Deamidation of Asparagine Residues: Direct Hydrolysis versus Succinimide-Mediated Deamidation Mechanisms

Saron Catak,[†,‡] Gérald Monard,[†] Viktorya Aviyente,[‡] and Manuel F. Ruiz-López*[,†]

Equipe de Chimie et Biochimie Théoriques, UMR CNRS-UHP No. 7565, Nancy-Université, BP 239, 54506 Vandoeuvre-lès-Nancy, France, and Department of Chemistry, Bogazici University, 34342 Bebek, Istanbul, Turkey

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Quantum chemical calculations are reported to provide new insights on plausible mechanisms leading to the deamidation of asparagine residues in proteins and peptides. Direct hydrolysis to aspartic acid and several succinimide-mediated mechanisms have been described. The catalytic effect of water molecules has been explicitly analyzed. Calculations have been carried out at the density functional level (B3LYP/6-31+G**). Comparisons of free energy profiles show that the most favorable reaction mechanism goes through formation of a succinimide intermediate and involves tautomerization of the asparagine amide to the corresponding imidic acid as the initial reaction step. Another striking result is that direct water-assisted hydrolysis is competitive with the succinimide-mediated deamidation routes even in the absence of acid or base catalysis. The rate-determining step for the formation of the succinimide intermediate is cyclization, regardless of the mechanism. The rate-determining step for the complete deamidation is the hydrolysis of the succinimide intermediate. These results allow clarification of some well-known facts, such as the isolation of succinimide or the absence of iso-Asp among the reaction products observed in some experiments.

Introduction

Asparagine (Asn) and glutamine (Gln) residues are known to undergo spontaneous nonenzymatic deamidation to form aspartic acid (Asp) and glutamic acid (Glu) residues under physiological conditions.[1−5] The conversion of the neutral amide side chain to the negatively charged carboxylate causes time-dependent changes in conformation and limits the lifetime of peptides and proteins.[1,6−8] Deamidation half-times in human proteins were shown to occur over a wide range of biologically relevant time intervals[9] and have been associated with the timed process of protein turnover, development, and aging.[10] Robinson has proposed the *molecular clock hypothesis*, which suggests that deamidation is a biological molecular timing mechanism that could be set to any desired time interval by genetic control of the primary, secondary, and tertiary structure surrounding the amide.[11] Recent experiments[12−19] and computations[20−25] have been in accord with this hypothesis and provided compelling evidence of its significance.

The deamidation reaction mechanism was initially believed to be an acid- or base-catalyzed direct hydrolysis, with a minimum reaction rate near neutral pH. Deamidation products for L-Asn and L-Gln were expected to be L-Asp and L-Glu with little racemization to D-Asp and D-Glu at basic pH. However, the pH minimum for deamidation was actually observed to be around 5 for both peptides and proteins and an L-iso-Asp product was observed in addition to the L-Asp.[13] The succinimide-mediated deamidation mechanism (Scheme 1) is suggested to be responsible for shifting the minimum to pH 5 and for the variety of reaction products observed.

Capasso et al. have proposed that deamidation of relatively unrestrained Asn residues goes through a succinimide intermediate (Scheme 1).[12,14] The cyclic imide then hydrolyzes at either one of the two carbonyls to give Asp and iso-Asp. The ratio of L-Asp to L-iso-Asp was experimentally found to be 3:1.[13]

Experimental evidence indicates that, at low pH, hydrolysis of the side-chain amide functionality occurs with ease and imide intermediates are not observed, contrary to neutral and basic pH conditions where succinimide derivatives have almost always been identified.[12−19] As pH decreases below 5, direct hydrolysis via acid catalysis takes place at an increasing rate. The fact that direct hydrolysis is most prevalent is indicated by the marked drop of the iso-Asp/Asp ratio.[5] Ordinary base catalysis also occurs at high pH, but the rapidity of the imide mechanism at high pH usually obscures this.

Because reaction rates are often related to transient chemical species that are difficult to observe and are subject to influences of solvent and other factors, there is often uncertainty concerning a proposed mechanism, as is the case with deamidation. Previous computational studies on deamidation of Asn include modeling the formation of the succinimide as a two-step process; cyclization followed by deamination.[20] Subsequent computations on this mechanism established the fact that water molecules in the vicinity of Asn residues catalyze deamidation.[26] Radkiewicz et al. have computationally explored the racemization of Asp and Asn via succinimide intermediates[23] and have studied the effect of neighboring side chains on backbone NH acidity.[24] Peters et al. explored pH dependence of the deamidation mechanism.[25]

Experimental findings have shown that the rate of deamidation of Asn residues is primarily controlled by the carboxyl side residue ($n + 1$) with smaller effects from the amino side residue ($n - 1$);[16] this is also consistent with the succinimide reaction mechanism. However, the relationship between the size and/or

* To whom correspondence should be addressed: E-mail: manuel.ruiz@cbt.uhp-nancy.fr.
† Nancy-Université.
‡ Bogazici University.

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charge of the $n+1$ residue and rate of deamidation is not clear. In peptides with substantial freedom of movement, sequence dependence has also been detected for other residues further along the peptide chain in both directions.[16] The effects of more distant residues are probably largely suppressed in proteins.

In light of the succinimide-mediated mechanism, one may expect deamidation reaction rates of ordinary Asn residues in physiological solvent conditions to be largely affected by their chemical environment. First, by the intrinsic acidity of the $n+1$ backbone nitrogen, which depends on inductive and electrostatic effects, and, therefore, upon peptide sequence. Second, by the amount of available conformational space and steric hindrance in the vicinity of the Asn residue, which may enhance or inhibit the formation of the cyclic intermediate. This is especially important in proteins where rearrangements in the protein 3D structure may be necessary to allow proper alignment of the side chain and/or the backbone for ring closure. Finally, by the availability of water molecules or a proton donor, which is crucial for the decomposition of the cyclic tetrahedral intermediate that may otherwise revert to the open form. Whereas protein structure usually inhibits deamidation, there are many instances in which protein structure near the amide allows deamidation to occur at its primary sequence controlled rate.[16] There are also relatively rare instances in which protein structure actually increases the deamidation rate.[19]

This study aims to get a deeper insight on plausible mechanisms leading to deamidation of Asn residues. Relative energetics and feasibilities of these pathways will be comparatively discussed. Theoretical calculations on several succinimide-mediated deamidation paths will be compared against a direct hydrolysis mechanism. Catalysis of the reactions by water molecules will be investigated. To make a decent comparison on all possible pathways, both new and already reported routes[20−],[26] have been recalculated at a consistent level of theory.

Computational Methodology. All gas-phase geometry optimizations were performed using the density functional theory (DFT)[27−30] at the B3LYP/6-31+G** level.[31−33] Diffuse and polarization functions are included on heavy atoms because they are especially necessary in the optimization of anionic systems; polarization functions were also added on hydrogen atoms in order to account for the presence of hydrogen-bonds. Geometries of stationary points were optimized without any constraints. All stationary points were characterized by a frequency analysis from which zero-point energy and thermal corrections were attained using the ideal gas approximation and standard procedures. Local minima and first-order saddle points were identified by the number of imaginary vibrational frequencies. The intrinsic reaction coordinate (IRC) approach,[34,35] followed by full geometry optimization, was used to determine the species connected by each transition structure. Energy values for gas-phase optimizations listed throughout the discussion include thermal free energy corrections at 298 K and 1 atm.

The catalytic effect of water molecules on deamidation was previously established[26] and has been taken into account in this study. For clarity, comparative discussion of energetics between different mechanisms is always made among species with identical molecularity, that is to say for species that are associated with the same n in the initial [peptide model, $(H_2O)_n$] complex. This choice minimizes the errors in the estimation of entropic contributions.[36] Relative free energies of activation ($\Delta G^{\ddagger}$) are calculated as the difference of free energies between transition states and the initial reactant. Following previous studies on amide hydrolysis[36] in water-assisted mechanisms, the initial reactant is taken as the solute−water complex with the relevant number of water molecules. Solvent effects on amide hydrolysis have been analyzed and commented in detail by Gorb et al.[36] The authors showed that 1) bulk solvent effects are small and 2) the main solvent effect comes from the participation of an auxiliary water molecule to the activation process, with other water molecules in the first solvation shell playing a secondary role. On the basis of those results, in our work, we have neglected solvent effects other than those introduced by explicit consideration of the interactions with one or two water molecules participating to the hydrolysis mechanism. Obviously, formation of several hydrogen bonds between water and the groups in our model peptide not directly involved in the reaction, is expected to occur, thereby stabilizing the peptide. We assume that such a stabilization is similar for all systems so that relative energies would not change too much if a much more elaborated solvation model is used.

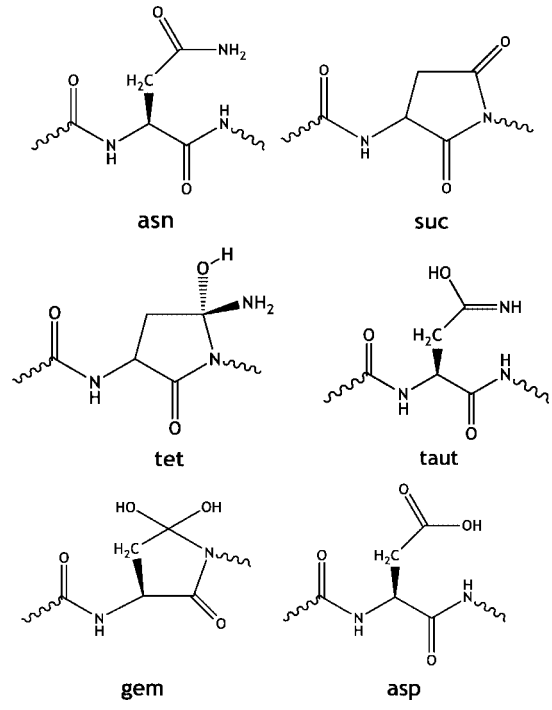
Nomenclature of each transition state corresponds to the name indicated in the relevant scheme and the explicit number of water molecules used in that particular step. All calculations were carried out using the *Gaussian 03* program package.[37]

Results and Discussion

In the first part of this section, we briefly describe different mechanisms for deamidation. The main characteristics of the transition structures will be commented. The direct hydrolysis of the Asn side chain amide is compared with mechanisms involving a succinimide intermediate. Afterward, the energetics and feasibilities of different mechanisms leading to deamidation are discussed.

Different number of explicit water molecules was used (0, 1, or 2) to analyze the effect of solvent on reaction mechanism and energetics. The initial structure for each system is the model compound or model compound−water complex (Figure 1).

The following abbreviations are used throughout the discussion: asparagine (***asn***), succinimide intermediate (***suc***), tetrahedral intermediate (***tet***), amidic acid tautomer (***taut***), gemdiol (***gem***), and aspartic acid (***asp***).



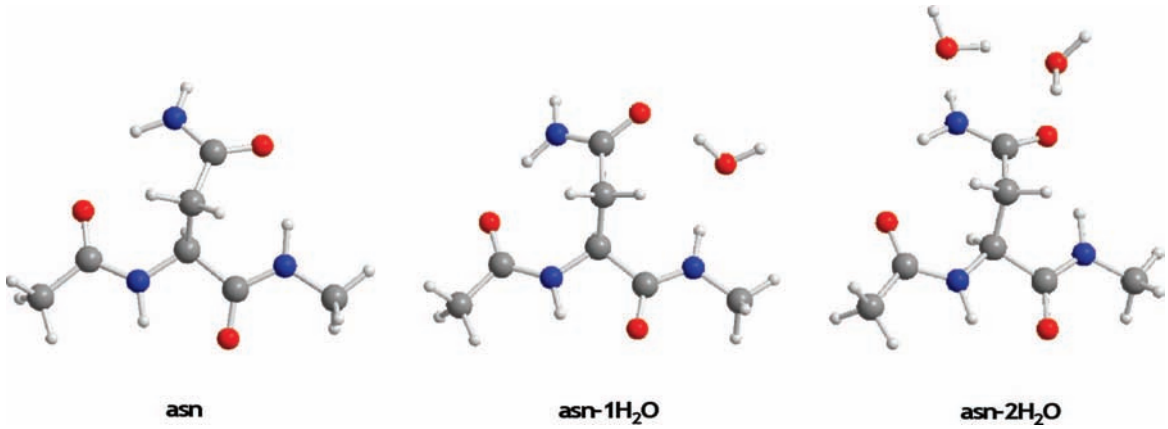
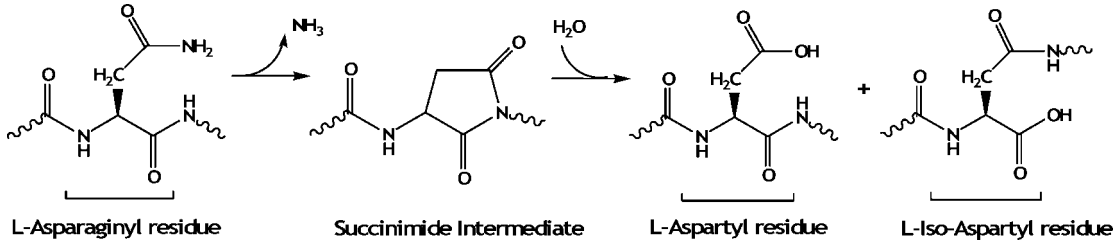
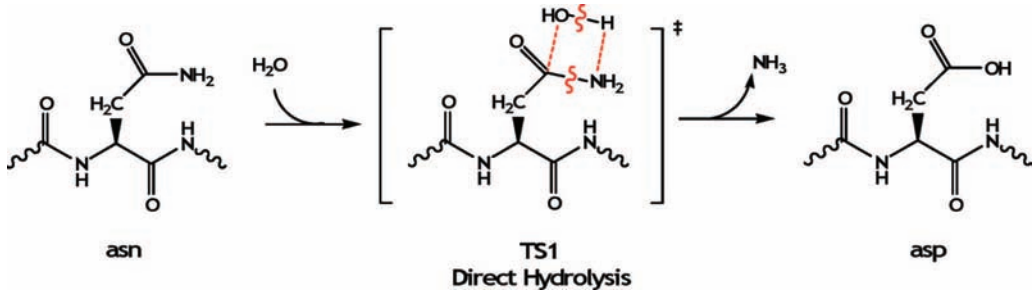


Figure 1. Optimized structures of model peptides with Asn residue.

SCHEME 1: Succinimide-Mediated Deamidation of Asn Residues



SCHEME 2: Direct Hydrolysis of Asn to Asp



As commented above, some reaction mechanisms have already been considered in previous theoretical studies.[20−26] However, different models of water environments were assumed in those investigations and comparison is not straightforward. Thus, for consistency, we have recalculated all reaction paths here. Slight changes in free energies of activation have been observed for some steps, but differences are within the range of 2 kcal/mol.

A. Direct Hydrolysis of Asparagine to Aspartate (*asn* → *asp*). Amide hydrolysis has been extensively studied by computational methods, formamide hydrolysis in particular.[36] The hydrolysis of the ***asn*** side chain to the carboxylic acid via a concerted mechanism was modeled with one or two water molecules. In the two-water case, the reaction involves a proton relay mechanism where solvent molecules serve as a conduit; amide hydrolysis involves the expulsion of an NH_3 group and deamidation takes place in a single step (Scheme 2).

Optimized structures are shown in Figure 2. Given that the water molecule is a reactant, **TS1−1H$_2$O** is in fact an unassisted case, whereas **TS1−2H$_2$O** shows one-water assistance. Transition-state structures show that the proton transfer from the water molecule to the leaving group ($-NH_2$) is underway in both cases. The C−N amide distances are rather short (approximately 1.5 Å), indicating early transition states.

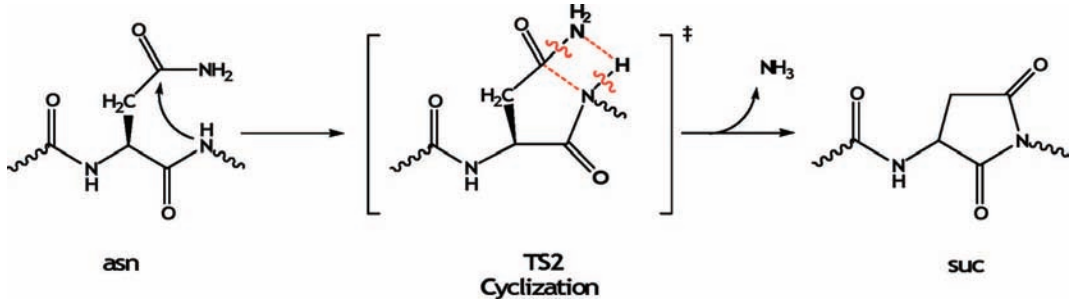
B. Succinimide-Mediated Deamidation of Asn to Asp. Succinimide-mediated deamidation consists of two main parts; the formation of the succinimide intermediate followed by the hydrolysis of the imide. Both may be achieved via several ways involving a different number of steps that are discussed below. The mechanisms already described in the literature will be only outlined. The corresponding structures to these mechanisms can be found in the original references.

Succinimide Formation. *Single Step (asn → suc).* The first mechanism is the concerted ring closure accompanied by deamination, in which the $n + 1$ backbone NH transfers its H to the Asn side chain NH_2. Ejection of an ammonia molecule and formation of the cyclic imide occur in a single step (Scheme 3).

The main difference between the water assisted (**TS2−1H$_2$O**) and nonassisted (**TS2**) cases (Figure 3) is the extent of ring closure, the C−N distance is considerably shorter in **TS2** (2.261 Å as opposed to 2.555 Å in **TS2−1H$_2$O**). In addition, the leaving group is further detached from the side-chain carbonyl carbon (1.605 Å as opposed to 1.556 Å in **TS2−1H$_2$O**).

Two Step (*asn → tet → suc*). This mechanism has been previously described.[26] The succinimide intermediate is formed via a tetrahedral intermediate (Scheme 4). A proton is transferred from the n +1 backbone NH to the **asn** side chain carbonyl

SCHEME 3: Succinimide Formation − Single-Step Mechanism (*asn* → *suc*)



oxygen instead of the side chain NH_2 as in the previous mechanism. In the second step (deamination), the tetrahedral intermediate transforms into a succinimide.

Three Step (*asn* → *taut* → *tet* → *suc*). This mechanism has also been previously described.[26] The first step is the tautomerization (*asn* → *taut*) of the Asn side chain amide into an amidic acid tautomer (Scheme 5). The tautomer then undergoes ring closure (*taut* → *tet*) to give the cyclic tetrahedral intermediate, which subsequently deaminates to form the succinimide intermediate (*tet* → *suc*) through a process identical to the one discussed above (Scheme 4).

Hydrolysis of the Succinimide Intermediate (*suc* → *asp*). Deamidation is completed when the succinimide intermediate undergoes hydrolysis and an Asp residue forms. It is noteworthy to indicate that the hydrolysis may take place at either one of the carbonyl groups on the succinimide. However, products will be different, Asp and iso-Asp may both form; when iso-Asp forms, the peptide backbone is altered (Scheme 1). An extra atom on the peptide chain will be an unstabilizing factor in a protein 3D structure, as it might disrupt intermolecular interactions within the chain. The formation of iso-Asp has been previously investigated computationally[22] and will not be discussed in this study because the aim is to find the most plausible pathway for the complete deamidation of Asn into Asp.

Previous studies on amide hydrolysis have shown that a stepwise mechanism going through a gemdiol intermediate has a considerably lower barrier than a concerted reaction.[36] These studies have also revealed that water molecules catalyze hydrolysis of an amide, as mentioned earlier. To check whether

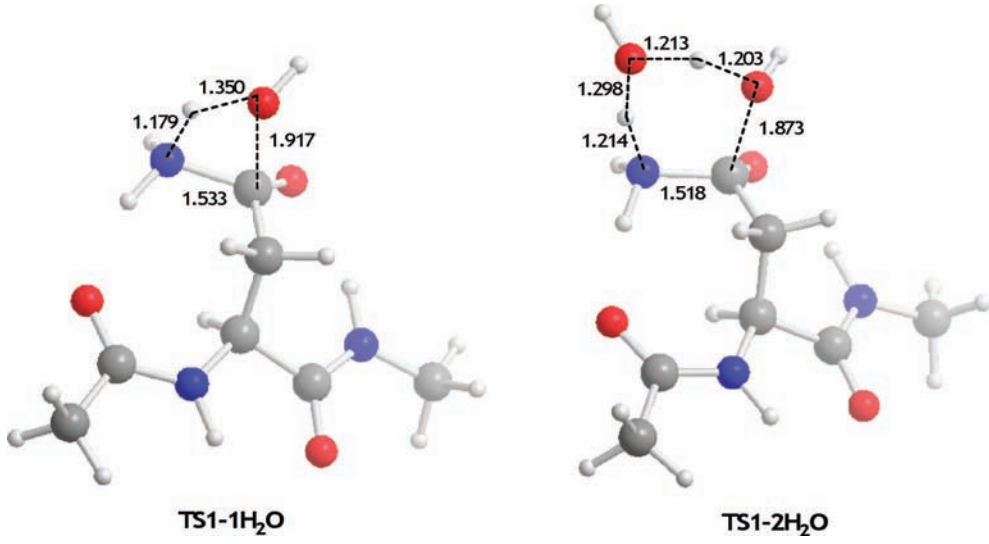


Figure 2. Optimized geometries and free energies of activation for the transition state of direct hydrolysis of Asn to Asp (*asn* → *asp*) with one (**TS1−1H$_2$O**) and two (**TS1−2H$_2$O**) water molecules, respectively.

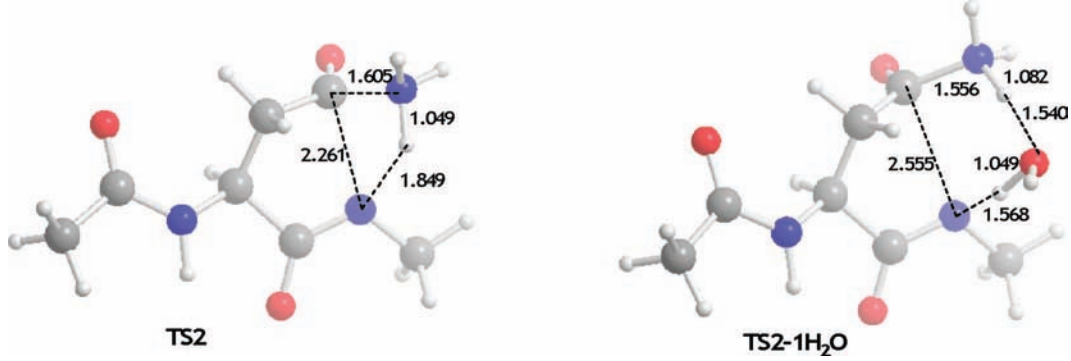
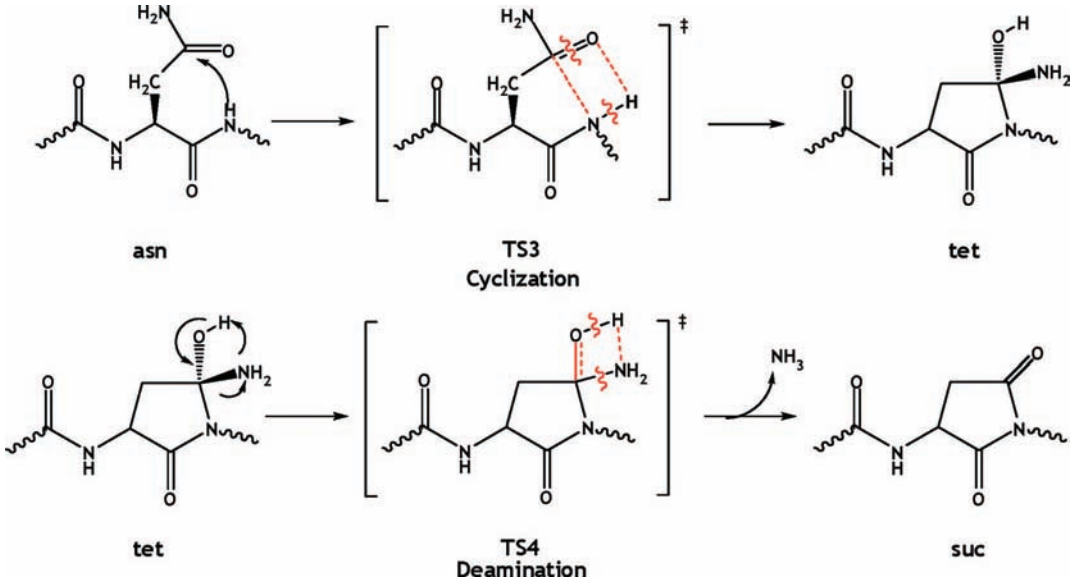
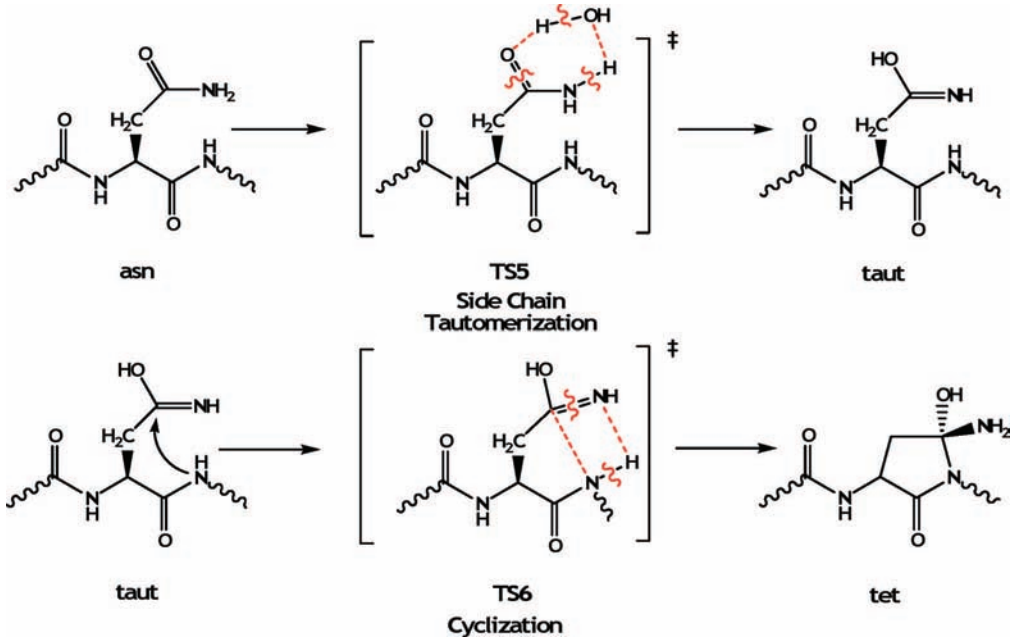


Figure 3. Optimized geometries for the transition state of succinimide formation through a single step (*asn* → *suc*), waterless (**TS2**), and one-water (**TS2−1H$_2$O**) assisted mechanisms.

SCHEME 4: Succinimide Formation − Two-Step Mechanism (*asn* → *tet* → *suc*)



SCHEME 5: Succinimide Formation − Three-Step Mechanism (*asn* → *taut* → *tet* → *suc*)



or not the same applies here, we have considered concerted and stepwise mechanisms, as well as nonassisted and water-assisted processes.

Single Step (*suc* → *asp*). In the concerted amide hydrolysis reaction, a water molecule attacks the ring carbonyl, the N−C bond breaks, as a proton is transferred from the water molecule to the ring NH. As a result, a carboxylic acid forms (Scheme 6).

Optimized geometries for the transition structures of concerted amide hydrolysis are depicted in Figure 4. Given that this is a *hydrolysis* reaction, transition state **TS7−1H$_2$O** corresponds to an unassisted process and **TS7−2H$_2$O** corresponds to a one-water *assisted* concerted hydrolysis reaction. The two transition state structures differ in several aspects. The unassisted hydrolysis of the imide (**TS7−1H$_2$O**) is a four-centered concerted − yet asynchronous − transition state, where the proton transfer from the water molecule to the ring nitrogen has already occurred (N−H distance 1.126 Å), the lengthening in the C−N distance is substantial (1.684 Å), but the attacking −OH is still rather far (C−O distance 1.954 Å). The one-water assisted mechanism (**TS7−2H$_2$O**), however, shows different geometrical features; in the six-centered transition state, the proton transfer is still not complete, the nucleophilic −OH group is quite close (C−O distance 1.670 Å) to the carbonyl under attack but the ring opening is still premature (C−N distance 1.693 Å).

Two Step (*suc* → *gem* → *asp*). In the gemdiol-mediated stepwise mechanism (Scheme 7), the initial step is the addition of a water molecule to the ring carbonyl, forming a gemdiol intermediate, which consequently undergoes ring opening in the second step to reveal the same product. In this way, the transformation of the Asn side chain amide into a carboxylate group is complete. Transition structures with one and two water molecules are shown in Figure 5. In the unassisted stepwise mechanism, a water molecule is added to the ring in step 1 (**TS8−1H$_2$O**), and the second step (**TS9**) is the ring-opening. In the two-water case, one of the solvent molecules is added to

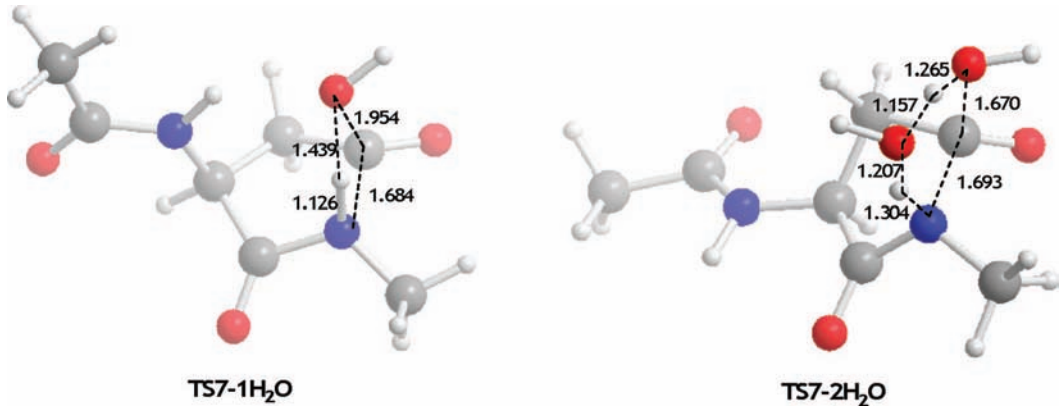
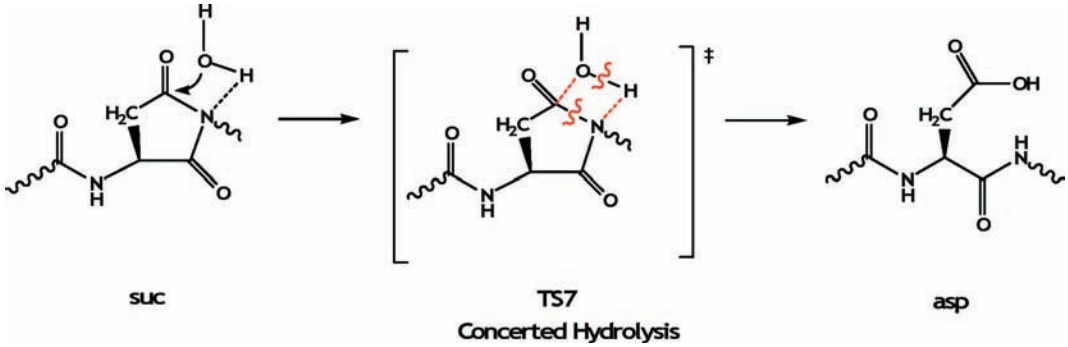
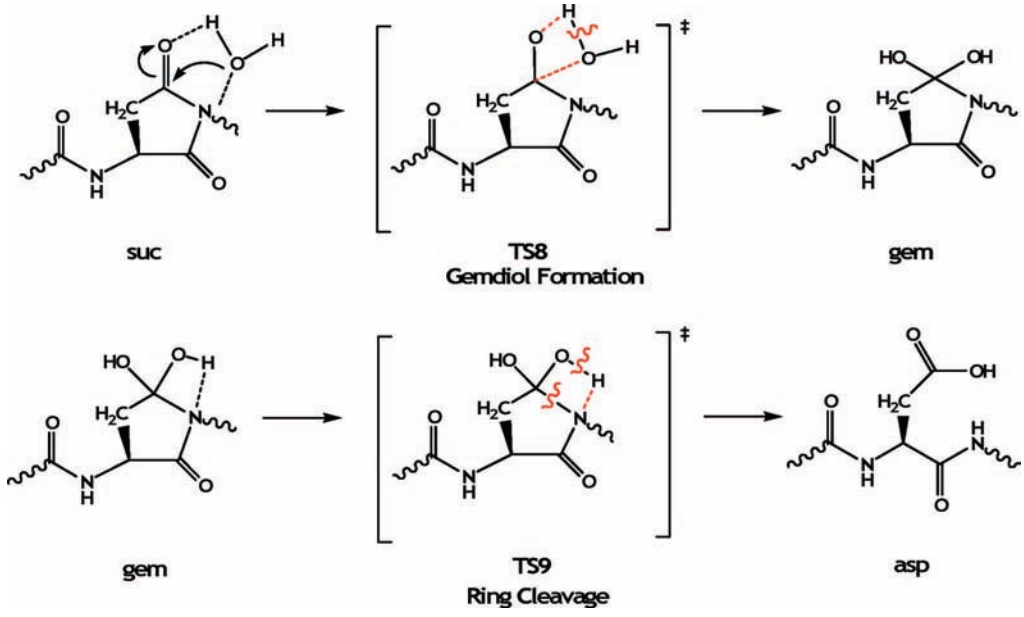


Figure 4. Optimized geometries for the transition state of concerted hydrolysis of the succinimide intermediate into Asp (***suc → asp***), unassisted (**TS7−1H$_2$O**) and one-water assisted (**TS7−2H$_2$O**) mechanisms, respectively.

SCHEME 6: Concerted Hydrolysis of the Succinimide Intermediate (*suc → asp*)



SCHEME 7: Stepwise Hydrolysis of the Succinimide Intermediate (*suc → gem → asp*)



the ring in step 1 (**TS8−2H$_2$O**), whereas the second solvent molecule assists this process. The following ring opening step (**TS9−1H$_2$O**) is also assisted by a water molecule. The two water case (**TS8−2H$_2$O** and **TS9−1H$_2$O**) is undoubtedly a better model to study the gemdiol mechanism, due to the water-assistance, which is absent in the former case (**TS8−1H$_2$O** and **TS9**). This will be more apparent in the next section, when energetics of these reactions are comparatively discussed. The main difference in the geometries of the gemdiol formation transition states (**TS8−1H$_2$O** and **TS8−2H$_2$O**) is the extent of proton transfer. In the water-assisted case (**TS8−2H$_2$O**), proton transfer from the attacking water molecule to the carbonyl oxygen is almost complete, contrary to the unassisted case (**TS8−1H$_2$O**). However, the carbonyl C−O distance is longer in **TS8−1H$_2$O** (1.313 Å as opposed to 1.293 Å in **TS8−2H$_2$O**). A strong H-bond network can be seen in the water-assisted case (**TS8−2H$_2$O**). In the second step, water-assistance (**TS9−1H$_2$O**) has enhanced the extent of proton transfer compared to the unassisted case (**TS9**). Ring opening is slightly more achieved in **TS9−1H$_2$O** (2.238 Å as opposed to 2.220 Å in **TS9**).

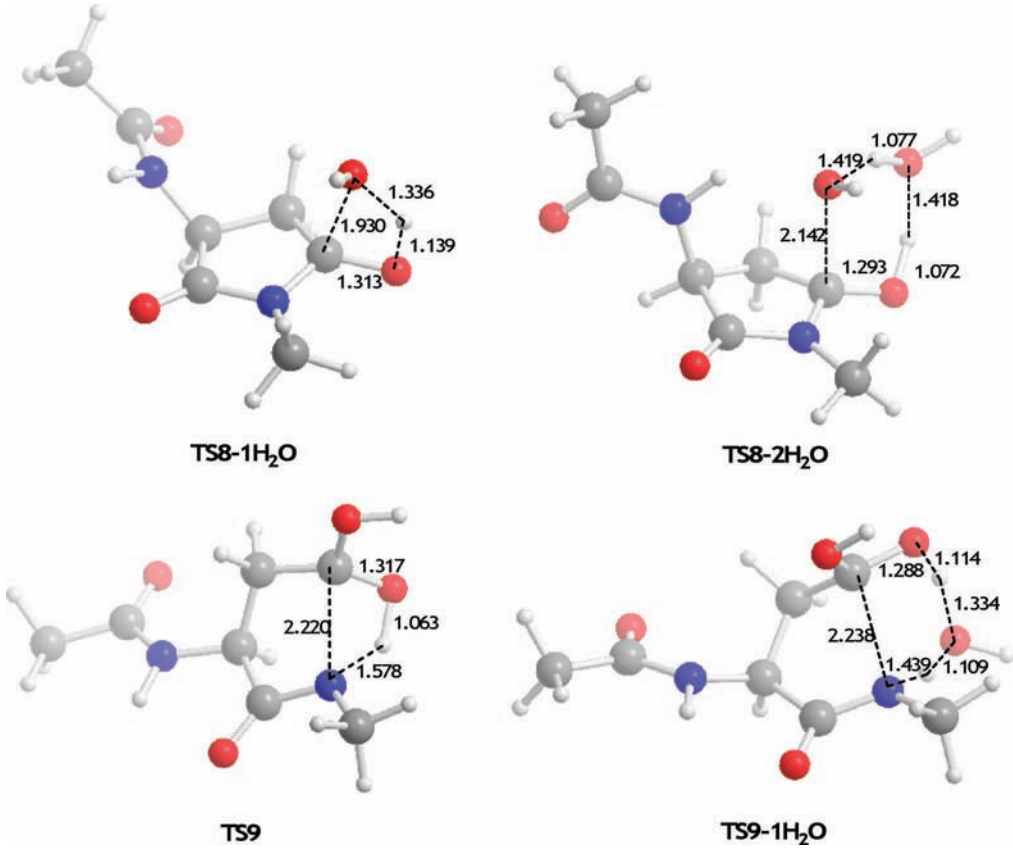


Figure 5. Optimized geometries for the transition state of gemdiol-mediated hydrolysis of the succinimide intermediate into Asp (***suc*** → ***gem*** → ***asp***), unassisted (**TS8−1H$_2$O** and **TS9**) and one-water assisted (**TS8−2H$_2$O** and **TS9−1H$_2$O**) mechanisms.

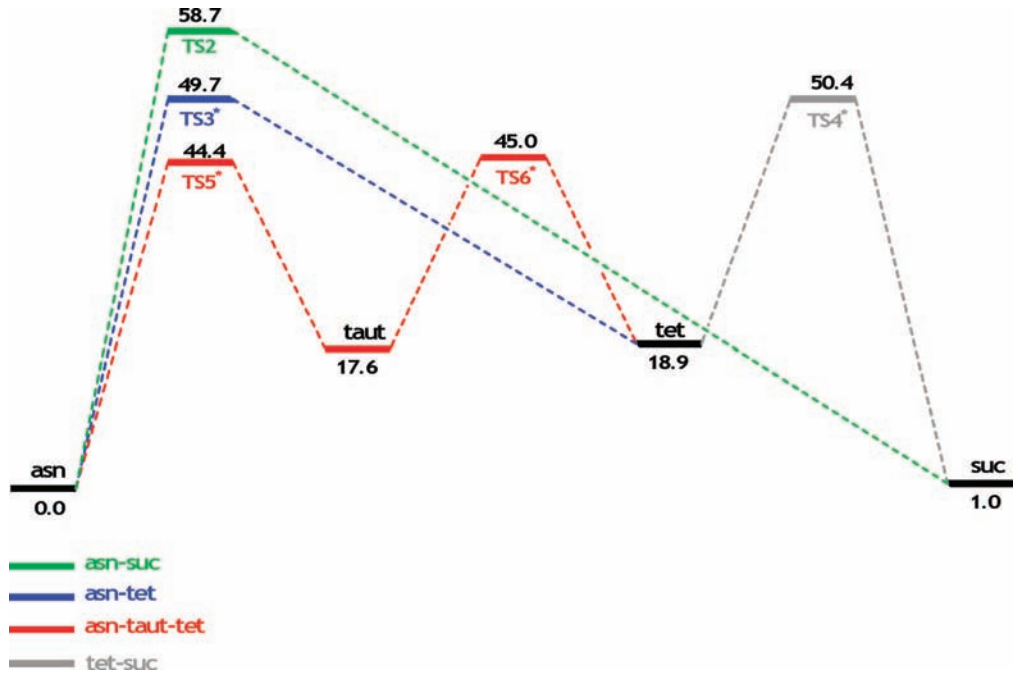


Figure 6. Reaction coordinate for deamidation − no water.

C. Comparison of Energetics and Mechanisms. In this part, imide-mediated routes will be energetically compared among each other as well as against the direct hydrolysis pathway. Relative barriers and feasibilities will be discussed. Competing mechanisms with identical molecularity (same number of atoms, depending on the number of water molecules in the initial complex, 0, 1, or 2) are grouped and presented in Figures 6 (no water), 7 (one-water) and 8 (two-water) along with energetics. All structures whose energy values are reproduced from previous deamidation studies[26] are labeled with an asterisk (*).

Although previously explored by Konuklar et al.,[20−22] the energetics for 1) the formation of the succinimide intermediate and 2) its decomposition into aspartic acid by means of water hydrolysis has not been analyzed with the same number of water molecules and within the same energetic scale. These reactions

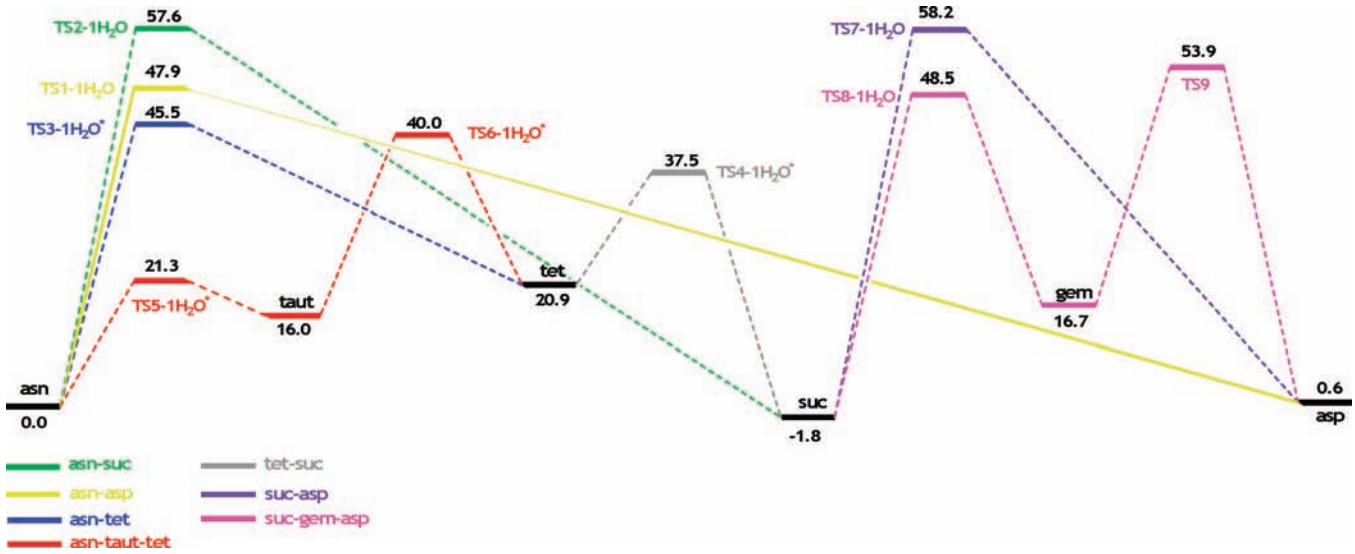


Figure 7. Reaction coordinate for deamidation − one water.

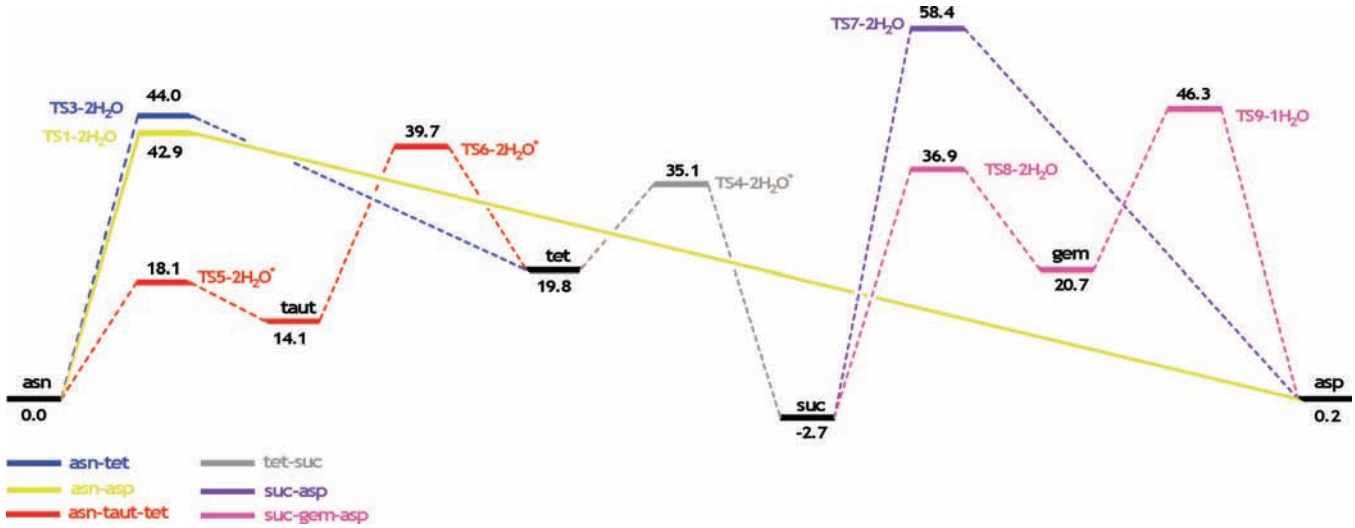


Figure 8. Reaction coordinate for deamidation − two water.

have been previously explored separately[20−22] and barrier heights have been calculated with respect to the initial structure of each individual reaction. Therefore, activation energies are not relative to one another but to the reactant of each step. In this study, we have evaluated all steps of the deamidation process with respect to a single reference point, the model peptide (Figure 1). Activation barriers calculated in earlier studies[26] for waterless, one- and two-water cases were recalculated with respect to this reference point for a legitimate comparison; slight changes in free energies of activation have been observed for some steps, but differences are within the range of 2 kcal/mol. The free energy of a single ammonia molecule was added to each component in the succinimide hydrolysis mechanism for scaling purposes.

The waterless mechanism (Figure 6) shows all three possibilities for succinimide formation, ***asn*** → ***suc***, ***asn*** → ***tet*** → ***suc***, and ***asn*** → ***taut*** → ***tet*** → ***suc*** (Schemes 3, 4 and 5, respectively). The concerted mechanism (***asn*** → ***suc***) has the highest barrier (58.7 kcal/mol). The tautomerization route (***asn*** → ***taut*** → ***tet*** → ***suc***) is the most plausible pathway for succinimide formation in the absence of solvent assistance. The deamination (***tet*** → ***suc***) step is rate-determining (50.4 kcal/mol) in both stepwise mechanisms. Please note that the waterless mechanism depicted in Figure 6 does not include the hydrolysis of the imide (***suc***) into an Asp because this requires a water molecule. Waterless mechanisms have been modeled as a benchmark to see the effect of water catalysis on each step, but the fact that a succinimide intermediate can form even in the absence of water molecules is remarkable.

Deamidation mechanisms involving one water molecule are depicted in Figure 7. Three possibilities for succinimide formation are shown; once again the concerted mechanism (***asn*** → ***suc***) has the highest barrier (57.6 kcal/mol) and the tautomerization route (***asn*** → ***taut*** → ***tet*** → ***suc***) is the most plausible pathway for succinimide formation with one water assistance. The deamination (***tet*** → ***suc***) step is no longer rate-determining (37.5 kcal/mol) for succinimide formation in the stepwise pathways (***asn*** → ***tet*** → ***suc*** and ***asn*** → ***taut*** → ***tet*** → ***suc***); the cyclization step is the bottleneck for the formation of the imide.

However, the hydrolysis of the succinimide also shows relatively high barriers (Figure 7). The concerted hydrolysis (***suc*** → ***asp***) was expected to have a higher barrier (58.2 kcal/mol) than the gemdiol-mediated stepwise route (***suc*** → ***gem*** → ***asp***); the barrier difference between the two mechanisms is approximately 5 kcal/mol. The ring-opening step (***gem*** → ***asp***) is rate-determining for the stepwise pathway (53.9 kcal/mol as opposed to 48.5 kcal/mol in ***suc*** → ***gem***).

When imide-mediated deamidation is considered, the most plausible pathways for succinimide formation and succinimide hydrolysis are the tautomerization (***asn*** → ***taut*** → ***tet*** → ***suc***) and gemdiol (***suc*** → ***gem*** → ***asp***) mechanisms, respectively. The rate-determining step for the complete imide-mediated deamidation process seems to be the ring opening step (***gem*** → ***asp***) in succinimide hydrolysis with a barrier much higher (53.9 kcal/mol) than the cyclization step. However, it should be noted that the succinimide hydrolysis barriers involve mechanisms with only one water molecule, that is the hydrolysis step depicted in Figure 7 is unassisted, unlike the succinimide formation steps. The water molecule is a reactant in hydrolysis, as mentioned earlier (Figure 4 and 5); therefore, the one-water case does not constitute for a fair comparison of energetics between the formation and hydrolysis of the succinimide. The two-water case (Figure 8) will provide more realistic grounds for comparison.

The direct hydrolysis mechanism (***asn*** → ***asp***) with a one-water molecule is relatively slower (47.9 kcal/mol) than the succinimide formation steps but, like the imide hydrolysis steps, direct hydrolysis with one water is unassisted, and therefore a legitimate comparison cannot be made.

Two-Water Reactions. As mentioned above, inclusion of two-water molecules allows water assistance in all steps. Figure 8 shows that activation barriers of all steps are lower than their uncatalyzed counterparts in Figure 6 and/or 7 (the concerted succinimide formation was not investigated here because the barrier height is considerably higher than the stepwise routes). Apart from the lowering of activation barriers, the deamidation processes are qualitatively close to those described for the one-water reactions. Thus, as before the tautomerization route (***asn*** → ***taut*** → ***tet*** → ***suc***) is the most plausible pathway for succinimide formation with the cyclization step being rate-determining (highest TS at 39.7 kcal/mol). Similarly, succinimide hydrolysis prefers the gemdiol-mediated stepwise route (***suc*** → ***gem*** → ***asp***); the barrier difference between the two mechanisms being approximately 12 kcal/mol below the concerted hydrolysis. Moreover the rate-determining step for the complete succinimide-mediated deamidation process is still the last step (***gem*** → ***asp***) (TS at 46.3 kcal/mol). Finally, the activation barrier for direct hydrolysis of Asn (42.9 kcal/mol, ***asn*** → ***asp***) is slightly below the succinimide hydrolysis barrier (and slightly higher than the one for succinimide formation), confirming that it can be a competitive deamidation reaction, even in the absence of acid or base catalysis.

Overall, our computations show that free energy barriers for succinimide hydrolysis are higher than those for succinimide formation and explain therefore the observation and isolation of succinimide intermediates during deamidation experiments. Previous computational studies have suggested[20,22] that the rate-determining step for the entire deamidation process is succinimide formation (specifically, the cyclization step leading to the tetrahedral intermediate). However, those studies did not take into account the effect of water assistance that, as shown in the present work, plays an important role in both succinimide formation (compare Figures 6 and 7) and in succinimide hydrolysis (compare Figures 7 and 8). The identity of neighboring residues or backbone orientation may influence the role played by water molecules and therefore could change the position of the bottleneck in the succinimide-mediated deamidation process.

Another novel result provided by the present calculations is that direct hydrolysis seems to be competitive with the usual succinimide-mediated route. The activation barrier for direct hydrolysis (***asn*** → ***asp***) with two-water molecules (42.9 kcal/mol) is slightly lower than the rate-determining step (***gem*** → ***asp***) in the imide mechanism (46.3 kcal/mol) and the energy difference (3.4 kcal/mol) is close to the expected computational error. Although it is well-known that direct hydrolysis is the dominant mechanism for deamidation of Asn under acidic conditions, it has been suggested that at neutral pH the succinimide mechanism is in effect.[12−19] The fact that these barriers are only 3 kcal/mol apart suggests that they are rather competitive and neither mechanism can be ruled out for the deamidation of Asn. Availability of solvent molecules, position of water bridges, intramolecular interactions within the peptide or protein, as well as spatial distribution of neighboring residues and 3D aspects such as hindrance by secondary structure may effect which mechanism will be at play. The absence of iso-Asp as a product of deamidation in some cases[5] may be a result of secondary structure inhibiting cyclization; in that case direct hydrolysis is destined to be the major pathway for deamidation.

Conclusions

The main conclusions that can be drawn from this study are as follows: 1) water assistance increases the rate of deamidation, a fact already established;[26] 2) the tautomerization route has the lowest barrier for the formation of the succinimide intermediate regardless of the number of water molecules that assist the reaction, including the waterless mechanism; 3) cyclization is the rate-determining step for succinimide formation in all water assisted mechanisms; 4) hydrolysis of the succinimide intermediate is likely to go through a stepwise mechanism, where a gemdiol intermediate is formed; 5) more importantly, succinimide hydrolysis barriers are higher than those for succinimide formation.

These conclusions contrast in part with previous calculations that had shown that cyclization is the rate-determining step for the formation of the succinimide intermediate.[20] Our study suggests that when the entire deamidation process is considered, the hydrolysis step is the actual rate-determining step. As shown in Figure 8, the stepwise hydrolysis barrier is the rate-determining step for the overall water-assisted deamidation process, which is likely to proceed through the tautomerization route. This also explains the isolation of succinimide intermediates during deamidation reactions. The bottleneck of this process is therefore proposed to be the hydrolysis of the succinimide intermediate.

Another important finding is the relative ease of direct hydrolysis. In all mechanisms involving the use of explicit solvent molecules (Figures 7 and 8), direct hydrolysis seems to be a competitive reaction to the imide-mediated route even in the absence of acid or base catalysis.

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Supporting Information Available: Cartesian coordinates and energy of B3LYP/6-31+G** optimized conformation for varioud compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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