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Conformations and Arrangement of Substrates at Active Sites of ATP- Utilizing Enzymes

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Conformations and arrangement of substrates at active sites of ATP-utilizing enzymes

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The phosphoryl transferring enzymes pyruvate kinase, cAMP-dependent protein kinase and the pyrophosphoryl transferring enzyme PP-Rib-P synthetase utilize the β, γ bidentate metal–ATP chelate (Δ-isomer) as substrate, as determined with substitution-inert $Cr^{III}ATP$ or $Co^{III}(NH_3)_4ATP$ complexes. In addition, these enzymes bind a second divalent cation, which is an essential activator for pyruvate kinase and PP-Rib-P synthetase and an inhibitor of protein kinase. The enzyme-bound metal has been used as a paramagnetic reference point in T_1 measurements to determine distances to the protons and phosphorus atoms of the bound nucleotide and acceptor substrates. These distances have been used to construct models of the conformations of the bound substrates. The activating metal forms a second sphere complex of the metal–nucleotide substrate on pyruvate kinase and PP-Rib-P synthetase while the inhibitory metal directly coordinates the polyphosphate chain of the metal–nucleotide substrate on protein kinase. Essentially no change is found in the dihedral angle at the glycosidic bond of ATP upon binding to pyruvate kinase (χ = 30°), an enzyme of low base specificity, but significant changes in the torsional angle of ATP occur on binding to protein kinase (χ = 84°) and PP-Rib-P synthetase (χ = 62°), enzymes with high adenine-base specificity. Intersubstrate distances, measured with tridentate CrATP or β, γ bidentate CrAMPPCP as paramagnetic reference points, have been used to deduce the distance along the reaction coordinate on each enzyme. The reaction co-ordinate distances on pyruvate kinase (3 ± 1 Å) and PP-Rib-P synthetase (not less than 3.8 Å) are consistent with associative mechanisms, while that on protein kinase (5.3 ± 0.7 Å) allows room for a dissociative mechanism.

Introduction

Enzyme-catalysed nucleophilic substitutions on the phosphorus atoms of ATP permeate all of biochemistry. Four key steps in glycolysis involve kinases that catalyse substitution at the γ-phosphorus of ATP and concomitant phosphoryl transfer. In recent years, several methods especially useful for the study of the mechanism of ATP reactions have been developed. These include the paramagnetic probe – longitudinal relaxation rate ($1/T_1$) method for measuring metal–substrate and intersubstrate distances on enzymes (Mildvan 1977), the synthesis and use of substitution-inert Co^{III} and Cr^{III} complexes of ATP of known structure in kinetic, structural and stereochemical studies (De Pamphilis & Cleland 1973; Janson & Cleland 1974; Gupta *et al.* 1976*a*; Cornelius *et al.* 1977; Cornelius & Cleland 1978; Li *et al.* 1978; Cleland & Mildvan 1979), and the synthesis and use of other chiral ATP derivatives for elucidating the stereochemical consequences of substitution at each of the three phosphorus atoms of ATP (Sheu *et al.* 1979; Pliura *et al.* 1980). These methods have been discussed in detail in the reviews cited above. Here we shall compare the results of our own studies with the paramagnetic probe – $1/T_1$ method and substitution-inert metal–ATP complexes on three reactions of ATP: the phosphoryl transfers catalysed by pyruvate kinase and cAMP-dependent protein kinase, and the pyrophosphoryl transfer catalysed by PP-Rib-P synthetase.

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Pyruvate kinase from rabbit muscle

This enzyme catalyses the enolization of pyruvate (Rose 1960) and the phosphorylation of the enolate of pyruvate or of other nucleophiles such as NH_2OH, F^- and glycollate (Boyer 1962; Kayne 1974). Two divalent cations (Gupta *et al.* 1976*b*) and a monovalent cation at the active site (Boyer 1962; Reuben & Kayne 1971) are required for each of the reactions catalysed by this enzyme. The monovalent cation and one of the two essential divalent cations are directly

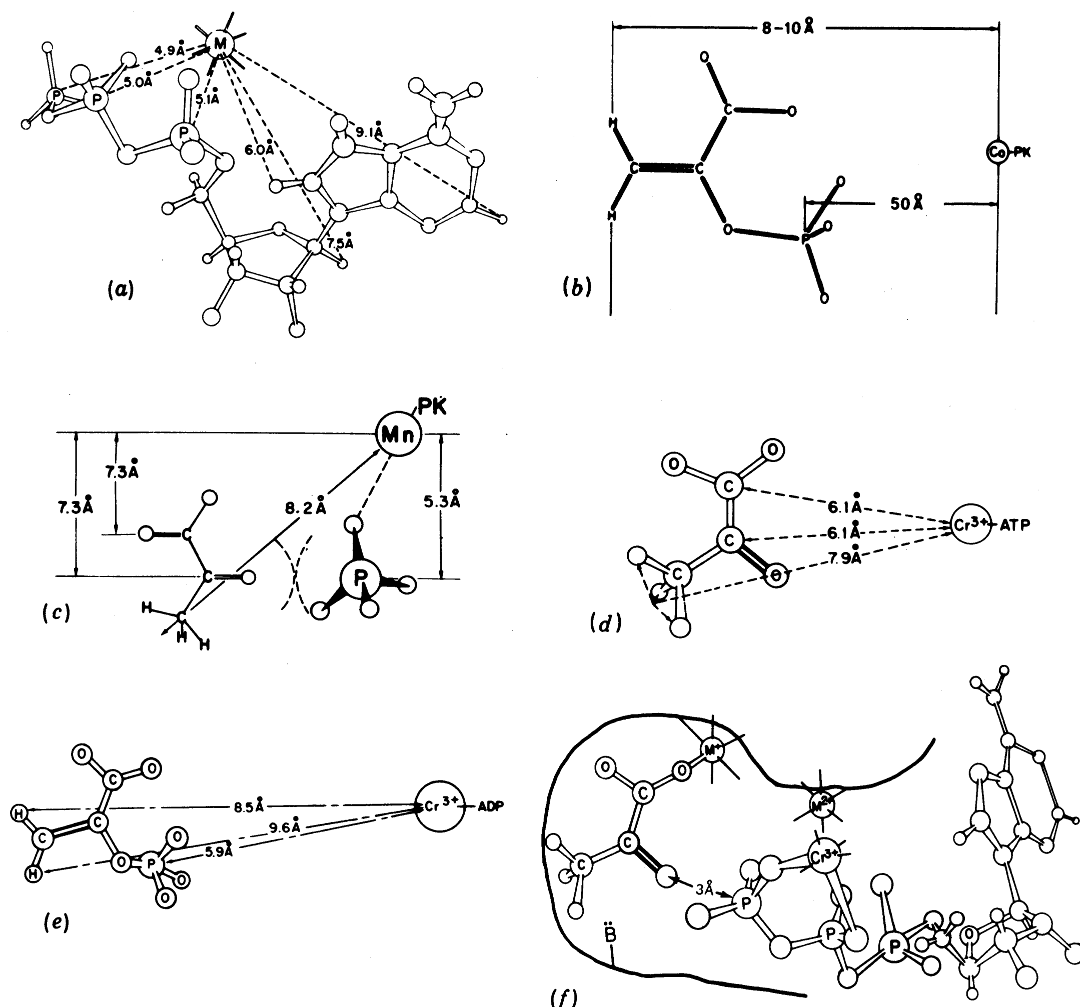

Figure 1. Conformations and arrangement of substrates on rabbit muscle pyruvate kinase. (*a*) ATP (Sloan & Mildvan 1976); (*b*) phosphoenolpyruvate (Melamud & Mildvan 1975); (*c*) pyruvate and P_i (Fung *et al.* 1973); (*d*) CrATP and pyruvate (Gupta *et al.* 1976*a*); (*e*) CrADP and phosphoenolpyruvate (Gupta & Benovic 1978); (*f*) composite of all.

bound to the enzyme, the latter via an imidazole ligand as indicated by n.m.r. studies of the protein (Meshitsuka *et al.* 1980). The other essential divalent cation is coordinated to the β and γ phosphoryl groups of ATP as found by kinetic studies with CrATP (Gupta *et al.* 1976*a*, *b*; Dunaway-Mariano *et al.* 1979) and Mg^{2+} binding studies with ^{31}P-n.m.r. (Gupta & Mildvan 1977). Much of our early confusion about the mechanism of pyruvate kinase resulted from our failure to appreciate the dual divalent cation requirement.

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Using Mn^{2+} and Co^{2+} as paramagnetic reference points at the site of the enzyme-bound divalent cation, we detect second sphere complexes of ATP (figure 1*a*) (Sloan & Mildvan 1976) and phosphoenolpyruvate (figure 1*b*) (Melamud & Mildvan 1975). These studies confirmed each other since the γ-phosphoryl group of ATP has long been known to compete with the phosphoryl group of phosphoenolpyruvate (Boyer 1962). The conformation of enzyme-bound ATP was found to be extended, with a dihedral angle at the glycosidic bond ($\chi = 30 \pm 10°$) (figure 1*a*) that differs little from that found for such nucleotides when free in solution (Granot *et al.* 1979*a*). This result is consistent with the high mobility of the etheno-adenine ring of a fluorescent ATP analogue on pyruvate kinase (Barrio *et al.* 1973), the substrate activity of another ATP analogue with χ locked at *ca.* 30° (Hampton *et al.* 1972) and with the low nucleotide base specificity of this enzyme (see below).

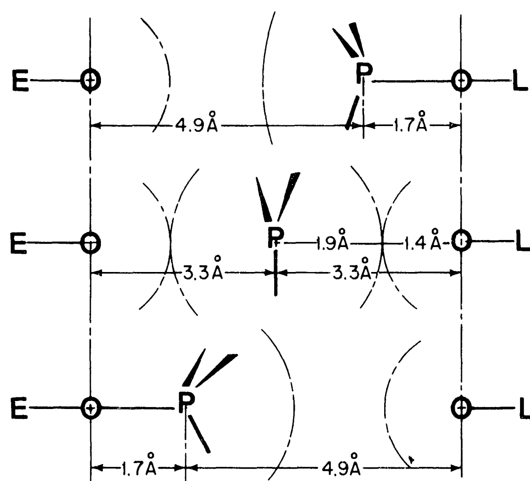

FIGURE 2. Limiting reaction coordinate distance for a dissociative mechanism. E–O represents the entering group and O–L the leaving group. Reaction coordinate distance less than 4.9 Å implies that the mechanism has associative character.

The enzyme-bound Mn^{2+} was also used to determine distances to both pyruvate and phosphate in a quaternary complex that is active in enolizing pyruvate (figure 1*c*) (Fung *et al.* 1973). Intersubstrate distances were determined from α, β, γ tridentate CrATP to the carbon atoms and protons of pyruvate in an enzyme complex that also was highly active in enolizing pyruvate (figure 1*d*) (Gupta *et al.* 1976*a*). Intersubstrate distances were also determined from α, β bidentate CrADP to the phosphorus and protons of phosphoenolpyruvate (figure 1*e*) (Gupta & Benovic 1978). All of the complexes studied were kinetically competent to function in catalysis as determined by n.m.r. A total of 20 distances, many of which were cross-checked as discussed above, together with the observation that the Δ-isomer of β, γ bidentate CrATP was the only species capable of phosphorylating glycollate in an enzyme-catalysed single turnover (Dunaway-Mariano *et al.* 1979) were used to derive the arrangement and conformations of the substrates at the active site (figure 1*f*). From the model of the bound substrates, the distance along the reaction coordinate between the γ-phosphorus of ATP and the carbonyl oxygen of pyruvate (3 ± 1 Å) approaches molecular contact (3.3 Å). This short reaction coordinate distance (Mildvan *et al.* 1976), the stereochemical inversion on phosphorus (Orr *et al.* 1978; Blattler & Knowles 1979; Pliura *et al.* 1980) and the substrate activity of CrATP (Dunaway-Mariano *et al.*

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1979) argue cogently against a phosphoenzyme intermediate. Further, as explained in figure 2, a reaction coordinate distance no less than 4.9 Å is needed to allow room for a classical metaphosphate intermediate in a dissociative mechanism. A distance less than 4.9 Å would require the formation of the new bond to begin before the breakage of the old bond was complete. Hence the reaction coordinate distance on pyruvate kinase, which is significantly less than 4.9 Å, suggests that the mechanism of phosphoryl transfer has associative character.

cAMP-dependent protein kinase from bovine heart

While not direct participants in glycolysis, cAMP-dependent protein kinase enzymes control the rates of glycolysis in many cell types (Walsh & Krebs 1973; Krebs & Beavo 1979; Rubin & Rosen 1975; Granot *et al.* 1980*b*). The kinase or catalytic subunit (C) is largely inhibited in cells owing to the presence of a tightly bound regulatory subunit (R_2) which forms an inactive holoenzyme (R_2C_2). We have recently shown the mechanism of inhibition by the regulatory subunit to be simple competition against peptide or protein substrates, but not against ATP or metal ions (Granot *et al.* 1980*a*). Indeed, the regulatory subunit has long been known to be a substrate for the catalytic subunit. The dissociation constant of the regulatory subunit from the catalytic subunit ($K_I = 10^{-10}$ M) is increased by an order of magnitude by phosphorylation of R_2 ($K_I = 10^{-9}$ M) and by an additional four orders of magnitude upon the binding of cAMP to R_2 ($K_I = 10^{-5}$ M). Since the cellular concentration of R_2C_2 in heart muscle is 10^{-6} M, phosphorylation of the regulatory subunit and the binding of cAMP would result in nearly complete dissociation of R_2C_2, releasing the active catalytic subunit (Granot *et al.* 1980*b*).

The active enzyme that we have studied catalyses the phosphorylation of serine or threonine residues in histones, or in simple heptapeptides of the type Leu-Arg-Arg-Ala-Ser-Leu-Gly, with the same low K_m (*ca.* 20 μM) and high turnover rate (30 s^{-1}). The amino acid residues that are required in the substrate for such favourable kinetic parameters are arginines at positions 2 and 3, a 'spacer' at position 4, a phosphoryl acceptor at position 5, and a hydrophobic amino acid at position 6 (Kemp *et al.* 1977; Feramisco *et al.* 1979). The enzyme also catalyses the hydrolysis of ATP, i.e. the phosphorylation of water, in a slow side reaction (Armstrong *et al.* 1979*a*).

In the absence of any added metal ions, the enzyme was found to catalyse the phosphorylation of the above heptapeptide (referred to as the Ser-peptide) by the Δ-isomer of β, γ bidentate $Co(NH_3)_4ATP$, establishing the coordination scheme of the active species to be a nucleotide-bridge or an enzyme–ATP–metal complex (Granot *et al.* 1979*b*). Accordingly, the enzyme did not bind Mn^{2+} unless a nucleotide was also present (Armstrong *et al.* 1979*b*). However, the binding of a nucleotide at the single active site of the catalytic subunit permitted two divalent cations to bind. One was directly coordinated to the β and γ phosphoryl groups of ATP, and was essential for activity. The second was bound to the enzyme and partly inhibited the kinase activity. To avoid the complexity of two paramagnetic probes at the same active site, the activating metal site on the nucleotide was blocked with β, γ bidentate $Co(NH_3)_4ATP$, and Mn^{2+} at the inhibitory site was then used as the paramagnetic reference point to determine the conformation of the enzyme-bound metal–ATP substrate (figure 3*c*, *d*) (Granot *et al.* 1979*a*). Interestingly, the inhibitory metal was found to bridge the enzyme to each of the three phosphoryl groups of $Co(NH_3)_4ATP$. As a control, the weak binary complex between Mn^{2+} and $Co(NH_3)_4ATP$ in free solution was also studied (figure 3*a*, *b*). A comparison of the dihedral angles at the glycosidic bond indicates a significant increase in χ from $44 \pm 10°$ in the free

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nucleotide to $84 \pm 10°$ for the enzyme-bound nucleotide (figure 3*b*, *d*) (Granot *et al.* 1979*b*). This change reflects a strong interaction of the enzyme with the adenine ring and is consistent with the high adenine base specificity of protein kinase.

Both Mn^{2+} at the inhibitory metal site and Cr^{III} at the activating site (as tridentate CrAMP-PCP) have been used to determine the intersubstrate distances to the Ser-peptide (figure 3*e*) and its analogues the Ala[5] heptapeptide and a Tyr[4] heptapeptide (Granot *et al.* 1980*b*, *c*).

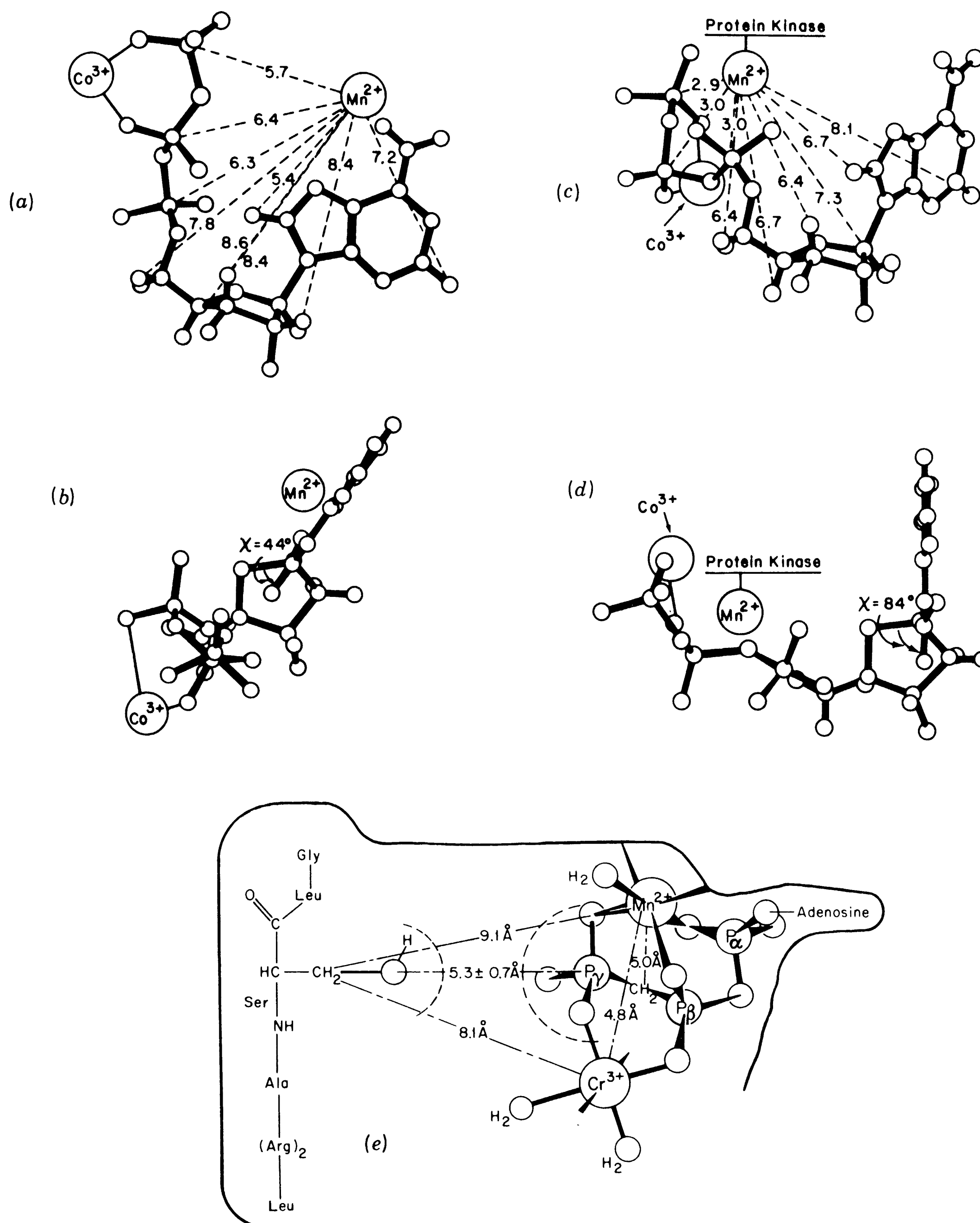

FIGURE 3. Conformations and arrangement of substrates on the catalytic subunit of cAMP-dependent protein kinase from bovine heart. (*a*, *b*) Binary Mn^{2+}–$Co(NH_3)_4ATP$ complex; (*c*, *d*) $Co(NH_3)_4ATP$ on Mn^{2+}–protein kinase (Granot *et al.* 1979*a*); (*e*) intersubstrate and reaction coordinate distances (Granot *et al.* 1980*c*).

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These distances have been used to rule out α-helical and β-pleated sheet conformations for the bound heptapeptide substrates.

Kinetic data on peptides and protein substrates from the literature in which amino acids 1 and 7 were omitted, in which prolines were placed at positions 4 or 6, or in which hydroxyproline was placed at position 5 were used to rule out the obligatory requirement for all of the β-turns possible within the Ser-heptapeptide (Granot *et al.* 1980*b*). Hence if protein kinase has an obligatory requirement for a specific secondary structure, then, by elimination, this structure must be a coil. If no such absolute requirement exists, then a β_{2-5} or β_{3-6} turn might be preferred. It is of interest that the phosphorylated serine of glycogen phosphorylase occurs in a coil structure, as shown by X-ray analysis (Fletterick *et al.* 1979).

The intersubstrate and intermetal distances were used to derive the reaction coordinate distance of 5.3 ± 0.7 Å (figure 3*e*) (Granot *et al.* 1980*c*), which exceeds 4.9 Å and is therefore large enough to permit an intermediate (figure 2). Since a phosphoenzyme intermediate is rendered unlikely by the substrate activity of the substitution-inert $Co(NH_3)_4ATP$ complex, a metaphosphate intermediate in a dissociative mechanism is an interesting possibility. Indeed, a dissociative mechanism would nicely explain two observations: (*a*) the profound inhibition produced by a second metal coordinating the γ-phosphoryl group, which is known not to alter the substrate geometry (Granot *et al.* 1980*b*), and (*b*) the preservation of the rate ratio between peptide phosphorylation and ATP hydrolysis despite an 18-fold decrease in their absolute rates observed upon replacing Mg^{2+} with Mn^{2+} as the activator (Armstrong *et al.* 1979*a*). Of course a dissociative mechanism is not established, since the reaction coordinate distance could decrease as the transition state is more closely approached.

PP-Rib-P synthetase from *S. typhimurium*

This is one of those rare enzymes that catalyse nucleophilic substitution at the electron-rich β-phosphorus of ATP. The reaction involves the transfer of the metal–pyrophosphoryl group of the metal–ATP substrate to the 1-hydroxyl group of the α-isomer of Rib-5-P with inversion at the β-phosphorus (figure 4*b*) (Li *et al.* 1978). Our observation that the Δ-isomer of β, γ bidentate $Co(NH_3)_4ATP$ is a substrate, only in the presence of an added divalent cation, established that this enzyme, like pyruvate kinase, requires a second divalent cation for activity. The enzyme itself was found to bind one Mn^{2+} ion per subunit tightly with a dissociation constant consistent with its activator constant (Li *et al.* 1978). With Mn^{2+} at this site as a paramagnetic reference point, the conformation of the enzyme bound metal–nucleotide substrate has been determined by using β, γ bidentate $Co(NH_3)_4ATP$, $Co(NH_3)_4AMPCPP$, as well as the nucleotide products AMP and AMPαS (Granot *et al.* 1980*d*). As with pyruvate kinase, the activating enzyme-bound metal forms a second sphere complex of the metal nucleotide substrate (figure 4*a*). As with protein kinase, but not with pyruvate kinase, a significant change in the dihedral angle χ of the nucleotide to $62 \pm 5°$ was found upon binding to PP-Rib-P synthetase, which is consistent with the high adenine base specificity of this enzyme. Upon binding of Rib-5-P to the enzyme–nucleotide complex, no movement of the adenosine portion of the bound metal–nucleotide substrate was observed, as indicated by the absence of changes in the Mn^{2+}–proton distances. However, a significant conformational change occurred in the polyphosphate chain as manifested in 1.2 and 0.6 Å increases in the distances from Mn^{2+} to the P_α and P_β atoms respectively. This represents a conformational change of the bound substrate, metal–ATP,

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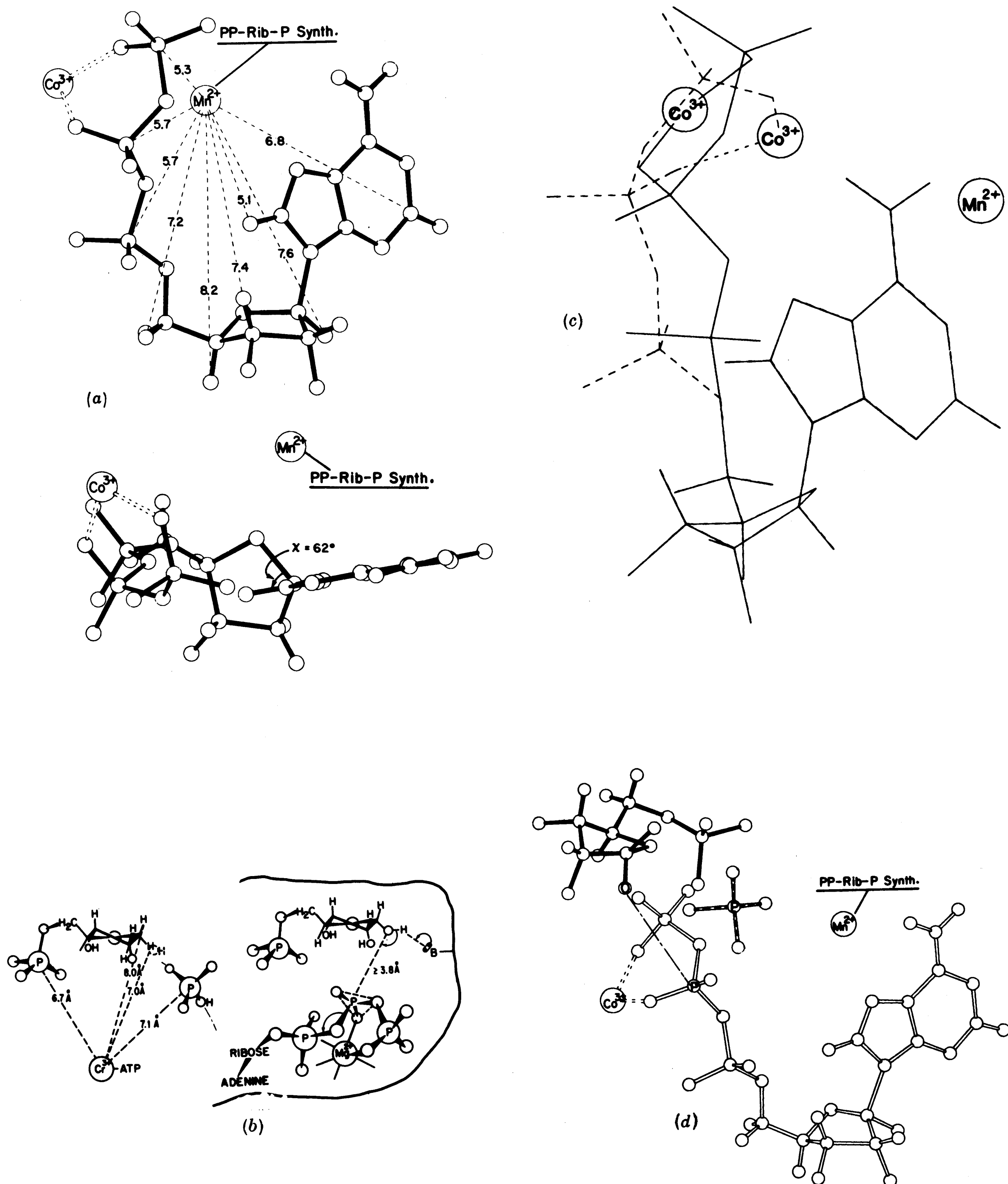

FIGURE 4. Conformation and arrangement of substrates on PP-Rib-P synthetase from *S. typhimurium*. (*a*) $Co(NH_3)_4$-ATP (Granot *et al.* 1980*d*); (*b*) intersubstrate and reaction coordinate distances (Li *et al.* 1979); (*c*) effect of Rib-5-P binding on the conformation of enzyme-bound metal–ATP (broken lines) (Granot *et al.* 1980*d*); (*d*) composite of all substrates and activators (Granot *et al.* 1980*d*).

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induced by the binding of the second substrate, Rib-5-P (see broken lines in figure 4*c*) (Granot *et al.* 1980*a*).

Based on the intersubstrate distances from tridentate CrATP to the anomeric protons and the phosphorus atom of Rib-5-P, a lower limit reaction coordinate distance between the α-1-hydroxyl group and the β-phosphorus of ATP of 3.8 Å was estimated (figure 4*b*) (Li *et al.* 1979).

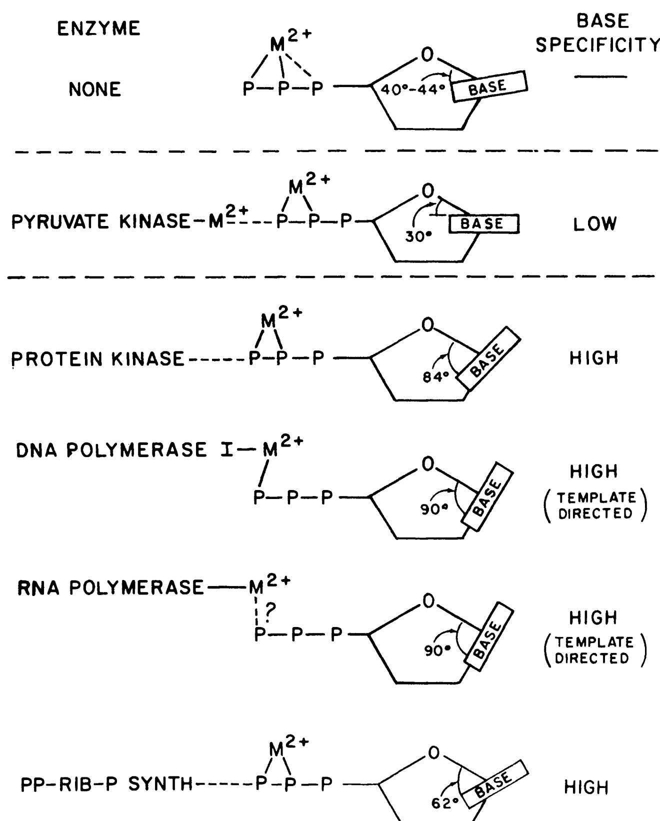

FIGURE 5. Correlation of glycosidic conformational angle (χ) with nucleotide base specificity of five enzymes studied by n.m.r. (Granot *et al.* 1980*b*).

This value, the location of the metal on the β-phosphoryl group of ATP, and the stereochemical inversion on the β-phosphorus (Li *et al.* 1978) are consistent with a mechanism with significant associative character. A model of the active site based on 14 distances from two reference points, the inversion on phosphorus, and the appropriate (Δ)-stereoisomer of the metal–nucleotide substrate is shown in figure 4*d* (Granot *et al.* 1980*d*). Also shown is the location of the enzyme-bound phosphate ion, an essential enzyme activator and stabilizer which, however, does not participate directly in the reaction. Its position is compatible with its possible role as the general base that deprotonates the α-1-hydroxyl group of Rib-5-P during the reaction (Li *et al.* 1979).

Preliminary studies of another pyrophosphoryl transferring enzyme, dihydropteridine pyrophosphorylase, by P. Rosevear in my laboratory, indicate that this enzyme, like PP-Rib-P synthetase, binds a divalent cation at the active site in addition to the nucleotide-bound metal and that both of these metal ions appear to be necessary for activity.

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Conclusions

Many, but not all, ATP-utilizing enzymes require two divalent cations at the active site for catalysis, one of which is directly coordinated to the β and γ phosphoryl groups of ATP while the other is coordinated to the enzyme and forms a second sphere complex of the metal–ATP substrate. Our n.m.r. studies of the nucleotide conformation on pyruvate kinase, protein kinase, PP-Rib-P synthetase and two other ATP utilizing enzymes (figure 5) (Granot *et al.* 1980*b*) indicate that four enzymes that significantly alter χ, the dihedral angle at the glycosidic bond of ATP, show high nucleotide base specificities, while the one enzyme studied that does not alter χ shows low base specificity. Two recent X-ray studies support this generality. Hexokinase, which alters χ to 0°, shows high adenine base specificity (T. Steitz, personal communication, 1980), and aspartate transcarbamoylase binds the *syn* conformation of the highly specific allosteric inhibitor, 5-BrCTP (Honzatko *et al.* 1979). Other X-ray data on bound nucleotides appear to have too low a resolution as yet to examine this generality further. Our measured intersubstrate distances and derived reaction coordinate distances suggest that ATP-utilizing enzymes position the acceptor substrates such that the entering atom is closest to the appropriate phosphorus atom of ATP, in some cases approaching molecular contact. It is this relative positioning of the attacking substrate relative to ATP, rather than the location of the activating metals, that determines qualitatively which phosphorus atom of ATP is to undergo substitution, and quantitatively, the extent of associative (or dissociative) character to the reaction mechanism.

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