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Metal/ADP complexes promote phosphorylation of ribonucleotides

Emilie Werner,^{a,†} Silvana Pinna,^{a,†} Robert J. Mayer,^a Joseph Moran^{a,b,c*}

^aUniversity of Strasbourg, CNRS, ISIS UMR 7006, 67000 Strasbourg, France; ^bInstitut Universitaire de France (IUF), 75005 Paris, France; ^cDepartment of Chemistry and Biomolecular Sciences, University of Ottawa, Ottawa, Ontario, K1N 6N5, Canada

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ABSTRACT: Under enzyme catalysis, adenosine triphosphate (ATP) transfers a phosphoryl group to the canonical ribonucleotide diphosphates (NDPs) to form ribonucleotide triphosphates (NTPs), the direct biosynthetic precursors to RNA. However, it remains unclear whether the phosphorylation of NDPs could have occurred in water before enzymes existed and why an adenosine derivative, rather than another canonical NTP, typically performs this function. Here, we show that adenosine diphosphate (ADP) in the presence of Fe³⁺ or Al³⁺ promotes phosphoryl transfer from acetyl phosphate to all canonical NDPs to produce the corresponding NTP in water at room temperature and in the absence of enzymes. No other NDPs were found to promote phosphorylation, giving insight into why adenosine derivatives specifically became used for this purpose in biology. The metal-ADP complex also promotes phosphoryl transfer to ribonucleoside monophosphates (NMPs) to form a mixture of the corresponding NDPs and NTPs, albeit less efficiently. This work represents a rare example where a single nucleotide carries out a function critical to biology without enzymes. ADP-metal complexes may have played an important role in nucleotide phosphorylation in prebiotic chemistry.

Introduction

Metabolic theories for the origin of life hypothesize that geochemical reactions originating under nonenzymatic catalysis ultimately led to the living state in a process continuous with modern biochemistry.^{1–10} Mirroring microbial metabolism, the reaction networks would have produced small molecules, including those of the genetic material, that acted as catalysts to reinforce existing chemical processes or to enable new ones. In this way, the system could gradually take control of its own catalysis. Recently, experimental work done along these lines has shown that many processes central to metabolism can indeed occur without enzymes under acid/base or metal catalysis.^{10–16} However, one of the crucial missing pieces has been the nonenzymatic reconstruction of phosphorylation processes central to metabolism. Furthermore, in the absence of an enzyme, a functional role of the ribonucleotide monomers typically associated with genetics has yet to be demonstrated in the context of metabolism, despite some recent examples in organic synthesis.^{17,18} This absence is rather surprising since metabolism revolves around adenosine derivatives such as nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD), coenzyme A and, perhaps most importantly, adenosine-5'-triphosphate (ATP)¹⁹. ATP drives metabolism through phosphorylation, which activates substrates toward metabolic reactions and polymerization.^{20,21} One important metabolic process that links phosphorylation and genetic molecules, with a special role for adenosine derivatives, is the phosphorylation of ribonucleotides at the 5'-position. Under enzyme catalysis, ribonucleoside monophosphates (NMPs) are phosphorylated to ribonucleoside diphosphates (NDPs), and further to the ribonucleoside triphosphates (NTPs), the key biosynthetic precursors for the

formation of RNA and DNA. The enzymatic mechanism generally involves an initial phosphoryl transfer from ATP to a histidine moiety within the enzyme and then from the resulting histidine phosphate to the receiving nucleotide.²² Importantly, parallels between histidine, its catalytic function, and adenine derivatives can be drawn. Histidine biosynthesis derives from adenine catabolism,²³ and adenosine-5'-diphosphate (ADP) has been shown to play the same catalytic role as histidine in chemically modified phosphoproteins.²⁴ These two observations suggest a plausible evolutionary scenario in which an adenine moiety preceded histidine in phosphorylation reactions.

Nonenzymatic phosphorylation of ribonucleoside mono- or diphosphates at the 5' position has been reported.^{25,26} The existing examples described within the context of prebiotic chemistry were carried out in the absence of water using wet-dry cycles, in the presence of an activating agent,^{27,28} or were limited to adenosine derivatives.²⁹ The sources of activated phosphate used were acetyl phosphate,³⁰ imidazole phosphate,²⁸ diamidophosphate,³¹ and other simple phosphorylating agents.^{27,32–34} Among these, only acetyl phosphate (AcP) is of direct biological significance. It is a conserved phosphorylating agent in some modern organisms and is regarded by many as the fulcrum between thioester and phosphate metabolism in bacteria and archaea.^{35–41} Although these studies might give insight into prebiotic phosphorylation, they do not shed light on why ATP became the universal energy currency, as none of the elements of ATP are involved. If phosphorylation began with simple phosphorylating agents and arrived at ATP-dependent enzymatic phosphorylation without chemical discontinuity, as the metabolic origins hypothesis would predict, one should expect to find an intermediary evolutionary state between these two different processes. Such a “missing link” would most likely involve a simple phosphorylating agent and an adenosine derivative as a promoter of

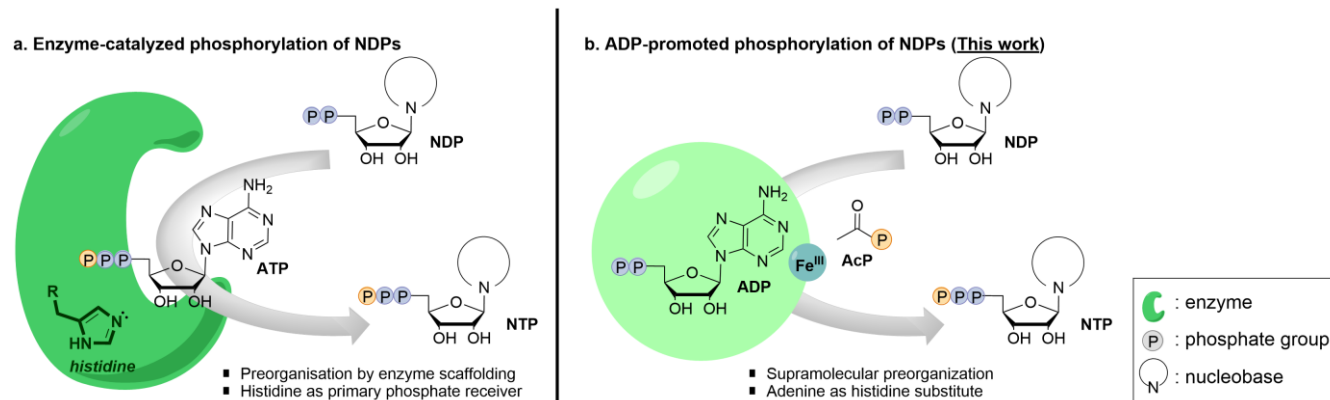


Figure 1. Phosphorylation of NDP to NTP. a. Enzyme-catalyzed phosphorylation. b. This work.

phosphoryl transfer. Along these lines, previous work has shown that phosphate can be transferred in water from AcP to ADP to give ATP in 15 – 20% yield solely in the presence of Fe^{3+} .^{42–44} Under these conditions, it was also possible to convert adenosine-5'-monophosphate (AMP) to ADP, albeit to a limited extent.⁴⁴ In contrast, the other three canonical NDPs did not undergo phosphorylation to their respective NTPs under the reaction conditions. The apparent specificity for ADP in this plausible prebiotic phosphorylation reaction hints at why metabolism is built around ATP and, more generally, adenine derivatives. However, it remains unclear how the flow of phosphate through adenosine derivatives could be efficiently transferred to other species before the advent of enzymes.

We hypothesized that, rather than simply acting as a substrate for phosphorylation, ADP might also play an active role in the nonenzymatic phosphoryl transfer process. As such, we suspected that an intermediate in the reaction of ADP with AcP and Fe^{3+} might be capable of passing on phosphate to other biomolecules in the absence of enzymes, in the same way as ADP can replace histidine's catalytic activity for phosphoryl transfer within chemically modified phosphoproteins.²² Here, we show that by adding another nucleoside diphosphate to the system containing ADP, AcP, and Fe^{3+} , phosphate is transferred nonenzymatically to form the corresponding nucleoside triphosphate without inhibiting the phosphorylation of ADP to ATP (Figure 1).

Results and Discussion

In our initial experiments (Figure 2b, **L1**), we observed that the addition of uridine-5'-diphosphate (UDP, 1 mM) to a mixture of ADP (1 mM), AcP (4 mM) and $\text{Fe}_2(\text{SO}_4)_3$ (0.5 mM, 1 mM in Fe^{3+}) resulted in the formation of ATP (14.1%, Table S8, Entry 3) and uridine-5'-triphosphate (UTP, 14.3%) at room temperature (22 °C) in unbuffered water (pH of the reaction ~ 5.5–6, yields calculated with respect to the initial concentration of ADP and UDP). We then systematically varied each component to understand their role in the reaction. Products of the reaction were identified and quantified by HPLC at $\lambda=260$ nm, against commercial standards (S.I., Materials and Methods, Experimental Data Section 1).

First, we explored the influence of the ADP promoter and its specific structure. Omitting ADP from the reaction precluded all phosphorylation of UDP to UTP, as had been previously observed.⁴⁴

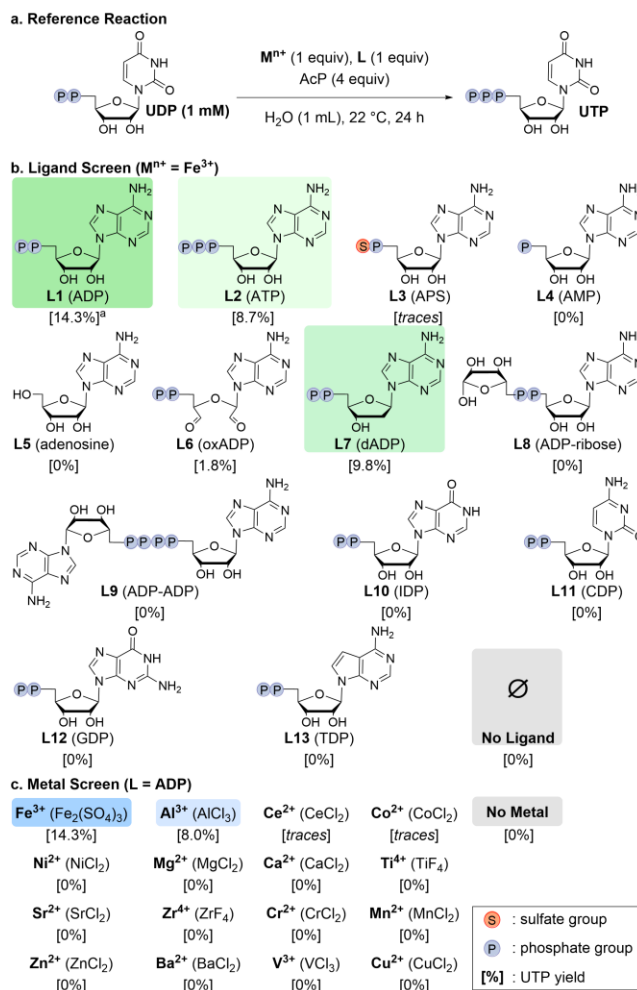


Figure 2. a. Reference reaction. b. Ligand screen (Table S23). c. Metal screen (Table S6). ^aaverage yield from triplicate. Yields determined by HPLC quantification ($\lambda=260$ nm, against a commercial standard). For detailed data, see S.I., Experimental Data.

When ATP was used instead of ADP while leaving the initial concentration of AcP and Fe^{3+} unchanged, the reaction gave lower yields of UTP (**L2**, Figure 2b). Therefore, ATP either is an inferior promoter compared to ADP, is driving the reaction by hydrolyzing *in situ* to ADP, or both. Attempting the phosphorylation of UDP to UTP using adenosine-5'-phosphosulfate (APS), AMP, or adenosine (Figure 2b: **L3**, **L4**, and **L5**, respectively; Table S23, Entries 9 and 10)

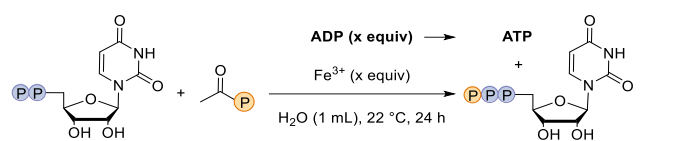
instead of ADP was unsuccessful, confirming the importance of the pyrophosphate motif of ADP. Finally, only trace conversion was obtained when periodate oxidized ADP (oxADP) was used instead of ADP (**L6**, Figure 2b). Using deoxy-ADP (dADP) instead of ADP only decreased the yield of UTP slightly (**L7**, Figure 2b). Taken together, these two results highlight the structural importance of the pentose fragment of ADP, but not its diol motif, for the reactivity. Bulky ADP derivatives proved to be ineffective (**L8** and **L9**). Furthermore, no phosphorylation was observed when IDP (**L10**), CDP (**L11**), or GDP (**L12**) replaced ADP as promoter (Figure 2b), showing complete specificity for the adenine derivative over other nucleobases. As the 5-membered ring of the adenine nucleobase resembles the imidazole moiety of histidine, the amino acid involved in the enzymatic phosphorylation of nucleotides (Figure 1), we hypothesized that, by analogy, the N7 of the adenine nucleobase plays a role in this nonenzymatic phosphorylation reaction. To evaluate this hypothesis, an experiment replacing ADP with tubercidin-5'-diphosphate (TDP), which has a carbon instead of nitrogen at the 7-position of the nucleobase, was performed (Figure 2b, **L13**). In this case, no product formation was observed, an expected outcome if phosphoryl transfer was occurring via N7.

Second, we investigated the effect of the metal ion on the reaction. Without added metal ions, no formation of NTP was observed (Figure 2c). Under the standard reaction conditions, a screening of salts corresponding to 15 other abundant metals in the Earth's crust was performed. NTP was not detected in the presence of most metal ions. Co^{2+} and Ce^{2+} gave trace amounts of product (<1%). Aside from Fe^{3+} , only Al^{3+} showed good reactivity, affording ATP and UTP in 11% and 8% yield, respectively (Table S6, Entry 5).

Third, we investigated the influence of acetyl phosphate. No conversion of UDP to UTP was observed in the absence of AcP, regardless of whether ADP or ATP was used, ruling out the adenosine nucleotides as the phosphate donor and confirming AcP as the source of active phosphate (Table 1, Entry 2, Figures S4-S5). In other words, ADP acts as a promoter for the reaction but is not a phosphorylating agent. Replacing AcP with other prebiotically relevant phosphorylating agents led to no product formation (Table S7). One limitation of AcP is that it readily hydrolyses to acetate and free phosphate under aqueous conditions. This problem was partially addressed by adding 4 additional equivalents of AcP to the reaction at $t = 24$ h. After 48 h, the yield of UTP and ATP increased to 18.0% and 19.2%, respectively (Table 1, Entry 3), in comparison to 14.1% and 14.4% when no additional AcP was added (Table 1, Entry 4). The moderate increase in yield indicates a potential detrimental effect of the hydrolysis products on the reaction. This will be discussed in more detail later in the paper.

Fourth, we varied the reaction concentration and the stoichiometry. When the stoichiometry was kept constant, the overall concentration of the reaction showed little influence on the yields over the range of 1 – 10 mM with respect to UDP (Table S8, Entry 1). However, when the concentration of UDP was kept constant at 1 mM, and the concentrations of the other reagents were increased by a factor of 5, the yield of UTP increased to 25.0% (Table 1, Entry 5). The reaction continues to occur at significantly lower ADP concentrations. A 2- to 20-fold decrease in the initial concentration of ADP only lowered the yield of UTP by 1.1- to 4.6-fold (Table 1, Entries 6-9), which suggests that ADP is likely not a stoichiometric reagent in the reaction.

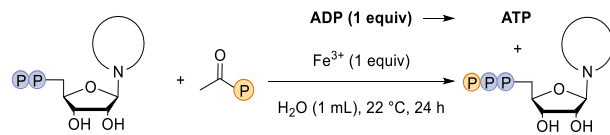
Table 1. Influence of stoichiometry on the reaction yield.

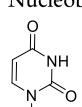
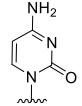
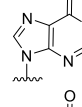



Entry	[ADP] ₀ (mM)	[AcP] ₀ (mM)	[Fe ³⁺] ₀ (mM)	ATP yield	UTP yield	ATP/UTP ratio
1	1	4	1	14.1% ^a	14.3% ^a	1:1
2	1	0	1	0%	0%	-
3 ^b	1	4+4	1	19.2%	18.0%	-
4 ^b	1	4	1	14.4%	14.1%	-
5	5	20	5	17.7%	25.0%	-
6	0.5	4	1	11.4%	12.6%	1:2
7	0.2	4	1	6.9%	7.9%	1:5
8	0.1	4	1	5.1%	5.7%	1:10
9	0.05	4	1	2.4%	3.1%	1:20

^aaverage yield from triplicates. ^bReaction time = 48 h; entries 3 and 4 started as a single reaction solution, then split into two vessels (A and B) after 24 h. Vessel A was left unchanged (entry 4), and 4 additional equiv of AcP were added to vessel B (entry 3), then both solutions were left to react for another 24 h. Yields of ATP and UTP were calculated relative to the initial quantity of ADP and UDP, respectively (HPLC quantification, $\lambda=260$ nm, see S.I., Experimental Data, Sections 2-4).

Table 2. Scope of the phosphorylation reaction. IDP: inosine-5'-diphosphate; UDP: uridine-5'-diphosphate; CDP: cytidine-5'-diphosphate; GDP: guanosine-5'-diphosphate.



Entry	Nucleobase	ATP yield ^a	NTP yield ^a
1		14.1 ± 0.4%	14.3 ± 1.2%
2		15.5 ± 1.5%	12.4 ± 1.0%
3		15.6 ± 1.0%	11.4 ± 0.3%
4		14.1 ± 1.7%	12.6 ± 0.4%

^aaverage yield from triplicates. Yields determined by HPLC quantification ($\lambda=260$ nm, against commercial standard). For detailed data, see S.I., Experimental Data, Sections 4 and 5.

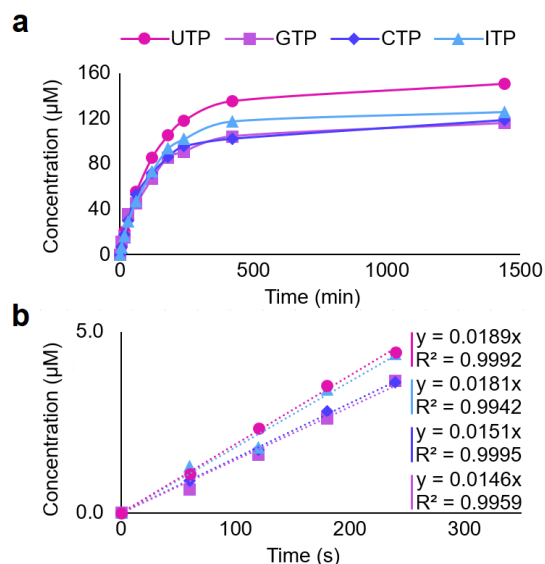


Figure 3. Phosphorylation of uridine-, inosine-, cytidine- and guanosine-5'-diphosphate. a. Advancement of the phosphorylation reaction of NDP to NTP. b. Initial rates of the phosphorylation reactions. Reaction conditions: ADP (1 mM), NDP (1 equiv), AcP (4 equiv), Fe^{3+} (1 equiv) at 22 °C in unbuffered H_2O (reaction pH ~ 5.5-6). For detailed data, see S.I., Experimental Data, Sections 5 and 8.

Fifth, we explored which nucleoside diphosphates could be phosphorylated under the reaction conditions. All four canonical NDPs, as well as inosine-5'-diphosphate (IDP), were successfully phosphorylated (Table 2). For all substrates, the reaction reached >50% of its ultimate yield in <2 h (Figure 3a). No clear difference in reactivity was observed between the nucleobases (Table 2 and Figure 3).

Lastly, we investigated whether the reaction could be extended to the phosphorylation of nucleosides and nucleoside monophosphates. Under the standard reaction conditions (Table 1, Entry 1), the nucleosides uridine, inosine, cytidine, and guanosine were phosphorylated to a mixture of their corresponding NMPs (traces - 1.2%), NDPs (traces - 1.8%), and NTPs (traces - 0.2%). Moreover, the reaction conditions converted UMP, CMP, GMP, and IMP to a mixture of their corresponding NDPs (4.4% - 8.3%) and NTPs (0.5% - 1.1%) (see SI, Sections 4 and 5). Although reactivity was still observed for nucleosides and NMPs, NDPs appear to be the optimal substrates for this reaction.

To rationalize our experimental observations, we propose the mechanism in Figure 4 – the details of which are discussed below. M^{3+} , ADP, and NDP first generate a mixture of several $\text{M}_x(\text{ADP})_y(\text{NDP})_z$ complexes (Figure 4a, step A). Among these complexes, $\text{M}_2(\text{ADP})(\text{NDP})$ (**1**) undergoes an irreversible phosphorylation on its adenine nucleobase by AcP follows to give the phosphorylated complex $\text{M}_2(\text{ADP})(\text{NDP})\text{P}^*$ (**2**, step B). Notably, the N-P bond on the adenine nucleobase (Figure 4b) is analogous to the one formed on histidine during enzymatic phosphorylation.²⁴ A nucleoside triphosphate heterodimer $\text{M}_2(\text{ADP})(\text{NTP})$ (**3**) is then formed via an irreversible intramolecular phosphoryl transfer (step C). Finally, the dissociation of complex **3** yields the product NTP (step D). ADP plays two different roles in the reaction: it is a promoter and a substrate, explaining why the valuable side-product ATP is always obtained.

Further observations corroborate and refine this mechanism. The experiments discussed below were performed with either Al^{3+} or Fe^{3+} , according to their compatibility with the analytical technique employed.

Aluminum complexes of adenine nucleotides have been previously studied and are described in the literature.⁴⁵⁻⁴⁹ Studies by Wang *et al.*⁴⁶ on 1:1 or 2:1 mixtures of ADP/Al identified two complexes by ^1H and ^{31}P NMR. One complex corresponds to $(\text{HO})_2\text{Al}_2(\text{ADP})_2$, where two ADP-Al motifs are bridged by two hydroxide ligands bound to both metal centers. This complex was associated with the most upfield-shifted set of aromatic ^1H signals. The nature of the second complex depends on the stoichiometry of the mixture. When the nucleotide and metal are present in a 1:1 ratio, the observed complex was assigned as the 2:2 $(\text{HO})\text{Al}_2(\text{ADP})_2$ dimer, whereas when the nucleotide is in excess, a 1:2 $\text{Al}(\text{ADP})_2$ dimer was identified. These results are supported by speciation studies using ^{31}P , ^1H , ^{13}C , and ^{27}Al NMR.⁴⁷ A more recent speciation diagram by Cardiano *et al.*⁴⁹ reports both the presence of the 2:1 and 2:2 complexes at pH 5. To identify whether the same complexes were formed in our reaction and to obtain further experimental indication of oligomeric complexes, DOSY NMR experiments were performed on different UDP/ADP/ Al^{3+} mixtures. We identified two ADP homodimers of type $\text{Al}_k(\text{ADP})_2$ (**A1** and **A2**), three UDP homodimers of type $\text{Al}_k(\text{UDP})_2$ (**U1**, **U2**, and **U3**), and one ADP/UDP heterodimer complex of the form $\text{Al}_k(\text{ADP})(\text{UDP})$ (**1'**), (Figure 4d). Due to their order of appearance in the titration experiments (Figures S9b and S11b) and based on the literature,⁴⁵⁻⁴⁹ the number of metal ions in each complex was determined. **A1** was assigned as a bimetallic complex of type $\text{Al}_2(\text{ADP})_2$. Similarly, **U2** and **U3** were assigned as bimetallic complexes of type $\text{Al}_2(\text{UDP})_2$. **A2** and **U1** were assigned as monometallic complexes of type $\text{Al}(\text{ADP})_2$ and $\text{Al}(\text{UDP})_2$, respectively. Taking all of the above experiments into account, the reactive heterodimer complex in UTP formation, **1'**, is most likely of the type $\text{Al}_2(\text{ADP})(\text{UDP})$ rather than the type $\text{Al}(\text{ADP})(\text{UDP})$. **A1**, **A2**, and **1'** were additionally observed by HRMS (SI, Section 9, Subsection j). The evolution of the complexes was monitored during the reaction by ^1H NMR (Figure S15, Table S36). The concentration of all homodimers and of the heterodimer decreased with time, with the exception of one UDP complex, which remained unchanged.

To obtain further insight into the reaction, the kinetics of UTP formation using Fe^{3+} were followed over 8 h (SI, Section 9, Subsection k). The conversion to UTP could be fitted to a mono-exponential curve, allowing the extraction of apparent first-order rate constants. A first experiment varying the reaction concentration showed no significant change in the apparent rate constant (Table S38), suggesting that the reaction proceeds via a unimolecular rate-determining step.

Similar experiments were performed by varying the number of equivalents of reagents or substrates and analyzing the kinetics of UTP formation. When the initial AcP concentration was increased from 1 to 3 equivalents, the conversion to UTP increased, with saturation behavior observed with a larger excess of AcP. However, the apparent rate constant did not vary significantly over the range of 1 to 4 equivalents of AcP. This supports the involvement of a phosphorylated species derived from AcP in the rate-determining step. Additionally, as mentioned previously, attempts to phosphorylate UDP to UTP using the bulky ADP derivative

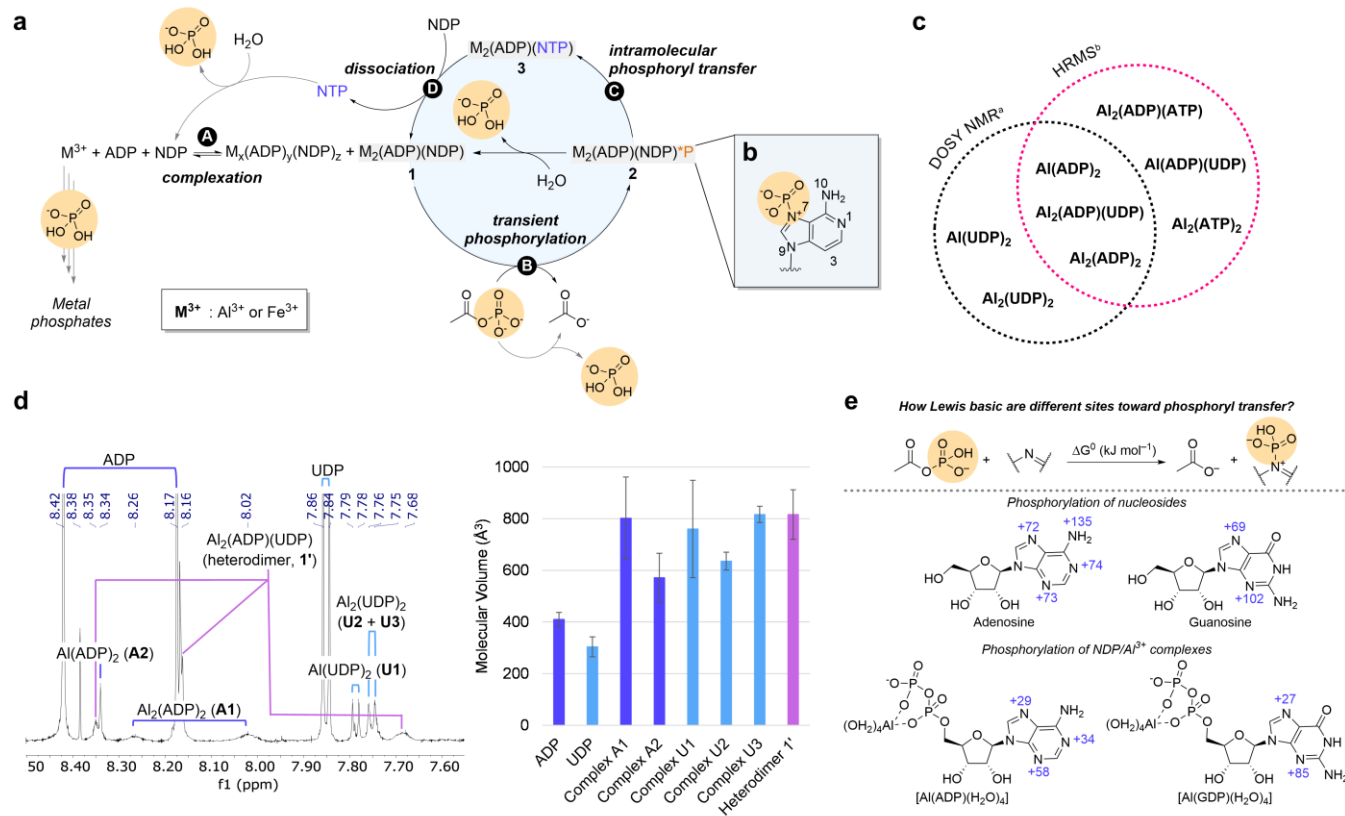


Figure 4. a. Proposed mechanism. For the sake of clarity, the charge of the different complexes and the aqua ligands are not specified. b. N-P bond on the adenine nucleobase of the phosphorylated complex $M_2(ADP)(NDP)P^*$ (2); c. Different complexes observed by DOSY NMR (circled in black) and HRMS (circled in pink); d. Molecular volume of the different species observed in Al^{3+} /NDP mixtures, calculated from the DOSY NMR results. The best-resolved signal of each species was chosen to calculate the representative molecular volume (for details, see S.I., Experimental Data, Section 9, Subsections d to h); e. Thermochemistry for phosphorylation by acetyl phosphate at the SMD(H_2O)/M06-2X/def2-SVP level of theory. ^aFor the full study, see S.I., Experimental Data, Section 9, Subsections d to i. ^bFor the full study, see S.I., Experimental Data, Section 9, Subsection j.

ADP-ribose or a promoter capable of higher denticity, such as P_i , P_4 -di-(adenosine-5')-tetraphosphate (Figure 2b, **L9** and **L10**) were unsuccessful. These ADP derivatives would be expected to hinder the binding of the incoming NDP, preventing it from forming a heterodimer. These two observations led us to hypothesize that the rate-determining step corresponds to the intramolecular phosphoryl transfer (Figure 4a, step C). These experiments were then repeated varying UDP, Fe^{3+} , and ADP concentrations, both under saturation conditions (4 equiv of AcP) and outside saturation (2 equiv of AcP). As UDP was increased from 0.75 to 2.5 equivalents under saturation conditions (4 equiv AcP), the apparent rate constant decreased (Table S40, Entry 1). Outside saturation conditions, going from 0.75 to 1.5 equivalents of UDP resulted in the same apparent rate constant, and a decrease was only observed at 2.5 equivalents (Table S40, Entry 2). These observations can be rationalized by the formation of unreactive UDP dimers $M_2(UDP)_2$.

When varying Fe^{3+} , experiments run under saturation conditions or outside saturation gave comparable trends (Table S41): the apparent rate constant increased upon going from 0.75 to 1.5 equivalents of Fe^{3+} and then decreased sharply at 2.5 equivalents. This drop, which coincided with only trace conversion to UTP, can be attributed to the formation of unreactive 2:1 metal-nucleotide monomers.

Finally, varying the equivalents of ADP showed little difference, whether under or outside saturation conditions. A steady apparent

rate constant was obtained over the range of 0.75 to 1.5 equivalents of ADP, and it decreased at 2.5 equivalents (Table S42). Conversion to UTP was comparable over this whole range. To determine whether the drop in apparent rate constant was due to competitive ATP formation, apparent rate constants were also calculated for the formation of ATP and for the combined formation of NTP (*i.e.*, ATP+UTP). A similar decrease was observed at 2.5 equivalents in both cases, ruling out the competing formation of ATP as a cause. The drop in the rate of formation of all NTPs when ADP is present in more than a two-fold excess compared to the metal can therefore be attributed to the formation of an unreactive 1:2 metal-ADP complex.

To obtain further insights into the phosphorylation reaction, we finally turned to DFT calculations. First, we calculated the thermochemistry for the phosphorylation reaction of nucleosides by acetyl phosphate to quantify the relative Lewis basicity of the different nitrogen sites towards phosphoryl transfer (Figure 4e). As GTP is responsible for phosphoryl transfer in a small number of metabolic reactions, adenosine and guanosine derivatives were compared. For adenosine, phosphorylation is substantially favored at the ring-nitrogen atoms over the primary amine. Adenosine and guanosine showed similar Lewis basicities, suggesting that additional factors are necessary to explain the specificity for ADP in the reaction. The thermochemistry for phosphorylation was next computed for the experimentally more relevant NDP/ Al^{3+} complexes for both ADP and GDP. Phosphorylation of any ring nitrogen within the NDP/ Al^{3+}

complexes was computed to be significantly more exergonic compared to the free nucleoside. For ADP, a small preference for the phosphorylation of the N7 site is observed compared to the N3 and N1 sites, in line with the experimental observations that phosphorylation of N7 in ADP seems to be crucial for the reaction (Figure 2b, **L11**). Notably, a similar Lewis basicity for phosphoryl transfer was computed for the GDP/ Al^{3+} complex, which was found to be experimentally inert. Thus, the thermochemistry of ring nitrogen phosphorylation alone cannot explain why the reaction was found to work exclusively with ADP, and further factors must be considered. For example, previous studies have shown that the guanine nucleobase of GMP was more likely to bind through its N7 to a metal than the N7 of AMP,^{50,51} which might block phosphorylation at this site and explain the observed unreactivity of GDP.

Further efforts were made to obtain computational insights into the structure of the active 2:2 complex. However, the extensive combinatorial space with respect to binding sites, the number of aqua ligands, protonation states, and the stereochemistry at the metal center, as well as the lack of experimental structural data preclude reliable insights. Nonetheless, some tentatively optimized structures for key complexes of the ADP/ Al^{3+} system are given as a visual aid in the supporting information (SI, Experimental Data, Section 10).

In a sense, M^{3+} and ADP can be viewed as co-catalysts⁵² for NDP phosphorylation, as the reaction occurs only in their presence and as both are reactants and products of the reaction. However, a turnover number (TON) greater than one was not observed during this study (Table 1, Entries 1, 3-6). To identify why this was the case, inhibition and deactivation experiments were performed.⁵³ The slightly different kinetic profiles between the same excess experiment and the reference reaction (Figure S8b) hint at product inhibition or catalyst deactivation. To confirm and discriminate between the two possibilities, additional experiments with added product were performed. Starting the reaction with concentrations of UDP (0.9 mM), UTP (0.1 mM), and AcP (3.9 mM), corresponding to what we observe at $t = 400$ min, *i. e.* 10% of conversion (Figure S8c), gave a different conversion profile than the reference reaction, indicating catalyst deactivation and ruling out product inhibition. One plausible explanation may be the loss of the available metal ions through complexation with free phosphate due to the high affinity of Fe^{3+} and Al^{3+} for this ligand.⁵⁴⁻⁵⁶ As all the phosphorylated species (AcP, ATP, and UTP) are prone to hydrolysis, the concentration of free phosphate in solution increases with time. This effect was confirmed by adding varying equivalents of free phosphate to the standard reaction conditions (Table S28, Entries 1-4). As expected, the presence of four equivalents led to a nearly complete loss of reactivity, confirming free phosphate as a catalyst deactivator. For a metal-ADP complex to achieve TON >1 and hence be functional within a protometabolism, the inevitable accumulation of free phosphate through hydrolysis would therefore need to be coupled to a process that removes or reactivates phosphate.

Conclusion

In conclusion, we have shown that in the presence of Fe^{3+} , ADP promotes nonenzymatic phosphoryl transfer from acetyl phosphate to all four canonical NDPs and IDP to produce their corresponding NTPs under mild conditions (22 °C and pH 5.5-6). To a lesser extent, the same conditions also promote the phosphorylation of

ribonucleosides and NMPs. This study shows for the first time that, without enzymes, phosphate can flow through adenosine derivatives to form all other canonical ribonucleoside triphosphates, as well as ITP in water. It also constitutes a rare example in which a single ribonucleotide is able to promote a metabolic function without enzymes, namely NDP phosphorylation. Interestingly, the only two metals that were found to co-promote nonenzymatic phosphoryl transfer with ADP (Fe^{3+} and Al^{3+}) were the same ones found to provide the largest cooperative effects in nonenzymatic pyridoxal-catalyzed transamination.⁵⁷ These two metals are both among the most abundant in the Earth's crust. While Al is known to be toxic to extant life, Fe is widely used in modern metabolism, strongly implicating this metal in the evolution of coenzymes.

The present study reinforces the already close structural, functional, and biosynthetic relationship between histidine and adenine ribonucleotides and hints at the histidine moiety as a functional remnant of adenine ribonucleotides in the context of enzymatic phosphorylation.⁵⁸ The observed reactivity may represent a plausible prebiotic ancestral process to enzymatic ribonucleotide phosphorylation.

ASSOCIATED CONTENT

Supporting Information. The Supporting Information is available free of charge on the ACS Publications website. Full experimental details and details on the individual kinetic experiments (PDF).

AUTHOR INFORMATION

Corresponding Author

*moran@unistra.fr

Author Contributions

*E.W. and S.P. contributed equally.

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ABBREVIATIONS

NAD, nicotinamide adenine dinucleotide; FAD, flavin adenine dinucleotide; ATP, adenosine-5'-triphosphate; NMP, nucleotide-5'-monophosphate; NDP, nucleotide-5'-diphosphate; NTP, nucleotide-5'-triphosphate; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; ADP, adenosine-5'-diphosphate; AcP, acetyl phosphate; AMP, adenosine-5'-monophosphate; UDP, uridine-5'-diphosphate; UTP, uridine-5'-triphosphate; APS, adenosine-5'-phosphosulfate; oxADP, periodate oxidized adenosine-5'-diphosphate; dADP, deoxyadenosine-5'-diphosphate; IDP, inosine-5'-diphosphate; CDP, cytidine-5'-

diphosphate; GDP, guanosine-5'-diphosphate; TDP, tubercidin-5'-diphosphate; UMP, uridine-5'-monophosphate; CMP, cytidine-5'-monophosphate; GMP, guanosine-5'-monophosphate; IMP, inosine-5'-monophosphate; GTP, guanosine-5'-triphosphate; TON, turnover number; ITP, inosine-5'-triphosphate.

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Table of Contents Graphics:

