N^5-Methylasparagine and Asparagine as Nucleophiles in Peptides: Main-Chain vs Side-Chain Amide Cleavage

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The chemistry of peptides containing N^5-methylasparagine (NMA) was investigated by incubating the synthetic peptides Ile-Ala-Pro-Gly-Gly-**Asn**-Gly-Tyr and Ile-Ala-Pro-Gly-Gly-**NMA**-Gly-Tyr at 60 °C in 0.1 M $NaPO_4$, pH 7.4, to assay for peptide deamidation. The Asn-Gly octapeptide deamidates to Ile-Ala-Pro-Gly-Gly-isoAsp/Asp-Gly-Tyr with a half-life of 2.17 h and activation energy of 18.6 kcal/mol. The NMA-Gly octapeptide partitions between main-chain cleavage and side-chain deamidation in 2.7:1 ratio. Analysis of products diagnostic for each of these NMA peptide reactions yields indistinguishable activation energies for each pathway: 22.6 kcal/mol. The half-life for NMA side-chain deamidation is 98 h, commensurate with a 2.5 kcal/mol difference in activation free energies for deamidation at Asn and NMA sites. These results indicate that methylation provides a substantial (45-fold) stabilization against intramolecular C–N cleavage. The identical activation energy for the alternative pathways of NMA peptide reactivity suggests the differences in the rates may be due to the preexponential portion of the rate equation reflecting small differences in ΔS^*. Molecular mechanics studies were performed to account for these patterns. The computational studies disclose 3-fold more conformers in the Boltzmann population for the tetrahedral intermediate leading toward main-chain cleavage. This result supports the hypothesis that the 2.7-fold difference in NMA peptide partitioning rates is attributable to differences in ΔS^*.

Introduction

Amide nucleophilicity is a subject of rapidly increasing relevance in biochemistry for asparagine *N*-glycosylation,[1] protein asparagine deamidation,[2] protein splicing reactions that may involve asparagine side-chain amides,[3] and a recent proposal for the mechanism of asparagine synthetase.[4] All these reactions use an unactivated amide nitrogen as the attacking nucleophile, but the bioorganic foundation for such reactions is rudimentary. Amides display modest reactivity as intramolecular nucleophiles primarily under basic conditions, when the leaving group is activated, or in five-membered ring formation.[5] *N*-Alkyl amides are superior to simple amides as nucleophiles in substitution reactions,[6] but the origins of this reactivity have not been systematically investigated. In the course of studying the biological chemistry of N^5-methylasparagine (γ-*N*-methylasparagine; NMA) in peptides we have reacquainted ourselves with the nucleophilic propensities of *N*-methyl amides, which are competent nucleophiles effecting intramolecular peptide bond cleavage.

Phycobiliproteins are extrinsic pigment–protein complexes attached to the thylakoid membrane in cyanobacteria and red algae which function as light-harvesting antennae to support photosynthesis.[7] These proteins are composed of nonidentical α and β subunits that each possess linear tetrapyrrole (bilin) prosthetic groups covalently linked to the primary structure via cysteine thioether linkages. The β subunits of most phycobiliproteins contain a posttranslationally methylated NMA residue at the β-72 position near the tetrapyrrole binding site. Asparagine methylation, which is nearly universal in phycobiliproteins, presumably serves an important function in these proteins because the distribution of methylated asparagine crosses the prokaryotic/eukaryotic boundary.[8] We have formulated the functional hypothesis that methylation offers protection against deamidation at a site which is sensitive to the introduction of a charged residue. In many systems glutamine and asparagine deamidation contribute to protein aging, accelerate protein turnover, and may control some developmental processes. Asparagine deamidation has recently gained recognition as one of the major routes of chemical damage to proteins[9] and is particularly relevant in long-lived proteins. This is potentially the case for phycobiliproteins where turnover is low under physiological conditions.

Short peptides containing the naturally occurring sequence NMA-Ala exclusively react to initiate peptide main-chain cleavage while the analogous Asn-Ala peptides only display side-chain deamidation.[10] The present report describes a peptide containing the sequence NMA-Gly which evidences competition between main-chain cleavage

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and side-chain deamidation. Consequently, partitioning between intramolecular nucleophilic attack by either the main-chain or side-chain amide nitrogen on the proximal amide bond can be observed in a single molecule. This affords the opportunity for more detailed kinetic and thermodynamic studies of both intramolecular reactions in the same structure.

Experimental Section

The peptides Ile-Ala-Pro-Gly-Gly-**NMA**-Gly-Tyr and Ile-Ala-Pro-Gly-Gly-**Asn**-Gly-Tyr were synthesized by solid-phase methods and purified to a single peak by reverse phase HPLC using our standard methods.[10] Analysis of each purified peptide was satisfactory. The NMA-Gly octapeptide was characterized by ^{1}H NMR (see supplementary material); mass spectrometry ($[M + H]^+$ = 762.4, $[M + Na]^+$ = 785.5, $[M + 2Na]^+$ = 808.1; exact mass, m/z 762.3776 for $[M + H]^+$, 762.3786 calcd for $C_{34}H_{52}N_9O_{11}$); and amino acid analysis (Asp, 1.0; Pro, 0.95; Gly, 2.9; Ala, 1.0; Ile, 1.0; Tyr, 1.0; methylamine, 0.95). The Asn-Gly octapeptide was characterized by ^{1}H NMR (see supplementary material); mass spectrometry ($[M + H]^+$ = 748.1, $[M + Na]^+$ = 771.8, $[M + 2Na]^+$ = 794.0; exact mass m/z 748.3638 for $[M + H]^+$, 748.3630 calcd for $C_{33}H_{50}N_9O_{11}$); and amino acid analysis (Asp, 1.0; Pro, 1.0; Gly, 3.0; Ala, 1.0; Ile, 1.0; Tyr, 1.0; NH_3, not determined).

Peptide incubations (n = 2–4) were performed in duplicate in 0.1 M $NaPO_4$, pH 7.4, at 60, 70, or 85 °C in Pierce reacti-vials sealed with a Teflon liner. Reactions were quenched by freezing to −20 °C and stored before analysis. The time points were acidified with an equal volume of 0.1 N HCl prior to chromatography and analyzed by reverse-phase HPLC utilizing a 5–20% linear gradient (15 min) of acetonitrile containing 0.06% TFA mixed with water containing 0.1% TFA at 1.5 mL/min on a Vydac 218TP54 column with detection at 215 nm. Control experiments indicated that sample acidification did not alter the product distribution or formation rate. However, the retention time and peak shape of early eluting components were dramatically improved with prior pH adjustment by acidification. Under these conditions the Asn-Gly octapeptide had a t_R = 12.6 min and the NMA-Gly octapeptide had a t_R = 13.5 min. Peak areas were obtained by computer integration and converted to molar values from response factors determined by quantitative amino acid analysis, and each peptide component was calculated as a molar percentage of the total. Data were fitted by nonlinear regression to a single exponential decay curve using ENZFITTER software.[11] The data obtained from computer fits are reported ± standard error of the mean from the regression program.

Peptide incubation products were isolated by semipreparative HPLC using very shallow acetonitrile gradients. The Asn-1 product was characterized by mass spectrometry ($[M + H]^+$ = 749.7, $[M + Na]^+$ = 772.4, $[M + 2Na]^+$ = 795.0); amino acid analysis (Asp, 1.0; Pro, 1.0; Gly, 3.2; Ala, 1.1; Ile, 1.0; Tyr, 0.94); and chiral aspartate analysis[12] (80.8% D/19.2% L). The Asn-2 product was characterized by ^{1}H NMR (see supplementary material); mass spectrometry ($[M + H]^+$ = 749.3, $[M + K]^+$ = 787.2; exact mass, m/z 749.3506 for $[M + H]^+$, 749.3470 calcd for $C_{33}H_{49}N_8O_{12}$); amino acid analysis (Asp, 1.0; Pro, 1.0; Gly, 3.2; Ala, 1.1; Ile, 1.0; Tyr, 1.0); and chiral aspartate analysis[12] (18.3% D/81.7% L). The Gly-Tyr peptide product was characterized by amino acid analysis (Gly, 1.0; Tyr, 0.96), leucine aminopeptidase digestion[10] (Gly, 1.0; Tyr, 1.0), and ^{1}H NMR (500 MHz, H_2O) δ 2.87 (d, J = 5 Hz, Tyr β-CH_2), 3.14 (d, J = 9 Hz, Tyr β-CH_2), 3.69 (d, J = 16 Hz, Gly α-CH_2), 3.79 (d, J = 16 Hz, Gly α-CH_2), 4.45 (m, Tyr α-CH), 6.87 (d, J = 9 Hz, Tyr 3,5-Ar-H), 7.15 (d, J = 9 Hz, Tyr 2,6-Ar-H), 8.17 (s, Tyr N-H). The NMA-4 product was characterized by mass spectrometry ($[M + H]^+$ = 749.2, $[M + K]^+$ = 787.2); amino acid analysis (Asp, 1.0; Pro, 1.0; Gly, 3.2; Ala, 1.1; Ile, 1.0; Tyr, 0.93); and chiral aspartate analysis[12] (81.3% D/18.7% L). The NMA-5 product was characterized by mass spectrometry ($[M + H]^+$ = 749.3, $[M + K]^+$ = 787.2); and amino acid analysis (Asp, 1.0; Pro, 1.0; Gly, 3.1; Ala, 1.1; Ile, 1.0; Tyr, 0.93). The individual purified peptides coeluted as Asn-1/NMA-4 and Asn-2/NMA-5 pairs when coinjected on the HPLC system.

Peptide ^{1}H NMR spectra were obtain on a Bruker AMX-500 spectrometer in D_2O or H_2O containing a TSP internal standard. Resonances were assigned through the use of standard pulse routines for two-dimensional DQF-COSY, TOCSY, and ROESY.[13]

Computational studies used PCMODEL (ver 4.0) and GMMX (ver 1.0), Serena Software, Bloomington, IN. 3-Amino-*N*-methylsuccinimidylglycine structures representing the tetrahedral intermediates for either peptide cleavage or side chain deamidation were constructed with an acetylated amino terminal and methylamide blocked carboxyl terminal. The global minimum was searched statistically in GMMX by generating structures from random coordinates and evaluating 1000 conformers each of either *R* or *S* stereochemistry at the new tetrahedral center. All structures within 6 kcal of the minima (D = 1.5) were reminimized with PCMODEL utilizing computational conditions of D = 80 and taking hydrogen bonding into account. Identical conformers were rejected using a structural rms cut-off of 0.5 Å^2 and the resulting Boltzmann distribution was recalculated using all structures within 3.5 kcal of the global minimum.

Results and Discussion

The peptides Ile-Ala-Pro-Gly-Gly-**NMA-Gly**-Tyr and Ile-Ala-Pro-Gly-Gly-**Asn-Gly**-Tyr were synthesized as part of our investigation into the influence of the n + 1 amino acid structure on peptide deamidation lability. Analysis of the Asn-Gly octapeptide incubations at 60 °C indicated the time-dependent disappearance of the parent peptide fitting a single exponential decay curve ($t_{0.5}$ = 2.17 ± 0.08 h) and production of a new peak eluting ~0.6 min later (Figure 1A). The peptide product peak could be separated into a minor peak eluting slightly prior to the major product (ratio = ~1:10) using a shallow gradient elution system. The minor peak, Asn-1, was assigned as Ile-Ala-Pro-Gly-Gly-(D-Asp)-Gly-Tyr by acid hydrolysis, amino acid analysis, chiral aspartate analysis, leucine aminopeptidase digestion (not shown), and mass spectrometry. The major product, Asn-2, was assigned as Ile-Ala-Pro-Gly-Gly-L-isoAsp/L-Asp-Gly-Tyr by amino acid analysis, chiral aspartate analysis, leucine aminopeptidase digestion (not shown), mass spectrometry, and ^{1}H NMR. Examination of the ^{1}H NMR signals confirmed that the isoaspartyl peptide linkage predominated over a normal aspartyl peptide linkage for the major product but an exact ratio could not be accurately determined (Table I). Our previously reported data[10] for the analogous Asn-Ala octapeptide ($t_{0.5}$ = 45 ± 1.0 h at 60 °C) indicate the expected ~15-fold increase in deamidation rate when Gly replaces Ala at the n + 1 position.[2c,d] Additional incubations at 70 and 85 °C permitted us to measure the activation energy, E_a, as 18.6 ± 0.5 kcal/mol for the Asn-Gly octapeptide reaction.

Analysis of the NMA-Gly octapeptide incubations under similar conditions revealed a more complex product profile. Product peaks attributable to both the octapeptide Ile-Ala-Pro-Gly-Gly-Asp-Gly-Tyr and the dipeptide Gly-Tyr accumulated as a function of incubation time (Figure 1B). The putative Asp peptide product peak could be further separated into a minor peak eluting slightly prior to the

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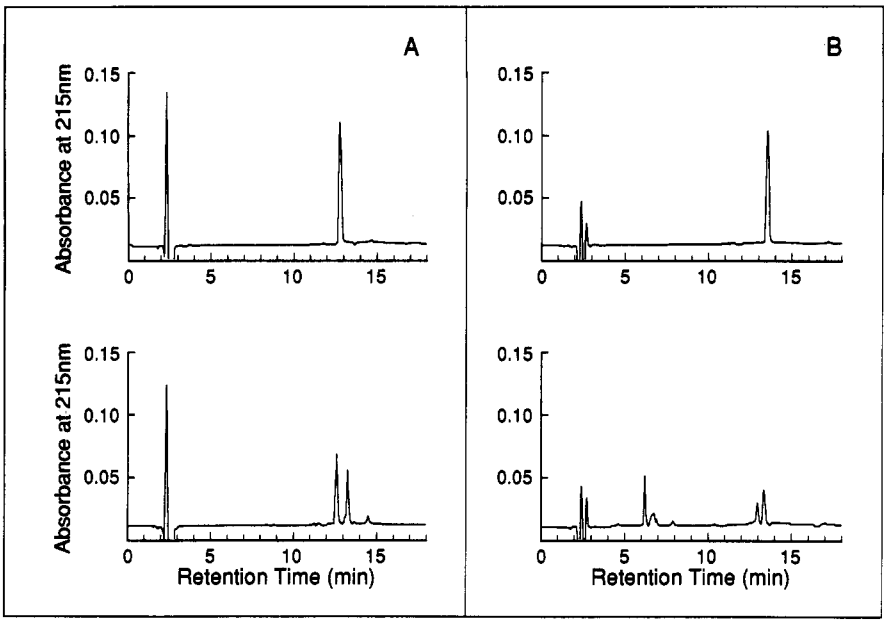

Figure 1. HPLC analysis of octapeptide incubations. Peptides were incubated at 60 °C in 0.1 M $NaPO_4$, pH 7.4, and then stored frozen until analysis. Uniform aliquots were acidified with an equal volume of 0.1M HCl and injected onto a Vydac C-18 column. The column was eluted over a 15-min period with a linear 5–20% acetonitrile gradient in 0.1% aqueous TFA and products were detected by absorbance at 215 nm. A: Asn-Gly octapeptide at time zero (top) and after a 2-h incubation (bottom). The first product peak (t_R = 13.2 min) was further resolved into Asn-1 (minor) and Asn-2 (major) peaks (see Experimental Section). The second product peak (t_R = 14.5 min) is unstable and does not accumulate further during the incubations; instead it subsequently disappears, suggesting it represents the peptidyl succinimide intermediate in the reaction. B: NMA-Gly octapeptide at time zero (top) and after a 48-h incubation (bottom) . The first product peak (t_R = 6.1 min) was isolated and assigned as glycyltyrosine (see Experimental Section). The product peak with a t_R = 12.9 min was further resolved into NMA-4 (minor) and NMA-5 (major) peaks (see Experimental Section). The other product peaks (t_R = 6.6–6.8 and 7.8 min) were not extensively characterized because material was limited. However these components have identical chromatographic behavior and amino acid compositions to the hexapeptide products Ile-Ala-Pro-Gly-Gly-NMA/isoNMA and related *N*-methylsuccinimide peptides examined in our earlier work with NMA-Ala peptide incubations.[10]

Table I. ^{1}H NMR Assignments for Ile-Ala-Pro-Gly-Gly-L-Asp/L-isoAsp-Gly-Tyr Product[a]

residue	NH	α-CH	β-CH	others
Ile-1		4.44	1.92	γ-CH_2 1.49, 1.50 γ-CH_3 1.20 δ-CH_3 0.90
Ala-2	8.67	4.67	1.39	
Pro-3		4.45	2.32, 1.96	γ-CH_2 2.03 δ-CH_2 3.67, 3.85
Gly-4				
(no. 6 = Asp)	8.40	4.02		
(no. 6 = isoAsp)	8.48	3.94		
(no. 6 = isoAsp)	8.51	4.02		
Gly-5				
(no. 6 = Asp)	8.25	3.97		
(no. 6 = isoAsp)	8.37	3.92		
Asp-6	8.41	4.65	2.73	
isoAsp-6	8.01	4.53	2.67	
	8.19	3.93	2.83	
Gly-7				
(no. 6 = Asp)	8.59	3.98		
(no. 6 = isoAsp)	8.22	3.84		
Tyr-8	7.65	4.42	2.91, 3.10	δ-CH 7.12 ε-CH 6.83

[a] Chemical shifts are reported relative to an internal TSP standard set to 0.0 ppm.

major product (ratio = ~1:10) using an shallow gradient elution system as with the Asn-Gly deamidation product. The minor peak, NMA-4, was assigned as Ile-Ala-Pro-Gly-Gly-(D-Asp)-Gly-Tyr by amino acid analysis, chiral aspartate analysis, and mass spectrometry. The major peak, NMA-5, was assigned as Ile-Ala-Pro-Gly-Gly-L-isoAsp/L-Asp-Gly-Tyr by amino acid analysis and mass spectrometry. A pathway to account for this partitioning is presented in Figure 2. Isolation of the individual product peaks and quantitative amino acid analysis allowed us to directly measure the partition ratio by quantitation of Ile-Ala-Pro-Gly-Gly-isoAsp/Asp-Gly-Tyr (deamidation pathway) and Gly-Tyr (peptide cleavage pathway) summarized in Figure 3. Application of the partition ratio to deconvolute the individual rate constants from the overall rate of parent peptide disappearance yielded $t_{0.5}$ = 98 ± 1.7 h for deamidation and 36 ± 0.63 h for cleavage at 60 °C. Construction of an Arrhenius plot led to the finding that the activation energy was identical for each reaction: E_a = 22.6 ± 0.6 kcal/mol for deamidation and 22.6 ± 0.5 kcal/mol for peptide cleavage.

We have performed these studies in 0.1 M $NaPO_4$, pH 7.4, because this is a common buffer used in the literature for peptide deamidation experiments.[2a-d] Buffer effects on the rate of reaction at carbonyl centers are known[14] but product ratios are unaffected for strictly amide systems such as these peptides.[2d] Nucleophilic reactions by amides can also be complicated by the distinction between the carboxamide oxygen and nitrogen as the initial nucleophile.[15] Other investigators while noting this confounding aspect have not unambiguously eliminated the mechanism of initial isoimide formation followed by facile rearrangement to an imide.[2a,b] Our data do not directly address the issue but we make the common assumption in the literature that nitrogen is the nucleophile.

We draw several conclusions from these data. The observed 45-fold decrease in deamidation (2.17 vs 98 h $t_{0.5}$) associated with methylation is greater than the 16-

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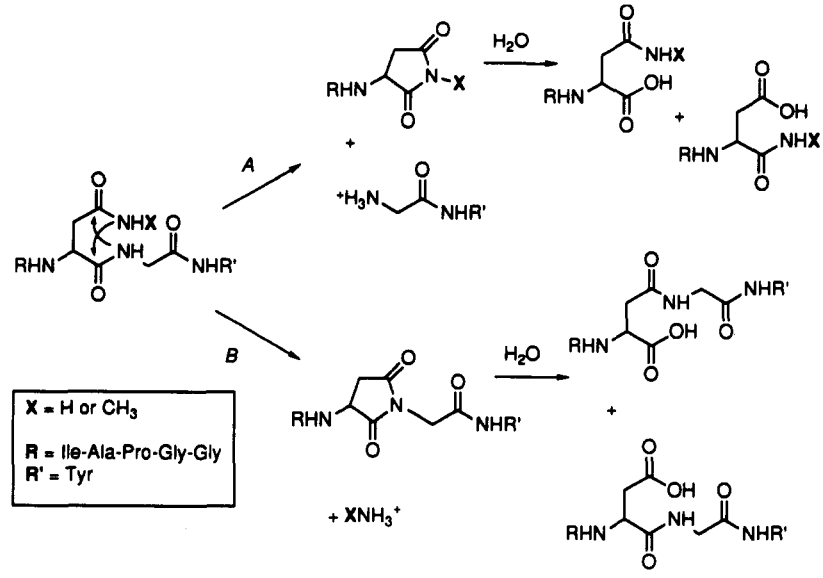

X	Path A	Path B
H	0%	100% (0.32 h^{-1})
CH_3	73% (0.019 h^{-1})	27% (0.007 h^{-1})

Figure 2. Reaction scheme for NMA/Asn-Gly peptides. For asparaginyl peptides pathway B is the exclusive route to product formation. The limit of detection for products signaling peptide chain cleavage is <3% of the deamidation rate for the Asn-Gly octapeptide. Partitioning occurs between scission of either the main-chain peptide bond (path A) or the side-chain methylamide (path B) when NMA is present. In all cases the peptidyl succinimide intermediates are unstable and hydrolyze to a mixture of iso and normal peptide bond isomers.

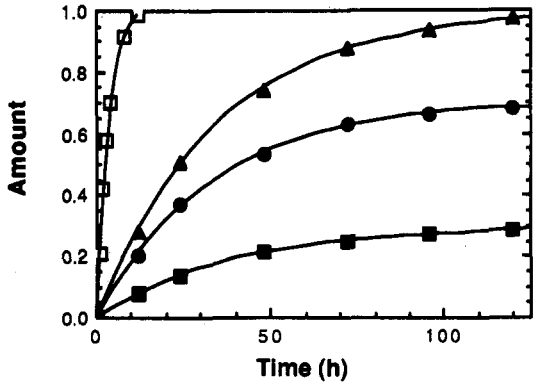

Figure 3. Incubation peptide product profile. The results of HPLC analysis for a single Ile-Ala-Pro-Gly-Gly-NMA/Asn-Gly-Tyr peptide incubation at pH 7.4 and 60 °C are displayed. Quantitation was based on the response factors for each peptide component derived from quantitative acid hydrolysis and amino acid analysis: (open squares) Ile-Ala-Pro-Gly-Gly-Asp/isoAsp-Gly-Tyr (Asn-1 + Asn-2) derived from incubation of Ile-Ala-Pro-Gly-Gly-Asn-Gly-Tyr; (filled squares) Ile-Ala-Pro-Gly-Gly-Asp/isoAsp-Gly-Tyr (NMA-4 + NMA-5) derived from incubation of Ile-Ala-Pro-Gly-Gly-NMA-Gly-Tyr; (filled circles) Gly-Tyr derived from incubation of Ile-Ala-Pro-Gly-Gly-NMA-Gly-Tyr; (filled triangles) sum of two products derived from incubation of Ile-Ala-Pro-Gly-Gly-NMA-Gly-Tyr.

18-fold differences found in hydrolyses such as acetamide vs *N*-methylacetamide[16] which lack the intramolecular aspect (see below). Generally, attack on an amide is base-catalyzed and tetrahedral adduct collapse is acid-catalyzed because protonation at nitrogen is required for breakdown of the tetrahedral intermediate at neutral pH.[5,6] However, intramolecular deamidation reactions at asparagine in peptides do not evidence specific base catalysis.[2d] Data comparing imide formation by an amide nucleophile via intramolecular ester cleavage (methyl *N*-methylphthalamate, methyl phthalamate) and intramolecular amide cleavage (*N,N'*-dimethylphthalamide, phthalamide) reveal a >1000-fold decrease in rate when the leaving group changes from an alcohol to an amine.[6] This is consistent with a change in rate-determining step from tetrahedral adduct formation when the leaving group is an alcohol to tetrahedral adduct collapse when the leaving group is an amine. Ammonia departure from a tetrahedral intermediate is expected to be thermodynamically driven by the large heat of hydration while cleavage of a primary amine such as methylamine in preference to ammonia would be kinetically driven.[17] Assuming that tetrahedral intermediate formation is not rate-determining[18] in our system implies that methylamine is indeed a poorer leaving group than ammonia and thus tetrahedral intermediate breakdown is subject to thermodynamic control. We further reason that collapse of the tetrahedral intermediate is rate-determining by referring to Figure 2. Although steric conflict may be a factor (see below), it is otherwise difficult to rationalize why pathway B is 45-fold more rapid when X = H unless ammonia is the superior leaving group.

The two competing reactions of the NMA-Gly peptide system have identical activation energies accompanying the 2.7-fold difference in rate. This suggests that the preexponential term of the rate equation accounts for differences in kinetics. Energy minimization studies of two unstable tetrahedral intermediates modeling the

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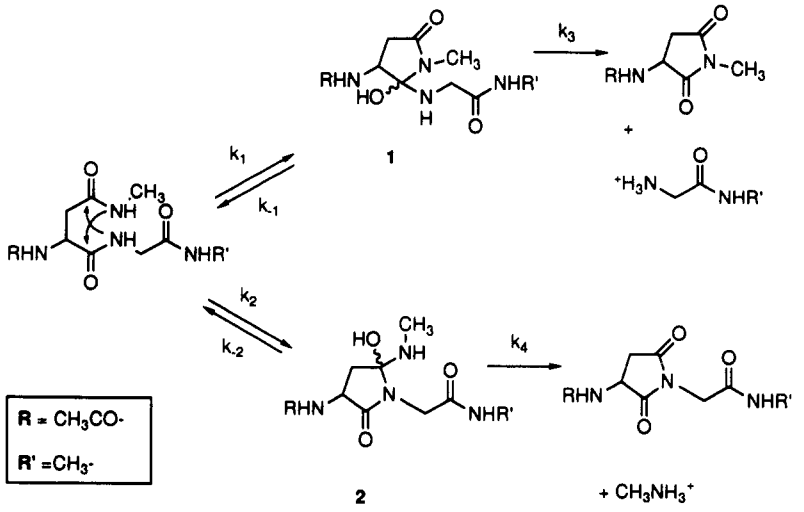

Figure 4. Schematic for the NMA-Gly peptide reaction. Tetrahedral intermediates 1 and 2 were built in PCMODEL and conformationally searched for global minima using GMMX. We assume that k_3, $k_4 < k_1$, k_2 and that k_4 is the step most sensitive to the presence of methylamine vs ammonia.

transition states (Figure 4) reveal that the tetrahedral intermediate (2) leading to side-chain deamidation has 14 conformers contributing ≥1% of the Boltzmann population (E = −180.09 kcal/mol). The tetrahedral intermediate (1) leading to main-chain cleavage has 42 conformers contributing ≥1% of the Boltzmann population (E = −178.26 kcal/mol). These calculations imply that intermediate 1 is stabilized by the large number of energetically feasible conformations and is characterized by a broad energy surface representing three rotatable bonds on the side chain. In contrast, intermediate 2 has a narrow envelope of minimal energy because nitrogen methylation has destabilized many of the ring conformations via unfavorable interactions with the side chain containing two rotatable bonds.[19] The result is fewer conformers that are close to E_{min}, consistent with the notion that the 3-fold difference in rates is attributable to entropic contributions in the transition state ($\Delta S^{\ddagger}/R$). Further, the potential steric conflicts in intermediate 2 may also account for the magnitude of stabilization achieved by methylation in the intramolecular reaction (45-fold) vs the 16–18-fold differences reported for hydrolyses.[16]

The literature contains limited examples[6] of relevant substitution reactions at carbonyl centers in that many of the studies were performed with esters where the leaving group is relatively activated. Amide hydrolysis studies have been largely performed using acid- and base-catalyzed conditions but rarely with an amide nucleophile.[14-18] The Curtin–Hammett principle[20] places limits on our kinetic analysis. In Figure 4 we assume that k_3 and k_4 are rate-determining irreversible steps and that k_4 is influenced by the diminished leaving group potential of methylamine vs ammonia. Under conditions where k_1, $k_2 > k_3$, k_4 a preequilibrium involving the tetrahedral intermediates 1 and 2 is established. Our conformational analysis would hold if k_3 and k_4 are equivalent such that the pathway which is better populated would predominate. However it is possible that k_1, $k_2 \gg k_3$, k_4, in which case the product pattern could be explained by positing that k_3/k_4 = 2.7 and our data do not distinguish between these limiting cases. It should be noted that the leaving groups in both cases are primary amines although the glycyl amino group has a substantially lower pK_a than that for methylamine.

As a consequence of these physical organic factors, amide methylation slows the rate of deamidation. Diminution of the deamidation rate allows alternative pathways to become kinetically significant and accentuates the nucleophilicity of the methyl amide. Other investigators have reported modest peptide bond cleavage at asparaginyl sites, generally on the order of 15% or less.[2c,d,21] Future studies on peptides containing Ser, Thr, or Leu at the $n+1$ position may provide further insight into the chemistry of NMA-associated cleavage and allow the design of peptides which display intramolecular assistance (such as $n+1$ = His or Ser)[2c,d] to catalyze the cleavage.

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Supplementary Material Available: ^{1}H NMR spectra for the octapeptides Ile-Ala-Pro-Gly-Gly-Asn-Gly-Tyr and Ile-Ala-Pro-Gly-Gly-NMA-Gly-Tyr (5 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(19) As predicted by this conjecture, calculations between the number of conformers for the homologs of 1 and 2 with asparaginyl structures (without the N-methyl group) do not display a sharp difference. Unfortunately we cannot directly compare the energies of the asparaginyl structures with those derived from NMA by these computations.

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