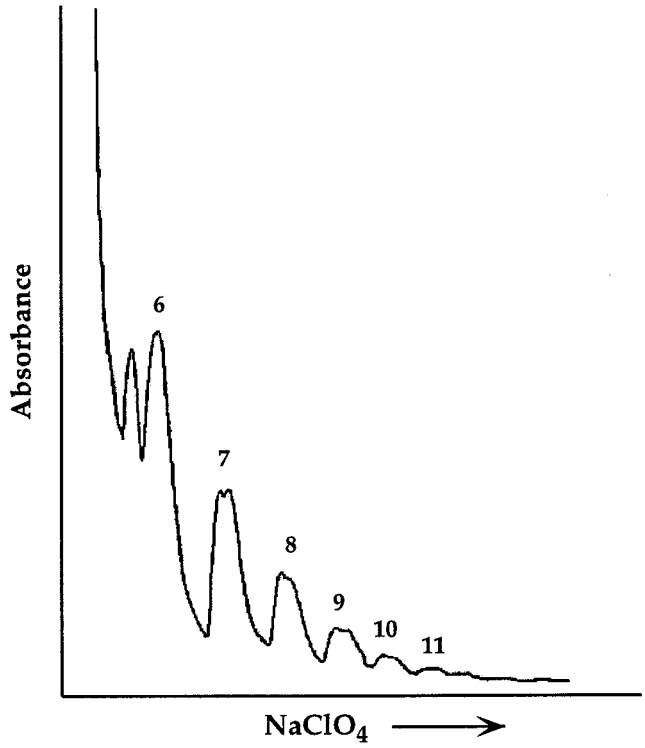
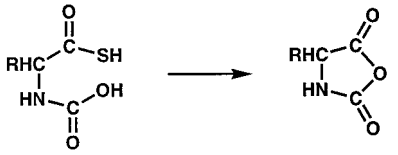


Figure 2. HPLC elution profile of the products from the oligomerization of GluSH in the presence of bicarbonate and ferricyanide after 14 days. GluSH (200 mM); $NaHCO_3$ (250 mM); $K_3Fe(CN)_6$ (100 mM) and $MgCl_2$ (50 mM). The HPLC run time is 20 min.

3.2. PRODUCTS FROM THE OLIGOMERIZATION OF GluSH

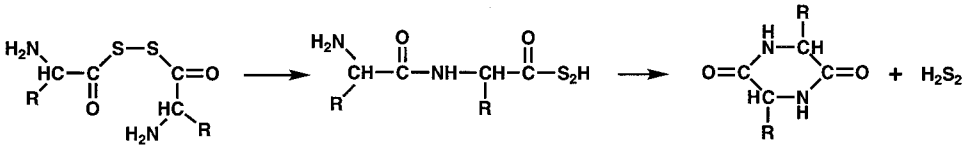
We have not analyzed in detail the products of the direct condensation reaction of GSH. It is clear that the efficiency of these reactions in the presence of various combinations of bicarbonate and ferricyanide parallels the efficiency observed for the elongation of a $(glu)_{10}$ primer, and that the main products are peptides. Under optimal conditions with bicarbonate and ferricyanide oligomers up to at least the 8mer are formed in substantial amounts (Figure 2) after 14 days, and oligomers up to the 11mer are detectable.

Samples of fractions attributed to $(glu)_5$ to $(glu)_8$ were analyzed by electrospray mass spectrometry. Signals at 663.3 and 645.2 can be attributed to $(glu)_5$ COOH and to a molecule derived from $(glu)_5$ COOH by loss of H_2O (theoretical values: 663.59 and 645.57). A similar pair of signals is observed for $(glu)_5$ (774.4 and 792.4); theoretical (774.68, 792.7). $(glu)_7$ is represented by a similar pair of mass peaks (921.5, 903.5; theoretical (921.82, 903.80) and a third peak corresponding to an Na^+ adduct (943.2, theoretical 943.82). $(glu)_8$ is represented by three corresponding peaks (1056.2, 1032.59, 1072.37; theoretical 1050.93, 1032.91, 1072.93).



R = $H_2C-CH_2-CO_2H$

Scheme 1.



R = $CH_2-CH_2-CO_2H$

Scheme 2.

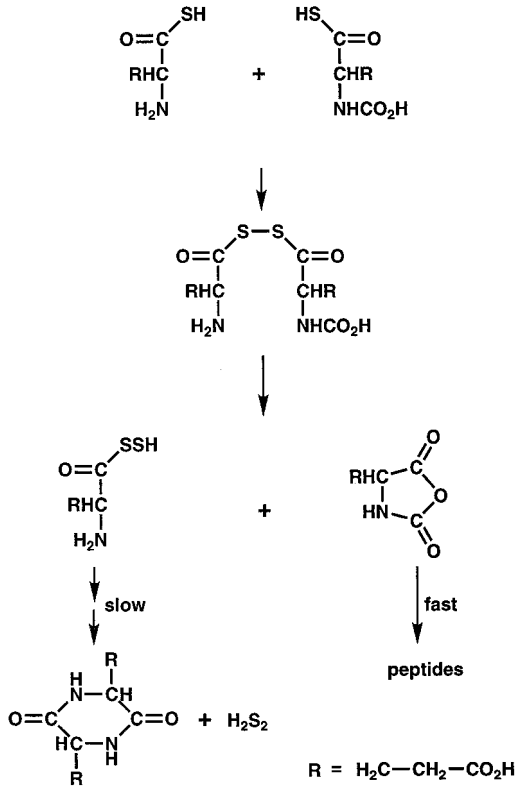
We conclude that we have identified condensation products, $(glu)_5$ to $(glu)_8$ and have probably identified the corresponding peptides terminated by a pyroglutamic acid residue.

4. Discussion

The direct oligomerization of a thioacid leads to the formation of peptides that still have a thioacid function at their C-terminus. This leads to the formation of a complex mixture of products, since the first-formed oligomer can hydrolyze or cyclize. We decided, therefore, to use a decaglutamic acid $(glu)_{10}$ 'primer', and to study its elongation. This avoids all the problems that are associated with the production of activated oligomers. Furthermore, the choice of oligoglutamic acid as the primer leads to products that are readily separated on an RPC5 column.

The extension of $(glu)_{10}$ by gluSH in the absence of bicarbonate is very slow because the thioacid is present as an anion at neutral pH, and is then a very poor electrophile. The acceleration of the reaction by the bicarbonate ion is due to the formation of a N-carboxyanhydride as suggested by Wieland (1988) (Scheme I). The absence of elongation products in the presence of ferricyanide when no bicarbonate is added is at first sight, surprising, since the oxidation products of thioacids are powerful acylating agents. We believe that a very rapid cyclization reaction competes effectively with elongation, and leads finally to the formation of a cyclic dipeptide (Scheme II). The proposed rearrangement of the diacyldisulfide is similar to the rearrangement that forms the basis of a much used peptide-ligation procedure (Dawson *et al.*, 1994).

The rapid component of the efficient reaction that occurs when both bicarbonate and ferricyanide are present is explained by the reactions in Scheme III. The first-



Scheme 3.

formed oxidation products include the unsymmetrical carbamate. This undergoes a rapid intramolecular reaction to yield an N-carboxyanhydride and a disulfide derivative of glutamic acid. The N-carboxyanhydride is a rapid and efficient acylating agent. The disulfide derivative reacts more slowly to generate a second equivalent of the N-carboxyanhydride.

Our results show that GluSH, and presumably other α-amino-thioacids are potential activated substrates for the synthesis of peptides. The reaction is too slow to study at neutral pH in the absence of CO_2, but proceeds more rapidly but not very efficiently in the presence of bicarbonate ions. In the presence of bicarbonate and an oxidizing agent the reaction is rapid and efficient. The relevance of these findings remain problematical, until the prebiotic synthesis of thioacids and their derivatives is established experimentally. If this can be done, thioacids together with a prebiotically plausible oxidizing agent such as nitrite or nitrate would be promising sources of peptides in the sulfur world (De Duve, 1991).

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