Depyrophosphorylation of Adenosine 5′-Triphosphate (ATP)

FIKRU TAFESSE and RONALD M. MILBURN*

Department of Chemistry, Boston University, Boston, Mass. 02215, U.S.A.

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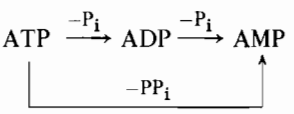
Abstract

The slow hydrolysis of free adenosine 5′-triphosphate (ATP) in both weakly and strongly basic aqueous solution (NaOH, 4 °C) is found to favor depyrophosphorylation over dephosphorylation. In the middle pH region (25 °C) the addition of $N_4Co(H_2O)_2^{3+}$ ($N_4 = tn_2$, trpn; $N_4Co(H_2O)(OH)^{2+}$ predominant species) to preformed N_4CoATP^- complexes ($N_4 = tn_2$, trpn, tren) results in increased rates in production of P_i, but not of PP_i. However, addition of Cu^{2+} or Ca^{2+} to preformed N_4CoATP^- complexes ($N_4 = tn_2$, trpn, tren) leads to increased rates in the production of PP_i as well as of P_i. Mechanistic implications are discussed. The production of P_i from ATP (dephosphorylation) has been monitored by quenching aliquots with Eu^{2+} (H^+) and rapidly replacing multivalent cations with Na^+ (ion exchange) before developing phosphomolybdate for analysis. The production of PP_i from ATP (depyrophosphorylation) has been monitored, following similar quenching with Eu^{2+} (H^+) and replacement of multivalent cations, by assaying for free PP_i using a coupled enzyme system.

Introduction

The nonenzymatic hydrolysis of ATP** involving transfer of the terminal PO_3^- to water, with production of P_i and ADP (dephosphorylation), has been extensively examined [1]. Considerable attention has been devoted to the roles of metal ions in this process [1–6]. The hydrolysis of ATP can also proceed with transfer of the terminal $P_2O_6^{2-}$ moiety to water to yield PP_i and AMP (depyrophosphorylation), but this process has been very little studied [7, 8]. In particular, factors which give rise to depyrophosphorylation in contrast to dephosphorylation and mechanistic details for the former process have remained unsettled.



Previous investigations on the hydrolysis of free ATP have shown that acidic conditions greatly favor dephosphorylation over depyrophosphorylation [9]. The dephosphorylation becomes much slower in the middle pH region where it falls to a plateau of apparent minimum rate at pH ~ 9 [7, 9–11]. The addition of divalent metal ions can result in large accelerations in ATP dephosphorylation [1], but divalent metal ions have not been seen as effective in promoting the depyrophosphorylation process, except for Ca^{2+} at pH 9 for which pyrophosphate production has been reported [8].

In the present study we have measured both P_i and PP_i produced from the hydrolysis of ATP under several sets of conditions: (a) free ATP in basic solution at 4 °C (pH 8.5 and pH 13.5 {0.3 M NaOH}); (b) preformed N_4CoATP^- complexes with added $N_4Co(H_2O)_2^{3+}$ at pH 6.5 and 25 °C ($N_4 = tn_2$, trpn, tren, 4,11-ct); (c) preformed N_4CoATP^- complexes with added Cu^{2+} and with added Ca^{2+} at pH 6.5 and 25 °C. For all systems the amounts of P_i and PP_i produced at various stages of the reactions were measured by quenching aliquots with Eu^{2+} and acid, rapidly removing multivalent cations by ion exchange, and analyzing for free P_i based on the development of phosphomolybdate [1, 12] and for free PP_i using a coupled enzyme system [13, 14]. Significant amounts of PP_i are formed in some systems, but not in others. The mechanistic implications of the results are discussed. In these experiments 4 °C was chosen to study the hydrolysis of free ATP because it has been a common practice, for investigations involving ATP hydrolysis, to store ATP solutions at pH 8.5 for several days in a refrigerator (0–5 °C). While such storage results in very little dephosphorylation, the effect of this practice on depyrophosphorylation was unknown.

*Author to whom correspondence should be addressed.

**Abbreviations: AMP, ADP, ATP: adenosine 5′-mono-, -di-, and triphosphate. The phosphate groups are labelled α, β and γ where γ refers to the terminal group. P_i = orthophosphate; PP_i = pyrophosphate; tn = 1,3-diaminopropane; trpn = tris(3-aminopropyl)amine; tren = tris(2-aminoethyl)amine; 4,11-ct = 5,7,7,12,14,14-hexamethyl-1,4,8,11-tetraazacyclotetradeca-4, 11-diene; bpy = 2,2′-bipyridine.

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Experimental

Analytical grade reagents were used except where otherwise indicated. Sodium hydroxide solutions were freshly prepared from J. T. Baker concentrates using CO_2 free water and were diluted as required. The ligands tris(3-aminopropyl)amine tetrahydrochloride hemihydrate (trpn·4HCl·1/2H_2O) and 5,7,7,12,14,14-hexamethyl-1,4,8,11-tetraazacyclotetradeca-4,11-diene dihydrobromide dihydrate (4,11-ct·2HBr·2H_2O) were obtained from Strem Chemicals, Inc. 1,3-Diaminopropane (tn) and tris(2-aminoethyl)-amine (tren) were purchased from Eastman Kodak Co. Adenosine 5′-triphosphate disodium salt (ATP, product number A 2383), pyrophosphate assay reagent and perchloric acid were supplied by Sigma Chemical Co. Bis-tris buffer (2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol) and spectroscopic grade acetone were obtained from Aldrich Chemical Co.

Measurements of pH were made with an Orion pH meter model 601A, using a combination electrode. Glass stick 'dotting' was used to adjust the reaction pH when buffers were not used. A Cary 210 UV-visible spectrophotometer (thermostatted cell compartment) was used to obtain spectra and collect rate data.

Synthesis of Tetraamine Complexes

The diaqua complexes, $[N_4Co(H_2O)_2]^{3+}$, where N_4 = tn_2, trpn, tren, and 4,11-ct, were prepared in solution from the carbonato complexes, $[N_4CoCO_3]ClO_4$, which in turn were prepared from $Na_3[Co(CO_3)_3]\cdot 3H_2O$ [15]. The conversion of the carbonato to the diaqua species was achieved by adding 2.5 mmol of $HClO_4$ per mmol of finely divided carbonato complex, and stirring under an aspirator vacuum for 20 min in the dark at a temperature of 50 °C. Characterization of the complexes was by UV–Vis spectroscopy; the extinction coefficients and absorbance maxima and minima matched well with recorded values [14, 16, 17].

Preparation and Subsequent Hydrolysis of N_4CoATP^- Solutions

The teraaminecobalt(III) ATP complexes, N_4CoATP^-, were prepared in solution in such a way that final reaction solutions would be 10^{-2} or 10^{-3} M in the desired complex, and 0.10 M in $NaClO_4$ (for ionic strength control). An example of the protocol is given below.

The preparation of a solution 10^{-3} M in N_4CoATP^- (1:1 Co to ATP ratio) was accomplished by mixing (in a thermostatted reaction vessel) 2 ml of ATP solution (5×10^{-3} M, pH 6.5) and 1 ml of $NaClO_4$ (1 M, pH 6.5). If the experiment required the use of bis-tris buffer, 1 ml of buffer (5×10^{-2} M, pH 6.5) was also added. Enough water was then added to make the volume up to 8 ml. Two ml of temperature-equilibrated diaquatetraamincecobalt(III) complex, (5×10^{-3} M, pH 6.5, hydroxoaqua form predominant) was now added to the reaction vessel over a period of 60 s to complete the volume to 10 ml. The solution was equilibrated for 15 min. For higher metal to ATP ratios (e.g., 2:1, 3:1, etc.) the solutions were prepared by preforming the 1:1 complex in a smaller volume of solution (e.g., 8 ml, 6 ml, etc.) and then adding the remaining cobalt reactant to complete the final volume to 10 ml.

Solutions prepared according to the above procedure were maintained at a given pH, if required, by addition of NaOH or $HClO_4$ (using a glass stick, 'dotting' [1]). The hydrolysis of the ATP was monitored by observing the development of hydrolysis products with time. Aliquots were withdrawn at intervals and quenched (see below) to stop the reaction and release all bound phosphate and pyrophosphate before assaying.

Europium(II) Quenching Method

A solution which was 0.1 M in Eu(III) was prepared by dissolving Eu_2O_3 in 2 M HCl. Eu(III) was reduced to Eu(II) by adding about 10 ml of the reagent to freshly amalgamated zinc in an atmosphere of nitrogen and leaving for 20 min. One ml aliquots from the ATP reaction solution were quenched with 0.1 ml of Eu(II) solution, which rapidly (~1 s) reduces Co(III) to Co(II) and releases all bound phosphate species. The reduction of Co(III) is seen by the decolorization of the solution. The remaining Eu(II) was then air oxidized to Eu(III) (process achieved in less than one minute) [18]. The multivalent cations (Eu^{3+}, Co^{2+}) were now replaced by Na^+ using ion exchange, and analysis for P_i or PP_i was carried out immediately. No significant differences in the P_i analyses were observed when this replacement of multivalent cations was omitted, but it was important to carry out this procedure before analyzing for PP_i.

Determination of P_i and PP_i

The amount of P_i produced was determined by a modified Hirata and Appleman procedure [1, 3, 12]. The amount of PP_i was determined using an enzymatic method for pyrophosphate assay [13, 14]. The latter procedure, which is based on a coupled enzyme system, allows the determination of PP_i from the NAD produced in a series of reactions which are pyrophosphate dependent. The yield of NAD (two moles are produced per mole of pyrophosphate) is monitored from the absorbance changes at 340 nm. For both the P_i and PP_i analyses, careful standardizations were first carried out.

Identification of Nucleotides

Identification of the nucleotides was possible by TLC, using a solvent consisting of n-butanol, acetone,

acetic acid, 5% ammonium hydroxide and water in the ratio 45:15:10:10:20 respectively [19]. The R_f values for the corresponding free and complexed nucleotides on 0.2 mm thick precoated silica TLC sheets with aluminum support were obtained. Estimation was possible by comparison of the intensities of the TLC spots under UV light with those of standard spots. The time for each run was 1 h.

Results and Discussion

Figure 1 summarizes results for the base induced dephosphorylation and depyrophosphorylation of free ATP at 4 °C for pH 8.5 and pH 13.5 (0.3 M NaOH). Inclusion of 0.10 M $NaClO_4$ in the solutions produced no measurable changes in rates. Table I provides results on the depyrophosphorylation of free ATP, of β,γ-tn_2CoATP^- alone, and of β,γ-tn_2CoATP^- in the presence of $trpnCo(H_2O)_2^{3+}$, $Cu(bpy)^{2+}$, Cu^{2+}, and Ca^{2+}, all at pH 6.5 (bis-tris buffer) and 25 °C, and including 0.10 M $NaClO_4$.

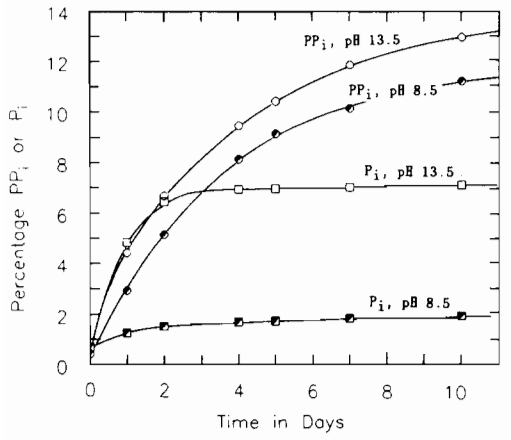

Fig. 1. Hydrolysis of free ATP at 4 °C.

For free ATP the amounts of P_i and PP_i present in the initially prepared solutions are very small; *i.e.*, 0.6% P_i and 0.4% PP_i (where 100% represents complete hydrolysis of ATP to P_i and ADP, or to PP_i and AMP, respectively). These initial amounts, which were determined for each solution following the same quenching and cation exchange procedure used for all reaction aliquots, were constant for a given ATP sample (within the limits of experimental error of a few tenths of 1%). The results for dephosphorylation at pH 8.5 and 4 °C thus confirm the previous recognized basis for storing ATP solutions under these conditions (*i.e.*, that the amount of P_i remains low over many days). On the other hand, at pH 13.5 (0.3 M NaOH) we see significant amounts of P_i being produced over 1 or 2 days, after which the level appears to saturate at 6–7%.

The amounts of PP_i continue to increase steadily, although at rates which appear to be essentially independent of pH. The relative amounts of PP_i and P_i produced (Fig. 1) are in agreement with the results from thin layer chromatography. Thus, the latter experiments show that after 10 days of reaction at pH 13.5 the molar ratio of AMP to ADP is about 2:1, while after the same period at pH 8.5 this molar ratio is about 5:1. No adenosine was detected.

Previous work on the dephosphorylation of free ATP shows decreases in rate as the pH is increased from low values to pH ~ 9 where a plateau of apparent minimum rate is reached [7, 9–11]. The present results (Fig. 1) indicate an increase in the initial rate as the pH is changed from 8.5 to 13.5; the reason for the subsequent fall-off in rate at the higher pH is not yet clear. Possibilities include direct reaction of P_i with ATP to produce PP_i and ADP [20] perhaps assisted by Na^+, but further studies will be needed to distinguish this from other possibilities. The interpretation of results for the depyrophosphorylation of free ATP is more clear-cut; here one sees very similar rate profiles for pH 8.5 and pH 13.5 (Fig. 1).

TABLE I. Summary of Results for ATP Depyrophosphorylation at pH 6.5 and 25 °C[a]

Reactants	% PP_i produced				
	0 min	5 min	10 min	20 min	30 min
ATP[b]	0.38	0.38	0.38	0.39	0.39
tn_2CoATP^- [c]	0.38	0.39	0.39	0.39	0.39
tn_2CoATP^- + trpnCo(III)(aq) [d]	0.31	0.32	0.31	0.32	0.32
tn_2CoATP^- + $Cu(bpy)^{2+}$ [e]	0.39	0.41	0.41	0.43	0.46
tn_2CoATP^- + Cu^{2+} [e, f]	0.41	3.98	4.72	5.06	5.18
tn_2CoATP^- + Ca^{2+} [e, f]	0.45	5.06	6.08	6.85	7.11

[a]Bis-tris buffer, [ATP] or $[tn_2CoATP^-] = 10^{-3}$ M, I = 0.1 M ($NaClO_4$). [b]Free ATP. [c]Similar results were obtained for N_4CoATP^- where N_4 = trpn and tren. [d]No observable change was noted with increased metal to ATP ratio up to 5:1. Similar results were obtained when tn_2CoATP^- was reacted with other $N_4Co(III)(aq)$ (N_4 = tn_2, tren, and 4,11-ct) and when preformed $trpnCoATP^-$ or $trenCoATP^-$ were reacted with various $N_4Co(III)(aq)$ (N_4 = tn_2, trpn, tren, and 4,11-ct). [e]Similar results were obtained for N_4CoATP^- where N_4 = trpn and tren. [f]1:1 M^{2+} to tn_2CoATP^- ratio.

This result shows that nucleophilic attack of ATP by OH^- cannot be an important path in the production of PP_i, and leaves the most obvious interpretation that the process involves nucleophilic attack by water. These experiments clearly show that appreciable depyrophosphorylation of ATP continues in weakly and strongly basic solution. Hence, for studies involving ATP hydrolysis we recommend the use of freshly prepared ATP solutions at all times.

The experimental findings for the $N_4Co(III)$/ATP systems show no appreciable production of PP_i for the reactions between preformed tn_2CoATP^- with $N_4Co(III)(aq)$ up to 5 to 1 metal to ATP ratio (N_4 = tn_2, trpn, tren or 4,11-ct; $[tn_2CoATP^-] = 10^{-3}$ M, pH 6.5, 25 °C, 0.1 M $NaClO_4$). A related set of results was obtained when preformed $trpnCoATP^-$ or $trenCoATP^-$ were reacted with other tetraamine complexes under similar conditions. It is evident from these studies that dephosphorylation rather than depyrophosphorylation is the preferred reaction for the tetraaminecobalt–ATP systems. This result can be attributed to the high affinity of the N_4Co^{3+} moiety towards the oxygens of the polyphosphate chain. Thus, on addition of the tetraaminecobalt(III)aqua complex to preformed β,γ-N_4CoATP^- the cobalt center will coordinate first to an α, β, or γ phosphate oxygen of the ATP to produce a monodentate complex, and this will then either form a double chelated six-membered species, or produce P_i as a result of intramolecular attack by the coordinated hydroxide on the β or γ phosphorus [2].

On addition of Cu^{2+} to preformed tn_2CoATP^-, we see the rapid production of a small amount of PP_i, followed by slower very small further increases. The production of PP_i could here be due to coordination of Cu^{2+} to the adenine moiety (N-7) of the β,γ-tn_2CoATP^-. Such coordination could provide increased opportunity for an appropriately positioned coordinated hydroxide to attack the α phosphorus to produce PP_i. The results obtained with $Cu(bpy)^{2+}$ reflect the increased coordination tendency of $Cu(bpy)^{2+}$ towards the phosphate oxygens [21–23]. $Cu(bpy)^{2+}$ has only two strongly coordinating equatorial sites, and the mechanism of hydrolysis of ATP by $Cu(bpy)^{2+}$ is expected to be similar to that proposed for the hydroxoaquatetraamine–cobalt(III) complexes [2]. For added Cu^{2+} the question of why the production of PP_i shows a dramatic slowdown remains. The results suggest the possibility that the Cu^{2+} complex initially formed provides the suitably positioned coordinated hydroxide, hence producing some PP_i, but that subsequent reaction ensues to produce a fully chelated Cu^{2+} which is much less reactive toward PP_i production.

Depyrophosphorylation by Ca^{2+} may possibly involve a water bridged structure in which the Ca^{2+} is linked indirectly to the N-7 of the adenine, in a manner analogous to that advanced by Glassman *et al.* [24]. As with Cu^{2+}, production of PP_i soon falls off. Similar considerations to those described for Cu^{2+} may also apply here. In all of the above reactions a competing dephosphorylation reaction was noted along with depyrophosphorylation.

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