# Crystal Structure of the Complex of UMP/CMP Kinase from *Dictyostelium discoideum* and the Bisubstrate Inhibitor $P^1$-(5′-Adenosyl) $P^5$-(5′-Uridyl) Pentaphosphate ($UP_5A$) and $Mg^{2+}$ at 2.2 Å: Implications for Water-Mediated Specificity‡

Klaus Scheffzek,§,|| Werner Kliche,§ Lisa Wiesmüller,§,⊥ and Jochen Reinstein*,||

*Max-Planck-Institut für Medizinische Forschung, Abteilung Biophysik, Jahnstrasse 29, 69120 Heidelberg, Germany, and Max-Planck-Institut für Molekulare Physiologie, Rheinlanddamm 201, 44139 Dortmund, Germany*

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ABSTRACT: The three-dimensional structure of the UMP/CMP kinase (UK) from the slime mold *Dictyostelium discoideum* complexed with the specific and asymmetric bisubstrate inhibitor $P^1$-(5′-adenosyl) $P^5$-(5′-uridyl) pentaphosphate ($UP_5A$) has been determined at a resolution of 2.2 Å. The structure of the enzyme, which has up to 41% sequence homology with known adenylate kinases (AK), represents a closed conformation with the flexible monophosphate binding domain (NMP site) being closed over the uridyl moiety of the dinucleotide. Two water molecules were found within hydrogen-bonding distance to the uracil base. The key residue for the positioning and stabilization of those water molecules appears to be asparagine 97, a residue that is highly specific for AK-homologous UMP kinases, but is almost invariably a glutamine in adenylate kinases. Other residues in this region are highly conserved among AK-related NMP kinases. The catalytic $Mg^{2+}$ ion is coordinated with octahedral geometry to four water molecules and two oxygens of the phosphate chain of $UP_5A$ but has no direct interactions with the protein. The comparison of the geometry of the $UK_{dicty}$·$UP_5A$·$Mg^{2+}$ complex with the previously reported structure of the $UK_{yeast}$·ADP·ADP complex [Müller-Dieckmann & Schulz (1994) *J. Mol. Biol. 236*, 361−367] suggests that $UP_5A$ in our structure mimics an ADP·Mg·UDP biproduct inhibitor rather than an ATP·Mg·UMP bisubstrate inhibitor.

Nucleoside monophosphate kinases (ATP·NMP phosphotransferases) catalyze the reversible transphosphorylation between nucleoside triphosphates and nucleoside monophosphates (NMP) according to the scheme:

$$\text{ATP·Mg} + \text{NMP} \rightleftharpoons \text{ADP·Mg} + \text{NDP}$$

They play a major role in the regulation of the level of nucleoside diphosphates in the cell that are important in a variety of metabolic processes (Noda, 1973). The best studied enzyme of this class is adenylate kinase (EC 2.7.4.3, AK)[1] which catalyzes the phosphoryl transfer from the donor ATP·Mg to the acceptor molecule AMP. NMP kinases are globular enzymes with a typical $\alpha/\beta$-fold of eight to nine α-helices that surround a five-stranded parallel $\beta$-sheet which constitutes the rigid core (CORE domain) of this otherwise flexible protein. Adenylate kinases undergo large domain movements upon substrate binding (Vonrhein et al., 1995). These conformational changes mainly involve the highly conserved phosphate binding loop (P-loop), the binding domain for the acceptor nucleoside monophosphates (NMP bind), and a domain that closes like a lid over the phosphate chains of the substrates (LID) and carries many of the catalytically important residues.

The presence of $Mg^{2+}$ is essential for the phosphoryl transfer reaction but not for the binding of ATP or ADP at the donor nucleotide binding site (Noda, 1958; Reinstein et al., 1990b). The NMP binding site of AK has a higher specificity for adenine nucleotides (as acceptor molecules) than the donor site that binds ATP·Mg but also various other nucleoside triphosphates (NTP) (Noda, 1973). In contrast, UMP/CMP kinase from the slime mold *Dictyostelium discoideum* (EC 2.7.4.14, $UK_{dicty}$) that shares up to 41% sequence homology with known adenylate kinases accepts both UMP and CMP as the acceptor species (Wiesmüller et al., 1990) and thus belongs to the group of pyrimidine monophosphate kinases. We are interested in the specificity of $UK_{dicty}$ in relation to other monophosphate kinases such as adenylate kinase (AK), guanylate kinase (GK), or thymidylate kinase (TmpK), and we therefore need to investigate the architecture of the active center, in particular, the monophosphate binding site. The structure of UMP kinase from yeast ($UK_{yeast}$) has been solved recently (Müller-Dieckmann & Schulz, 1994). However, comparative complementation analysis of enzymatic activity indicated that

‡ The coordinates were deposited in the Brookhaven Protein Data Bank with the accession code 1UKD.

* To whom correspondence should be addressed. Tel: (0231)-1206366. Fax: (0231)-1206229. E-mail: joachim.reinstein@mpi-dortmund.mpg.de.

§ Max-Planck-Institut für Medizinische Forschung.

|| Max-Planck-Institut für Molekulare Physiologie.

⊥ Present address: Heinrich-Pette-Institut für experimentelle Virologie und Immunologie an der Universität Hamburg, Martinistrasse 52, 20251 Hamburg, Germany.

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[1] Abbreviations: UK, UMP/CMP kinase in general (EC 2.7.4.14); $UK_{dicty}$, UMP/CMP kinase from *Dictyostelium discoideum*; $UK_{yeast}$, UMP/CMP kinase from *Saccharomyces cerevisiae*; AK, adenylate kinase in general (EC 2.7.4.3); $AK_{eco}$, adenylate kinase from *Escherichia coli*; $AP_5A$, $P^1$, $P^5$-(5′-diadenosyl) pentaphosphate; $UP_5A$, $P^1$-(5′-adenosyl) $P^5$-(5′-uridyl) pentaphosphate.

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$UK_{yeast}$ has different properties than $UK_{dicty}$ since it accepts AMP almost as well as UMP or CMP as the phosphate acceptor. It is therefore much less specific for pyrimidine nucleotides (Schricker et al., 1992). The crystallized $UK_{yeast}$ contained two adenine nucleotide molecules and not just one ATP or ADP at the donor site, as would be expected for a kinase that is highly specific for pyrimidines at the acceptor site. It thus appears that the enzyme referred to as UMP kinase from yeast rather represents a nonspecific NMP kinase.

Recently, the amino acid sequence of another UMP/CMP kinase from porcine brain was reported that shares a higher homology with $UK_{dicty}$ than $UK_{yeast}$ and also like $UK_{dicty}$ has a higher specificity for UMP and CMP versus AMP (Okajima et al., 1995).

To understand better the structural basis of pyrimidine nucleotide specificity of this AK-related enzyme we have crystallized $UK_{dicty}$ (Wiesmüller et al., 1995) with its specific bisubstrate inhibitor $P^1$-(5′-adenosyl) $P^5$-(5′-uridyl) pentaphosphate ($UP_5A$) and report here the structure at a resolution of 2.2 Å. $UP_5A$ was shown recently by fluorescence measurements to bind 50 times tighter than $AP_5A$ (Wiesmüller et al., 1995), the bisubstrate inhibitor that is specific for adenylate kinases (Lienhard & Secemski, 1973; Feldhaus et al., 1975). This is the first structure of a NMP kinase with an asymmetric bisubstrate inhibitor, and therefore the assignment of binding sites does not necessitate comparisons to structures of homologous kinases. The model is in agreement with the previous assignments for the location of the binding sites of ATP and NMP, respectively. The detailed information of the coordination of the phosphates relative to catalytic amino acid residues and specifically the catalytic $Mg^{2+}$ ion allows us to draw some conclusions on the mechanism of phosphoryl transfer and specifically how the simultaneous specificity of UMP/CMP kinase for UMP *and* CMP as phosphate acceptors may be achieved.

## MATERIALS AND METHODS

*Crystallization, Data Collection, and MIR Analysis.* Crystals of $UK_{dicty}$ complexed with $UP_5A$ were grown as described (Wiesmüller et al., 1995). Since molecular replacement searches carried out with several AK models were not successful, the structure was determined by the multiple isomorphous replacement (MIR) method. Data sets of crystals mounted in thin-walled glass or quartz capillaries were collected using an oscillation camera with a Siemens/Nicolet area detector and a GX-18 rotating anode (Elliott/Enraf-Nonius, Delft) with Francks double mirror optics as the X-ray source. Processing of diffraction data was carried out with XDS (Kabsch, 1993). Analyses of heavy atom derivatives and phase determination were done with a program suite written by W. Kabsch (unpublished). Statistical information for data collection of native and two heavy atom derivatized crystals used for phasing is summarized in Table 1. Fifteen heavy atom compounds were tested in 40 soaking experiments. The phasing statistics for a four-site heavy atom model derived from a mercury and a platinum derivative are given in Table 1.

*Structure Determination.* Solvent flattened MIR electron density maps calculated at 5 and 3 Å resolution, in space group $P4_32_12$, showed the basic features of known AK structures including the bisubstrate inhibitor. However, the

Table 1: Data Collection and Structure Determination

| | heavy atom compound | | |
|---|---|---|---|
| | native | $K_2HgI_4$ | $K_2PtBr_4$ |
| Crystal Space Group $P4_12_12$; $a = b = 78.5$ Å, $c = 101.5$ Å, $\alpha = \beta = \gamma = 90°$; 1 Molecule/Asymmetric Unit | | | |
| concn (mM) | | 0.2 | 5 |
| soaking time (h) | | 15 | 22 |
| data statistics | | | |
| max resolution (Å) | 2.2 | 3 | 3 |
| no. of reflections | 119529 | 18065 | 19841 |
| no. of unique reflections (all refl $>2\sigma$) | 16642/13025 | 5576/3745 | 6252/4070 |
| $R_{sym}$ (%)[a] | 7.5 | 10.7 | 9.4 |
| completeness (%; all refl $>2\sigma$) | 99.6/78.0 | 81.4/54.7 | 91.3/59.4 |
| MIR | | | |
| $R_F$ (%)[b] | | 36.1 | 31.3 |
| resolution range (Å) | 15−5/15−3 | 15−3 | 15−4 |
| no. of sites | | 2 | 2 |
| $R_C$ (%)[c] | | 56.2 | 49.9 |
| $F_{H/E}$[d] | | 1.6 | 1.8 |
| $\langle m \rangle$ (mean figure of merit) of map (15−5 Å/15−3 Å) | 0.64/0.32 | | |

[a] $R_{sym} = \sum_h \sum_i |I_{hi} - I_h| / \sum_h I_{hi}$, where $I_{hi}$ is the scaled intensity of the $i$th symmetry-related observation of reflection $h$ and $I_h$ is the mean value. [b] $R_F = 2\sum_h |F_{PH} - F_P| / \sum_h |F_{PH} + F_P|$, where $F_{PH}$ and $F_P$ are the derivative and native structure amplitudes. [c] $R_C = \sum_h |F_{PH,obs} - F_{PH,calc}| / \sum_h |F_{PH,obs} - F_P|$. [d] $F_H/E = [f_H^2 / \sum (F_{PH,obs} - F_{PH,calc})^2]^{1/2}$; $f_H 2$ are the heavy atom scattering factors.

$AK1_{pig}$ model (1adk) could be accommodated only after its inversion through the origin of the coordinate system. Reprocessing of the heavy atom derivative data using the symmetry of the enantiomorphous space group $P4_12_12$ allowed us to position the $C_\alpha$ backbone of $AK1_{pig}$ (Dreusicke et al., 1988) correctly in the resulting solvent-flattened MIR map. The uridyl- and adenylyl part of the inhibitor could be distinguished clearly in this and subsequent maps at higher resolution, indicating that the crystalline enzyme was indeed complexed with $UP_5A$. It was immediately clear that $UK_{dicty}$ was in the closed conformation, with helices α3 and α4 (AK1 numbering) being in contact with the uridine part of the nucleotide.

A starting model of UK was established with the program O (Jones et al., 1991) using the $C_\alpha$-backbone of $AK1_{pig}$ as a template for chain tracing in a 3 Å solvent flattened MIR map. Refinement of this model with X-PLOR 3.1 (Brünger, 1992) resulted in an *R*-factor of 38%, omitting residues for which electron density was ill-defined (1−6, 104−112, 131−137) and truncating all side chains except glycine to alanine if they could not be identified in the map. Heavy atom positions were found close to M54/M58 and M192 for the platinum and to C23 and M124 for the mercury compound. C23 is located in proximity of the phosphate binding loop and has been inferred as the target of the SH-blocking agent 5,5′-dithiobis(2-nitrobenzoic acid) which inactivates $UK_{dicty}$ and $AK1_{pig}$ but not $AK_{eco}$ in which the corresponding residue is an alanine (Wiesmüller et al., 1990). It might also be a target of mercury since several mercury compounds diminished the diffraction power of $UK_{dicty}$ crystals to zero within minutes after exposure, possibly by destroying the integrity of the nucleotide binding region.

As discussed previously (Wiesmüller et al., 1995) it was not possible to decide from the preliminary characterization of $UK_{dicty}$ crystals if there were one or two molecules in the asymmetric unit. Therefore, a molecular replacement search

was done using the program AMORE (Navaza, 1993) with the prerefined $UK_{dicty}$ coordinates as a search model; only one independent peak could be detected in the rotation function map. Further inspection of the electron density maps showed large regions of very low density, which is consistent with the high solvent content to be postulated for one $UK_{dicty}$ molecule per asymmetric unit and with the rotation function result. All model building and refinement were done with the programs O and X-PLOR 3.1, respectively, as referenced above. $2F_o - F_c$ and $F_o - F_c$ maps were calculated using combined MIR and model phases (program COMBINE, W. Kabsch).

To confirm the presence of the pyrimidine base, subsequent model building and refinement were done using only the ATP part of the dinucleotide as ligand coordinates. During the following 13 rounds of alternate crystallographic refinement and model building the resolution was gradually increased to up to 2.2 Å. Previously unclear residues were added during refinement, and the detailed structure of the uridylate moiety with uracil in an anti conformation became clear. In addition, a spherical density peak bridging the $\beta$- and $\gamma$-phosphoryl oxygens of the ATP could be detected which for reasons discussed below made us believe that $Mg^{2+}$ is present in the crystalline $UK_{dicty}$. ATP was now replaced by $UP_5A$ [which was built from the coordinates of the $AP_5A$ ligand in $AK_{eco}$ (Müller & Schulz, 1992)]. After another round of refinement we were able to model residues 105−112.

The *R*-factor then dropped to 24.3% for an atomic model comprising residues 2−194 of UK, $UP_5A$, and $Mg^{2+}$ using data between 8 and 2.2 Å. At this stage $F_o - F_c$ maps contoured at 40−60% of the maximum electron density were used to add water molecules in positions where at least one hydrogen bond to the protein could be inferred. Solvent molecules that did not reappear in subsequent $2F_o - F_c$ maps or had *B*-factors higher than 70 Å$^2$ after refinement were discarded from the model. The present model contains 43 water molecules, of which were found close to the $Mg^{2+}$ position. Together with oxygens of the $\beta$- and $\gamma$-phosphates of $UP_5A$ they establish an almost perfect octahedral coordination pattern, which confirms the assignment of the "bridging" density to $Mg^{2+}$. Electron density of the refined $UK_{dicty}$ model, which shows excellent geometry as indicated by the Ramachandran plot (Figure 3B) with almost all non-glycine residues with allowed $\Phi/\Psi$ combinations, is well-defined except for the side chains of several lysines (3, 5, 50, 60, 72, 106, 136, 146), E2, E53, F108, and residues 135−137 of the "LID" domain which is assumed to be flexible in the protein. A summary of the refinement results together with model statistics is given in Table 2.

Table 2: Structure Refinement and Model Statistics

| | |
|---|---|
| refinement | |
| resolution range (Å) | 8−2.2 |
| no. of unique reflections | 15781 |
| $R_{cryst}$ (%)[a] | 21.5 |
| $R_{free}$ (%)[a] | 27.6 |
| model | |
| $UK_{dicty}$ | residues 2−194 |
| $UP_5A$ | 1 |
| $Mg^{2+}$ | 1 |
| no. of water molecules | 43 |
| rms deviation from ideal geometry | |
| bond lengths (Å) | 0.009 |
| bond angles (deg) | 1.2 |
| average *B*-factor (Å$^2$) | 28 |
| estimated coordinate error (Å): | 0.3/0.27 |
| $\sigma_A$ (Read, 1986)/(Luzzati, 1952) | |

[a] $R_{cryst} = \sum_h |F_o - F_c| / \sum_h F_o$. $F_o$ and $F_c$ are the observed and calculated structure factor amplitudes for reflection *h*. 10% of the reflections were put aside for the calculation of $R_{free}$ and were not included in the refinement.

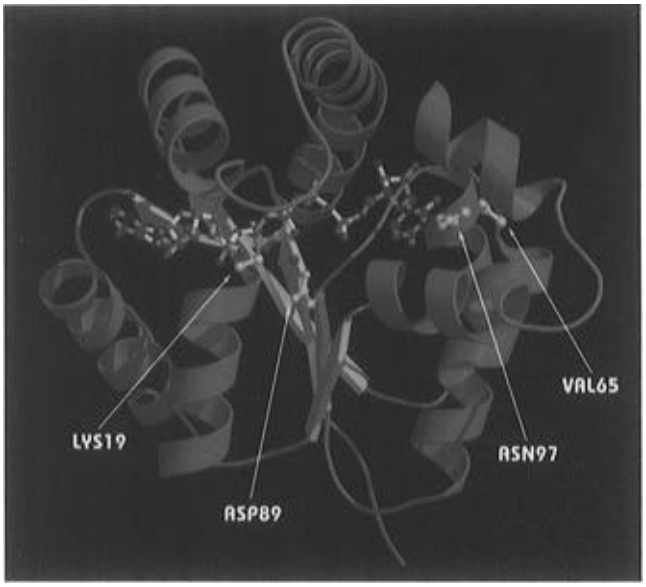


FIGURE 1: Ribbon diagram of the structure of UMP/CMP kinase generated with MOLSCRIPT (Kraulis, 1991). The five central parallel $\beta$-sheets of $UK_{dicty}$ are shown in yellow, and the eight $\alpha$-helices are shown in red. The bisubstrate inhibitor $UP_5A$ is shown with the ADP part on the left side and the UDP part on the right side. This standard orientation of displaying the structure of NMP kinases is comparable to those in Figures 4 and 6. The catalytic $Mg^{2+}$ ion is indicated in green, and four key residues are highlighted in bright yellow.

## RESULTS AND DISCUSSION

*General Description of the Structure.* UMP/CMP kinase from *D. discoideum* ($UK_{dicty}$) has 194 amino acid residues and a calculated molecular weight of 21 943. $UK_{dicty}$ belongs to the class of small NMP kinases like adenylate kinase from muscle cytosol (Schulz et al., 1974) with 194 residues (AK1) or guanylate kinase from *Saccharomyces cerevisiae* (Stehle & Schulz, 1992; Konrad, 1992) with 186 residues (GK). This is in contrast to representatives of the class of large NMP kinases like $AK_{eco}$ with 214 residues (Brune et al., 1985) or $AK_{yeast}$ with 220 residues (Tomasselli et al., 1986). In addition to residues 2−194 of $UK_{dicty}$, the positions of the bisubstrate inhibitor $UP_5A$, the catalytic $Mg^{2+}$, and 43 water molecules were identified at a resolution of 2.2 Å; the average *B*-factor is 28 Å$^2$. A summary of the model statistics is given in Table 2. The globular enzyme shows the known $\alpha/\beta$-fold of AKs and consists of eight $\alpha$-helices that surround a five -stranded parallel $\beta$-sheet (Figure 1) which constitutes the rigid core (CORE domain) of this otherwise flexible protein. A homology plot of $UK_{dicty}$, $UK_{pig}$, $UK_{yeast}$, $AK1_{pig}$, and $AK_{eco}$ is shown in Figure 2 and indicates the relative positions of conserved and important residues and also the position of the secondary structural elements of $UK_{dicty}$. The helices are labeled starting with helix $\alpha$2 to be compatible with the numbering system introduced for AK1 which has an additional short $\alpha$-helix at the N-terminus.

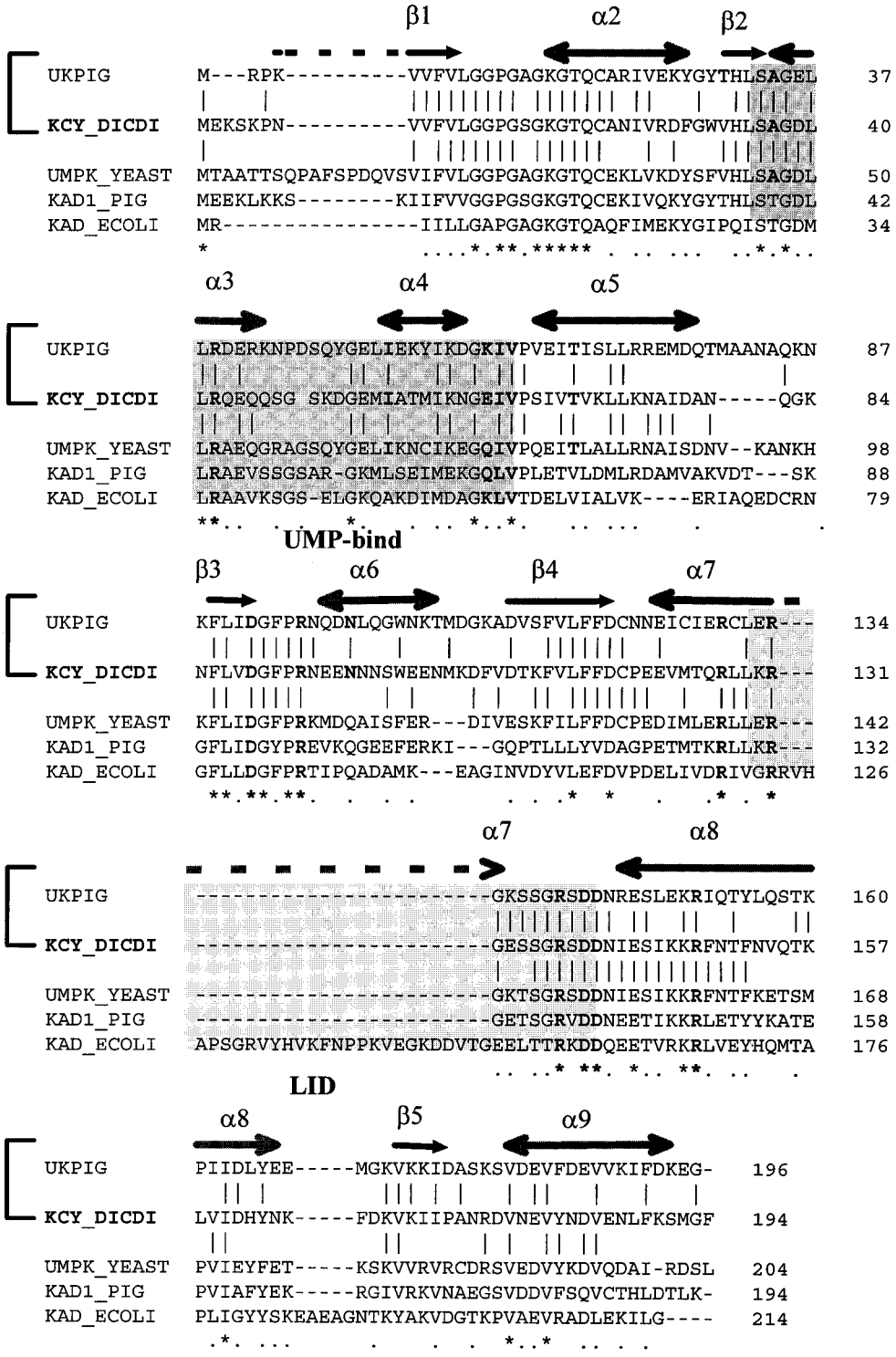


FIGURE 2: Homology plot of various NMP kinases. The amino acid sequences of $UK_{pig}$ (UKPIG), $UK_{dicty}$ (KCY_DICDY), $UK_{yeast}$ (UMP_YEAST), $AK1_{pig}$ (KAD1_PIG) and $AK_{eco}$ (KAD_ECOLI) were aligned with the program PCGene according to the algorithm of Higgins and Sharp (1988). The secondary structural elements of $UK_{dicty}$ as derived with the program DSSP (Kabsch & Sander, 1983) are indicated. Amino acid residues that are emphasized in the text because they are common in all NMP kinases and are important for the catalytic function or specificity of $UK_{dicty}$ are indicated by bold print. The regions of the highly flexible NMP binding domain and the catalytic LID region are underlined by shaded regions. The general homology of amino acid residues is indicated as follows: *, highly conserved residues; ·, conserved residues (conservative replacements); |, residues that are identical in $UK_{dicty}$, $UK_{pig}$, and $UK_{yeast}$. The secondary structural elements are indicated as follows: α-helix (↔) and β-sheet (→).

The overall structure of $UK_{dicty}$ resembles that of adenylate kinases (Dreusicke et al., 1988; Egner et al., 1987; Müller & Schulz, 1992; Diederichs & Schulz, 1991) specifically considering the CORE domain, but nevertheless molecular replacement with these known structures failed to assist the solution of the $UK_{dicty}$ structure as described in the Materials and Methods section. Only the structure of $UK_{yeast}$ (Müller-Dieckmann & Schulz, 1995) that is closest to $UK_{dicty}$ is apparently similar enough to allow molecular replacement which we included as a control, although the structure of $UK_{dicty}$ had been solved when the coordinates of $UK_{yeast}$ were released.

It has been shown that adenylate kinases undergo large domain movements upon substrate binding. A comparative

study on the structures of various NMP kinases with different occupancies at the substrate binding sites and different packing forces showed transitions from an open conformation in the ligand-free enzyme to a closed state with both substrates (mimicked by $AP_5A$) bound to the protein (Vonrhein et al., 1995). The conformational changes involve the NMP binding domain, essentially consisting of helices α3 and α4 (residues 36−65 in $UK_{dicty}$), and the LID domain which in $UK_{dicty}$ extends from residues 130 to 140 but is much longer in the large variants of NMP kinases like $AK_{eco}$. The additional sequence in the LID domain of the longer adenylate kinases is also called INSERT. Both parts are closed upon the substrate binding sites in the $AP_5A$-complexed $AK_{eco}$ and $AK_{yeast}$ (Schulz et al., 1990) as well as in $AK_{eco}$ complexed with AMPPNP and AMP (Berry et al., 1994). These movements have been summarized in a movie that represents an interpolation of different structures of NMP kinases (Vonrhein et al., 1995). Similar structural changes were observed in $UK_{yeast}$ complexed with two ADP molecules and essentially confirmed by our study of $UK_{dicty}$.

The structure of $UP_5A$ complexed to $UK_{dicty}$ confirms the current picture of domain movements in AK-like NMP kinases upon substrate binding (induced fit) (Schulz & Schirmer, 1979; Schulz et al., 1990; Reinstein et al., 1988); domain NMP is closed upon the UMP moiety of the bound dinucleotide in a similar way to that observed for the AMP group in $UK_{yeast}$ or $AK_{eco}$, and domain LID closes over the negatively charged phosphate groups of the bound nucleotides with a battery of highly conserved, positively charged arginines (Müller-Dieckmann & Schulz, 1994; Müller & Schulz, 1992). The high flexibility of these two functional domains is indicated by the *B*-factor plot in Figure 3A.

The LID domain has an orientation comparable to that observed in $UK_{yeast}$ and appears to be flexible in the crystals as concluded from high *B*-factors and weak electron density for corresponding residues. As a consequence, interactions of the conserved R137 that is part of the LID domain are not well-defined. This residue was shown to be a key catalytic residue in $AK1_{chick}$ (R138) and its function could not be replaced by the positively charged lysine (Yan et al., 1990). Two aspartate residues, namely D140 and D141 of $AK1_{chick}$ (D139 and D140 of $UK_{dicty}$) that are also part of the LID domain are believed to be important for transition state stabilization but not for substrate-induced conformational changes (Dahnke & Tsai, 1994). According to our structure, this would leave R131 as the prime candidate to be the trigger that causes closure of the LID domain over bound substrate since it interacts with oxygens of Pα and Pγ of the ATP binding site.

The N-terminal β-strand (starting at N7) is preceded by six residues which seem to be held in place by few interactions with a neighboring molecule. Increasing *B*-factors toward the first residue M1 which could not be modeled into the electron density suggest a high flexibility of this region. This first β-strand is connected with α2 via the highly conserved P-loop sequence (Saraste et al., 1991). Specifically, the lysine residue of this sequential and structural motif (K19 in $UK_{dicty}$) was recognized to be essential for the catalytic function of adenylate kinases, and its possible roles in phosphoryl transfer have been discussed (Reinstein et al., 1990a; Tian et al., 1990; Byeon et al., 1995).

The two tryptophan residues of $UK_{dicty}$ are located between α2 and β2 (W32) and in the C-terminal half of α6 (W101).

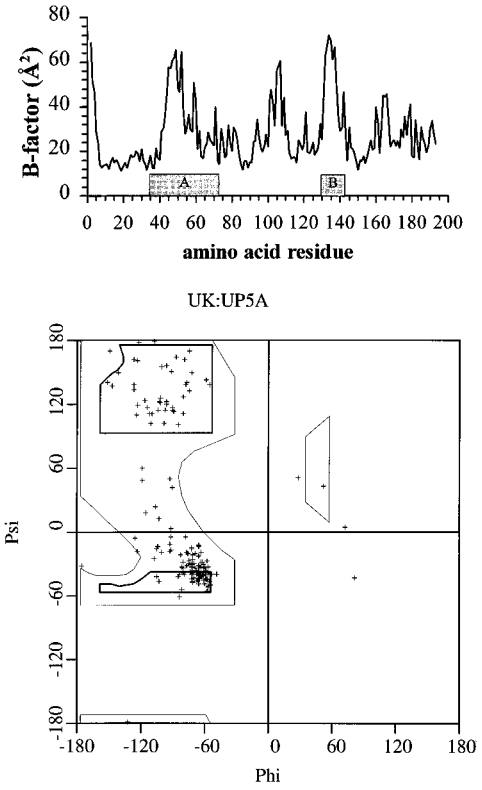


FIGURE 3: (A, top) Temperature factors (*B*-factors) as a function of the residue number for the $UK_{dicty}$·$UP_5A$ structure. The temperature factors and thus the mobility of parts of $UK_{dicty}$ are higher at the positions of the NMP (residues 36−65) and LID domains (residues 130−140). These domains are flexible and are involved in movements that are connected to the induced-fit mechanism of NMP kinases. (B, bottom) Ramachandran plot of the main-chain dihedral angles for the $UK_{dicty}$·$UP_5A$ structure at 2.2 Å resolution. Fully allowed Φ and Ψ angles are enclosed by thick lines; those only partially allowed are enclosed by thin lines. The Ramachandran plot shows that, except for the proline and glycine residues, only 4 out of the 193 amino acid residues of $UK_{dicty}$ are outside the common regions of the dihedral angles Φ and Ψ.

The ring systems of both residues are far away from the aromatic ring systems of the bases of $UP_5A$, which suggests that changes in the intrinsic tryptophan fluorescence observed upon ligand binding represent structural rearrangements of the protein rather than direct interactions of the fluorescent residues with the substrate (unpublished results).

*ATP Site.* The active center is well-defined with many conserved arginines and residues of the conserved P-loop forming interactions with the 5 phosphoryl groups of $UP_5A$. Of special interest is the $NH_3$ group of K19 in $UK_{dicty}$ which interacts strongly with oxygens of P2 and P4 and is stabilized additionally by the main-chain carbonyl group of G14. The interaction pattern in the structure of $AK_{eco}$ differs markedly from this situation, such that the corresponding group is more than 5 Å from each of the P4 oxygen atoms.

The adenine base of the ATP part of $UP_5A$ is in the anti conformation as observed with many nucleotides bound to proteins. It is sandwiched by the guanidinium group of R127 with a hydrophobic contact to V178 on the opposite side of the base. The contact between the R127 side chain and the adenosine ring appears to represent a typical aromatic-charged group contact as described by Burley and Petsko (1986, 1988) because one of the two terminal amino groups of R127 (NH2) is located above the center of the six-membered ring of adenine with a distance of 3.5 Å. This

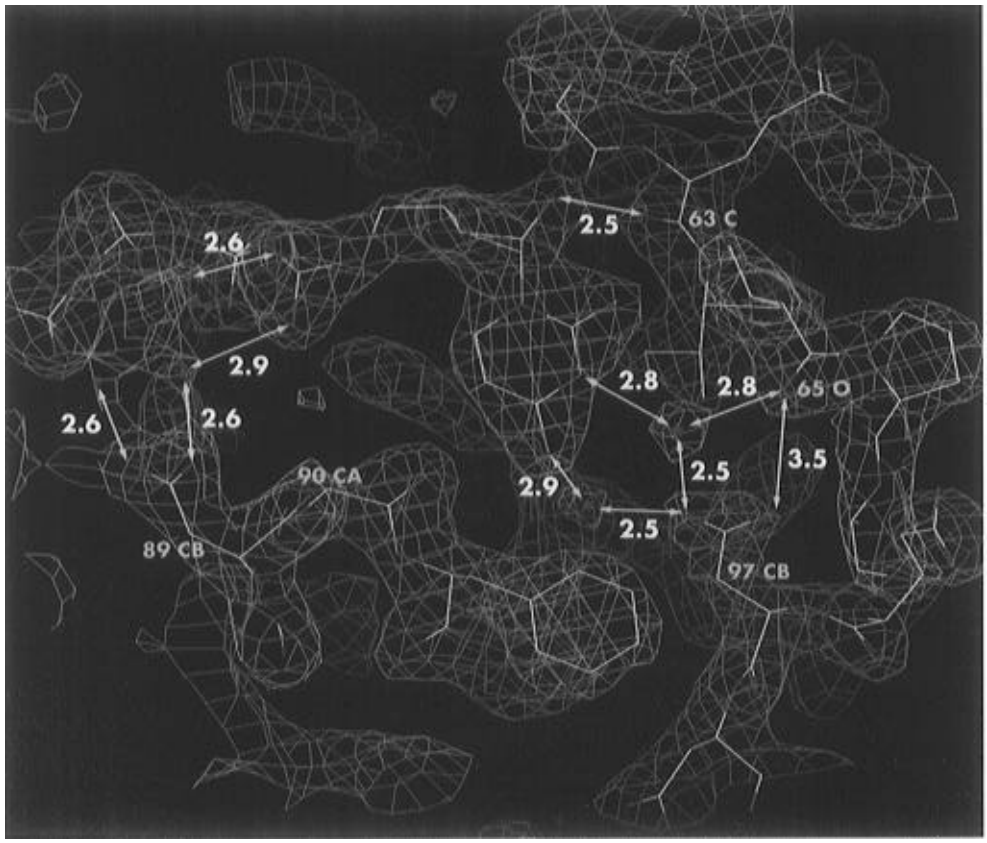


FIGURE 4: $2F_o - F_c$ electron density map of the UMP binding region, contoured at 15% of the maximum. The interactions of the two water molecules that presumably mediate specificity are emphasized by solid gray arrows that lead to N97 and V65 and to O2, N3, and O4 of uracil (see also Figure 5). The catalytic $Mg^{2+}$ ion (Mg196) is clearly visible with its well-defined density and octahedral arrangement of six interacting ligands, four of them being water molecules (Wat220, Wat227, Wat198, and Wat215) and the oxygen atoms O2 of P2 and P3 (see also Figure 5).

could explain why the adenosine base of $UP_5A$ is positioned despite the few interactions with the protein and thus gives reasonable electron density. Besides this interaction, the electron density map shows that except for the exocyclic amino group at C6 which is in hydrogen-bonding distance to the main-chain carbonyl oxygen of R176 only water-mediated interactions of the base atoms with the protein are observed with one water molecule also shared by the 3′-hydroxyl group of the ribose. There appear to be no aromatic residues that stack to either base, a situation that is found in thymidine kinase of herpes simplex virus where Y172 stacks against the thymine base (Brown et al., 1995). Few contacts with the base of the donor nucleotide (ATP) appear to be a common theme in NMP kinases and are consistent with the low specificity toward the donor nucleotide.

*Binding of UMP*. The UMP-part of $UP_5A$ is clearly surrounded by various residues of $UK_{dicty}$ that could form contacts with the base, ribose, and the phosphate (see Figure 4). The uracil base is in anti conformation and is located in a hydrophobic pocket that is formed by A37, L42, I64, V65, T70, F92, and R94 with a pronounced polar interaction of O2 with the backbone amide group of V65. Two water molecules with *B*-factors of 21 and 14 Å$^2$ are located close to the pyrimidine ring, and both contact the carboxamide group of N97. Wat200 is nearly in the plane of the aromatic ring and forms hydrogen bonds with the side chain of N97, the N3 H of uracil, the hydroxyl group of T70, and the carbonyl oxygen of V65. It is near the region occupied by the imidazole part of the adenine ring in $AK_{eco}$ (Müller & Schulz, 1988) or $UK_{yeast}$ (Müller-Dieckmann & Schulz, 1994). The second water molecule, Wat199, is below the plane of the ring when viewed in the standard orientation of AK (Schulz et al., 1974) and has a distance to Wat200 and R93 that also allows the formation of a hydrogen bond.

V65 contributes in three different ways to the binding of the base: it is part of the hydrophobic pocket, it interacts with the O2 oxygen atom of uracil via its amide nitrogen, and it stabilizes Wat200 by forming a hydrogen bond with its carbonyl oxygen. The ribose is in a 3′-endo conformation and shows the characteristic interactions of the main-chain carbonyl of E63 with the 2′-OH group. The 3′-OH group is also in contact with Wat212 that in turn interacts with the main-chain carbonyl group of I59 and the highly flexible D139 (domain LID), as has also been observed for the structures of $AK_{eco}$·AMPPNP·AMP (Berry et al., 1994), $AK_{eco}$·$AP_5A$ (Müller & Schulz, 1992), and also $UK_{yeast}$·ADP·ADP (Müller-Dieckmann & Schulz, 1994, 1995).

*Substrate Specificity.* The most interesting point is the question of how $UK_{dicty}$ achieves specificity toward the pyrimidine nucleotides UMP and CMP as acceptors, where UMP is mimicked by the UMP part of the bisubstrate inhibitor $UP_5A$ in the structure described here. To understand the specificity of $UK_{dicty}$, we compared its structure with the $UK_{yeast}$ model (1uky). However, the atomic model of the less specific $UK_{yeast}$ includes the purine nucleotides ADP or AMP bound at the NMP site (Müller-Dieckmann & Schulz, 1994). In an overlay done with the program O (Jones et al., 1991) 181 corresponding $C_\alpha$ atoms (which represent more than 90% of the residues in each molecule) were aligned to give an rms difference of 1.1 Å. In a shift

plot (not shown) only the region 80−110 shows marked differences from the situation in $UK_{yeast}$; there a similar conformation would lead to steric clashes with the N-terminus which comprises 12 residues more than in $UK_{dicty}$. However, both structures resemble the closed conformation of known NMP kinases. The adenine base at the NMP site of $UK_{yeast}$ is bound in a comparable way to that of $AK_{eco}$ (Müller & Schulz, 1992) or $AK3_{bov}$ (Diederichs & Schulz, 1991) where the carbonyl group of the highly conserved V76 ($UK_{yeast}$), the N3 of the base, Q111 (that is invariably present in all adenylate kinases), and N1 from the purine ring form characteristic contacts. The O2 atom of the uracil base interacts with the backbone amide of V65 of $UK_{dicty}$. This interaction is comparable to the V76/N3 contact of adenylate kinases. There are no direct contacts of uracil with side-chain atoms of the protein. The carbonyl group of the conserved glycine G90 is 3.4 Å apart from the O4 atom of the uracil base. If the base is cytosine, however, this contact could result in an additional albeit weak hydrogen bond with the amino group of cytidine and thus explain the slight preference (some 3-fold) of $UK_{dicty}$ for CMP (Wiesmüller et al., 1990).

The two water molecules that are described in the previous section appear to play a central role for the specificity of the NMP site of $UK_{dicty}$. They are part of a network where the aromatic N3 H proton, the O4 atom, the hydroxyl group of T70, and specifically the carboxamide group of N97 are connected. The presence of these well-located water molecules, Wat199 and Wat200, constitutes the most pronounced difference to the less specific $UK_{yeast}$ that accommodates purines at the NMP site (Müller-Dieckmann & Schulz, 1994; Schricker et al., 1992). Interestingly, all known adenylate kinases as well as $UK_{yeast}$ have the longer glutamine instead of asparagine at the position comparable to N97. The glutamine of AKs interacts with the exocyclic amino group and N1 of the adenine base and is highly conserved among adenylate kinases. It appears paradoxical that the shorter asparagine of $UK_{dicty}$ should increase the selectivity toward the smaller pyrimidines. We propose that N97 of $UK_{dicty}$ allows the localization and stabilization of Wat199 and Wat200 in a way a glutamine at the same position could not achieve. The binding of a purine base like adenine would, however, be highly energetically unfavorable since it would disturb the water−protein network of $UK_{dicty}$ and hence the positioned water molecules could provide specificity. The binding of purine to the NMP site of $UK_{yeast}$ is obviously not energetically unfavorable since the enzyme accepts AMP as well as UMP or CMP (Schricker et al., 1992). It would be interesting to see if according to our prediction the two positioned water molecules are also missing in a structure of $UK_{yeast}$ if it is cocrystallized with UMP or UDP. Another interesting aspect would be to see whether the $UK_{dicty}$ apoenzyme still contains these water molecules. This point could be clarified with a structure of the ligand-free $UK_{dicty}$ which we were not able to obtain yet due to crystallization problems.

An additional contribution to the stabilization of Wat200 is provided by the hydroxyl group of T70, whereas a valine that is present in the homologous position of many AKs would not tolerate Wat200. Some AKs, however, have threonine at this position, but this is obviously not sufficient to render the binding of purines unfavorable.

The observed Wat200 in $UK_{dicty}$ agrees with the suggestion of Müller-Dieckmann and Schulz derived from the ADP/AMP structure of $UK_{yeast}$ that a pyrimidine could be accommodated at the UMP binding site of $UK_{yeast}$ (Müller-Dieckmann & Schulz, 1995). In contrast to this proposal, however, a rotation of 180° of the carboxamide group would not be necessary to bind the O4 keto form of UMP. This is obvious from the pattern of interactions, specifically with Wat199 as indicated in Figure 5. A direct interaction of N97 (Q111 of $UK_{yeast}$) with O4 of the pyrimidine base appears to be unlikely with a distance of 3.9 Å. Instead, the orientation observed with the carboxamide group of N97 allows an additional stabilizing hydrogen bond of the amide proton to the carbonyl group of V65 besides fixing Wat199.

A particularly important aspect of the water-mediated specificity described above is the fact that this mechanism also explains how UMP and simultaneously CMP may be bound with high specificity although they have opposing hydrogen-bonding properties at positions 3 and 4 of the pyrimidine ring. UMP and CMP differ at these positions where UMP has an oxygen and thus a proton acceptor and CMP has an amino group and thus a hydrogen donor. U and C have thus complementary hydrogen-bonding properties at positions 3 and 4 of the pyrimidine ring whereas water molecules have both properties simultaneously. Since N97 can also switch to be either hydrogen bond acceptor or donor in dependence of its orientation, the water-mediated specificity of $UK_{dicty}$ provides a flexible way to accommodate CMP and UMP in the NMP binding site without loss in specificity.

A homology plot of various NMP kinases (see Figure 2) shows that A37 and I64 are specific for UMP kinases and are replaced by threonine/serine or leucine, respectively, in AKs. The structure of $UK_{dicty}$ indicates that a replacement of A37 with threonine would be sterically unfavorable if the conformation of the side chain would be comparable to that of AKs. It was shown recently, however (Bucurenci et al., 1996), that A37 can be replaced by threonine in $UK_{dicty}$ without a change in specificity of the enzyme. We therefore assume that the position of threonine in this mutated $UK_{dicty}$ must be different from the one observed with AKs. The specific presence of I64, however, could not be rationalized with our structure. It is also interesting to note that CMP kinases from *Escherichia coli*, *Mycobacterium leprae*, and *Bacillussubtilis* also have a threonine like AKs (Bucurenci et al., 1996). On the other hand, mutagenesis of the corresponding amino acids of $AK1_{chick}$ to alanine or isoleucine did indeed increase the specificity of the enzyme for pyrimidine nucleotides and decreased the AK activity (Okajima et al., 1991, 1993a, 1993b).

*Discrimination of Other NMP Substrates.* Substrate specificity involves the recognition of a particular substrate and the rejection of a structurally or chemically similar compound. Can we rationalize with the structure of $UK_{dicty}$ why the specificity constant ($k_{cat}/K_m$) of AMP is 250 lower than UMP and that of TMP (2′-deoxy-5-methyluridine monophosphate) is even some $10^4$ lower (unpublished results). As mentioned above, N97 appears to play a major role in the discrimination of AMP versus UMP since it is substituted by glutamine in all known adenylate kinases and plays a major role in positioning the water molecules which would interfere with the binding of a purine base. TMP is most likely excluded for two reasons. First, the lack of a 2′-hydroxyl group of the ribose does not allow for the

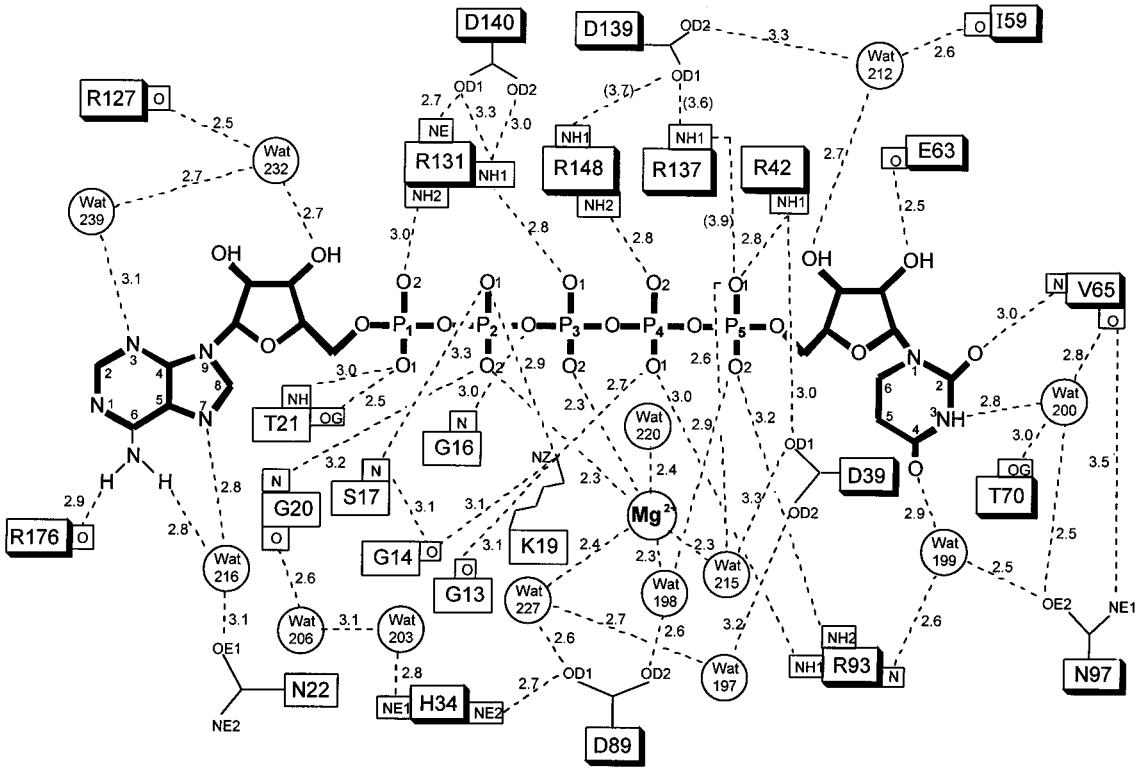


FIGURE 5: Distance map of the active center of $UK_{dicty}$ and the bisubstrate inhibitor $UP_5A$. The circled residues are water molecules, amino acid residues are boxed, and individual atoms are indicated by small boxes. The catalytic $Mg^{2+}$ ion is circled and in bold print. The part of $UP_5A$ with the adenosine moiety and P1, P2, and P3 that corresponds to ATP is on the left side. The UMP part of $UP_5A$ with P5 and the uridine moiety is on the right side. Two water molecules (Wat199 and Wat200) are positioned by N97 and presumably mediate the specificity for pyrimidine nucleotides (see text). Distances of the corresponding atoms are indicated by labels attached to the dashed lines connecting these atoms.

favorable interaction with the backbone carbonyl atom of E63, and second, the additional methyl group at position 5 of the ring system would sterically interfere with the methyl group of A37.

*$UK_{dicty}$ and the Binding of Pyrimidines by Other Proteins.* The three-dimensional structures of some proteins that bind pyrimidine nucleotides were described recently. The nature of the nucleotide binding sites seems to be determined by the particular requirements of the specific recognition process. The dUMP-specific thymidylate synthase has a conserved asparagine that acts as proton donor and acceptor for O4 and NH3 of the uracil base, respectively (Montfort et al., 1990). Uracil−DNA glycosylase has a similarly oriented asparagine, a histidine, and some main-chain amide groups that mediate the binding of uracil (Savva et al., 1995). Uracil is formed in DNA through the spontaneous deamination of cytosine. The excision of uracil by this enzyme requires a highly accurate recognition of uracil in DNA to exclude the removal of thymidine since otherwise highly abasic DNA strands would be formed. Besides the recognition of the 2′-OH group this specificity also excludes RNA as substrate since it contains uracil as a component. Nucleoside diphosphate kinase (NDP kinase), on the other hand, is not very specific for the acceptor nucleotide and accepts pyrimidine and purine nucleotides at the same site without forming many direct interactions with the purine bases (Cherfils et al., 1994).

*Phosphate and $Mg^{2+}$ Binding.* Since $Mg^{2+}$ is essential for the phosphoryl transfer reaction catalyzed by NMP kinases, special attention has been focused on the magnesium binding site which was identified in the structure of the $AK_{yeast}$·$AP_5A$ complex (Egner et al., 1987) with limited resolution and described recently in detail at a resolution of 1.96 Å (Abele & Schulz, 1995). The $Mg^{2+}$ ion in the $AK_{yeast}$ structure was reported to show distorted octahedral coordination geometry with one of the six ligands not being identified (Abele & Schulz, 1995). As the coordination pattern of $Mg^{2+}$ in the structure of the AK-related $UK_{dicty}$ has a pronounced octahedral character with all ligands present, however, it is doubtful that the distorted geometry observed in $AK_{yeast}$ is a special feature of adenylate kinases as stated by the authors (Abele & Schulz, 1995).

$Mg^{2+}$ is located between the second and third phosphate of $UP_5A$ (P2 and P3; see Figure 5) which mimics the $\beta$- and $\gamma$-phosphate of ATP and bridges the O2 oxygens (OS oxygens of P2 and P3; see also Figure 5). The octahedral coordination pattern of $Mg^{2+}$ with the phosphoryl groups and four water molecules as coordinating ligands could be inferred clearly from the electron density map; interestingly, no atoms of the protein are directly involved in this coordination. These ligands together with additional water molecules are part of a network of interactions which are supported mainly by D89 binding to Wat198 and Wat227 in a bidentate manner and by D39 which contacts the first coordination sphere of $Mg^{2+}$ directly (OD1−Wat215) and indirectly through a further water molecule (OD2−Wat197−Wat227). Both aspartate residues are involved in additional stabilizing interactions. In the case of D89 it is H34 (NE2) which also interacts with the carbonyl oxygen of G20 through a chain of two water molecules. D39 contacts the guanidinium group NH1 of R42 and the $\beta$-hydroxyl group of S36 thereby being held in a relatively fixed orientation. D39 is

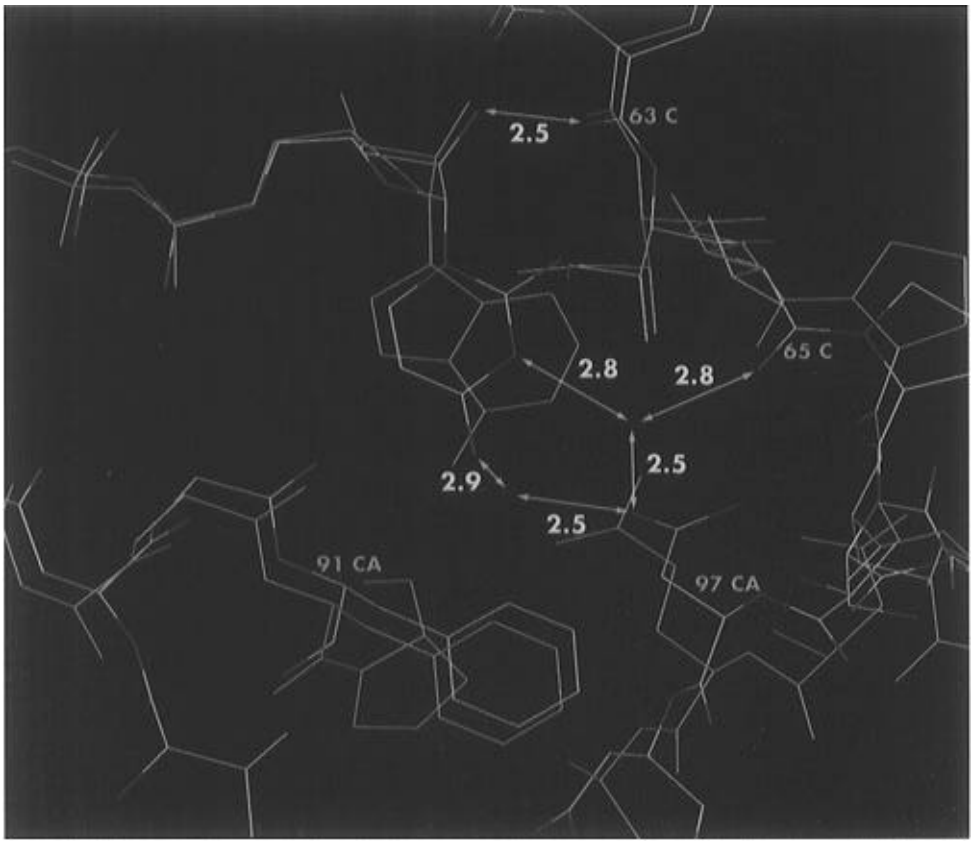


FIGURE 6: Overlay of the NMP binding regions of the $UK_{dicty}$·$UP_5A$ and $UK_{yeast}$·ADP/ADP structures (Müller-Dieckmann & Schulz, 1995). The structure of $UK_{dicty}$ and the NMP part of $UP_5A$ are colored according to atom types, and the ADP molecule bound at the NMP site of $UK_{yeast}$ and the NMP binding site of $UK_{yeast}$ are shown in uniformly light blue. Clearly visible are the two water molecules, Wat199 and Wat200, that are positioned by R93, V65, and N97. The latter residue is specific for UMP kinases but is a glutamine in UMP kinase from yeast and adenylate kinases. The comparison of the two structures indicates that the binding of purines should interfere with these water molecules of $UK_{dicty}$ and thus be unfavorable.

located at the N-terminus of helix α3, which together with α4 forms domain NMP that closes the NMP binding site when substrate is bound. Whether changes in the coordination sphere of $Mg^{2+}$ are involved in domain closure of the NMP region or opening when products are released remains to be determined.

The involvement of D39 and D89 in $Mg^{2+}$ coordination provides a structural rationale for their high degree of conservation in homologous kinases (Figure 2); it is replaced by the functionally comparable E in $UK_{pig}$. Furthermore, mutagenesis studies with AK1 from chicken showed that D89 (D93 in $AK1_{chick}$) is involved in $Mg^{2+}$ binding since a mutant protein with alanine at that position was reported to have a lower affinity for $Mg^{2+}$ (Yan & Tsai, 1991), but specifically $k_{cat}$ decreased by a factor of 650 whereas the affinity for nucleotides was practically unaltered with $K_d$ staying the same and $K_m$ increasing 4-fold. The authors concluded from these observations that $Mg^{2+}$ is mainly involved in orienting the phosphate groups and in stabilizing the transition state. The $Mg^{2+}$ coordination shell also interacts with the phosphoryl groups of the UMP moiety of the bound dinucleotide via two of its water molecules [Wat215−O1(OR), Wat198−O2(OS)]. This interaction may be important in helping to establish the proper geometry for the phosphoryl transfer reaction as described in more detail below.

The phosphate chain of $UP_5A$ mainly makes contacts with residues of the P-loop, namely, K19 as described before and conserved arginines (see also Figure 5). The residue R137 of the LID domain also makes contacts to the phosphates but has a rather high flexibility as indicated by poor electron density and high *B*-factors. We could not observe any water molecule that would be positioned properly for apical nucleophilic attack of the β-phosphate of the ATP part of $UP_5A$ by an in-line mechanism consistent with the low ATPase activity generally observed with NMP kinases.

Threonine 21 that is part of the P-loop of $UK_{dicty}$ appears to contact the OP1R oxygen atom of the ATP part of $UP_5A$. Kinetic studies with the diastereomers of ($R_p$)- and ($S_p$)-adenosine 5′-(1-thiotriphosphate) (ATPαS) (Shi et al., 1993) indicated that this residue contributes to catalysis presumably via hydrogen bond interaction. The authors of this careful study pointed out that this contribution was not apparent from site-directed mutagenesis studies and kinetic studies with ATP alone.

*What Structure Does the $Mg^{2+}$-Bound Conformation of $UP_5A$ Represent? General Implications for Models of Phosphoryl Transfer Mechanisms.* An interesting and important question is what the structure of $UP_5A$ in the $UK_{dicty}$·$UP_5A$·Mg complex represents. It would be most informative if $AP_5A$ or $UP_5A$, like complexes of myosin S1 and ADP·$AlF_4$ (Fisher et al., 1995) or the α-subunit of transducin with GDP·$AlF_4$ (Sondek et al., 1994), could show us the structure of the transition state. However, the following points need to be considered:

First, with the assumption that all AK-like NMP kinases share a common structure of the active site and therefore a common mechanism of enzyme-catalyzed phosphoryl transfer, there should be a common conformation of $AP_5A$ or

$UP_5A$ bound to the enzyme in the presence of $Mg^{2+}$ since there should be a common transition state. In contrast to this, the conformations of all bisubstrate inhibitor structures of AK-related NMP kinases solved until now are significantly different, with high variabilities in the positions of P3 and P4 relative to the consistent positioning of P1 and P2 (Pα and P$\beta$ of ATP) as well as P5 (Pα of NMP) (Abele & Schulz, 1995; this work). Second, the binding of $AP_5A$ and $UP_5A$ should be much tighter if $AP_5A$ or $UP_5A$ indeed did resemble transition state analogs. Instead, their tight binding can be accounted for solely by the entropic advantage that is gained by covalently linking the two substrates (Jencks, 1981; Reinstein et al., 1990b).

If one accepts that $AP_5A$ and $UP_5A$ do not represent the transition state, then what structures do they actually mimic?

There is no per se reason that a bisubstrate inhibitor should mimic the two substrate situation (ATP·NMP) rather than the two product situation (ADP·NDP), since the chemical equilibrium of the substrates and products of NMP kinases is close to unity in free solution and on the enzyme (Rhoads & Lowenstein, 1968; Reinstein et al., 1990b). Structural evidence that allows the assignment of the conformational state of the bisubstrate inhibitors may, however, be gained from overlays of structures of NMP kinase−bisubstrate inhibitor complexes with structures of the individual substrates.

In an overlay of the structures of $UK_{yeast}$ (Müller-Dieckmann & Schulz, 1994) and $UK_{dicty}$ (program O) the two ADP molecules of $UK_{yeast}$ superimpose well onto the corresponding parts of $UP_5A$ bound to $UK_{dicty}$. In this overlay, most of the residues have similar conformations in $UK_{dicty}$ and $UK_{yeast}$. One of the arginines at the active site, R142 (R131 in $UK_{dicty}$) together with its stabilizing D151 (D140 in $UK_{dicty}$), is close to the $\beta$-phosphates in $UK_{yeast}$. A similar position of the corresponding residues in $UK_{dicty}$ would be sterically hindered by the $\gamma$-phosphate (P3) of the ATP group in $UP_5A$. This superposition indicates that $UP_5A$ bound to $UK_{dicty}$ may represent the situation of ADP·Mg and UDP bound to $UK_{dicty}$ rather than ATP·Mg and UMP and would thus mimic a biproduct inhibitor.

On the other hand, the structures of $AP_5A$ complexed to $AK_{eco}$ (Müller & Schulz, 1988; Müller & Schulz, 1992) and AMP together with the nonhydrolyzable ATP analog AMP-PNP (Berry et al., 1994) superimpose well, indicating that $AP_5A$ in this case rather mimics a bisubstrate inhibitor. The apparently high variability of the structures of bisubstrate (or biproduct) inhibitors may reflect the equal likelihood of these two states being occupied. However, there might be a bias toward one direction that is mainly determined by arrangements caused by the additional bridging phosphate of the inhibitor or the presence of $Mg^{2+}$. Consistent with this interpretation is the variability of the catalytically important lysine residue of the P-loop (Reinstein et al., 1990a; Tian et al., 1990; Byeon et al., 1995); in $UK_{dicty}$ the amino group of K19 has distances of 2.8 Å to the oxygens of P2 and P4 whereas K13 in $AK_{eco}$ is more than 5 Å away from the oxygen atoms of P4.

*Mechanism of Phosphoryl Transfer.* Phosphoryl transfer of AKs has been shown to proceed via an in-line mechanism (Richard & Frey, 1978). Although the principal mechanisms of phosphoryl transfer were discussed extensively in excellent reviews (Sigel, 1990; Knowles, 1980), it is not widely recognized, unfortunately, that an in-line mechanism does not necessarily have to be associative as was often assumed with the phosphoryl transfer reaction of NMP kinases but could potentially also be dissociative.

The principal difference between these two mechanisms is that a dissociative mechanism of phosphoryl transfer has a small amount of bond formation to the incoming nucleophile and a large amout of bond cleavage to the outgoing leaving group. An associative mechanism, on the other hand, has a large amount of bond formation to the incoming nucleophile and a small amount of bond cleavage to the outgoing leaving group (Admiraal & Herschlag, 1995). The two mechanisms therefore differ in respect to charge accumulation during the reaction and therefore may require different strategies by which enzymes stabilize the transition state (Admiraal & Herschlag, 1995).

Model reactions for phosphoryl transfer specifically in the presence of $Mg^{2+}$ actually indicated that the phosphoryl transfer reaction has a dissociative character (Herschlag & Jencks, 1990; Admiraal & Herschlag, 1995), and further analysis of enzyme-catalyzed phosphoryl transfer also showed the mechanism to be dissociative in the case of alkaline phosphatase in the presence of $Zn^{2+}$ (Hollfelder & Herschlag, 1995). The main role of metal ions in this mechanism is to provide a template for the transition state that holds the reacting phosphoryl groups in place through electrostatic interactions. This does not mean that the transfer catalyzed by NMP kinases has to be dissociative, but it definitely requires that this mechanism is taken into account when models are derived specifically if a pure associative mechanism cannot satisfactorily explain kinetic or structural observations.

Detailed analysis of a high-resolution structure of the $Mg^{2+}$-bound form of CheY indicated that an associative phosphoryl transfer mechanism would be difficult to explain but a dissociative mechanism was in agreement with the experimental data (Stock et al., 1993).

In the structure of the $AK_{eco}$·$AP_5A$ complex (Müller & Schulz, 1992) one of the phosphate oxygens of AMP is reported to be almost in-line with P3 and O23 of the ATP corresponding part of $AP_5A$. The authors argue that this geometry, particularly the atoms surrounding the P3 (except the additional phosphate P4, OP4R, OP4S), is close to the postulated pentacoordinated transition state. A model of the transition state was constructed (Müller-Dieckmann & Schulz, 1995) on the basis of the $AK_{eco}$ and $UK_{yeast}$ structures, and slight deviations of the exact geometry of an in-line associative mechanism were explained by the missing $Mg^{2+}$ ion.

However, in our structure of $UK_{dicty}$ with $Mg^{2+}$ we do not see the corresponding atoms of $UP_5A$ in positions comparable to the situation in $AK_{eco}$; the P3 phosphate of $UP_5A$ is displaced (ca. 1.5 Å) with respect to that of $AP_5A$ in $AK_{eco}$, and we find even higher deviations from the proposed transition state geometry.

Besides the fact that we do not observe the geometry of in-line attack as described before (Abele & Schulz, 1995), a purely associative mechanism also appears to be in conflict with a reasonable explanation for the reverse reactions for the following reasons. If the mechanism were purely associative, then the attacked phosphate could be activated by electron-withdrawing groups like positive charges of basic residues (lysine or arginine) or $Mg^{2+}$—a mechanism that explains reasonably the transfer of P$\gamma$ of ATP to AMP as

described (Abele & Schulz, 1995). But the back-reaction is difficult to explain since now a $Mg^{2+}$ ion at the P$\beta$ of ADP at the ATP site would hamper the reaction if it were a pure associative mechanism, but presumably less if the phosphoryl transfer had a more dissociative character. In addition, it is also known that $Mg^{2+}$ is not accepted at the AMP site. This makes it highly unlikely that $Mg^{2+}$ accompanies the transferred phosphoryl group as suggested before (Abele & Schulz, 1995) on the basis of the $AK_{yeast}$·$AP_5A$·$Mg^{2+}$ complex in which space for this kind of movement appears to be present. In our structure, however, this position is occupied by the sixth ligand (Wat215), which is missing in the $AK_{yeast}$·$AP_5A$·$Mg^{2+}$ structure.

On the basis of the observations described above, we suggest that $Mg^{2+}$ together with the important lysine residue of the P-loop (K19 in the case of $UK_{dicty}$) is responsible for the positioning of the reacting phosphoryl groups. Additionally, $Mg^{2+}$ could provide a template for the transferred phosphoryl group that would have metaphosphate character for a more dissociative mechanism, and it appears likely to us that one of the four water molecules that form inner-sphere complexes with $Mg^{2+}$ could be replaced by coordination with one of the oxygens of this metaphosphate group. The same water molecule could potentially replace the coordination of $Mg^{2+}$ with O2 from P$\gamma$ that is formed in the ground state of the reaction but has to be broken at least when the product state is reached. This would provide a snug fit for the transition state of the phosphoryl transfer reaction with a well-positioned catalytic residue, a general feature that was described before to be important for enzymatic reaction in general (Jencks, 1975). This hypothesis is supported by an interesting correlation of the rate of ligand exchange of nucleotides with $Mg^{2+}$ and the fastest rates of phosphoryl transfer catalyzed by kinases that use $Mg^{2+}$ as a catalytic ion. The rate constant for ligand exchange of ADP·Mg was measured as 2500 $s^{-1}$ and with ATP it is 1200 $s^{-1}$ at 25 °C, (Eigen & Hammes, 1960), whereas one of the fastest kinases has a turnover rate of 1500 $s^{-1}$ (AK1). In this case the central step of the phosphoryl transfer reaction would be rate limiting as was observed before (Rhoads & Lowenstein, 1968).

To summarize, we believe that a model for the geometry of the transition state close to the one proposed for $AK_{eco}$ is difficult (if not impossible) to derive from our $Mg^{2+}$-bound structure since any complex with the bisubstrate inhibitor AP5A or $UP_5A$ represents rather the bisubstrate or biproduct state.

## CONCLUSIONS

The structure of UMP/CMP kinase may show an interesting way in which nature managed to convert a nucleotide binding site that was specific for purine nucleotides to a site that is specific for pyrimidines. This switch in specificity would only involve the change of basically one amino acid residue in order to bind and position two water molecules such that they interact favorably with pyrimidine bases in general but disfavor the binding of the bulkier purine bases.

In general, on the basis of the available structures of AK-related NMP kinases it is doubtful whether bisubstrate−inhibitors are suitable candidates for detecting transitions states or for deriving firm conclusions on the mechanism of phosphoryl transfer. Their conformations are likely to be the result of steric arrangements between the modified phosphate chain and the protein rather than of enzyme−substrate driven attempts of catalysis. It appears, however, that based on the available structures of NMP kinases and the fundamental experimental evidence on the phosphoryl transfer mechanism in free solution in the presence of $Mg^{2+}$ as well as for the enzyme-catalyzed reaction (Herschlag & Jencks, 1987; Admiraal & Herschlag, 1995; Hollfelder & Herschlag, 1995) a dissociative mechanism is an attractive model to explain how the forward and backward reactions are catalyzed by NMP kinases.

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