

Non-enzymatic coupling of protometabolic reactions with a prebiotic redox cofactor

Corresponding Author: Professor Andrés de la Escosura

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

SUMMARY:

This work investigates whether simple, prebiotically plausible pyridinium/dihydropyridine pairs could have acted as redox cofactors, similar to NAD⁺/NADH, in early metabolism. Using pyruvate as a model substrate, the authors show that these proto-cofactors can mediate both oxidative decarboxylation (catabolic) and reductive amination (anabolic) reactions non-enzymatically, yielding acetate and alanine respectively. The authors support their conclusions through 1H-NMR spectroscopy, cyclic voltammetry, and DFT calculations, proposing that these simple molecules could have linked primitive redox chemistry to early metabolic networks through mechanisms similar to those observed in modern biology.

Overview:

The identification of redox chemistry that could have driven early metabolism prior to the emergence of complex enzymes is a significant scientific inquiry with broad implications for the origins of life.

As the authors cite, non-enzymatic NAD⁺-mediated α -keto acid oxidation to carboxylates and NADH-mediated reduction to amino acids/hydroxy acids have been demonstrated previously (citations 25–28). Thus, the major contributions provided by this new work are:

- 1) Nicotinamide cofactors and their analogs may be “capable of linking catabolic and anabolic transformations” under non-enzymatic conditions. This would entail cycling/turnover of the redox cofactors.
- 2) An evaluation of how prebiotically plausible “simpler analogues may have served a similar function in early prebiotic chemistry, acting as reversible redox agents capable of coupling oxidative and reductive transformations, thus contributing to the emergence of primitive protometabolic networks.” This work also enabled the authors to evaluate non-enzymatic reaction mechanisms that were unconfirmed in the current literature.

Related to Contribution 1, the authors demonstrated that both the oxidative and reductive half reactions proceed under common conditions (Figure 2c), yielding 6-8% of both acetate and alanine. However, the linked/cycling aspect of the redox cofactors could have been more compelling; for example, the paper would have been improved by a clear demonstration that 1aRed produced from the α -keto acid oxidation is capable of subsequent α -keto acid reduction. For example: pyruvate + 1aOx (deuterated) $\rightarrow$ acetate + 1a Red (deuterated), then subsequently in situ pyruvate + 1a Red (deuterated) $\rightarrow$ alanine (deuterated). I am not suggesting that this type of experiment is a requirement, but without further experimental demonstration and/or controls as noted below, I am not 100% certain that redox cycling has been fully demonstrated.

As the authors note, there is data in the current manuscript consistent with the turnover/redox cycling. For example, data in 2C 100:0 indicates that small amounts of alanine were detected, even though only oxidant was included in the reaction (potentially indicating that in situ generated 1aRed was able to reduce pyruvate). However, this quantification needs to be verified with controls showing no trace alanine generated without 1aOx added. I appreciate the extensive supplementary materials (131 pages!), but I could not find that relevant control reaction. If I missed it, I withdraw my concern, but suggest they mention it in the main manuscript.

The 2C 0:100 data may also demonstrate linked/cycling redox reactions by indicating ~3% acetate formation with no added oxidant. However, I again would need a control to feel confident about this interpretation; α -ketoacids are readily oxidized by even trace amounts of O_2 , which are hard to remove even under the most stringent inert conditions. A control showing no acetate production without added 1aOx would be required to safely interpret this data (again I could not find this control in the extensive supplementary data, but could have overlooked it). This also pertains to the data in the last column of Table 1, which is consistent with cycling.

For contribution 2, the extensive table (Table 1) of nicotinamide analogs shows the effect of N and C3 substituents on overall redox potential. The data nicely indicates that electron-deficient substituents on C3 (like the amide in native NAD⁺) are necessary to oxidize α -ketoacids, and that the amide is in a goldilocks zone of electron density so that the reaction is facile, but not irreversible. The amide was also found to resist hydrolytic degradation compared to other electron-withdrawing groups. This data was further corroborated by DFT calculations. As far as I am aware, the presented cv data is also the first experimental exploration of the non-enzymatic pyridinium-mediated oxidative decarboxylation reaction, and confirms a previous theoretical radical mechanism. The use of multiple α -ketoacids (pyruvate, α -ketoglutarate, glyoxylate) also shows that the results are not substrate-specific.

Combined, I find these two contributions to be significant and worthy of publication.

Minor Weaknesses

- Some required control experiments appear to be lacking.
- The differing quantities of acetate and dihydropyridine are concerning. More experimentation might help account for the degradation of the NADH analog, but this may be beyond the scope of the work.

Minor Notes and Edits:

- Fig. 2C - typo (piruvate)
- List reaction temperature more prominently (e.g. main text and Fig 2 legend.)
- I suggest revisiting the graphical abstract; it is an efficient overview of the work, but not an good introduction to the work. Without first reading the paper, it is challenging to interpret, particularly the left half.

Reviewer #2

(Remarks to the Author)

Gonzalez-Martinez et al. investigated the ability of a simpler analogue of NAD⁺/NADH to participate in the capture and donation of electrons from/to α -ketoacids, producing amino acids in the process (during the reduction). The authors identify a molecule (1a) as a somewhat ideal prebiotic analogue. This molecule retains the carbamoyl group at the 3 position of the pyridinium ring. Instead of an ADP moiety (referred to here as ADPR), the molecule has a CH₂CONH₂ N-substituent. Results are supported by cyclic voltammetry and DFT calculations.

This work builds on previously published studies. The reduction of NAD⁺ and NAD⁺ analogues by α -ketoacids was reported by the Mansy group (refs 25 & 26), and the reductive amination of α -ketoacids by NADH was reported on by the group of the corresponding author (ref 28). Related reactions were also previously published, such as the reduction of NAD⁺ by H₂ and a mineral catalyst (ref 10, Preiner group) and the nonenzymatic reduction of α -ketoacids by NADH to produce α -hydroxy acids (ref 27, Moran group).

The key insight here seems to be identification of a simpler analogue that when placed under the proper conditions (pH 8.5 and 600 mM ammonium) can mediate both oxidation and reduction reactions (as in refs 25, 26, and 28) resulting in the synthesis of amino acids, which is undoubtedly a quite nice result.

It would be helpful if the authors could comment on why acetate yield keeps increasing while PyH does not in Figure 2, panel a. Similarly, why does Py⁺ outpace Alanine in panel b. Finally, the shapes of the curves in panel c are different when comparing the reduction of the NAD analogue and the synthesis of alanine. These are all worth commenting on.

On page 4 when discussing these results, it is stated that "Pyridinium 1a_{ox} was also formed in 17% yield..." I don't understand why "also" is used. This refers to panel b. In panel a, 17% of acetate was formed. In panel b it's 17% of oxidized pyridinium 1a. If panel a showed 17% reduced pyridinium 1a or if panel b showed 17% production of alanine, I would understand better the comparison. The reason for comparing the yield of acetate and oxidized pyridinium 1a from two different reactions is not clear to me.

It is stated on page 6 that "among the redox pairs studied (Figure 1B) only 1a operated efficiently in a reversible manner..." 1a certainly performed well. My problem is that no indications of error were mentioned. I cannot understand if 1a is really different from 1c or 1e (or even from NAD⁺/NADH) or if everything is within error.

page 6: "Notably, the reaction did not proceed at pH 7.0 and gave only low yields at pH 8.0." In reference 26 this was also observed, and it was shown that activity could be recovered in the presence of Mg²⁺. Reference 27 also noted a role of metals (Al³⁺, Fe^{2+/3+}) in reduction. Have the authors investigated or noted an affect of metals on this observed chemistry? Would an effect be expected? When considering prebiotic conditions, wouldn't the presence of metals be expected?

The structure of the oxidized Py⁺-pyruvate adduct in scheme 1 of the supplemental section was previously observed by mass spectrometry in reference 26 (figures 3, S10-S12).

The observation of the intermediates by cyclic voltammetry is impressive.

I was a bit disappointed by the lack of discussion of the effect of N-substitution. There are just a couple of sentences at the end of page 8 saying that this position is important, whereas a "bare nicotinamide" is not reactive. The demonstration of "bare nicotinamide" not being able to accept electrons from α -ketoacids was already shown in reference 26 (as well as several other derivatives). I make this point simply because the introduction states that the ADPR part of NAD⁺/NADH is primarily necessary in modern biology as an anchoring group. It seems that a little more attention to the interpretation of the N-substituted derivatives is warranted, particularly since references 10 and 26 make the argument that this part of the molecule is important. Unless I missed it, I do not believe any of the presented calculations explored the N-substituents.

To be clear, I think this is nice work and makes a meaningful contribution to the field. The demonstration that under a single condition we can observe the nonenzymatic acceptance of electrons from α -ketoacids to a NAD⁺ analogue and that these electrons can support the synthesis of amino acids from α -ketoacids is instructive for how early metabolic-like chemistry could have emerged. However, I also believe that my points above should be considered during revisions to make this manuscript stronger.

Reviewer #3

(Remarks to the Author)

The manuscript "Non-enzymatic coupling of protometabolic reactions with a prebiotic redox cofactor", submitted to Nature Communications, investigates the potential for simple pyridinium/dihydropyridine pairs to act as non-enzymatic redox cofactors in a primordial protometabolism. As I was explicitly asked by the editor to provide an expertise on the theoretical calculation part, I do not discuss much here the experimental demonstration of coupled oxidative decarboxylation and reductive amination, which is definitely very interesting. Instead, I perfectly understand that the theoretical framework is a complement to the massive experimental data, and thus intended to provide the "physicochemical rationale" for the selection of nicotinamide, but I do not find it completely convincing. The authors rely on static, gas-phase-like DFT calculations to claim a sort of "Goldilocks" behavior for the carbamoyl group. However, the exclusion of dynamical effects and explicit solvent interactions, factors that are crucial in the high-entropy, condensed-phase environments of prebiotic chemistry, seriously limits, I believe, the reliability and accuracy of the reported free energies and the validity of the resulting conclusions.

More specifically, my main criticism concerns the reliability of free energies in static DFT. One of the central theses of the paper rests on the precision of the calculated $\Delta G^\ddagger$ values (e.g., the 9.4 kJ/mol difference between 1ared and 1dred) to explain reactivity trends. However, the use of static stationary point analysis (M06-2X/cc-pVTZ) completely ignores the entropic and dynamical contributions of the solvent. In prebiotic aqueous environments, particularly those hypothesized at hydrothermal vents or surface ponds, the reorganization energy of water is a dominant term in the activation barrier.

Indeed, the authors employ the SMD implicit model to represent water, but the reactions involve highly charged or polar species, including iminium ions, pyridinium rings, and enolates. Implicit models fail to capture the local hydrogen-bonding networks that stabilize these transition states. For a paper aiming for Nature Communications impact, the absence of at least a hybrid implicit/explicit solvation shell is a major oversight.

Similarly, the "vertical" ionization potentials reported (Fig 3F) are calculated in a static framework. Without accounting for the solvent's electronic and nuclear relaxation during the electron transfer event, the reported values (4.57 eV for the enolate) cannot be considered accurate representations of the process in a prebiotic aqueous medium.

Also, the barrierless decarboxylation, which the authors claim once the keto radical is formed, cannot be considered completely convincing. Again, this conclusion is derived from a static potential energy surface scan. In a dynamical system with explicit solvent, "barrierless" steps on an electronic surface frequently manifest as diffusion-limited or solvent-reorganization-limited processes. By characterizing this step as barrierless without dynamical verification, the authors bypass the very kinetic constraints they later cite to explain their low experimental yields.

Finally, the study correlates reactivity with LUMO energies and NBO charges derived from static structures. While these trends are qualitatively suggestive, the attempt to provide a "physicochemical rationale" for natural selection requires a more rigorous assessment of the reaction coordinate that goes beyond static electronic properties. The balance is asserted based on a narrow set of substituents without exploring how environmental fluctuations (temperature, pH shifts, mineral surfaces) would perturb these static energy levels.

Again, I understand that theoretical calculations are presented here just in support, and they take a very minor part of the paper (a little more than a paragraph in the main manuscript, 5 pages out of 130 of the SI). Still, in its current state, with respect to such theoretical calculations, I feel that the manuscript does not meet the technical rigor required for Nature Communications. The DFT results, which are intended to provide the mechanistic "why" behind the experimental "what," are way too simplified to be predictive or conclusive for prebiotic chemistry.

If the authors consider them to be a necessary validation/interpretation of their experimental results, I recommend them to provide, at the very least, some calculations including explicit solvent molecules, at least a first solvation shell, for the transition states of the hydride transfer to assess the impact of hydrogen bonding on the reported $\Delta\Delta G^\ddagger$. More generally, in the absence of proper *ab initio* molecular dynamics simulations and enhanced-sampling calculations, a more nuanced discussion of the errors inherent in static DFT must be put out, when estimating free energies for biological cofactors in condensed phases.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have satisfied my concerns with their edits and additions, and I now strongly recommend publication.

Reviewer #2

(Remarks to the Author)

I am satisfied with the authors responses and changes to the manuscript. I have no further criticisms.

Reviewer #3

(Remarks to the Author)

The authors have revised the theoretical section and the Supporting Information following my previous comments, and I appreciate the effort to clarify the scope and the limitations of the calculations. In particular, the paragraph added at the beginning of Section 14 of the SI makes clear that the reported free energies come from static DFT with implicit solvation, and that the results should be interpreted only in a qualitative way. This improves the transparency of the work.

I also note that additional explicit-solvent cluster calculations were included for the ionization potentials, and that the interpretation of the results has been moderated accordingly. These changes go in the right direction.

At the same time, the level of theory remains limited to static DFT, and more advanced calculations mentioned in my previous report (for example explicit solvent treatment of transition states or dynamical sampling) were not performed. The authors explain that this is beyond the scope of the present study, which is acceptable given that the theoretical part represents only a small portion of the manuscript and is mainly used to support the experimental results.

Since I was asked to evaluate mainly the theoretical aspects, I consider that my concerns about clarity and interpretation are now sufficiently addressed, even if the methodology itself has not changed. I therefore leave the final judgement to the editor and to the other referees, who are more qualified to evaluate the experimental part and the overall impact of the work.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

SUMMARY:

This work investigates whether simple, prebiotically plausible pyridinium/dihydropyridine pairs could have acted as redox cofactors, similar to NAD^+/NADH , in early metabolism. Using pyruvate as a model substrate, the authors show that these proto-cofactors can mediate both oxidative decarboxylation (catabolic) and reductive amination (anabolic) reactions non-enzymatically, yielding acetate and alanine respectively. The authors support their conclusions through ^1H -NMR spectroscopy, cyclic voltammetry, and DFT calculations, proposing that these simple molecules could have linked primitive redox chemistry to early metabolic networks through mechanisms similar to those observed in modern biology.

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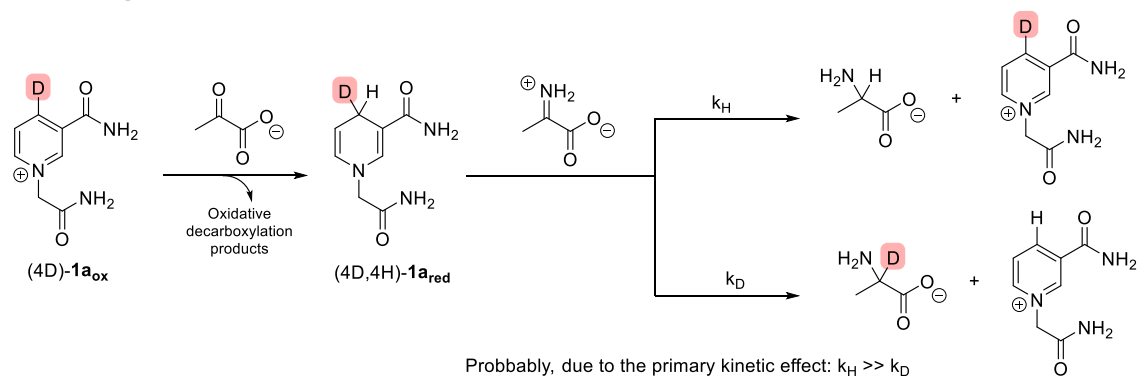
We sincerely thank the reviewer for this thoughtful and comprehensive assessment of our work. We greatly appreciate the recognition of the significance of investigating prebiotically plausible redox systems.

Related to Contribution 1, the authors demonstrated that both the oxidative and reductive half reactions proceed under common conditions (Figure 2c), yielding 6-8% of both acetate and alanine. However, the linked/cycling aspect of the redox cofactors could have been more compelling; for example, the paper would have been improved by a clear demonstration that 1aRed produced from the α -keto acid oxidation is capable of subsequent α -keto acid reduction. For example: pyruvate + 1aOx (deuterated) $\rightarrow$ acetate + 1a Red (deuterated), then subsequently in situ pyruvate + 1a Red (deuterated) $\rightarrow$ alanine (deuterated). I am not suggesting that this type of experiment is a requirement, but without

further experimental demonstration and/or controls as noted below, I am not 100% certain that redox cycling has been fully demonstrated.

We agree that labelling experiments in which the labelled element “travels” through the molecules involved in both reactions would provide compelling evidence that the two reactions are coupled, as shown in the cycle described in Figure 1C. Indeed, the reductive amination reaction involves a hydride transfer and, in principle, a deuterium could certainly pass from **1a_{red}** to the substrate. In contrast, in the oxidative decarboxylation, **1a_{ox}** receives electrons from the α -ketoacid while protonation occurs from the aqueous medium. Therefore, there is not a direct nucleus transfer from the α -ketoacid to **1a_{ox}**.

As suggested by the reviewer, one possible approach involves starting from 4-deuterated **1a_{ox}**, i.e., (4D)-**1a_{ox}**. Once this **4D-1a_{ox}** acts as oxidant of the α -ketoacid, it will be transformed into the monodeuterated (4D,4H)-**1a_{red}**. At best, the *in situ* generated (4D,4H)-**1a_{red}** will promote the reductive amination, forming a mixture of alanine-2-*d* and non-deuterated alanine (see Scheme below). However, the primary isotopic kinetic effect might favour considerably the transfer of the non-deuterated hydride over the deuterated one, so the absence of alanine-2-*d* would not definitively rule out the coupling of the two reactions shown Figure 1C.



Considering these mechanistic and interpretative challenges for the labelling experiment, the reviewer’s request of providing more proofs of redox cycle occurrence has been addressed through carefully designed control experiments, which we believe fully support for redox cycling under the conditions investigated (see next comment).

As the authors note, there is data in the current manuscript consistent with the turnover/redox cycling. For example, data in 2C 100:0 indicates that small amounts of alanine were detected, even though only oxidant was included in the reaction (potentially indicating that *in situ* generated **1a_{red}** was able to reduce pyruvate). However, this quantification needs to be verified with controls showing no trace alanine generated without **1a_{ox}** added. I appreciate the extensive supplementary materials (131 pages!), but I could not find that relevant control reaction. If I missed it, I withdraw my concern, but suggest they mention it in the main manuscript.

We apologize for missing the inclusion of the control experiments in the SI document due to the large amount of data already included in the previous draft. We agree with the reviewer about the importance of such controls, which have now been added for pyruvate as substrate in the most relevant conditions of this work: NaHCO₃/Na₂CO₃, (200 mM, pH 9.0), NH₄Cl/NH₃ (600 mM, pH= 8.5), NH₄Cl/NH₃ (600 mM, pH= 9.0). The results are now

collected in Table S1 and Figure S2 (pages S14-S15). Importantly, as we have recalled in this section of the revised SI (page S15, first paragraph), none of the controls, including those in ammonia/ammonium solution, showed any trace of alanine formation. Therefore, the presence of alanine in the reactions of pyruvate and **1a_{ox}** must necessarily be consequence of the action of the reductant **1a_{red}** being formed *in situ*.

The 2C 0:100 data may also demonstrate linked/cycling redox reactions by indicating ~3% acetate formation with no added oxidant. However, I again would need a control to feel confident about this interpretation; α -ketoacids are readily oxidized by even trace amounts of O₂, which are hard to remove even under the most stringent inert conditions. A control showing no acetate production without added **1a_{ox}** would be required to safely interpret this data (again I could not find this control in the extensive supplementary data, but could have overlooked it). This also pertains to the data in the last column of Table 1, which is consistent with cycling.

This is again a good point of the reviewer. As a matter of fact, control reactions of pyruvate in carbonate or phosphate buffer (Table S1, page S14) always show small amounts of acetate, probably caused by the presence of traces of O₂. However, as shown in the newly added Figure S5B (page S18), this background contribution (below 0.16%) is negligible when compared to the acetate formation caused by the cofactor analogue **1a_{ox}** (16.8%).

The background formation of acetate is somewhat more relevant in ammonia/ammonium solution, being slightly higher at pH 8.5 compared to pH 8.0 (Table S1). Figure S7 (page S19) and Figure S55 (page S69) compare, for pH 8.0 and pH 8.5 respectively, the background yield of acetate in NH₃/NH₄Cl media with that obtained in the reaction of **1a_{red}** + **2a** under the same conditions. In both cases, while the background reaction is detectable, the substantial increase in acetate yield in the reaction mixtures is attributable to the action of the *in situ* generated **1a_{ox}**.

The inclusion of these control experiments in the SI and their explicit mention (note 38) in the revised manuscript provides a clearer and more transparent interpretation of the results. In addition, we have included control experiments of α -ketoacids **2b** and **2c** (Figure S3 and Figure S4 in pages S16-S17) in ammonia/ammonium media for a more accurate interpretation of all results in Table 1.

For contribution 2, the extensive table (Table 1) of nicotinamide analogs shows the effect of N and C3 substituents on overall redox potential. The data nicely indicates that electron-deficient substituents on C3 (like the amide in native NAD⁺) are necessary to oxidize α -ketoacids, and that the amide is in a goldilocks zone of electron density so that the reaction is facile, but not irreversible. The amide was also found to resist hydrolytic degradation compared to other electron-withdrawing groups. This data was further corroborated by DFT calculations. As far as I am aware, the presented cv data is also the first experimental exploration of the non-enzymatic pyridinium-mediated oxidative decarboxylation reaction, and confirms a previous theoretical radical mechanism. The use of multiple α -ketoacids (pyruvate, α -ketoglutarate, glyoxylate) also shows that the results are not substrate-specific.

Combined, I find these two contributions to be significant and worthy of publication.

We appreciate the reviewer's insightful comments, which have helped us further clarify and strengthen the manuscript.

Minor Weaknesses

- Some required control experiments appear to be lacking.

As described previously, the pertinent control experiments have been added to the Supporting Information (Figures S5, S7 and S55) and mentioned in note 38 of the main text. We have reordered some sections of the SI document to accommodate the data of these new control experiments

- The differing quantities of acetate and dihydropyridine are concerning. More experimentation might help account for the degradation of the NADH analog, but this may be beyond the scope of the work.

Indeed, we are currently performing investigations in which these NAD⁺/NADH analogues are studied in more complex protometabolic networks, and relevant data about their stability is being collected. We hope that the future publication of this ongoing project will shed light about this question.

Minor Notes and Edits:

- Fig. 2C - typo (piruvate)

Thank you for this indication. The typo has been corrected. We have corrected other typos in the Supporting Information that were found during the revision.

- List reaction temperature more prominently (e.g. main text and Fig 2 legend.)

The information regarding the reaction temperature has been added in the main text (page 4, 2nd paragraph, line 2; and page 4, 3rd paragraph, line 2), and at the caption of Figure 2 and Table 1.

- I suggest revisiting the graphical abstract; it is an efficient overview of the work, but not a good introduction to the work. Without first reading the paper, it is challenging to interpret, particularly the left half.

We have modified the left half of the graphical abstract, with the intention to clarify the rationale that brought us from NAD⁺/NADH to the pyridinium/dihydropyridine pairs.

In addition to reviewer's requirements, given the importance of control experiments for interpreting the results, we replaced the NAD⁺ control in carbonate originally performed at 15 mM with the corresponding experiment at 30 mM (the real concentration in the reactions), enabling a more accurate comparison with the studied reaction. Another typo in Table 1, related to **1a_{red}** formation in the control, has also been corrected.

Reviewer #2 (Remarks to the Author):

Gonzalez-Martinez et al. investigated the ability of a simpler analogue of NAD⁺/NADH to participate in the capture and donation of electrons from/to alpha-ketoacids, producing amino acids in the process (during the reduction). The authors identify a molecule (**1a**) as a somewhat ideal prebiotic analogue. This molecule retains the carbamoyl group at the 3 position of the pyridinium ring. Instead of an ADP moiety (referred to here as ADPR), the molecule has a CH₂CONH₂ N-substituent. Results are supported by cyclic voltammetry and DFT calculations.

This work builds on previously published studies. The reduction of NAD⁺ and NAD⁺ analogues by alpha-ketoacids was reported by the Mansy group (refs 25 & 26), and the reductive amination of alpha-ketoacids by NADH was reported on by the group of the corresponding author (ref 28). Related reactions were also previously published, such as the reduction of NAD⁺ by H₂ and a mineral catalyst (ref 10, Preiner group) and the nonenzymatic reduction of alpha-ketoacids by NADH to produce alpha-hydroxy acids (ref 27, Moran group).

The key insight here seems to be identification of a simpler analogue that when placed under the proper conditions (pH 8.5 and 600 mM ammonium) can mediate both oxidation and reduction reactions (as in refs 25, 26, and 28) resulting in the synthesis of amino acids, which is undoubtedly a quite nice result.

We thank the reviewer for this careful contextualization and evaluation of our work.

It would be helpful if the authors could comment on why acetate yield keeps increasing while PyH does not in Figure 2, panel a. Similarly, why does Py⁺ outpace Alanine in panel b. Finally, the shapes of the curves in panel c are different when comparing the reduction of the NAD analogue and the synthesis of alanine. These are all worth commenting on.

We thank the reviewer for these insightful questions and we agree that all these issues require more discussion:

1) Why does acetate yield keep increasing while PyH does not in Figure 2?

Control experiments with **1a_{red}** (PyH) (Table S6, page S23) indicate that even in carbonate buffer alone, gradual formation of **1a_{ox}** (Py⁺) is observed, highlighting the intrinsic redox sensitivity of this dihydropyridine.

Under the reaction conditions, **1a_{red}** is formed continuously from **1a_{ox}** through oxidative decarboxylation of the α-ketoacid (and additionally through the formation of **Py⁺-Pyruv**; see Scheme S1, page S22). However, it can also undergo secondary redox processes, likely reacting with minor byproducts present in the reaction mixture and returning to **1a_{ox}**. As these byproducts accumulate over time, the rate of **1a_{red}** consumption can progressively compete with its rate of formation. This dynamic behaviour explains why acetate formation continues to increase, while the steady-state concentration of **1a_{red}** does not accumulate proportionally at medium-long reaction times. We have clarified this point in page 4, 2nd paragraph, line 11, claiming that this behaviour provides "... a temporal window with **1a_{red}** steady-state concentration for its participation in subsequent anabolic transformations." In light of the reviewer's comment, we have also moved the note regarding the physiological

NAD⁺/NADH cytoplasmatic ratio (10^3) to the main text (page 4, 2nd paragraph, line 12), to better contextualize the potential functional relevance of low but sustained concentrations of the NADH analogue in protometabolic settings.

2) Why does Py⁺ outpace alanine in panel b?

Control experiments with **1a_{red}** (PyH) in ammonia/ammonium solution (Table S27, page S46) indicate a higher tendency toward oxidation to **1a_{ox}** (Py⁺) compared to carbonate buffer (Table S6, page S23), reflecting the greater redox lability of the dihydropyridine under these conditions.

As discussed above, the reaction medium constitutes a dynamic redox environment in which **1a_{red}** is both consumed and formed. In addition to its role in reductive amination, **1a_{red}** can undergo partial oxidation through interaction with reactive intermediates or minor byproducts present in the mixture. Consequently, the observed accumulation of **1a_{ox}** reflects not only the productive reductive amination pathway but also these competing oxidative processes.

At the same time, a fraction of the generated **1a_{ox}** is further engaged in oxidative decarboxylation to form acetate and in the formation of **Py⁺-Pyruv** adducts, contributing to the complex but internally consistent redox balance observed in panel b of Figure 1.

3) The shapes of the curves in panel C are different when comparing the reduction of the NAD analogue and the synthesis of alanine.

In fact, in Figure 2, the experiment shown in panel B and the experiment shown in the rightmost figure of panel C are the same, with the only difference than the former is carried out at pH 8.0 and the latter at pH 8.5. Up to 72 hours, alanine yields are comparable under both conditions. At longer reactions times, alanine increases notably in the pH 8.0 reaction, while the curve approaches to a plateau for pH 8.5. In our previous work (<https://doi.org/10.1039/d4qo00050a>), we showed that NADH-mediated reductive amination is optimal around pH 8 and decreases at higher pH, which we attributed to the lower steady-state concentration of the corresponding iminium intermediate under more basic conditions (<https://doi.org/10.1002/anie.202212237>). The present system appears to follow the same tendency, which is likely behind this subtle differences in curve shape. Unfortunately, as described in Figure S6 (pages S18-S19), the influence of pH in this reductive amination could not be properly addressed due to experimental issues.

On page 4 when discussing these results, it is stated that "Pyridinium 1a_{ox} was also formed in 17% yield..." I don't understand why "also" is used. This refers to panel b. In panel a, 17% of acetate was formed. In panel b it's 17% of oxidized pyridinium 1a. If panel a showed 17% reduced pyridinium 1a or if panel b showed 17% production of alanine, I would understand better the comparison. The reason for comparing the yield of acetate and oxidized pyridinium 1a from two different reactions is not clear to me.

We apologize for the ambiguity in this sentence. Our intention was not to compare the 17% yield of pyridinium **1a_{ox}** in panel B with the 17% yield of acetate in panel A, as these values are unrelated. Rather, the term "also" was intended to indicate: "in addition (to alanine formation mentioned in the preceding sentence), pyridinium **1a_{ox}** was formed in 17% yield". We have revised this sentence to remove the ambiguity (see page 4, last paragraph, line 4 of the revised version).

It is stated on page 6 that "among the redox pairs studied (Figure 1B) only 1a operated efficiently in a reversible manner..." 1a certainly performed well. My problem is that no indications of error were mentioned. I cannot understand if 1a is really different from 1c or 1e (or even from NAD⁺/NADH) or if everything is within error.

We thank the reviewer for this important observation. We agree that the sentence "among the redox pairs studied (Figure 1B) only 1a operated efficiently in a reversible manner" does not accurately reflect the full set of results and we have changed it for a more precise description. The sentence now reads as "Among the redox pairs studied (Figure 1B), **1a** was optimal in terms of efficiency, reversibility and stability" (page 6, last paragraph, 1st line). This statement is supported by the following considerations:

Under the investigated conditions, three redox pairs exhibit reversible behaviour: **1a_{ox}/1a_{red}**, **1c_{ox}/1c_{red}** and **1e_{ox}/1e_{red}**. While Table 1 reports data at 168 h for clarity, the complete kinetic profiles, including experimental errors, are provided in the Supporting Information, in section 6 (pages S20-S43) and in section 7 (pages S44-S64). This clarification has been included in the caption of Table 1, so that the reader can check those errors in the corresponding sections of the SI document.

Comparison of both oxidative decarboxylation and reductive amination profiles indicates that **1c_{ox}/1c_{red}** consistently affords lower yields for both transformations, with differences exceeding experimental errors. In contrast, **1a_{ox}/1a_{red}** and **1e_{ox}/1e_{red}** display comparable efficiencies within the margin of experimental uncertainty. However, the **1e_{ox}/1e_{red}** pair shows lower stability under the reaction conditions. **1a_{ox}/1a_{red}** is therefore the optimal redox pair in terms of yield, stability and reversibility.

Finally, NAD⁺/NADH exhibits efficiencies comparable to those of **1a_{ox}/1a_{red}**, and the data are given for comparison. Nevertheless, the objective of this work is to evaluate whether structurally simple pyridinium/dihydropyridine systems could plausibly operate under prebiotic conditions, rather than outperform the biological cofactor.

page 6: "Notably, the reaction did not proceed at pH 7.0 and gave only low yields at pH 8.0." In reference 26 this was also observed, and it was shown that activity could be recovered in the presence of Mg²⁺. Reference 27 also noted a role of metals (Al³⁺, Fe^{2+/3+}) in reduction. Have the authors investigated or noted an effect of metals on this observed chemistry? Would an effect be expected? When considering prebiotic conditions, wouldn't the presence of metals be expected?

We thank the reviewer for this suggestion. We had actually conducted some studies of the activity of metal ions in the reaction promoted by the **1a** pyridinium/dihydropyridine system, and we have now completed them with additional experiments. All these results have been compacted in the new Figure S56 (page S71) and discussed in the Supporting Information with the following text.

"These experiments reveal that metal ions interact with the redox pair and substrates, influencing several aspects of the observed reactivity. While a comprehensive investigation of metal-cofactor interactions is beyond the scope of the present work, our preliminary findings demonstrate that the redox activity of **1a_{ox}/1a_{red}** is compatible with the presence of prebiotically plausible metal ions and provide a basis for identifying further beneficial metal-mediated effects.

Accordingly, we have introduced a new sentence in page 11, 3rd paragraph, line 5 to summarize these conclusions.

The structure of the oxidized Py⁺-pyruvate adduct in scheme 1 of the supplemental section was previously observed by mass spectrometry in reference 26 (figures 3, S10-S12).

Thank you for this observation: we have added the correct reference and mentioned the adduct in note 39 of the references section.

The observation of the intermediates by cyclic voltammetry is impressive.

We appreciate these encouraging words.

I was a bit disappointed by the lack of discussion of the effect of N-substitution. There are just a couple of sentences at the end of page 8 saying that this position is important, whereas a "bare nicotinamide" is not reactive. The demonstration of "bare nicotinamide" not being able to accept electrons from alpha-ketoacids was already shown in reference 26 (as well as several other derivatives). I make this point simply because the introduction states that the ADPR part of NAD⁺/NADH is primarily necessary in modern biology as an anchoring group. It seems that a little more attention to the interpretation of the N-substituted derivatives is warranted, particularly since references 10 and 26 make the argument that this part of the molecule is important. Unless I missed it, I do not believe any of the presented calculations explored the N-substituents.

We agree with the reviewer that this aspect deserves to be emphasized. Its discussion in the previous version was brought to the Supporting Information for not extending excessively the article length. In response to the reviewer's suggestion, we have now moved it from the SI document to the main text, and constitutes a whole new paragraph between page 8 and page 9 (see manuscript version with changes highlighted).

In addition, we have expanded the computational analysis and included new calculations in the SI specifically addressing the effect of N-substitution (Figure S76, page S103), in parallel to the 3-substitution studies presented in the main text.

To be clear, I think this is nice work and makes a meaningful contribution to the field. The demonstration that under a single condition we can observe the nonenzymatic acceptance of electrons from alpha-ketoacids to a NAD⁺ analogue and that these electrons can support the synthesis of amino acids from alpha-ketoacids is instructive for how early metabolic-like chemistry could have emerged. However, I also believe that my points above should be considered during revisions to make this manuscript stronger.

Reviewer #3 (Remarks to the Author):

The manuscript "Non-enzymatic coupling of protometabolic reactions with a prebiotic redox cofactor", submitted to Nature Communications, investigates the potential for simple pyridinium/dihydropyridine pairs to act as non-enzymatic redox cofactors in a primordial protometabolism. As I was explicitly asked by the editor to provide an expertise on the theoretical calculation part, I do not discuss much here the experimental demonstration of coupled oxidative decarboxylation and reductive amination, which is definitely very interesting. Instead, I perfectly understand that the theoretical framework is a complement to the massive experimental data, and thus intended to provide the "physicochemical rationale" for the selection of nicotinamide, but I do not find it completely convincing. The authors rely on static, gas-phase-like DFT calculations to claim a sort of "Goldilocks" behavior for the carbamoyl group. However, the exclusion of dynamical effects and explicit solvent interactions, factors that are crucial in the high-entropy, condensed-phase environments of prebiotic chemistry, seriously limits, I believe, the reliability and accuracy of the reported free energies and the validity of the resulting conclusions.

We thank the reviewer for the technically detailed and constructive assessment of our theoretical work. We fully agree that modeling redox chemistry in aqueous prebiotic environments presents substantial challenges, particularly regarding solvent reorganization and dynamical effects. As correctly pointed out, the theoretical calculations were intended to provide a mechanistic and physicochemical interpretation of experimentally observed trends rather than a quantitatively predictive model.

In response to the reviewer's concerns, we have substantially strengthened the theoretical section by new calculations of the ionization potential including explicit solvent waters, through a systematic cluster-continuum approach (from 1 to 5 water molecules, including conformational search) and several changes in the text and methodological details within the SI document, to clarify the scope and limitations of static DFT free energies.

We detail these implemented changes below.

More specifically, my main criticism concerns the reliability of free energies in static DFT. One of the central theses of the paper rests on the precision of the calculated $\Delta G^\ddagger$ values (e.g., the 9.4 kJ/mol difference between 1ared and 1dred) to explain reactivity trends. However, the use of static stationary point analysis (M06-2X/cc-pVTZ) completely ignores the entropic and dynamical contributions of the solvent. In prebiotic aqueous environments, particularly those hypothesized at hydrothermal vents or surface ponds, the reorganization energy of water is a dominant term in the activation barrier.

Indeed, the authors employ the SMD implicit model to represent water, but the reactions involve highly charged or polar species, including iminium ions, pyridinium rings, and enolates. Implicit models fail to capture the local hydrogen-bonding networks that stabilize these transition states. For a paper aiming for Nature Communications impact, the absence of at least a hybrid implicit/explicit solvation shell is a major oversight.

Effect of the explicit solvation and limitation of continuum models

We agree that absolute activation free energies obtained from static DFT in solution may carry uncertainties on the order of several kJ/mol. Our mechanistic argument, however, relies primarily on relative trends computed consistently at the same level of theory.

The calculated $\Delta\Delta G^\ddagger$ between **1a_{red}** and **1d_{red}** (almost 10 kJ/mol) is in good agreement with our measurements. Experimentally, **1d_{red}** shows no detectable activity over one week, while **1a_{red}** is clearly reactive. Even allowing for typical DFT uncertainties, the energy difference is relevant enough to assume that the trend is correct.

To make this clearer, we have revised the manuscript to state explicitly that the computed activation free energies are intended as qualitative descriptors of relative substituent effects rather than quantitatively predictive values, given the inherent uncertainty of static DFT treatments in aqueous solution. Consequently, we have removed the statement that one reaction is 44 times faster than another, as this represents an overly quantitative interpretation of the calculated activation energy difference, and have replaced it with a more appropriate qualitative description of the observed trend. The sentence (in page 8, 2nd paragraph, line 9) now reads as “The difference ($\Delta\Delta G^\ddagger = 9.4$ kJ/mol) is consistent with the considerable decrease of reactivity observed in these (already slow) reactions as the electron-withdrawing character of the substituent increases.”.

Solvent effects in hydride-transfer transition states

We agree that the hydride transfer step involves highly polar species and that hydrogen bonding may influence transition-state stabilization. However, the transition state depends on the microsolvation and, since the aim of the DFT calculations is to provide qualitative explanation to the experimental results, we think that keeping the implicit model is a good way to treat all the steps of the reactions at the same level.

While a full ab initio molecular dynamics treatment is beyond the scope of this study, we have clarified (in page 8, 2nd paragraph, line 6) that: “These reported $\Delta G^\ddagger$ values should be interpreted as qualitative trends and, although solvent reorganization may contribute additional stabilization not fully captured by implicit models, the purpose of the calculations is comparative across substituents rather than predictive of absolute rates”.

Similarly, the "vertical" ionization potentials reported (Fig 3F) are calculated in a static framework. Without accounting for the solvent's electronic and nuclear relaxation during the electron transfer event, the reported values (4.57 eV for the enolate) cannot be considered accurate representations of the process in a prebiotic aqueous medium.

Explicit solvation in ionization potential calculations

The reviewer correctly noted implicit solvent neglects explicit hydrogen bonding and solvent relaxation effects, which may lead to errors in the calculated energies.

To address this, we have now performed a systematic explicit-solvent study for pyruvate in its keto, enol, and enolate forms, according to the following guidelines:

- 1) For each species, clusters containing 1, 2, 3, 4, and 5 explicit water molecules were generated.
- 2) Conformational exploration was carried out using CREST at the xTB level (considering also implicit solvent).
- 3) The ten lowest-energy conformers for each hydration level were reoptimized at the DFT level.
- 4) Vertical ionization potentials were then computed for the optimized structures.

The results show that, as pointed by the referee, absolute ionization energies increase moderately in the presence of explicit water and convergence of the value is observed

around 4-5 water molecules. However, the relative ordering of ionization potentials (enolate < enol < keto) remains unchanged across all hydration levels.

Thus, although explicit hydrogen bonding affects the absolute magnitude of the ionization energies, it does not alter the qualitative conclusion that the enolate and enol forms are more susceptible to oxidation than the keto form and the importance of these species in the reaction.

We have added these new results to the Supporting Information (page S104, Figure S77) and revised the main text, in note 54 of the references section, to clarify that ionization energies are interpreted as relative electronic trends, and explicit-solvent cluster calculations confirm that the ordering of oxidizability remains robust even when hydrogen-bonding interactions are included.

Also, since several structures were computed for each system (a total of 150 structures), our new calculations address the concern regarding solvent electronic and nuclear relaxation at the level of first solvation shell effects.

Also, the barrierless decarboxylation, which the authors claim once the keto radical is formed, cannot be considered completely convincing. Again, this conclusion is derived from a static potential energy surface scan. In a dynamical system with explicit solvent, "barrierless" steps on an electronic surface frequently manifest as diffusion-limited or solvent-reorganization-limited processes. By characterizing this step as barrierless without dynamical verification, the authors bypass the very kinetic constraints they later cite to explain their low experimental yields.

Barrierless decarboxylation

We acknowledge that the term "barrierless" can be misleading in a condensed-phase context. Our calculations show no intrinsic electronic barrier along the C-C bond cleavage coordinate once the keto radical is formed.

However, as correctly noted, solvent reorganization or diffusion constraints may influence the effective rate. Therefore, in Figure 3F, we have replaced "Barrierless decarboxylation" by "Lowest barrier decarboxylation", and the corresponding revision in the main text (page 11, line 2), which now states that "among the radical intermediates in equilibrium, the keto radical undergoes decarboxylation through the lowest kinetic barrier". In the Supporting Information (page S105), we have specified that "No intrinsic electronic barrier was identified on the calculated potential energy surface. Although solvent reorganization in the condensed phase may still influence the effective kinetics, the calculations suggest that C-C bond cleavage is more favorable for the keto radical form than for the other radical intermediates."

Finally, the study correlates reactivity with LUMO energies and NBO charges derived from static structures. While these trends are qualitatively suggestive, the attempt to provide a "physicochemical rationale" for natural selection requires a more rigorous assessment of the reaction coordinate that goes beyond static electronic properties. The balance is asserted based on a narrow set of substituents without exploring how environmental fluctuations (temperature, pH shifts, mineral surfaces) would perturb these static energy levels.

Use of static descriptors (LUMO energies and NBO charges)

We agree that frontier orbital energies and atomic charges alone cannot justify evolutionary selection. In the revised manuscript, we clarify that these quantities are used as qualitative electronic descriptors to rationalize observed substituent trends.

The mechanistic interpretation is not based only on static electronic properties, but integrates: i) experimental reactivity data, ii) electrochemical measurements and iii) DFT trends.

Although our theoretical results are in very good agreement with the experimental observations, as pointed out by the referee, he/she is right when saying that environmental aspects such as temperature, pH shifts or mineral surfaces could play a role. However, the objective of the present study is to isolate and examine the intrinsic electronic effects of 3- and N-substituents within a controlled chemical framework. A comprehensive treatment of the environment would require a different methodological approach and it is beyond the mechanistic scope of this work. Precisely, we are currently developing a research project in which we study the interaction of this pyridinium/dihydropyridine system with more complex protometabolic networks, and we evaluate the influence of temperature, addition of metals that favours the global process, pH, etc.

In line with these considerations, and in order to avoid overinterpretation of the data, we have moderated the language in the manuscript and now frame our conclusions in terms of physicochemical tendencies that may have contributed to chemical persistence and reversibility, rather than deterministic evolutionary selection (see manuscript version with changes highlighted).

Again, I understand that theoretical calculations are presented here just in support, and they take a very minor part of the paper (a little more than a paragraph in the main manuscript, 5 pages out of 130 of the SI). Still, in its current state, with respect to such theoretical calculations, I feel that the manuscript does not meet the technical rigor required for Nature Communications. The DFT results, which are intended to provide the mechanistic "why" behind the experimental "what," are way too simplified to be predictive or conclusive for prebiotic chemistry.

If the authors consider them to be a necessary validation/interpretation of their experimental results, I recommend them to provide, at the very least, some calculations including explicit solvent molecules, at least a first solvation shell, for the transition states of the hydride transfer to assess the impact of hydrogen bonding on the reported $\Delta\Delta G^\ddagger$. More generally, in the absence of proper ab initio molecular dynamics simulations and enhanced-sampling calculations, a more nuanced discussion of the errors inherent in static DFT must be put out, when estimating free energies for biological cofactors in condensed phases.

Following this last suggestion of the reviewer, we have now included a paragraph at the beginning of section 14 of the SI (page S102, second paragraph), where the methodology of our calculations is presented, dedicated to discuss the key sources of error that are relevant here: (i) absence of explicit hydrogen-bond networks and solvent reorganization; (ii) lack of configurational sampling of solute/solvent microstates, and (iii) limitations of harmonic free-energy corrections in solution. We emphasize that absolute $\Delta G^\ddagger$ values may shift with solvent configuration, and we therefore restrict interpretation to relative trends among closely related cofactors, where partial error cancellation is expected.