at C-1 vs. C-2 and might also control access of a basic species to abstract a proton from C-1. Furthermore, a number of investigators have shown that olefin oxygenation leads to suicide inactivation in P-450 systems,[29] and we have recently reported an olefinic suicide substrate for the copper enzyme dopamine-β-hydroxylase.[48] Thus, although analogous mechanisms may be operative for various monooxygenases, it appears that the distribution of products and the extent, if any, of suicide inactivation reflects differences in the active site and metal ligation environments and the particular electronic nature of the substrate being examined.

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General Considerations on Transphosphorylations: Mechanism of the Metal Ion Facilitated Dephosphorylation of Nucleoside 5′-Triphosphates, Including Promotion of ATP Dephosphorylation by Addition of Adenosine 5′-Monophosphate[2]

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Abstract: First-order rate constants (50 °C; I = 0.1, $NaClO_4$) for the dephosphorylation of uncomplexed nucleoside 5′-triphosphates (= NTP = ATP, GTP, ITP, CTP, UTP, or TTP) are virtually identical at the same pH, reaction occurring by water attack as is evident from the specific rate constants; the reactivity decreases in the phosphate-protonated series $H_2(NTP)^{2-} > H(NTP)^{3-} > NTP^{4-}$. Rate constants are also compared for NTP systems containing metal ions [M^{2+} = Mg^{2+}, Mn^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, or Cd^{2+}] by combining new results with previous data. The effectiveness of the metal ions to promote ATP dephosphorylation decreases in the order $Cu^{2+} > Cd^{2+} > Zn^{2+} > Ni^{2+} > Mn^{2+} > Mg^{2+}$, when the rates at the pH of the maximum promotion are compared. This pH, beginning with Cu^{2+} at pH 6.7, increases within the series $Cu^{2+} < Zn^{2+} < Ni^{2+} < Cd^{2+} < Mn^{2+}$ ($< Mg^{2+}$). The latter order reflects the tendency of these ions to form hydroxo complexes (which occurs in the reverse order) and implies that the nucleophilic attack occurs in an *intra*molecular fashion via an M–OH unit. This view is supported by the calculated specific rate constants and by other experimental results. The sum of all experimental data for the determination of the initial rate of the dephosphorylation ($v_0 = d[PO_4]/dt$) gives evidence that the most reactive species for the pyrimidine–NTP systems has the composition $M_2(R\text{-}TP)(OH)^-$, where R-TP represents any triphosphate (including methyltriphosphate) with a noncoordinating terminal organic residue. The most reactive species for the purine–NTP/Cu^{2+} systems at pH <6 has the composition $[M_2(NTP)]_2(OH)^-$; this holds also (including the higher pH range) for the ATP systems with Ni^{2+}, Zn^{2+}, and Cd^{2+}. The most reactive species for Cu^{2+}/ATP at pH ≥6.7 is also a dimer with the composition $[Cu(ATP)]_2(OH)^{5-}$. For 1 mM Cu^{2+}/NTP 1:1 systems the reactivity decreases in the pH range 2–8 in the order ATP > GTP > ITP > CTP ~ UTP ~ TTP; this can only be explained by the decreasing stacking tendency in this series. An explanation based on the N-7/metal ion interaction, which is crucial for the reactivity of the dimers as shown by NMR experiments, is not applicable because the N-7 coordination tendency follows the order adenosine < inosine < guanosine. The larger dephosphorylation rates of the purine–NTPs compared to the pyrimidine–NTPs in the presence of M^{2+} have their origin in the additional M^{2+}/N-7 interaction in purine–NTPs which facilitates the formation of the reactive species. Experiments at pH 5.5 with increasing amounts of Cu^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, Mg^{2+}, $Cu(dien)^{2+}$, or $Cu(dpa)^{2+}$ [dien = diethylenetriamine; dpa = di(2-picolyl)amine] added to a 1 mM Cu^{2+}/ATP mixture [$Cu(ATP)^{2-}$ is formed to a high degree] indicate that an *inter*molecular attack by water on $[Cu(ATP)(M)]_2$ is also possible if M^{2+} = Mg^{2+}, Ni^{2+}, Cd^{2+}, $Cu(dien)^{2+}$, or $Cu(dpa)^{2+}$. Addition of ligands including several nucleoside 5′-monophosphates (NMP) to a Cu^{2+}/ATP system at pH 6.7 supports the view that one purine–NTP in the reactive dimeric species is needed to facilitate transfer of the other into the reactive state. Addition of AMP enhances the reactivity by formation of mixed AMP/ATP stacks, forcing more ATP into the reactive form. However, the "structuring" role of a purine–NTP can only be taken over by a NMP^{2-} having the N-7 and a phosphate group; the effectiveness decreases in the series AMP > GMP > IMP (which is again the order of decreasing stacking tendency). All other ligands (2,2′-bipyridyl, L-tryptophan, phosphate, dien, or dpa) including adenosine, ribose 5′-monophosphate, and tubercidin 5′-monophosphate (= 7-deaza-AMP) inhibit the reaction. The delicacy of the structural arrangement in the stacked dimeric intermediates is evident from addition experiments with adenosine 5′-monophosphate $N(1)$-oxide and 1,N^6-ethenoadenosine 5′-monophosphate: both these AMP derivatives have an enhanced base/metal ion coordination tendency (compared with AMP) because they chelate Cu^{2+} to their base, and both are strong inhibitors of the reaction. The proposed structures for the most reactive species have the common feature that a nucleophilic intramolecular attack via a M^{2+}-bound OH^- occurs. The connection between the described dephosphorylations in vitro, i.e., trans phosphorylations to H_2O, and related reactions in vivo are outlined.

Nucleoside 5′-triphosphates (NTP)[6] play a central role in the metabolism of living cells.[7] They serve as substrates for the enzyme-catalyzed transfers of nucleotidyl or phosphoryl groups—reactions which depend on the presence of divalent metal

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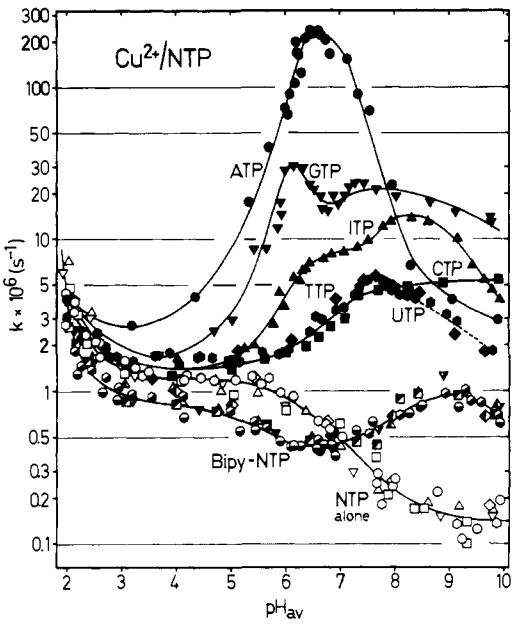


Figure 1. Comparison of the Cu^{2+} promoted dephosphorylation of ATP (●),[31] ITP (▲),[32] GTP (▼),[32] CTP (■),[32] UTP (●),[3] and TTP (◆)[3] (always in the ratio 1:1) as a function of pH, characterized as the first-order rate constants k (s^{-1}). In addition is given ATP (◯; see also Figure 3), ITP (△), GTP (▽), CTP (□), UTP (○), and TTP (◇) alone and ATP (◒), ITP (▲), GTP (▼), CTP (◧), UTP (◕), and TTP (◆) in the presence of Cu^{2+} and 2,2′-bipyridyl (1:1:1). The concentration of each reagent was always 10^{-3} M, when present; I = 0.1, $NaClO_4$; 50 °C. The broken line portion indicates uncertainty due to precipitation.

ions.[8,9] For example, DNA polymerase[10] and RNA polymerase[11] are both Zn^{2+} metalloenzymes and integral components of nucleic acid metabolism.[12] To fulfill its function, DNA polymerase must be activated by a cation such as Mg^{2+} or Mn^{2+}, and there is evidence[13] that these metal ions bind the NTP substrates to the

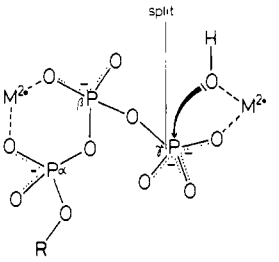
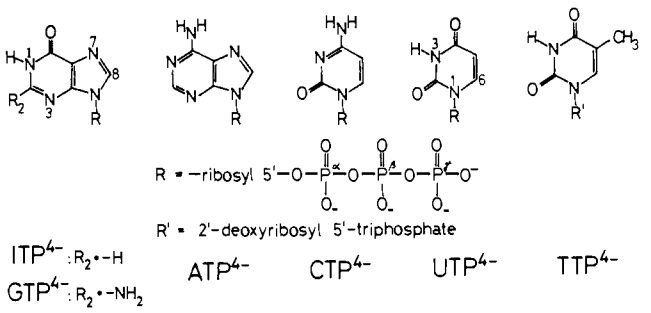


Figure 2. Likely structure of the reactive species formed during the metal ion promoted dephosphorylation of organic triphosphates undergoing only a metal ion-phosphate coordination, like pyrimidine-nucleoside 5′-triphosphates or methyltriphosphate.[3]

Chart I



enzyme.[14-16] Consequently, metal ions are also involved in the selectivity processes.

Considering this, it is not surprising that the formation,[17,18] stability,[19] structure,[20-23] and reactivity[3,4,24-26] of NTP–metal ion complexes are receiving much attention. Indeed, the metal ion promoted dephosphorylation of NTPs in aqueous solution to the corresponding diphosphates and orthophosphate has long been recognized.[27-29] Study of the transfer of a phosphoryl group to a water molecule appears likely to provide some insight into transphosphorylations. This insight has long been delayed[30] by incomplete knowledge of the stability and structure of NTP complexes in solution; however, recent studies have improved the situation.[20,23]

A summary of the dephosphorylating properties of the NTPs[6] shown in Chart I is provided by Figure 1, which includes some of the more recent results.[3,30-32] The main conclusions are the following: (i) The dephosphorylation rates for all six NTPs are identical within experimental error in the absence of metal ions, hence, here the nucleic bases have no influence. (ii) Different properties become apparent only in the presence of metal ions, like Cu^{2+} (Figure 1) or Zn^{2+} (ref[3,30,33]), and the base moieties must

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(6) Abbreviations and Definitions: ε-AMP, 1,N^6-ethenoadenosine 5′-monophosphate; AMP•NO, adenosine 5′-monophosphate N(1)-oxide; AMP, ADP, and ATP, adenosine 5′-mono-, -di-, and -triphosphate; bpy, 2,2′-bipyridyl; CTP, GMP or GTP, IMP or ITP, UTP, and TTP, cytidine, guanosine, inosine, uridine, and thymidine 5′-mono- or -triphosphate; dien, diethylenetriamine [= bis(2-aminoethyl)amine = 1,4,7-triazaheptane]; dpa, di(2-picolyl)amine; M^{2+}, bivalent metal ion; NMP or NTP, nucleoside 5′-mono- or -triphosphate; RiMP, ribose 5′-monophosphate; R-TP, triphosphate with one terminal organic residue; Trp, L-tryptophan; TuMP, tubercidin 5′-monophosphate [= 7-deaza-AMP]. The phosphate groups in NTP are labeled as α, β, and γ, where the latter refers to the terminal phosphate group (see Chart I). If nothing else is specified, the formula PO_4 represents all related species which may be present in solution, i.e., H_3PO_4, $H_2PO_4^-$, HPO_4^{2-}, and PO_4^{3-}. The term "dephosphorylation" is used for the transfer of a phosphate group to a water molecule; the term "hydrolysis" is mainly used in connection with the formation of hydroxo complexes of metal ions. The terms monomeric or dimeric complexes mean that one or two NTP^{4-} together with at least the corresponding equivalents of M^{2+} are within the considered complex; hence, e.g., $M_2(NTP)$ is a monomeric (but dinuclear) nucleotide complex whereas $[M(NTP)]_2^{4-}$ is a dimeric one.
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be responsible for these differences. (iii) A more careful comparison reveals that only the purine–NTPs differ markedly, while the pyrimidine–NTP/Cu^{2+} systems have the same properties up to pH 8. (iv) Indeed, there is no metal ion–base interaction in M(pyrimidine–NTP)$^{2-}$ complexes[23] and consequently the pyrimidine–NTP/Cu^{2+} systems[3] have essentially the same dephosphorylating properties as the methyltriphosphate/Cu^{2+} system.[29] The differences at pH >8 are due to the ionization[23,34] at N-3 which can occur only in M(UTP)$^{2-}$ and M(TTP)$^{2-}$ but not in M(CTP)$^{2-}$ (see Chart I). The kinetic properties showed[3] that the reactive species in the metal ion promoted dephosphorylation of triphosphates with a *non*coordinating organic residue (R–TP^{4-}) has the composition M_2(R–TP)(OH)$^-$; a likely structure and proposed reaction mode is shown in Figure 2. (v) For the purine–NTP/M^{2+} systems the metal–ion base interaction[23] is important for the reactivity, and for ATP it was shown[30] for several M^{2+} that the reaction involves dimeric $[M(ATP)]_2^{4-}$ and $[M_2(ATP)]_2$ complexes. (vi) The properties of the 2,2′-bipyridyl-(bpy)/M^{2+}/NTP systems[3,30,33] are in accord with points ii–v: the formation of ternary M(bpy)(NTP)$^{2-}$ complexes leads to intramolecular stacks between the base residues and the pyridyl rings;[35-37] consequently the metal ion–base interaction is prevented and indeed all bpy/Cu^{2+}/NTP systems have the same dephosphorylating properties (Figure 1).

With the indicated results in mind, the aim of this study is to shed some light on the following questions: (i) Why do the various divalent metal ions differ in their efficiency to promote the dephosphorylation of triphosphates? (ii) What are the detailed compositions and the structures of the reactive intermediates in the M^{2+}/ATP systems where the reaction involves dimeric $[M(ATP)]_2^{4-}$ and $[M_2(ATP)]_2$ complexes? (iii) Why do the Cu^{2+}/purine–NTP systems differ in their reactivity? (iv) Are there any common features between the reactive intermediates for those M^{2+}/NTP systems in which the base moieties do not participate in the reaction (Figure 2) and those in which they do participate? In other words, are there some features which appear to be generally valid for phosphoryl and nucleotidyl transfers?

In seeking answers to these questions we now have investigated the Cd^{2+}/ATP system and have carried out extended studies on the Zn^{2+} and Cu^{2+}/ATP systems, including an examination of the influence on dephosphorylation of additionally added metal ions and of added AMP, other nucleoside 5′-monophosphates, and several additional ligands. The new results are considered in conjunction with previously obtained data.

Experimental Section

Materials. The disodium salts of ATP (>98%; *p.A.*), CTP (for biochemistry), GMP (*p.A.*), and ribose 5′-monophosphate were from Serva Feinbiochemica GmbH, Heidelberg, FRG, and those of AMP (*puriss. CHR*) and IMP (*purum*) from Fluka AG, Buchs, Switzerland. Tubercidin 5′-monophosphate (with a molecular weight of 372 as determined by us via potentiometric pH titration) and the sodium salt of 1,N^6-ethenoadenosine 5′-monophosphate (ε-AMP) (>97%) were purchased from Sigma Chemical Co., St. Louis, MO. Adenosine 5′-monophosphate *N*(1)-oxide was from the preparation described earlier.[38]

Adenosine, 2,2′-bipyridyl, L-tryptophan, NaH_2PO_4, Na_2HPO_4, and $NaClO_4$ (all *p.A.*), diethylenetriamine (98%), and 2-cyanopyridine (98%) were from Merck AG, Darmstadt, FRG. D_2O (≥99.8%) was from CIBA-Geigy AG, Basel, Switzerland. $Cu(ClO_4)_2$ (*purum*) and $Mg(ClO_4)_2$ (*puriss.*) were from Fluka AG; $Cd(ClO_4)_2$ was from Ventron GmbH, Karlsruhe, FRG, and $Zn(ClO_4)_2$ from K&K Laboratories, Cleveland, OH. The exact concentrations of the M^{2+} stock solutions were determined by EDTA titrations.

The tris(hydrochloride) of diethylenetriamine was prepared similarly to the method previously described.[39] dien, purified by spinning band distillation, was slowly added to 6 equiv of ice cooled concentrated hydrochloric acid. The trihydrochloride was precipitated with ethanol and recrystallized three times from a HCl/ethanol mixture (colorless plates; mp 230–232 °C). A neutralized sample of dien·3HCl gave a single spot by thin-layer chromatography on silica gel (liquid phase: 2-propanol/concentrated ammonia 2:1).

Di(2-picolyl)amine was synthesized similarly as described:[40] 30 g of 2-cyanopyridine were dissolved in 140 mL of ethanol/water (6:1), 1.6 g of catalyst (10% Pd on charcoal) was added, and the mixture was treated with H_2 under pressure (20–30 atm) for 9 h at room temperature. The mixture was filtered, the filtrate evaporated to dryness, and the residual oil distilled batchwise in a "Kugelrohr" apparatus to give 15 g of di(2-picolyl)amine as a pale yellow oil (bp 130–145 °C at 0.1 torr). The trinitrate was prepared[41] by careful addition of 30 mL of concentrated HNO_3 to an ice-cooled solution of 15 g of dpa in 100 mL of ethanol. The precipitate was filtered off and recrystallized three times from a mixture of ethanol/water containing a few drops of concentrated HNO_3: 16.8 g of dpa·3HNO_3 were obtained as colorless needles (mp 152–154 °C). Anal. Calcd for $C_{12}H_{16}N_6O_9$: C, 37.12; H, 4.15; N, 21.65. Found: C, 37.15; H, 4.14; N, 21.44.

Apparatus. The ^1H NMR spectra for Figure 4 were recorded with a Bruker WH-90 FT spectrometer (90.025 MHz) at 27 °C in D_2O.[42] The potentiometric pH titrations were carried out with a Metrohm potentiograph E536 and a Metrohm macro EA 121 glass electrode.[39,42] The buffers (pH 4.64 and 7.00) used for calibrations were also from Metrohm AG. Optical absorption measurements were made with Bausch and Lomb Spectronic 88, Varian Techtron 635, and Cary 219 spectrophotometers.

Measurement of Nucleotide Hydrolysis and Evaluation. All dephosphorylation experiments were carried out at 50 °C and I = 0.1 M ($NaClO_4$). As buffers inhibit the metal ion accelerated dephosphorylation of NTPs,[33] the pH was adjusted with NaOH or $HClO_4$ with use of a glass stick (the change in volume was negligible).[30,31,33]

The concentration of liberated phosphate from NTP was determined with molybdate reagent in samples taken at suitable intervals.[31] [NTP] at time t is given by $[NTP]_t = [NTP]_0 - [PO_4]_t$, where $[NTP]_0$ is the initial concentration of NTP, and $[NTP]_t$ and $[PO_4]_t$ are at time t. The free phosphate initially present (1–2%) was taken into account.

The (pseudo) first-order rate constant, k (s^{-1}), which is generally used for comparisons, was determined from the slope of the straight-line portion of a log $[NTP]_t$/time plot. The corresponding pH_{av} was obtained by averaging the pH values measured for those samples that gave points on the straight-line portion. Examples are shown in the figures of ref 31, 32, and 33.

For mechanistic considerations, where an exact relationship between rate and pH is essential,[30] the initial rate of dephosphorylation, $v_0 = d[PO_4]/dt$ (M s^{-1}), was determined from the slope of the tangent of the $[PO_4]_t$/time curve at the time $t = 0$. The corresponding initial pH of the reaction solution, i.e., pH_0, was determined analogously.[31] To obtain v_0 for a given system at a particular pH_0, two experiments were carried out in this range of pH (one slightly above and the other below the desired value) and these results were then interpolated to the desired pH_0. An example of this procedure is shown in Figure 1 of ref 30.

The two quantification methods for the dephosphorylation rates may be transferred into each other by the relation $v_0 = d[PO_4]/dt = k[NTP]$, and by also taking into account that pH_0 is usually by about 0.2 log units larger than pH_{av}.

Results and Discussion

Metal ion facilitated dephosphorylation of all NTPs decreases within the series $Cu^{2+} > Zn^{2+} > Ni^{2+}$.[3,30,33] This cannot originate in different formation degrees for the M(NTP)$^{2-}$ complexes, as these are rather similar.[19] However, the decrease in reactivity corresponds to (i) the decreasing tendency to form hydroxo complexes[34] and (ii) the decrease in the metal ion substitution rates;[17] a further reason (iii) could be sought in different complex structures. A distinction between the first two points seems possible by including Cd^{2+} in the considerations: Cd^{2+} has a water substitution rate[17] similar to that of Cu^{2+} while its tendency to form hydroxo complexes is much smaller.[42] The necessary

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knowledge on the stability and structure of the complexes present in the Cd^{2+}/ATP system has been accumulated in a recent study.[42] However, to appreciate the possible importance of hydroxo complexes for the reactivity of a system we have first evaluated in a quantitative way some of the pH–rate profiles shown in Figure 1.

1. The Dephosphorylation Process in Free Nucleoside Triphosphates. All the common NTPs including ATP (see Chart I) and also methyltriphosphate[3,29] display virtually identical dephosphorylation rates in the absence of metal ions (Figure 1). The entire pH–rate profile from pH 2 to 10 may be fitted by only two equilibrium and three rate constants. Since deprotonations from the nucleic base portion do not alter the hydrolysis rate, we consider only the phosphate protonations, i.e., the reasoning holds for any triphosphate with one terminal organic residue (R–TP).

For free NTPs the observed first-order rate constant (k_{obsd}) for hydrolysis to the nucleoside diphosphate and phosphate is given by eq 1[43]

$$k_{obsd}[R\text{–}TP]_{tot} = k_2[H_2(R\text{–}TP)^{2-}] + k_4[H(R\text{–}TP)^{3-}] + k_9[R\text{–}TP^{4-}] \quad (1)$$

The acidity constants of the protonated R–TP species are defined by eq 2 and 3

$$K^{H}_{H_2(R\text{–}TP)} = (H^+)[H(R\text{–}TP)^{3-}]/[H_2(R\text{–}TP)^{2-}] \quad (2)$$

$$K^{H}_{H(R\text{–}TP)} = (H^+)[R\text{–}TP^{4-}]/[H(R\text{–}TP)^{3-}] \quad (3)$$

The value of the constant for eq 3 is $pK^{H}_{H(R\text{–}TP)} = 6.5$,[19] whereas the one for $pK^{H}_{H_2(R\text{–}TP)}$ (eq 2) is less certain but it is about 2.[44]

The three rate constants in eq 1 may be evaluated from three different regions in the pH–rate profile of Figure 1. From the high pH 9–10 plateau one obtains $k_9 = 0.14 \times 10^{-6}$ s^{-1} (see also Figure 3) and from the pH 4–5 plateau follows $k_4 = 1.2 \times 10^{-6}$ s^{-1}. Thus protonation of the terminal γ-phosphate group increases the specific rate constant by a factor of about 9. For the rise in rate at pH <3 one obtains from eq 1 the expression

$$k_{obsd} = \frac{k_2(H^+)}{(H^+) + K^{H}_{H_2(R\text{–}TP)}} + \frac{k_4 K^{H}_{H_2(R\text{–}TP)}}{(H^+) + K^{H}_{H_2(R\text{–}TP)}} \quad (4)$$

Figure 1 shows that at pH 2.0 $k_{obsd} = 5 \times 10^{-6}$ s^{-1}. The uncertainty in $K^{H}_{H_2(R\text{–}TP)}$ makes an exact calculation impossible, but for $pK^{H}_{H_2(R\text{–}TP)} \simeq 2$ (cf. ref 44) we estimate, based on eq 4, $k_2 \simeq 9 \times 10^{-6}$ s^{-1}. Again a decrease in negative charge on the triphosphate group increases the specific rate constant.[45]

2. Evidence That the Nucleophilic Attack in Free NTPs Occurs by H_2O and Not by OH^-. It may be shown that R–TP dephosphorylation as described in Section 1 is unlikely to proceed by the kinetically equivalent nucleophilic OH^- attack on a species with one more proton, as unreasonably high rate constants result.

For the pH 9–10 plateau, where the OH^- pathway would be relatively more important than in the pH 4–5 plateau (Figure 1), the observed first-order rate constant is given by the sum of the water and OH^- pathways:

$$k_{obsd}[R\text{–}TP]_{tot} = k_9[R\text{–}TP^{4-}] + k_9'(OH^-)[H(R\text{–}TP)^{3-}]$$

From this expression follows

$$k_{obsd} = k_9 + k_9' \frac{K_w}{K^{H}_{H(R\text{–}TP)}} \quad (5)$$

where k_9' is the specific rate constant for nucleophilic OH^- attack on the $H(R\text{–}TP)^{3-}$ species. If $K_w = 10^{-13.05}$ (cf ref 46a) and $k_{obsd} = 0.14 \times 10^{-6}$ s^{-1}, and if the OH^- pathway alone occurs then $k_9' = 0.50$ s^{-1} M^{-1}.

The ratio of the specific rate constants for nucleophilic OH^- to H_2O attack on the $H(R\text{–}TP)^{3-}$ species is $k_9'/k_4 \simeq 4 \times 10^5$ M^{-1}. This large ratio corresponds to that found for esters,[46b] and a negatively charged triphosphate should be much less susceptible to nucleophilic attack by a negative OH^- ion than by the neutral H_2O molecule. This comparison rules out OH^- attack as a competitive pathway, and we conclude that the last term in eq 5 does not contribute significantly to k_{obsd}.

The same treatment for the pH 4–5 plateau yields for $H_2(R\text{–}TP)^{2-}$ an even higher OH^- to H_2O calculated rate constant ratio of 10^{11} M^{-1}. Therefore, throughout the entire pH–rate profile of Figure 1 free NTP dephosphorylation proceeds by nucleophilic water attack and nowhere does hydroxide contribute significantly as a nucleophile.

3. The Dephosphorylation Process for Pyrimidine–NTPs in the Presence of Cu^{2+}. From pH 2 to 8 all three pyrimidine–NTPs (Chart I) display virtually identical pH–rate profiles (Figure 1), and they may therefore be treated together. In fact, there is no base moiety participation in complex formation,[23] and consequently the Cu^{2+}/pyrimidine–NTP systems[3,30] show the same properties as the Cu^{2+}/methyltriphosphate system.[29] Hence, the following reasoning holds for any system containing Cu^{2+} and a triphosphate with one noncoordinating terminal organic residue (R–TP).

The observed dephosphorylation rate in the pH range 5 to 6 as shown in Figure 1 for the 1:1 solutions ($k_{obsd} = 1.5 \times 10^{-6}$ s^{-1}) may be assigned to nucleophilic water attack on the $Cu(R\text{–}TP)^{2-}$ complex. As above pH 5 the formation degree of $Cu(R\text{–}TP)^{2-}$ is high,[47] we obtain for the specific rate constant of this species the estimate $k_6 \simeq 1.5 \times 10^{-6}$ s^{-1}.[48] This value is close to that given in section 1 for water attack on the free $H(R\text{–}TP)^{3-}$ species ($k_4 = 1.2 \times 10^{-6}$ s^{-1}).

As described elsewhere,[3] in a 2:1 solution at pH 5.2 the reactive species is $Cu_2(R\text{–}TP)(OH)^-$. With an estimated[49] stability constant of $10^{3.7}$ for addition of a second Cu^{2+} to $Cu(R\text{–}TP)^{2-}$ and the rough estimate of 10^{-6} M for the acidity constant of $Cu_2(R\text{–}TP)$ to yield the reactive species, eq 6 to 8 may be written

$$Cu(R\text{–}TP)^{2-} + Cu^{2+} \overset{10^{3.7}}{\rightleftharpoons} Cu_2(R\text{–}TP) \quad (6)$$

$$Cu_2(R\text{–}TP) \overset{10^{-6}}{\rightleftharpoons} H^+ + Cu_2(R\text{–}TP)(OH)^- \quad (7)$$

$$Cu_2(R\text{–}TP)(OH)^- \xrightarrow{k_{MOH}} \text{products} \quad (8)$$

A solution which is 1 mM in R–TP and 2 mM in Cu^{2+} contains about 65% $Cu_2(R\text{–}TP)$ and 35% $Cu(R\text{–}TP)^{2-}$; the corresponding observed first-order rate constant is about 5×10^{-6} s^{-1} (see Figure 3 in ref 3). Combination of this information[50] yields for intramolecular nucleophilic attack by Cu^{2+}-bound hydroxide the specific rate constant for eq 8, $k_{MOH} = 5 \times 10^{-5}$ s^{-1} (see also section 10).

4. Evidence for an Intramolecular Attack by Coordinated OH^- in Cu^{2+}/R–TP 2:1 Complexes. Although OH^- is ineffective as a nucleophile compared to H_2O in NTP hydrolysis without metal ions (section 2), metal ion bound OH^- is an effective nucleophile with pyrimidine–NTPs and methyltriphosphate (section 3). This contrast provides another example of the relatively high nucleo-

(43) The subscripts 2, 4, and 9 for the rate constants k refer to the corresponding pH ranges in the pH-rate profile of free NTP in Figure 1.

(44) Phillips, R., S. J. *Chem. Rev.* **1966**, *66*, 501-527.

(45) (a) Each of the three rate constants k_2, k_4, and k_9 may characterize either of two kinetic pathways or a combination of both. In the addition-elimination pathway each rate constant refers to nucleophilic attack of water on the corresponding species in brackets in eq 1 to yield a pentacoordinate oxyphosphorane intermediate. In the elimination-addition pathway monomeric metaphosphate anion, PO_3^-, is eliminated and undergoes water addition to give phosphate. The metaphosphate pathway with a tricoordinate phosphorus becomes more favored the more anionic the reacting species. The contribution of the two pathways to hydrolysis of the several ATP species has not been definitively resolved.[45b] For the purposes of the following comparison with hydroxide as a possible nucleophile it is assumed that the pentacoordinate addition-elimination pathway predominates. (b) Ramirez, F.; Marecek, J. F.; Szamosi, J. *J. Org. Chem.* **1980**, *45*, 4748-4752. Ramirez, F.; Marecek, J. F. *Pure Appl. Chem.* **1980**, *52*, 1021-1045, 2213-2227.

(46) (a) Harned, H. S.; Hamer, W. J. *J. Am. Chem. Soc.* **1933**, *55*, 2194-2206. (b) Conley, H. L., Jr.; Martin, R. B. *J. Phys. Chem.* **1965**, *69*, 2923-2935.

(47) See Figure 2 in ref 3.

(48) The rate constant subscripts are defined analogously to footnote 43.

(49) Estimate based on the information given on pp 7393 and 7395 in ref 30.

(50) From $k_{obsd}[R\text{–}TP]_{tot} = k_{MOH}[Cu_2(R\text{–}TP)(OH)^-]$ follows $(5 \times 10^{-6})/0.65 = k_{MOH}K_a/(H^+) = k_{MOH}10^{-6}/10^{-5.2}$ which gives $k_{MOH} = 5 \times 10^{-5}$ s^{-1}.

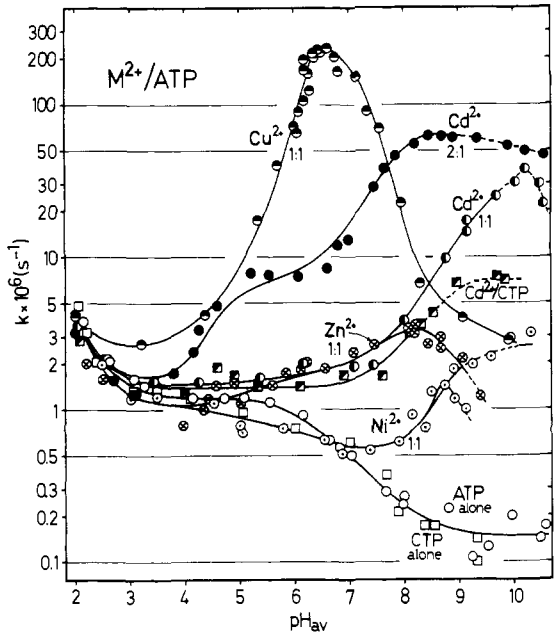

Figure 3. First-order rate constant, k (s^{-1}), for the dephosphorylation of ATP as a function of pH: ATP alone (○, part of the data are from ref 31), Ni^{2+}:ATP = 1:1 (⊙;[33] regarding the extension of the two broken line portions see text in section 5), Cu^{2+}:ATP = 1:1 (◓),[31] Zn^{2+}:ATP = 1:1 (⊗),[33] Cd^{2+}:ATP = 1:1 (◐), and Cd^{2+}:ATP = 2:1 (●). For comparison the corresponding data are also given for CTP alone (□)[32] and Cd^{2+}:CTP = 1:1 (▣). $[NTP]_{tot} = 10^{-3}$ M; I = 0.1, $NaClO_4$; 50 °C. The broken line portions indicate uncertainty due to precipitation.

philic capability of metal ion bound hydroxide.[51-53]

The ratio of specific second-order rate constants for OH^- and M–OH nucleophiles has been expressed[51] by eq 9 where the

$$\log (k_{OH}/k^*_{MOH}) = s(3.8 - 0.24pK_a) \quad (9)$$

sensitivity, s, to a change in nucleophilicity varies only from about 0.7 to 1.1, and pK_a refers to the acidity constant of the conjugate water ligand bound to a metal ion.

For $pK_a = 6$ (eq 7) and a sensitivity $s = 0.7$ at the low end of the scale chosen for triphosphate hydrolysis, we find $k_{OH}/k^*_{MOH} = 50$. We divide the intramolecular first-order rate constant k_{MOH} of section 3 by 10 (equivalent to 10 M of a second reactant) to convert it to an estimated intermolecular second-order rate constant $k^*_{MOH} \simeq 5 \times 10^{-6}$ s^{-1} M^{-1}.

Combination with the given ratio yields $k_{OH} \simeq 2 \times 10^{-4}$ s^{-1} M^{-1} for the attack by unbound OH^- on Cu_2(R–TP). This constant may now be compared with that calculated in section 3 for nucleophilic water attack on Cu(R–TP)$^{2-}$ to give $k_{OH}/k_6 = 10^2$ M^{-1}. This ratio is reasonable; further it is much smaller than the 4×10^5 M^{-1} calculated in section 2 for H(R–TP)$^{3-}$, and it thus confirms the conclusions of section 2 about the ineffectiveness of unbound OH^- as a nucleophile for free NTP. Most important, this reasoning strongly supports also the *intra*molecular OH^- attack indicated in Figure 2.

5. Dephosphorylation Rate of the Cd^{2+}/ATP 1:1 System in Comparison with Other M^{2+}/NTP Systems. In Figure 3 the first-order rate constants, k (s^{-1}), for the dephosphorylation of ATP in the presence of Ni^{2+}, Cu^{2+}, Zn^{2+}, and Cd^{2+} are plotted as a function of pH. In the 1:1 system, Cd^{2+} is a relatively poor promotor of the dephosphorylation in the neutral pH range despite the fact that Cd(ATP)$^{2-}$ reaches a formation degree of about 90% already at pH 6 (see Figure 4 in section 7). At pH below 5 Cd^{2+}, like Ni^{2+} and Zn^{2+}, shows no significant promotion of the reaction, but in the alkaline pH region it becomes a good promotor.

Mg^{2+} is the poorest promotor, as far as this can be judged (see Table S1);[54] that Mg^{2+} inhibits the reaction at pH 5.5 somewhat less than Ni^{2+} is simply due to a lower formation degree of its complexes. This low reactivity is in agreement with the earlier conclusion[30] that metal ion/N-7 interactions are important for this reaction, and Mg^{2+} binds exclusively to the triphosphate chain,[55] with no direct N-7 coordination.[23]

The lack of a Cd^{2+}/base interaction[23,42] accounts for the fact that the dephosphorylation rate in the Cd^{2+}/CTP 1:1 system reaches only about 1/5 of the maximal rate of the Cd^{2+}/ATP 1:1 system (Figure 3). On the other hand, due to the rather pronounced interaction with N-7 in Cd(ATP)$^{2-}$ (cf. ref 23 and 42), Cd^{2+} in the alkaline pH range is remarkably effective in facilitating the ATP dephosphorylation though not quite as much as Cu^{2+} in the neutral pH range (Figure 3; Table S1).

With these results in mind, the following questions arise: What are the main differences between Cu^{2+} and Cd^{2+} regarding complex formation with ATP? Why is Cd^{2+} only an effective promotor in the alkaline pH range? An important difference is that Cu^{2+} already develops M(ATP)(OH)$^{3-}$ (eq 10)[56,57] at pH about 8 while Cd^{2+} does so only at pH about 10. Indeed, the values

$$M(ATP)^{2-}{}_{aq} \rightleftharpoons M(ATP)(OH)^{3-} + H^+$$

$$K^H_{M(ATP)(H_2O)} = (H^+)[M(ATP)(OH)^{3-}]/[M(ATP)^{2-}] \quad (10)$$

of $pK^H_{M(ATP)(H_2O)}$ follow the order:[31,34,42] Cu^{2+} (8.17) < Zn^{2+} (8.87) < Ni^{2+} (9.4) < Cd^{2+} (10.1) < Mn^{2+} (10.7) < Mg^{2+} (>11). This order parallels exactly the order of the pH values at which the maximum rates of dephosphorylation are observed at the M^{2+}/ATP 1:1 ratio, despite the fact that the maximum in the Ni^{2+}/ATP system is obscured due to precipitation; fortunately this problem was less serious in the Cd^{2+}/ATP system. The tendency to form hydroxo complexes decreases of course in the reverse order.

These results indicate then that in the reactive intermediate a $M(OH)^+$ unit seems to be involved, just as has been concluded for the Cu^{2+}/pyrimidine–NTP systems (section 4). On the other hand it is also evident, by comparing the given values of $pK^H_{M(ATP)(OH)}$ with the peaks in Figure 3, that too much hydroxo-complex formation inhibits the reaction, a problem to be addressed further in section 7.

6. Detailed Kinetic Properties of the Cd^{2+}/ATP System. The results of the preceding section indicate that Cd^{2+} fits into the general picture obtained so far for other divalent metal ions.[30] This is confirmed by the higher reactivity of the Cd^{2+}/ATP 2:1 system (Figure 3), an observation in accord with conclusions that the coordination of more than one metal ion to an oligophosphate promotes the hydrolysis of a P–O–P bond.[4,5,58,59] For several M^{2+}/NTP systems it has been proven[3,30] that the reactive intermediate contains the metal ion and the NTP in a 2:1 ratio.

Job's method[60] was used to prove that also with Cd^{2+} the most reactive species has two metal ions bound to ATP. The initial rates, v_0 (M s^{-1}), were measured and plotted vs. the ratios $[Cd^{2+}]/([Cd^{2+}] + [ATP])$, keeping $[Cd^{2+}] + [ATP]$ constant (Figure S1).[54] A maximum rate at values of 0.33, 0.5, or 0.67 would indicate a composition for the reactive species of Cd^{2+}:ATP = 1:2, 1:1, or 2:1, respectively. The results obtained at pH_0 7.20, 8.20, and 9.00 confirm that the most reactive species indeed contains two Cd^{2+} and one ATP^{4-}. In fact, the formation of

(51) Martin, R. B. *J. Inorg. Nucl. Chem.* **1976**, *38*, 511-513.
(52) Buckingham, D. A.; Keene, F. R.; Sargeson, A. M. *J. Am. Chem. Soc.* **1974**, *96*, 4981-4983.
(53) Brown, R. S.; Huguet, J.; Curtis, N. J. *Met. Ions Biol. Syst.* **1983**, *15*, 55-99.
(54) Supplementary material. Related experimental data for other M^{2+}/NTP systems are plotted in the figures of ref 3 and 30.
(55) Bishop, E. O.; Kimber, S. J.; Orchard, D.; Smith, B. E. *Biochim. Biophys. Acta* **1981**, *635*, 63-72.
(56) For Cu^{2+}/ATP 1:1 systems[31] hydroxo-complex formation is well described up to pH 9.5 by eq 10,[57] despite the formation of dimeric species.[57] Hydroxo-complex formation is connected with a release of N-7 from Cu^{2+} (see section 7).
(57) Werner, E. R.; Rhode, B. M. *Inorg. Chim. Acta* **1983**, *80*, 39-46.
(58) Miller, D. L.; Westheimer, F. H. *J. Am. Chem. Soc.* **1966**, *88*, 1514-1517.
(59) Hübner, P. W. A.; Milburn, R. M. *Inorg. Chem.* **1980**, *19*, 1267-1272.
(60) Job, P. *C. R. Hebd. Seances cad. Sci.* **1933**, *196*, 181-183.

M_2(NTP) species is well-known.[3,17,30,55,61]

As unbound Cd^{2+} in a 2×10^{-3} M solution begins to hydrolyze at pH about 7 to 7.5, it is to be expected that in a species containing two Cd^{2+} and one ATP^{4-} Cd^{2+} will also be partly hydrolyzed in this pH region, because ATP^{4-} is not able to saturate the coordination spheres of both Cd^{2+}. This view is supported by an evaluation of the shape of the 2:1 curve in Figure 3: a plot of log k vs. log $[H^+]$ gives a straight line in the pH range 7 to 8 with a slope close to −1, indicating that the reaction is proportional to $1/[H^+]$, or to $[OH^-]$. This then is also evidence that a $M(OH)^+$ unit is involved in the reactive intermediate.

Further experiments, leading to plots of log v_0 vs log [ATP], were carried out at pH_0 7.20, 8.20, and 9.00 to learn more about the dependence of the initial rate v_0 on the concentration of the Cd^{2+}/ATP 1:1 and 2:1 systems. At these pH values, the formation degree of $Cd(ATP)^{2-}$ at the 1:1 ratio exceeds 90%, and at the 2:1 ratio significant amounts of $Cd_2(ATP)$ are formed; this latter conclusion is based on the known stability constants of related systems.[30] For both ratios the data of all experiments fit straight lines with a slope of 2 at [ATP] $\gtrsim 10^{-3}$ M (Figure S2).[54,62] Hence, under these conditions the rate depends on the square of the reactant concentration what may be rationalized with the monomer–dimer equilibria 11 and 12:[63,64]

$$2M(ATP)^{2-} \rightleftharpoons [M(ATP)]_2^{4-} \quad (11)$$

$$2M_2(ATP) \rightleftharpoons [M_2(ATP)]_2 \quad (12)$$

This result that the most efficient promotion of the dephosphorylation reaction of ATP by Cd^{2+} proceeds via dimeric complexes is important; it also corresponds to the earlier result[30] for the Ni^{2+}, Cu^{2+}, and Zn^{2+}/ATP systems (see also section 8). However, all these results contrast with the metal ion facilitated dephosphorylation of pyrimidine–NTPs in which the rate-determining step always occurs in a monomeric intermediate (Figure 2 and section 3).

7. Interplay between Hydroxo-Complex Formation and Reaction Rate in M^{2+}/ATP Systems. In the metal ion promoted dephosphorylation of those triphosphate systems in which *no* metal ion/base interaction occurs, the most reactive intermediate has the composition $M_2(R\text{-}TP)(OH)^-$ (Figure 2),[3] and consequently the reaction rate levels off at about that pH at which the hydroxo $M(R\text{-}TP)(OH)^{3-}$ complex is fully developed, as is nicely seen for Cu^{2+}/CTP in Figure 1. An alteration occurs only in those M^{2+}/R–TP systems which undergo with increasing pH a side reaction, such as the ionization at N-3 in UTP or TTP; for the latter two nucleotides, this leads to macrochelated $M(NTP\text{–}H)^{3-}$ species.[23]

The properties of the M^{2+}/purine–NTP systems differ in several respects: (i) In all cases the dephosphorylation rate reaches at a certain pH a *maximum* (see, e.g., Figures 1, 3, and S3). (ii) Plots of log k vs. pH give, for a certain pH range on the ascending side of the peak, straight lines with slopes close to 1.[65] A slope of 1 means that the reaction rate is proportional to $[OH^-]$, while a slope below 1 indicates that H_2O also acts as a nucleophile;[33] a slope greater than 1 is only observed in the Cu^{2+} 2:1 system indicating that two reactive sites are present in the reactive dimeric intermediate (see Figure 7 and section 9). (iii) That the ascending sides of the peaks are observed in different pH ranges in the individual systems, despite the fact that from pH 5.5 on the formation degree of $M(ATP)^{2-}$ has always reached 80% (or more), suggests that the metal ion is not simply needed for the neutralization of the negatively charged phosphate groups; this suggests further that the attack of OH^- is not *inter–* but *intra-*

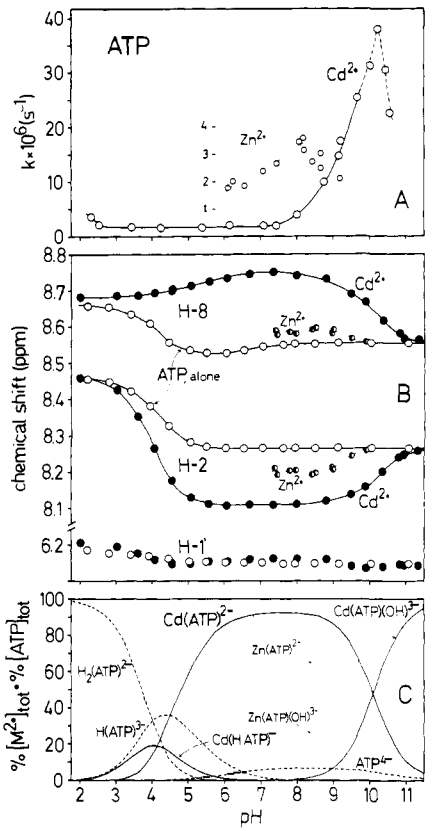

Figure 4. Comparison of the variation of (A) the Cd^{2+} promoted dephosphorylation of ATP (each 10^{-3} M) in aqueous solution at 50 °C as a function of pH (I = 0.1, $NaClO_4$; cf. Figure 3), with (B) the chemical shift of H-2, H-8 and H-1′ in the ^1H NMR spectra of ATP (○, 5×10^{-3} M) and of ATP in the presence of Cd^{2+} (●, 1:1, 5×10^{-3} M)[42] in D_2O at 27 °C (I = 0.1, $NaNO_3$) as a function of "pH" (i.e., no correction according to pD = pH meter reading +0.40 was applied),[66] and with (C) the effect of pH at 25 °C (I = 0.1, $NaNO_3$) on the concentration of the species present in an aqueous solution of Cd^{2+} and ATP (each 10^{-3} M); the results were computed with the constants listed in ref 42, and they are given as the percentage of the total Cd^{2+} present (= total ATP). The broken lines indicate the free ATP species and the solid lines the ATP complexes.[67] Further comparisons are shown with *dotted lines* inserted: (A) the Zn^{2+} promoted dephosphorylation of ATP (note the 5-fold expanded scale; cf. Figure 3), (B) the chemical shift of H-2 and H-8 for Zn^{2+}/ATP (◐),[42] and (C) the formation of $Zn(ATP)^{2-}$ and $Zn(ATP)(OH)^{3-}$ (calculated with the constants listed in ref 19 and with $pK^H_{Zn(ATP)(H_2O)}$ = 8.87 of ref 34). The experimental conditions for the measurements with Zn^{2+} correspond to those given for Cd^{2+}. Diprotonated complexes of the type $M(H_2ATP)$ were ignored in the calculations as the appropriate constants are unknown; however, such species would probably exist only below pH 3.

(61) (a) Mohan, M. S.; Rechnitz, G. A. *Arch. Biochem. Biophys.* **1974**, *162*, 194–199. (b) Glassman, T. A.; Cooper, C.; Kuntz, G. P. P.; Swift, T. J. *FEBS Lett.* **1974**, *39*, 73–74. (c) Arena, G.; Cali, R.; Musumeci, S.; Purrello, R.; Rizzarelli, E.; Sammartano, S. *Proc. Int. Conf. Coord. Chem., 21st* **1980**, 263.

(62) At [ATP] $\lesssim 10^{-3}$ M the slopes move toward 1. This is not surprising because for the monomeric $Cd(ATP)^{2-}$ complex without an N-7 interaction (this species comprises about 50%)[42] a reactivity similar to the Cd^{2+}/CTP 1:1 system must be expected, and from Figure 3 it is evident that in 10^{-3} M solutions at pH 7 to 9 the reactivity of Cd^{2+}/ATP is indeed not much higher than that of Cd^{2+}/CTP. In other words, at low Cd^{2+}/ATP concentrations (see Figure S2) the reactivity is partly governed by the monomeric complex (slope moves towards 1) while at high concentrations the dimer determines the reactivity (slope of 2).

(63) Assuming eq 11 and 12 are far to the left (i.e. the concentration of the dimers is small) then the dimer concentration will be proportional to the square of the total concentration because K_D = [dimer]/[monomer]^2. Hence, if the dimers are the reactive complexes, the slopes of the plots, log v_0 vs. log $[\text{reactant}]_{tot}$, will be 2 while for the monomer as the reactive species it would be 1. Calculations based on the known self-association of the Cd^{2+}/ATP 1:1 system[23] indicated that in a 5×10^{-3} M reactant solution still about 85% are present in the monomeric $Cd(ATP)^{2-}$ form;[64] clearly, at higher concentrations the slope of 2 is expected to deviate again toward 1.

(64) The calculations with K = 17 M^{-1} (see Table II in ref 23) give for $[Cd^{2+}]_{tot}$ = $[ATP]_{tot}$ = 5×10^{-3} M 85.9% monomer, 12.5% dimer, 1.4% trimer, and 0.13% tetramer. The calculations with K^*_D = 20 M^{-1} and K_{st} = 4 M^{-1} (see legend of Figure 5 in ref 23) give for the mentioned conditions 85.1% monomer, 14.5% dimer, 0.37% trimer, and 0.04% tetramer.

(65) A slope of 1 is observed for the Cu^{2+} 1:1 systems of ATP,[31] ITP,[32] and GTP;[32] for the 1:1 systems of ATP and Zn^{2+}, Ni^{2+}, or Cd^{2+} (Figure 3) the slopes are somewhat below 1, while for the 2:1 systems the slope in case of Cu^{2+} is slightly larger than 1, it is about 1 for Cd^{2+} (see Section 6), and for Zn^{2+} and Ni^{2+} it is somewhat less than 1 (see Figure 2 in ref 30).

molecular. This is further confirmed by the parallelism between reactive pH range and hydroxo-complex formation as discussed in section 5, i.e., a M–OH^+ unit is part of the reactive intermediate (see also section 9).

On the other hand it is evident that the peaks of the rates in the 2:1 systems (Figures 3 and S3 and Figure 2 in ref 30) occur always well before the pH equals the values of $pK^H_{M(ATP)(H_2O)}$ for the corresponding $M(ATP)^{2-}$ complexes, i.e., well before $M(ATP)(OH)^{3-}$ reaches its maximum concentration. However, the descending sides of all peaks, be it a 2:1 or 1:1 system, correlate closely with the $pK^H_{M(ATP)(H_2O)}$ values (eq 10), indicating that the formation of $M(ATP)(OH)^{3-}$ is connected with a structural rearrangement which inhibits the reactivity. As the formation of certain ternary complexes, based on $M(ATP)^{2-}$, leads to a release of N-7 from the coordination sphere of the metal ion,[36] the same could be surmised for $M(ATP)(OH)^{3-}$. In accord with this view, the rate constants for the Cu^{2+}/ATP and Cu^{2+}/CTP systems become quite close at pH >8.5 (Figure 1).

Furthermore, the pH dependence of the chemical shifts of H-2, H-8 and H-1′ of a 5×10^{-3} M 1:1 mixture of Cd^{2+} and ATP in D_2O is compared in Figure 4 (middle)[66] with the Cd^{2+} facilitated ATP dephosphorylation in the 1:1 system (upper) and the distribution of complex species (lower).[67] With increasing concentration of $Cd(ATP)(OH)^{3-}$ the shifts of H-2 and H-8 move back to the position in free ATP (middle), showing a release of N-7 from the coordination sphere of Cd^{2+}, which is paralleled by the decreasing rate of dephosphorylation (upper). Moreover, as the formation degree of $Cd(ATP)^{2-}$ reaches more than 90% (lower) the acidity constant $pK^H_{Cd(ATP)(H_2O)} \simeq 10.2$ (expressed in "pH")[66] may directly be read from the shifts of H-2 and H-8; the result is in excellent agreement with the value of 10.1 from the potentiometric pH titrations,[42] thus confirming that alterations of the chemical shift are coupled with the formation of $Cd(ATP)(OH)^{3-}$.

Alterations of the chemical shifts by the coordination of Zn^{2+} to ATP^{4-} are less pronounced. This agrees with earlier observations.[23,42] Even so, it is evident (Figure 4, dotted lines) that all the conclusions outlined for Cd^{2+}/ATP are also valid for Zn^{2+}/ATP. Interestingly, substitution of Cd^{2+} for the native Zn^{2+} in human carbonic anhydrase B shifts the apparent pK_A for esterase activity from 7.0, in the native enzyme, to 9.1, in the Cd^{2+} substituted enzyme.[68] This shift corresponds exactly to the 2.1 pH units between the two peaks in the upper part of Figure 4.

Figure 5 assembles some results obtained for Cu^{2+}/ATP systems. A comparison of the upper and the lower parts confirms again that with increasing concentration of $Cu(ATP)(OH)^{3-}$ the dephosphorylation rate decreases. Selective line broadening ^1H NMR experiments[69] by paramagnetic metal ions give little information about the structure of species present under 1:1 conditions,[70] but despite these severe limitations it is evident from the middle part of Figure 5 that the effect of Cu^{2+} on the signals of H-2 and H-8 disappears at pH >8. Hence, these experiments confirm that N-7 becomes unaccessible for Cu^{2+} in this upper pH range. The apparent pK_A estimated from the effect of Cu^{2+} on the signal of H-8 is 8.5, a value which is of the same order as $pK^H_{Cu(ATP)(H_2O)} \simeq 8.0$ (see legend of Figure 5).

To conclude, these results demonstrate that the metal ion/N-7 interaction plays a crucial role in the formation of the reactive species, that a M–OH^+ unit is involved, and that an excess of OH^- destroys the reactive complex.

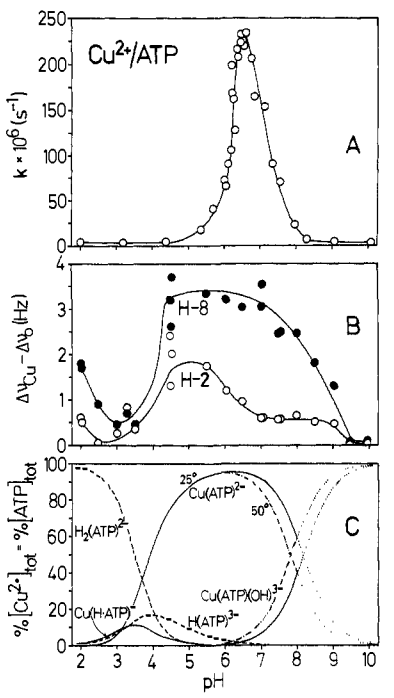

Figure 5. Comparison of the variation of (A) the Cu^{2+} promoted dephosphorylation of ATP (each 10^{-3} M) in aqueous solution at 50 °C as a function of pH (I = 0.1, $NaClO_4$; cf. Figure 1) with (B) the influence of Cu^{2+} (5×10^{-5} M) on the line width of the signals due to H-2 (○) and H-8 (●) in the ^1H NMR spectra of ATP (0.1 M) measured at 37 °C in aqueous solution as a function of pH (see text; the broadening, $\Delta\nu_{Cu} - \Delta\nu_0$, at a particular pH was determined[69] by the difference in line width at the half-height of the signal in the presence, $\Delta\nu_{Cu}$, and absence, $\Delta\nu_0$, of $Cu(ClO_4)_2$), and with (C) the effect of pH at 25 °C (I = 0.1, $NaClO_4$) on the concentration of the species present in an aqueous solution of Cu^{2+} and ATP (each 10^{-3} M); the results are given as the percentage of the total Cu^{2+} present (= total ATP). The broken lines indicate the free ATP species and the solid lines the ATP complexes; the dotted line portions indicate uncertainty due to the fact that $pK^H_{Cu(ATP)(H_2O)} = 8.17$ describes the situation well only in the pH range up to 8.0 (ref 31; see also ref 57). The additional lines (–·–) represent the distribution of the complexes at 50 °C; they were calculated with $pK^H_{Cu(ATP)(H_2O)} = 7.82$.[31] The other constants used in the calculations are from ref 19 (25 °C); $[ATP^{4-}] \leq 1.5\%$. The diprotonated $Cu(H_2ATP)$ complex was ignored in the calculations as the appropriate constant is unknown; however, such species would probably exist only below pH 3.

8. Further Studies on the Cu^{2+}/ATP System. Since the most reactive species contain M^{2+} and ATP in the ratio 2:1, one may expect that under M^{2+}/ATP = 0.5:1 conditions the reactivity will be considerably reduced. With Cu^{2+} being the most effective promotor (Figure 3), this expectation was checked and confirmed (Figure S3 contains[54] the first-order rate constants in dependence on pH for the ratios Cu^{2+}/ATP 2:1, 1:1, and 0.5:1; the total ATP concentration was constant at 10^{-3} M),

As the reactive species was so far always dimeric (section 6 and ref 30), the next question is, if under an unfavorable Cu^{2+}/ATP 0.5:1 condition this is still true. In fact, for all the ratios (studied in Figure S3) slopes of two are obtained at pH 5.50, 6.70, and 7.60 from plots of log v_0 vs. log $[ATP]_{tot}$ (Figure S4);[54] hence, the reactive complexes are dimers for all conditions.

Job's series carried out earlier[30] have shown that in the Cu^{2+}/ATP system in the lower pH range a 2:1 complex is the most reactive species. This is also evident from the upper part of Figure 6: the addition of 1 equiv of Cu^{2+} to a Cu^{2+}/ATP 1:1 system promotes the reaction already to its maximum rate indicating that this 2:1 complex is relatively stable (although reactive).[30] In the hope to learn more about the role of the second metal ion we added to the Cu^{2+}/ATP 1:1 system Mg^{2+}, Ni^{2+}, Zn^{2+}, or Cd^{2+}, as well as the 1:1 complexes of Cu^{2+} and diethylenetriamine (dien) or di(2-picolyl)amine (dpa); the latter

(66) (a) The expression "pH" represents the direct pH meter reading for a D_2O solution; pD means that the following correction was applied:[66b] pD = pH meter reading + 0.40. As the difference between pK_A values determined in H_2O and D_2O corresponds approximately to the given correction, it is realistic to compare experiments carried out in H_2O and D_2O on the basis of the direct pH meter reading.[42] (b) Glasoe, P. K.; Long, F. A. *J. Phys. Chem.* **1960**, *64*, 188-190.

(67) The comparison is valid despite the different experimental conditions needed for Figure 4. Indeed, the calculations show[64] that in a 5 mM reactant solution still about 85% is in the monomeric $Cd(ATP)^{2-}$ form.

(68) (a) Coleman, J. E. *Nature (London)* **1967**, *214*, 193-194. (b) Bauer, R.; Limkilde, P.; Johansen, J. T. *Biochemistry* **1976**, *15*, 334-342. (c) Galdes, A.; Vallee, B. L. *Met. Ions Biol. Syst.* **1983**, *15*, 1-54.

(69) Naumann, C. F.; Prijs, B.; Sigel, H. *Eur. J. Biochem.* **1974**, *41*, 209-216.

(70) Espersen, W. G.; Martin, R. B. *J. Am. Chem. Soc.* **1976**, *98*, 40-44.

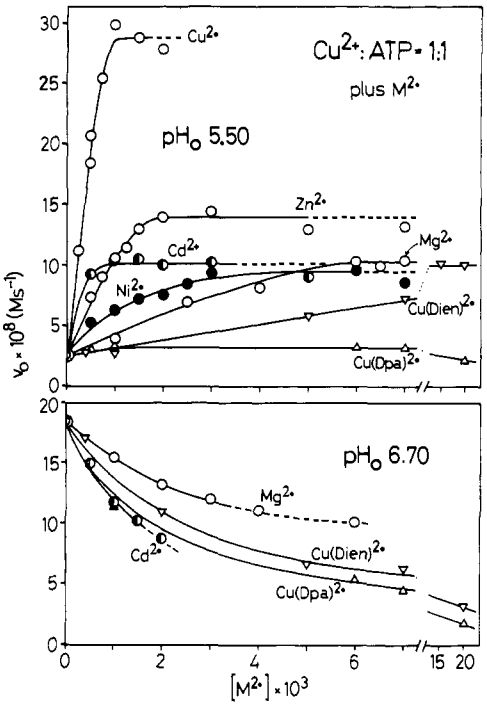

Figure 6. Dependence of the initial rate v_0 of the Cu^{2+} promoted dephosphorylation of ATP ($[Cu^{2+}]_{tot} = [ATP]_{tot} = 10^{-3}$ M) on the addition of divalent metal ions (○, ◐, ●) or Cu^{2+} 1:1 complexes with dien (▽) and dpa (△) at pH_0 5.50 (upper part) and 6.70 (lower part); $I = 0.1$, $NaClO_4$; 50 °C. The broken line portions indicate uncertainty due to precipitation. The experimental data shown in the upper part for Ni^{2+}, Cu^{2+}, and Zn^{2+} are taken from the lowest part of Figure 5 in ref 30.

are nearly 100% formed under the conditions used.[71,72]

Evidently the addition of other metal ions than Cu^{2+} is less effective, although Zn^{2+} still promotes the activity of the Cu^{2+}/ATP system considerably. As the second Cu^{2+} acts in the reactive complex in the form of Cu–OH^+ (cf. ref 65), it seems reasonable to assume that Zn^{2+} acts as Zn–OH^+, but in this system part of the reactivity occurs also via nucleophilic water attack (see also section 7 and footnote 65). The latter assumption agrees with the properties of the systems containing Cd^{2+}, Ni^{2+}, or Mg^{2+}; these ions also promote the reactivity but they do not form hydroxo complexes at pH 5.5; they could therefore carry only water as a nucleophile. However, in agreement with the reasoning given in sections 2 and 3 we propose that water attack does not occur here in an intramolecular fashion (as suggested for other systems),[73-75] because of the following: (i) It is difficult to see why all three metal ions should "activate" or "position" the bonded attacking water molecule in exactly the same way. That they reach the same limiting rate value is easier to understand by assuming the same structure of the reactive species and an intermolecular water attack. Different concentrations are needed to reach the limiting value because these ions have different coordination tendencies toward phosphate groups ($Cd^{2+} > Ni^{2+} > Mg^{2+}$).[19,42] (ii) Cu(dien)$^{2+}$ also reaches the same limiting rate value (Figure 6)[76] even though there is no water molecule left in the equatorial coordination sphere of Cu^{2+} after coordination to $Cu(ATP)^{2-}$ [for the apical water molecules again the argument of point i would hold]. Hence, we conclude that $[Cu(ATP)(M)]_2$ is the most reactive species under these conditions and that it undergoes intermolecular H_2O attack when $M^{2+} = Mg^{2+}$, Ni^{2+}, Cd^{2+}, or Cu(dien)$^{2+}$.

With Cu(dpa)$^{2+}$ it seems that the promoting and inhibiting effects offset each other.[77] This means the promotion occurs as with Cu(dien)$^{2+}$, while the inhibition is probably due to stacking between the pyridyl rings and the purine system. Such stacking interactions are well-known[35-37] and they are expected to influence the structure of the dimeric intermediate. That both Cu(dien)$^{2+}$ and Cu(dpa)$^{2+}$ are able to coordinate to $Cu(ATP)^{2-}$ is evident from the results at pH 6.70 shown in the lower part of Figure 6 (which will be discussed further in section 10).

These results allow one further important conclusion: in Cu(dien)$^{2+}$ and Cu(dpa)$^{2+}$ only a single equatorial binding site is left for coordination to $Cu(ATP)^{2-}$. As the coordination tendency of the apical Cu^{2+} positions is very weak, we conclude that the second metal ion (or complex) coordinates to $Cu(ATP)^{2-}$ (or its dimeric derivative) only in a monodentate fashion and that a 4-membered chelate is not formed in the reactive species (see sections 9 and 10).

Furthermore, from the properties of the Cu^{2+}/ATP system in the presence of Mg^{2+} or Cu(dien)$^{2+}$ it is clear that the second metal ion does not interact with N-7, because such role could not be taken over by either of these two ions. However, it is revealing to see, regarding biological systems, that Mg^{2+} promotes the dephosphorylation, provided it is correctly positioned by "outside" forces.

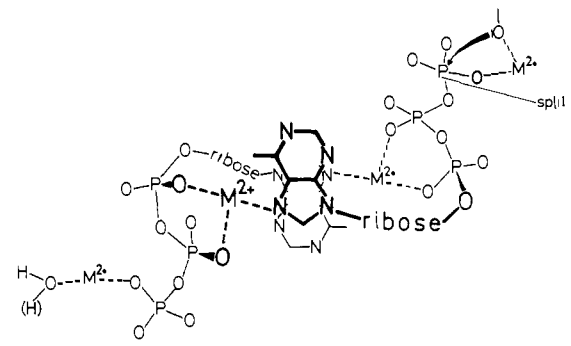

Figure 7. Proposed structure of the reactive $[M_2(ATP)]_2(OH)^-$ dimer, which occurs in low concentrations during the metal ion promoted dephosphorylation of ATP (section 9). The intramolecular attack of OH^- is indicated on the right side, while the left side is ready to transfer also into the reactive state by deprotonation of the coordinated water molecule or to undergo an intermolecular water attack.

9. Composition and Tentative Structure of the Reactive 2:1 Intermediate in the M^{2+} Facilitated Dephosphorylation of ATP. We now summarize the facts outlined in the preceding sections regarding the composition of the reactive intermediate: (i) the M^{2+}:ATP ratio is 2:1 for all metal ions studied (with the single exception of Cu^{2+} at higher pH; see section 10); (ii) the intermediate is of a dimeric nature; and (iii) the attack occurs preferably via OH^- in an *intra*molecular fashion through a M–OH^+ unit (section 7). Hence, the most reactive intermediate has the composition $[M_2(ATP)]_2(OH)^-$, although an *inter*molecular attack of water on $[M_2(ATP)]_2$ may also occur to some extent (section 8).

That a correctly positioned OH^- in a M–OH unit can be a potent nucleophile[51-53] despite its reduced basicity[51,52] is well-known and has been implied for OH^- bound to, e.g., Zn^{2+},[78] Co^{3+},[4,5,52,73] and Cu^{2+}.[74]

Taking into account the crucial metal ion/N-7 interaction (section 7), as well as the recent evidence from an ^1H NMR shift

(71) In a 1:1 mixture of dien and Cu^{2+} complex formation is complete from pH ≥5; see the upper part of Figure 1 in ref 39.

(72) The acidity constants of $H_3(dpa)^{3+}$ were determined by potentiometric pH titrations: $pK^H_{H_3(dpa)} = 0 \pm 0.5$, $pK^H_{H_2(dpa)} = 2.47 \pm 0.02$, $pK^H_{H(dpa)} = 7.23 \pm 0.01$ ($I \sim 0.1$, $NaNO_3$; 25 °C). From $Cu^{2+}/H_3(dpa)^{3+}$ titrations it was evident that complex formation is complete already from pH ≥2.5 on ($I = 0.1$, $NaNO_3$; 25 °C).

(73) Boreham, C. J.; Buckingham, D. A.; Keene, F. R. *J. Am. Chem. Soc.* **1979**, *101*, 1409-1421; *Inorg. Chem.* **1979**, *18*, 28-38.

(74) Suh, J.; Cheong, M.; Suh, M. P. *J. Am. Chem. Soc.* **1982**, *104*, 1654-1657.

(75) Kunugi, S.; Hirohara, H.; Ise, N. *Eur. J. Biochem.* **1982**, *124*, 157-163.

(76) Cu(dien)$^{2+}$ forms the hydroxo complex[39] only at high pH: $pK^H_{Cu(dien)(H_2O)} = 9.5$.

(77) The addition of 1 equiv of dien or dpa at pH_0 5.50 to the Cu^{2+}/ATP 1:1 system reduces the rate dramatically to about 1/20.

(78) (a) Breslow, R.; McClure, D. E.; Brown, R. S.; Eisenach, J. *J. Am. Chem. Soc.* **1975**, *97*, 194-195. (b) Lipscomb, W. N. *Acc. Chem. Res.* **1982**, *15*, 232-238.

study[23] that Zn^{2+} and Cd^{2+} promote the self-association of ATP by bridging neighboring ATP species, the structure shown in Figure 7 is proposed for the reactive intermediate. As the coordination of one metal ion to a NTP leads commonly to an $(\alpha),\beta,\gamma$ coordination,[20,79] we postulate now that the coordination of a second metal ion forces one metal ion to the γ phosphate group (which is the most basic site) and the other into the α,β position, causing in this way a labilization of the γ group. The shift of the one metal ion into the α,β position is facilitated by its simultaneous coordination to N-7 of the neighboring ATP. We conclude that this additional N-7 interaction is the reason why purine–NTPs are dephosphorylated much faster than pyrimidine–NTPs (Figure 1); the latter do not have this additional support in achieving the intermediate (Figure 2).

Ni^{2+} is a rather poor promotor of the reactivity, despite the fact that $Ni(ATP)(OH)^{3-}$ is formed even at a somewhat lower pH than $Cd(ATP)(OH)^{3-}$. This might indicate that the substitution rate is crucial with Ni^{2+}, but it could also mean that there is a further structural feature. Indeed, the low reactivity of Ni^{2+}/purine–NTP systems has been explained[30,33] by an α,β,γ coordination of this hexacoordinate metal ion in $Ni(NTP)^{2-}$. This binding type would render the formation of an α,β coordinated Ni^{2+} more difficult compared to that of Cu^{2+}, Zn^{2+}, and Cd^{2+}, if these latter ions coordinate initially only β,γ because then a shift into an α,β position would not alter the denticity in the coordination sphere.

The split indicated in Figures 2 and 7 is in agreement with ^{18}O incorporation from water into PO_4^{3-} derived from the γ phosphate group of NTPs in the metal ion promoted dephosphorylation and in the enzyme-catalyzed cleavage of the terminal P–O–P bond.[80] As the nucleophilic attack by a metal ion bound hydroxide occurs in an intramolecular fashion, the development of a pentacoordinated[81] transition state is implied. A metaphosphate-type[82] intermediate is not expected to develop during a metal ion promoted phosphoryl transfer process as considered here.

It seems worthwhile to include also some mechanistic considerations using Cu^{2+} as an example, because this will allow comparisons with the reasoning in sections 3 and 4. As indicated above, in a 2:1 solution of Cu^{2+} and ATP in the pH region around 5.3 the most reactive species possesses the composition $[Cu_2ATP]_2(OH)^-$. Under the present conditions (Figure S3), the predominant complex in solution is $Cu_2(ATP)$ (see section 8 and the upper part of Figure 6), so that only dimerization and coordinated water deprotonation need to be considered further:

$$2Cu_2(ATP) \rightleftharpoons [Cu_2(ATP)]_2 \quad (13)$$

$$[Cu_2(ATP)]_2 \rightleftharpoons [Cu_2(ATP)]_2(OH)^- + H^+ \quad (14)$$

$$[Cu_2(ATP)]_2(OH)^- \rightarrow \text{products} \quad (15)$$

For eq 13 the dimerization constant is taken as $K_D \simeq 10^2\ M^{-1}$ (cf. ref 83) and for eq 14 a rough estimate is $pK_a \simeq 6$. In a 1 mM ATP solution the observed first-order rate constant from Figure S3 (see also Figure 2 in ref 30) at pH 5.3 is $300 \times 10^{-6}\ s^{-1}$. We then have for Cu–OH attack within the hydroxo dimer $300 \times 10^{-6}\ s^{-1} = k_{obsd} = k_{5.3}K_aK_D[Cu_2(ATP)]/(H^+)$ from which $k_{5.3} \simeq 0.02\ s^{-1}$.[48] This $k_{5.3}$ rate constant applies to intramolecular nucleophilic attack by metal ion bound hydroxide; the value will be compared with related data in the next section.

(79) (a) Connolly, B. A.; Eckstein, F. *J. Biol. Chem.* **1981**, *256*, 9450–9456. (b) Goody, R. S.; Hofman, W.; Konrad, M. *FEBS Lett.* **1981**, *129*, 169–172.

(80) (a) Moll, H.; Schneider, P. W.; Brintzinger, H. *Helv. Chim. Acta* **1964**, *47*, 1837–1839. (b) Rohrbach, M. S.; Dempsey, M. E.; Bodley, J. W. *J. Biol. Chem.* **1974**, *249*, 5094–5101. (c) Cohn, M.; Hu, A. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 200–203. (d) Webb, M. R.; McDonald, G. G.; Trentham, D. R. *J. Biol. Chem.* **1978**, *253*, 2908–2911. (e) Meyerson, S.; Kuhn, E. S.; Ramirez, F.; Marecek, J. F. *J. Am. Chem. Soc.* **1982**, *104*, 7231–7239.

(81) (a) Westheimer, F. H. *Acc. Chem. Res.* **1968**, *1*, 70–78. (b) Eiki, T.; Horiguchi, T.; Ono, M.; Kawada, S.; Tagaki, W. *J. Am. Chem. Soc.* **1982**, *104*, 1986–1991.

(82) Westheimer, F. H. *Chem. Rev.* **1981**, *81*, 313–326.

(83) Estimate based on the known self-association of several $M(ATP)^{2-}$ complexes.[23]

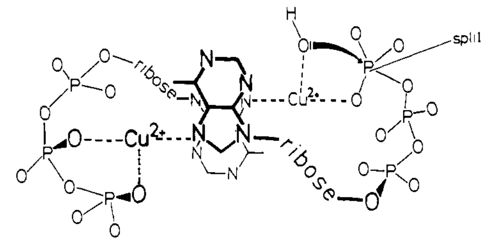

Figure 8. Tentative structure of the reactive $[Cu(ATP)]_2(OH)^{5-}$ dimer, which occurs in low concentrations during the Cu^{2+} promoted dephosphorylation of ATP at pH ≥6.5 (section 10). The intramolecular attack of OH^- is indicated on the right side, while the left side shows a Cu^{2+} stabilizing the dimer by coordination to the γ,β phosphate groups of one ATP and to N-7 of the other.

10. Reactivity in M^{2+}/ATP 1:1 Systems Including the Special Case of $[Cu(ATP)]_2^{4-}$. For all M^{2+}/ATP systems studied (with the single exception of Cu^{2+}; Figure S3)[30] the 2:1 mixture is always more reactive than the 1:1 mixture over the whole range from pH 2 to 10 (Figure 3 and ref 30), and Job's series showed throughout a 2:1 composition for the reactive dimeric species. Hence, based on Figure 7 the reactivity in M^{2+}/ATP 1:1 systems (see, e.g., Figure 3) may be well explained, assuming that the position of equilibria 16 to 18 or of any related equilibria is such that few

$$2\ M(ATP)(OH)^{3-} \rightleftharpoons M_2(ATP)(OH)^- + ATP^{4-} + OH^- \quad (16)$$

$$M(ATP)(OH)^{3-} + M(ATP)^{2-} \rightleftharpoons M_2(ATP)(OH)^- + ATP^{4-} \quad (17)$$

$$M_2(ATP)(OH)^- + M(ATP)^{2-} \rightleftharpoons M_3(ATP)_2(OH)^{3-} \quad (18)$$

percent of $M_3(ATP)_2(OH)^{3-}$ are formed. In this species two metal ions stabilize the dimer via bridging and the third coordinates to one of the two γ phosphate groups to facilitate the nucleophilic attack. This explanation holds for the 1:1 systems with Ni^{2+}, Zn^{2+}, or Cd^{2+} and for the Cu^{2+}/ATP 1:1 system at pH <6.5; it does, however, not satisfy the observations in the Cu^{2+}/ATP 1:1 system at pH ≥6.5 as indicated below.

Previous Job's series (Figure 6 in ref 30) have shown that from pH >6.7 the Cu^{2+}/ATP ratio is 1:1 in the reactive dimer. Indeed, at pH >6.5 the 2:1 and 1:1 system share the same reactivity (Figure S3).[30] This indicates that the second Cu^{2+} simply hydrolyzes away in this pH range and is no longer available for a promotion of the dephosphorylation. In accord with this view are the results summarized in the lower part of Figure 6. The addition of Mg^{2+} or Cd^{2+} and $Cu(dpa)^{2+}$ or $Cu(dien)^{2+}$ inhibits the reactivity at pH_0 6.70 in the Cu^{2+}/ATP 1:1 system. The explanation is that any additional metal ion coordination to the triphosphate impairs the coordinated Cu^{2+} so that it becomes in part released. As the added metal ions or metal ion complexes promote the reaction less effectively than Cu^{2+}, the dephosphorylation rate in the system is therefore decreased.

The kinetic studies (Figures S3 and S4, and ref 30) indicate that the reactive species has the composition $[Cu(ATP)]_2(OH)^{5-}$. We assume also for this complex that both Cu^{2+} bridge an ATP–dimer and that a reaction is initiated by a *partial* release of the $(\alpha),\beta$ group from the coordination sphere of Cu^{2+} upon deprotonation of a coordinated water molecule. This latter Cu^{2+} would then be coordinated in the reactive intermediate to N-7 of one ATP and the γ phosphate group of the other ATP, allowing an intramolecular attack of a coordinated OH^- (Figure 8). It is evident that with progressing hydroxo-complex formation the bridging to N-7 and the stability of the dimer will be affected (section 7) leading to $Cu(ATP)(OH)^{3-}$ (cf. ref 56 and 57) and a decreasing reactivity.

The reactive $[Cu(ATP)]_2(OH)^{5-}$ may be derived from the predominant complex in an equimolar solution, $Cu(ATP)^{2-}$, by the following reactions:

$$2Cu(ATP)^{2-} \rightleftharpoons [Cu(ATP)]_2^{4-} \quad (19)$$

$$[Cu(ATP)]_2^{4-} \rightleftharpoons [Cu(ATP)]_2(OH)^{5-} + H^+ \quad (20)$$

$$[Cu(ATP)]_2(OH)^{5-} \rightarrow \text{products} \quad (21)$$

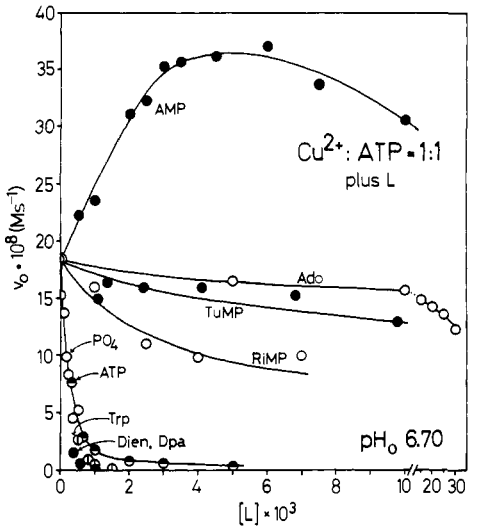

Figure 9. Dependence of the initial rate v_0 of the Cu^{2+} promoted dephosphorylation of ATP ($[Cu^{2+}]_{tot} = [ATP]_{tot} = 10^{-3}$ M) at pH_0 6.70 on the addition of ligands: L = AMP, adenosine, TuMP, RiMP, ATP, PO_4, L-tryptophan, dien, and dpa. $I = 0.1$, $NaClO_4$; 50 °C. The results of PO_4 are taken from Figure 8 of ref 30.

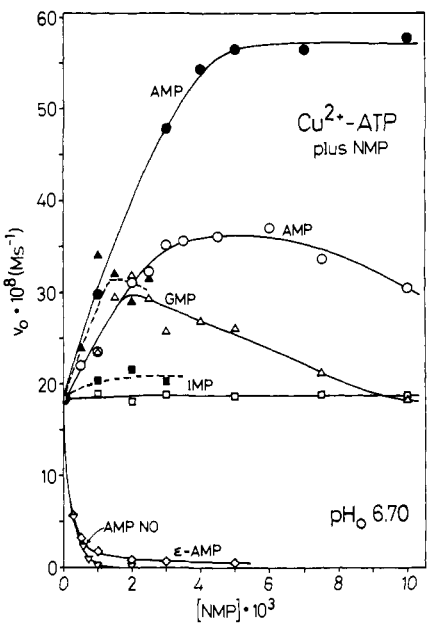

Figure 10. Influence of purine–nucleoside 5′-monophosphates and derivatives (NMP) on the rate of dephosphorylation of the 1:1 (empty marks) and 2:1 (full marks) Cu^{2+}:ATP systems. The dependence is given of the initial rate v_0 of the Cu^{2+} promoted dephosphorylation of ATP in 1:1 ($[Cu^{2+}]_{tot} = [ATP]_{tot} = 10^{-3}$ M; ○, △ □, ◇, ▽) and 2:1 systems ($[Cu^{2+}]_{tot} = 2 \times 10^{-3}$ M and $[ATP]_{tot} = 10^{-3}$ M; ●, ▲, ■) at pH_0 6.70 on the addition of NMP: AMP (○, ●), GMP (△, ▲), IMP (□, ■), ε-AMP (◇), and AMP·NO (▽). The broken lines indicate uncertainty due to precipitation. $I = 0.1$, $NaClO_4$; 50 °C.

For eq 19 the dimerization constant is estimated[83] as $K_D' \simeq 10^{1.3}$, while for the deprotonation in eq 20 $pK_a' \simeq 8$.[31] For a 1 mM solution we read from Figure 1 at pH 6.7 $k_{obsd} = 230 \times 10^{-6}$ s^{-1}. We then have for Cu–OH attack within the hydroxo dimer 230×10^{-6} s^{-1} = $k_{obsd} = k_{6.7}K_a'K_D'[Cu(ATP)^{2-}]/(H^+)$ from which $k_{6.7} \simeq 0.2$ s^{-1}.[48]

No special meaning should be attached to the apparent difference between $k_{6.7}$ ($\simeq 0.2$ s^{-1}) and $k_{5.3}$ ($\simeq 0.02$ s^{-1}; section 9), because the error is large due to the approximate nature of the equilibrium constants used in the calculation. However, both these values for intramolecular metal ion bound hydroxide attack are definitely much larger than the corresponding value calculated for eq 8 ($k_{MOH} = 5 \times 10^{-5}$ s^{-1}) in section 3, suggesting therefore special structural features: these are born out by a comparison of Figures 7 and 8 with Figure 2.

11. Promotion of the Reactivity in M^{2+}/ATP Systems by AMP and Inhibition by Other Ligands, Including 7-Deaza-AMP. Considering the delicacy of the structures in Figures 7 and 8 it is evident that the addition of any ligand with a larger coordination tendency toward the metal ion than the one of N-7 will inhibit the reactivity. Consequently, ligands like 2,2′-bipyridyl[30] or tryptophan (Figure 9) inhibit the reaction by forming ternary complexes of the type $M(bpy)(ATP)^{2-}$ (ref 35–37) and $M(ATP)(trp)^{3-}$.[84,85] dien and dpa also inhibit the reaction drastically.

The addition of ATP also inhibits the reactivity very strongly (Figure 9) in accordance with the results of Figure S3, and this implies that the most probably formed $Cu(ATP)_2^{6-}$ species, which would be a stacked complex, shows no reactivity. However, addition of AMP^{2-} promotes the reaction. By viewing Figure 8 this can only mean that in the $[Cu(ATP)]_2^{4-}$ dimers part of ATP^{4-} is replaced by AMP^{2-} to give a $Cu_2(ATP)(AMP)^{2-}$ complex and that this species has a higher stability thus leading to a larger concentration of a reactive species. This assumption is quite feasible because charge repulsion will be less and the stack will thus be stabilized; in addition, bridging of the two ligands by Cu^{2+} will probably be easier on one side of the "dimer" as the phosphate chain is shorter, and in this way the Cu^{2+} on the other side can possibly reach its reactive state more readily.

The importance of bridging on *both* sides of the dimer is confirmed with adenosine (Figure 9) which can only stack or undergo a weak N-7 interaction, but it cannot lead to a double bridge. Correspondingly, ribose 5′-monophosphate ($RiMP^{2-}$) and phosphate also inhibit the reaction. That $RiMP^{2-}$ inhibits less indicates that phosphate coordinates as PO_4^{3-}. However, most important is the observation that tubercidin 5′-monophosphate (=TuMP = 7-deaza-AMP; i.e., N-7 in AMP is replaced by —CH=) also *inhibits* the reaction;[86] this proves definitely that simultaneous stacking and phosphate coordination is *not* enough for a promotion: N-7 is a crucial part of the base moiety. Only metal ion bridging of the dimer via N-7 leads to the reactive intermediate!

The corresponding experiments in the Zn^{2+}/ATP 1:1 system were strongly hampered by precipitates, but the data (Figure S5)[54] still allow the conclusion that the overall properties are a reflection of those described for the Cu^{2+} system.

It is evident that one could view the reactive species shown in Figures 7 and 8 also by saying that one ATP is needed to bring the other into the reactive state. This view is further supported by the results described in this section for AMP: AMP^{2-}, having the N-7 and one phosphate group, is able to take over the role of the "structuring" ATP, thus creating mixed AMP/ATP stacks and forcing more ATP into the reactive form. The addition of too much AMP will of course lead to unreactive AMP stacks, which will bind Cu^{2+}, and in this way inhibit the reaction, as is actually observed (Figure 9).

12. The Influence of AMP on the Dephosphorylation Rate of the Cu^{2+}/ATP System in Comparison with Other Purine–Nucleoside 5′-Monophosphates. Based on the results of Figure 9 it is to be expected that the effect of AMP on a Cu^{2+}/ATP 2:1 system should be even more dramatic than that in a 1:1 system. Indeed, the addition of a 5-fold excess of AMP^{2-} leads to a 3-fold increase of the rate in the 2:1 system at pH 6.7 as is seen in Figure 10.

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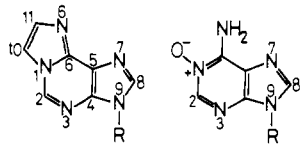
(86) (a) That TuMP is a slightly less effective inhibitor than RiMP (Figure 9) is probably due to steric hindrance, the participation of the base moiety in stacking (like in case of adenosine), and the higher basicity of TuMP leading probably to a somewhat lower formation degree of TuMP complexes at pH 6.7. By potentiometric pH titration ($I = 0.1$, $NaClO_4$; 25 °C) of a 6.5×10^{-4} M solution of H_2(TuMP) we determined $pK^H_{H_2(TuMP)} = 5.25 \pm 0.01$ (deprotonation of N-1) and $pK^H_{H(TuMP)} = 6.34 \pm 0.01$. For H(RiMP)$^-$ $pK^R_{H(RiMP)} = 6.22$ ($I = 0.1$, KNO_3; 15 °C).[86b] (b) Frey, C. M.; Stuehr, J. E. *J. Am. Chem. Soc.* **1972**, *94*, 8898–8904.

Chart II



R = ribosyl 5'-monophosphate

ε-AMP AMP·NO

With these results it was of course interesting to see if IMP^{2-} and GMP^{2-} could take over the role of AMP^{2-}. Both these nucleotides have a purine unit and hence a N-7. Figure 10 shows that IMP and GMP can take over the role of AMP only in a less efficient way. This result cannot be due to a smaller coordination tendency of N-7 in IMP and GMP, because the corresponding nucleoside complexes are somewhat more stable than those with adenosine.[23,87,88] The lower efficiency must be due to the stacking properties; indeed, the self-association tendency of the nucleosides and nucleotides decreases in the series[23,89] adenosine > guanosine > inosine, and this reflects exactly the order of the decreasing efficiency of $AMP^{2-} > GMP^{2-} > IMP^{2-}$. These results confirm further that indeed N-7 is involved in the bridging process by the metal ion (sections 7–9 and 11), because N-1 of IMP and GMP is not available for coordination in this pH range.

Stacking properties are most probably also the reason for the different reactivities of the Cu^{2+}/purine–NTP 1:1 systems shown in Figure 1. The rate of dephosphorylation decreases in the pH range from 2 to 8 in the order ATP > GTP > ITP. There is no M^{2+} or H^+ binding property that accounts for this order of the dephosphorylation rates. All H^+ and M^{2+} binding to the base moieties follow the order adenosine < inosine < guanosine,[87] whereas the tendency of the bases to stack follows the order adenosine > guanosine > inosine.[23,89] Hence, this comparison supports the conclusion that stacking interactions are important in forming the kinetically active dimers.[90]

How sensitive the structure of the reactive intermediate is also becomes clear from the addition experiments (Figure 10) with 1,N^6-ethenoadenosine 5'-monophosphate (ε-AMP) and adenosine 5'-monophosphate N(1)-oxide (AMP·NO) (Chart II). ϵ-AMP^{2-} has stacking properties[92] very similar to those of AMP^{2-} and the same may be surmised for AMP·NO. However, both AMP derivatives form chelates with Cu^{2+} at pH 6.7: in Cu(ε-AMP) the metal ion is bound to the N-6/N-7 site[92,93] and in Cu(AMP·NO) to the ionized O-amino N-oxide group.[38,94] This leads to different orientations of the metal ion in space, and consequently both nucleotide derivatives are strong inhibitors of the dephosphorylation in the Cu^{2+}/ATP system (Figure 10).

Some Conclusions with Regard to Enzymic Systems

The present results give evidence for three striking features, which seem to be generally valid for transphosphorylations:

(i) The observation of a highly facilitated reaction is connected with a very sensitive and delicate structure (Figures 7 and 8 and sections 11 and 12). The role of the "structuring" ATP in the reactive dimers will be taken over in nature by enzymes; this allows via different enzymes activation of a given substrate selectively for different but special purposes.

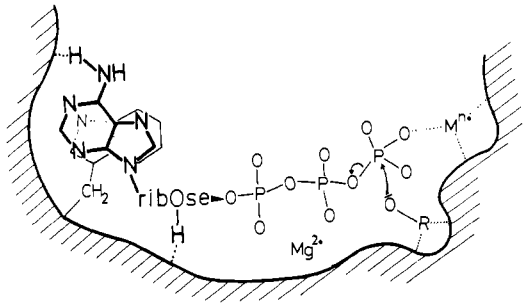


Figure 11. Tentative view of a reactive $Mg(ATP)^{2-}$ complex at the enzyme, the γ phosphate group being ready for the transfer to R-O which is sitting in a groove of the enzyme. ATP may be orientated at the enzyme surface by stacking (e.g., with an indole residue) and hydrogen bonding. Obviously, Mg^{2+} and M^{n+} could interchange their positions, and one of the two metal ions could even be replaced by an ionic interaction (e.g., with an arginyl group) and a reactive intermediate would still result. The release of ADP from the enzyme could in the example shown be initiated by a stronger coordination of the metal ion to the now twofold negatively charged β group leading thus to a release of $Mg(ADP)^-$ from the enzyme surface.

(ii) The nucleophilic attack at the terminal γ phosphate group occurs in the most reactive species in an intramolecular fashion. It is to be expected that not only coordinated OH^- remains a nucleophile of high quality but that this also occurs with other groups, e.g., alcoholic and related residues, to which transphosphorylation occurs in nature.

(iii) With the single exception of the Cu^{2+}/ATP system at pH >6.7, *all* other metal ion/NTP systems studied showed the property that the most reactive species contains two metal ions for each NTP. Coordination of two metal ions (Figures 2 and 7) is also expected to yield reactive species in enzymic systems. In fact, a growing number of enzymic systems are becoming known which contain two or more metal ions.[14–16,95]

On the basis of the present results one is tempted to say that "nature made a good choice" in selecting Mg^{2+} for so many reactions which involve nucleotides. In $Mg(ATP)^{2-}$ the metal ion is mainly coordinated to the β, γ phosphate groups,[20,96] no direct Mg^{2+}/N-7 interaction occurs,[23] and because there is little tendency for Mg^{2+} to form a hydroxo complex in the physiological pH range (section 5) no "self-activation" of the $Mg(ATP)^{2-}$ substrate is possible. With most other metal ions, including Zn^{2+} and Mn^{2+}, which have a tendency to interact with N-7, this self-activation will occur to some extent. Nature possesses, however, a tool to prevent this self-activation, if one of these metal ions should be desired for a special task: the formation of a mixed ligand complex, as with tryptophanate or even phosphate, will strongly inhibit a dephosphorylation reaction.

Figure 11 depicts a proposal of how nature could achieve selective transphosphorylation. The first step could be to anchor the nucleotide or its complex to the enzyme–protein, e.g., by stacking with an indole residue or by hydrophobic interactions with an isopropyl moiety,[84,85] but hydrogen-bond formation (e.g., with histidine)[97] and ionic interactions (e.g., with an arginyl residue)[98] also appear feasible. Either of the two latter interactions, if it occurs with the phosphate chain, could also replace one of the two metal ions shown in Figure 11 in leading to the reactive

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(90) At pH_0 5.7 plots of log v_0 vs. log [Cu^{2+}/NTP] yield straight lines with a slope of two for ITP and GTP. At pH >6 the situation is more complicated but corresponding plots at pH_0 8.4 give for both NTPs slopes of m = 1.4, indicating that now a monomeric complex gains influence. As in this pH range the proton from N-1 is liberated in $Cu(ITP)^{2-}$ and $Cu(GTP)^{2-}$, we presume that $Cu_2(NTP\text{-}H)(OH)^{2-}$ is the reactive monomeric intermediate and that the higher reactivity compared with $Cu_2(R\text{-}TP)(OH)^-$ is due to ring back-binding of one of the two Cu^{2+}. This would then be the third reactive species, besides the two characterized in Figures 2 and 7; moreover, it would correspond to a long sought reactive complex.[29,91] However, before a final decision can be made more work is needed.
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intermediate. If the anchoring process occurs in such a sterically orientated way that the protein offers binding sites which facilitate a shift of Mg^{2+}, either into an α,β position or to the γ group, an activation would result. There are indeed examples where the specific function of a divalent metal ion is the formation of an enzyme-metal ion-nucleotide bridged complex.[14,99]

This reasoning indicates that the anchoring process of a nucleotide to the enzyme-protein is crucial for an activation. As purine-nucleotides are much more easily anchored via stacking and hydrophobic interactions than pyrimidine-nucleotides, this difference may be the main reason why pyrimidine-NTPs, e.g., UTP, are not able to substitute effectively for ATP in many enzymic systems.[15,100]

From Figure 11 it is evident that the two activation "partners", e.g., the two metal ions, may interact not only in a M(α,β)-M-(γ)-like way (as is shown in the figure) but that a M(α)-M(β,γ) coordination can also be enforced and this would then lead to a reactive species ready for the transfer of either a nucleoside monophosphate or a diphosphate group. In fact the concept outlined allows selective activation for the transfer of all groups which can be derived from a nucleoside triphosphate, including the transfer of a nucleoside group.

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Registry No. Dien, 111-40-0; Dpa, 1539-42-0; AMP, 61-19-8; IMP, 131-99-7; GMP, 85-32-5; ϵ-AMP, 37482-16-9; AMP·NO, 4061-78-3; ATP, 56-65-5; CTP, 65-47-4; TuMP, 16719-46-3; RiMP, 4300-28-1; Cu/ATP (1:1), 18925-86-5; Cd/ATP (2:1), 74114-69-5; Cd/ATP (1:1), 72052-13-2; Cd/CTP (1:1), 75898-71-4; Zn/ATP (1:1), 6602-83-1; Ni/ATP (1:1), 18839-84-4; Cu^{2+}(dien), 45520-77-2; Cu^{2+}(Dpa), 65956-54-9; Mg, 7439-95-4; Ni, 7440-02-0; Zn, 7440-66-6; Cd, 7440-43-9; adenosine, 58-61-7; L-tryptophan, 73-22-3.

Supplementary Material Available: Table S1 and Figures S1-S5 giving additional kinetic details (6 pages). Ordering information is given on any current masthead page.

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Chain-Folding Initiation Structures in Ribonuclease A: Conformational Analysis of *trans*-Ac-Asn-Pro-Tyr-NHMe and *trans*-Ac-Tyr-Pro-Asn-NHMe in Water and in the Solid State

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Abstract: In order to investigate the role of β-bends with non-native *trans* peptide bonds preceding Pro^{114} and Pro^{93} in the mechanism of folding of bovine pancreatic ribonuclease A, we have examined the conformations of the synthetic peptides Ac-Asn-Pro-Tyr-NHMe (I) and Ac-Tyr-Pro-Asn-NHMe (II) in water and in the solid state. These sequences occur at residues 113-115 and 92-94, respectively, in ribonuclease A. Evidence for a significant population of I with a *trans*-Asn-Pro peptide bond and a β-bend at Pro-Tyr in water includes backbone/backbone and side chain/backbone NOE's, N-H bending frequencies characteristic of a β-bend, and hydrogen bonds detected by solvent spin-saturation transfer measurements involving the TyrN*H* and N*H*Me amide protons. Comparison of the Raman spectrum in water with that of the crystal suggests that the major backbone conformation in solution is similar to that in the solid state. Populations of II with a *trans*-Tyr-Pro peptide bond also have a hydrogen bond involving the N*H*Me amide. The crystal structure of I ($R \leq 0.064$), determined in two different crystalline forms, is a type I β-bend at Pro-Tyr with a *trans* peptide bond at Asn-Pro and intramolecular hydrogen bonds involving both the TyrN*H* and N*H*Me amide protons. The crystal structure of II ($R < 0.076$) also has a *trans* peptide bond (at Tyr-Pro), but an extended backbone conformation. The relevance of these results to the mechanism(s) of chain-folding initiation in ribonuclease A is discussed.

It is generally believed that local short- and medium-range interactions dominate in determining the native structure of globular proteins.[1-12] In the case of bovine pancreatic ribonuclease A, this principle is supported by the observations that reduced ribonuclease retains ~0.04% of native enzymatic activity[13] and ~6% native antigenic activity.[14] Spectroscopic measurements also demonstrate that the reduced protein,[15-17] the C-peptide

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