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# Glutathione as a Prebiotic Answer to α-Peptide Based Life

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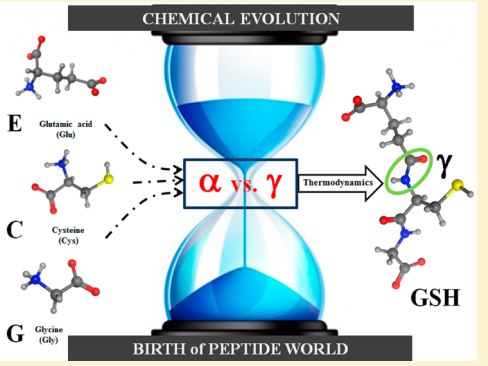
Supporting Information

**ABSTRACT:** The energetics of peptide bond formation is an important factor not only in the design of chemical peptide synthesis, but it also has a role in protein biosynthesis. In this work, quantum chemical calculations at 10 different levels of theory including G3MP2B3 were performed on the energetics of glutathione formation. The strength of the peptide bond is found to be closely related to the acid strength of the to-be N-terminal and the basicity of the to-be C-terminal amino acid. It is shown that the formation of the first peptide activates the amino acid for the next condensation step, manifested in bacterial protein synthesis where the first step is the formation of an N-formylmethionine dipeptide. The possible role of glutathione in prebiotic molecular evolution is also analyzed. The implications of the thermodynamics of peptide bond formation in prebiotic peptide formation as well as in the preference of α- instead of β- or γ-amino acids are discussed. An empirical correction is proposed for the compensation of the error due to the incapability of continuum solvation models in describing the change of the first solvation shell when a peptide bond is formed from two zwitterions accompanied by the disappearance of one ion pair.




## INTRODUCTION

In the biosynthesis of bacterial proteins, the amino acid (AA) polymerization always begins with formation of a peptide bond to the carboxyl group of a modified methionine, *N*-formylmethionine (fMet). In the first step of bacterial protein synthesis, the amino group of methionine is protected by enzymatic formylation of the $NH_2$ group so that the next residue can attack only its carboxyl group[1] (Scheme 1).

The first amino acid residue that will connect to fMet later will be the N-terminal end of the protein. The peptide chain is then built step by step, each new peptide bond being formed by the carboxyl group of the C-terminus amino acid whose α-amino group is involved in an existing peptide bond. Finally, the methionine is removed from the N-terminus of the protein. In fact, *N*-formylmethionine acts like a catalyst or an activator: connecting to the amino group, it makes the would-be N-terminal amino acid capable of forming a new peptide bond at the C-terminus. Similar "activation" seems to operate in the biosynthesis of other peptides, too. For example, in the synthesis of glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH, Figure 1, bottom right), in spite of being performed by completely different enzymes in different organisms, the first step is always formation of the peptide bond involving the γ-carboxyl group of glutamic acid and the amino group of cysteine. The common features of these processes indicate that the chemistry, in particular, the themodynamical characteristics, can be similar. Investigation of the simpler case, the energetics of glutathione formation, can help one to understand how this "activation" works.

GSH is accumulated in several cellular compartments such as the cytosol, nucleus, and mitochondria (in as high concentration as 1−11, 3−15, and 5−10 mM, respectively).[2] Besides many of its other features, it is one of the most important antioxidants,[3−5] and it contributes to amino acid transport through the cell membrane.[6,7] GSH has an essential role in numerous biochemical processes like cell differentiation, proliferation, apoptosis, signal transduction, and gene expression.[8,9] A large variety of human diseases like cystic fibrosis, cancer, and neurodegenerative diseases are closely related to the irregular GSH homeostasis.[10−13] Its omnipresence indicates that it has some structural element that lends it the capability of performing a special function, as well as of surviving and remaining active in drastically different environments.

What is unique in GSH is that the energetics of its formation, together with those of its α analogue (L-glutamyl-L-cysteinyl-

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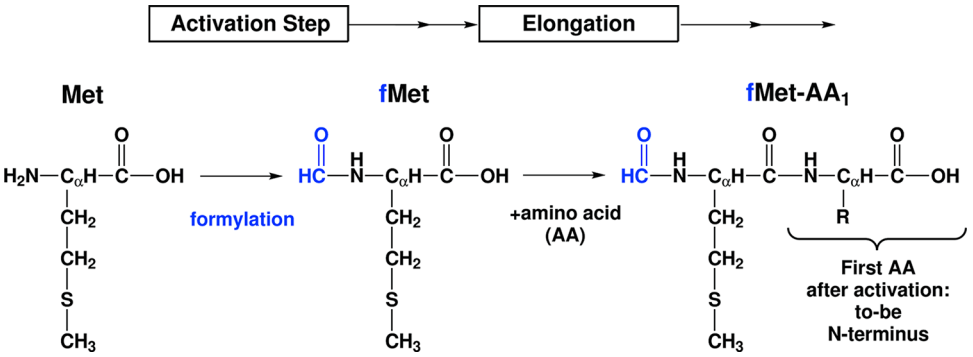
**Scheme 1. Initial Steps of the Bacterial Protein Synthesis**[a]




[a]Met, methionine; fMet, *N*-formylmethionine.

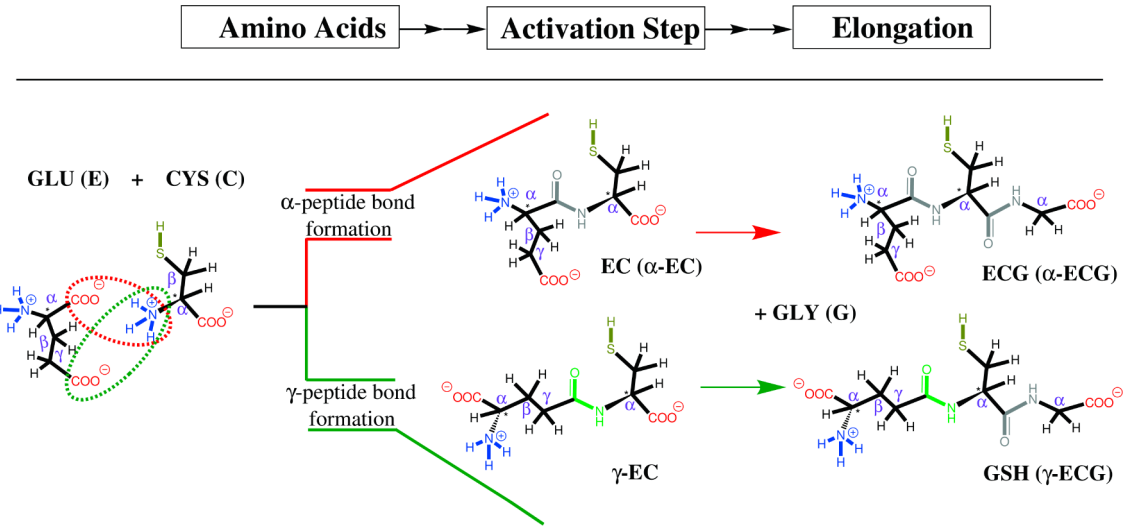



**Figure 1.** Scheme of peptide formation and the steps leading to $\alpha$ (upper row) and $\gamma$ (lower row) di- and tripeptides from glutamic acid (Glu, E), cysteine (Cys, C), and glycine (Gly, G). The pharmacophore groups are marked by colors: blue, amino; red, carboxyl; yellow, SH; gray, $\alpha$-peptide bond; green, $\gamma$-peptide bond. The $\alpha$-, $\beta$-, and $\gamma$-carbons are also marked.

glycine, ECG, also shown in Figure 1), provide all information needed to understand the general features of the thermodynamics of peptide formation.

Using quantum chemical methods, we intend to contribute to the elucidation of the factors determining the strength of peptide bonds that may explain the role of an existing peptide bond in the formation of a new one and the energetic factors that contribute to the preference of the $\alpha$-peptide bond in proteins. In the following, we summarize the known properties of GSH, and then present the results of a comparative theoretical study of the thermodynamics of the formation of the $\alpha$- and $\gamma$-peptide bonds and show the probable factors responsible for the primary accumulation of GSH. Finally, we address the consequences of the relative strength of regular $\alpha$- vs $\beta$- and $\gamma$-peptide bonds in the assembly of proteins. Thereafter, the connection between the thermodynamics of peptide and protein formation and the acidity and basicity of amino acids will be discussed.

## METHODS

Molecular structures, standard reaction enthalpies ($\Delta_r H°$), and reaction Gibbs free energies ($\Delta_r G°$) were calculated for the di- and tripeptide formation by quantum chemistry calculations. Since peptides can assume numerous conformations, it is important to decide which molecular geometry is used in the calculations. Conformation analysis of glutathione carried out by NMR spectroscopy and molecular dynamics (MD)[14−18] shows that GSH does not adopt a preferred conformation in solution. This suggests that the solvent−solute interactions are preferred instead of (internal) solute−solute interactions. In order to describe the solvent−solute interactions for different functional groups in a comparable environment, the electronic structure calculations were performed at the optimized geometry of the extended zwitterion forms of the amino acids and peptides. At the extended linear geometry, the intramolecular interactions are small and not specific to the substituents on the peptide backbone. This kind of standardization then allows one to separate local thermodynamic factors such as bond strength from secondary effects such as intramolecular interactions and different solvation due to differences of the environment.

The thermodynamics of the peptide formation reaction

$$H_3^+N{-}Q{-}COO^- + H_3^+N{-}Q'{-}COO^- \rightarrow H_3^+N{-}Q{-}C(O)NH{-}Q'{-}COO^- + H_2O \quad \text{(R1)}$$

was studied using the G3MP2B3 method[19,20] and two density functional sets, namely, B3LYP, as it is implemented in the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J. Phys. Chem. B XXXX, XXX, XXX−XXX

Gaussian 09 program package,[21] and the hybrid meta-GGA (generalized gradient approximation) functional M05-2X,[22] combined with two split-valence basis sets, 6-31G(d) and 6-311+G(d,p). The G3MP2B3 method and each functional−basis-set pair was combined with two implicit water models, the conductor-like polarizable continuum model, CPCM,[23,24] and the continuum solvation model "D", SMD,[25] to mimic the solvent effects of bulk water. The four different DFT levels of theory combined with two solvent models provided very similar molecular geometries. The method dependence of the energy is larger, but each model provides the same qualitative picture. Among the DFT methods, the M05-2X/6-311+G(d,p) level of theory provides energy differences closest to the benchmark G3MP2B3 data.

Reaction enthalpies and reaction Gibbs free energies were calculated using the standard rigid rotor−harmonic oscillator (RRHO) approximation. The calculated relative enthalpies realistically reflect the differences in the bond strength of different peptides. The calculation of reaction entropies, however, is more sensitive to anharmonicities and solvent effects on vibrations. In addition, during the formation of a peptide formation in neutral water, one $NH_3^+$ and one $COO^-$ ion disappear, involving extensive changes in solvation, especially in the first solvation shell that is not considered explicitly by continuum solvation models. As a consequence, the reaction free energies calculated with continuum solvation models and the RRHO approximation cannot be expected to accurately reproduce the available experimental values. In order to provide a more realistic picture, we corrected the reaction Gibbs free energies based on the following reasoning: The experimental free energy change for formation of the zwitterionic form of an amino acid from the neutral is −30.4 kJ/mol.[26] The formation of a peptide bond from two zwitterionic amino acids results in a zwitterionic peptide so that one zwitterionic structure disappears. The free energy change then involves the free energy of the peptide bond formation reaction from neutral peptides plus the free energy of annihilation of an ion-pair structure. We correct our calculated peptide formation reaction Gibbs free energies $\Delta G_{pf}$ by +30.4 kJ/mol for each new peptide bond (each corresponding to the disappearance of one ion pair), i.e., once for dipeptide formation from two amino acids plus once for the tripeptide formation from a dipeptide and an amino acid. As a test of our correction procedure, we calculated the reaction Gibbs free energy of formation of zwitterionic diglycine from two glycine zwitterions with the G3MP2B3 composite method combined with the SMD solvation model. We obtained $\Delta G_{GG,calc} = -16.3$ kJ/mol. Correction of the calculated value according to our scheme yields $\Delta G_{GG,corr} = 14.1$ kJ/mol. The experimental value is $\Delta G_{GG,exp} = 15.1$ kJ/mol,[27] which means that the correction reproduces the experimental reaction free energy within 1 kJ/mol.

All computations were carried out using the Gaussian 09 program package.[21]

## RESULTS AND DISCUSSION

**Structure and Functionality of Glutathione.** Let us first investigate the properties of glutathione that indicate its possible prebiotic origin. The most conspicuous in the structure of GSH is the nonregular peptide bond that is formed between the side-chain carboxyl group (located in the $\gamma$ position, i.e., two carbons away from the $\alpha$-carbon carrying the amino group) of glutamic acid (E) and the amino group of the cysteine residue (C), a $\gamma$-peptide bond (Figure 1). Remarkably, free ECG, the analogue of GSH in which there is an $\alpha$-peptide bond between E and C (Figure 1), has not been found to appear in living organisms. On the other hand, the antioxidant activity of GSH is retained if the third residue in the tripeptide is changed. In these GSH analogues, glycine is replaced by $\beta$-alanine,[28−30] serine,[29,30] or glutamate.[30] Furthermore, another GSH derivative, trypanothione, in which two GSH units are connected by a polyamine linker, also acts similarly to glutathione in kinetoplastids,[29,31] just as the dipeptide $\gamma$-L-glutamyl-L-cysteine ($\gamma$-EC, Figure 1) does in halobacteria.[32] All analogues of glutathione that can perform its functions share a common structural unit, the $\gamma$-peptide bond. These analogues operate and perform the same function in rather different organisms, inspiring one to surmise that the $\gamma$-EC structural element is an ancient motif and has a special role in living organisms. There is another fact supporting this conjecture. When GSH is synthesized in cells, the first step is always the formation of the $\gamma$-peptide bond.[33,34] However, in different organisms, the synthesis of GSH is facilitated by different enzymes that, while fulfilling analogous functions, do not display any detectable similarity in their structure and sequence.[32,35] On the basis of these facts, it seems reasonable to assume that the $\gamma$-EC motif is more ancient than the proteins synthesizing it. Apparently, once Nature found a small peptide that performs very well the job of an antioxidant, very probably there were several "attempts" to synthesize them, and under different conditions different pathways were found to be successful. Finally, some of those that proved to be "useful" were retained during evolution.

It is curious why Nature relies on the unusual $\gamma$-peptide bond in GSH and its analogues when the standard in biology is the $\alpha$-peptide linkage. Molecular biological studies showed that, if GSH did not contain the nonregular peptide bond, the peptidases would degrade the molecule, thus preventing it from accumulating in concentrations needed to fulfill its function.[32] This means that, at the current stage of evolution, it is the $\gamma$-peptide bond that ensures that in living organisms GSH remains in the arsenal of antioxidant agents. Accepting that GSH and its role are ancient, probably prebiotic, it is reasonable to assume that the chemistry of its formation is responsible for its appearance in living organisms in the first place. From the observation that different enzymes synthesize GSH via the same first step, one can assume that the decisive factor is the thermodynamics, not the kinetics of the $\gamma$-peptide bond formation. The most straightforward assumption is that the relative stability of the possible isomers of the key building unit, glutamyl-cysteine, determines the actual reaction mechanism.

**Energetics of Peptide Bond Formation.** At the early stages of evolution, dipeptides from glutamic acid and cysteine obviously were formed without the assistance of enzymes that would control the relative rate of formation of the possible isomers, because at that time enzymatic catalysis was obviously less developed. In other words, dipeptide "synthesis" took place under thermodynamic control. Thermodynamic control operates when neither of the isomers is formed significantly faster than the other, and the result is that their formation leads to a mixture being in thermodynamic equilibrium. Accordingly, when the formation of both $\alpha$- and $\gamma$-dipeptides (EC and $\gamma$-EC, Figure 1) is possible from their two constituents, their relative stability governs their relative population. The dominance of one of them will then be passed on to the tripeptide formation step. Thus, if $\gamma$-EC and GSH are thermodynamically more


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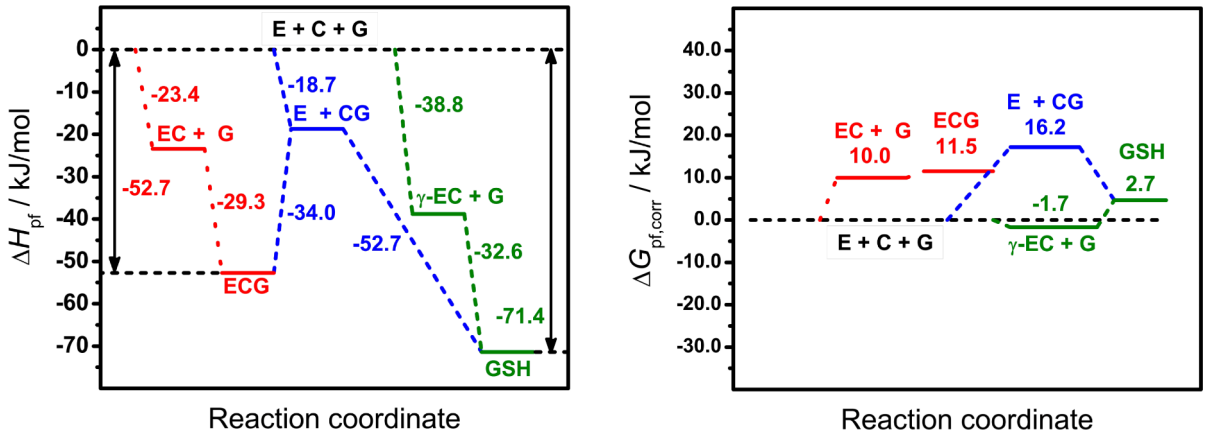


**Figure 2.** Standard reaction enthalpy ($\Delta H_{pf}$) and corrected Gibbs free energy ($\Delta G_{pf,corr}$) profile of peptide bond formation leading to ECG and GSH, respectively, calculated at the G3MP2B3 level of theory combined with the SMD implicit (continuum) solvent model. Red: Both steps form $\alpha$-peptide bonds. Blue: First step is $\alpha$-, second is $\gamma$- or $\alpha$-peptide formation. Green: first step is $\gamma$-, second is $\alpha$-peptide formation.

**Table 1. Standard Reaction Enthalpies $\Delta H_{pf}$ (in kJ/mol) of Peptide Bond Formation Involving the $\alpha$- and $\gamma$-Carboxyl Groups of Glutamic Acid, and Other Acids with and without the $\alpha$-Amino Group[a]**

| $\Delta H_{pf}$ | level of theory | | | |
|---|---|---|---|---|
| formed peptides | M05-2X | G3MP2B3 | p$K_a$ (COOH) | N-terminal acids |
| EC | −14.7 | −23.4 | 2.16 | E-$\alpha$-COOH |
| $\gamma$-EC | −31.0 | −38.8 | 4.15 | E-$\gamma$-COOH |
| *N*-glutaryl-L-cysteine | −33.5 | −39.7 | 4.34 | glutaric acid I. |
| DC | −12.7 | −22.2 | 1.95 | D-$\alpha$-COOH |
| $\beta$-DC | −23.9 | −30.3 | 3.71 | D-$\beta$-COOH |
| *N*-succinyl-L-cysteine | −34.1 | −32.5 | 4.21 | succinic acid I. |
| AA | −13.5 | −21.8 | 2.33 | A-$\alpha$-COOH |
| *N*-propionyl-L-alanine | −33.1 | −41.4 | 4.87 | propionic acid |
| AG | −18.1 | −23.7 | 2.33 | A-$\alpha$-COOH |
| *N*-propionyl-glycine | −37.8 | −43.6 | 4.87 | propionic acid |
| GA | −11.9 | −18.1 | 2.34 | G-$\alpha$-COOH |
| *N*-acetyl-L-alanine | −33.0 | −37.3 | 4.76 | acetic acid |

[a]Results of calculations at the G3MP2B3 and M05-2X/6-311+G(d,p) levels of electronic structure theory combined with the SMD implicit solvent model. The experimental acidity constants of N-terminal acids forming the bond are also shown.[36] The abbreviations of acid residues are E, $\alpha$-glutamyl; $\gamma$-E, $\gamma$-glutamyl; D, $\alpha$-asparagyl; $\beta$-D, $\beta$-asparagyl; A, alanyl; G, glycyl.

stable compared to EC and ECG, respectively, one can understand why the former were easily available when an SH-group based antioxidant was needed.

In quantitative terms, the relative amount of the $\alpha$- and $\gamma$-peptide products is determined by the equilibrium constant $K$ of the formal $\alpha$- to $\gamma$-isomerization:

$$\text{EC} \leftrightarrows \gamma\text{-EC}$$

We computed $K$ using quantum chemical methods at 10 different levels of theory. At all theoretical levels, $K$ is obtained to be over 100, indicating that the formation of the $\gamma$-isomer is found to be more favorable than its $\alpha$ counterpart.

The analysis of the energetics of the sequential formation of di- and tripeptides provides a more comprehensive picture. The relative strength of a peptide bond is reflected in the enthalpy of the reaction in which the bond is formed, namely, when the two amino acids react and the peptide is formed, with water as a byproduct (see reaction R1). Since the byproduct is always the same, the difference between the reaction enthalpies of two different peptide-formation channels will be the same as the difference between the strengths of the peptide bonds formed in the two processes. (It should be noted that the picture can be refined by considering acidic dissociation and proton take-up, but the leading term remains the relative bond strength.) Figure 2 shows the reaction enthalpies ($\Delta H_{pf}$) of all possible steps of tripeptide formation: (a) formation of the EC peptide bond first leading to ECG (red) via EC and GSH (green) via $\gamma$-EC and (b) formation of the CG bond first (blue) yielding both ECG or GSH; the numerical values are listed in Tables S1 and S2 (Supporting Information). The comparison of the pathways shows that the enhanced stability of GSH comes from the larger strength of the $\gamma$-peptide bond, the formation of which either in the first or the second step brings the system to lower enthalpy than the corresponding step in ECG formation. $\Delta H_{pf}$ for E + C → $\gamma$-EC as well as E + CG → GSH is −38.8 and −52.7 kJ/mol, significantly more negative than $\Delta H_{pf}$ for the corresponding $\alpha$-peptide bond formation steps, −23.4 and −34.0 kJ/mol, respectively. The reaction Gibbs free energies reflect the same features. For example, $\Delta G_{pf,corr}$ for the reaction forming $\gamma$-EC is negative, that for EC is not negligibly positive, and, similarly, the formation of GSH from CG is much more favorable than that of ECG. The equilibrium constants for peptide formation, $K_{pf,corr}$, obtained from the corrected Gibbs free energy changes are consistently much higher for the formation of the $\gamma$-peptides than for their $\alpha$ counterparts ($K_{pf,corr,\gamma}/K_{pf,corr,\alpha}$ = 109 for di- and 34 for tripeptides).


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J. Phys. Chem. B XXXX, XXX, XXX−XXX

Consequently, in thermal equilibrium, the GSH concentration is much higher compared to that of ECG, similarly to the γ-EC−EC pair. Accordingly, at ancient times, very probably more γ-EC than EC and more GSH than ECG was available to fulfill the role of a redox agent. By the time the regular α-peptide bond became standard for peptides and proteins, GSH probably performed its function satisfactorily efficiently not to be replaced later. This is the possible reason why such a curious structure was retained by evolution, and also supports the assumption that GSH is a prebiotic relict.

**The Correlation between the Enthalpy of Peptide Bond Formation with the Acidity and Basicity of the Contributing Amino Acids.** The enhancement of the reaction enthalpy, i.e., the larger strength of γ- versus α-peptide bonds, can be traced back to the difference of their environment in the molecule. In α-peptides, the amino group is close to the carboxyl functional group involved in the peptide bond. The inductive effect of the α-$NH_2$ group polarizes the carboxyl group, and one can expect that the strength of the peptide bond it forms will be different from that made by the essentially unpolarized carboxyl in the γ-position to the amino group. The degree of polarization of the carboxyl group will be reflected in other properties, for example, in its acidity. Indeed, the acid dissociation constant $K_a$ corresponding to the γ-carboxyl group in glutamic acid is 2 orders of magnitude smaller than that of the α-carboxyl (Table 1). The acidic strength is connected to the covalent character of the O−H bond, which is more expressed in weak acids, making the bond relatively strong, less easy to dissociate into ions. One can expect that weak acids would form stronger covalent bonds not only with the H atom but also with other functional groups. The opposite is the expectation for the connection between the base strength of the C-terminus acid and the strength of the peptide bond: strong bases hold the proton more strongly because the covalent character of their N−H bond is more expressed. Overall, the weaker acid is the would-be N-terminal amino acid and the stronger base is the would-be C-terminal amino acid, the stronger will be the peptide bond they form.

If one of the constituents, either the one that becomes the N-terminal or the one that turns into the C-terminal, is kept the same, one can expect a linear free energy relationship between the reaction enthalpy of peptide formation, $\Delta H_{pf}$ (and bond strength), and the $pK_a$ or $pK_b$ of the respective amino acids. Table 1 shows the acidic dissociation constants of the constituents (in the form of $pK_a$) and the calculated reaction enthalpies of the first peptide bond formation steps shown in Figure 2, supplemented by those of peptide or amide bonds formed by a number of related acids. The linearity of the correlation is displayed in Figure 3.

Recalling the general tendencies of changes of acidity with molecular structure, one may attempt to extend the applicability of the rule sketched above. For example, it is known that α-amino acids are stronger acids than the analogous organic acids lacking the amino group from the α-carbon atom. To explore the correlation, the reaction enthalpies for peptide/amide formation from a number of amino-acid/amino-acid versus organic-acid/amino-acid pairs were calculated. The results, together with the experimental acid dissociation constants of the N-terminal acids, are listed in Table 1. In the absence of the amino group from the α-position, the peptide bond is consistently stronger. When the $NH_2$ group is built into the α-position of the N-terminal acid, the acid dissociation constant $K_a$ increases by about 2 orders of magnitude, and $\Delta H_{pf}$ as well as the strength of the peptide bond decreases by around 20 kJ/mol. For example, glutaric acid, formally obtained when the amino group of glutamic acid is replaced by a hydrogen atom, is a much weaker acid than the α-carboxyl unit and somewhat weaker than the γ-carboxyl unit in glutamic acid, and forms a peptide bond with cysteine that is about 20 kJ/mol stronger than that in EC. Similar energetic order is observed when the chain length of the N-terminal acid is reduced by a $CH_2$ group. In dipeptides with cysteine as the C-terminal and aspartic acid (aminosuccinic acid, denoted here as D, formally obtained by removing the central carbon of glutamic acid, resulting in "moving" the side-chain carboxyl to the β-position) as well as succinic acid as the "N-terminal", β-DC and *N*-succinyl-L-cysteine, respectively, are more stable than the α-dipeptide, DC. This follows the order of acidic strength of the carboxyl group participating in the formation of the peptide/amide bond.

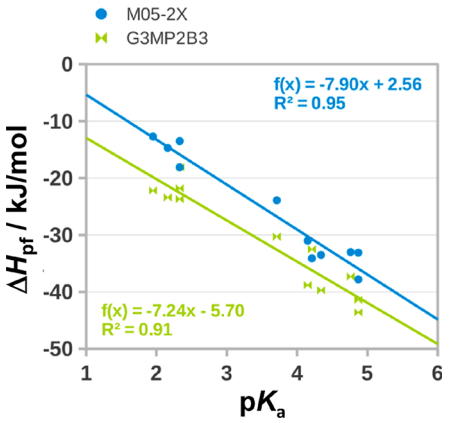


**Figure 3.** Correlation between the standard reaction enthalpies of peptide bond formation $\Delta H_{pf}$ (in kJ/mol) and the experimental acidity constants $pK_a$ of the bond-forming N-terminal acids. Results of calculations at the G3MP2B3 (green) and M05-2X/6-311+G(d,p) (blue) levels of electronic structure theory combined with the SMD implicit solvent model.

A similar correlation was found between $\Delta H_{pf}$ and the basicity of the C-terminal acid when the latter is varied and the N-terminal unit is kept constant. For example, the α-peptide bond formed between E and C ($pK_b$ = 10.78)[36] is weaker than that between E and CG ($pK_b$ = 8.67)[37] (Figure 2, blue lines). As Table 1 shows for AG and GA as well as for *N*-propionyl-glycine and *N*-acetyl-alanine, the weaker base alanine ($pK_b$ = 9.87)[36] forms weaker peptide/amide bonds than glycine ($pK_b$ = 9.78).[36] The same tendency is obtained with alanine and glycine as with cysteine and glutamic acid. The listed examples confirm that the assumed connection between the acid and base strength and that of the peptide bond is general.

**The Mechanism of Bacterial Protein Synthesis.** Figure 2 shows a remarkable fact that is also related to the acidic strength of acids participating in peptide bonds. Namely, the formation of the first peptide bond is always less exothermic than that of the second. In other words, the presence of an existing peptide bond in dipeptides makes the new peptide bond weaker. This is not surprising if one recalls that amino acid monomers are always stronger acids and bases than the peptides already containing a peptide bond. The exothermicity of the formation of further peptide bonds is also larger than that of the first (in general, it is similar to that of the second one). Consequently, the formation of the first α-peptide bond


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is the bottleneck in amino acid polymerization. One can expect that, in the biosynthesis of proteins, the formation of the first peptide bond is also the key obstacle because of its relative weakness. The astonishing mechanism of the biosynthesis of bacterial proteins is obviously a smart way of circumventing the bottleneck. The first peptide bond is formed between a modified methionine, *N*-formylmethionine (fMet), and some other amino acid. The amino group of methionine is not only protected by the enzymatic formylation, ensuring that only its carboxyl group is available for condensation with the next amino acid,[1] but since the $NH_2$ group of methionine is involved in a peptide bond (in fact, an amide bond), the "dipeptide" fMet will be a weaker acid than methionine. This is an ingenious application of the weak acid−strong peptide bond principle. This facilitates the formation of the first real peptide bond with the next residue. In terms of thermodynamics, the formylation of methionine reduces the Gibbs free energy of the peptide-formation reaction, shifting the equilibrium to a more favorable dipeptide concentration. This way, the bottleneck of the first peptide bond is bypassed. The initiator fMet is later removed, indicating that its only role is the facilitation of the formation of the first peptide bond.

The bottleneck of the first peptide bond must have had a role in prebiotic formation of peptides. Since enzymatic catalysis was not yet available, thermodynamics controlled the outcome of chemical reactions. If a reaction was thermodynamically unfavorable, such as the formation of a regular peptide bond, the concentration of its products was very small, giving little chance for AA polymerization. However, if there was a way to bypass the bottleneck, the dimerization step, then the polymerization forming longer peptides became thermodynamically more favored and relatively easy. For example, the first amide bond might have been formed from any weak organic acid. Another alternative is that the acidity of an amino acid was reduced by a "lucky" substitution or by binding to a surface, making the formation of the first dipeptide accessible, which, in a sense, initiated polymerization.

**The Strength of the Peptide Bond and the Preference of α-Amino Acids.** As we have seen, the presence of the $NH_2$ group next to a peptide bond always reduces the strength of the latter. This factor is in fact missing when the $NH_2$ group is two carbon atoms farther away. The difference between the acidic strengths of α- versus γ-amino acids is reflected in facts observed in natural peptides. For example, peptide bonds involving the γ-carboxyl group of glutamic acid are very rare in natural peptides and proteins. Obviously, the mechanism of peptide formation and metabolism Nature has developed handles efficiently the "standard" α-peptide bonds. It is not surprising that the individual steps in this "regular" mechanism do not operate for γ-peptides because of their enhanced strength. This is in agreement with GSH being found stable in cells: the large strength of the γ-peptide bond protects it from being degraded by peptidases. This explains why the too strong γ-peptide bond appears only in exceptional cases like GSH.

The comparison of the stability of α-peptides and proteins with those based on β- or γ-peptide bonds shows that, if the amino group in amino acids were not next to the carboxyl group but, instead, in the β- or γ-position, the formed peptide bond would be much stronger. However, while such peptides would be easier to make, they would have a strong disadvantage: they would be less easy to transform because the peptide bond would be too strong. This can explain Nature's preference of α-amino acids with respect to β- or γ-amino acids.

## CONCLUSIONS

The factors influencing the thermodynamics of the formation of peptide bonds was studied on the example of glutathione and related compounds with quantum chemical methods. A correction procedure is proposed for the calculation of the Gibbs free energy of peptide formation using standard quantum chemical techniques and continuum solvent models. The enthalpy of the peptide formation reaction R1, reflecting the strength of peptide bonds, was shown to be linearly related to the acidity and basicity of the N- and C-terminal amino acids. The stronger acid the N-terminal and the weaker base the C-terminal amino acid, the weaker is the peptide bond. Since the γ-carboxyl group of glutamic acid is weaker than the α-COOH, the dipeptide γ-EC and the tripeptide GSH are more stable than their regular, α-peptide isomers. The preference for formation of γ-EC and GSH is due to the enhanced stability of the γ-peptide bond.

The correlation between peptide bond strength and acidic strength proves to be a key factor in protein synthesis. Under thermodynamic control, the stronger the peptide bond the larger is the equilibrium concentration of the peptide. The closer the biosynthesis of proteins can follow the thermodynamics of the individual reaction steps the less effort and less smart technology is needed to make and break bonds efficiently. All essential amino acids are relatively strong acids and the α-peptide bonds they form as N-terminal partners are relatively weak. Dipeptides, on the other hand, are weaker acids and make stronger peptide bonds, so that once a peptide bond has been created, the formation of the next one is favored. This explains why the relatively strong γ-peptide bond is formed first in glutathione synthesis. More importantly, by considering the thermodynamics of peptide formation, one can understand why the biosynthesis of bacterial proteins starts with the formation of a peptide bond to the carboxyl group of *N*-formylmethionine. The role of fMet is similar to a catalyst or rather to an activator. The acidic strength of methionine is reduced by formylation, enabling it to form a stronger peptide bond whose strength is similar to that of α-peptide bonds in a peptide chain. This way the "bottleneck of the first peptide bond" is circumvented and fMet can be released from the N-terminus. The energetics of peptide bond formation also adds an item to the list why Nature relies on α-amino acids in proteins: β- or γ-peptide bonds are stronger than the α-peptide bond. While the enhanced strength is favorable in the first step of the synthesis, later too strong peptide bonds would be more difficult to manipulate in living organisms.

The weak acid−strong peptide bond principle can also be considered as one of the factors why prebiotic relicts like glutathione are still among the chemical agents in living organisms. At the beginning of chemical evolution, the larger exothermicity of the γ-dipeptide formation was probably one of the reasons why GSH has appeared, and when it successfully fulfilled its role as an antioxidant, it was retained in the redox arsenal of cells.

Finally, the correlation between the acid and base strength and the reaction enthalpy of peptide bond formation offers the possibility of being utilized in the design of new synthetic pathways for peptide synthesis, allowing one to control the steps of the process by varying the acidity and/or basicity of the amino acids.


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J. Phys. Chem. B XXXX, XXX, XXX−XXX

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Reaction enthalpies and Gibbs free energies for peptide formation. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Notes

The authors declare no competing financial interest.

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