Glutathione as a Prebiotic Answer to the α-peptide Based Life

Béla Fiser[a,b], Balázs Jójárt[a], Milán Szőri[a], György Lendvay[c], Imre G. Csizmadia[a,d], Béla Viskolcz[a,*]

[a]Department of Chemical Informatics, Faculty of Education, University of Szeged, Boldogasszony sgt. 6, Szeged, Hungary-6725
[b]University of the Basque Country/UPV-EHU, Department of Organic Chemistry I, Manuel de Lardizabal 3, Donostia-San Sebastián, Spain-20018
[c]Institute for Materials and Environmental Chemistry, Research Center for Natural Sciences, Hungarian Academy of Sciences, Magyar tudósok krt. 2, Budapest, Hungary-1117
[d]Department of Chemistry, University of Toronto, 80 St. George Str, Toronto, Ontario, Canada, M5S 3H6

Abstract

The energetics of peptide bond formation is an important factor not only in the design of chemical peptide synthesis but also has a role in protein biosynthesis. In this work quantum chemical calculations at ten 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.

Keywords: peptide bond formation, G3MP2B3, molecular evolution, N-formylmethionine, DFT.

Introduction

In the biosynthesis of bacterial proteins the amino acid (AA) polymerisation 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**).
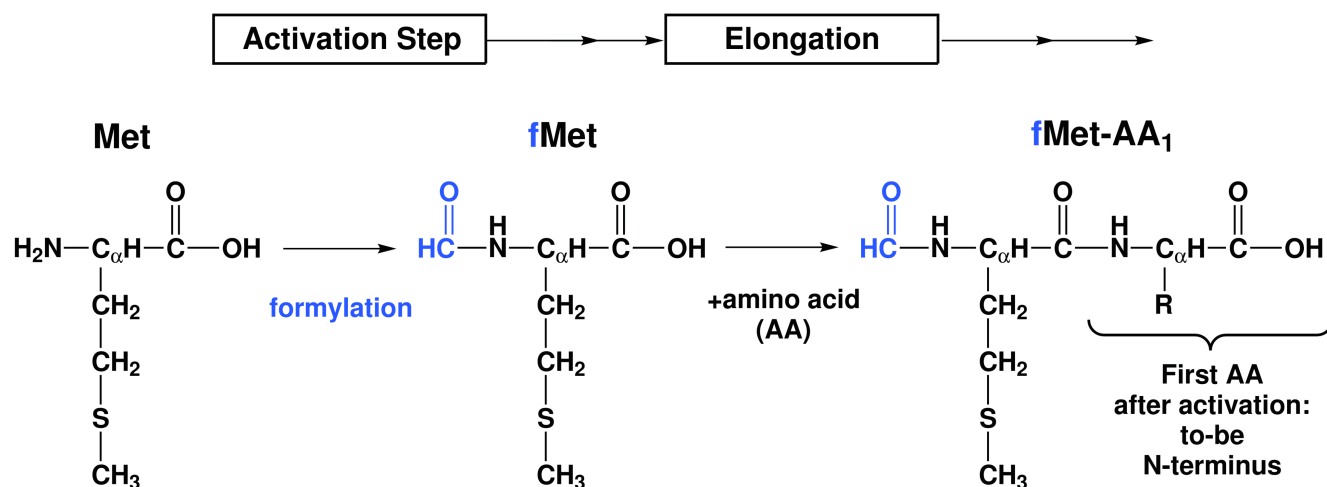

Scheme 1 The initial steps of the bacterial protein synthesis (Met – Methionine, fMet – N-formylmethionine).

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 thermodynamical 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 mM, 3-15 mM and 5-10 mM, respectively)[2]. Beside 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]. Disorders in GSH homeostasis are involved in the etiology and/or progression of a number of human diseases (e.g. cancer, cystic fibrosis, diseases of aging, cardiovascular, inflammatory, immune, metabolic and neurodegenerative diseases).[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-glycine, ECG, also shown in **Figure 1**), provide all information needed to understand the general features of the thermodynamics of peptide formation.
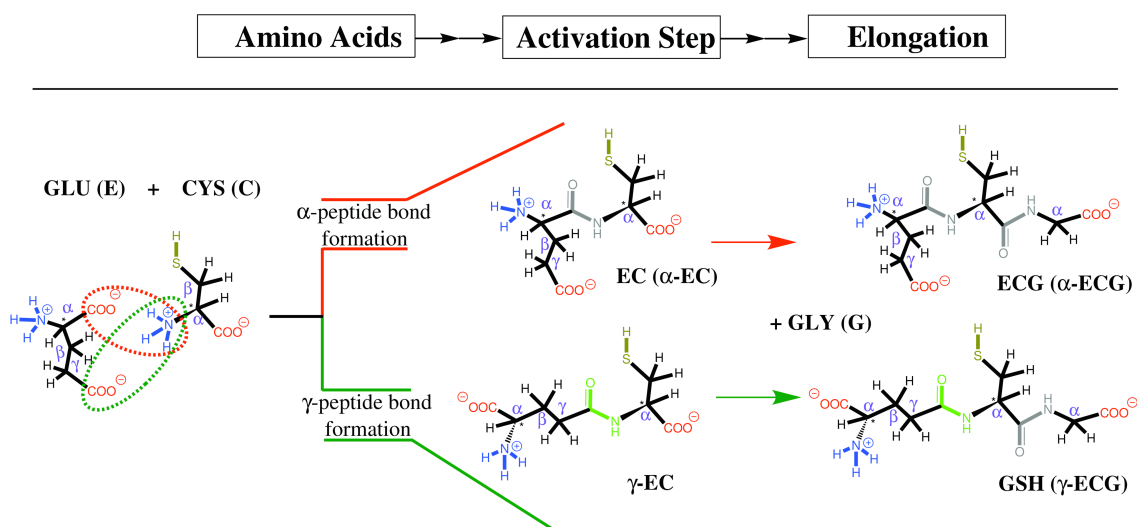
Figure 1 The scheme of peptide formation and the steps leading to α− (upper row) and γ− (lower row) di- and tripeptides from glutamic acid (Glu, E), cysteine (Cys, C) and glycine (Gly, G). The pharmacophore groups are marked by colors: blue – amino, red – carboxyl, yellow – SH, grey – α−peptide bond, green – γ–peptide bond. The α, β and γ-carbons are also marked.

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 α-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 α- and γ-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 α- *vs.* β- and γ-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 peptide formation reaction

$$H_3^+N\text{-}Q\text{-}COO^- + H_3^+N\text{-}Q'\text{-}COO^- + \rightarrow H_3^+N\text{-}Q\text{-}C(O)NH\text{-}Q'\text{-}COO^- + H_2O \qquad (R1)$$
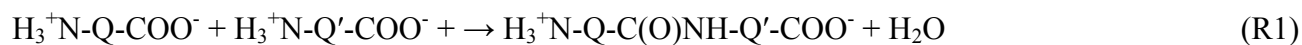
was studied using the G3MP2B3 method[19-20] and two density functional sets, namely B3LYP, as it is implemented in the Gaussian 09 program package[21] and the hybrid meta-GGA (Generalized Gradient Approximation) functional M05-2X[22], combined with two split-valence basis sets, 6-31G(d) and 6-311+G(d,p). 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 ΔG_{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 free enthalpy of formation of zwitterionic diglycine from two glycine zwitterions with G3MP2B3 composite method combined with 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's first investigate the properties of glutathione that indicate its possible prebiotic origin. The most conspicuous in the structure of GSH is the non-regular peptide bond that is formed between the side-chain carboxyl group (located in γ position, i.e. two carbons away from the α-carbon carrying the amino group) of glutamic acid (E) and the amino group of the cysteine residue (C), a γ-peptide bond (**Figure 1**). Remarkably, free ECG, the analogue of GSH in which there is an α-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 analogs, glycine is replaced by β-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 γ-L-glutamyl-L-cysteine (γ-EC, **Figure 1**) does in halobacteria[32]. All analogues of glutathione that can perform its functions share a common structural unit, the γ-peptide bond. These analogues operate and perform the same function in rather different organisms, inspiring one to surmise that the γ-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 γ-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]. Based on these facts, it seems reasonable to assume that the γ-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

7

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 γ-peptide bond in GSH and its analogues when the standard in biology is the α-peptide linkage. Molecular biological studies showed that if GSH did not contain the non-regular 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 γ-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 γ-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.
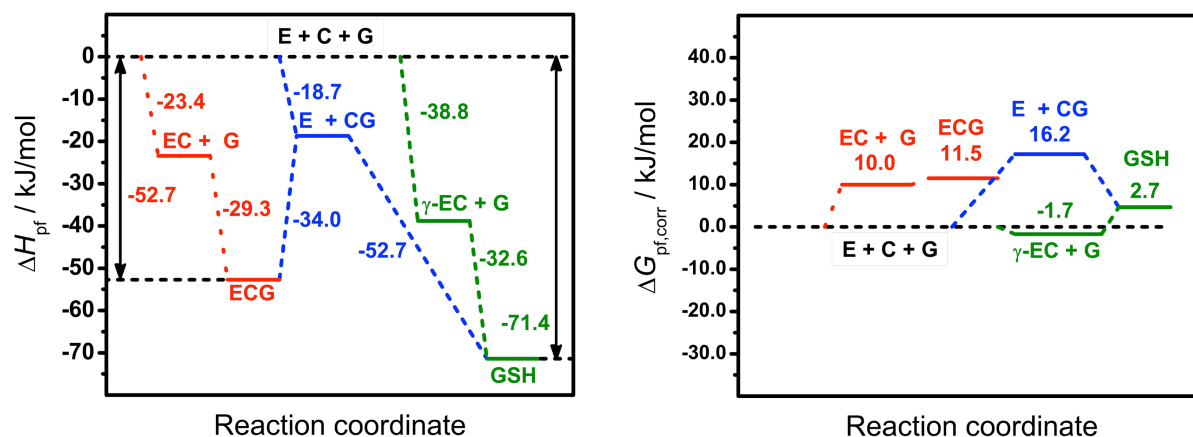
Energetics of peptide bond formation

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 α- and γ-dipeptides (EC and γ-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. So, if γ-EC and GSH are thermodynamically more 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 α- and γ-peptide products is determined by the equilibrium constant K of the formal α- to γ-isomerization:

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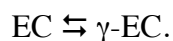
We computed K using quantum chemical methods at 10 different levels of theory. At all theoretical levels, K is obtained to be over 100 indicating that the formation of the γ-isomer is found to be more favorable than its α counterpart.

Figure 2 Standard reaction enthalpy (ΔH_{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 α–peptide bonds; blue: first step is α–, second is γ– or α–peptide formation; green: first step is γ–, second is α–peptide formation.

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 by-product (see reaction (R1)). Since the by-product 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 (ΔH_{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 γ–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**. The comparison of the pathways shows that the enhanced stability of GSH comes from the larger strength of the γ-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. ΔH_{pf} for E + C → γ–EC as well as E + CG → GSH is -38.8 and -52.7 kJ/mol, significantly more negative than ΔH_{pf} for the corresponding α-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 γ–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 constant for peptide formation, $K_{pf,corr}$ obtained from the corrected Gibbs free energy changes are consistently much higher for the formation of the γ-peptides than for their α counterparts ($K_{pf,corr,\gamma}/K_{pf,corr,\alpha}$ = 109 for di- and 34 for tripeptides). 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 fulfil 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 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 two 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 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- or 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, ΔH_{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**.
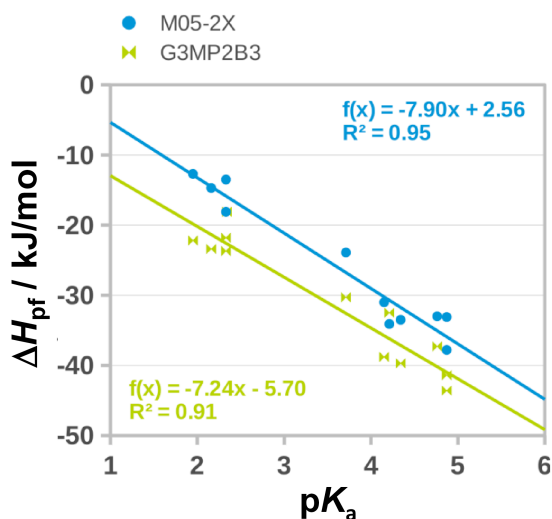
Figure 3 The correlation between the standard reaction enthalpies of peptide bond formation ΔH_{pf} (in kJ/mol) and the experimental acidity constants pK_a of the bond-forming N-terminal acids. Results of calculations at the G3MP2B3 (green) and M05-2X/6-311+G(d,p) (blue) levels of electronic structure theory combined with the SMD implicit solvent model.

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 calculated reaction enthalpies for peptide/amide formation from a number of amino-acid/amino-acid versus organic-acid/amino-acid pairs. 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 two orders of magnitude, and ΔH_{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 much weaker acid than the α-carboxyl- 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 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 β-position) as well as succinic acid as "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.

Similar correlation was found between ΔH_{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 exotherm 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 is the bottleneck in amino acid polymerisation. 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 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 free enthalpy of the peptide-formation reaction, shifting the equilibrium to 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 polymerisation. 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

14

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 alpha 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 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 free enthalpy 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.

ASSOCIATED CONTENT

Supporting Information.

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

AUTHOR INFORMATION

Corresponding Author

*Béla Viskolcz, viskolcz@jgypk.u-szeged.hu, Department of Chemical Informatics, Faculty of Education, University of Szeged, Boldogasszony sgt. 6, Szeged, Hungary-6725.

Notes

The authors declare no competing financial interest.

References

1. Laursen, B. S.; Sørensen, H. P.; Mortensen, K. K.; Sperling-Petersen, H. U. Initiation of Protein Synthesis in Bacteria. *Microbiol. Mol. Biol. Rev.* **2005**, *69*, 101–23.

2. Schafer, F. Q.; Buettner, G. R. Redox Environment of the Cell as Viewed through the Redox State of the Glutathione Disulfide/glutathione Couple. *Free Radic. Biol. Med.* **2001**, *30*, 1191–1212.

3. Fiser, B.; Szőri, M.; Jójárt, B.; Izsák, R.; Csizmadia, I. G.; Viskolcz, B. Antioxidant Potential of Glutathione: a Theoretical Study. *J. Phys. Chem. B* **2011**, *115*, 11269–77.

4. Morgan, B.; Ezeriņa, D.; Amoako, T. N. E.; Riemer, J.; Seedorf, M.; Dick, T. P. Multiple Glutathione Disulfide Removal Pathways Mediate Cytosolic Redox Homeostasis. *Nat. Chem. Biol.* **2012**, *9*, 119–125.

5. Winther, J. R.; Jakob, U. Redox Control: A Black Hole for Oxidized Glutathione. *Nat. Chem. Biol.* **2013**, *9*, 69–70.2.

6. Meister, A.; Anderson, M. E. Glutathione. *Annu. Rev. Biochem.* **1983**, *52*, 711–760.

7. Huijing, F. Why is glutathione, a tripeptide, synthesized by specific enzymes, while the TSH-

releasing hormone TRH or thyroliberin, also a tripeptide is produced as part of a pro-hormone peptide? *IUBMB Life,* **2003**, *55,* 695–696.

8. Arrigo, A. P. Gene expression and the thiol redox state. *Free Radic. Biol. Med.* **1999**, *27,* 936–944.

9. Voehringer, D. W. BCL-2 and glutathione: alterations in cellular redox state that regulate apoptosis sensitivity. *Free Radic. Biol. Med.* **1999,** *27,* 945–950.

10. Fierro, S. et al. In vivo assessment of cancerous tumors using boron doped diamond microelectrode. *Sci. Rep.* **2012**, *2,* 1–6.

11. Di Monte, D. A.; Chan, P.; Sandy, M. S. Glutathione in Parkinson's disease: a link between oxidative stress and mitochondrial damage? *Ann. Neurol.* **1992,** *32 Suppl*, S111–S115.

12. Schulz, J. B.; Lindenau, J.; Seyfried, J.; Dichgans, J. Glutathione, oxidative stress and neurodegeneration. *Eur. J. Biochem / FEBS.* **2000,** *267*, 4904–4911.

13. Ballatori, N.; Krance S. M.; Notenboom S.; Shi S.; Tieu K.; Hammond C. L. Glutathione dysregulation and the etiology and progression of human diseases. *Biol. Chem.* ***2009,*** *390,* 191–214.

14. Fujiwara, S.; Formicka-Kozlowska, G.; Kozlowski, H. Conformational study of glutathione by NMR. *Bull. Chem. Soc. Jpn.* **1977,** *50,* 3131–3135.

15. York, M.; Beilharz, G.; Kuchel, P. W. Conformation of reduced glutathione in aqueous solution by 1H and 13C nmr. *Int. J. Pept. Protein Res.* **1987,** *29,* 638–646.

16. Lampela, O.; Juffer, A. H.; Rauk, A. Conformational analysis of glutathione in aqueous solution with molecular dynamics. *J. Phys. Chem. A.* **2003,** *107,* 9208–9220.

17. Zhang, R.; Wu, W. Studies on the structures and interactions of glutathione in aqueous solution by molecular dynamics simulations and NMR spectroscopy. *J. Mol. Liq.* **2011,** *162,* 20–25.

18. Fiser, B.; Jojart, B.; Csizmadia, I. G.; Viskolcz, B. Glutathione – Hydroxyl Radical Interaction:

A Theoretical Study on Radical Recognition Process. *PLOS ONE*, **2013,** *8(9),* e73652.

19. Janoschek, R.; Rossi, M. J. Thermochemical properties of free radicals from G3MP2B3 calculations. *Int. J. Chem. Kinet.* **2002,** *34,* 550–560.

20. Baboul, A. G.; Curtiss, L. A.; Redfern, P. C.; Raghavachari, K. Gaussian-3 theory using density functional geometries and zero-point energies. *J. Chem. Phys.* **1999,** *110,* 7650-7657.

21. Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Scalmani, G.; Barone, V.; Mennucci, B.; Petersson, G. A.; et al. *Gaussian 09*, revision A.1; Gaussian, Inc.: Wallingford, CT, USA, 2009.

22. Zhao, Y.; Schultz, N. E.; Truhlar, D. G. Design of Density Functionals by Combining the Method of Constraint Satisfaction with Parametrization for Thermochemistry, Thermochemical Kinetics, and Noncovalent Interactions. *J. Chem. Theory Comput.* **2006,** *2,* 364–382.

23. Barone, V.; Cossi, M. Quantum calculation of molecular energies and energy gradients in solution by a conductor solvent model. *J. Phys. Chem. A.* **1998,** *102,* 1995–2001.

24. Cossi, M.; Rega, N.; Scalmani, G.; Barone, V. Energies, structures, and electronic properties of molecules in solution with the C-PCM solvation model. *J. Comput. Chem.* **2003,** *24,* 669–681.

25. Marenich, A. V.; Cramer, C. J.; Truhlar, D. G. Universal solvation model based on solute electron density and on a continuum model of the solvent defined by the bulk dielectric constant and atomic surface tensions. *J. Phys. Chem. B.* **2009,** *113,* 6378–6396.

26. Wada, G.; Tamura, E.; Okina, M.; Nakamura, M. On the ratio of zwitterion form to uncharged form of glycine at equilibrium in various aqueous media. *Bull. Chem. Soc. Jpn.* **1982,** *55,* 3064–3067.

27. Martin, R. B. Free Energies and Equilibria of Peptide Bond Hydrolysis and Formation. *Biopolymers*, **1998,** *45,* 351–353.

28. Yi, H.; Ravilious, G. E.; Galant, A.; Krishnan, H. B.; Jez, J. M. From sulfur to homoglutathione:

thiol metabolism in soybean. *Amino Acids.* **2010**, *39,* 963–978.

29. Hermes-Lima, M. Oxygen in biology and biochemistry: role of free radicals. In *Functional Metabolism Regulation and Adaptation;* Storey, K. B., Ed.; Wiley-Liss, Inc., Hoboken, New Jersey, USA, 2004; pp 319–368.

30. Meuwly, P.; Thibault, P.; Rauser, W. E. γ–Glutamylcysteinylglutamic acid-a new homologue of glutathione in maize seedlings exposed to cadmium. *FEBS Letters.* **1993**, *336,* 472–476.

31. Fairlamb, A. H.; Cerami, A. Metabolism and functions of trypanothione in the Kinetoplastida. *Annu. Rev. Microbiol.* **1992,** *46,* 695–729.

32. Ganguly, D.; Srikanth, C. V.; Kumar, C.; Vats, P.; Bachhawat, A. K. Why is glutathione (a tripeptide) synthesized by specific enzymes while TSH releasing hormone (TRH or thyroliberin), also a tripeptide, is produced as part of a prohormone protein? *IUBMB Life,* **2003,** *55,* 553–554.

33. Dringen, R.; Gutterer, J. M.; Hirrlinger, J. Glutathione metabolism in brain metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. *Eur. J. Biochem / FEBS.* **2000,** *267,* 4912–4916.

34. Noctor, G.; Mhamdi, A.; Chaouch, S.; Han, Y.; Neukermans, J.; Marquez-Garcia, B.; Queval, G.; Foyer, C. H. Glutathione in plants: an integrated overview. *Plant Cell Environ.* **2012***, 35,* 454–484.

35. Galperin, M. Y.; Walker, D. R.; Koonin, E. V. Analogous enzymes: independent inventions in enzyme evolution. *Genome Res.* **1998**, *8,* 779–790.

36. Haynes, W. M.; Lide, D. R.; Bruno, T. J. *CRC Handbook of Chemistry and Physics.* 93rd ed. CRC Press, Boca Raton, FL, USA, 2012.

37. Dolinsky, T. J.; Nielsen, J. E.; McCammon, J. A.; Baker, N. A. PDB2PQR: an automated pipeline for the setup, execution, and analysis of Poisson-Boltzmann electrostatics calculations.

Nucleic Acids Res. **2004**, *32*, W665-W667.

Tables

Table 1 Standard reaction enthalpies ΔH_{pf} (in kJ/mol) of peptide bond formation involving the α- and γ-carboxyl groups of glutamic acid, and other acids with as well as without the α-amino group. 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 – α-glutamyl, γ-E – γ-glutamyl, D – α-asparagyl, β-D – β-asparagyl, A – alanyl, G – glycyl.

ΔH_{pf}	Level of theory			
Formed peptides	**M05-2X**	**G3MP2B3**	pK_a **(COOH)**	**N-terminal acids**
EC	-14.7	-23.4	2.16	E-α-COOH
γ-EC	-31.0	-38.8	4.15	E-γ-COOH
N-glutaryl-L-cysteine	-33.5	-39.7	4.34	glutaric acid I.
DC	-12.7	-22.2	1.95	D-α-COOH
β-DC	-23.9	-30.3	3.71	D-β-COOH
N-succinyl-L-cysteine	-34.1	-32.5	4.21	succinic acid I.
AA	-13.5	-21.8	2.33	A-α-COOH
N-propionyl-L-alanine	-33.1	-41.4	4.87	propionic acid
AG	-18.1	-23.7	2.33	A-α-COOH
N-propionyl-glycine	-37.8	-43.6	4.87	propionic acid
GA	-11.9	-18.1	2.34	G-α-COOH
N-acetyl-L-alanine	-33.0	-37.3	4.76	acetic acid

Table of Content