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Nuclear relaxation and Overhauser effect studies of enzyme-substrate interactions*

Estudios de relajación nuclear y efecto Overhauser de las interacciones enzima-sustrato

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INTRODUCTION

We have long been interested in the mechanisms of enzyme-catalyzed reactions of ATP, and NMR has been especially useful in studying these mechanisms (1, 2). The combined use of paramagnetic effects on T_1 to measure metal-nucleus distance (3), and the nuclear Overhauser effect to measure interproton distances (4-9), has provided detailed information on the locations, conformations, and arrangement of enzyme-bound metal-ATP and other substrates. Active site binding of substrates in NMR experiments has been established by quantitative studies of the displacement of substrates by competitive inhibitors, by measurements of substrate exchange rates at equilibrium, and by comparing these results with those of kinetic studies of the enzyme in action (2). This paper will review our NMR studies of three enzymes, cAMP-dependent protein kinase, adenylate kinase, and DNA polymerase I.

cAMP-dependent protein kinase

The catalytic subunit of cAMP-dependent protein kinase catalyzes the phosphorylation of Ser and Thr residues by MgATP in peptides such as Leu-Arg-Arg-Ala-Ser-Leu-Gly and similar sequences in proteins. Such phosphorylation activates some enzymes and inhibits others, thereby controlling the rates of entire metabolic pathways.

Based on distances from Mn^{2+} bound at an inhibitory site of protein kinase to $Co^{3+}(NH_3)_4$ ATP, and interproton distances in MgATP, the enzyme-bound metal-ATP substrate has a high-anti glycosyl torsional angle (χ = 81 ± 13°) and a ribose pucker which is either 01' endo, or an interconverting mixture of C-2' or C-3' endo (10, 11) (Figure 1). The activating metal is coordinated by the β and γ phosphoryl groups of ATP but not by the protein. The chiral β-P shows the Δ-configuration (12).

The enzyme-bound pentapeptide (Arg-Arg-Ala-Ser-Leu) and heptapeptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) substrates exist in highly extended coil conformations, with no internal hydrogen bonds, as determined by a total of 31 distances from the inhibitory Mn^{2+} and from Cr^{3+}ATP to protons of the peptides, including backbone Cα and amide NH protons (13-15) (Figure 2). The reaction coordinate distance between the serine hydroxyl oxygen and the γ-P of MgATP, estimated as 5.3 ± 0.7 Å from the intersubstrate distances (16) would allow room for an intermediate (2, 17). Hence the NMR data suggest either a dissociative mechanism with a metaphosphate intermediate or an associative mechanism with a highly elongated bipyramidal transition state (2, 16, 17).

Adenylate kinase

Adenylate kinase catalyzes the phosphorylation of AMP by MgATP, each substrate binding at a separate and specific site (18-21). On rabbit muscle adenylate kinase (194 amino acids), and on a 45 amino acid

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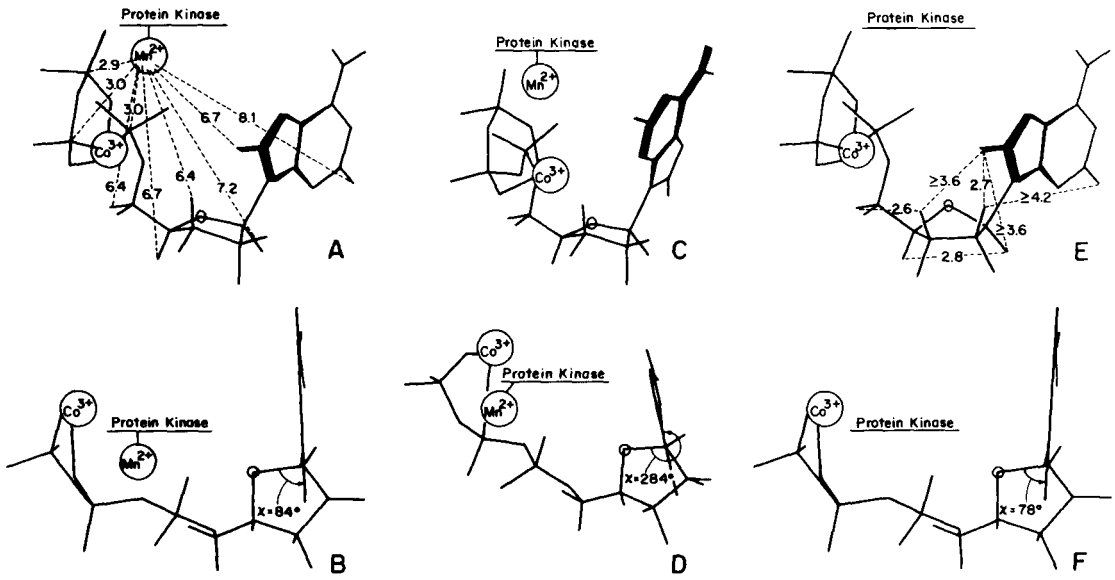

Fig. 1: Comparison of the conformations of $CO(NH_3)_4$ ATP bound to the catalytic subunit of protein kinase determined by the paramagnetic probe T_1 method (A,B or C,D) and the NOE method (E,F) (10, 11). A,B and C,D are alternative fits based on measured distances from Mn^{2+} bound at the inhibitory site.

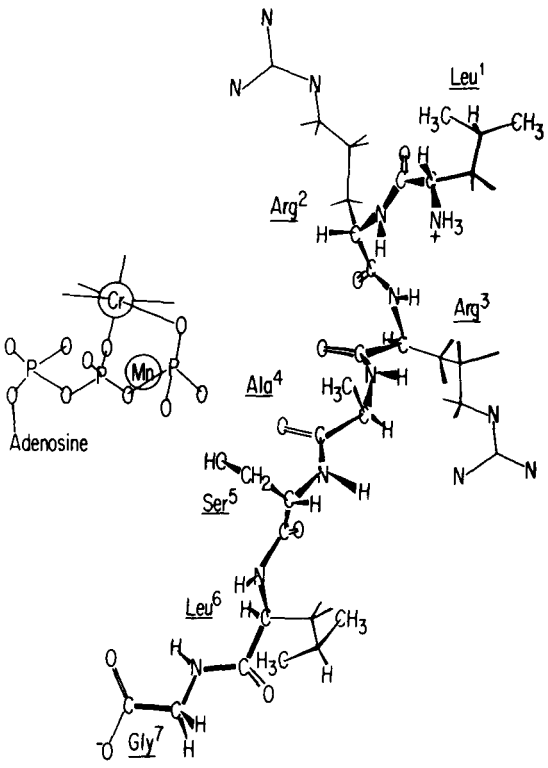

Fig. 2: Coil conformation of the enzyme-bound heptapeptide substrate of protein kinase consistent with distances from Mn^{2+} and Cr^{3+} to peptide protons (13, 14) and with the kinetic properties of N-methylated peptides (15).

fragment of this enzyme (residues 1-45) which binds metal-ATP with comparable affinity, MgATP has a glycosyl angle $\chi = 63 \pm 12^{o}$ and a 3'-endo ribose pucker (22). 2D NMR, CD, and FTIR spectroscopy indicate this peptide to be structured in solution with regions of α-helix and β-sheet, very similar to the X-ray structure of this portion of the enzyme (23). On both the enzyme and the peptide, bound MgATP interacts with two hydrophobic residues, probably Ile 28 and Leu 37, as detected by intermolecular NOE's, and the Cr^{3+} of bound Cr^{3+}ATP is ~13 Å from both His 30 and His 36, as determined by paramagnetic effects on T_1 (22). These and other distances and interactions permitted the positioning or 'docking' of the proper conformation of MgATP into the X-ray structure of the enzyme (22) (Figure 3).

The conformation of enzyme-bound AMP, based on intersubstrate distances from Cr^{3+}AMPPCP and on interproton NOE's within AMP, is somewhat strained, with a very high anti-glycosyl angle ($\chi = 110 \pm 10^{o}$) and a 3'-endo/2'-exo ribose pucker (24) (Figure 4). Such strained conformations of bound nucleotide sub-

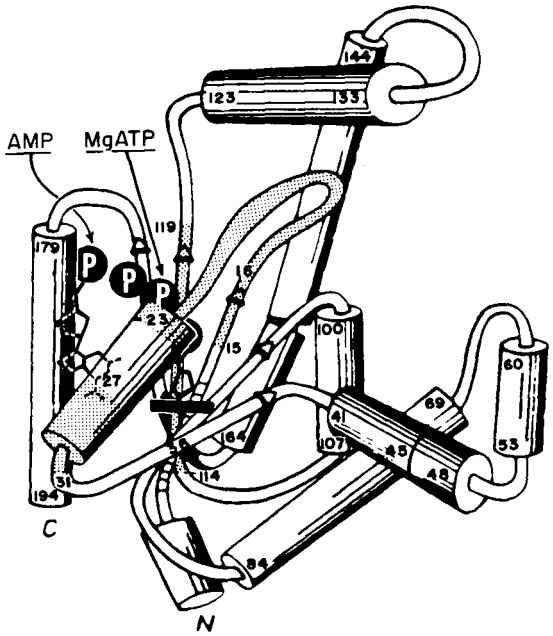

Fig. 3: Representation of the crystal structure of the entire adenylate kinase molecule showing the location of bound MgATP and AMP as determined by NMR docking. The stippling shows regions of amino acid homology with other nucleotide binding enzymes (22, 24).

strates correlate with high substrate specificity (17). The intersubstrate distances, as well as intermolecular NOE's from the enzyme to AMP were used to position AMP into the X-ray structure of adenylate kinase (24) (Figures 3, 5). From the positions of the enzyme-bound substrates,

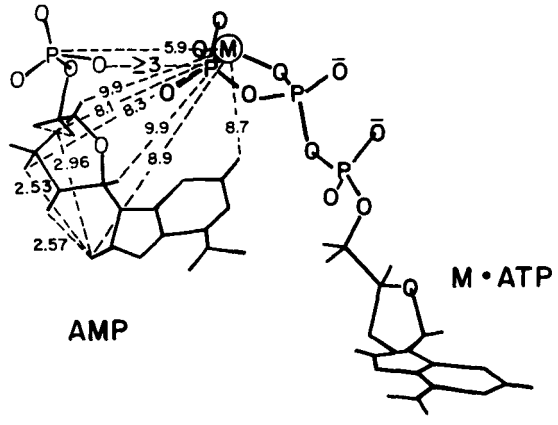

Fig. 4: Conformation of metal-ATP and of AMP on adenylate kinase, showing intersubstrate distances and interproton distances in angstrom units (22, 24). Also shown is the lower limit reaction coordinate distance (≥ 3 Å) based on model building (24).

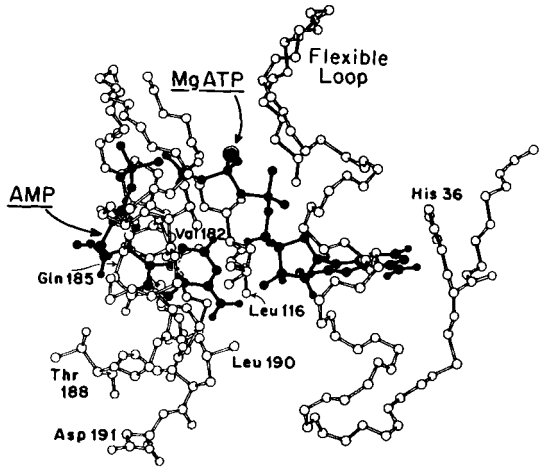

Fig. 5: Molecular details of the binding sites of AMP and MgATP on adenylate kinase as determined by NMR docking (22, 24).

a reaction coordinate distance of 3 ± 1 Å is estimated between the γ-P of MgATP and the phosphate oxygen of AMP (24), which is consistent with an associative nucleophilic substitution at phosphorus (2, 17).

Large fragment of DNA polymerase I

DNA polymerase catalyzes the central reaction in the replication of DNA, a nucleophilic substitution on the α-P of deoxynucleoside triphosphates with the displacement of pyrophosphate as the leaving group (25, 26). The enzyme utilizes four deoxynucleotide substrates, binding one at a time (27, 28), and elongates the growing DNA chain or primer in a manner consistent with Watson-Crick base pairing between the incoming substrate and the template. In addition to the polymerase reaction, DNA polymerase I from *E. coli* (pol I) catalyzes a 3'-5' exonuclease reaction which contributes to the fidelity of template copying. Pol I also catalyzes a 5'-3' exonuclease reaction on a site located on a 35,000 molecular weight domain at the N-terminus of the enzyme which can be removed by proteolysis (25). The remaining large fragment or Klenow fragment of molecular weight 68,000, retains the polymerase and 3'-5' exonuclease activities. This simpler enzyme has been cloned (29), crystallized, and an X-ray structure has

been reported (30), which shows the molecule to have a claw-like structure suitable for binding double-stranded DNA. The substrate binding site has not been clearly located in the crystal structure.

We have used NMR to study the conformations of the bound substrates, bound templates, and the interactions of both the substrates and templates with the enzyme (28, 31). To avoid hydrolysis by the 3',5' exonuclease we have used oligoribonucleotide templates.

On the large fragment of DNA polymerase I, the substrates MgdATP and MgTTP and the substrate analog AMPCPP have anti glycosyl torsional angles (χ = 50 ± 15°) and O_1' endo deoxyribose puckers (δ = 98 ± 12°), appropriate for nucleotide residues of B-DNA. Templates and primers do not greatly alter these conformations. In contrast, enzyme-bound dGTP exists in at least two conformations in the absence of template, 60% anti (χ = 32 ± 10°, 3'-endo) and 40% syn (χ = 222 ± 10°, 2'-endo). The complimentary template $(rC)_{30}$ and the non-complimentary template $(rU)_{43}$ simplify the conformations of bound deoxyguanine nucleotide substrates to a single anti-species, suggesting base-pairing with the template (31, 32). In the case of $(rU)_{43}$ a G-U wobble base pair may form. The enzyme-bound templates $(rU)_{54}$ and $(rA)_{50}$ are partially immobilized, and are held in a conformation such that their average nucleotides are more B-like than A- or Z-like (31).

Intermolecular NOE's indicate that the enzyme binds substrates near at least two hydrophobic residues including Ile, and an aromatic residue, probably Tyr (28, 31). These NMR results are consistent with photoaffinity labeling of the enzyme with the substrate analog 8-azido dATP which modifies Tyr 766 in the sequence Leu-Ile-Tyr (33). Intermolecular NOE's from the enzyme to the templates $(rU)_{54}$ and $(rA)_{50}$ indicate the proximity of cationic Arg and/or Lys residues and a hydrophobic residue. These NMR results are consistent with the observation that the pol A6 mutant, which binds templates less tightly, results from the conversion of Arg 690 to His (34). The holding of substrates and templates in a B-like conformation may contribute to the high fidelity of template replication by pol I. A stepwise mechanism of action for pol I consistent with the NMR data is shown in Figure 6.

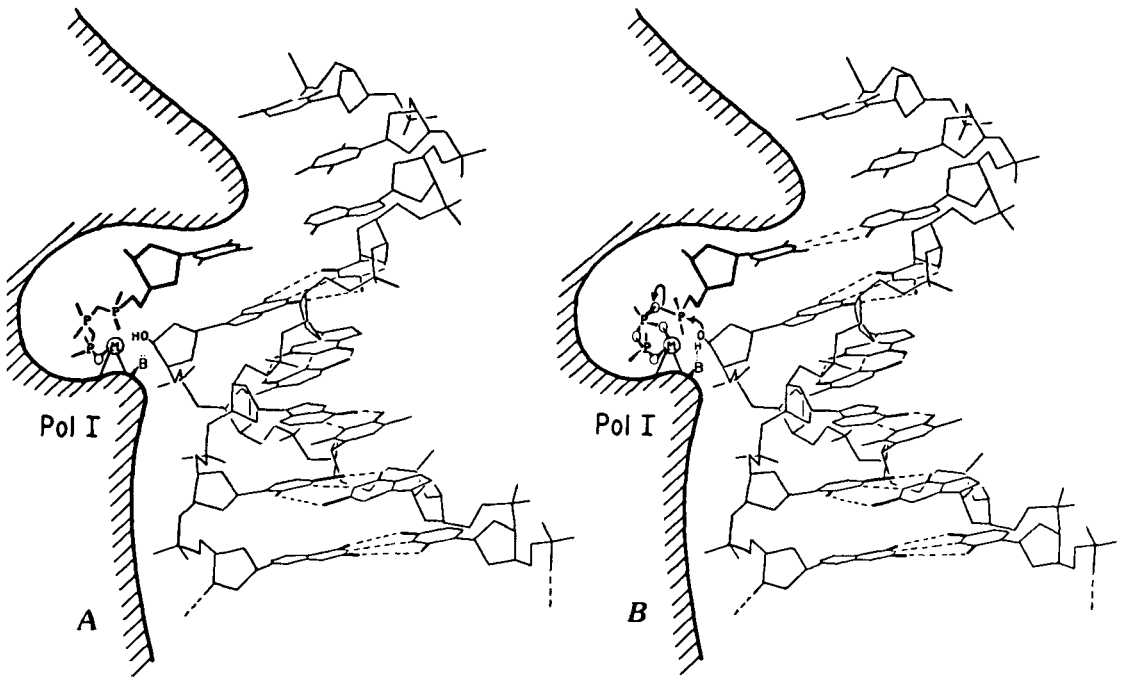

Fig. 6: Interactions of substrate, template and primer with DNA polymerase I based on NMR studies (2, 28, 31, 35). A. Initial monodentate substrate complex. B. Base-paired and bidentate substrate complex and mechanism of reaction.

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