from the $\Delta M_s = \pm 2$ transition. This result indicates that copper has migrated from the native copper site of one subunit to the vacant zinc site of another subunit, forming the imidazolate-bridged binuclear unit found in Cu_2Cu_2SOD. The disappearance of the Cu_2E_2SOD signal fits a titration curve for a single ionizable group with a pK_a of 8.2. This pK_a could represent the deprotonation of histidine-61, a necessary step in forming the bridged species. The visible absorption spectrum of Cu_2E_2SOD changes at high pH to that characteristic of Cu_2Cu_2SOD, providing additional confirmation of the interpretation of the ESR results.

The migration of copper at pH >7 means that studies of Cu_2E_2SOD conducted at alkaline pH were actually carried out on dicopper subunits and apoprotein subunits. Earlier work on the activity and thermal stability of Cu_2E_2SOD must therefore be reevaluated. The specific activity of Cu_2E_2SOD at pH 6.0, where no dicopper active sites are present, was found to be 80% of that of Cu_2Zn_2SOD, confirming that zinc is not essential to enzymatic activity.

Visible and ESR spectra indicate that, below pH 4, Cu_2Zn_2SOD and Cu_2Cu_2SOD lose the metal in the zinc site giving Cu_2E_2SOD.[43] The different metal binding preferences of the copper and zinc sites at low pH makes possible the preparation of metal-substituted derivatives of SOD having specific metals in each site. The difference in metal binding properties of the two sites and the metal migration observed at pH >7 may be important if the true function of the protein is something other than superoxide dismutation.[65]

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Toward the Future

We have shown the value of model compounds in elucidating the properties of the imidazolate-bridged bimetallic center in SOD. Especially important were the binucleating macrocycles A and A′ which stabilized the $Cu_2(im)^{3+}$ unit over the physiological pH range in aqueous solution. Using ligands of this kind it should be possible to build models for other ligand bridged bimetallic centers in biology. A step in this direction has been taken with the synthesis and characterization of $[Cu_2(OH)(ClO_4)\subset A]^{2+}$. This monohydroxo-bridged strongly antiferromagnetically coupled dicopper(II) complex has spectroscopic and magnetic properties similar to those of binuclear copper sites in other proteins.[66] Two metals in a binucleating macrocycle could also promote new chemistry of significance to areas apart from biological ones. The future of this field holds considerable promise.

The work described here was in large part carried out in the laboratory of S.J.L. with generous support from the National Institute of General Medical Sciences and the National Science Foundation. Several talented graduate students and postdoctoral associates cited in the individual references contributed in a major way to its success. S.J.L. is especially grateful to Professor Joan Valentine for stimulating discussions at the outset and fruitful collaboration in some of the biochemical studies. K.G.S. gratefully acknowledges leave time from Bryn Mawr College to carry out experiments on SOD. We also thank Professors Jean-Marie Lehn, John Bulkowski, and Dr. Andrea Martin for providing generous quantities of the ligands A and A′. Figure 1 was prepared by Matthew Eichner.

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Some Properties of the Phosphorothioate Analogues of Adenosine Triphosphate as Substrates of Enzymic Reactions

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Nucleoside triphosphates, most frequently adenosine triphosphate, play a central role in the bioenergetics of the cell. As shown in Figure 1, the formation of ATP from ADP and inorganic phosphate is coupled with energy-producing reactions such as oxidation through the electron-transport system, photophosphorylation, and glycolysis. Conversely, the expenditure of the chemical potential energy of the nucleoside triphosphates by conversion to diphosphates and inorganic phosphate, or alternatively the monophosphate and inorganic pyrophosphate, is coupled to many energy-requiring processes. Those processes that are characterized by the transfer of chemical energy derived from hydrolysis of nucleoside triphosphate to other forms of energy, such as mechanical work in muscle contraction, translocation during protein synthesis, or ion transport by the Na^+,K^+-ATPase, are of necessity complex processes of cellular organelles. However, those processes in which chemical energy is transferred for endergonic chemical reactions, such as biosynthesis

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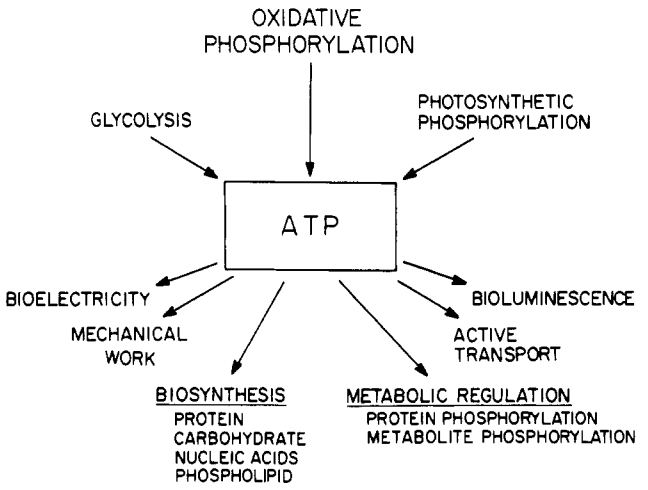

Figure 1. Role of ATP in cellular processes.

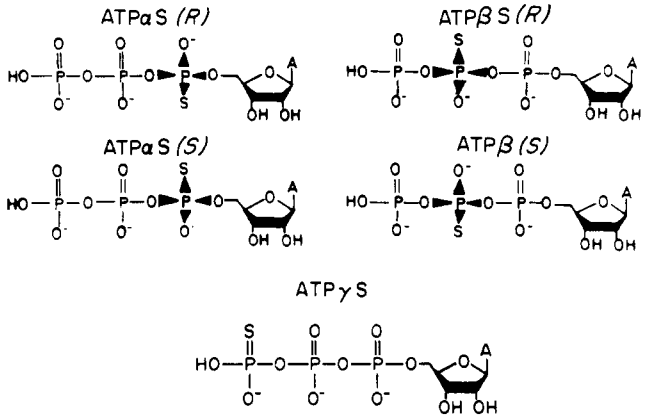

Figure 2. Structures of phosphorothioate analogues of ATP: ATPαS, *R* and *S* diastereomers, ATPβS, *R* and *S* diastereomers, and ATPγS.

of macromolecules and phosphoryl transfer in metabolic regulation, are in general simpler and, consequently, more amenable to detailed analysis at a molecular level.

Among the attempts to specify the detailed mechanism of the multitudinous enzymic reactions with adenosine 5′-triphosphate (ATP) as substrate, the substitution of the obligatory divalent Mg ion by other active divalent ions and of ATP by active analogues of ATP has been highly rewarding. The phosphorothioate analogues of ATP shown in Figure 2 were first introduced by Eckstein and co-workers,[1] and many of their properties and applications to biochemical problems have been reviewed.[2] The unique characteristics of these analogues that have proven particularly useful include the following: (a) the phosphorus bearing the substitution in ATPαS[3] and ATPβS (see Figure 2) is chiral in nature; (b) for a given diastereomer the metal chelates differ in their geometric configuration for hard and soft metal ions with their respective preference for O and S ligands as shown in Figure 3; (c) three analogues of ATP become available by sulfur substitution

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(2) (a) Eckstein, F. *Acc. Chem. Res.* **1979**, *12*, 204. (b) Eckstein, F. *Trends Biochem. Sci., Pers. Ed.* **1980**, *5*, 157. (c) Knowles, J. R. *Annu. Rev. Biochem.* **1980**, *49*, 877. (d) Frey, P. A. **1982**, *Tetrahedron*, in press. (e) Webb, M. R. *Methods Enzymol.* **1982**, in press.
(3) The following abbreviations are used: ADPβS, adenosine 5′-*O*-(2-thiodiphosphate); ATPαS, adenosine 5′-*O*-(1-thiotriphosphate); ATPβS, adenosine 5′-*O*-(2-thiotriphosphate); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate).

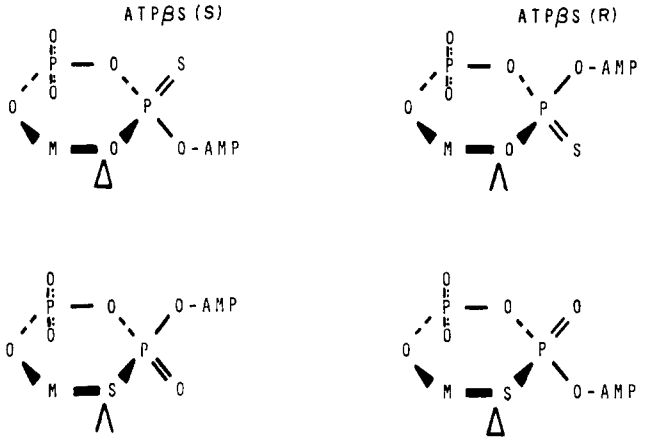


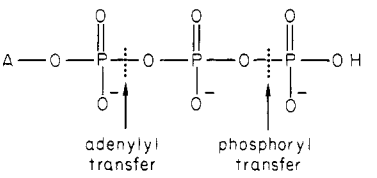
Figure 3. The metal chelate configurations, Δ or Λ, of β,γ bidentate ATPβS diastereomers with oxygen and sulfur ligands, respectively.

on a nonbridge oxygen of α- or β- or γ-phosphate (see Figure 2); and (d) the free energy difference between MgATPβS and MgADPβS differs from that between MgATP and MgADP by more than 2 kcal.

Investigations of the stereochemical course of a variety of enzymic ATP reactions, i.e., retention or inversion of configuration at a chiral phosphorus, using diastereomeric phosphorothioate analogues have been reviewed[2] and will not be discussed further. This Account will focus on three other aspects of enzymic mechanism that are revealed by (a) stereoselectivity and its metal ion dependence for the diastereomeric forms of ATPαS and ATPβS, (b) the effects that different metal ions have on reaction rates resulting from substitution of an oxygen by S on α-, β-, or γ-P, and (c) the shift in equilibrium constant for ATPβS relative to ATP in reactions where the triphosphate is converted to the diphosphate.

Stereoselectivity

The absolute configurations have been established for both ATPαS[4] and ATPβS[5] diastereomers. Many examples of stereoselectivity have accumulated in the past 5 years for both major types of cleavage of ATP(S) species (1) at the terminal O–P bond (phosphoryl transfer) and (2) at the α,β-P–O bond (adenylyl transfer) as shown:



The results of early work showing low stereospecificity should be viewed with caution because the diastereomers were not always sufficiently stereochemically pure. Contamination of an inactive diastereomer with even a few tenths of 1% of the active one is particularly serious in cases of high selectivity: a factor of 100–1000 in the rates is not unusual, but the Michaelis–Menten constants (K_M) usually vary only by a factor of 2–5.

For the dozen phosphoryl-transfer reactions (kinases and synthetases) investigated, there is no consistency

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Table I
Stereoselectivity and Its Metal Dependence of Some Kinase Reactions for the ATPαS and ATPβS *R* and *S* Diastereomers

	ATPαS			ATPβS			
	V_S/V_R		selectivity[a]	V_S/V_R		selectivity[a]	
kinase	Mg	Cd	Cd/Mg	Mg	Cd	Cd/Mg	ref
Type I [Metal Dependence α-S(−), β-S(+)]							
acetate	4.6	52.5	11.4	<0.0006	1.1	0.00066	8
adenylate	8.9	9.7	1.16	8.9	0.035	3.2	22
hexokinase	20.4	23	1.13	0.0017	36.7	0.062	5
Type II [Metal Dependence α-S(+), β-S(−)]							
	Mg	Zn	Zn/Mg	Mg	Zn	Zn/Mg	
3-P-glycerate	235	0.98	0.0043	1000	3000	3	7
Type III [Metal Dependence α-S(+), β-S(+)]							
	Mg	Cd	Cd/Mg	Mg	Cd	Cd/Mg	
arginine	0.092	9.8	0.90	0.041	137	5.6	23
creatine	0.12	3000+		0.016	41.7[b]		24

[a] Stereoselectivity is defined as the ratio of the V_{max} values of the more active substrate to the less active substrate and the ratio of selectivities for Cd to Mg are listed. [b] Initial rates at 1 mM nucleotide concentration.

either qualitatively or quantitatively in the stereoselectivity. The preference is almost evenly divided as shown in Table I between the *R* and *S* diastereomers of both MgATPαS and MgATPβS (see Figure 2). Quantitative values of stereoselectivity, i.e., ratios of rates, are meaningful only if K_M and V_{max} (the rate at substrate saturation) have been determined for highly purified diastereomers; values at a single concentration may be misleading because of differences in K_M and in substrate inhibition. Evaluation of stereoselectivity by measurement of relative amounts of each diastereomer of ATP(S) formed from ADP(S) is invalid since the ratio will be time dependent and will approach the equilibrium value of 1 in all cases. Wide variations in the degree of stereoselectivity have been reported for kinase reactions (cf. Table I). The stereoselectivity ratio for MgATPαS in the fructose-6-P kinase reaction[6] is about 2 and in the 3-P-glycerate kinase reaction[7] it is 235. With MgATPβS as substrate, in the fructose-6-P kinase reaction[6] again a preference of about 2 is found for one diastereomer whereas in the acetate kinase reaction the preference is greater than 1600.[8]

The relative rate patterns of ATP and the ATPβS diastereomers in reactions involving phosphorylated enzyme (E–P) intermediates have proven to be especially informative. For example, in the hydrolysis at the terminal O–P bond of nucleoside triphosphates catalyzed by sarcoplasmic reticulum Ca^{2+},Mg^{2+}-ATPase[9] and by *Escherichia coli* alkaline phosphatase[10] the rates are the same for ATP and for the two diastereomers of ATPβS. In both cases, the identity of rates for the three nucleotides implies that nucleoside tri- or diphosphate is not involved in the rate-determining step. However, not all phosphoryl transfer reactions that involve an E–P intermediate exhibit this pattern. For example, dog kidney Na^+,K^+-ATPase[9] shows no stereoselectivity between the ATPβS diastereomers, but the rate for both is about half that for ATP. This suggests that a step involving the nonchiral ADPβS is probably rate determining for this ATPase. Nucleoside diphosphokinase, which also involves an E–P intermediate, reveals yet another pattern, namely high stereoselectivity for ATPβS *R*.[10] This finding suggests that the formation of E–P may be the rate-determining step in this case.

For those hydrolytic reactions where no evidence exists for a phosphorylated intermediate and stereochemical inversion[2e] indicates a one-step reaction, stereoselectivity is invariably observed. One such example is provided by myosin subfragment S1[11] involved in muscle contraction: $k_{cat}(S)/k_{cat}(R)$ is 0.058 for ATPαS and >70 for ATPβS. Similarly for the chloroplast light-induced, proton-transporting ATPase,[12] the ratio V_S/V_R is 34 for ATPαS and 19 for ATPβS. For the mitochondrial ATPase and the chromaffin granule ATPase, the rates for ATPβS *R* were 3% and 30% of ATP, respectively, and the activity of ATPβS *S* was undetectable.[9] In all of these cases, a step involving the chiral substrate does contribute to the overall rate. A detailed analysis of the rates of individual steps in the ATPase reaction catalyzed by myosin subfragment S1 has been reported.[11]

In an examination of the stereoselectivity of the adenylyl transfer reactions, some interesting patterns emerge. With MgATPαS as substrate for macromolecular synthesis including DNA,[13] RNA[14] polymerases, polynucleotide phosphorylase,[15] and tRNA nucleotidyl transferase,[16] the *S* isomer is greatly preferred. The aminoacyl-tRNA synthetases catalyze two partial reactions, an adenylyl transfer to form enzyme-bound aminoacyladenylate followed by an acyl transfer to tRNA.

$$E + ATP + \text{amino acid} \rightleftharpoons E\text{–aminoacyl-AMP} + PP_i \quad (1)$$

$$E\text{–aminoacyl-AMP} + tRNA \rightleftharpoons \text{aminoacyl-tRNA} + AMP + E \quad (2)$$

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Table II
Relative Rates (V_{max}) of Mg(II)-Activated Reactions Catalyzed by *E. coli* Aminacyl-tRNA Synthetases

	valyl enzyme[17c]		methionyl enzyme[18]	
substrate	amino-acylation	interchange	amino-acylation	interchange
ATP	100		100	
ATPγS	21.4	nd[a]	57.6	
ATPβS *R*	11.4	nd	nd	nd
ATPβS *S*	3.3	3.6	10.7	69.5

[a] nd, *not* detectable.

For about 20 such synthetases investigated, preference for MgATPαS *S* or *R* is about equally divided, with a few showing very low stereoselectivity and a few very high selectivity in the overall aminoacylation reaction.[17] With the normal substrate, MgATP, the first partial reaction (eq 1) is generally 1 or 2 orders of magnitude faster than the second. With MgATPαS as substrate, the relative rates of the two partial reactions have not been determined. Consequently, it is not known whether the observed stereoselectivity in the overall reaction has contributions from both partial reactions or only one of the two.

The interpretation of observed stereoselectivity for ATPβS in adenylyl-transfer reactions is complicated by the possibility of a rapid stereoselective conversion of ATPβS to ATPγS concurrent with the adenylyl transfer and pyrophosphate formation. Such a complication arose in the aminoacyl-tRNA synthetase investigation from a novel reaction designated the interchange reaction catalyzed by these enzymes,[17c,18] namely an interchange of the β-P and γ-P groups resulting in the conversion of ATPβS to ATPγS. The equilibrium between ATPβS and ATPγS lies far toward ATPγS, and the latter's conversion to ATPβS is not observable. The interchange reaction occurs in the absence of tRNA, and it has been shown[18] that it is not due to the reversal of the first partial reaction (eq 1), i.e., E–aminoacyl-AMP + $PP(S)_i \rightleftarrows$ ATPγS + amino acid + AMP. It will be noted from Table II that (a) the rate of aminoacylation with ATPγS as substrate is faster than with ATPβS and (b) for the valyl enzyme, the *S* isomer is preferred in the interchange reaction but the *R* isomer is preferred in the overall aminoacylation reaction. The consequence of these properties is that on the one hand, the observed rate of aminoacylation of the *R* isomer of ATPβS is a valid measure of the reaction rate for this substrate since this isomer does not undergo interchange (cf. Table II), and on the other hand for the *S* isomer which converts readily to ATPγS via the interchange reaction, the observed rate of aminoacylation is in fact a reflection of the rate of the interchange reaction. If other adenylyl-transfer reactions also catalyze an interchange reaction, straightforward interpretation of the observed stereoselectivity may not be possible. When the stereoselectivities of the overall reaction and of the interchange reaction are different, the observed stereoselectivity due to both reactions need not represent the preference in the reaction of interest. This possibility has not been ruled out in the case of RNA polymerase. An alternative explanation for the lack of stereoselectivity with the MgATPβS isomers based not on rates but on different modes of binding of the two isomers has been invoked.[14]

Metal Chelate Structures

The structures of the Λ or Δ diastereomers of bi- and tridentate metal–ATP complexes and of the *R* and *S* diastereomers of ATPαS, ATPβS, and their metal complexes have been depicted and discussed in an earlier review.[2a] Jaffe and Cohn[19] presented NMR evidence that Mg(II) chelates via oxygen and Cd(II) via sulfur of the thiophosphate groups of the nucleotide thio analogues as would be expected for hard and soft metals, respectively. As shown in Figure 3, the same diastereomer of ATPβS yields opposite stereochemical configurations of the β,γ bidentate chelates Λ or Δ, when complexed either to Mg(II) through O or to Cd(II) through S. If reversal of stereoselectivity occurs in a Mg-*vs.* Cd-activated ATP enzymic reaction, for example, if MgATPβS, *R*, and CdATPβS, *S*, are the preferred substrates in a particular enzymic reaction, such reversal indicates unequivocally that in a rate-determining step in the reaction the metal is chelated to the phosphate group bearing the sulfur. This criterion for determining the metal chelate structure of nucleoside triphosphate substrates in enzymic reactions was first applied to the hexokinase reaction[20] by measuring the steady-state kinetic parameters, K_M and V_{max}, for a series of metal ions: Mg(II), Ca(II), Mn(II), Co(II), Ni(II), Zn(II), and Cd(II). The V_S/V_R ratio for ATPαS of ≃20 varied little with metal ion, but for ATPβS the V_S/V_R ratio of 0.0017 with Mg(II) increased monotonically in the series above to a value of 37 for Cd(II) (cf. Table I). It was concluded that the metal ion is chelated to the β-phosphate group of the nucleotide and probably not to the α-phosphate.

When there is no change of stereoselectivity, as in the above case with ATPαS, the interpretation is equivocal. The simplest explanation is that the α-phosphate group is not a ligand for the metal. The possibility that metal *is* chelated to the α-phosphate group in spite of the absence of metal-dependent stereoselectivity cannot be excluded. The enzyme may impose other constraints, e.g., obligatory binding to an α-phosphate oxygen atom, thus forcing all metals, regardless of their normal preference, to bind to the same ligand. For the reaction catalyzed by hexokinase, the interpretation that β,γ-bidentate ATP is the substrate was given credence from the prior results with substitution-inert metal complexes that established the bidentate $Co^{III}(NH_3)_4$–ATP as a substrate in the reaction but not the tridentate $Co^{III}(NH_3)_3$–ATP.[21]

Subseqently, a number of phosphoryl-transfer reactions were investigated with hard and soft metal ions, and some representative systems for which K_M and V_{max} values have been determined are listed in Table I.[22-24] In general, the K_M values vary only 2- or 3-fold but the V_{max} values may vary over 3 orders of magnitude. Metal dependency of stereoselectivity as measured by V_{max}

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Table III
Relative Rates of ATP and Its Active Thio Analogues with Mg(II) and Cd(II) or Zn(II)

	adenylate kinase			arginine kinase			3-P-glycerate kinase		
substrate	Mg	Cd	Mg/Cd	Mg	Cd	Mg/Cd	Mg	Zn	Mg/Zn
ATP	100	25.6	3.90	100	10.1	10	100	70.9	1.41
ATPαS	64.0	17.3	3.69	0.38	2.0	0.19	58.0	87.0	1.15
ATPβS	0.19	0.024	7.38	13.6	38.9	0.35	3.0	65.8	1.52
ATPγS	5.9	0.95	6.2	10.6	0.63	17	1.2		

ratios of the diastereomers manifests itself either by a decrease in the ratio of 1 or 2 orders of magnitude (relaxation of stereoselectivity) in a series from hard to soft metals or by a reversal of the stereoselectivity. Three patterns of metal-dependency of the three phosphates of ATP are observed: type I, reversal or relaxation on β-phosphate and not on α-phosphate; type II, α-phosphate and not on β-phosphate; and type III, on both α- and β-phosphate. Qualitative data available for additional systems not listed in Table I indicate that many adenylyl transfer reactions[14,15,17b,c] show a type I pattern; pyruvate kinase,[5] glutamine synthetase,[25] and carbamoyl synthetase[26] fall in the type III category; carbamate kinase[25] may be type II since its stereoselectivity for ATPβS like that of 3-P-glycerate kinase appears to have no metal ion dependence.

It is apparent from Table I that the degree of stereoselectivity and its metal dependence for each thio analogue vary greatly with enzyme and for any given enzyme vary with the position of sulfur substitution. Within the group of enzymes where reversal of stereoselectivity occurs only with ATPβS (type I, Table I) the relative stereoselectivities for Cd and Mg complexes of ATPβS vary more than three orders of magnitude (from 3.2 for adenylate kinase to <0.0007 for acetate kinase). With myosin ATPase,[27] there is a striking difference between the αS and βS analogues for both the degree of stereoselectivity with a given metal ion and in the degree of metal dependence of stereoselectivity. With Mg(II) rates between diastereomers vary 3-fold for αS and >3000-fold for βS and the ratio of stereoselectivities with Mg(II) and Cd(II) is 1.7 and 690 for αS and βS, respectively.

Although the preference of Cd(II) for S ligands and Mg(II) for O ligands is usually the dominant contribution to their effects on stereoselectivity, other contributions may also play a role. Such contributions may arise from interactions of the active site with a particular oxygen of the substrate through hydrogen bonding and from steric effects deriving from the ionic radius of the metal. In some cases the steric effects become very significant, and since they may also exist for diastereomeric metal chelates of ATP itself, they should influence the stereoselectivity for metal–ATP chelates. Hopefully, spectroscopic studies of enzymic complexes of labeled isotopic oxygen diastereomers[28] of metal–ATP chelates will aid in evaluating these aspects of stereospecificity.

Of the approaches used to specify the chelate structure of the metal nucleotide substrates, two are kinetic and one is structural. Cleland and co-workers[21,29] investigated the relative reactivities of bidentate (β,γ) and tridentate (α,β,γ) exchange-inert ATP complexes with Co(III) or Cr(III) and the relative inhibitory constants of bidentate (α,β) and monodentate (β) exchange-inert ADP complexes. These investigators concluded that there are two classes of metal chelation for kinase substrates and products, both of which utilize the β,γ bidentate metal–ATP as substrate rather than the α,β,γ tridentate form, the two classes being differentiated by the chelate structure of the metal–ADP product. One class, represented by hexokinase, produces β monodentate ADP, and the other exemplified by creatine kinase, yields the α,β bidentate ADP as product.[29] The second kinetic approach evaluates the metal dependence of stereoselectivity for the diastereomeric ATP thio analogues and yields the three patterns of behavior discussed above (see Table I). The interpretation from both kinetic approaches for type I enzymes, exemplified by hexokinase, leads to the conclusion that the β,γ bidentate metal chelate of ATP is the substrate.

Insofar as type III enzymes have been compared by the two methods, all evidence indicates that the β-phosphate is a ligand for the metal, but there is disagreement on the α-phosphate of ATP. Since the dissociation of metal–ADP may be the rate-determining step in these reactions, it may be that only the α-phosphate of ADP but not of ATP is coordinated to the metal ion. Burgers and Eckstein[24] favor the β,γ bidentate metal chelate structure of ATP as substrate and α,β bidentate metal ADP as product for creatine kinase, a type III enzyme. Their choice rests on the finding[29] that bidentate β,γ Cr^{III}–ATP is a substrate, albeit a very poor one, but that tridentate α,β,γ Cr^{III}–ATP is unreactive in the creatine kinase reaction. For another very similar type III enzyme, arginine kinase,[23] we preferred to interpret the stereoselectivity data in terms of metal ligation to the α-phosphate as well as the β-phosphate of both ADP and ATP. In this case,[23] there is also cogent kinetic evidence that the metal is liganded to the γ-phosphate as well, based on the following reasoning. When Cd(II) is substituted for Mg(II), the V_{max} for ATP as substrate decreases by a factor of 10, but for ATPαS and ATPβS as substrates, V_{max} with Cd(II) actually increases by factors of 5 and 3, respectively, as shown in Table III. This result is plausible if Cd(II) chelates to both α- and β-phosphate and the foreign atom is in the chelate ring not exposed to direct interaction with the enzyme. For ATPγS, however, the rate with Cd(II) is 17-fold slower than for MgATP, strongly suggesting that the rate-determining step has become the breaking of the strong Cd(II)–S ligand bond in the formation of the thiophosphoarginine product.

The establishment of α,β,γ tridentate metal–ATP as the true substrate for type III enzymes is most firmly

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supported by the ^{17}O hyperfine coupling observed in Mn(II) EPR spectroscopy of metal–enzyme–substrate complexes. The spectroscopic method is not subject to the ambiguities of the kinetic methods. By appropriate labeling with ^{17}O, the six ligands to Mn(II) in a creatine kinase transition-state analogue complex (enzyme–MnADP–formate–creatine) have been shown to include the two phosphate groups of ADP and the formate anion.[30] The latter is an analogue of the γ-phosphate of ATP in the transition state. Furthermore, Reed and Leyh have also shown[31] that in an equilibrium mixture of the central ternary complexes, all three phosphate groups are liganded in both the E–Mn–ATP–creatine complex and in the E–Mn–ADP–P-creatine complex. As discussed by Reed and Leyh,[30] the apparent discrepancy between their conclusion and those from the Cr^{III}–ATP experiments[29] may arise from the inability of the Cr(III) tridentate complex to undergo puckering motion of the chelate ring formed by the α- and β-phosphate groups and the metal ligand, a motion that facilitates the cleavage of the ATP β,γ bridge bond. Although Mn(II)–^{17}O studies are not available for arginine kinase, it is highly probable that the substrate for arginine kinase is also the tridentate α,β,γ ATP chelate, in view of the similar behavior to creatine kinase by both kinetic approaches.

Displacement of Equilibria of Kinase Reactions with ATPβS as Substrate

It has been noted that when ATPβS replaces ATP as a substrate in the 3-P-glycerate kinase reaction, the equilibrium appears to be displaced toward the formation of ADPβS and 1,3-bis-P-glycerate.[32,33] Since the only components of the equilibrium system that change are the nucleoside di- and triphosphates, the same shift in equilibrium constant between oxynucleotides and thionucleotides should be observed in all kinase reactions. Thus the equilibrium constants, measured by ^{31}P NMR at pH 8.0 and 30 °C, for both the creatine and arginine kinase reactions were shifted about 60-fold in the direction toward ADPβS formation relative to ADP formation.[34] The finding that the equilibrium shifts in favor of formation of a terminal thiophosphate group from an internal thiophosphate group is consistent with the earlier observation on aminoacyl-tRNA synthetase, where ATPβS was converted completely to ATPγS and the reverse reaction was not detected.[18]

The thermodynamic property of the nucleoside phosphorothioates described above, namely that the free energy difference between ATPβS and ADPβS is more exergonic by about 2.4 kcal/mol than between ATP and ADP, can be used in a number of different applications. First, it becomes possible to determine the equilibrium constants of those phosphoryl-transfer reactions where direct determination is not feasible because the oxynucleotide equilibrium lies too far toward ATP. The apparent thionucleotide equilibrium constant is readily measurable, and the oxynucleotide equilibrium constant, K_{oxy}, may be calculated since $K_{oxy} = K_{thio}/60$. The measured values of K_{thio} for the pyruvate and 3-P-glycerate kinase reactions in the direction of ADPβS formation were 0.019 and 0.018, respectively, and the corresponding values of K_{oxy} are therefore 3.1×10^{-4} and 2.9×10^{-4}.[34] Second, if it is desirable to increase the yield of a phosphorylated product, e.g., a phosphorylated protein, it would be advantageous to use ATPβS rather than ATP as substrate since the equilibrium will always be shifted significantly toward the phosphorylated product and ADPβS.

Perhaps the most promising application lies in studies of energy transduction systems where an endergonic process such as the formation of an ion gradient is coupled to the exergonic hydrolysis of ATP. The nature of that coupling may be elucidated by using ATPβS as the energy source. Such a study has been done for the transport of Ca(II) in the sarcoplasmic reticulum coupled to the hydrolysis of ATP.[9] For the sarcoplasmic reticulum system, it had been calculated[35] that the free energy change involved in the maintenance of the Ca(II) gradient was very close to that predicted from the free energy change derived from the concomitant hydrolysis of ATP to ADP and P_i. The question posed was whether an increased Ca(II) gradient would result when ATPβS replaced ATP as substrate, corresponding to the increased free energy of hydrolysis of the thionucleotide compared to the oxynucleotide. Fortunately, both ATPβS *R* and *S* are hydrolyzed as rapidly as ATP itself so that there are no kinetic limitations introduced in comparing the efficacy of the substrates. The Ca(II) gradients produced and maintained were the same regardless of substrate, thus demonstrating the absence of a 1:1 correspondence between the free energy change of the hydrolytic reaction and the free energy change associated with the Ca(II) ion gradient.

Concluding Remarks

The versatility of the thio analogues of ATP in probing mechanistic aspects of ATP reactions has been amply demonstrated. (1) the chirality of α-P and β-P in ATPαS and ATPβS, respectively, has permitted the stereochemical course of many enzymic reactions to be followed. (2) Wide variability has been revealed in the degree of stereoselectivity within a class of enzymic reactions such as kinases, indicating a variability in the geometric configuration at the active sites even though the grosser aspects of kinase structures in the crystalline state often show many common features. The stereoselectivity of the metal chelates of the thio analogues has stimulated interest in the question of whether stereoselectivity also exists for the diastereomeric species of metal chelates of ATP itself. (3) Multiple reactions catalyzed by the same enzyme become distinguishable if their stereoselectivities differ significantly. The reversal of stereoselectivity for ATPβS in the β,γ phosphate interchange from that in the aminoacylation reaction was the most direct evidence for the existence of a hitherto unsuspected reaction catalyzed by *E. coli* valyl-tRNA synthetase. Differences in rates of reaction of the thio analogue substrates and in their stereoselectivities are useful for differentiating ATP reactions in complex or multienzyme systems. For example, in the sarcoplasmic reticulum system, the

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Ca(II)-dependent ATPase proceeds at the same rate with ATP and ATPβS (*R* and *S*) as substrates but the Ca(II)-independent ATPase is not detectable with ATPβS as substrate. (4) The metal dependence of stereoselectivity of ATPαS and of ATPβS has contributed significantly to the elucidation of the metal chelate structures of the nucleotide but has often raised as many questions as it has answered. Again, the variability of the patterns among the phosphoryl-transfer reactions is striking. (5) The use of the difference in free energy change between the nucleoside di- and triphosphate pairs of the β-S analogues relative to the oxynucleotide as a probe in energy transduction systems has only begun. In addition to studies of equilibria and coupling mechanisms, the ability to manipulate concentrations of products in metabolic and biosynthetic pathways by replacement of ATP by ATPβS may yet prove the most valuable property of the thionucleotide.

Formation of Carbon–Hydrogen Bonds by Reductive Elimination

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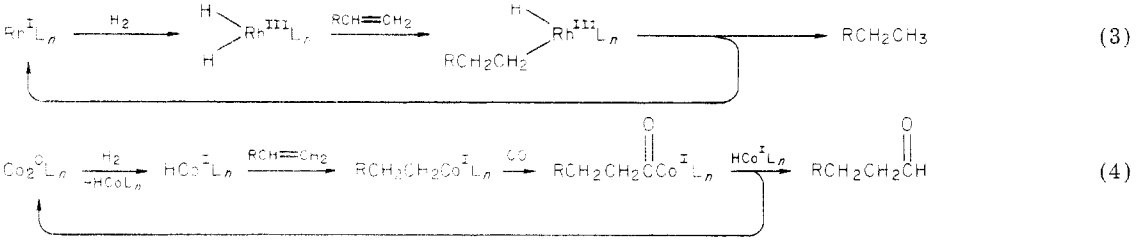
Formation of a C–H bond by coupling of a metal-bonded alkyl, aryl, or acyl ligand with a hydride ligand constitutes an important and widespread class of reactions in organometallic chemistry. Such reactions commonly are referred to as *reductive eliminations* because they typically are accompanied by a decrease in the formal oxidation states of the metal complexes. Reductive elimination reactions may be either intramolecular or intermolecular, specific examples being

$$\underset{1}{cis\text{-}[Pt^{II}H(CH_3)(PPh_3)_2]} \rightarrow CH_4 + [Pt^0(PPh_3)_2] \quad (1)$$

$$\underset{2}{[C_6H_5CH_2Mn^I(CO)_5]} + [HMn^I(CO)_5] \rightarrow C_6H_5CH_3 + [Mn_2^0(CO)_{10}] \quad (2)$$

Such reactions commonly constitute the product-forming steps in catalytic processes such as hydrogenation[1] and hydroformylation[2] as exemplified by the simplified mechanistic schemes of eq 3 and 4 (where $RhL_n = [Rh^ICl(PPh_3)_3]$ and $CoL_n = [Co(CO)_4]$).

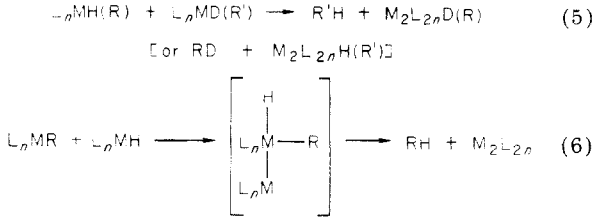


(3)

(4)

While the distinction between intramolecular and intermolecular reductive elimination reactions, exemplified by eq 1 and 2, is readily apparent from a stoichiometric standpoint, the mechanistic implications of the distinction are less clear. For example, mononuclear hydridoalkyl complexes may undergo reductive elimination through intermolecular mechanisms (demonstrable by double labeling)[3,4] as in the schematic example of eq 5. On the other hand, apparently intermolecular reductive elimination may proceed through an intramolecular step (eq 6).

This Account is concerned with the scope of such reductive elimination reactions, with their kinetic and mechanistic aspects, and with the factors that influence their rates and reactivity patterns. The study of such reactions also is of obvious relevance to an understanding of the reverse process, i.e., the oxidative addition of C–H bonds, a topic of considerable current



(5)

(6)

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