Accelerated Racemization of Aspartic Acid and Asparagine Residues via Succinimide Intermediates: An ab Initio Theoretical Exploration of Mechanism

Jennifer L. Radkiewicz, H. Zipse, Steven Clarke,* and K. N. Houk*

Contribution from the Department of Chemistry and Biochemistry, University of California, Los Angeles, Los Angeles, California 90095-1569

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Abstract: Aspartic acid and asparagine residues racemize rapidly relative to other amino acid residues in proteins and peptides. This has been attributed to the increased acidity of the α-carbons of succinimide residues derived from the spontaneous cyclizations of these residues. To understand the basis of this effect, the acidities of model compounds were calculated using ab initio quantum mechanics (RHF/6-31+G*). The results were also checked with DFT (Becke3LYP/6-31+G*) and solvent cavity models (IPCM and SCIPCM). The geometries of succinimide, 2-pyrrolidinone, and the derived enolate anions were optimized, and the gas phase deprotonation energies were calculated. The imide is more acidic than the amide by 18 kcal/mol in the gas phase. Since there is a qualitative correlation between gas phase and aqueous acidities, this result provides an explanation for the experimental observations that the rate of peptidyl succinimide racemization can be $\sim 10^5$ times greater than that of unmodified aspartic acid residues. To quantitate the source of the succinimide acidity, the geometries and CH acidities of various conformations of *N*-formylacetamide and acetamide, acyclic models of succinimide and 2-pyrrolidinone, and 3-oxobutanal and acetone, acyclic models lacking the nitrogen atom, were studied. The importance of resonance effects for increasing the acidity of the α-carbon of succinimide was established, but electrostatic and inductive effects also have an important influence on acidities. The acidity of succinimide is compared to the acidities of several peptide models. Isosuccinimide, an alternative degradation product of aspartic acid and asparagine residues, is also be expected to be racemization prone by similar mechanisms.

Introduction

Amino acids are incorporated into cellular proteins via ribosomal synthesis solely in the L-configuration. However, spontaneous racemization reactions can result in the generation of D-residues during the life span of the protein. There is an extensive literature that shows the accumulation of D-aspartic acid in acid hydrolysates of stable tissue proteins from aging cells and organisms. For example, the ratio of D-aspartic acid to L-aspartic acid in hydrolysates of polypeptides from human tissues can reach values of 0.06 in tooth dentin[1] and erythrocyte membrane proteins,[2] 0.13 in aortic elastin,[3] 0.21 in specific peptides of brain myelin basic protein,[4a] 0.25 at specific residues in Alzheimer disease plaque *β*-amyloid,[4b] and 1.1 at specific residues in eye lens αA-crystallin.[5] Such accumulation can contribute to the loss of tissue functions during the biological aging process. D-Aspartate accumulation has also been used to date archeological[6] and paleontological[7] materials. Although the formation of the D-isomers of other types of amino acid residues has been also observed, the rate of accumulation of D-aspartic acid is generally greater than those of the other species.[8] For example, when the D/L ratios of amino acids from hydrolysates of casein incubated at 65 °C in 0.1 M NaOH for 40 min were measured, the value for aspartic acid (0.303) was much greater than that of alanine (0.07), valine (0.04), leucine (0.03), proline (0.04), glutamic acid (0.08), and phenylalanine (0.09).[9] A more extensive study of alkaline-treated soybean proteins revealed that the rate of racemization of 13 amino acid residues generally followed their side-chain inductive constants (σ^*) with the exception that the rate for asparagine and aspartic acid residues was 4−25-fold greater than expected.[10]

It was initially assumed that racemization proceeds from L-aspartic acid residues in amino acids and polypeptides by deprotonation of the α-carbon atom of the amino acid, generating a planar α-carbanion, and then reprotonation on the other side (Figure 1).[8,11] The relative rates of racemization of various amino acids would thus reflect the electron-withdrawing capacities of the side chains. This relationship has been experimentally demonstrated with free amino acids.[12] However, the situation with amino acid residues in polypeptides appears to be more complex. For example, the simple mechanism described above would not explain the experimental observation that aspartic acid residues appear to racemize more rapidly than serine or threonine residues, the side chains of which have greater electron-withdrawing properties than the deprotonated aspartyl

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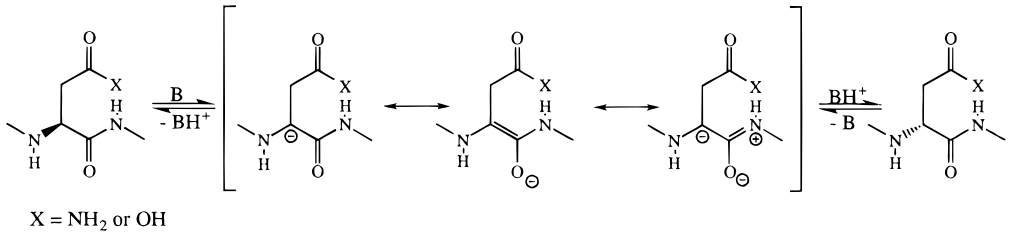


Figure 1. Principal resonance contributors to the intermediate enolate in direct racemization of Asx residues.

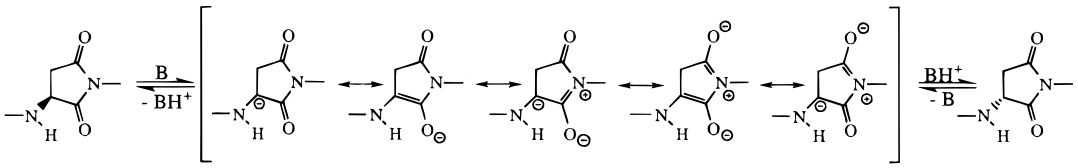
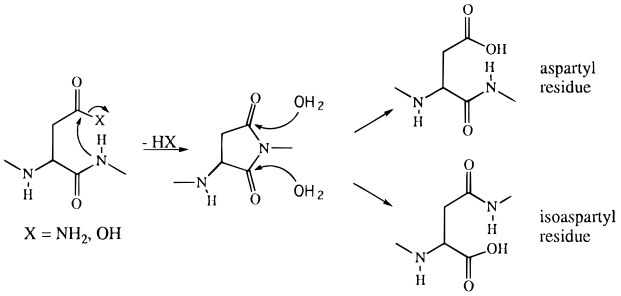


Figure 2. Principal resonance contributors to the intermediate in racemization of succinimidyl residues.

Scheme 1



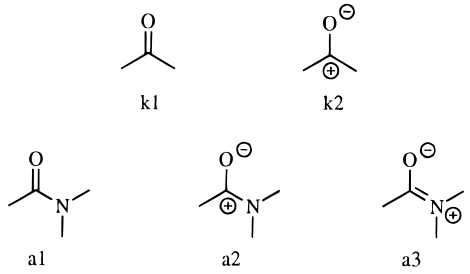


Figure 3. Resonance structures for a ketone and an amide.

side chain.[13] In addition, the hydroxy group of Ser or Thr can form a hydrogen bond to the negatively charged oxygen, further stabilizing the anion.

More recent studies have indicated that the D-aspartic acid that accumulates in hydrolysates of aged proteins can be derived from a number of species in addition to L-aspartic acid residues, including L-asparagine residues.[2,14] Both aspartic acid and asparagine residues are subject to spontaneous degradation reactions via succinimide intermediates (Scheme 1).[15] These intermediates can be readily hydrolyzed to give a mixture of isoaspartyl (also called β-aspartyl) and aspartyl peptides in a ratio of ~3:1.[15] The structure of the succinimide suggested that it may itself be racemization prone.[15a,16] In amino acid residues in proteins or peptides, stabilization of the α-carbanion by the carbonyl group would be expected to be limited by competing amide resonance (Figure 1), the same effect that makes amides less acidic than ketones. It has been postulated that the resonance interaction of the nitrogen lone pair with the β-carbonyl group in the succinimide causes the nitrogen to be a poorer donor to the second carbonyl, making it a better anion stabilizer (Figure 2).

The nature—indeed, even the existence—of lone-pair resonance with the carbonyl group has been the subject of extensive discussion in the recent literature.[17] It has been noted that the C−O bond length of an amide is only 0.01 Å longer than that of a ketone, which belies the traditional resonance formulation. Traditionally, the decreased acidity of an amide compared to a ketone is attributed to resonance between the carbonyl group and nitrogen. A description which better reflects modern analysis[17] is that the resonance is between the carbon and nitrogen without the oxygen being involved. This conclusion comes from extensive calculations on the source of the high rotational barrier about the C−N bond in amides.[17] Upon rotation of the NH_2 group, calculations show a 0.01 Å change in the C−O bond length, little change in the charge on oxygen, and no change in the electrostatic potential around oxygen.[17b] The polarized π-system of a ketone may be considered to be made up of comparable amounts of resonance structures k1 and k2 (Figure 3). A nonplanar amide would have similar contributions of a1 and a2, respectively. The calculations indicate that the contribution of a1 is not changed much in the planar conformation. Instead, resonance form a3 becomes much more important, but at the expense of a2, not a1. Thus, the bond order between the carbon and oxygen is not affected.

The involvement of succinimide residues in racemization has been verified experimentally: the rate of succinimide racemization in the hexapeptide Val-Tyr-Pro-Asu-Gly-Ala (where Asu represents a succinimide residue) at pH 7.4[15a] is 117 000 times more rapid than that of aspartic acid itself under similar conditions.[12] Furthermore, it was shown that racemization of the succinimide intermediate could account for the bulk of the total racemization observed for the corresponding asparagine-containing peptide.[15a] The enhanced racemization of succinimide residues has also been confirmed with other peptides.[18] Finally, it has become clear that the succinimide pathway is the major spontaneous degradative route of aspartyl and

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asparaginyl residues in proteins at neutral pH values. Succinimidyl forms of a number of bioactive proteins have been shown, including epidermal growth factor,[19] lysozyme,[20] basic fibroblast growth factor,[21] hirudin,[22] pro-opiomelanocortin,[23] somatotropin,[24] and methioninyl growth hormone.[25]

These results, taken together, suggest that D-aspartic acid in hydrolysates of aged proteins largely originates from protein-bound D-succinimide residues and their hydrolysis products D-isoaspartate and D-aspartate residues. All of these species can be derived from either L-aspartic acid or L-asparagine residues, via the intermediate succinimide. The rate of succinimide racemization may thus control the rate of protein degradation to D-aspartic acid residues. Understanding this process may allow a better understanding of the reactions that result in spontaneous protein degradation in aging, as well as in the inactivation of protein pharmaceuticals.[26]

The rate of racemization of the succinimide intermediate is also a crucial factor in a recently discovered pathway that can catalyze a protein repair reaction. D-Aspartyl residues can be methyl esterified by the protein L-isoaspartate-(D-aspartate) *O*-methyltransferase (EC 2.1.1.77).[16,27] The D-aspartate β-methyl ester formed can be rapidly hydrolyzed under physiological conditions to generate the D-succinimide derivative.[15d] The facile racemization of this species, followed by hydrolysis, can result in the reformation of aspartate derivatives in the L-configuration. This pathway thus represents a mechanism for converting D-aspartyl residues to L-aspartyl residues. A similar pathway, utilizing the same enzyme, has been clearly demonstrated to convert L-isoaspartyl residues to L-aspartyl residues.[28]

In this paper, ab initio and density functional (DFT) quantum mechanics have been used to identify quantitatively the factors that lead to enhanced succinimide α-carbon acidity and racemization. Previously, similar methods were used to explain the origin of the anomalous acidity of Meldrum's acid, a cyclic anhydride, as compared to its acyclic analogs.[29] The same theoretical techniques are employed here to quantitate the acidity of the α-carbon atom in succinimide versus amide and acyclic derivatives and to identify the origins of the large differences in acidity. The computational methodology employed for these calculations is explained in the next section of the paper. The third section examines the relationship between gas phase acidities and aqueous pK_a's. Next, the results of various calculations on the source of the increased acidity of succinimide and the conclusions that can be drawn from them are presented. These RHF gas phase results are then compared to DFT calculations of acidities and to solvation calculations using two cavity models. Finally, the calculated proton affinities of model acyclic and cyclic peptide residues are compared.

Table 1. Calculated RHF/6-31+G* Energies (au) of Neutral Carbonyl Compounds and Their Enolates

compound	neutral	enolates
succinimide (**1**)	−358.613 510	−358.013 614
2-pyrrolidinone (**2**)	−284.887 434	−284.258 883
isosuccinimide	−358.578 948	−357.974 535
acetamide (**3**)[a]		
pyramidal nitrogen	−207.983 987	−207.358 769
planar nitrogen		−207.353 900
N-formylacetamide (**4**) conformations		
4a	−320.714 137	−320.109 692
4b	−320.727 535	−320.133 272
4c	−320.723 595	−320.128 754
4d	−230.724 213	−320.118 915
4e	−230.699 923	−320.087 460
4f	−320.698 247	−320.087 705
acetone	−191.967 613	−191.352 886
3-oxobutanal	−304.691 141	−304.097 529
propanamide	−247.019 293	−246.388 287
succinamic acid	−434.634 208	−434.040 311
succinamate	−434.099 019	−433.378 405
methyl succinamate	−473.669 851	−473.054 331

[a] The conformation of the nitrogen in the anion; the nitrogen remains planar in neutral acetamide.

Computational Methodology

Geometries were optimized with RHF theory and the 6-31+G* basis set using the GAUSSIAN 92 program.[30] The energies obtained from these optimizations are given in Table 1. It has been established that calculations at this level give good predictions of relative gas phase acidities.[29,31] Frequency calculations were performed on all nonconstrained structures to prove that they are energy minima. DFT optimizations and single-point calculations with solvent cavity models were performed on the proton affinity of succinimide and three other compounds. The DFT calculations used the Becke3LYP functional with the 6-31+G* basis set. Solvation calculations were performed at the RHF/6-31+G* level using the IPCM model (static isodensity cavity) and the SCIPCM model (self-consistent isodensity cavity) with a dielectric constant of 78.54.

Results and Discussion

Most of the calculations performed here are for the gas phase. This provides good evidence for the inherent acidity of different species. For a number of related compounds, the level of theory used provides similar relative acidities when compared to gas phase measurements.[29,31] For example, the calculations described later in this paper show a 6.6 kcal/mol difference in acidity for acetone and acetamide compared to a 6.0 kcal/mol difference between acetone and *N*,*N*-dimethylacetamide found experimentally in the gas phase.[32] There can also be good

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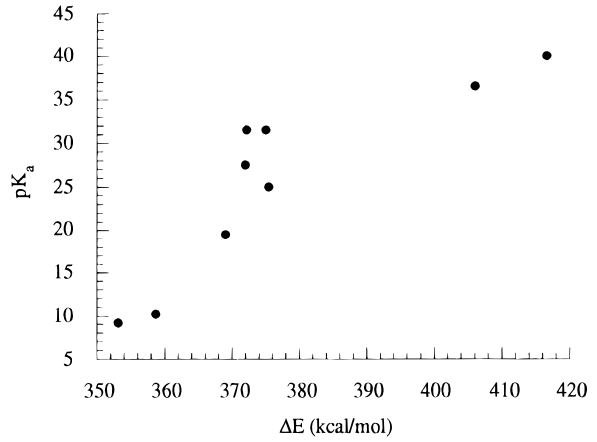

Figure 4. Plot of the proton affinity, ΔE, (gas phase) vs pK_a (aqueous) for the following series of carbon acids (left to right): hydrogen cyanide, nitromethane, acetone, ethyl acetate, acetonitrile, *N*,*N*-dimethylacetamide, acetylene, ethylene, and methane. Least-squares correlation pK_a = $0.61\Delta E - 203.1$ ($R = 0.87$). Data from refs 32, 35, and 36.

correlations between gas phase and solution acidities. Taft and Bordwell reported correlations between DMSO and gas phase acidities for a series of acids of a given type,[33] although their comparisons of gas phase acidities to aqueous acidities showed no clear correlation, and in some cases, the order of acidities was even reversed.[34] However, if only acidities of carbon acids to form carbanions are considered, a qualitative correlation does exist between gas phase and aqueous solution acidities,[35] as can be seen in Figure 4. Of greater relevance, the trend of acidities for acetone > methy/ethyl acetate > *N*,*N*-dimethylacetamide is the same in both the gas phase and in aqueous solution.[32,36] The proton affinity values are 369.0, 371.9, (methyl acetate), and 375.0 kcal/mol, respectively, in the gas phase. The pK_a's are 19−20, 27−28 (ethyl acetate), and 31−32, respectively. Since our calculations deal only with carbanions, our gas phase proton affinity values should parallel the aqueous phase pK_a values. Solvation calculations, reported below, were performed to verify this conclusion.

2-Pyrrolidinone and Succinimide. To compare the acidities of succinimide with an analogous amide, the structures of succinimide (**1**), 2-pyrrolidinone (**2**), and their corresponding enolate ions were optimized. These geometries are shown in Figure 5. Proton affinities of these and related structures are given in Table 2, along with relevant experimental data.

Succinimide is 18 kcal/mol more acidic than the amide (Table 2). The β-carbonyl has a sugnificant effect on the acidity! This translates to a difference in aqueous pK_a of 10.8 units, using the equation obtained from the graph in Figure 4. Clearly, succinimide will be much more acidic and thus more prone to racemization than 2-pyrrolidinone in aqueous solution.

Geometrical changes upon deprotonation of 2-pyrrolidinone are consistent with the resonance interaction of the carbanion with the carbonyl group. The C−C bond contracts by 0.14 Å and the C−O bond lengthens by 0.04 Å. Note that, just as with amides, the lone-pair interaction (here on C) with the carbonyl carbon is much larger than the decrease of carbonyl carbon interaction with oxygen. At the same time, the C−N bond length increases 0.08 Å in the anion and the nitrogen

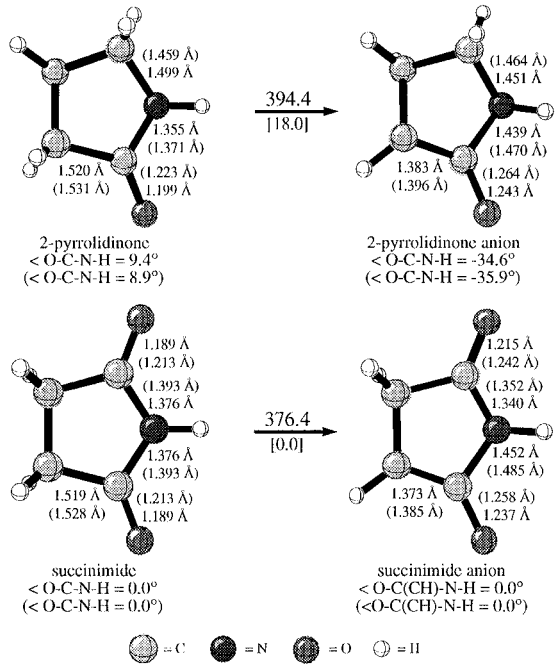

Figure 5. Optimized geometries of succinimide, 2-pyrrolidinone, and the corresponding enolate anions. The proton affinities are given (kcal/mol). The numbers in brackets are the relative proton affinities (kcal/mol); (RHF/6-31+G*). The numbers in parentheses are the optimized geometrical parameters from the B-3LYP/6-31+G* calculations.

becomes partially pyramidalized. These changes signify a reduction in the interaction between the carbonyl carbon and the nitrogen. This competition between N lone-pair and α-carbanion resonance interaction with the carbonyl is generally considered to be the origin of the decreased acidity of amides vs ketones.

Examination of succinimide bond lengths show that there is less interaction of the nitrogen lone pair with either carbonyl carbon than in a simple amide. The C−N imide bond lengths are 0.02 Å longer in succinimide than in 2-pyrrolidinone. The carbonyl bond lengths of succinimide are 0.01 Å shorter than those of 2-pyrrolidinone. Upon anion formation, one carbonyl becomes part of an enolate. The resonance between the nitrogen and that carbonyl carbon becomes very small, as evidenced by the C−N bond length increase to 1.452 Å. The enolate C−C bond length in succinimide is 1.373 Å, 0.01 Å shorter than in the 2-pyrrolidinone enolate. There is increased resonance between the nitrogen and the β-carbonyl in the anion, signaled by a 0.04 Å decrease in the C−N bond length and an 0.03 Å increase in the β-carbonyl bond length.

Taken together, these results demonstrate that the β-carbonyl of succinimide increases the acidity of the α-carbanion relative to a simple amide. This can be attributed to resonance, but the β-carbonyl is also capable of acidifying the amide by inductive or through-space dipolar field effects. In order to understand the origin of these geometrical changes and the relatively high acidity of succinimide, we have studied a number of model systems, described below.

Acetamide and *N*-Formylacetamide. Calculations were performed on acetamide (**3**) and *N*-formylacetamide (**4**), acyclic analogs of 2-pyrrolidinone and succinimide, in various conformations to gain better estimates of the importance of resonance and inductive factors. Wong and Wiberg previously calculated the geometry of acetamide at the RHF/6-31+G* level of theory.[37] This geometry and that found here for the enolate anion are given in Figure 6. The lowest energy conformation

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Table 2. Proton Affinities (kcal/mol) Calculated as the Energy of the Enolate Anion Minus the Energy of the Neutral Compound (RHF/6-31+G*)

compound	proton affin	rel energy of neutral compd	rel energy of enolate anion	rel proton affin	exptl proton affin[b]
succinimide (**1**)	376.4			0.0	
2-pyrrolidinone (**2**)	394.4			18.0	
isosuccinimide	379.3			2.9	
acetamide (**3**)[a]					
pyramidal nitrogen	392.3			19.4	
planar nitrogen *N*-formylacetamide (**4**) conformations	395.4			22.5	
4a	379.3	8.4	14.8	6.4	
4b	372.9	0.0	0.0	0.0	
4c	373.3	2.5	2.8	0.4	
4d	379.8	2.1	9.0	6.9	
4e	384.3	17.3	28.7	11.4	
4f	383.1	18.4	28.6	10.2	
acetone	385.7			13.2	369.0
3-oxobutanal	372.5			0.0	369.0
N,*N*-dimethyl-acetamide					375.0
methyl acetate					371.9
acetaldehyde					365.9

[a] The conformation of the nitrogen in the anion; the nitrogen remains planar in neutral acetamide. [b] Reference 32.

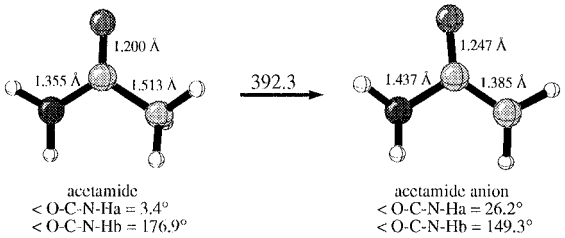

Figure 6. Optimized geometries of acetamide and the enolate anion. The proton affinity is given (kcal/mol); (RHF/6-31+G*).

of the anion of acetamide has a partially pyramidal nitrogen group, as in the anion of 2-pyrrolidinone. The anion with a planar amino group was also optimized, but this structure was a transition state, 3.0 kcal/mol above the pyramidal minimum.

The four conformational minima of *N*-formylacetamide are given in Figure 7. Table 2 gives the calculated relative energies of the amides, the calculated relative energies of the corresponding enolate anions, and the calculated relative proton affinities. The four conformers of **4** are planar or nearly planar and can be described by usual peptide nomenclature as follows: when the acetamide methyl carbon is *anti* to the N−formyl bond, this is called Me-*trans*; similarly, when the formyl CH bond is *anti* to the N−Ac bond, this is called H-*trans*. In conformer **4a**, both amides are *trans*. The most stable and acidic conformer **4b**, H-*cis* and Me-*trans*, is 6.4 kcal/mol more acidic than **4a**. In conformer **4c**, a relatively acidic conformer, the amides are H-*trans* and Me-*cis*. The least acidic conformer **4d** has both amides *cis* and is 6.9 kcal/mol less acidic than **4b**.

The relative acidities of acetamide and *N*-formylacetamide along with their bond length changes indicate that the conclusions drawn from these calculations can be reliably applied to succinimide. Conformer **4c** of *N*-formylacetamide, which is conformationally similar to succinimide, is more acidic than acetamide by 19 kcal/mol. This is in good agreement with the calculated difference between the acidities of succinimide and 2-pyrrolidinone (18 kcal/mol). Acetamide undergoes similar geometrical changes upon anion formation as calculated for 2-pyrrolidinone, whereas all four conformers of *N*-formylacetamide undergo similar bond length changes as calculated for succinimide. The nitrogen becomes pyramidal in the acetamide anion, the C−C bond contracts by 0.13 Å (0.14 Å in **1**), the C−O bond lengthens by 0.05 Å (0.04 Å in **1**), and the C−N bond length increases from 1.36 to 1.44 Å a change of 0.08 Å

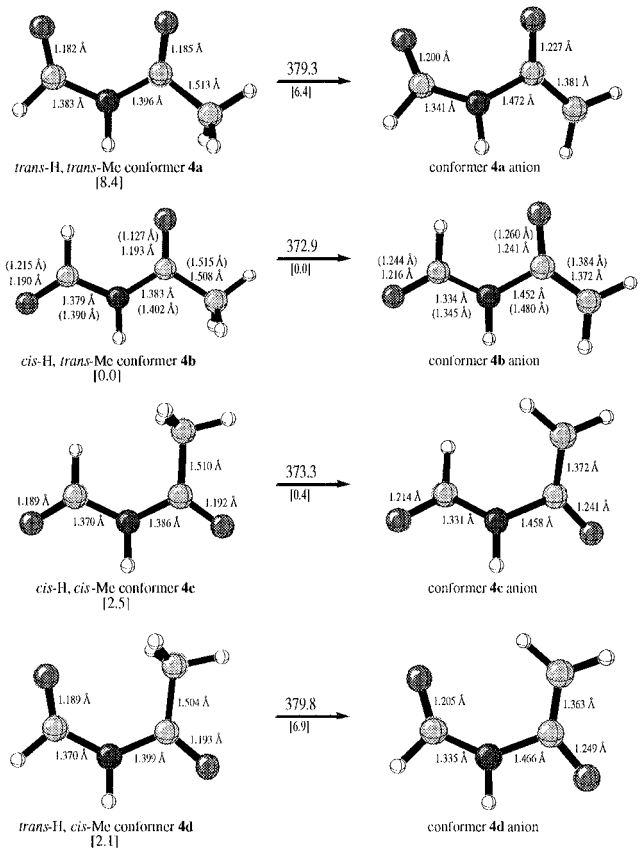

Figure 7. RHF/6-31+G* optimized conformations of *N*-formylacetamide and the corresponding enolate anions. The proton affinities are given (kcal/mol). The relative proton affinities (kcal/mol) are in brackets under the arrows. The bond lengths in parentheses are from the B-3LYP/6-31+G* calculations.

(0.08 Å in **1**). In the anion of conformer **4c**, the C−N bond length increases by 0.07 Å (0.08 Å in **2**), the C−C bond decreases by 0.14 Å (0.15 Å in **2**), the imide C−N bond length decreases by 0.04 Å (0.04 Å in **2**), and the β-C−O bond increases by 0.03 Å (0.03 Å in **2**).

As expected, resonance also plays a role in the increased acidity of *N*-formylacetamide compared to acetamide. However, there does not appear to be any relationship between the amount of amide resonance (as indicated by bond length changes) and

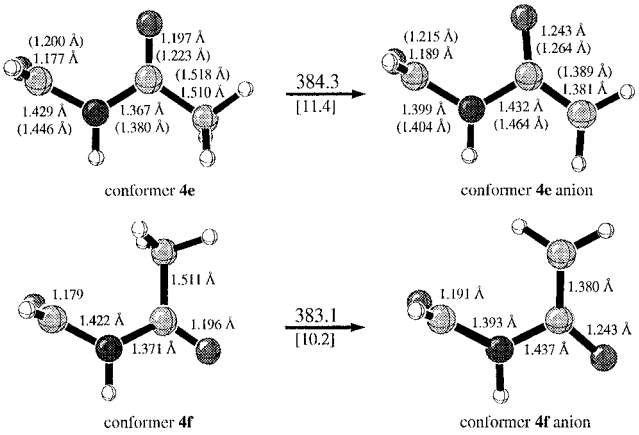

Figure 8. *N*-Formylacetamide constrained conformations with the β-carbonyl group rotated 90° from the plane of the acetamide function. Proton affinities are given (kcal/mol). The numbers in brackets are the relative proton affinities with respect to conformer **4b** (kcal/mol). (RHF/6-31+G*) The bond lengths in parentheses are from the B-3LYP/6-31+G* optimized calculations.

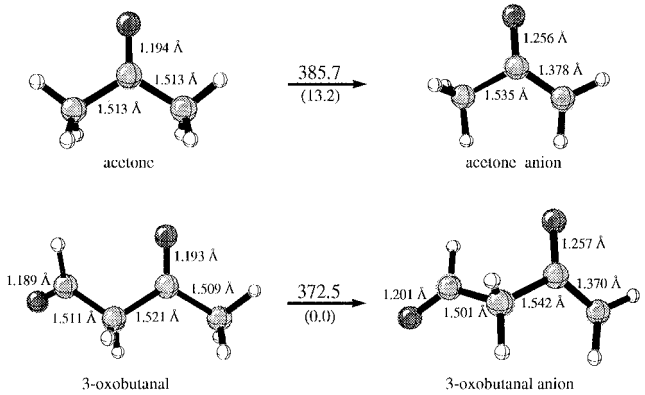

Figure 9. Optimized geometries of acetone, 3-oxobutanal, and the corresponding enolate anions. The proton affinities are given (kcal/mol). The numbers in parentheses are relative proton affinities (kcal/mol); (RHF/6-31+G*).

the relative acidities of the four *N*-formylacetamide conformers. For example, enolate **4b** has a shorter C−N bond length of 1.45 Å, while conformer **4a**, which is 6.4 kcal/mol less acidic, has a longer 1.47 Å C−N bond length, indicative of less amide resonance. If resonance between β-C and N corresponded to increased acidity, the β-C−N bond should be shortest in the most acidic amides. Figure 7 shows no such correlation. Instead, examination of the various electrostatic and dipole−dipole interactions present in the four conformers and the corresponding anions provides an explanation for the observed relative acidities. These results (discussed below) also indicate that a stabilizing alignment of the dipoles in succinimide and the absence of any destabilizing electrostatic interactions in the anion help to contribute to the high acidity of succinimide.

Conformers **4b** and **4c** are more acidic by 6 kcal/mol than conformers **4a** and **4d**. Structure **4b** is the most stable conformer among both the neutral compounds and the carbanions. The two carbonyl dipoles of **4b** point in opposite directions in the neutral, stabilizing this structure. In the anion, the second carbonyl is also oriented in a stabilizing manner. The dipoles in conformer **4c** are roughly perpendicular, resulting in some destabilization of the amide, but stabilization of the anion by the β-carbonyl dipole. The decreased acidities of conformers **4a** and **4d** can be attributed to unfavorable electrostatic interactions in their anions. In conformer **4a**, the dipoles point in the same direction causing a destabilizing electrostatic interaction between the two partially negative oxygens in the neutral compound. This repulsion is more pronounced in the anion, due to the increased electron density on each oxygen. In conformer **4d**, the dipoles point in opposite directions, but there is some slight steric hindrance between the oxygen of the β-carbonyl and the methyl group. In the anion, the β-oxygen is now near the partially negatively charged carbon, destabilizing this structure.

Constrained Conformers of *N*-Formylacetamide. To further quantify the relative importance of electrostatic, inductive, and resonance effects, two additional constrained conformations of *N*-formylacetamide were optimized. In these conformers, the β-carbonyl group is rotated 90° from the plane of the α-carbonyl group by constraining the O−C(H)−N−C(CH_3) dihedral angle to 90°. To eliminate all possible resonance between the β-carbon and the nitrogen lone pair, the dihedral angles C(HO)−N−C(CH_3)−O and H−N−C(CH_3)−O were also fixed to 0 and 180°, respectively, for conformer **4e** and to 180 and 0°, respectively, for conformer **4f**. The results of these calculations are given in Figure 8 and Table 2. The acidities of these conformations were compared to those of acetamide. Both amide and enolate were constrained in the same way. The relative proton affinity of acetamide with this planar anion constraint is given in Table 2.

The increase in the acidities of conformers **4e** and **4f** of *N*-formylacetamide over planar acetamide can be attributed to electrostatic interactions and the inductive effect of the β-carbonyl. Electrostatics plays a role by placing a partial negative charge of the β-carbonyl oxygen near the positive end of the CO dipole. The β-carbonyl may also have an inductive effect by pulling electron density through bonds away from the α-carbonyl. This will increase the facility of forming the anion. The increased acidity of the *N*-formylacetamide conformers cannot be attributed to resonance since the β-carbonyl group is perpendicular to the plane of the molecule. Conformer **4e** is 11.1 kcal/mol more acidic and conformer **4f** is 12.3 kcal/mol more acidic than acetamide with the planarity constraint. Thus, a surprisingly large amount of the increased acidity of succinimide is caused by the electrostatic interaction and inductive effects.

Nonresonance effects, accounting for a 12 kcal/mol increase in acidity, appear to be as important if not more important than resonance in explaining the increased acidity of succinimide over acyclic peptides. To estimate the effect of resonance, an upper limit can be obtained by comparing conformers **4b** (resonance with β-carbonyl) and **4e** (no resonance with β-carbonyl). Conformer **4b** is more acidic than **4e** by 11.4 kcal/mol. A lower limit for the effect of resonance on acidity can be obtained by comparing the acidity of *N*-formylacetamide conformer **4b** with that of acetamide. Here, 12 kcal/mol of the 19 kcal/mol difference in acidity between **4b** and acetamide can be attributed to nonresonance effects. The remaining 7 kcal/mol difference in acidity can be attributed to resonance. Thus, resonance only accounts for a 7−11 kcal/mol increase in acidity, about half the effect in succinimide. There is a large conformational dependence of the acidification by the β-carbonyl.

Acetone and 3-Oxobutanal. An additional model system was chosen to obtain an independent estimate of electrostatic and inductive effects on acidities. Acetone and its enolate anion were compared to formylacetone (3-oxobutanal) and its anion. In these compounds, the absence of the nitrogen atom precludes amide-type resonance structures and only inductive and dipolar effects are possible. The results are given in Figure 9 and Table 2.[38] The β-formyl group increases the acidity of acetone by

Table 3. Proton Affinities (kcal/mol) and Relative Energies (kcal/mol) for RHF, B3LYP, and Solvation Calculations with the 6-31+G* Basis Set

	RHF		B3LYP		IPCM ($E = 78.54$)		SCIPCM ($E = 78.54$)	
compound	proton affin	rel energy	proton affin	rel energy	proton affin	rel energy	proton affin	rel energy
2-pyrrolidinone	394.4	18.0	384.6	18.3	338.6	10.8	349.7	13.5
succinimide	376.4	0.0	366.3	0.0	327.8	0.0	336.3	0.0
N-formylacetamide								
conformer **4b**	372.9	0.0	362.3	0.0	324.9	0.0	333.8	0.0
conformer **4e**	384.3	11.4	376.3	14.0	332.5	7.6	342.2	8.4

13.2 kcal/mol. Here, only inductive and dipolar effects are possible. This is similar to the 12 kcal/mol increase in acidity caused by the nonresonating formyl group in conformer **4e** of *N*-formylacetamide.

DFT Calculations of Acidities. RHF calculations tend to underestimate the extent of delocalization or resonance. This problem can be corrected for by including electron correlation. Although the Becke3LYP method tends to overemphasize delocalization, it is an inexpensive and relatively accurate way to include electron correlation in the calculations. Succinimide, 2-pyrrolidinone, and *N*-formylacetamide conformers **4b** and **4e** were optimized using B3LYP. The results are summarized in Table 3. As stated earlier, the comparison of the proton affinities of succinimide and 2-pyrrolidinone give the increase in acidity caused by the addition of a β-carbonyl group, while the comparison of the proton affinities of **4b** and **4e** provides an upper limit estimation for the effect of resonance on the increased acidity.

The B3LYP method indicates that resonance stabilization of the anion has a larger effect on acidity than predicted by the RHF calculations. The increase in acidity due to an added carbonyl group remains similar to the gas phase value with an 18.3 kcal/mol difference between the acidities of succinimide and 2-pyrrolidinone. However, the increase in acidity of *N*-formylacetamide upon rotation of the carbonyl group is 14.0 kcal/mol, 2.4 kcal/mol higher than the RHF calculated value. This 14.0 kcal/mol can be attributed to resonance effects. Electrostatic and inductive effects then only contribute 4.3 kcal/mol of stabilization to the anion.

The contribution of resonance to the acidity probably lies between the predicted RHF and B3LYP values. This would place the upper limit of resonance contribution between 11 and 14 kcal/mol. If B3LYP calculations have a similar effect on the lower limit for the contribution of resonance, it will be between 7 and 10 kcal/mol. Thus, resonance has a larger impact on the acidity than predicted by RHF, but electrostatic interactions and the inductive effect still affect the acidity.

Solvation Effects on Acidities. The effect of solvation on the relative acidities was also investigated. Calculations were performed on succinimide, 2-pyrrolidinone, and *N*-formylacetamide conformers **4b** and **4e**. The results are presented in Table 3. The SCIPCM calculation results give lower proton affinities than the gas phase calculations and lower relative energies. For the solvation model, the difference in acidity between succinimide and 2-pyrrolidinone is only 13.5 kcal/mol. This number is significantly lower than the 18.0 and 18.3 kcal/mol calculated by RHF and B3LYP, respectively. The increase in acidity obtained by rotation of the carbonyl group in *N*-formylacetamide is also lower: 7.6 kcal/mol for SCIPCM. The electrostatic interactions and inductive effect then contribute 5.1 kcal/mol.

To compare these results with the RHF and DFT calculations, we compare the ratios of the decrease in acidity caused by resonance to the total decrease in acidity from the additional carbonyl group. Similar ratios would indicate that the resonance and nonresonance effects have the same amount of importance for each type of calculation. The ratios for the three types of calculations are 0.63 for RHF, 0.76 for DFT, and 0.62 for SCIPCM. The SCIPCM ratio is the same as the RHF ratio, indicating that solvent has had no effect on the importance of resonance as determined by the RHF calculations. The solvent calculation results support the proposal we advanced at the beginning of this paper that the main effect solvation would have on our gas phase results would be to lower the proton affinities and the relative energies but would have a small effect on the ratios of the relative acidities.

(38) The acidity of acetone was previously calculated by Saunders.[25a]

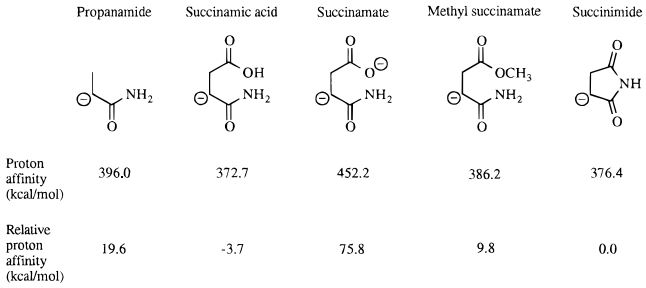

Figure 10. Proton affinities and relative proton affinities of acyclic and cyclic peptide residue models.

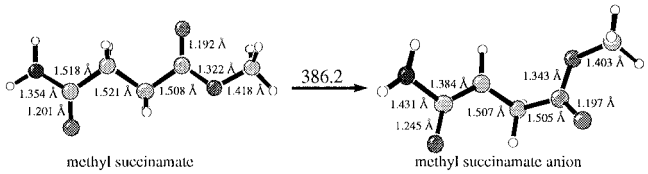

Figure 11. Optimized geometries of methyl succinamate and its enolate anion. The proton affinity is given (kcal/mol); (RHF/6-31+G*).

Peptide Residue Model Systems. Calculations were performed on model systems of peptide residues to determine whether the increase in acidity upon succinimide formation is large enough to account for the observed higher rates of racemization. The model systems calculated were propanamide, succinamic acid, succinamate, methyl succinamate, and succinimide. The results are given in Figure 10, and the optimized structures of methyl succinamate and its α-carbanion are given in Figure 11. Propanamide is used as a model for an alanine residue, and succinimide is used as a model for a succinimidyl residue. Succinamic acid and succinamate were originally conceived as models of aspartic acid and aspartate, but due to hydrogen-bonding interactions in both of these systems, the gas phase calculations of their proton affinities gave unrealistic results for solution considerations. For this reason, the proton affinity of methyl succinamate, which does not form these hydrogen bonds, was calculated and this was used as the model for an aspartyl residue.

Comparison of the acidities of model acyclic and cyclic peptide residues indicates that the addition of a carboxylate group to the side chain increases acidity but that formation of a succinimide causes a much larger increase in acidity. Methyl succinamate is 9.8 kcal/mol more acidic than propanamide. This

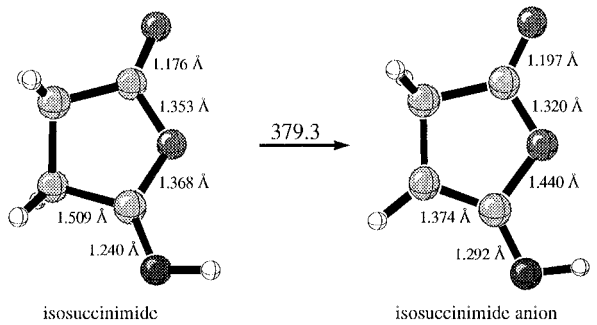

Figure 12. Optimized geometries of isosuccinimide and the corresponding enolate anion. The proton affinity is given (kcal/mol); (RHF/6-31+G*).

is yet another indication that the inductive effect of a carbonyl group has a large effect on acidity. These calculations agree with the experimental observation that free aspartic acid racemizes more quickly than alanine.[12] However, the rates of racemization of aspartyl residues in peptides or proteins are higher than expected on the basis of free aspartic esters.[8,13] Succinimide is 9.8 kcal/mol more acidic than methyl succinamate. Thus, formation of succinimide will increase the acidity and the rate of racemization of the α-C of aspartyl residues.

Other Possible Cyclic Structures. There are other cyclic intermediates from peptides that may contribute to the overall racemization processes.[39,40] One of these involves the formation of five-membered cyclic intermediates, where the peptide bond oxygen atom, rather than the nitrogen atom, of the residue attacks the side-chain carbonyl.[39] This reaction forms an imino-δ-lactone ring that has been termed an "anhydride" or "isoimide". This structure may share the electronic properties of succinimide intermediates that lead to rapid racemization.[15a] Both decreased resonance and through-space effects that place the anion at the positive end of a carbonyl dipole can increase acidity. To test this hypothesis, the proton affinity of isosuccinimide was calculated and compared to that of succinimide. The results are given in Figure 12 and Table 2. Isosuccinimide is only 2.9 kcal/mol less acidic than succinimide and should also be racemization prone. The high acidity of isosuccinimide is also attributed to both resonance and nonresonance effects, since the geometrical changes it undergoes upon anion formation are very similar to the those observed for succinimide.

Experimental studies have implicated electrostatic interactions between the two peptide units of diketopiperazine as the cause of a 740-fold increase in the rate of NH exchange, as compared to 2-piperidone.[41] This interaction has been invoked as an important influence on rates of NH exchange in peptides. The electrostatic effects found here provide an explanation of this trend.

Conclusions

The theoretical calculations in this work provide quantitative rationalization of the rapid racemization observed at succinimide residues in proteins. Such residues have been shown to be the major intermediates in the deamidation and isomerization of L-asparagine residues and in the isomerization of L-aspartic acid residues. Succinimide residues are calculated to be 18 kcal/mol more acidic than their acyclic counterparts in the gas phase and 13 kcal/mol more acidic in solution. As expected, the resonance interaction between the β-carbonyl carbon and nitrogen contributes to the higher acidity of succinimide. This accounts for about one-half to two-thirds of the calculated increased acidity where the remaining acidity increase is due to electrostatic interactions and the inductive effect of the β-carbonyl group.

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