This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

nature portfolio

Peer Review File

Kinetic, thermodynamic, and ab initio insights of AsnGly isomerisation as a ticking time bomb for protein integrity

Corresponding Author: Professor András Perczel

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors used NMR and simulations to elucidate the Asn isomerization.

A general comment: The manuscript and the information presented is very complex and readability of the manuscript for a non-protein/peptide expert needs further improvement. The use of abbreviations which might or might not have all been introduced is a major source to the difficulties in accessing the content. There is a list of abbreviations at the end of the manuscript but this is not complete. I advise the authors to rather not use an abbreviation. MeCN is only used once in the methods section i.e. it doesn't need an abbreviation.

Some of the figures should be revised for readability e.g. the molecular structures in Figure 5. Please revise that the figure in the actual size is readable.

Comments:
Abstract: The authors use abbreviations like TS and IRC. Please revise for readability and clarity for non-experts. Communications chemistry publishes in all areas of the chemical sciences and has a broad and diverse readership.

Introduction:
Please provide a reference to your first sentence. "It has been shown that a protein would remain stable for years under neutral and sterile conditions in water, with the typical half-life of uncatalysed amide hydrolysis reaching hundreds of years."

What is the meaning of Amino acid Xxx in this sentence? L 52 Amino acid Xxx, with a small side chain following Asn promotes isomerisation, probably due to the increased flexibility of the backbone atoms. I assume the authors refer to the sequence but please clarify.

L64 delete a comma

Methods:
In L120, the authors claim that the selected 1H resonances are proportional to the concentration. No information about the experimental settings for the 1H NMR experiments are given. For 1H resonances to be quantitative, the spectrum needs to be recorded with a repetition delay of 5*T1. The T1 may depend in addition on temperature. The authors in addition do not show any NMR spectrum as an example to judge the quality of the recorded data for example the signal to noise. Was a baseline correction performed? Window function? Automatic integration? Resonance shifts with pH and temperature. Was the assignment redone at different pH and temperatures? H exchange and dynamics are dependent on pH and temperatures and the resonances will shift.
Please provide an example of a spectrum and the procedure of how it has been recorded and processed and analysed in the SI.
Why was DSS used?

Please provide a reference to "IEFPCM water model".

Results:

Fig 1a The y-axis showing the integrals versus time shows mol/dm^-3.
How were the integrals converted to concentration? DSS is know to also interact with some species. Was this checked in case DSS was used as an internal standard? In this case also the T1 of DSS (which should be rather long due to the content

of Si) needs to be estimated at all these conditions. 100 mol/L is a lot, please make sure that these numbers are correct.

Table 1:
What was the reasoning behind choosing these specific temperatures?
Why is there such a huge variation in the data of points? For some settings, only 3 data points were recorded and for others 245!
Why was the pH only changed for -NGAA-?

L254: The authors conclude that NG isomerisation is spontaneous and can be controlled by adjusting the pH. The authors changed pH only for the -NGAA-. Could the authors clarify how they arrive at this conclusion?

L298 Has the release of NH3 been detected? I am not 100% sure but it should be visible in the 1H NMR spectra.

Reviewer #2

(Remarks to the Author)
Pilhál et al. aim at determining a kinetic and mechanistic model of Asn-Gly dipeptide bond isomerization. They use 1H NMR to follow the reaction in real time and derive a kinetic model. Further, they use quantum chemistry calculations to provide potential mechanisms of the rate-limiting step. Finally, they investigate the effect of pH, temperature, and amino acid type (net charge) at the n+2 position. The manuscript is interesting for a broad chemistry audience but suffers from readability issues and (I assume) from a lack of explanation on the main findings and how they are supported by experimental data. Novelty is also a concern that should be adressed before potential publication. In conclusion, major revision should be made before it can be assessed if the manuscript can be published in Communications Chemistry.

It is not very clear what key conclusion is driven by the experimental results. The authors go in great details about kinetic models and reaction micro-steps but do not really provide statements of their main findings, and they add to previous knowledge. What did they find that explain isomerization in a new light? Do they think they provide clear evidence for a specific kinetic model and reaction mechanism?

I would advice the shape the manuscript around main findings:
- Kinetic model for isomerization: succinimide formation is the rate-limiting step
- The most probable mechanisms for succinimide formation is water-assisted?
- Reaction is dictated by side chain rotation angles, which explains influence of n+2 residue type

The novelty of findings is also not clear. A lot of aspects of the work were already discussed in the 2020 review (ref. 1) such as the effect of pH, temperature, local conformation, etc. The Introduction and Discussion should clearly state what is new in the current manuscript compared to previous knowledge.

To me some the claims made are not fully supported by the data. Maybe they are by previous litterature but then the novelty of the current work would be greatly diminished. Mainly, many other kinetic models for the reaction could be imagined. Why do the authors only consider one? Please either explain why no other model are possible, or provide a comparison of several different models. It is not enough to state that one model fits the data, especially since some of the rates do not seem to fit the data that well visually (for instance beta-Asp on Figure S4). A fair comparison of different model with a proper goodness of fit assessment and selection criterion between models is necessary to claim such statement.

It is sometimes difficult to follow the authors train of thoughts because of the language and wording. There are many instances of poorly chosen words or typos that give an impression of draft rather than finished manuscript, and at worst make it difficult to understand what the authors actyually meant. Please carefully read through and correct these. A few examples:

L51 – I assume we are talking about ‘polypeptide’ rather than ‘polyamide’ chain.
L52 – ‘Xxx’ shall be ‘Gly’, probably? Else, this sentence needs further explanation.
L54 – ‘smallest random coil shift’ does not mean much to me, I think the authors are simply talking about small chemical shift in respect to the concept of electron ‘shielding’. This has nothing to do with random coil.
L63 – Not sure what the authors define as transport molecules, but I assume something along the lines of ‘ion-binding proteins’?
L76 – Is a more complex explanation a good thing? Do the authors mean a more complete model or a more exhaustive view?
L116 – TOCSY and COSY are also 1H-NMR experiments.

Other minor comments:

L90 – A figure should be referenced so that the reader knows what reactions 1, 2 and 3 are.

L116 – The NMR method section is not sufficently well written to judge the quality of the procedure. Assignments do not provide quantitative data… Please explain seperately how assignment, and how kinetic data were acquired and analysed.

L175 – On what data is that claim based? Fitting NMR kinetics? Please elaborate.

L231 – On what ground does it remain valid, a goodness of fit value?

L245 – It would help to write the values obtained for Gibbs free energy of different contructs in the text.

Reviewer #3

(Remarks to the Author)
In the manuscript, the combined experiments and computational analysis have been performed to understand the kinetics and mechanism of the -Asn-Gly- (-Asn-Xxx-) isomerisation reactions in polypeptides. It was found that the formation of succinimide (Suc) from the Asn-Gly sequence is the rate-limiting step in the isomerisation process, which can be influenced by pH and temperature. In addition, the electrostatic catalysis from the positively charged residues at n+2 site can speed-up the reactions. I think most of the key conclusions are convincing to me. In addition, the work adds important insights on this biological process.
1. The unit of rate constant in Table 1 should be provided.
2. In Figure 1c, I guess that the free energy profile is obtained from experiments? This is should be clarified.
3. The calculated barriers (210.13 kJ/mol and 243.70 kJ/mol in the presence and absence of catalytic water) is much higher than the experimental value of 110.8 kcal/mol (Figure 1c), suggesting other factors involved in stabilizing transition states could be ignored. The authors can test the effect of more implicit waters, as done before (J. Comput. Chem. 2013, 34, 372–378; ACS Catal., 2015, 5, 7077–7090).
4. Can the isomerisation process be affected by other factors? Such as the ionic strength and types of pH buffer, which could be relevant to the natural process.
5. In order to better understand the electrostatic catalysis of positively charged (n+2) residues, it is better to calculate the dipole moment change from the reactant to the rate-determining TS state. With this information, the electrostatic catalysis of positively charged (n+2) residues can be analyzed quantitively (J. Phys. Chem. B 2023, 127, 4245-4253).
6. The detailed information in Table 2 can be moved to SI.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I am pleased with the response of the authors and the changes in the manus. In my opinion, the manuscript is publishable now.

Reviewer #2

(Remarks to the Author)
Most of my questions and concerns have been answered appropriately. I still think that an extra table providing quantitative statistics (AIC) about different kinetic models would greatly contribute to the strength of the paper's claims and novelty. However, I consider that the manuscript can be published.

Reviewer #3

(Remarks to the Author)
The authors have addressed my concerns. I think the manuscript can be accepted for publication

Response to Reviewer #1 comments:

Dear Reviewer #1,

Thank you for your questions and comments about this article. Please see our carefully considered responses below. They are also highlighted in blue in the revised manuscript and Supporting Information.

The authors used NMR and simulations to elucidate the Asn isomerization.

A general comment: The manuscript and the information presented is very complex and readability of the manuscript for a non-protein/peptide expert needs further improvement. The use of abbreviations which might or might not have all been introduced is a major source to the difficulties in accessing the content. There is a list of abbreviations at the end of the manuscript but this is not complete. I advise the authors to rather not use an abbreviation. MeCN is only used once in the methods section i.e. it doesn't need an abbreviation.

Thank you for your suggestion. Apart from our most commonly used abbreviations, such as NMR - nuclear magnetic resonance, or the one/tree-letter codes of amino acids, we have tried to avoid abbreviations in the revised version of the manuscript.

Some of the figures should be revised for readability e.g. the molecular structures in Figure 5. Please revise that the figure in the actual size is readable.

Thank you for your comment. We have improved the readability of our figures both in the manuscript and in the Supporting Information. We hope to get your approval.

Comments:

Abstract:
The authors use abbreviations like TS and IRC. Please revise for readability and clarity for non-experts. Communications chemistry publishes in all areas of the chemical sciences and has a broad and diverse readership.

Thank you for your helpful suggestions. We have updated the current version accordingly.

Introduction:

Please provide a reference to your first sentence. "It has been shown that a protein would remain stable for years under neutral and sterile conditions in water, with the typical halflife of uncatalysed amide hydrolysis reaching hundreds of years."

Thank you for pointing out this deficiency. We have added it to the current version of the manuscript. Please see the first literature reference highlighted blue: "Radzicka, A. & Wolfenden, R. Rates of Uncatalyzed Peptide Bond Hydrolysis in Neutral Solution and the Transition State Affinities of Proteases. *J. Am. Chem. Soc.* **118**, 6105–6109 (1996)."

What is the meaning of Amino acid Xxx in this sentence? L 52 Amino acid Xxx, with small side chain following Asn promotes isomerisation, probably due to the increased flexibility of the backbone atoms. I assume the authors refer to the sequence but please clarify.

Thank you for pointing this out. Similarly to *x* in mathematics, which represents a variable; we intended to use *Xxx* to represent an arbitrary three-letter code of amino acids. In the referred sentence we meant glycine, and rephrased it on page 3 highlighted blue:

"Glycine, following asparagine with a small side chain, promotes isomerisation, probably due to the increased flexibility of the backbone atoms [2 4 5]."

The next occurrence on page 5 we gave the meaning of *Xxx* highlighted blue:

"To follow the isomerisation reaction, time-dependent 1D ^{1}H-NMR measurements of Ac-Asn-Gly-*Xxx*-Ala-NH_2 peptides were completed (where *Xxx* was used as an arbitrary three-letter abbreviated amino acid)."

L64 delete a comma

Thank you for the comment. We have done it on page 4.

Methods:
In L120, the authors claim that the selected 1H resonances are proportional to the concentration. No information about the experimental settings for the 1H NMR experiments are given. For 1H resonances to be quantitative, the spectrum needs to be recorded with a repetition delay of 5*T1. The T1 may depend in addition on temperature. The authors in addition do not show any NMR spectrum as an example to judge the quality of the recorded data for example the signal to noise. Was a baseline correction performed? Window function? Automatic integration? Resonance shifts with pH and temperature. Was the assignment redone at different pH and temperatures? H exchange and dynamics are dependent on pH and temperatures and the resonances will shift. Please provide an example of a spectrum and the procedure of how it has been recorded and processed and analysed in the SI.

Thank you for pointing this out.
Please see the new and detailed NMR section in the Supporting Information, Part I./B. chapter.

Why was DSS used?

DSS (sodium 3-(trimethylsilyl)propane-1-sulfonate) is widely used as an NMR reference or standard molecule during protein and peptide measurements in aqueous buffers. The main reasons for its use are that it is considered to be an inert molecule, it gives a singlet signal, and it resonates at higher field. In other words, its sharp resonance line is well separated from the other signals. In addition, the pulse program we use (excitation sculpting for water suppression) has no effect on the DSS signal, so its integral value can be used as a reference. Finally, DSS is insensitive to pH changes.

Please provide a reference to "IEFPCM water model".

Thank you for your comment. We have added this reference as well (marked in blue, page 7, and reference number 35): Tomasi, J., Mennucci, B. & Cancès, E. The IEF version of the PCM solvation method: an overview of a new method addressed to study molecular solutes at the QM ab initio level. *Journal of Molecular Structure: THEOCHEM* **464**, 211–226 (1999).

Results:
Fig 1a: The y-axis showing the integrals versus time shows mol/dm^-3. How were the integrals converted to concentration?

Thank you for pointing this out. In fact, it is an incorrect axis label that has now been changed. We can only plot relative concentration values, between 0 and 1, as a function of time. Please see the corrected panels of Figure 1b and the figures in the Supporting Information: Part II/B.

DSS is know to also interact with some species. Was this checked in case DSS was used as an internal standard?

Thank you for this comment. Although there are indeed some examples in the literature where DSS is reported to interact with some compounds such as cyclodextrins, very specific cationic peptides, etc., it is still widely used as a reference standard for NMR data processing for the reasons described above and below. As expected, the DSS signal was set to 0 ppm for all processed spectra. No such resonance perturbation was observed for any of the current peptides used in this study. Furthermore, the ongoing isomerization reaction was successfully monitored by the decrease or increase of selected ^{1}H resonance integrals with respect to that of the DSS.

In this case also the T1 of DSS (which should be rather long due to the content of Si) needs to be estimated at all these conditions.

Thank you for this comment. We believe that the relaxation time of the DSS is mainly determined by that of the protons in solution, since they were the only nuclei excited, and there is no magnetization/coherence transfer from ^{1}H to Si in the pulse sequences we used. Furthermore, among the Si isotopes, only ^{29}Si is NMR active, with a natural abundance below 5%, whose contribution to the DSS spin-lattice relaxation is probably negligible. ^{28}Si, with a natural abundance of more than 92%, is an NMR inactive nucleus, so it does not couple to other nuclei and thus has no effect on relaxation. For the current proton relaxation, we believe that a total length of the pulse sequence including the acquisition time plus the d1 time of 2.1 seconds is more than sufficient.

100 mol/L is a lot, please make sure that these numbers are correct.

Thank you for your concern. Because the axis label was incorrect, the axis scale was also incorrect. The corrected version now uses relative values between 0 and 1 according to the relative concentration, where 1 corresponds to the starting point of the curve fitted to the asparagine consumption.

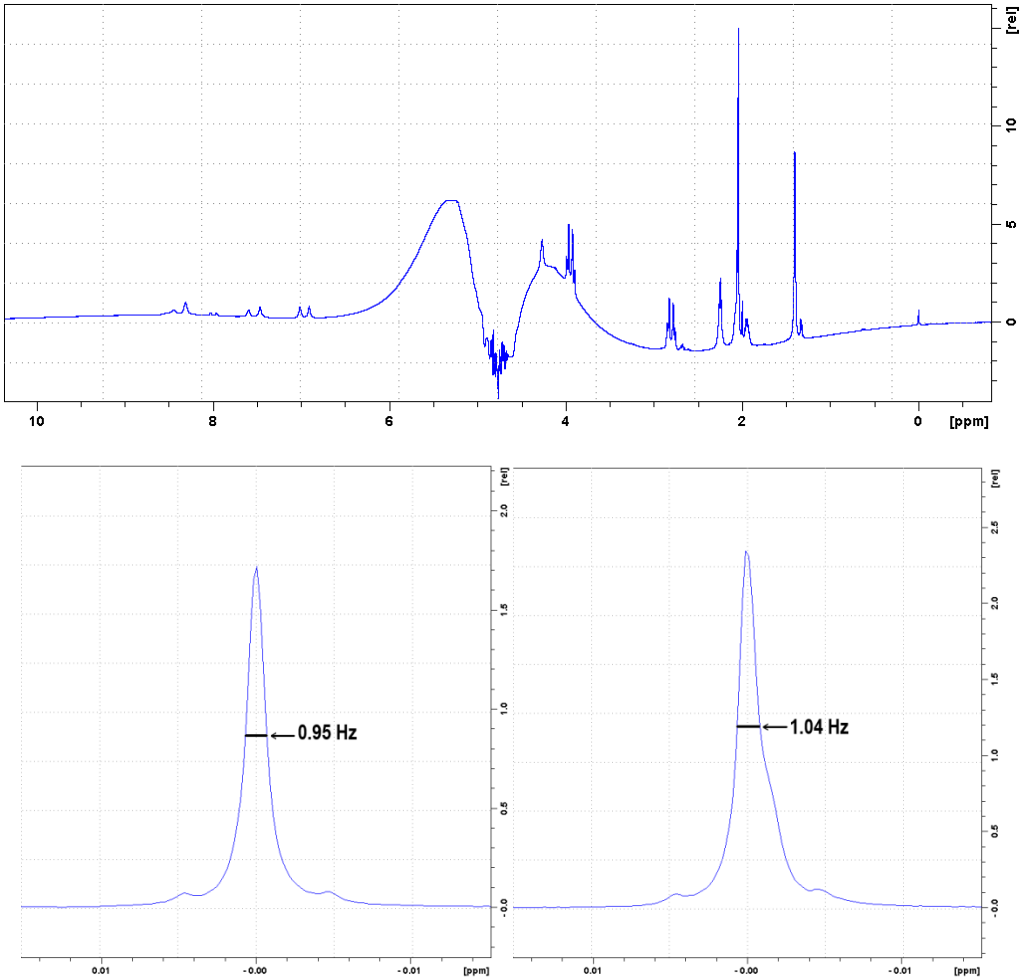
Table 1:
What was the reasoning behind choosing these specific temperatures?

The physiological temperature of 37 °C was chosen because of its biological relevance. The other temperatures were chosen to be equidistant from 37 °C and not too close to each other, as we planned to measure at more than two temperatures to determine the activation Gibbs free energies more reliably. Additional measurements below the lowest temperature of 28 °C were not performed because of the increasing time required for the NMR experiments. Furthermore, it is better for reaction monitoring (e.g. for shimming, baseline, integration) if the sample is not removed from the NMR magnet until the end of the isomerization reaction. As a result, experiments performed at higher temperatures (46, 55 °C) went faster, saving us and our scientific community NMR time.

Why is there such a huge variation in the data points? For some settings, only 3 data points were recorded and for others 245!

Thank you for your question. To make it clearer "# of data points [c]" in Table 1 was modified. Now it contains the number of the relative integral values used for parameter estimation by the COPASI software. Earlier the number of NMR experiments performed was given as "# of data points [c]" in Table 1.

One of the reasons for the lower number of NMR experiments is that we experienced bubble formation during some of the measurements, which distorted the water suppression and thus also the baseline. We tried to get rid of this by sonication before the start of the experiment or during the experiment when we noticed their appearance. Another reason for the selection was that for spectra where we left the sample in the NMR magnet overnight, the automatic shimming system did not always provide signals suitable for quantitative analysis. These spectra were either corrected or discarded. One such example is shown below (Ac-NGRA-NH_2, 46 °C, sodium phosphate buffer, pH~7.4, 700 MHz Bruker NMR instrument, 1D ^{1}H-NMR measurement) with water suppression and baseline "error". In addition, we present an example of a well and a poorly shimmed sample, shown on the "well separated" singlet signal of the DSS (Ac-NGRA-NH_2, 46 °C, sodium phosphate buffer, pH~7.4, 700 MHz Bruker NMR instrument, 1D ^{1}H-NMR measurements).



Why was the pH only changed for -NGAA-?

In the case of the protected Ac-NGAA-NH_2 tetrapeptide, only the glycine N-H carries a "somewhat labile" proton, so a change in pH will mostly affect only this proton, making Ac-NGAA-NH_2 the simplest possible model for monitoring proton dissociation and

deamidation, which is the rate-determining step. Therefore, pH was monitored only for this model system. However, -NGRA-, -NGKA-, and -NGEA- peptides containing a charged amino acid at the (n+2) position were examined to study the effect of the charge of the amino acid closest to the reaction center. Furthermore, we do not expect any protonation/charge state changes for these three tetrapeptides in this pH range, based on their pK_a values.

L254: The authors conclude that NG isomerisation is spontaneous and can be controlled by adjusting the pH. The authors changed pH only for the -NGAA-. Could the authors clarify how they arrive at this conclusion?

We consider this reaction to be spontaneous, since isomerization has occurred in all cases studied so far. We could only influence the reaction rate by changing the external and internal conditions and parameters, but we could not stop the reaction to proceed. The effect of changing pH over this pH range was determined by considering the reaction rate coefficients and half-lives of the peptides, which consistently followed the direction of the pH change. In simple terms, if the pH is more acidic and the temperature is lowered, the isomerization reaction slows down, but it doesn't stop!

L298 Has the release of NH3 been detected? I am not 100% sure but it should be visible in the 1H NMR spectra.

Thank you for your question. We agree with the reviewer that an NH_3 release signal could be seen in unbuffered medium, but we believe that it is not or not clearly detectable in 50 mM phosphate buffer.

Response to Reviewer #2 comments:

Dear Reviewer #2,

Thank you for your comments and suggestions about this article. Please see our carefully considered responses below. They are also highlighted in yellow in the revised manuscript and Supporting Information.

Pilhál et al. aim at determining a kinetic and mechanistic model of Asn-Gly dipeptide bond isomerization. They use 1H NMR to follow the reaction in real time and derive a kinetic model. Further, they use quantum chemistry calculations to provide potential mechanisms of the rate-limiting step. Finally they investigate the effect of pH, temperature, and amino acid type (net charge) at the n+2 position. The manuscript is interesting for a board chemistry audience but suffers from readability issues and (I assume) from a lack of explanation on the main findings and how they are supported by experimental data. Novelty is also a concern that should be addressed before potential publication. In conclusion, major revision should be made before it can be assessed if the manuscript can be published in Communications Chemistry.

It is not very clear what key conclusion is driven by the experimental results. The authors go in great details about kinetic models and reaction micro-steps but do not really provide statements of their main findings, and they add to previous knowledge. What did they find that explain isomerization in a new light? Do they provide clear evidence for a specific kinetic model and reaction mechanism?

To the best of our current knowledge, our current results add the following specific discoveries to the NG literature:
1) No quantitative kinetic model was previously available. No information about k_2, k_{-2}, k_3, k_{-3}, ΔG_2, ΔG_{-2}, ΔG_3, and ΔG_{-3} was previously available, as only the rate constant of the first step (k_1) was determined for some cases. We have constructed a complete kinetic model of the ring closure and coupled reactions, including the rate constants k_1, k_2, k_{-2}, k_3 and k_{-3}, and the activation Gibbs free energies ΔG_1, ΔG_2, ΔG_{-2}, ΔG_3, and ΔG_{-3}. All parameters were determined using the COPASI program, based on the quantitative integral values of selected NMR resonances, which are proportional to the concentration of the different species.
2) We determined the elementary steps of the most probable deamidation pathway by performing a detailed and complete NBO analysis. The key steps such as the proton transfer step, the TS, the ring closure and the deamidation were determined. The evolution and coupling of these elementary steps are explained by using the second-order perturbation energies extracted from the NBO analysis and by investigating which natural bond orbitals interact with each other.
3) Finally, we investigated the driving force of the isomerization reaction for peptides containing both charged and neutral (n+2) amino acid residues. Contrary to previous conclusions suggesting that this reaction is charge-driven, we found that molecular geometry strongly influences the success of the reaction.
Based on our chemical knowledge and the acquired quantitative NMR data, we have identified the components involved in this isomerization reaction, which is in agreement with the previous literature data, at the same time, through these large number of new data, it allows clarifications and leads to a deeper understanding. The isomerization reaction takes place between Asn, via Suc, to obtain both α-Asp and β-Asp. This reaction mixture involves the following interconversions, namely the initial and fully irreversible deamidation of Asn during the formation of Suc, and the reversible hydrolysis of Suc to α-Asp and/or to β-Asp.

We have developed a quantitative kinetic model that includes all of the above 5 reactions, based on data fitting of the quantitative NMR data, and thus back-validated the established model.

I would advice the shape the manuscript around main findings:
- Kinetic model for isomerization: succinimide formation is the rate-limiting step
- The most probable mechanisms for succinimide formation is water-assisted?
- Reaction is dictated by side chain rotation angles, which explains influence of n+2 residue type

The novelty of findings is also not clear. A lot of aspects of the work were already discussed in the 2020 review (ref. 1) such as the effect of pH, temperature, local conformation, etc. The Introduction and Discussion should clearly state what is new in the current manuscript compared to previous knowledge.

Thank you very much for this advice, we have revised the text to make this point clearer. Following your recommendation, we have highlighted most of the novelties of the paper in the Abstract, Introduction, Aims, Results and Discussion and in the Conclusion. In summary, see the above 3 points where we have described 1) the quantitative kinetic model, 2) the key intermediate steps of deamidation and the explanation of them now revealed by NBO analysis, and 3) for peptides containing both charged and neutral (n+2) amino acid residues, geometry plays a more important role in their isomerisation reaction rates.

To me some the claims made are not fully supported by the data. Maybe they are by previous literature but then the novelty of the current work would be greatly diminished. Mainly, many other kinetic models for the reaction could be imagined. Why do the authors only consider one? Please either explain why no other model are possible, or provide a comparison of several different models. It is not enough to state that one model fits the data, especially since some of the rates do not seem to fit the data that well visually (for instance beta-Asp on Figure S4). A fair comparison of different model with a proper goodness of fit assessment and selection criterion between models is necessary to claim such statement.

Regarding the choice of the kinetic model described in Eq. (1), we can answer the following. 1) In ref. (2) of this manuscript, the same scheme was used to interpret the data, which resulted in a very good fit to the experimental results. This was the reason to use this model in the present study. 2) The current mechanism includes at least five key kinetic parameters (k_1, k_2, k_{-2}, k_3, and k_{-3}) to estimate and sometimes – in case there are no data measured early enough during the reaction – also a few initial concentrations as well. Thus, using five to eight parameters to estimate and having satisfactory fits in the overwhelming cases of experiments, we did not want to try more complicated mechanisms with more parameters. Of course there could be tested lots of more complicated mechanisms; the more complicated, the better fit could have been achieved. However, calculating e. g. the Akaike information criterion, it would turn out that this metrics would not have improve significantly by increasing the complexity of the model and the number of parameters. 3) In addition to the rate coefficients (k_1-k_{-3}), the model also allowed us to determine the corresponding Gibbs free energies (ΔG_1-ΔG_{-3}). In conclusion, the established model works well enough to give a coherent picture of both the kinetics and thermodynamics of the isomerization in focus. 4) The current first-order kinetic model is also supported by the chemical composition of the reaction mixture identified by NMR. We found the presence of four substances, namely Asn, Suc, β-Asp and α-Asp. Considering the trends of the 1D ^{1}H-NMR signal intensities, we can conclude that these 4 molecules are (only) involved in the coupled reactions. The initial and

irreversible deamidation starts with the formation of succinimide, followed by the reversible hydrolysis, linking α-Asp, succinimide and β-Asp moieties. 5) Finally, by plotting and analyzing the relative concentration-time curves of the reactant (Asn) and products, we obtained exponential curves typical of first-order reactions. By plotting the natural logarithm of the relative concentration values versus time, we obtained a "straight line" whose linearity is considered to prove that the kinetic is first order.

It is sometimes difficult to follow the authors train of thoughts because of the language and wording. There are many instances of poorly chosen words or typos that give an impression of draft rather than finished manuscript, and at worst make it difficult to understand what the authors actually meant. Please carefully read through and correct these. A few examples:

L51 – I assume we are talking about 'polypeptide' rather than 'polyamide' chain.

Thank you for pointing this out. We have corrected the text.

L52 – 'Xxx' shall be 'Gly', probably? Else, this sentence needs further explanation.

Thank you for pointing this out. The completed and corrected sentence is marked in blue on page 3: "Glycine, following asparagine with a small side chain, promotes isomerisation, probably due to the increased flexibility of the backbone atoms [2 4 5]."

L54 – 'smallest random coil shift' does not mean much to me, I think the authors are simply talking about small chemical shift in respect to the concept of electron 'shielding'. This has nothing to do with random coil.

Thank you for your comment. We have changed the text for better understanding (page 3 highlighted in yellow): "Comparison of the average chemical shifts of the different residue types shows that the smallest residues typically have smaller ^{15}N shifts at the amide bond, suggesting that these backbone N atoms have lower electron densities and their protons dissociate more readily, a prerequisite for nucleophilic attack."

L63 – Not sure what the authors define as transport molecules, but I assume something along the lines of 'ion-binding proteins'?

Thank you for your question. We wanted to emphasize here that even transporter molecules can be subject to deamidation, but the type of cargo molecule in this case seems irrelevant.

L76 – Is a more complex explanation a good thing? Do the authors mean a more complete model or a more exhaustive view?

Thank you for your comment. We have corrected the sentence accordingly (page 4 highlighted in yellow): "Here, an NMR-based kinetic analysis and a QM (quantum mechanical) derived reaction pathway with key intermediates provide reasonably but sufficiently detailed explanation for this reaction."

L116 – TOCSY and COSY are also 1H-NMR experiments.

We agree with the reviewer. We have clarified the text accordingly (page 5 highlighted in yellow): "Both 1D (Table S1) and 2D ^{1}H-NMR (COSY, TOCSY) experiments were used for resonance assignment."

Other minor comments:
L90 – A figure should be referenced so that the reader knows what reactions 1, 2 and 3 are.

Thank you for pointing this out. We have referenced it in the current version (page 4 highlighted in yellow): "Under different conditions, the data allowed to explain and characterize all coupled reactions of isomerisations k_1, k_2, k_{-2}, k_3, k_{-3}, ΔG_1, ΔG_2, ΔG_{-2}, ΔG_3, ΔG_{-3} (Figure 1-2)."

L116 – The NMR method section is not sufficiently well written to judge the quality of the procedure. Assignments do not provide quantitative data... Please explain separately how assignment, and how kinetic data were acquired and analysed.

Thank you for your comment. The NMR Methods section does indeed not describe in detail how the relative concentrations - needed for the kinetic model fitting with COPASI - are obtained from the NMR measurements. Please see the new and detailed NMR section in the Supporting Information, Part I./B. chapter. In the following section it is written that the integration of peak areas of ^{1}H-NMR signals is considered proportional to the concentration of the species belonging to that specific signal. These relative concentration values are the inputs to the kinetic model fitting described in detail in the chapter Kinetic inference of NMR data.

L175 – On what data is that claim based? Fitting NMR kinetics? Please elaborate.

Thank you for your questions. The reaction rate coefficients are derived by parameter estimation using the Copasi program, based on the integral values obtained from the 1D ^{1}H-NMR experiments. For a detailed explanation, please refer to our previous answer.

L231 – On what ground does it remain valid, a goodness of fit value?

Thank you for your question. Yes, we draw this conclusion based on the fitting errors of the kinetic model. Please refer to Table 1 for the exact values of the errors.

L245 – It would help to write the values obtained for Gibbs free energy of different contructs in the text.

Thank you for your advice. We added the values to the referred sentence highlighted with yellow (page 11):
"The activation Gibbs free energies of the -NGAA-, -NGE(-)A-, -NGK(+)A- and -NGR(+)A- peptides (110.8, 94.7, 87.3 and 97.3 kJ/mol, respectively) show that the activation energy of the rate-determining initial step decreases for -NGRA-, -NGEA- and -NGKA- when compared to -NGAA- (Table S2)."

Response to Reviewer #3 comments:

Dear Reviewer #3,

Thank you for your questions and suggestions about this article. Please see our thoughtful answers below. They are also highlighted in green in the revised manuscript and Supporting Information.

In the manuscript, the combined experiments and computational analysis have been performed to understand the kinetics and mechanism of the -Asn-Gly- (-Asn-Xxx-) isomerisation reactions in polypeptides. It was found that the formation of succinimide (Suc) from the Asn-Gly sequence is the rate-limiting step in the isomerisation process, which can be influenced by pH and temperature. In addition, the electrostatic catalysis from the positively charged residues at n+2 site can speed-up the reactions. I think most of the key conclusions are convincing to me. In addition, the work adds important insights on this biological process.

1. The unit of rate constant in Table 1 should be provided.

Thank you for your comment, it was indeed omitted from the table. It has now been corrected in the current version of the manuscript. Please note the dimension of the green highlighted rate constants in the new header of Table 1.

2. In Figure 1c, I guess that the free energy profile is obtained from experiments? This is should be clarified.

Thank you for pointing this out. Yes, the free energy profiles - now in Figure 2b - are based on parameter estimation of the experimental NMR data obtained at different temperatures, as described in Section II.A of the Supporting Information. In the current version, you will find the corrected and supplemented figure caption for Figure 2b (highlighted in green on page 10): "**b)** $\Delta G^{\ddagger}$ profile of the -α-NGAA- isomerisation reaction at pH 7.4, based on selected non-overlapping NMR signal integral data from ^{1}H-NMR experiments."

3. The calculated barriers (210.13 kJ/mol and 243.70 kJ/mol in the presence and absence of catalytic water) is much higher than the experimental value of 110.8 kcal/mol (Figure 1c), suggesting other factors involved in stabilizing transition states could be ignored. The authors can test the effect of more implicit waters, as done before (J. Comput. Chem. 2013, 34, 372-378; ACS Catal., 2015, 5, 7077-7090).

Thank you for your suggestion. The effect of the number of added water molecules (Asn, Asn-1H_2O and Asn-2H_2O) has been studied in the literature. (Catak et al. 2009) It was concluded that the reaction pathways containing one water molecule have a lower activation energy barrier than the reaction pathways without water. However, the activation energy barrier was lowest for reactions involving two water molecules. It is necessary to find a reasonable balance between chemical accuracy and computational requirements. Considering the above, we decided to use a simpler one-water model with a smaller number of possible conformations, while providing a suitable reaction environment for the deamidation reaction.

4. Can the isomerisation process be affected by other factors? Such as the ionic strength and types of pH buffer, which could be relevant to the natural process.

Thank you for your thoughtful question. According to the literature, both the ionic strength and the type of pH buffer can affect deamidation. Deamidation can be accelerated by increasing the ionic strength or at higher pH by using phosphate buffer instead of Tris buffer (37°C - pH 7.6, 40°C - pH 7.4).

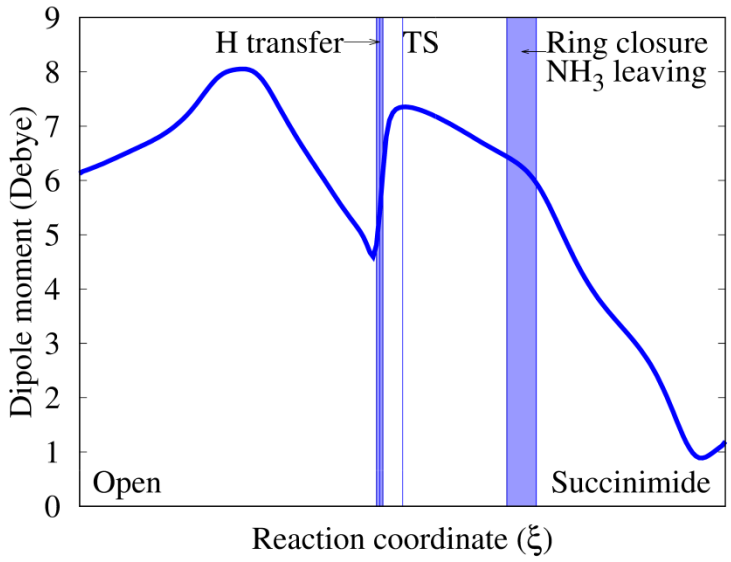
(A.L. Pace, R.L. Wong, Y.T. Zhang, Y.H. Kao, Y.J. Wang, Asparagine deamidation dependence on buffer type, pH, and temperature, *J. Pharm. Sci.* 102 (6) (2013) 1712–1723.;

J.W. Scotchler, A.B. Robinson, Deamidation of glutaminyl residues: dependence on pH, temperature, and ionic strength, *Anal. Biochem.* 59 (1) (1974) 319–322.;

Tyler-Cross, R., & Schirch, V., Effects of amino acid sequence, buffers, and ionic strength on the rate and mechanism of deamidation of asparagine residues in small peptides. *The Journal of biological chemistry*, *266*(33), (1991) 22549–22556.)

5. In order to better understand the electrostatic catalysis of positively charged (n+2) residues, it is better to calculate the dipole moment change from the reactant to the rate-determining TS state. With this information, the electrostatic catalysis of positively charged (n+2) residues can be analysed quantitively (J. Phys. Chem. B 2023, 127, 4245-4253).

Thank you, we have considered your suggestion. The cited article emphasizes the importance of the external electrostatic field for TS stabilization, which is indeed a critical factor, so we've now included it in the main text (highlighted in green on page 19), and in the Supporting Information (highlighted in green on page 36).
We verified our case by collecting the dipole moments of the water-assisted Ac-Asn-NH-CH_3 model along the IRC path. Please see the corresponding figure below.



Comparing the change in dipole moment with the change in ESP charge differences between the C^{γ}_{Asn} and N_{Gly} atoms (Figure 6j), we see that they follow a very similar path, but with a slight phase shift. The dipole moment values of the Ac-Asn-Gly-Arg(+)-NH-CH_3, Ac-Asn-Gly-Ala-NH-CH_3 and Ac-Asn-Gly-Glu(-)-NH-CH_3 one-water systems are 19.7472 Debye, 10.7260 Debye and 6.2423 Debye, respectively. Meanwhile, the local ESP charge and other geometry parameters (see Table S22 in the Supplementary Information) are similar. It can be concluded that although the global electrostatic environment around the reaction center is different, no significant local dipole moment difference is expected, since these numbers are basically the same. This shows that the neighboring (n+2) amino acids

exert their effect on the reaction by influencing the local geometry rather than the electrostatics of the molecule.

6. The detailed information in Table 2 can be moved to SI.

Thank you for this advice, it has been transferred to SI as Table S22.