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NUCLEOSIDE MONOPHOSPHATE KINASES: STRUCTURE, MECHANISM, AND SUBSTRATE SPECIFICITY

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CONTENTS

I. Introduction

In our 1991 "Perspectives in Biochemistry" article (Tsai and Yan, 1991), we summarized and critically analyzed the highly controversial state of the

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knowledge on the structure–function relationship of adenylate kinase (AK). We also emphasized the importance of the interplay between kinetic and structural analyses. Since then many new studies have been reported that are mostly supportive of the picture that was emerging at the time of the last review. In addition, significant knowledge has been advanced in other nucleoside monophosphate (NMP) kinases: guanylate kinase (GK), uridylate kinase (UK), and cytidylate kinase (CK). It is timely to review the subject again, with the scope expanded to include all NMP kinases. However, this report focuses on the NMP kinases that are homologous to AKs because functional studies can be correlated with the wealth of structural information on these enzymes.

II. Primary Structure

A. ADENYLATE KINASE

AK has been cloned from a variety of sources. All known AKs can be divided into short and long types (Fukami-Kobayashi et al., 1996). The difference between the short and long types of AK is that the long type has an insert of approximately 27 residues. Three isoforms are found in vertebrates: AK1 in the cytosol, AK2 in the mitochondrial intermembrane space, and AK3 in the mitochondrial matrix. AK1 belongs to the short type. AK2, AK3, plant, and most microbial AKs belong to the long type. The amino acid sequences of some extensively studied AKs are aligned in Figure 1. Interestingly, most AKs from gram-positive bacteria contain a zinc ion and have a zinc finger-like motif in the insert region (Glaser et al., 1992; Perrier et al., 1994). Surprisingly, AKs from archaebacteria have very low homology with any other AKs (Kath et al., 1993; Ferber et al., 1997). Many critical catalytic residues, including the "invariant lysine" in the P-loop, are not conserved in the methanococcal AKs. It will be very interesting to determine the three-dimensional structures and catalytic mechanisms of these enzymes.

B. GUANYLATE KINASE

The amino acid sequences of the known GKs and some related proteins are aligned in Figure 2. GKs are only distantly related to AKs. They are most similar to AK1, but even the sequence identity between GKs and AK1 is rather low. For example, there is only about 13% identity between GKy and AK1p (Stehle and Schulz, 1992). Most of the amino acid residues that


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AK1p  1-54      MEEKLKKSKIIFVVGGPGSGKGTQCEKIVQKYGYTHLSTGDLLRAEVSS-GSARG
AK1c  1-54      STEKLKHHKIIFVVGGPGSGKGTQCEKIVHKYGYTHLSTGDLLRAEVSS-GSERG
AK1h  1-54      MEEKLKKTKIIFVVGGPGSGKGTQCEKIVQKYGYTHLSTGDLLRSEVSS-GSARG
AK2b  1-62   APNVPAAEPVPESPKGVRAVLLGPPGAGKGTQAPKLAKNFCVCHLATGDMLRAMVAS-GSELG
AK3b  1-52      GASARLLRAAIMGAPGSGKGTVSSRITKHFELKHLSSGDLLRDNMLR-GTELG
AKy   1-52      MSSSESIRMVLIGPPGAGKGTQAPNLQERFHAAHLATGDMLRSQIAK-GTQLG
AKe   1-46         MRIILLGAPGAGKGTQAQFIMEKYGIPQISTGDMLRAAVKS-GSELG
AKst  1-46         MNLVLMGLPGAGKGTQAEKIVAAYGIPHISTGDMFRAAMKE-GTPLG
UKp   1-50       MRPKVVFVLGGPGAGKGTQCARIVEKYGYTHLSAGELLRDERKNPDSQYG
UKy   1-63   MTAATTSQPAFSPDQVSVIFVLGGPGAGKGTQCEKLVKDYSFVHLSAGDLLRAEQGRAGSQYG
UKd   1-52      MEKSKPNVVFVLGGPGSGKGTQCANIVRDFGWVHLSAGDLLRQEQQS-GSKDG
Ukel  1-49         MHNVVFVLGPPGSGKGTICAKIQENLNYVHLSAGDLLRAERQREGSEFG

AK1p  55-110   KMLSEIMEKGQLVPLETVLDMLRDAMVAKV---DTSKGFLIDGYPREVKQGEEFER----KIG
AK1c  55-110   KKLQAIMEKGELVPLDTVLDMLRDAMLAKA---DTSKGFLIDGYPREVKQGEEFEK----KIA
AK1h  55-110   KKLSEIMEKGQLVPLETVLDMLRDAMVAKV---NTSKGFLIDGYPREVQQGEEFER----RIG
AK2b  63-121   KKLKATMDAGKLVSDEMVLELIEKNLET----PPCKNGFLLDGFPRTVRQAEMLDDLMEKRKE
AK3b  53-105   VLAKTFIDQGKLIPDDVMTRLVLHELKNL-----TQYNWLLDGFPRTLPQAEALDR-----AY
AKy   53-112   LEAKKIMDQGGLVSDDIMVNMIKDELTNN---PACKNGFILDGFPRTIPQAEKLDQMLKEQGT
AKe   47-101   KQAKDIMDAGKLVTDELVIALVKERIAQ----EDCRNGFLLDGFPRTIPQADAMKE----AGI
AKst  47-105   LQAKQYMDRGDLVPDEVTIGIVRERLSK----DDCQNGFLLDGFPRTVAQAEALETMLADIGR
UKp   51-113   ELIEKYIKDGKIVPVEITISLLRREMDQTMAANAQKNKFLIDGFPRNQDNLQGWNKTMDGKAD
UKy   64-120   ELIKNCIKEGQIVPQEITLALLRNAISDNV--KANKHKFLIDGFPRKMDQAISFER----DIV
UKd   53-109   EMIATMIKNGEIVPSIVTVKLLKNAIDAN-----QGKNFLVDGFPRNEENNNSWEENM-KDFV
UKel  50-107   ALIESHIKNGSIVPVEITCSLLENAMKAC----GDAKGFLVDGFPRNEDNLQGWNKQM-DGKA

AK1p  111-146  QPTLLLYVDAGPETMTKRLLKRGETS----------------------------GRVDDNEETI
AK1c  111-146  PPTLLLYVDAGKETMVKRLLKRGETS----------------------------GRVDDNEETI
AK1h  111-146  QPTLLLYVDAGPETMTQRLLKRGETS----------------------------GRVDDNEETI
AK2b  122-184  KLDSVIEFSIPDSLLIRRITGRLIHPQSGRSYHEEFNPPKEPMKDDITGEPLIRRSDDNKKAL
AK3b  106-168  QIDTVINLNVPFEVIKQRLTARWIHPGSGRVYNIEFNPPKTMGIDDLTGEPLVQREDDRPETV
AKy   113-175  PLEKAIELKVDDELLVARITGRLIHPASGRSYHKIFNPPKEDMKDDVTGEALVQRSDDNADAL
AKe   102-164  NVDYVLEFDVPDELIVDRIVGRRVHAPSGRVYHVKFNPPKVEGKDDVTGEELTTRKDDQEETV
AKst  106-168  KLDYVIHIDVRQDVLMERLTGRRICRNCGATYHLIFHPPAKPGVCDKCGGELYQRADDNEATV
UKp   114-148  VSFVLFFDCNNE-ICIERCLERGKSS----------------------------GRSDDNRESL
UKy   121-156  ESKFILFFDCPEDIMLERLLERGKTS----------------------------GRSDDNIESI
UKd   110-145  DTKFVLFFDCPEEVMTQRLLKRGESS----------------------------GRSDDNIESI
UKel  108-141  LVQFVLFLSCPVSICIERCLNRGQ-----------------------------GRTDDNEESL

AK1p  147-194  KKRLETYYKATEPVIAFYEK-----RGIVRKVNAEGSVDDVFSQVCTHLDTLK
AK1c  147-193  KKRLETYYKATEPVIAFYKG-----RGIVRQLNAEGTVDEVFQQVCSYLDKL
AK1h  147-194  KKRLETYYKATEPVIAFYEK-----RGIVRKVNAEGSVDEVFSQVCTHLDALK
AK2b  185-240  KIRLEAYHTQTTPLVEYYSK-----RGIHSAIDASQTPDVVFASILAAFSKATCKDLVMFI
AK3b  169-226  VKRLKAYEAQTEPVLEYYRK-----KGVLETFSGTETNKIWPHVYAFLQTKLPQRSQETSVTP
AKy   176-222  KKRLAAYHAQTEPIVDFYKK-----TGIWAGVDASQPPATVWADILNKLGKD
AKe   165-214  RKRLVEYHQMTAPLIGYYSKEAEAGNTKYAKVDGTKPVAEVRADLEKILG
Akst  169-217  ANRLEVNMKQMKPLVDFYEQ-----KGYLRNINGEQDMEKVFADIRELLGGLAR
UKp   149-196  EKRIQTYLQSTKPIIDLYEE-----MGKVKKIDASKSVDEVFDEVVKIFDKEG
UKy   157-204  KKRFNTFKETSMPVIEYFET-----KSKVVRVRCDRSVEDVYKDVQDAIRDSL
UKd   146-194  KKRFNTFNVQTKLVIDHYNK-----FDKVKIIPANRDVNEVYNDVENLFKSMGF
UKel  142-191  KKRVETYNOOTFPIIEHFEK-----SGLVREVKSERPVDVVYADVEKVFDAANKK
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Figure 1. Amino acid sequence alignment of adenylate kinases and UMP/CMP kinases modified from Kukami-Kobayashi et al. (1996). The polar active site residues are *boldface*. The NMPbind and LID domains are *underlined* and *double-underlined,* respectively, for AK1p and AKe. The definition of the domains is according to Vonrhein et al. (1995). AK1p, pig AK1; AK1c, chicken AK1; AK1h, human AK1; AK2b, AK from bovine mitochondrial intermembrane space; AK3b, AK from bovine mitochondrial matrix; AKy, yeast cytosolic AK; AKe, *E. coli* AK; AKst, *B. stearothermophilus* AK; UKp, pig UK; UKy, yeast UK; UKd, *D. discoideum* UK; UKel, *C. elegans* UK.

```
GKy      1-53          SRPIVISGPSGTGKSTLLKKLFAEYP-DSFGFSVSSTTRTPRAGEVNGKDYNFV
GKh      1-56        MSGPRPVVLSGPSGAGKSTLLKRLLQEHS-GIFGFSVSHTTRNPRPGEENGKDYYFV
GKp      1-55         GSPRPVVLSGPSGAGKSTLLKKLLQEHS-SIFGFSVSHTTRDPRPGEENGKDYYFV
GKb      1-55         SGPRPVVLSGPSGAGKSTLLKKLLQEHG-SIFGFSVSHTTRDPRPGEENGKDYYFV
GKm      1-56        MAGPRPVVLSGPSGAGKSTLLKKLFQEHS-SIFGFSVSHTTRNPRPGEEDGKDYYFV
GKe      1-57        MAQGTLYIVSAPSGAGKSSLIQALLKTQPLYDTQVSVSHTTRQPRPGEVHGEHYFFV
DlgA   768-819         NYTRPVIILGPL---KDRINDDLISEYP-DKFGSCVPHTTRPKREYEVDGRDYHFV
ZO-1   630-677         GFLRPVTIFGPI---ADVAREKLAREEP-DIYQIAKS----EPRDAGTDQRSSGYI
PSD95  531-582         HYARPIIILGPT---KDRANDDLLSEFP-DKFGSCVPHTTRPKREYEIDGRDYHFV
SAP102 657-708         HYARPVIILGPM---KDRVNDDLISEFP-HKFGSCVPHTTRPRRDNEVDGQDYHFV
p55    280-334         FKRKTLVLIGASGVGRSHIKNALLSQNP-EKFVYPVPYTTTRPPRKSEEDGKEYHFI
AK1p     1-59      MEEKLKKSKIIFVVGGPGSGKGTQCEKIVQKY--GYTHLSTGDLLRAEVSSGSARGKMLSE

GKy     54-110     -SVDEFKSMIKNNEFIEWAQFSGNYYGSTVASVKQVSKSGKTCILDIDMQGVKSVKAI---
GKh     57-112     -TREVMQRDIAAGDFIEHAEFSGNLYGTSKVAVQAVQAMNRICVLDVDLQGVRNIKA----
GKp     56-111     -TREVMQRDIAAGDFIEHAEFSGNLYGTSKAAVRAVQAMNRICVLDVDLQGVRNIKK----
GKb     56-111     -TREVMQRDIAAGDFIEHAEFSGNLYGTSKAAVRAVQAMNRICVLDVDLQGVRNIKK----
GKm     57-112     -TREMMQRDIAAGDFIEHAEFSGNLYGTSKEAVRAVQAMNRICVLDVDLQGVRSIKK----
GKe     58-113     -NHDEFKEMISRDAFLEHAEVFGNYYGTSREAIEQVLATGVDVFLDIDWQGAQQIRQ----
DlgA   820-876     SSREQMERDIQNHLFIEAGQYNDNLYGTSVASVREVAEKGKHCILDVSGNAIKRLQV----
ZO-1   678-706     ----------------------------RLHTIKQIIDQDKHALLDVTPNAVDRLNY----
PSD95  583-639     SSREKMEKDIQAHKFIEAGQYNSHLYGTSVQSVREVAEQGKHCILDVSANAVRRLQA----
SAP102 709-765     VSREQMEKDIQDNKFIEAGQFNDNLYGTSIQSVRAVAERGKHCILDVSGNAIKRLQQ----
p55    335-390     STEE-MTRNISANEFLEPGSYQGNMFGTKFETVHQIHKQNKIAILDIEPQTLKIVRT----
AK1p    60-107     IMEKGQLVPLETVLD-----------MLRDAMVAKVDTSKGFLIDGY-PREVKQGEEFER

GKy    111-161     -PELNARFLFIAPPSVEDLKKRLEGRG------TETEESINKRLSAAQA-ELAYA-ETGA-
GKh    113-164     -TDLRPIYISVQPPSLHVLEQRLRQRN------TETEESLVKRLAAAQA-DMESSKEPGL-
GKp    112-163     -TDLQPIYIFVQPPSLDVLEQRLRQRN------TETEESLAKRLAAAKA-DMESSKEPGL-
GKb    112-163     -TDLRPIYIFVQPPSLDVLEQRLRQRN------TETEESLAKRLAAARA-DMESSKEPGL-
GKm    113-164     -TDLCPIYIFVQPPSLDVLEQRLRLRN------TETEESLAKRLAAART-DMESSKEPGL-
GKe    114-165     -KMPHARSIFILPPSKIELDRRLRGRG------QDSEEVIAKRMAQAVA-EMSHYAEYDY-
dlgA   877-925     -AQLYPVAVFIKPKSVDSVMEMNRRMT------EEQAKKTYERAIKMEQ-EFGEYFT----
ZO-1   707-761     -AQWYPIVVFLNPDSKQGVKTMRMRLCPES---RKSARKLYERSHKLAK-NNHHLFTTTI-
PSD95  640-688     -AHLHPIAIFIRPRSLENVLEINKRIT------EEQARKAFDRATKLEQ-EFTECFS----
SAP102 766-814     -AQLYPIAIFIKPKSIEALMEMNRRQT------YEQANKIFDKAMKLEQ-EFGEYFT----
p55    391-438     -AELSPFIVFIAPTDQGTQTEALQQLQ------KDSEAIRSQYAHYFDL-SLVNNG-----
AK1p   108-166     KIGQPTLLLYVDA-GPETMTKRLLKRGETSGRVDDNEETIKKRLETYYKATEPVI-AFYEK

GKy    162-186     --HDKVIVMD-DLDKAYKELKDFIFAEK
GKh    165-197     --FDVVIIMD-SLDQAYAELKEALSEEIKKAQRTGA
GKp    164-197     --FDLIIIMD-SLDKAYWALKEALSEEIKKAQATGHS
GKb    164-197     --FDLIIVMD-SLDKAYWALKEALSEEIKKAQGTGQS
GKm    165-198     --FDLVIIMD-DLDKAYATLKQALSEEIKKAQGTGHA
GKe    166-207     --LIVNDDFDTALTDLKTIIRAERLRMSRQKQRHDALISKLLAD
DlgA   926-960     --GVVQGDT--IEEIYSKVKSMIWSQSGPTIWVPSKESL
ZO-1   762-797     --NLNSMND--GWYGALKEAVQQQQNQLVWVSEGKADGAT
PSD95  689-723     --AIVEGDS--FEEIYHKVKRVIEDLSGPYIWVPARERL
SAP102 815-849     --AIVQGDS--LEEIYNKIKQIIEDQSGHYIWVPSPEKL
p55    439-466     --VDETLKK--LQEAFDQACSSPQWVPVSWVY
AK1p   167-194     RGIVRKVNAEGSVDDVFSQVCTHLDTLK
```

Figure 2. Amino acid sequence alignment of guanylate kinases and related proteins. The polar active site residues are *boldface*. The NMPbind and LID domains of GKy and AK1p are *underlined* and *double-underlined*, respectively. There is no structural homology between the NMPbind domains of GK and AK. GKy, yeast GK; GKh, human GK; GKp, pig GK; GKb, bovine GK; GKe, *E. coli* GK; DlgA, *Drosophila* discs-large tumor suppressor protein; ZO-1, human tight junction protein type-1; PSD95, a human postsynaptic protein; SAP102, a rat synapse-associated protein; p55, a human erythrocyte membrane protein; AK1p, pig AK1.

interact with the phosphoryl groups of adenosine triphosphate (ATP) are identical between GKs and AKs. However, the amino acid sequence that constitutes the guanosine monophosphate (GMP) binding domain is not homologous to the amino acid sequence that forms the AMP binding domain of AK. Interestingly, several membrane-associated proteins have a domain that is significantly homologous to GK. The sequence identities between these proteins and GKy are approximately 30%. The so-called GK homologues include the *Drosophila* discs-large tumor suppressor protein (dlgA) (Woods and Bryant, 1991), the protein encoded by *Caenorhabditis elegans* vulvaless gene *lin*-2 (Hoskins et al., 1996), the mammalian zonula ocludens or tight junction proteins ZO-1 (Willott et al., 1993) and ZO-2 (Jesaitis and Goodenough, 1994), the erythrocyte membrane protein p55 (Ruff et al., 1991), and several synapse-associated proteins (PSD-95/SAP90, SAP97/hdlg, chapsyn-110/PSD-93, and SAP-102) (Cho et al., 1992; Kistner et al., 1993; Lue et al., 1994; Müller et al., 1995, 1996; Brenman et al., 1996; Kim et al., 1996). However, all these proteins lack the conserved residues required for binding of ATP and catalysis. Some of the proteins also miss the key residues involved in binding of GMP. Thus, these GK homologues are unlikely to be enzymatically active. It has been shown that the recombinant SAP90 binds GMP with high affinity and specificity but has no GK activity (Kistner et al., 1995). It appears that the GK domains in these proteins may be involved in protein–protein interactions. It has recently been found that a synaptic protein binds specifically to the GK domains of the synapse-associated proteins PSD-95/SAP90, SAP97/hdlg, chapsyn-110/PSD-93, and SAP-102 (Kim et al., 1997).

C. URIDYLATE AND CYTIDYLATE KINASES

All known eukaryotic UKs catalyze efficient phosphorylation of both UMP and CMP, making them UMP/CMP kinases. As shown in Figure 1, their amino acid sequences are highly homologous to those of AKs (Liljelund et al., 1989; Wiesmuller et al., 1990; Okajima et al., 1995). In prokaryotes, there are two distinct enzymes for phosphorylation of UMP and CMP. Surprisingly, bacterial UKs have no homology with AKs (Serina et al., 1995). They are members of the aspartokinase family instead. Bacterial CKs are only remotely related to AKs (Bucurenci et al., 1996; Schultz et al., 1997).

III. Tertiary Structure

A. BACKBONE FOLDING

To date, approximately 26 crystal structures have been determined for NMP kinases (Vonrhein et al., 1995; Abele and Schulz, 1995; Müller et al., 1996b; Schlauderer and Schulz, 1996; Schlauderer et al., 1996; Schlichting and Reinstein, 1997; M.B. Berry and G.N. Phillips, unpublished observations). All the structures are highly similar, as evidenced by the overlays of AK1p with AKe, GKy, and UKy (Fig. 3), and contain a central five-stranded parallel β-sheet with helices on both sides. The polypeptides are conveniently divided into three parts termed CORE, NMPbind and LID domains (Vonrhein et al., 1995) as indicated in Figures 1 and 2. The CORE domain includes the central parallel β-sheet and the immediately packed helices. This domain is most similar among NMP kinases. It contains the P-loop that plays an important role in binding of the triphosphate of ATP. The NMPbind domain binds to NMP and consists of a 47-residue segment in GKs and a 29-residue segment in other NMP kinases. The domain is helical among all NMP kinases except GK. The chain fold of the NMPbind domain of GKy is grossly different from those of other NMP kinases (Stehle and Schulz, 1990). It is composed of a four-stranded β-sheet and a short helix. The LID domain consists of a 37-residue segment in the long-type AKs and only a short loop in other NMP kinases. Since the amino acid sequences of the LID domains are highly conserved among the long-type AKs, it is not surprising that the structures of the domains are highly similar. Interestingly, the LID domain of AKst contains a Zn^{2+}, but its chain fold is the same as those of other LID domains (M. B. Berry and G. N. Phillips, unpublished observations).

B. SUBSTRATE BINDING SITES

The controversy on the assignment of the substrate binding sites has been documented in our previous review (Tsai and Yan, 1991). The remaining question at the time was whether the ATP site of AK1 is identical to that of the long-type AKs. Although the ATP site of the long-type AKs has been determined by X-ray crystallography, the adenosine moiety of ATP is surrounded mainly by the residues of the insert segment, which is absent in

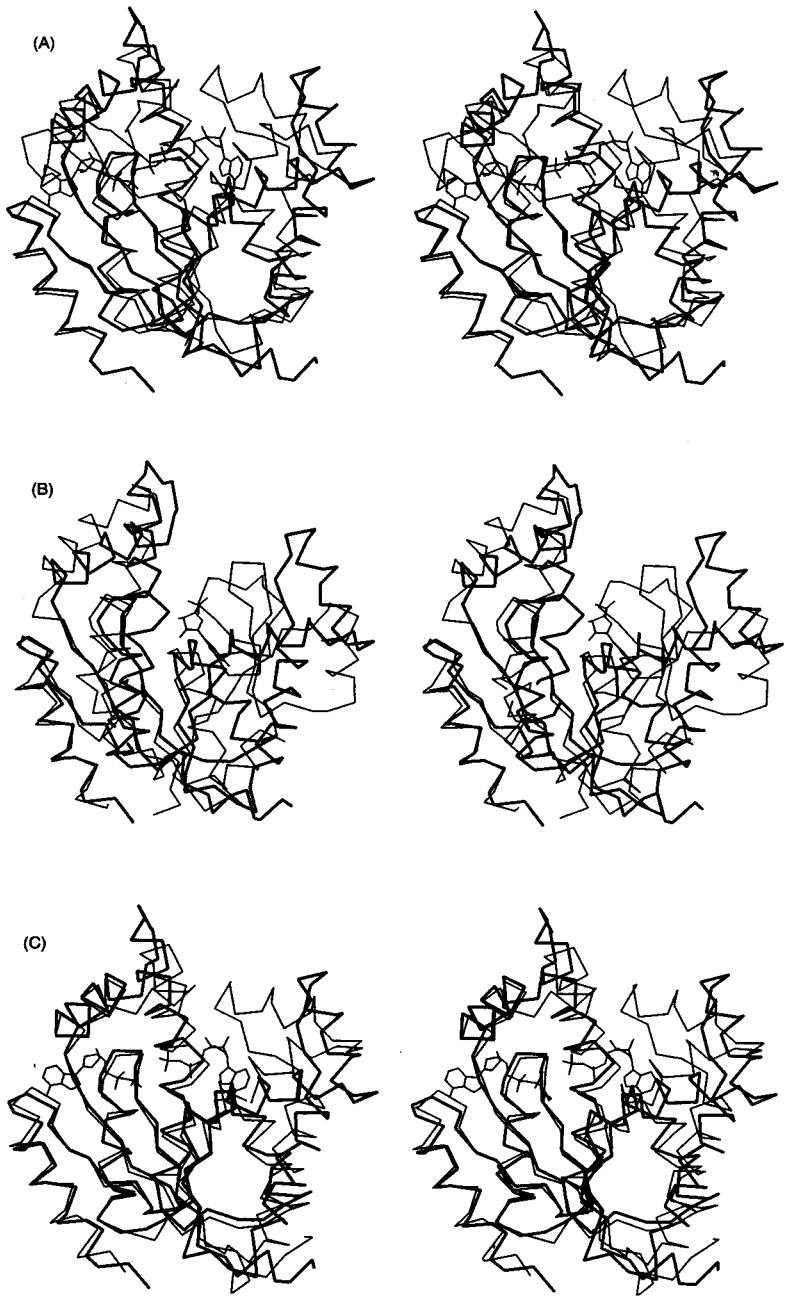


Figure 3. Overlays of the C_α trace of AK1p with those of AKe·AP_5A **(A)**, GKy·GMP **(B)**, and UKy·ADP·ADP **(C)**. AK1p is in thick line.

AK1. There was no physical evidence at the time for the assignment of the ATP site of AK1. A guanidino group was found to stack on the adenine ring at the ATP site of the long-type AKs (Egner et al., 1987). Because the arginine residue is conserved among all AKs, mutagenesis study of the residue might help locate the ATP site in AK1. Unfortunately, substitution of the residue in AK1c (Arg-128) with alanine caused moderate (<20-fold) perturbations on all kinetic parameters (Yan et al., 1990a). The conformation of the mutant was also perturbed to some extent. Thus the mutagenesis result was not conclusive regarding the assignment of the ATP site in AK1.

Recent multidimensional nuclear magnetic resonance (NMR) study of AK1c·$MgAP_5A$ has suggested that the substrates indeed bind to AK1 in the same manner as to the long-type AKs (Byeon et al., 1993). As many as nine residues have shown NOEs with AMP. The observed NOEs are in full agreement with those predicted by the crystal structure of AKe·AP_5A. Only Thr-23 has displayed NOEs with ATP. The result is also in agreement with the prediction based on the crystal structure of AKe·AP_5A. Gly-20 is the other residue predicted to have NOEs with ATP but the resonances of Gly-20 have not been assigned. Although AK1 has not been crystallized with any substrates or substrate analogues, the structures of the complexed forms of the closely related UKy and UKd have recently been reported (Müller-Dieckmann and Schulz, 1994; Scheffzek et al., 1996). The structures have shown that the interactions of the ATP adenine ring with UKy and UKd are indeed very similar to those between the ATP adenine ring and the long-type AKs, notably a hydrogen bond between the exocyclic amino group of the adenine and a backbone carbonyl oxygen and the stacking of the adenine ring with the guanidino group of the conserved arginine. Molecular modeling on the basis of the crystal structure of AKe·AP_5A has suggested that the carboxamide of Asn-168 in GKy is hydrogen-bonded to N7 of the adenine ring of ATP (Stehle and Schulz, 1992). Indeed, substitution of Asn-168 with alanine has resulted in significant increases in both $K_{m(MgATP)}$ and $K_{i(MgATP)}$ with little perturbations on $K_{m(GMP)}$ and $K_{i(GMP)}$ (Y. Li, Y. Zhang, and H. Yan, unpublished observations). The results taken together indicate that the ATP site is in the same location in all these NMP kinases.

IV. Catalytic Mechanism

A. ITERATIVE STRUCTURE-FUNCTION STUDIES

Although site-directed mutagenesis is a very powerful technique for studying enzymatic catalytic mechanisms, it has often generated new controversies rather than solved problems. A recent site-directed mutagenesis study of AK1h by Ayabe et al. (1997) is a case in point. In this study, seven lysine residues have been replaced by various amino acids. On the basis of the mere kinetic data, the authors have concluded that all the lysine residues play a role in substrate binding and/or catalysis. However, except Lys-21, which has been shown to play an important role in catalysis (Tian et al., 1990; Byeon et al., 1995), all other lysine residues are unlikely to have direct interactions with the substrates according to the crystal structure of the closely related AK1p (Dreusicke et al., 1988). Furthermore, except Lys-21, none of the lysine residues is conserved. Therefore, the lysine residues, with the exception of Lys-21, are unlikely to be directly involved in either substrate binding or catalysis. The effects of the mutations on the structure of the enzyme could be the cause for the perturbations on the kinetic parameters. Thus, in our last review on AKs (Tsai and Yan, 1991), we have advocated an iterative approach to structure–function studies of enzymes. Briefly, it involves interplay (or iteration) between kinetic analysis of site-directed mutant enzymes, structural analysis of the mutant enzymes and their substrate complexes, and further tests of the interpretations by conventional bioorganic and biophysical techniques. It has been illustrated by the site-directed mutagenesis studies of Lys-21, Thr-23, Arg-44, Asp-93, and Arg-97 of AK1c (Tian et al., 1990; Byeon et al., 1995; Shi et al., 1993; Yan et al., 1990a; Jiang et al., 1991; Yan and Tsai, 1991; Dahnke et al., 1991, 1992). Even with such a rigorous approach, it always difficult to relate structural perturbations to functional changes: a major structural change may not necessarily be responsible for the perturbation in function, whereas a minor conformational perturbation near the active site could have a substantial impact on the activity. To complicate the matter further, as exemplified by the iterative structure-function studies of Thr-23 of AK1c, lack of significant perturbation in steady-state kinetic parameters of a mutant may not necessarily mean that the mutated residue does not participate directly in catalysis (Shi et al., 1993). In other words, site-directed mutagenesis could fail to identify catalytic residues unless rigorous biochemical and physical characterizations, including pre-steady-state kinetics, can be performed. Thus, we have emphasized designing additional experiments, whenever possible and justified, to test whether the catalytic properties of mutant enzymes are consistent with the interpretations.

B. ROLES OF THE ACTIVE SITE RESIDUES IN CATALYSIS

Among NMP kinases, only AK and GK have been extensively studied by site-directed mutagenesis. The roles of most active site residues in AK have

been described in our previous review (Tsai and Yan, 1991) and will not be repeated in this discussion. A major development since has been to use unnatural amino acids to probe the functional roles of active site residues beyond the conventional site-directed mutagenesis (Zhao et al., 1996). Unnatural amino acid mutagenesis has been used to probe the importance of the aromaticity of Tyr-95 and the ring size of Pro-17 in the catalysis by AK1c. Previous studies indicated that replacement of Tyr-95 with phenylalanine did not cause a perturbation in the activity, whereas replacement with nonaromatic natural amino acids caused a significant decrease in activity. 2,5-Dihydrophenylalanine (DiHPhe) was incorporated into AK to replace Tyr-95. The Tyr95DiHPhe mutant showed k_{cat} and $K_{m(AMP)}$ similar to those of WT, suggesting that the aromaticity at Tyr-95 is not critically important for the catalysis by AK. Four ring analogues of proline, pipecolic acid, homopipecolic acid, 3,4-dehydroproline, and azetidine-2-carboxylic acid were used to replace Pro-17. Among the four proline mutants, only Pro17Aze exhibited a large decrease in activity. A possible interpretation of the results is that the rigidity of the four-membered ring of Aze could restrict the movement of the P-loop and impair the conformational changes obligatory for the catalysis by AK.

Structure-function studies of GK have been focused on the polar residues at the active center, as shown in Figure 4 (Zhang et al., 1997a,b; Li et al., unpublished observations). The active site residues have been identified by X-ray crystallography and homologous modeling with AKe·AP_5A (Stehle

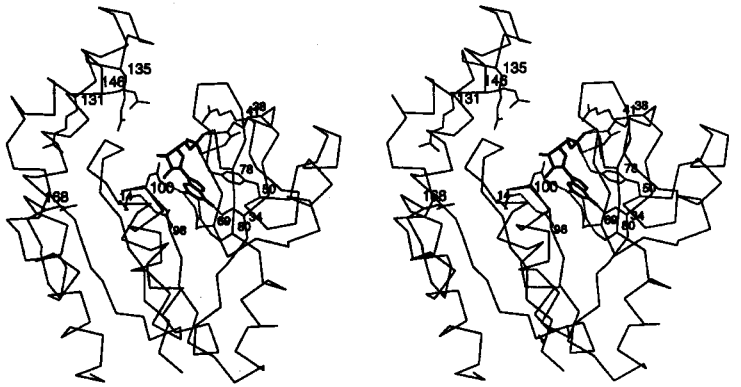


Figure 4. Stereo view of the C_α trace of GKy (thin line) and the bound GMP (thick line), along with the side chains at the active center. The ATP site was modeled based on the structure of AKe·AP_5A (Stehle and Schulz, 1992).

TABLE 1
Apparent Energetic Contributions of the Active Site Residues to Substrate Binding and Catalysis

Enzyme	Residue	E·AMP or E·GMP	E·MgATP	Ternary Complex	Transition State
		(kcal/mol)			
AKc	Lys-21	−0.5	0.1	0.1	6.3
GKy	Lys-14	−0.2	0.2	0.1	4.5
AKc	Thr-23	0.8	0.9	1.2	1.1
AKc	Thr-39	−1.0	−0.9	0.1	0.3
AKc	Arg-44	1.8	−0.2	2.1	2.8
AKc	Asp-93	−0.1	−0.2	0.8	4.6
GKy	Asp-98	0.4	0.7	−0.3	1.2
AKc	Arg-97	1.1	−0.5	2.0	4.0
AKc	Gln-101	1.6	−0.3	2.8	5.0
AKc	Arg-128	1.3	1.4	1.6	3.3
GKy	Arg-131	0.8	2.0	1.9	3.2
AKc	Arg-132	−0.5	0.1	0.2	5.3
GKy	Arg-135	1.3	1.9	1.2	7.2
AKc	Arg-138	0.1	−0.4	1.9	6.2
AKc	Asp-140	−0.5	0	1.1	3.6
AKc	Asp-141	−0.3	0	1.5	3.2
AKc	Arg-149	0.3	0.4	2.9	7.2
GKy	Arg-146	0.9	0.8	0.7	6.0
GKy	Ser-34	2.3	1.0	1.9	5.4
GKy	Arg-38	0.9	−0.4	1.4	3.6
GKy	Arg-41	0.7	0.4	0.3	4.6
GKy	Tyr-50	2.2	0.4	2.1	3.2
GKy	Glu-69	2.1	0.3	1.5	6.9
GKy	Tyr-78	1.7	0.4	1.8	4.6
GKy	Ser-80	1.3	0.3	1.1	3.0
GKy	Asp-100	1.2	0.4	1.3	2.5
GKy	Asn-168	0.6	2.2	2.2	3.9

and Schulz, 1992). The apparent energetic contributions of the residues to substrate binding and catalysis are listed in Table 1, along with those of the active site residues of AK1c for comparison. The roles of the residues at the phosphate binding region are summarized below, and the roles of the residues at the base binding pockets are described in the next section.

Eight residues are found at the phosphate binding region of GKy: Lys-14, Arg-38, Arg-41, Tyr-50, Tyr-78, Asp-98, Arg-135, and Arg-146. Arg-38, Arg-41, Tyr-50 and Tyr-78 are characteristic of GKs (Fig. 2). All these

residues except Arg-41 are hydrogen bonded to the phosphate of GMP in the GK·GMP complex (Stehle and Schulz, 1992) (Fig. 4). The guanidino group of Arg-41 is only 3.2 Å away from a phosphate oxygen of GMP. Site-directed mutagenesis studies indicate that Arg-38, Tyr-50, and Tyr-78 make significant contributions to both GMP binding and transition state stabilization. Arg-41 is mainly involved in the transition state stabilization, indicating that its interaction with GMP becomes much stronger as the reaction progresses to the transition state.

The other four residues at the phosphate binding region are conserved between GKs and AKs. Lys-14 is the "invariant" lysine of the P-loop in GKy and corresponds to Lys-21 in AK1. Its role in catalysis is very similar to that of Lys-21 of Ak1 but its contribution to the transition state stabilization is ~2 kcal/mol less that of the AK residue (Tian et al., 1990; Byeon et al., 1995). Arg-135 and Arg-146 correspond to Arg-132 and Arg-149, respectively, in AK1. Like the arginine residues in AK1 (Yan et al., 1990a; Dahnke et al., 1992), they are the key residues in transition state stabilization. Asp-98 corresponds to Asp-93 in AK1, a residue that plays critical roles in the binding and function of Mg^{2+} (Yan and Tsai, 1991). However, its contribution to the transition state stabilization is much less than that of the corresponding residue in AK1.

C. MECHANISM OF PHOSPHORYL TRANSFER

NMP kinases generally follow a random Bi–Bi kinetic mechanism (Roads and Lowenstein, 1968). It is unlikely there is a phosphoenzyme intermediate because the stereochemical course is inversion (Richard and Frey, 1978). However, the transition state, and how NMP kinases stabilize the transition state, remain unclear.

Like any reactions involving two reactants, phosphoryl transfer can follow a dissociative path with a metaphosphate-like transition state, an associative path with a pentacoordinate transition state, or anything in between. The major difference between the dissociative and associative transition states is the nature of the bonding of the transferred phosphate to the leaving group and the nucleophile (Thatcher and Kluger, 1989). Recent chemical studies using model compounds suggest that nonenzymatic or enzymatic hydrolysis of ATP and GTP follows a path with a transition state of more dissociative character (Admiraal and Herschlag, 1995; Hollfelder and Herschlag, 1995; Maegley et al., 1996). Do the reactions catalyzed by NMP kinases also proceed with a dissociative transition state? Although a dissociative mechanism has been proposed for NMP kinases (Scheffzek et al., 1996), the wealth of the structural and site-directed mutagenesis data suggests that NMP kinases catalyze phosphoryl transfer through an associative transition state.

First, a fully associative mechanism can be distinguished from a fully dissociative mechanism by the distances between the tranferred phosphorus and the oxygen atoms of the leaving group and the nucleophile. As shown in Figure 5A, in the crystal structure of UKd in complex with the transition state analogue ($MgADP+AlF_3+CMP$), the distances between Al^{3+} and the phosphate oxygen atoms are significantly shorter than the van der Waals contacts (Schlichting and Reinstein, 1997). A stepwise dissociative mechanism with a metaphosphate intermediate may be ruled out based on the structural information. However, the resolution of the data (the estimated mean coordinate error is 0.2 A) is insufficient to distinguish between concerted dissociative and associative mechanisms.

Second, the differences in bonding between a dissociative and an associative transition state are also manifested in the electronic distributions (Admiraal and Herschlag, 1995; Maegley et al., 1996). Thus, in a dissociative transition state, there are a loss of charge on the phosphoryl group being transferred and a gain of charge on the leaving group. The oxygen bridging the phosphate and the leaving group, namely the β-γ bridging oxygen of ATP in the forward reaction or the α-β bridging oxygen of NDP in the reverse reaction, gains most charge. On the other hand, a gain of charge on the phosphoryl group is transferred in an associative transition state. The differences in the electronic distributions of the two transition states require different strategies for catalysis. If the phosphoryl transfers proceed with a dissociative transition state, the enzymes must stabilize the charges developed on the β-γ bridging oxygen of ATP in the forward reaction and the α-β bridging oxygen of NDP in the reverse reaction. If NMP kinases catalyze the phosphoryl transfers with an associative transition state, they need to stabilize the charge developed on the γ-phosphate of ATP in the forward reaction and the β-phosphate of NDP in the reverse reaction. Site-directed mutagenesis have identified four positively charged residues in AKs (Lys-21, Arg-132, Arg-138, and Arg-149 according to AK1p numbering) that are involved in the transition state stabilization (Tsai and Yan, 1991). In the crystal structures of the ternary complexes, these residues are poised to form hydrogen bonds to the phosphate being transferred (Abele and Schulz, 1995; Schlichting and Reinstein, 1997; M. B. Berry and G. N. Phillips, unpublished observations). Indeed, in the crystal structure of UKd in complex

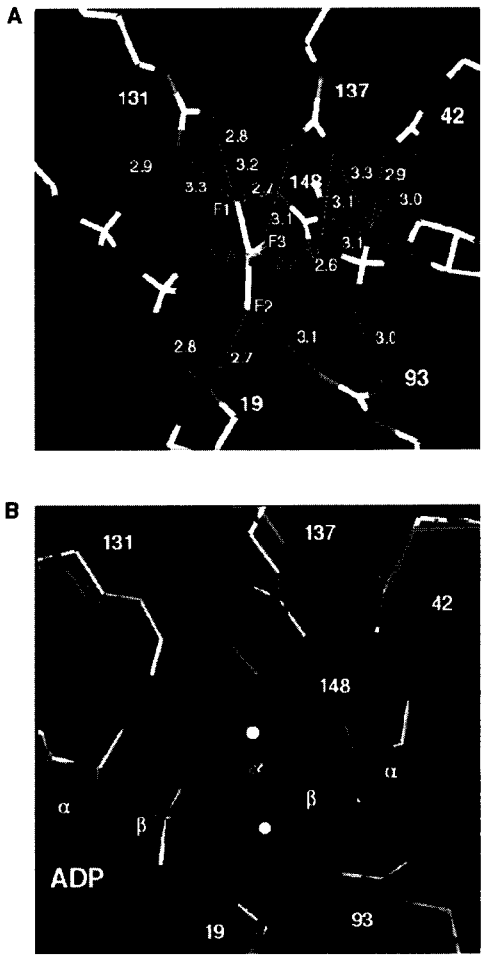


Figure 5. **(A)** Active site of UKd in complex with MgADP, CMP, and AlF_3 showing the distances of the phosphates and AlF_3 to the surrounding arginines and lysine. The magenta sphere represents the bound magnesium ion. **(B)** Overlay of the active sites of the various UKd complexes: UKd·MgADP·CMP *(yellow line)*, UKd·MgADP·CMP·AlF_3 *(cyan line)*, UKd·MgADP·UDP·BeF_2 *(green line)*, and UKd·$MgAP_5U$ *(magenta line)*. The crosses represent magnesium ions. Water molecules are shown as *yellow spheres*. The different position of Arg-131 in the $MgAP_5U$ complex is due to the steric hindrance caused by the bridging phosphate as indicated by the magenta arrow. The different position of Arg-93 in the MgADP·UDP·BeF_2 complex is due to the steric hindrance caused by the presence of β-phosphate at the NMP site *(green arrow)*. From Schlichting and Reinstein (1997). (See color plates.)

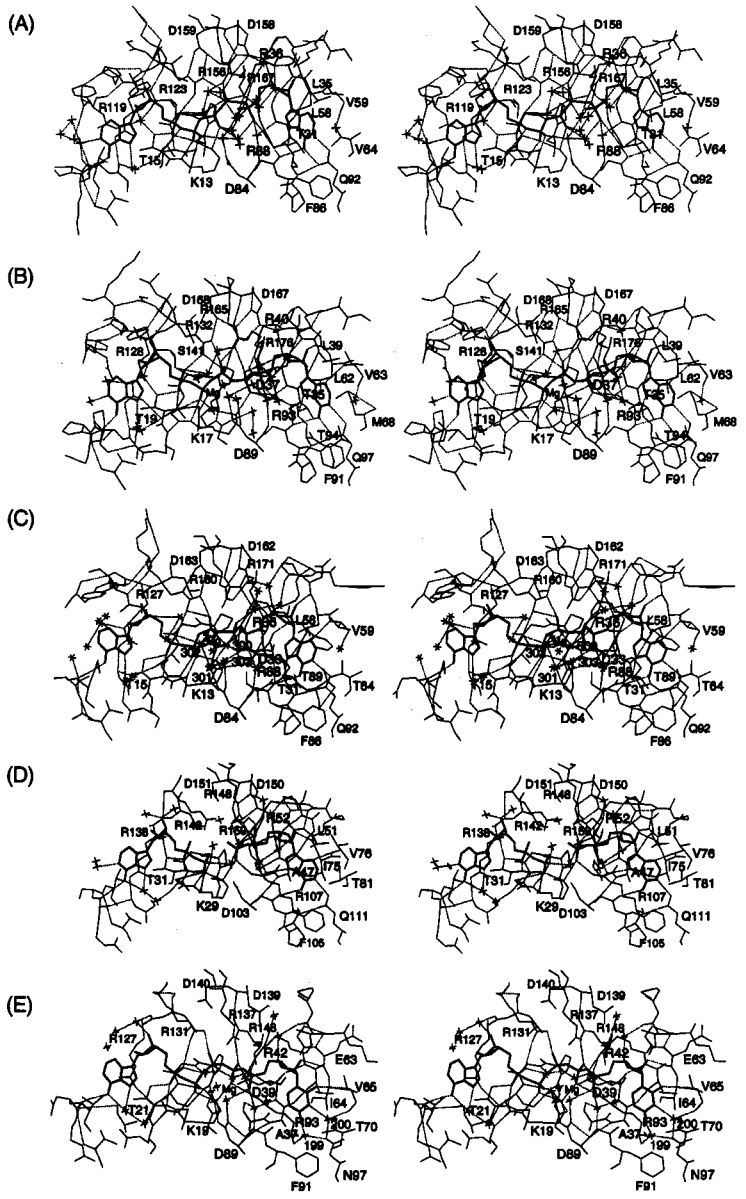
with the transition state analogue, Arg-137 and Arg-148 (equivalent to Arg-138 and Arg-149 in AK1) move to interact with AlF_3 (Figure 5B) (Schlichting and Reinstein, 1997). It suggests that the two arginine residues play a role in neutralizing the negative charge developed on the transferred phosphoryl group at the transition state.

Third, the position and/or orientation of the phosphate acceptor relative to the phosphate being transferred are very critical in an associative mechanism but are less so in a dissociative mechanism. Thus, an amino acid residue may play an important role in transition state stabilization by orienting the phosphate donor or acceptor. Lys-21 of AK1c has been shown to stabilize the transition state by orienting the triphosphate of ATP. Arg-132 may play a similar role because its position is essentially the same at the ground state and the transition state.

Fourth, the main role of Mg^{2+} is to orient the phosphate donor and acceptor for phosphoryl transfer. As shown in Figure 6C, in the AKst·AP_5A complex, the bound Mg^{2+} is coordinated with the β- and γ-phosphates of ATP and four water molecules (300–303) (M. B. Berry and N. G. Phillips, unpublished observations). Water molecules 300 and 303 are in turn hydrogen bonded to the phosphate of AMP. Water molecule 302 is within hydrogen bond distance of the two ATP oxygens that are coordinated with Mg^{2+}. Water molecule 300 is also hydrogen bonded to the guanidino group of Arg-36, a conservative residue that also interacts with the phosphate of AMP. Water molecules 301 and 303 are hydrogen bonded to the carboxyl group of Asp-84. It has been suggested that this coordination network helps orient both the phosphate donor and acceptor in the reaction and facilitates the progression of the ternary complex to the transition state. Indeed, it has been shown that the role of Asp-93 of AK1c (corresponding to Asp-84 of Akst) is to assist the bound Mg^{2+} in orienting the phosphoryl groups of the substrates (Yan and Tsai, 1991).

Fifth, as shown in Figure 6, the active sites of NMP kinases are not completely shielded from water, even when both substrates are bound to the enzymes. Thus, it is difficult to envisage how NMP kinases can avoid hydrolytic activity if the enzymes catalyze the reactions with a metaphosphate-like transition state.

Primary and secondary ^{18}O isotope effects have been recently developed by Cleland's group as a tool for determining the mechanisms and transition structures of phosphoryl transfer (Cleland, 1990; Cleland and Hengge, 1995). In their systematic studies of phosphoryl transfer reactions using heavy atom isotope effects, Cleland and coworkers have observed that



phosphoryl transfers from phosphate monoesters generally take highly dissociative paths, whereas phosphate triesters follow more associative mechanisms (Cleland and Hengge, 1995, and references therein). Phosphate diesters use S_N2 mechanisms with slightly dissociative or associative characters. A dissociative transition state has been suggested for the phosphoryl transfer reaction catalyzed by hexokinase (Jones et al., 1991). The mechanisms of phosphoryl transfer-catalyzed NMP kinases are apparently different from those for the phosphate monoesters studied by Cleland and coworkers. While support for an associative mechanism is substantial for the reactions catalyzed NMP kinases, further studies using physical organic methods are needed to address the problem unequivocally. Brönsted analysis may not be applicable because of the high substrate specificity of the enzymes. Primary and secondary ^{18}O isotope effects could be the best tool to unravel the transition state bonding and therefore the chemical mechanisms of phosphoryl transfer catalyzed by NMP kinases.

V. Structural Basis of Substrate Specificity

A. NTP SITE

In general, NMP kinases are not very specific with respect to phosphate donors. All NMP kinases except AK3 prefer ATP at the NTP site (Noda, 1973; Font and Gautheron, 1980; Hall and Kuhn, 1986; Konrad, 1992; Wiesmuller et al., 1990). Although few systematic studies use the specificity constant (k_{cat}/K_m) as a measure of specificity for phosphate donors, generally a preferred phosphate donor is an order of magnitude more active than the other phosphate donors. AK3 can use both GTP and ITP as effective phosphate donors, but GTP is probably the physiological substrate (Tomasselli and Noda, 1979). ATP and CTP do not serve as phosphate donors for AK3. The low specificity at the NTP site is not surprising because most of the binding energy comes from the triphosphate moiety (Sanders et al., 1989) and the adenosine moiety of ATP is only loosely

Figure 6. Stereo view of the amino acid residues *(thin line)* and bound water molecules within 5 Å of the bound substrates or substrate analogues *(thick line)*. Water molecules and magnesium ions are shown as *stars*. Possible hydrogen bonds with donors and acceptors <3.1 Å apart are indicated by *dotted lines*. **(A)** AKe·AP_5A (Müller and Schulz, 1992); **(B)** AKy·$MgAP_5A$ (Abele and Schulz, 1995); **(C)** AKst·$MgAP_5A$ (M.B. Berry and G.N. Phillips, unpublished observations); **(D)** UKy·ADP·ADP (Müller-Dieckmann and Schulz, 1995); **(E)** UKd·$MgAP_5U$ (Scheffzek et al., 1996).

bound in all known crystal structures (Müller and Schulz, 1992; Abele and Schulz, 1995; Müller-Dieckmann and Schulz, 1995; Scheffzek et al., 1996) (Fig. 6A–E). There is only one hydrogen bond observed between the adenine moiety and the enzymes. Apparently, it is this hydrogen bond between the exocyclic amino group of adenine and a backbone oxygen of AK that make the enzymes prefer ATP over other NTPs. As mentioned earlier, Asn-168 of GKy has been suggested to form a hydrogen bond to N7 of the adenine ring of ATP (Stehle and Schulz, 1992). Indeed, the kinetic data of N168A indicate that Asn-168 is important for binding of MgATP. It stabilizes both the MgATP complex and the ternary complex by 2.2 kcal/mol and the transition state by 3.9 kcal/mol. However, it is unlikely that Asn-168 plays a role in discriminating ATP and GMP.

B. NMP SITE

In contrast to NTP, the NMP substrate interacts extensively with NMP kinases. Consequently, extensive mutagenesis studies have been performed to investigate how different NMP kinases control the NMP specificity and to modify the NMP specificity of these enzymes.

AKs are very specific with respect to phosphate acceptors (Noda, 1973; Font and Gautheron, 1980; Okajima et al., 1995). As shown in Figures 6A–C and 7A, the adenine moiety is fixed in the AMP binding pockets by as many as five hydrogen bonds. A strictly conserved glutamine residue plays critical roles not only in NMP specificity but in catalysis as well. IMP may form hydrogen bonds to the enzyme by a 180° rotation of the carboxamide of the glutamine. However, the orientation of the carboxamide is fixed by the hydrogen bonds to a backbone amide and a conserved carboxylate. GMP is further discriminated by the presence of the exocyclic amino group at position 2 of guanine that may result in steric hindrance. Substitution of the glutamine residue in AK1c (Gln-101) with glutamate has resulted in not only a dramatic decrease in the catalytic efficiency for the native substrate but in an alteration in NMP specificity as well (Beichner et al., 1996). While the WT AK1c shows no measurable activities toward GMP and IMP, the activities of Q101E toward GMP and IMP are significant. It has been proposed that the glutamate in the mutant may form a hydrogen bond to the NH at position 1 of IMP or GMP. Glu-70 (corresponding to Asp-61 in AKe) may form a hydrogen bond to the exocyclic amino group of GMP. Random mutagenesis studies have shown that substitution of the glutamine residue with histidine has caused a significant increase in the activ-

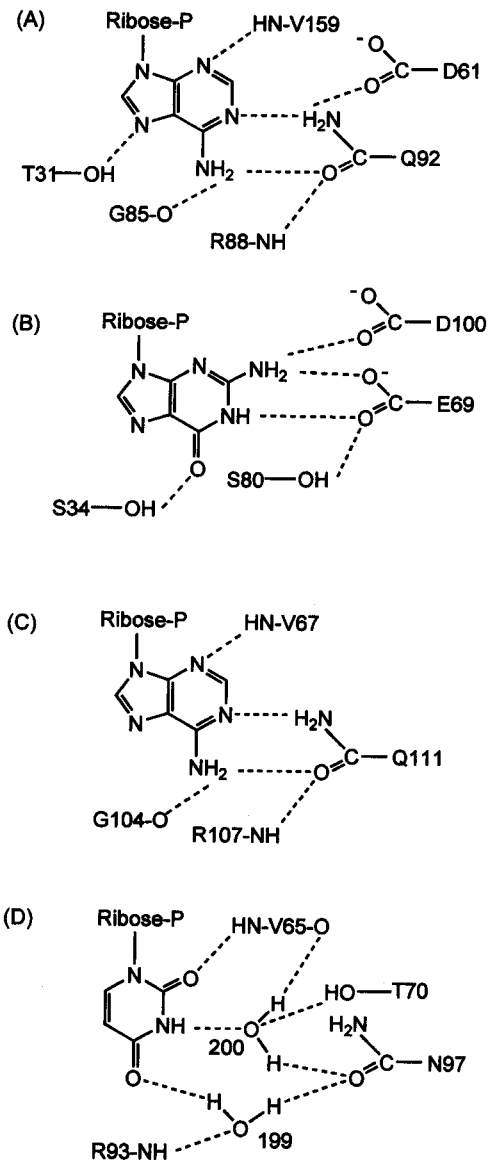


Figure 7. Schematic diagrams showing hydrogen bonding interactions of NMP kinases with the base moieties of NMPs. **(A)** AKe with adenine; **(B)** GKy with guanine; **(C)** UKy with adenine; and **(D)** UKd with uracil. Hydrogen bonds shown by *dotted lines*.

ity toward UMP and only a moderate decrease in the catalytic efficiency for AMP (Okajima et al., 1993b).

Surprisingly, the threonine residue, which is hydrogen bonded to the N7 nitrogen of adenine, also plays an important role in the specificity of the AMP binding site. Substitution of the threonine residue in AK1c (Thr-39) with alanine has resulted in significant increases in the catalytic efficiencies for CMP and UMP (Okajima et al., 1993a). The result was fully understood only after the determination of the crystal structures of UKy and Ukd.

UKs are least specific with respect to phosphate acceptors. They catalyze phosphoryl transfer not only to UMP and CMP, but to AMP as well (Wiesmuller et al., 1990; Okajima et al., 1995; Li et al., unpublished observations). The catalytic efficiency for AMP is about two orders of magnitude lower than those for UMP and CMP in the cases of UKd and UKp (Scheffzek et al., 1996; Okajima et al., 1995). UKy catalyzes phosphoryl transfer to AMP almost as efficiently as to UMP and CMP (Li et al., unpublished observations).

AMP binds to UKy essentially with the same hydrogen bonding pattern as its binding to AKs (Figs. 6D and 7C) (Müller-Dieckmann and Schulz, 1995). The hydrogen bonds appear to be sufficient to discriminate against GMP and IMP as described earlier, but it is unclear why UKy catalyzes more efficient phosphorylation of UMP and CMP. Sequence comparison suggests that Ala-47, Ile-75, and Thr-81 in UKy are characteristic of UMP/CMP kinases. These substitutions make the NMP binding pocket of UKy slightly larger than those of AKs. It is surprising that UKy needs a larger pocket to accommodate the smaller pyrimidines of UMP and CMP. Molecular modeling has suggested that a water molecule may play a pivotal role in determining the substrate specificity (Müller-Dieckmann and Schulz, 1995). The larger volume is needed to hold both the water molecule and the base moiety of UMP or CMP.

The proposed water-mediated substrate specificity has been largely confirmed by the crystal structure of UKd in complex with AP_5U (Scheffzek et al., 1996). Indeed, there is only one hydrogen bond between the uracil moiety of AP_5U and the protein, namely the exocyclic keto group at position 2 of uracil to the backbone amide of Val-65 (Figs. 6E and 7D). There are no direct interactions between the uracil moiety and the side-chains of the enzyme. The endocyclic amino group and the exocyclic keto group at positions 3 and 4 are connected to the enzyme by a hydrogen bond network mediated by two water molecules (199 and 200). Since water molecules can serve as both hydrogen bond donors and acceptors, the hydrogen bond network is well suited to accommodate the cytosine moiety of CMP. Thus, the two water molecules appear to play an essential role in the recognition of UMP and CMP.

The conservation of Ala-37 and Thr-70 among the UKs may be rationalized because substitution of them with threonine and valine, as seen in AKs, would render the binding pocket too small to accommodate the two water molecules. Indeed, substitution of Thr-39 of AK1c (corresponding to Ala-37 of UKd) with alanine has caused a significant increase in the catalytic efficiency toward CMP and UMP as mentioned earlier (Okajima et al., 1993a). The reason for conservation of Ile-64 is unclear because the volume of isoleucine is the same as that of leucine. Substitution of Leu-66 of AK1c (corresponding to Ile-64 of UKd) with isoleucine has caused an increase in K_m for AMP by a factor of 17 but no increase in the catalytic efficiency for UMP or CMP (Okajima et al., 1993a). The result is consistent with the high K_m for AMP of UKy (Li et al., unpublished observations). Thus the isoleucine residue in UKs may play a role in discriminating against AMP. Indeed, for UKp, there was a significant increase (~20-fold) in the catalytic efficiency for AMP only when all these three residues were replaced by the corresponding residues of AKs (Okajima et al., 1996). Since UKd and UKp have an asparagine residue at the position corresponding to Gln-111 of UKy and both enzymes have a much lower activity for AMP than the yeast enzyme (Okajima et al., 1995; Scheffzek et al., 1996), the asparagine residue may also play a role in discriminating AMP.

Among NMP kinases, GKs have the highest specificity at the NMP binding site (Hall and Kuhn, 1986; Agarwal et al., 1978; Konrad, 1992; Li et al., unpublished observations). Besides GMP, among the NMPs tested, including AMP, UMP, CMP, IMP, and XMP, only IMP can serve as a phosphate acceptor for yeast GK. However, the catalytic efficiency decreases by a factor of $\sim 10^5$ when GMP is substituted by IMP (Li et al., unpublished observations).

The high specificity of GK is achieved with a NMP binding domain that is grossly different from those of other NMP kinases, as described earlier. The guanine moiety of GMP is fixed in the GMP binding domain by Ser-34, Glu-69, Ser-80, and Asp-100 in GKy (Figs. 4 and 7B). The hydrogen bonding pattern is similar to those observed in H-ras-p21 (Pai et al., 1990), elongation factor Tu (Berchtold et al., 1993), T4 deoxynucleotide kinase (Teplyakov et al., 1996), and ribonuclease T1 (Nonaka et al., 1993). All proteins have a critical carboxylate (aspartate or glutamate) that forms hydrogen bonds to both the endocyclic and exocyclic amino groups at positions 1 and 2 of guanine.

Rejection of AMP by GKy is obvious because AMP cannot have any of the hydrogen bonds shown in Figures 4 and 7B, and its exocyclic amino group may clash with the hydroxyl group of Ser-34. XMP is discriminated because of the electrostatic repulsion between the enolate anion at position 2 of XMP (pK_a ~5.5) and the carboxylates of Glu-69 and Asp-100. IMP is a very poor substrate because it lacks the exocyclic amino group at position 2 of GMP that forms hydrogen bonds to both Glu-69 and Asp-100.

The four residues at the guanine binding pocket all play important roles in GMP binding and catalysis (Table 1). Glu-69 is the most important to transition state stabilization. In fact, it is one of the most critical residues to the transition state stabilization by GKy. However, none of the mutants except those of Asp-100 shows any activity for other nucleotides. Substitution of Asp-100 with alanine has resulted in a detectable activity for XMP. The catalytic efficiency of D100A for XMP is comparable to that of the WT GK for IMP. Thus, Asp-100 plays an important role in substrate specificity, although it only makes a moderate contribution to catalysis by GK.

VI. Substrate-Induced Conformational Changes

A. CONFORMATIONAL CHANGES

Substrate-induced fit is believed to be the mechanism by which kinases avoid hydrolytic activity (Koshland, 1994). It has been suggested that the active centers of NMP kinases are assembled with large domain movements upon binding of both substrates (Müller-Dieckmann and Schulz, 1995). As NMP kinases generally follow a random Bi–Bi mechanism, they can exist at least in four forms: a free form, an NMP-bound form, an ATP-bound form and a ternary complex. No NMP kinase structures have been determined in all forms. Substrate-induced conformational changes were first identified by comparison of the various forms of homologous AKs (Schulz et al., 1990; Gerstein et al., 1993). Comparison of the crystal structures of the free AK1p with those of the AK3b·AMP complex and the AKe·AP_5A complex has indicated that both ATP and AMP induce substantial conformational changes upon binding to the enzymes. Binding of AMP results in the closure of the NMPbind domain, whereas binding of ATP causes the closure of the LID domain. The induced conformational changes mainly involve hinge motions. The results have been extended by analysis of 17 crystal structures of homologous AKs (Vonrhein et al., 1995). Interpolation of the structural differences has created a movie showing possible substrate-induced motions.

Although comparison of the structures of homologous proteins is informative, the analysis is complicated by many sequence differences and therefore necessarily coarse. Recently, the crystal structure of an AKy mutant (D89V, R165I) in complex with an ATP analogue (AMPPCF_2P) has been determined at high resolution (Schlauderer et al., 1996). Comparison of the structure of the mutant with that of the wild-type AKy·AP_5A complex suggests that binding of ATP induces the closure of the LID domain. However, the LID domain is not completely closed as revealed in the crystal structure of the wild-type AKy·AP_5A complex. It is not surprising because binding of the second substrate may induce additional conformational changes. The difference may also be due to the mutations (especially R165I) and/or the difference between ATP and AMPPCF_2P. The NMPbind domain is apparently in an "open" state. It is unclear whether binding of ATP induces any movement of the NMPbind domain at all because the structure of the free form is unknown. Determination of the crystal structure of the free AKe has made it possible for detailed structural comparison of the free form and the ternary complex of the same enzyme (Müller et al., 1996b). The results show that the closure of the LID domain is well described by rigid body movements. However, the conformational changes in the NMPbind domain is caused by not just a movement of the domain as a whole but also a change of the angle between helices α2 and α3 (Fig. 8A).

Substrate-induced conformational changes have also been studied by energy transfer measurements. Using the steady-state energy-transfer technique, Bilderback et al. (1996) measured the distances from fluorescence donors at positions 41, 86, 133 and 137 to an acceptor at position 77 of AKe. The results indicate that either ATP or AMP can induce a partial closure of the LID domain. However, most of the distances measured by this study are inconsistent with the crystal coordinates of the free AKe and the AKe·AP_5A complex. The large differences (8–17 Å) are probably the result of errors in the steady-state energy transfer measurements and/or conformational changes caused by mutagenesis and addition of the fluorescence probe.

Using the method of time-resolved energy transfer, Sinev and colleagues have measured the distances of pairs of fluorescence donors and acceptors at residues 169/55 (Sinev et al., 1996a) and 73/142 (Sinev et al., 1996b) in AKe. The distance between the donor and acceptor at residues 169/55 is shortened by approximately 8 Å upon binding of MgATP, by about 12 Å upon binding of AMP, and by about 20 Å upon binding of AP_5A. The distances in the free form and the AP_5A complex measured by the time-resolved energy transfer experiments are in remarkable agreement with those

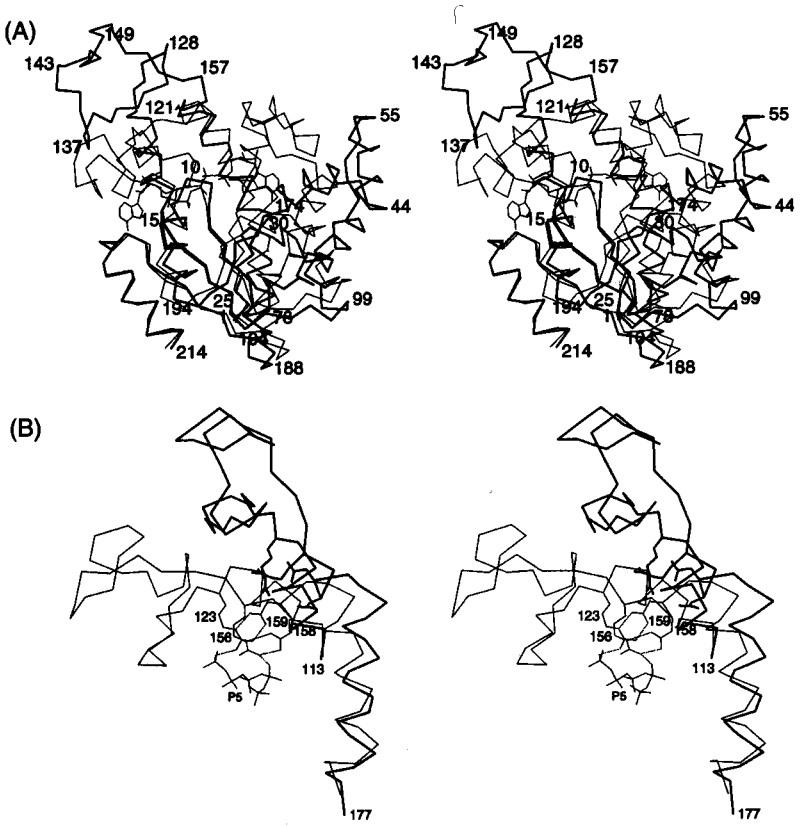


Figure 8. **(A)** Superposition of the C_α traces of the free AKe (Müller et al., 1996b) *(thick line)* and the AKe·AP_5A complex (Müller and Schulz, 1992) *(thin line)* showing the differences in the conformations of the NMPbind and LID domains. **(B)** Stereo view of C_α traces of the LID domain in the free AKe *(thick line)* and the AKe·AP_5A complex *(thin line)*, showing the rearrangement of the side chains of Arg-123, Arg-156, Asp-158, and Asp-159 upon binding of AP_5A (represented by the polyphosphate). Adapted from Müller et al. (1996b).

derived from the corresponding crystal coordinates. Because residue 169 moves only about 1 Å upon binding of AP_5A and residue 55 is at the tip of the NMPbind domain (Müller and Schulz, 1992; Müller et al., 1996b), the results suggest that binding of either AMP or MgATP can induce substantial movement of the NMPbind domain, although the movement induced by AMP is larger. For the pair of the fluorescence probes, the movement induced by binding of AP_5A is approximately the sum of the movements induced by binding of AMP and MgATP. The distance between the donor and acceptor at residues 73/142 is shortened by approximately 9 Å upon binding of either MgATP or AMP and ~12 Å upon binding of AP_5A. The measured distances are also in line with those derived from the crystal coordinates of the free AKe and AKe·AP_5A. The results indicate that both MgATP and AMP induce a movement of the LID domain upon binding to AKe and the LID domain movements induced by MgATP and AMP are approximately of the same magnitude. Binding of the second substrate causes an additional movement of the domain.

B. DYNAMICAL CHANGES

Assuming that crystallographic B-factors reflect molecular motions, Müller et al. have noted significant changes in the mobility of AKe at the free and AP_5A-bound states (Müller et al., 1996b). At the free state, the LID and NMPbind domains are rather mobile while the rest of the molecule is relatively well fixed. Upon binding of AP_5A, the LID and NMPbind domains are stabilized, and the loops $\alpha4$–$\beta3$ and $\alpha5$–$\beta4$ are mobilized. The mobility of the loops is proposed to be an energetic counterweight that keeps the ternary complex from dropping into an energy well. This is an interesting proposal. However, changes in the B factors could be due to static disorders. It needs to be further investigated by other means such as NMR relaxation measurements. The stabilization of the NMPbind domain by binding of the substrates and the bisubstrate analogue AP_5A is supported by the time-resolved energy transfer study as described earlier (Sinev et al., 1996a). The full width at half-maximum of the interprobe distance distribution between the fluorescence probes at positions 169 and 55 is 29 Å. It decreases to 23, 14, and 11 Å upon binding of MgATP, AMP, and AP_5A, respectively. The results indicate that the NMPbind domain becomes less flexible upon binding of the substrates and the bisubstrate analogue.

C. KEY RESIDUES IN INDUCED FIT

While the structures and dynamics of different conformational states can be learned from biophysical analysis, it is difficult to deduce which residues are critical for the substrate-induced fit mechanism. X-ray crystallographic studies have suggested that Arg-123, Arg-156, Asp-158, and Asp-159 (AKe numbering) may be important for the closure of the LID domain (Müller and Schulz, 1992; Müller-Dieckmann and Schulz, 1995; Müller et al., 1996b). It has been proposed that the rearrangement of the residues upon binding of

MgATP triggers the movement of the LID domain (Fig. 8B). Although this is an attractive proposal, it is difficult to test. The best approach will be to make site-directed mutants at these positions and to determine the structures of the free mutant enzymes, the binary and the ternary complexes. If a residue is involved in the substrate-induced fit mechanism, the structure of the mutant in the binary and the ternary complexes would be the same as that of the free form. The difficulty with this approach lies in the structural analysis. X-ray crystallography has the best resolution for structure determination, but no NMP kinases have been crystallized in all four forms as mentioned earlier. NMR is another powerful technique for structure determination. However, the size of any NMP kinase (>20 kDa) is at the cutting edge for structure determination by NMR. While the total resonance assignment and secondary structures for the AKc·MgAP$_5$A complex have been reported in 1993 (Byeon et al., 1993), the tertiary structure is still being refined to improve the resolution (I.-J. Byeon and M.-D. Tsai, unpublished observations). As demonstrated by Sinev et al. (1991), fluorescence energy transfer could be a good alternative to X-ray and NMR methods for characterization of substrate-induced conformational changes. However, the technique can only measure the distance between a pair of fluorescence probes and can not provide structural information on other residues. Furthermore, the analysis may be complicated by additional mutations and fluorescence labeling.

Even if the structures of the mutants at different stages of catalysis can be determined, a fundamental problem remains: the induced fit mechanism may involve a large number of residues and cannot be disrupted by single or double mutations. In our view, the conformational change and catalysis are integrated processes and some residues may play both functions. Dissecting the conformational changes and the catalytic steps may be difficult or impossible to accomplish. However, any knowledge gained in this effort will be valuable in the in-depth understanding of enzyme catalysis. The roles of Arg-132, Arg-138, Asp-140, and Asp-141 of AK1c (corresponding to Arg-123, Arg-156, Asp-158, and Asp-159 of AKe) in catalysis and substrate-induced fit have been studied by site-directed mutagenesis in conjunction with NMR (Yan et al., 1990b; Dahnke et al., 1992; Dahnke and Tsai, 1994). On the basis of the kinetic and NMR data of the single and double mutants, it is concluded that these residues are directly or indirectly involved in transition state stabilization. Their roles in substrate-induced fit are uncertain.

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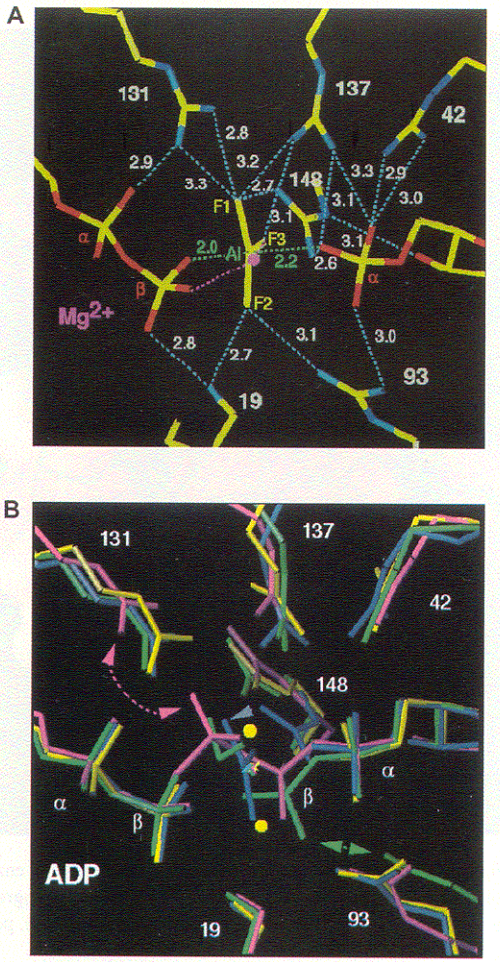

Yan and Tsai, Figure 5. **(A)** Active site of UKd in complex with MgADP, CMP, and AlF_3 showing the distances of the phosphates and AlF_3 to the surrounding arginines and lysine. The magenta sphere represents the bound magnesium ion. **(B)** Overlay of the active sites of the various UKd complexes: UKd·MgADP·CMP *(yellow line)*, UKd·MgADP·CMP·AlF_3 *(cyan line)*, UKd·MgADP·UDP·BeF_2 *(green line)*, and UKd·$MgAP_5U$ *(magenta line)*. The crosses represent magnesium ions. Water molecules are shown as *yellow spheres*. The different position of Arg-131 in the $MgAP_5U$ complex is due to the steric hindrance caused by the bridging phosphate as indicated by the magenta arrow. The different position of Arg-93 in the MgADP·UDP·BeF_2 complex is due to the steric hindrance caused by the presence of β-phosphate at the NMP site *(green arrow)*. From Schlichting and Reinstein (1997).

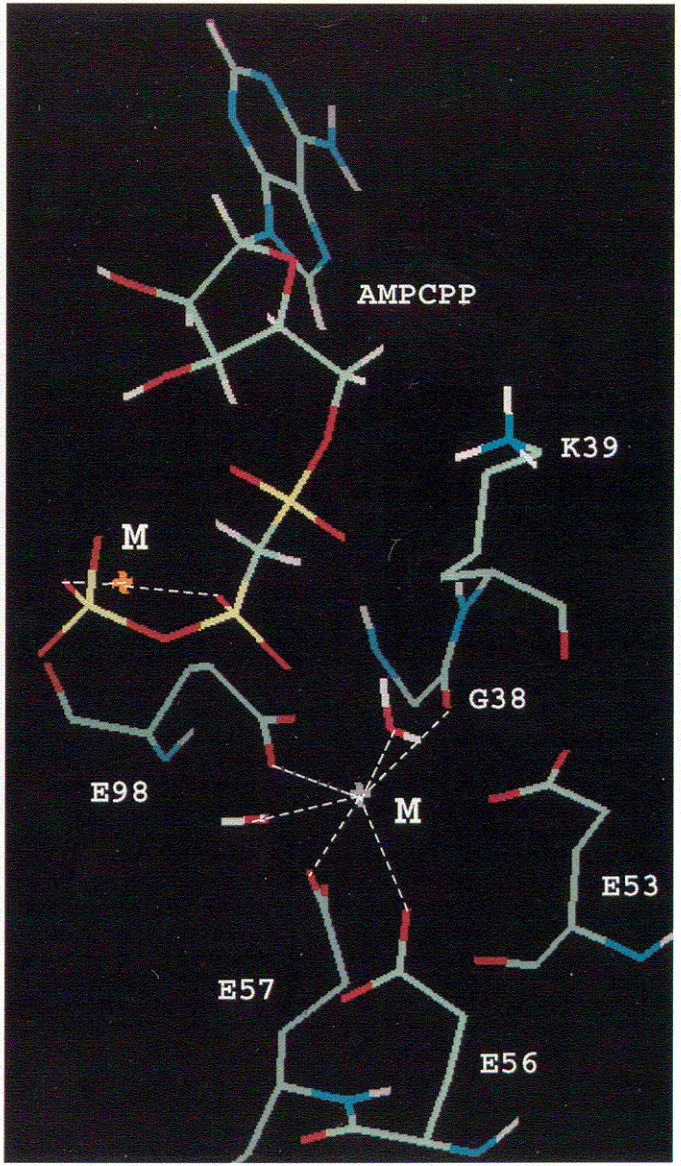

Figure 10. Reaction center of the MutT enzyme from the lowest-energy structure of the quaternary complex, showing residues and water molecules that interact with the enzyme-bound metal and the triphosphate group of M–AMPCPP. The water ligand *cis* to Gly-38 and near both Pβ and Glu-53 is well positioned to be the attacking nucleophile (Lin et al., 1997).

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ENZYMOLOGY OF NAD$^+$ SYNTHESIS

By GIULIO MAGNI, ADOLFO AMICI, MONICA EMANUELLI, and NADIA RAFFAELLI, *Istituto di Biochimica, Facoltà di Medicina, Università di Ancona, Italy,* and SILVERIO RUGGIERI, *Dipartimento di Biotecnologie Agrarie ed Ambientali, Facoltà di Agraria, Università di Ancona, Italy*

CONTENTS

I. Introduction

The importance of the role of NAD$^+$ in living organisms has grown increasingly since its function as a coenzyme in redox processes was fully

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