Peptide Models of Protein Metastable Binding Sites: Competitive Kinetics of Isomerization and Hydrolysis†

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ABSTRACT: α_2-Macroglobulin and the complement components C3 and C4 each contain a metastable binding site that is essential for covalent attachment. Two cyclic peptides are useful models of these unusual protein sites. Five-membered lactam **1** (CH_3CO-Gly-Cys-Gly-Glu-Glp-Asn-NH_2) contains an internal residue of pyroglutamic acid (Glp). Fifteen-membered thiolactone **2** (CH_3CO-Gly-Cys-Gly-Glu-Glu-Asn-NH_2 15-thiolactone) contains a thiol ester bond between Cys-2 and Glu-5. These isomeric hexapeptides are spontaneously interconverted in water. Competing with the two isomerization reactions are three reactions involving hydrolysis of **1** and **2** (Figure 2). These five processes were found to occur simultaneously under physiologic conditions (phosphate-buffered saline, pH 7.3, 37 °C). Best estimates of the five rate constants for these apparent first-order reactions were obtained by comparing the observed molar percentages of peptides **1**–**4** with those calculated from a set of exponential equations. Both isomerization reactions (ring expansion of **1** to **2**, $k_1 = 6.4 \times 10^{-5}$ s^{-1}; ring contraction of **2** to **1**, $k_{-1} = 69 \times 10^{-5}$ s^{-1}) proceeded faster than any of the hydrolysis reactions: α-cleavage of **1** with fragmentation to form dipeptide **3** ($k_2 = 3.3 \times 10^{-5}$ s^{-1}), γ-cleavage of **1** with ring opening to yield mercapto acid **4** ($k_3 = 0.35 \times 10^{-5}$ s^{-1}), and hydrolysis of **2** with ring opening to give **4** ($k_4 = 1.9 \times 10^{-5}$ s^{-1}). The isomeriztion rate ratio ($k_1/k_{-1} = 10.9$) agreed with the isomer ratio at equilibrium (**1**:**2** = 11 starting from **1** and 10 starting from **2**). The α/γ regioselectivity ratio ($k_2/k_3 = 9.7$) for hydrolysis of the internal Glp residue of **1** was consistent with results for model tripeptides. Part of the chemistry of the protein metastable binding sites can be explained by similar isomerization and hydrolysis reactions.

Activation of the classical pathway of the complement system, the major humoral immune defense against infection, involves enzymic cleavage of the proteins C3[1] and C4. The activation peptides C3a and C4a induce inflammatory responses by binding to a shared cellular receptor through their COOH-terminal active sites (Hugli et al., 1983; Unson et al., 1984; Lu et al., 1984). The newly activated proteins C3b and C4b are very briefly able to bind covalently to receptive surfaces (Law et al., 1979, 1980, 1984), such as cellular surfaces and immune complexes, through exposure and chemical reaction (Tack et al., 1980; Harrison et al., 1981) of their metastable binding sites (Müller-Eberhard & Schreiber, 1980). In addition, proteolytic activation of the serum protease inhibitor α_2-macroglobulin (α_2M) exposes a metastable binding site whose local structure and chemistry are similar to those of the sites in nascent C3b and C4b (Swenson & Howard, 1980a,b; Sottrup-Jensen et al., 1980, 1981).

The five-residue segement -Gly-"Cys"-Gly-Glu-"Gln"- is present in the metastable binding sites of each of these human serum proteins. The abbreviations "Cys" and "Gln" represent modified forms of cysteine (Cys) and glutamine (Gln), respectively, which are the residues encoded by the mouse genes for C3 (Wetsel et al., 1984) and C4 (Belt et al., 1984; Nonaka et al., 1985). Two plausible alternatives are available for the chemical nature of the "Cys" and "Gln" residues. First, reaction of the unactivated proteins with methylamine converts "Cys" into a free thiol-bearing Cys residue and "Gln" into a residue of N^{γ}-methylglutamine (Swenson & Howard, 1980a; Tack et al., 1980; Sottrup-Jensen et al., 1980; Howard, 1981; Harrison et al., 1981; Eccelston & Howard, 1985). These results are consistent with the proposal (Tack et al., 1980; Sottrup-Jensen et al., 1980) that the "Cys" and "Gln" residues are covalently joined by a thiol ester bond to form a 15-membered thiolactone ring (partial structure II of Figure 1). Second, under denaturing conditions the metastable binding sites of unactivated C3 (Howard, 1980; Janatova et al., 1980; Sim & Sim, 1981), C4 (Gorski & Howard, 1980; Janatova & Tack, 1981), and α_2M (Harpel et al., 1979; Howard et al., 1980) undergo spontaneous hydrolysis of a peptide bond. As a result, the "Gln" residue of α_2M becomes an NH_2-terminal pyroglutamic acid (Glp, 5-oxoproline) residue (Howard et al., 1980). These results are consistent with the proposals (Swenson & Howard, 1980b; Howard et al., 1980) that "Gln" is an internal Glp residue containing a five-membered lactam ring (I). Since the unactivated proteins do not react with thiol reagents, the free thiol group of partial structure I would need to be inaccessible to these reagents until the proteins undergo enzymic activation.

Lactam **1** and thiolactone **2** of Figure 2 are useful peptide models (Khan & Erickson, 1981, 1982; Erickson & Khan, 1983, 1985) of the proposed protein structures I and II. These cyclic hexapeptides are based on the hexapeptide segment

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[1] Abbreviations: α_2M, α_2-macroglobulin; C3, C3a, C3b, third component of serum complement, its activation peptide, and its activated form (similarly for C4, C4a, and C4b); "Cys", form of Cys in metastable binding site; DTT, dithiothreitol; Glp, pyroglutamic acid; "Gln", form of Gln in metastable binding site; ppm, parts per million; *S*, performance function (eq 26, Appendix).

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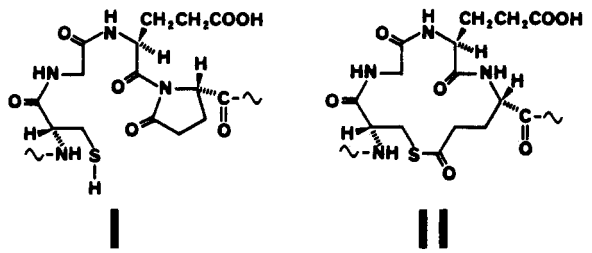

FIGURE 1: Two partial structures proposed for protein metastable binding sites.

common to C3 and α_2M (Tack et al., 1980; Swenson & Howard, 1980b; Thomas et al., 1982). Model peptides **1** and **2** are chemically interconverted under physiologic conditions (Khan & Erickson, 1982). The present detailed kinetic study of the five-reaction network shown in Figure 2 involves competitive isomerization and hydrolysis of lactam **1** and thiolactone **2**. The results are consistent with our proposal (Khan & Erickson, 1982) that part of the chemistry of the metastable binding sites of C3, C4, and α_2M can be explained by interconversion of isomeric forms of these proteins having partial structures I and II.

Experimental Procedures

Cyclic Hexapeptides. Lactam **1** (Khan & Erickson, 1981) and thiolactone **2** (Khan & Erickson, 1982) were prepared as described previously.

L-*Pyroglutamyl*-L-*asparagine Amide* (**3**). Dipeptide **3** was prepared by mixed-anhydride coupling of *N*-benzyloxycarbonyl-L-pyroglutamic acid (Bachem, Torrance, CA) and asparagine amide (76% yield) followed by hydrogenolysis of the amino protecting group (94% yield). It was homogeneous by reversed-phase liquid chromatography (Table I) and by thin-layer chromatography on silica gel [Analtech, Newark, DE; R_f 0.32 in 4:1:1 (v/v/v) 1-butanol/acetic acid/water; R_f 0.16 in 23:10:3 (v/v/v) ethanol/acetic acid/water], gave the required $(M + Na)^+$ ion [*m/e* ($C_9H_{14}O_4N_4Na$), calcd 265.09, found 265.05] in the positive ion portion of the ^{252}Cf fission fragment-induced mass spectrum (Chait et al., 1981), and showed a satisfactory 300-MHz proton NMR spectrum: [$(CD_3)_2SO$; ppm from $(CH_3)_4Si$] δ 1.91 (1 H, m, Glp γ), 2.12 (2 H, m, Glp β), 2.26 (1 H, m, Glp γ), ca. 2.5 (Asn β, masked by solvent), 4.03 (1 H, d, J = 8 Hz, d, J = 4.5 Hz, Glp α), 4.50 (1 H, d, J = 8 Hz, t, J = 7 Hz, Asn α), 6.87 and 7.30 (each 1 H, s, Asn β-$CONH_2$), 7.85 (1 H, s, Glp NH), and 8.21 (1 H, d, J = 8 Hz, Asn α-NH).

Table I: Reversed-Phase Liquid Chromatography[a]

peptide	k'[b]	relative molar area[c]
lactam **1**	2.45	1.00
thiolactone **2**	3.16	0.60
dipeptide **3**	0.52	0.11
mercapto acid **4**	1.52	0.24

[a] High-pressure liquid chromatography on a 0.78 × 30 cm column of octadecylsilica by isocratic elution with water containing 1% CH_3CN and 0.05% CF_3CO_2H at 4.0 mL/min (see Figure 1). [b] Relative retention time $k' = (t_{peptide}/t_{solvent} - 1)$, where t is the retention time. [c] Relative integrated area of absorbance at 220 nm/nmol of authentic compound.

N-Acetylglycyl-L-*cysteinylglycyl*-L-*glutamyl*-L-*glutamyl*-L-*asparagine Amide* (**4**). An authentic sample was prepared by treatment of a protected derivative of **4** having an *S*-(4-methylbenzyl) group on Cys-2 and an *O*-benzyl group on Glu-5 (compound **5**; Khan & Erickson, 1982) with 9:1 (v/v) HF/anisole for 40 min at 0 °C. After purification by liquid chromatography on octadecylsilica, **4** was homogeneous by reversed-phase chromatography (Table I) and by thin-layer chromatography on silica gel [R_f 0.63 in 4:1:1 (v/v/v) 1-butanol/acetic acid/water; R_f 0.14 in 3:1:1:1 (v/v/v/v) 1-butanol/ethyl acetate/acetic acid/water]. It gave satisfactory amino acid ratios (acid hydrolysis: Asp, 1.06; Gly, 2.01; Glu, 1.91; Cys, lost through oxidation), $(M + Na)^+$ ion [*m/e* ($C_{23}H_{36}O_{12}N_8SNa$), calcd 671.23, found 671.31], and 300-MHz proton NMR spectra: [$(CD_3)_2SO$; ppm from $(CH_3)_4Si$] δ 1.77 (4 H, m, Glu β), 1.86 (3 H, s, CH_3CO), 1.91 (1 H, s, SH) 2.25 (4 H, m, Glu γ), ca. 2.5 (Asn β, masked by solvent), 2.74 (2 H, m, Cys β), 3.74 (4 H, d, J = 5 Hz, Gly α), 4.18 and 4.29 (each 1 H, m, Glu α), 4.40 (2 H, m, Cys and Asn α), 6.86 and 7.31 (each 1 H, s, Asn β-$CONH_2$), 7.04 and 7.06 (each 1 H, s, Asn α-$CONH_2$), 7.97 and 7.99 (each 1 H, d, J = 7 Hz, m, Glu NH), 8.11 (3 H, m, Gly and Asn α-NH), 8.29 (1 H, m, Cys NH), and 12.09 (2 H, s, Glu γ-CO_2H). [CF_3CO_2H; ppm from $(CH_3)_4Si$] δ 2.27 (4 H, m, Glu β), 2.30 (1 H, s, SH), 2.42 (3 H, s, CH_3CO), 2.75 (4 H, m, Glu γ), 3.05 (1 H, d, J = 14 Hz, d, J = 6.5 Hz, Cys β), 3.15 (1 H,

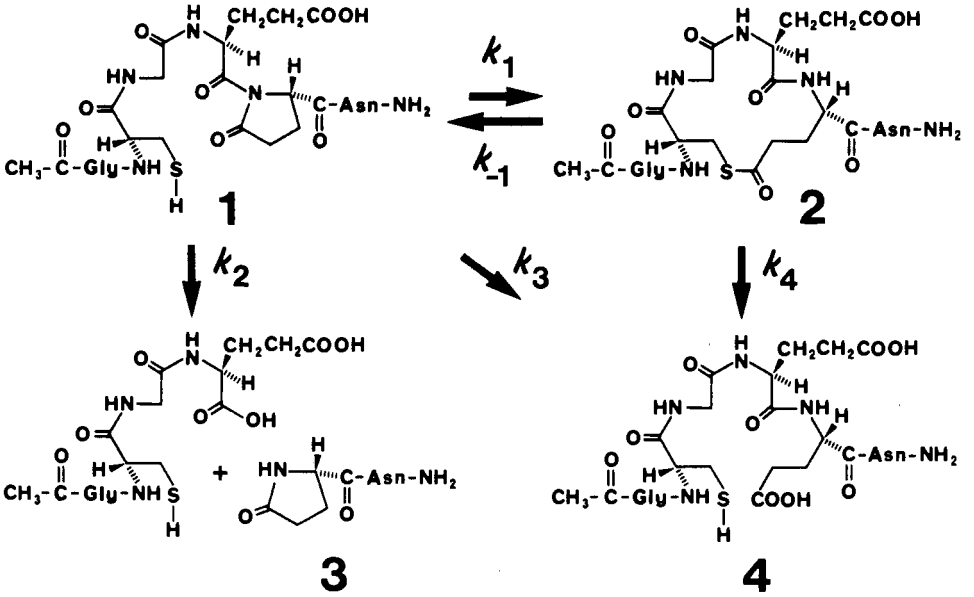

FIGURE 2: Reaction network for lactam **1** and thiolactone **2**, models of partial structures I and II of Figure 1. Five apparent first-order processes are shown: two isomerization reactions (k_1, k_{-1}) and three hydrolytic reactions (k_2, k_3, k_4).

d, J = 14 Hz, d, J = 5.5 Hz, Cys β), 3.20 (2 H, d, J = 6 Hz, Asn β), 4.36 and 4.40 (each 2 H, s, Gly α), 4.89 (2 H, m, Glu α), 4.99 (1 H, t, J = 6 Hz, Cys α), and 5.24 (1 H, t, J = 6 Hz, Asn α).

Kinetics. (*A*) *With DTT.* In experiment 1, a 1.0 mM solution of **1** was prepared in freshly prepared phosphate-buffered saline (10 mM phosphate, 145 mM NaCl). The buffer contained 10 mM dithiothreitol (DTT) and was previously adjusted to pH 7.30, 8.00, or 8.70 (±0.01). This solution was maintained at 37.0 ± 0.1 °C for 5 h. Every 30 min, part (20–100 μL) was injected onto an octadecylsilica column (0.78 × 30 cm, μBondapak C_{18}, Waters Associates, Milford, MA) and eluted with water (purified by reverse osmosis and ion exchange) containing 1% acetonitrile (glass-distilled, HPLC grade, EM Science) and 0.05% trifluoroacetic acid (sequanal grade, Pierce). Peptides were detected by absorbance at 220 nm (Model 635M, Hitachi Scientific) and quantitated by electronic integration (Model 730, Waters Associates). A typical reversed-phase chromatogram is shown in Figure 3. The compounds corresponding to the peaks labeled 1–4 were isolated and shown by amino acid analysis, molecular mass analysis, and 300-MHz proton NMR spectroscopy to be the peptides **1–4**, respectively. Their relative retention times and relative integrated areas per nanomole are given in Table I. Experiment 2 was conducted identically with experiment 1 except that **1** was replaced by **2**.

(*B*) *With Tributylphosphine.* In another set of experiments, DTT was replaced by 10 mM tributylphosphine.

Rate Constant Estimation. For both experiments 1 and 2, the molar percentages of peptides **1–4** were determined at 8–10 time points. Initial estimates for the five first-order rate constants (k_1, k_{-1}, k_2, k_3, k_4) were independently determined from the initial changes in the concentrations of peptides **1–4**. Better estimates were calculated with a curve-fitting algorithm written in the C programming language and implemented on a minicomputer (VAX 11-780, Digital Equipment, Maynard, MA). Performance function S (eq 26, Appendix) was repetitively evaluated by using the current rate constant estimates and 64–80 molar percentages. The current estimate of k_1 was successively changed by a preset relative increment of 0.5% while keeping the other rate constant estimates fixed until both increasing and decreasing this estimate failed to decrease S. In similar fashion, local minima of S with respect to each of the other four rate constants were found one at a time. This entire process was repeated until changing the current estimates of *each* rate constant by the present relative increment failed to decrease S. Further precision was achieved by decreasing the size of the preset relative increment and repeating the program starting from the current estimates. The preset relative increment was changed to 0.1%, then to 0.05%, and finally to 0.01%.

Results

Reaction Network. When either lactam **1** or thiolactone **2** was kept under physiologic conditions (phosphate-buffered saline, pH 7.30, 37.0 °C), four peptides were detected by reversed-phase liquid chromatography (Figure 3): isomers **1** and **2** and their hydrolysis products dipeptide **3** and mercapto acid **4**. The reaction network of Figure 2 accounts for each of these products by five apparent first-order reactions. First, the five-membered lactam **1** undergoes ring expansion (k_1) to the 15-membered thiolactone **2**. Second, **2** undergoes ring contraction (k_{-1}) back to **1**. While both of these isomerization reactions are occurring, three hydrolysis reactions are also proceeding. The diacylamino group of **1** is attacked at either

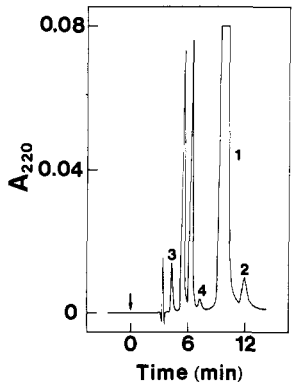

FIGURE 3: Reversed-phase liquid chromatography of peptides **1–4** on octadecylsilica. A solution of lactam **1** in phosphate-buffered saline containing DTT was sampled after 90 min at pH 7.30 at 37.0 °C. See Table I for chromatographic details. The absorbance at 220 nm is plotted against time. The two peaks between **3** and **4** are DTT and its cyclic disulfide, which is slowly formed by air oxidation.

carbonyl group to generate in both cases a carboxylic acid group and an amide group. Third, **1** suffers hydrolysis (k_2) with chain fragmentation but without ring opening by undergoing attact at the α-carbonyl group of Glu-4 (α-cleavage) to yield a tetrapeptide acid (CH_3CO-Gly-Cys-Gly-Glu-OH) and dipeptide amide **3** (Glp-Asn-NH_2). Fourth, **1** also undergoes hydrolysis (k_3) without chain fragmentation but with ring opening by suffering attack at the γ-carbonyl of Glp-5 (γ-cleavage) to give mercapto acid **4**. Fifth, **2** suffers hydrolysis (k_4) with ring opening by undergoing attack at the carbonyl group of the thiol ester bond between Cys-2 and Glu-5 to yield **4**.

Kinetic Curves. Experiments were normally conducted in the presence of excess DTT in order to keep the thiol groups of **1**, **4**, and the tetrapeptide in their reduced forms. Since the tetrapeptide coeluted during liquid chromatography with the disulfide oxidation product of DTT, this peptide was not observed in the presence of this reducing agent. In one set of experiments conducted in the absence of DTT, however, the tetrapeptide was detected by liquid chromatography and characterized by amino acid analysis.

The experimental molar percentages of each peptide during the firt 5 h are plotted against time in Figure 4. Panel a shows experiment 1 starting with only lactam **1** present; panel b shows experiment 2 with just thiolactone **2** present initially. Also shown are the theoretical curves that best fit all of the experimental points. These curves were calculated from the best estimates of the five rate constants obtained from a direct search method using the data from experiments 1 and 2 and a final preset relative increment of 0.01%.

The close agreement seen in Figure 4 between the 80 experimental points and the eight calculated curves provides good evidence that the reaction network of Figure 2, the kinetic model of Figure 5, and the corresponding eight exponential equations (eq 18–25, Appendix) adequately describe the competitive kinetics of isomerization and hydrolysis of **1** and **2**. In panel b of Figure 4, the visually greater disagreement between the experimental points and theoretical curve for thiolactone **2** arises because they are plotted on the 10-fold-expanded scale at the right.

Rate Constants. A set of best estimates of the five first-order rate constants at pH 7.30 in the presence of DTT is given in Table II. The reproducibility of these values is shown by the good agreement of two independent determinations. Also shown are rate constant estimates for single determinations conducted at pH 8.00 and 8.70 in the presence of DTT. For

Table II: Isomerization and Hydrolysis of Lactam **1** and Thiolactone **2** in Phosphate-Buffered Saline at 37.0 ± 0.1 °C: Apparent First-Order Rate Constants, Isomerization Rate Ratio, and α/γ Regioselectivity Ratio for Hydrolysis of **1** at Three pH Values

		rate constant (10^{-5} s^{-1})						
pH	agent[a]	k_1	k_{-1}	k_2	k_3	k_4	k_{-1}/k_1	α/γ, k_2/k_3
7.30[b]	DTT	6.36 ± 0.72	69.3 ± 4.6	3.34 ± 0.09	0.35 ± 0.02	1.94 ± 0.15	10.9	9.7
7.30	TBP	6.93	66.6	3.56	0.21	2.16	9.6	16.9
8.00	DTT	7.00	71.4	4.40	0.31	3.8	10.2	15.2
8.70	DTT	10.7	103	6.0	0.33	5.8	9.6	18
8.70	TBP	9.1	101	6.0	0.26	5.9	11.1	23

[a] Dithiothreitol (DTT, 10 mM) or tributylphophine (TBP, 10 mM) was present to reduce any disulfide bonds that formed during the experiment. [b] Mean and deviation of two determinations.

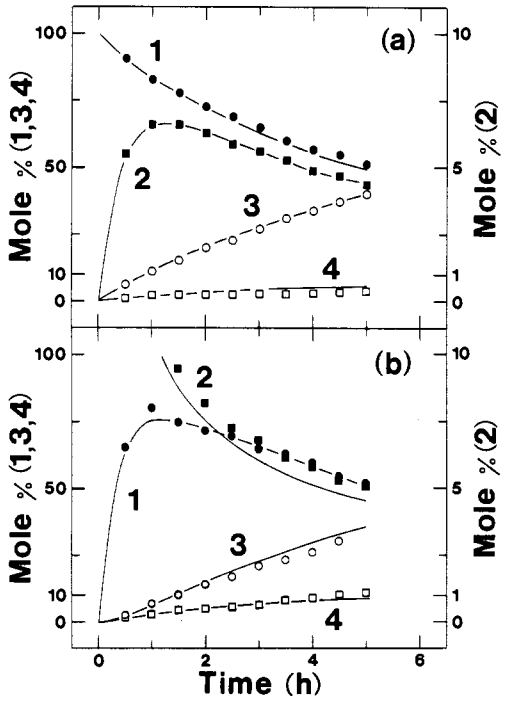

FIGURE 4: Molar percentages of peptides **1**–**4** vs. time under physiologic conditions (pH 7.30, 37.0 °C). The *experimental points* for lactam **1** (●), thiolactone **2** (■), dipeptide **3** (○), and mercapto acid **4** (□) were measured by reversed-phase chromatography (Table I; Figure 3). The *theoretical curves* were calculated from eq 18–25 (Appendix) and one set of estimated rate constants. Note that **2** is plotted against the 10-fold-expanded scale at the right. (a) Experiment 1 with only **1** present initially; (b) experiment 2 with only **2** present initially.

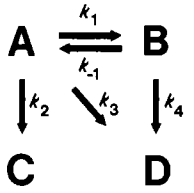

FIGURE 5: General kinetic model for the reaction network of Figure 2. Five apparent first-order processes are assumed.

each of the five reactions, the ratio of the rate constant estimate at pH 7.30 to that at pH 8.70 was less than 2 except for k_4, which was about 3. Since the hydroxide concentration is 25 times greater at pH 8.70 than at pH 7.30, the rates of these five reactions show little or no dependence on the hydroxide ion concentration.

At all three pH values examined, the rank order of the magnitude of the rate constants was $k_{-1} > k_1 > k_2 \sim k_4 > k_3$. After 3 at pH 7.30, the **1**/**2** isomer ratio was about 11 starting from **1** and 10 starting from **2** (Figure 4). These values are consistent with the isomerization rate ratio (k_{-1}/k_1) of 10.9 (Table II). This ratio was about 10 at both pH 8.00 and pH 8.70. Finally, the α/γ regioselectivity ratio (k_2/k_3) for hydrolysis of lactam **1** was 9.6 at pH 7.30 and 15–17 at the higher pH values.

The possibility that the reaction rates might be different in the absence of the thiol groups of DTT was examined by replacing this efficient disulfide reducing agent by a non-thiol reducing agent. Specifically, DTT was replaced by an equimolar amount of tributylphosphine (Khan & Erickson, 1981). As shown in Table II, at both pH 7.30 and pH 8.70 essentially the same results were observed in the presence of either reducing agent. Thus, neither of these additives perturbed the reaction rates.

The rate of appearance of thiol groups from **2** was measured for 5 min by continuous spectrometric monitoring of the reaction of thiol with 2,2′-dithiodipyridine. In barbital-buffered saline at pH 7.30 and 37.0 °C, the apparent first-order rate constant was 6.0×10^{-4} s^{-1} and the half-life was 19 min (Khan & Erickson, 1982). This process corresponds in Figure 2 to the sum of the isomerization of **2** to **1** (k_{-1}) and hydrolysis of **2** to **4** (k_4). In phosphate-buffered saline at pH 7.30 and 37.0 °C, the sum of k_{-1} and k_4 was 7.1×10^{-4} s^{-1}, and the half-life was 16 min. Thus, the sum of these rate constants measured directly by liquid chromatography agreed well with that determined indirectly by the appearance of thiol groups.

Discussion

Competitive Isomerization and Hydrolysis of **1** *and* **2**. We have reported previously (Khan & Erickson, 1982) that under physiologic conditions the five-membered lactam **1** undergoes ring expansion to the 15-membered thiolactone **2**, **2** undergoes ring contraction to **1**, and in both cases the **1**/**2** isomer ratio reaches a constant value of 10–11 after 3 h. Direct determination of the apparent first-order rate constants for these isomerization reactions was hindered by two or three competing hydrolysis reactions. Pyroglutamylasparagine amide (**3**), which was isolated in major amounts from **1** and in minor amounts from **2**, evidently arose by hydrolysis of the peptide bond between Glu-4 and Glp-5 of lactam **1**. In addition, hydrolysis of **1** and/or **2** could have furnished mercapto acid **4**.

The present kinetic study has confirmed and extended these observations. Authentic samples of peptides **3** and **4** were synthesized, characterized, and shown to be identical with hydrolysis products isolated from **1** and **2** under physiologic conditions. A kinetic model involving five first-order processes among four compounds (Figure 5) was described mathematically, and time-dependent exponential equations were derived that express the concentration of each compound in terms of the five rate constants (Appendix). By use of these equations, the rate constants for isomerization and hydrolysis of **1** and **2** were estimated from experimental measurement of the molar percentages of peptides **1**–**4** at several times. A reaction network (Figure 2) involving two isomerization reactions (k_1, k_{-1}) and three hydrolysis reactions (k_2, k_3, k_4) was

found to be necessary and sufficient to describe the competitive isomerization and hydrolysis of **1** and **2** under physiologic conditions and over the pH range 7.30–8.70.

Interconversion of isomers **1** and **2** proceeded faster than hydrolysis of either isomer. At any given moment at pH 7.30, lactam **1** was kinetically partitioned three ways, so that 64% underwent ring expansion to **2**, 33% suffered chain fragmentation to **3**, and only 3% reacted by ring opening to **4**. Similarly, thiolactone **2** was kinetically partitioned two ways, so that 97% underwent ring contraction to **1** and again only 3% reacted by ring opening to **4**. For both **1** and **2**, the kinetic competition between isomerization and hydrolysis favored isomerization. After the first 3 h, the isomer mixture was 92% lactam **1** and 8% thiolactone **2**.

Lactam **1** is structurally unusual because it contains an *internal* residue of pyroglutamic acid (Glp). The diacylamino group of the Glu–Glp segment underwent hydrolysis by two competing reactions. The major reaction was cleavage of the Glu–Glp peptide bond (k_2) to form a tetrapeptide and dipeptide **3**. The minor reaction was cleavage of the Glp lactam ring (k_3) to produce mercapto acid **4**. the α/γ regioselectivity ratio (k_2/k_3) for hydrolysis of **1** at 37.0 °C was 9.7 at pH 7.30 and 15.2 at pH 8.00 (Table II). These values agree well with those determined for a series of model tripeptides containing internal Glp residues. For example, at pH 8.30 the α/γ regioselectivity ratio was 13.2 for hydrolysis of Boc-Glu-Glp-Val-NH_2 and 10.2 for hydrolysis of Boc-Glu-Glp-Asn-$NHCH_3$ (Khan & Erickson, 1984). The rate constants for hydrolysis of lactam **1** also agree well with those of the model peptides. For example, the rate constants for hydrolysis of **1** at pH 8.00 were $k_\alpha = 4.4 \times 10^{-5}$ s^{-1} and $k_\gamma = 0.31 \times 10^{-5}$ s^{-1}. The corresponding values for hydrolysis of Boc-Glu-Glp-Asn-$NHCH_3$ at pH 8.30 were $k_\alpha = 3.6 \times 10^{-5}$ s^{-1} and $k_\gamma = 0.35 \times 10^{-5}$ s^{-1} (Khan & Erickson, 1984). Thus regioselective hydrolysis of **1** proceeded normally for a peptide containing an internal residue of pyroglutamic acid.

Protein Metastable Binding Sites. The present results provide a peptide model for the metastable binding sites of three serum proteins, the complement components C3 and C4 and the protease inhibitor α_2M. They are consistent with our proposal (Khan & Erickson, 1982) that part of the chemistry of these metastable binding sites can be explained by interconversion of isomeric proteins having lactam I and thiolactone II as partial structures.

Under certain nonphysiologic conditions (heat, denaturing agents), the metastable binding sites of unactivated C3 (Howard, 1980; Janatova et al., 1980; Sim & Sim, 1981), C4 (Gorski & Howard, 1980; Janatova & Tack, 1981), and α_2M (Harpel et al., 1979; Howard et al., 1980) react by spontaneous cleavage of a Glu–Gl? peptide bond. The COOH-terminal fragment of α_2M contains an NH_2-terminal Glp residue (Howard et al., 1980). Since possible intermediates bearing an NH_2-terminal amino group could not be detected in trapping experiments with amino reagents, it was proposed that the activated Gl? residue is a preformed, internal Glp residue (Howard et al., 1980).

At pH 7.30 and 37.0 °C, the reaction network shown in Figure 2 would result in net hydrolysis of the Glp-containing lactam **1** to yield dipeptide **3** and mercapto acid **4** in the final ratio of **3**/**4** = 86:14. But since equilibration of **1** and **2** is rapid relative to hydrolysis of either isomer, this same reaction network would result in net hydrolysis of **2** to give the same products in the final ratio of **3**/**4** = 84:16. These ratios are too similar to be distinguished experimentally, so partial structure I or II of Figure 1 cannot be assigned to a protein metastable binding site on this basis alone. In analogy to hydrolysis of lactam **1** with partial fragmentation, denaturation of α_2M in hot water exposes the hidden metastable binding site to hydrolysis with partial peptide chain fragmentation (Harpel et al., 1979). At pH 6.8 and 80 °C, the final ratio of fragmented to nonfragmented products is 77:23 (Howard, 1981). Considering that peptides **1** and **2** each contain less than 0.5% of the residues present in human α_2M (Sottrup-Jensen et al., 1984), this protein ratio is in good agreement with the final **3**/**4** peptide ratios calculated above. The present kinetic results strongly suggest that protein chain fragmentation occurs not by direct hydrolysis of thiolactone II but by direct hydrolysis of the Glu–Glp peptide bond of lactam I, as proposed previously (Sottrup-Jensen et al., 1980; Howard et al., 1981).

A major physiologic mechanism for exposing the metastable binding site of C3, C4, or A_2M is selective enzymic cleavage of a remote peptide bond. The exposed site survives very briefly until it decays to protein species that can no longer bind covalently to external nucleophiles. The apparent first-order rate constant for decay of activated, metastable α_2M is 6.2×10^{-3} s^{-1} at pH 7.5 and 23 °C (Salvesen et al., 1981). Figure 2 indicates that, depending on the initial structure of the metastable binding site, four mechanisms for consumption of peptides **1** and **2** are candidates for this protein decay process. If the metastable binding site of α_2M contains lactam structure I, protein decay would be analogous to lactam **1** undergoing either hydrolysis to **3** and **4** or isomerization to **2**. Similarly, if the site contains thiolactone structure II, decay would be analogous to thiolactone **2** undergoing either hydrolysis to **4** or isomerization to **1**. Under physiologic conditions, these four peptide processes occurred 100, 170, 330, and 9 times slower, respectively, than decay of metastable α_2M. Thus, the first three processes are probably too slow to correspond to protein decay. But the rate constant for ring contraction of the 15-membered thiolactone **2** to the five-membered lactam **1** (0.69×10^{-3} s^{-1}) is within an order of magnitude of the rate constant for decay of activated, metastable α_2M. Considering the substantial differences in structural complexity and solvent accessibility between the six-residue chain of macrocyclic thiol ester **2** and the 1451-residue chain of monomeric α_2M (Sottrup-Jensen et al., 1984), these rates are similar and may correspond to the same type of chemical reaction. Specifically, decay of the metastable binding site of activated α_2M might occur by ring contraction of thiolactone II to lactam I. The present kinetic results are consistent with the possibility (but do not prove) that the correct parital structure for the newly exposed metastable binding site of α_2M is the macrocyclic thiol ester II. Thus, chain fragmentation of unactivated α_2M, C3, and C4 under denaturing conditions probably occurs by isomerization of thiolactone II into lactam I followed by hydrolysis of the latter with α-cleavage of the Glu–Glp peptide bond.

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APPENDIX

Kinetic Model. The general kinetic scheme of Figure 5 shows interconversion of compounds A and B, conversion of A irreversibly into C or D, and conversion of B irreversibly into D but not C. Mathematical expressions are derived for the concentrations of A, B, C, and D in terms of the five apparent first-order rate constants (k_1, k_{-1}, k_2, k_3, k_4) and time

t for the case of an isothermal, constant-volume, well-stirred batch reactor. Let $a(t) = [A]$, $b(t) = [B]$, $c(t) = [C]$, and $d(t) = [D]$. The four kinetic equations are

$$da/dt = -fa + k_{-1}b \quad (1)$$

$$db/dt = k_1a - gb \quad (2)$$

$$dc/dt = k_2a \quad (3)$$

$$dd/dt = k_3a + k_4b \quad (4)$$

where constants $f = k_1 + k_2 + k_3$ and $g = k_{-1} + k_4$.

General Solution. The following expression is useful for eliminating b from eq 1 and 2:

$$j = ga + k_{-1}b \quad (5)$$

Differentiation gives

$$dj/dt = g(da/dt) + k_{-1}(db/dt)$$

Substitution of eq 1 and 2 provides

$$dj/dt = -ha \quad (6)$$

where constant $h = fg - k_1k_{-1}$. Combining eq 1 and 5 yields

$$da/dt = j - ka \quad (7)$$

where constant $k = k_1 + k_{-1} + k_2 + k_3 + k_4$. Differentiation of eq 7 and substitution of eq 6 gives

$$d^2a/dt^2 + k(da/dt) + ha = 0 \quad (8)$$

Concentration a can be expressed by the general exponential equation

$$a(t) = C_1e^{-mt} + C_2e^{-nt} \quad (9)$$

Differentiation furnishes

$$da/dt = -mC_1e^{-mt} - nC_2e^{-nt}$$

$$d^2a/dt^2 = m^2C_1e^{-mt} + n^2C_2e^{-nt}$$

Substitution of the last three equations into eq 8 yields

$$(m^2 - km + h)C_1e^{-mt} + (n^2 - kn + h)C_2e^{-nt} = 0$$

which is valid only for $m = [k \pm (k^2 - 4h)^{1/2}]/2$ and $n = [k \pm (k^2 - 4h)^{1/2}]/2$. For computation, a convenient equivalence is $k^2 - 4h = (f - g)^2 - 4k_1k_{-1}$. Substitution of eq 9 into eq 6 and integration gives

$$j = (h/m)C_1e^{-mt} + (h/n)C_2e^{-nt} + C_3$$

From this equation and eq 5 and 9

$$b(t) = rC_1e^{-mt} + sC_2e^{-nt} + C_3/k_{-1} \quad (10)$$

where constants $r = [(h/m) - g]/k_{-1}$ and $s = [(h/n) - g]/k_{-1}$. Substitution of eq 9 into eq 3 and integration affords

$$c(t) = -(k_2/m)C_1e^{-mt} - (k_2/n)C_2e^{-nt} + C_4 \quad (11)$$

Substitution of eq 9 and 10 into eq 4 and integration yields

$$d(t) = -uC_1e^{-mt} - vC_2e^{-nt} + (k_4/k_{-1})C_3t + C_5 \quad (12)$$

where constants $u = (k_3 + rk_4)/m$ and $v = (k_3 + sk_4)/n$. Equations 9–12 are general exponential equations for concentrations a, b, c, and d in terms of time t, the five rate constants, ten constants defined in terms of these rate constants, and five other constants.

Initial Conditions. The five constants C_1 through C_5 are determined by the initial kinetic conditions. Since C and D are not converted into A or B, $b(t)$ approaches zero as t approaches infinity. Since both exponential terms on the right side of eq 10 approach as t approaches infinity, for all initial conditions

$$C_3 = 0 \quad (13)$$

Consider the case that A and/or B is present initially but C and D are absent, so that $a(0) = a_0$, $b(0) = b_0$, and $c(0) = d(0) = 0$. At $t = 0$, from eq 9, 10, and 13

$$C_1 = w(sa_0 - b_0) \quad (14)$$

$$C_2 = w(b_0 - ra_0) \quad (15)$$

where constant $w = 1/(s - r)$. From eq 11, 14, and 15

$$C_4 = k_2w[(sa_0 - b_0)/m + (b_0 - ra_0)/n] \quad (16)$$

From eq 12–15

$$C_5 = w[u(sa_0 - b_0) + v(b_0 - ra_0)] \quad (17)$$

Substitution of eq 13–17 into eq 9–12 generates exponential equations for the concentrations of compounds A, B, C, and D in terms of the initial concentrations a_0 and b_0, the rate constants, and time t:

$$a(t) = w[(sa_0 - b_0)e^{-mt} + (b_0 - ra_0)e^{-nt}]$$

$$b(t) = w[r(sa_0 - b_0)e^{-mt} + s(b_0 - ra_0)e^{-nt}]$$

$$c(t) = k_2w[(sa_0 - b_0)(1 - e^{-mt})/m + (b_0 - ra_0)(1 - e^{-nt})/n]$$

$$d(t) = w[u(sa_0 - b_0)(1 - e^{-mt}) + v(b_0 - ra_0)(1 - e^{-nt})]$$

Specific Solutions. These equations can be simplified when only A or B is initially present. For *case 1* (subscript c1), $b_0 = 0$, so that

$$a_{c1}(t) = a_0w(se^{-mt} - re^{-nt}) \quad (18)$$

$$b_{c1}(t) = a_0rsw(e^{-mt} - e^{-nt}) \quad (19)$$

$$c_{c1}(t) = a_0k_2w[s(1 - e^{-mt})/m - r(1 - e^{-nt})/n] \quad (20)$$

$$d_{c1}(t) = a_0w[su(1 - e^{-mt}) - rv(1 - e^{-nt})] \quad (21)$$

For *case 2* (subscript c2), $a_0 = 0$, so that

$$a_{c2}(t) = b_0w(-e^{-mt} + e^{-nt}) \quad (22)$$

$$b_{c2}(t) = b_0w(-re^{-mt} + se^{-nt}) \quad (23)$$

$$c_{c2}(t) = b_0k_2w[-(1 - e^{-mt})/m + (1 - e^{-nt})/n] \quad (24)$$

$$d_{c2}(t) = b_0w[-u(1 - e^{-mt}) + v(1 - e^{-nt})] \quad (25)$$

Rate Constant Estimation. Equations 18–25 can be used to estimate the values of the five rate constants that best fit experimental results. *Experiment 1* (subscript e1) is conducted with only A present initially, and *experiment 2* (subscript e2) is carried out starting with only B. Then the concentrations of A, B, C, and D are determined at several time points for each experiment. Finally the values from experiment 1 [$a_{e1}(t)$, $b_{e1}(t)$, $c_{e1}(t)$, and $d_{e1}(t)$ for all times t_{e1}] and experiment 2 [$a_{e2}(t)$, $b_{e2}(t)$, $c_{e2}(t)$, and $d_{e2}(t)$ for all times t_{e2}] are compared with two corresponding sets of concentrations calculated from estimates of the five rate constants and eq 18–25. A direct search method (Côme, 1983) is used to improve the estimates of the five rate constants. The performance function $S(k_1, k_{-1}, k_2, k_3, k_4)$ is the sum of the squares of the relative deviations of the calculated values from both sets of experimental values, where summations are taken over the time points t_{e1} and t_{e2}:

$$S = \sum_{t_{e1}}\left[\left(\frac{a_{e1}(t) - a_{c1}(t)}{a_{c1}(t)}\right)^2 + \left(\frac{b_{e1}(t) - b_{c1}(t)}{b_{c1}(t)}\right)^2 + \left(\frac{c_{e1}(t) - c_{c1}(t)}{c_{c1}(t)}\right)^2 + \left(\frac{d_{e1}(t) - d_{c1}(t)}{d_{c1}(t)}\right)^2\right] + \sum_{t_{e2}}\left[\left(\frac{a_{e2}(t) - a_{c2}(t)}{a_{c2}(t)}\right)^2 + \left(\frac{b_{e2}(t) - b_{c2}(t)}{b_{c2}(t)}\right)^2 + \left(\frac{c_{e2}(t) - c_{c2}(t)}{c_{c2}(t)}\right)^2 + \left(\frac{d_{e2}(t) - d_{c2}(t)}{d_{c2}(t)}\right)^2\right] \quad (26)$$

The value of S is calculated for a set of initial estimates of the five rate constants. Additional values of S are calculated for subsequent sets of estimates until increasing or decreasing *any* of the estimates by a preset relative increment fails to decrease S.

Registry No. **1**, 83799-57-9; **2**, 80699-49-6; **3**, 103322-29-8; **4**, 103322-30-1; **5**, 103322-31-2; C3, 80295-41-6; C4, 80295-48-3; L-Asn-NH_2, 16748-73-5; *N*-benzyloxycarbonyl-L-pyroglutamic acid, 32159-21-0.

References

Belt, K. T., Carroll, M. C., & Porter, R. R. (1984) *Cell (Cambridge, Mass.) 36*, 907–914.

Chait, B. T., Agosta, W. C., & Field, F. H. (1981) *Int. J. Mass Spectrom. Ion Phys. 39*, 339–366.

Côme, G. M. (1983) *Compr. Chem. Kinet. 24*, 249–332.

Eccleston, E. D., & Howard, J. B. (1985) *J. Biol. Chem. 260*, 10169–10176.

Erickson, B. W., & Khan, S. A. (1983) *Ann. N.Y. Acad. Sci. 421*, 167–177.

Erickson, B. W., & Khan, S. A. (1985) in *Molecular Architecture of Proteins and Enzymes* (Bradshaw, R. A., & Tang, J., Eds.) pp 3–14, Academic, Orlando, FL.

Gorski, J. P., & Howard, J. B. (1980) *J. Biol. Chem. 255*, 10025–10028.

Harpel, P. C., Hayes, M. B., & Hugli, T. E. (1979) *J. Biol. Chem. 254*, 8669–8678.

Harrison, R. A., Thomas, M. L., & Tack, B. F. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7388–7392.

Howard, J. B. (1980) *J. Biol. Chem. 255*, 7082–7084.

Howard, J. B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2235–2239.

Howard, J. B., Vermeulen, M., & Swenson, R. P. (1980) *J. Biol. Chem. 255*, 3820–3823.

Hugli, T. E., Kawahara, M. S., Unson, C. G., Molinar-Rode, R., & Erickson, B. W. (1983) *Mol. Immunol. 20*, 637–645.

Janatova, J., & Tack, B. F. (1981) *Biochemistry 20*, 2394–2402.

Janatova, J., Lorenz, P. E., Schechter, A. N., Prahl, J. W., & Tack, B. F. (1980) *Biochemistry 19*, 4471–4478.

Janatova, J., Tack, B. F., & Prahl, J. W. (1980) *Biochemistry 19*, 4479–4485.

Khan, S. A., & Erickson, B. W. (1981) *J. Am. Chem. Soc. 103*, 7374–7376.

Khan, S. A., & Erickson, B. W. (1982) *J. Biol. Chem. 257*, 11864–11867.

Khan, S. A., & Erickson, B. W. (1984) *J. Am. Chem. Soc. 106*, 798–799.

Law, S. K., Lichtenberg, N. A., & Levine, R. P. (1979) *J. Immunol. 123*, 1388–1394.

Law, S. K., Lichtenberg, N. A., Holcombe, F. H., & Levine, R. P. (1980) *J. Immunol. 125*, 634–639.

Law, S. K., Minich, T. M., & Levine, R. P. (1984) *Biochemistry 23*, 3267–3272.

Lu, Z. X., Fok, K. F., Erickson, B. W., & Hugli, T. E. (1984) *J. Biol. Chem. 259*, 7367–7370.

Müller-Eberhard, H. J., & Schreiber, R. D. (1980) *Adv. Immunol. 29*, 1–53.

Nonaka, M., Nakayama, K., Yuel, Y. D., & Takahashi, M. (1985) *J. Biol. Chem. 260*, 10936–10943.

Salvesen, G. S., Sayers, C. A., & Barrett, A. J. (1981) *Biochem. J. 195*, 453–461.

Sim, R. B., & Sim, E. (1981) *Biochem. J. 193*, 129–141.

Sottrup-Jensen, L., Petersen, T. E., & Magnusson, S. (1980) *FEBS Lett. 121*, 275–279.

Sottrup-Jensen, L., Hansen, H. F., Mortensen, S. B., Petersen, T. E., & Magnusson, S. (1981) *FEBS Lett. 123*, 145–148.

Sottrup-Jensen, L., Stepanik, T. M., Kristensen, T., Wierzbicki, D. M., Jones, C. M., Lonblad, P. B., Magnusson, S., & Petersen, T. E. (1984) *J. Biol. Chem. 259*, 8318–8327.

Swenson, R. P., & Howard, J. B. (1980a) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4313–4316.

Swenson, R. P., & Howard, J. B. (1980b) *J. Biol. Chem. 255*, 8087–8091.

Tack, B. F., Harrison, R. A., Janatova, J., Thomas, M. L., & Prahl, J. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5764–5768.

Thomas, M. L., Janatova, J., Gray, W. R., & Tack, B. F. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1054–1058.

Unson, C. G., Erickson, B. W., & Hugli, T. E. (1984) *Biochemistry 23*, 585–589.

Wetsel, R. A., Lundwall, A., Davidson, F., Gibson, T., Tack, B. F., & Fey, G. H. (1984) *J. Biol. Chem. 259*, 13857–13862.