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Phosphotransferase and substrate binding mechanism of the cAMP-dependent protein kinase catalytic subunit from porcine heart as deduced from the 2.0 Å structure of the complex with Mn^{2+} adenylyl imidodiphosphate and inhibitor peptide PKI(5-24)

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The crystal structure of the porcine heart catalytic subunit of cAMP-dependent protein kinase in a ternary complex with the MgATP analogue MnAMP-PNP and a pseudosubstrate inhibitor peptide, PKI(5-24), has been solved at 2.0 Å resolution from monoclinic crystals of the catalytic subunit isoform C_A. The refinement is presently at an R factor of 0.194 and the active site of the molecule is well defined. The glycine-rich phosphate anchor of the nucleotide binding fold motif of the protein kinase is a β ribbon acting as a flap with conformational flexibility over the triphosphate group. The glycines seem to be conserved to avoid steric clash with ATP. The known synergistic effects of substrate binding can be explained by hydrogen bonds present only in the ternary complex. Implications for the kinetic scheme of binding order are discussed. The structure is assumed to represent a phosphotransfer competent conformation. The invariant conserved residue Asp166 is proposed to be the catalytic base and Lys168 to stabilize the transition state. In some tyrosine kinases Lys168 is functionally replaced by an Arg displaced by two residues in the primary sequence, suggesting invariance in three-dimensional space. The structure supports an in-line transfer with a pentacoordinate transition state at the phosphorus with very few nuclear movements.

Key words: crystal structure/manganese/nucleotide binding/phosphotransferase mechanism/protein kinase A

Introduction

Adenosine 1',5'-triphosphate, the universal energy exchange medium of living cells, serves also as a phosphate donor in the regulation of protein activity through specific sidechain phosphorylation. Almost four decades ago, Krebs and Fischer (1956) discovered protein phosphorylation used for enzyme activity control. The protein kinases, of which about 200 are already known (Hunter, 1991), catalyse the reversible transfer of the γ phosphate from ATP to a protein or peptide, and thereby play a central role in the regulation of most cellular processes. All protein kinases so far known share a common catalytic core, which comprises nine invariant amino acids and many other highly conserved residues (Hanks and Quinn, 1991), highlighting the residues directly involved in phosphoryl transfer.

The cAMP-dependent protein kinase, or protein kinase A, formerly called phosphorylase kinase kinase (Walsh *et al.*, 1968) serves as a prototype for the whole family and has been studied intensively, particularly to characterize substrate recognition and binding, and the mechanism of phosphoryl transfer. There are two main catalytic mechanisms proposed which differ in the educt binding order: a steady state ordered mechanism and a steady state random kinetic mechanism, both with ordered release of products (Granot *et al.*, 1981; Cook *et al.*, 1982; Whitehouse and Walsh, 1983; Whitehouse *et al.*, 1983; Adams and Taylor, 1992). The substrate recognition consensus sequence of the protein kinase A is characterized by two basic residues, usually arginines, separated by one amino acid from the site of the phospho-acceptor, and a large hydrophobic residue (Kemp *et al.*, 1977). The binding is enhanced by MgATP, and vice versa. The reasons for this synergism have been speculative. The activity of the catalytic subunit is efficiently inhibited by the natural heat stable protein kinase inhibitor or by its derivative peptide, PKI(5-24) (Cheng *et al.*, 1986). The high inhibitory potential of the PKI arises from an N-terminal binding helix and its pseudosubstrate binding sequence, where an alanine has replaced the phosphoryl group accepting serine. The structure of the inhibitor in solution has been determined by circular dichroism and NMR techniques (Reed *et al.*, 1987, 1989). An X-ray structure of a recombinant catalytic subunit from mouse Cα in complex with inhibitor peptide PKI(5-24) at 2.7 Å resolution has recently been reported (Knighton *et al.*, 1991a,b).

We present here the crystal structure of isoform C_A of the native catalytic subunit from porcine heart in complex with a pseudosubstrate PKI(5-24) and the MgATP substitute MnAMP-PNP at 2.0 Å resolution. In addition to the improvement of the model of the recombinant enzyme structure, the MgATP binding site is here resolved in great detail and allows functional assessment of active site amino acids and conserved structural elements. Furthermore, this structure and comparison with the structure of the mouse enzyme reveals the basis for the synergistic effects of peptide and MgATP binding.

Results and Discussion

Overall structure

The crystal structure of the catalytic subunit of the cAMP-dependent protein kinase from porcine heart includes the ATP analogue, adenylyl imidodiphosphate (AMP-PNP), two Mn^{2+} ions, a peptide PKI(5-24) (TTYADFIASGRTGRR-NAIHD) derived from the protein kinase inhibitor (Cheng *et al.*, 1986) and 169 water molecules. The overall architecture is identical to the recombinant catalytic subunit from mouse expressed in *Escherichia coli* (Knighton *et al.*, 1991a,b). The enzyme consists of a smaller (upper) lobe, mainly formed by the N-terminal part of the enzyme and having a predominantly β sheet structure, and a larger (lower) predominantly helical lobe, formed by residues of the C-terminal portion (Figure 1). A deep cleft between the

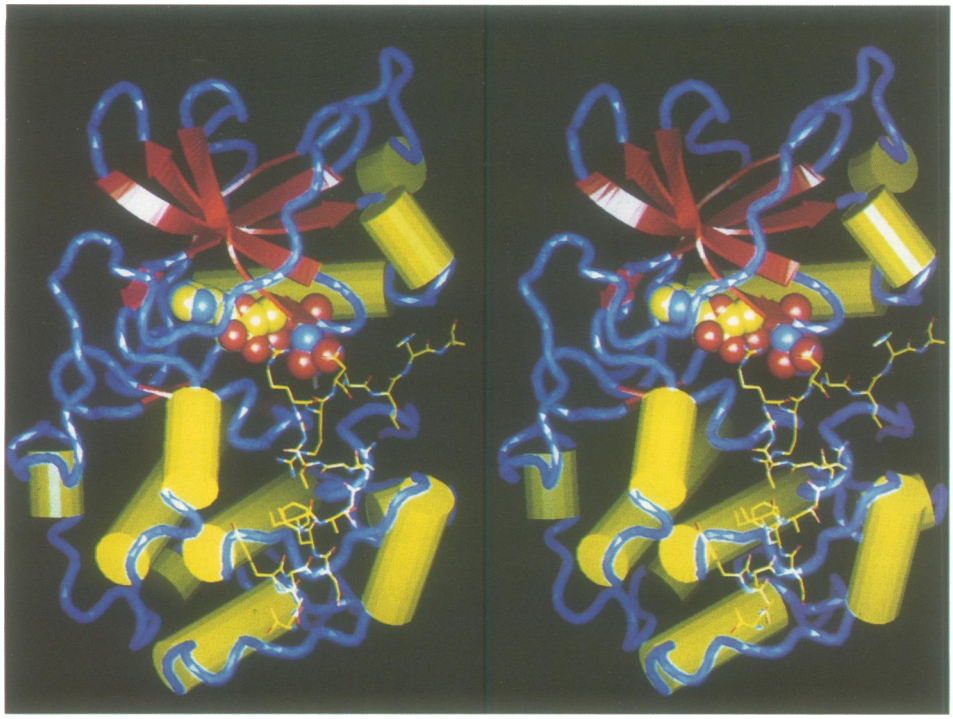


Fig. 1. Stereo representation of the overall architecture, made with the computer program O (Jones *et al.*, 1991). Helices are drawn in yellow, β sheets in red and the rest in blue. The MnAMP-PNP molecule is shown as a space filling CPK model; the inhibitor peptide as amino acids with sidechains. Alpha helixes: A: 10–31; 40–42 (3_{10} helix); B: 76–81; C: 85–97; D: 128–135; E: 140–159; E1:207–211; F: 218–233; G: 243–252; H: 263–272; I: 289–292; 295–297 (3_{10} helix); J: 302–306; K: 344–347. Beta strands: 1: 43–52; 2: 55–62; 3: 67–75; 4: 106–111; 5: 115–120; 6: 162–163; 7: 172–174; 8: 180–182; 9: 189–190. The algorithm of Kabsch and Sanders (1983) was used for secondary structure determination of the ternary complex.

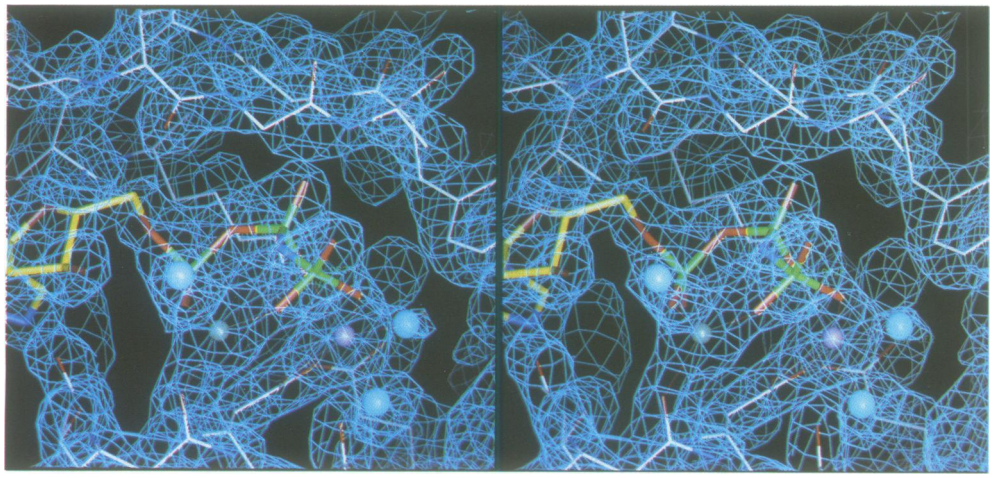


Fig. 2. Stereo representation of the electron density around the triphosphate group superimposed with the refined model. Water molecules are cyan balls, the activating metal (right) is in light red, the inhibiting metal in grey.

two lobes comprises the nucleotide and the pseudosubstrate binding site of the inhibitor peptide. (The secondary structure nomenclature has been adapted from Knighton *et al.*, 1991a.) The recombinant enzyme and the porcine heart enzyme differ most in two regions: in the nucleotide consensus binding motif (residues 54–64), and close to the C-terminus (residues 310–340). In each region, the two model structures are out of register by one residue. Thus, in the nucleotide binding region, the glycine-rich sequence forms a well defined β strand, turn, β strand structure in our model, rather than the loop as interpreted for the mouse model (Knighton *et al.*, 1991a). Also, the frame shift in the C-terminal region alters residues in contact with the glycine-rich sequence, including Tyr330, which in the new structure makes several direct and indirect contacts to residues at the nucleotide binding site, including the invariant Gly50 of the nucleotide binding flap. This is thus a candidate for the tyrosine residue involved in structural changes which accompany substrate binding, as proposed from CD experiments (Reed and Kinzel, 1984a).

The first seven amino acids are undefined, compared with 14 of Knighton *et al.* (1991b). High temperature factors of both C-terminal and N-terminal regions indicate flexibility of these non-catalytic regions. The bound inhibitor peptide

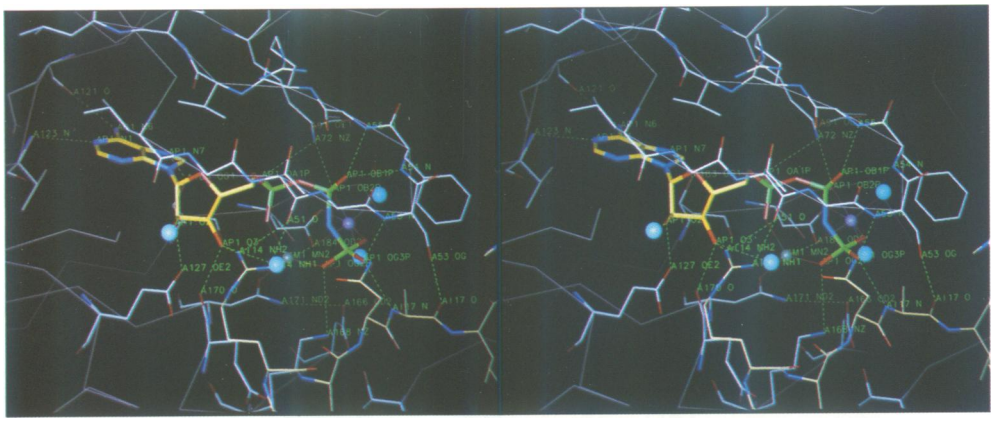


Fig. 3. Stereo representation of the binding of AMP-PNP with the glycine-rich flap. Green dashed lines represent intermolecular hydrogen bonds. Water and metal molecules as in Figure 2.

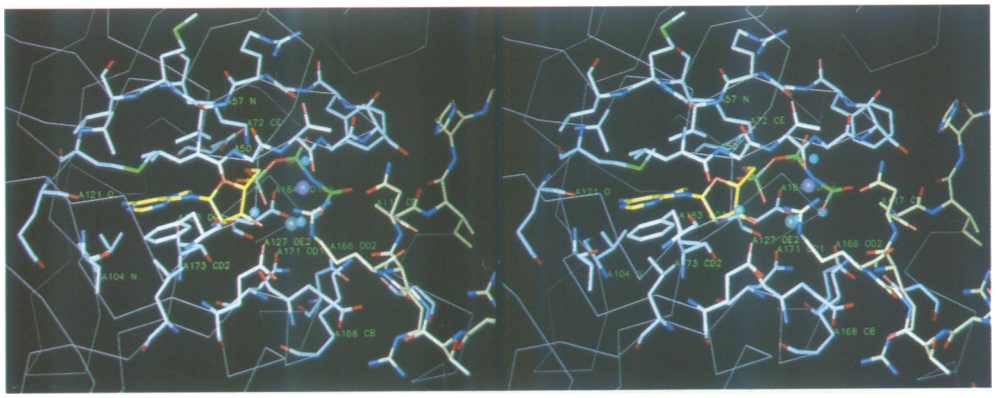


Fig. 4. Stereo representation of the AMP-PNP binding site, showing the hydrophobic packing of the adenosine ring, the principal conserved residues and solvent molecules (small cyan CPK spheres) at the binding site, and the inhibitory peptide (on the right, with grey shaded carbon atoms.

PKI(5-24) consists of an N-terminal helix, a turn and the extended coil of the substrate consensus region (Reed *et al.*, 1987, 1989; Glass *et al.*, 1989; Knighton *et al.*, 1991b). The inhibitor structure within the ternary complex is nearly identical; a small shift of AlaI17 towards the γ phosphate of the nucleotide, and a different orientation of the two C-terminal residues of the peptide are the only significant differences.

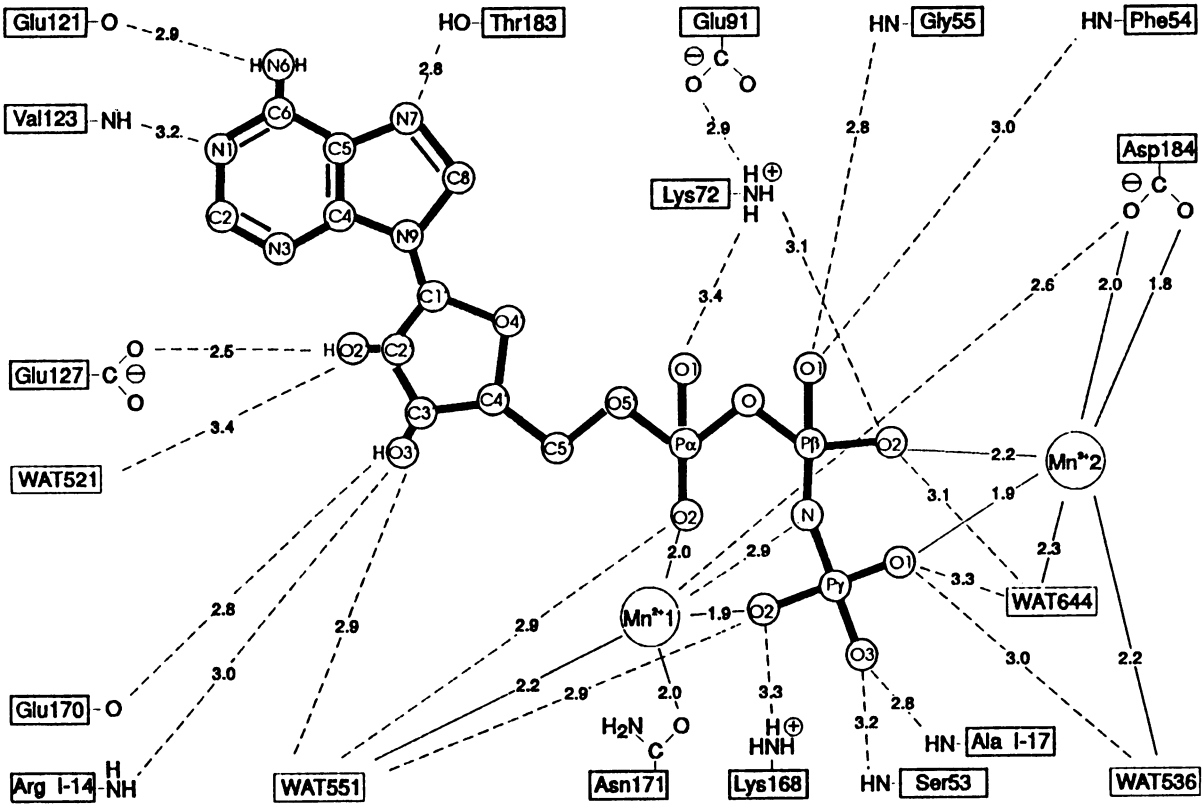
The nucleotide fold

The catalytic core of protein kinases contains a nucleotide binding motif unique among nucleotide binding proteins, as proposed by Benner and Gerloff (1991) before the structural confirmation by Knighton *et al.* (1991a). The specific interactions of the nucleotide binding region with the nucleotide are now revealed in detail (Figure 2).

Other mononucleotide binding proteins share a characteristic consensus sequence, Gly-X-X-Gly-X-Gly-Lys (for a recent review see Schulz, 1992), which, together with a subsequent α helix, links two parallel β strands. The amide groups of the glycines form a positively charged, 'giant anion hole' for the phosphoryl groups (Dreusicke and Schulz, 1986; Pai *et al.*, 1990), while the consensus lysine residue binds to the β and γ phosphoryl groups and is proposed to stabilize the transition state (Schulz, 1992). In contrast, the protein kinase consensus sequence, Gly50-X-Gly52-X-X-Gly-X-Val57, forms a β strand, turn, β strand structure, part of a highly twisted β sheet (Figure 3). The consensus sequence forms a flap over the nucleotide. The β strands are stabilized by backbone hydrogen bonds between Gly50 and Val57, both invariant residues, and between the invariant Gly52 and highly conserved Gly55 (Hanks and Quinn, 1991). A distorted type I turn is formed by Ser53 and Phe54. The role of the conserved glycines and invariant residues is now apparent: to form a flap-like β ribbon with conformational fexibility which can serve as the phosphoryl group anchor but avoid steric clash with ATP. Ser53, which is highly conserved in Ser/Thr kinases, also shares a hydrogen bond with the backbone of the phosphate accepting amino acid, as discussed below.

The flap β ribbon lies parallel to the nucleotide, covering it almost completely (Figure 3). In contrast, the glycine-rich loop of other nucleotide binding proteins lies on the phosphate side of the ATP, with the glycines hydrogen bonded to the phosphoryls, as in adenylate kinase or H-ras p21 (Schulz, 1992; Pai *et al.*, 1990). The enclosure of the MgATP may be essential for protein kinases, since they must accommodate a variety of large substrate proteins, and must thus orient the ATP to avoid steric restrictions around the active site for any potential substrate.

The protein kinase consensus sequence is similar to that of dinucleotide binding proteins, and likewise does not contain a lysine, unlike the mononucleotide binding proteins (Hanks *et al.*, 1988; Schulz, 1992). However, located on the third β strand is the invariant residue Lys72. This residue was proposed earlier to be involved in ATP−phosphoryl



Scheme I. Schematic diagram of the interactions between MnAMP-PNP and catalytic subunit, inhibitor peptide or water molecules. Dashed lines correspond to hydrogen bonds (<3.5 Å), thin solid lines indicate metal−ligand bonds.

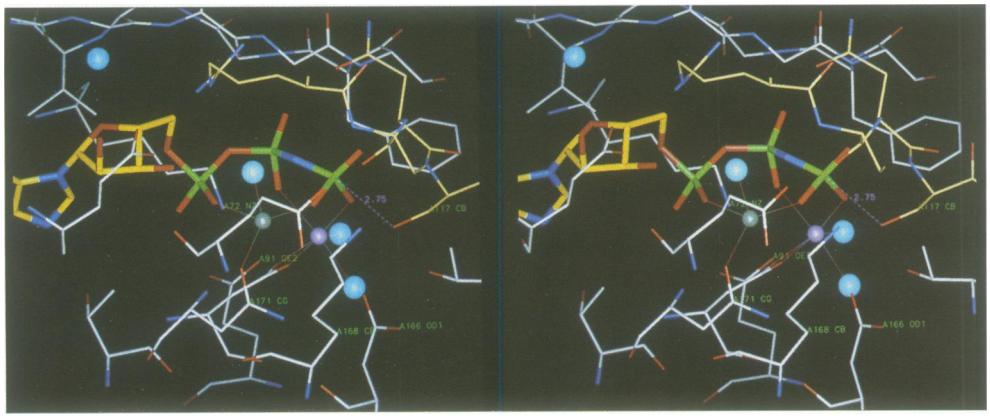


Fig. 5. Stereo representation of the catalytic site with the model of the replacement AlaI17 to Ser. Bonds of inner sphere metal ligands are thin solid lines. Water molecules are light cyan balls, the activating manganese is light pink, the inhibitory grey green.

interaction (Zoller and Taylor, 1979) in a region close to the β and γ phosphoryl groups (Bhatnagar, 1984). The detailed structure of the nucleotide binding site reveals that Lys72 does indeed have a function in phosphoryl interaction, but one quite different from the proposed role of the conserved Lys in other nucleotide binding proteins.

The MgATP binding site

AMP-PNP, the analogue for ATP for crystallization, is bound in anti-conformation with an anti-clinal glycosidic angle $\chi = -133°$, according to the nomenclature of Saenger (1984). The ribose has an unsymmetrical twist with major 3′ endo and minor 2′ exo puckering. MnAMP-PNP in the crystal structure is bound as the D stereoisomer, which is the preferred β γ bidentate metal ATP complex for the catalytic subunit (Granot *et al.*, 1979a; Bolen *et al.*, 1980). To our knowledge, no other high resolution structure of an enzyme bound Mn or MgATP complex has been determined.

Interactions with enzyme residues. The high affinity of MgATP in an enzyme inhibitor complex with a K_d in the nM range (Whitehouse and Walsh, 1983), is reflected in the large number of polar interactions between Mn^{2+}AMP-PNP and enzyme residues. The invariant and conserved residues involved in binding are apparently brought into appropriate orientations by neighbouring residues with conserved hydrophobicity, such as Leu167, Leu172 and Phe185, which are found loosely packed in a hydrophobic

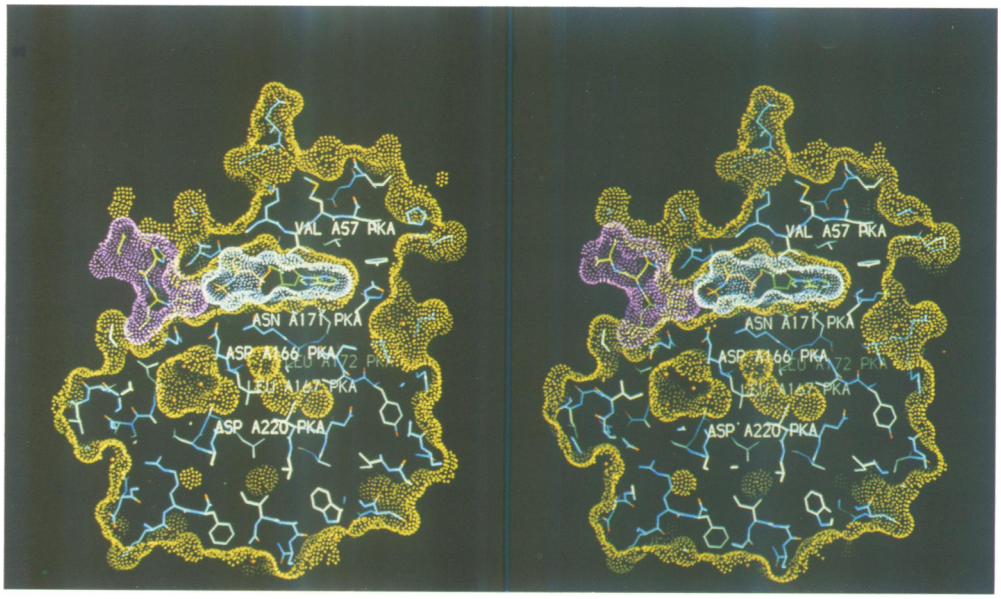


Fig. 6. Cross section of the enzyme complex with a Connolly surface, produced by the program MAIN (Turk, 1992). The enzyme surface is yellow, the peptide surface is purple and the surface of AMP-PNP is white. Water molecules are orange. A hydrophilic hole and the loosely packed hydrophobic region underneath the nucleotide binding are depicted.

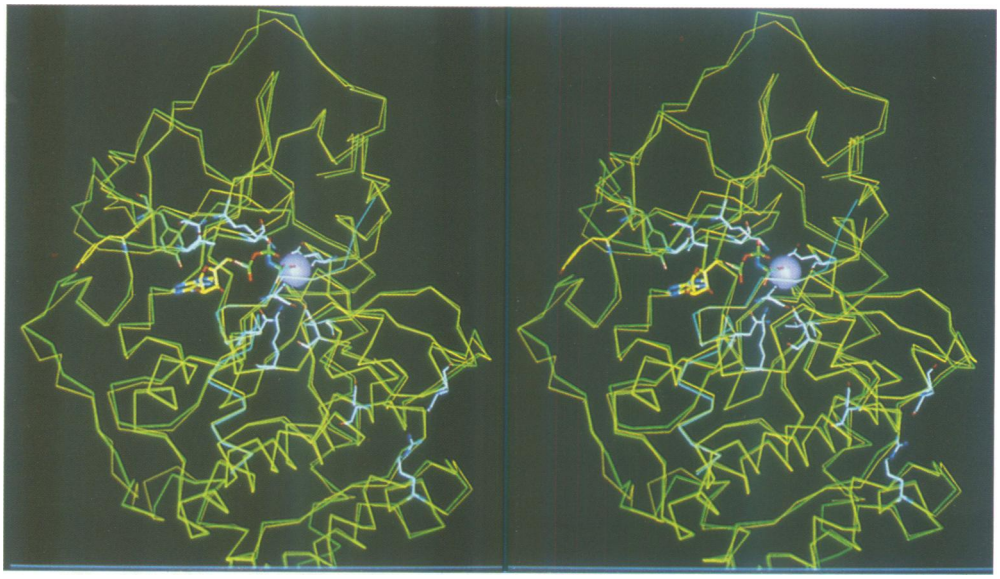


Fig. 7. Stereo figure of the best fit of the binary (yellow, Knighton *et al.*, 1991a,b) and ternary (green) complexes, showing differences in α chain tracing between them. Invariant residues and Lys168 of the ternary complex are drawn with their sidechains. The old and new positions of Tyr330 on the C-terminal strand are depicted with the corresponding Cα colours. The activating metal ion is shown in a CPK representation, and the inhibitory metal ion is omitted.

region underneath the MgATP site (Figure 6); the loose packing may further enhance mobilities seen upon substrate docking (Reed and Kinzel, 1984a; Adams and Taylor, 1992).

The purine base. The adenine base is bound to enzyme residues by three hydrogen bonds (Scheme I). The hydrogen bond between N6 and the backbone carbonyl of Glu121 and the hydrogen bond between N1 and the backbone amide of Val123 connect the purine to the extended coil stretch joining the two lobes of the enzyme. The third interaction is a hydrogen bond between N7 and the hydroxyl group of Thr183, the residue preceding the invariant and essential amino acid Asp184. Non-polar interactions with one side of the purine ring occur with Leu49 and invariant Val57 of the conserved glycine-rich flap (Figure 4). Non-polar interactions from the opposite direction come from Leu173 in β strand 7 near the invariant residue Asn171. Cook *et al.* (1982) and Bhatnagar *et al.* (1984) found similar affinities of adenosine and ADP or ATP but not of AMP to the enzyme, indicating specific interaction between the enzyme and the adenosine moiety.

The ribose. The ribose hydroxyl groups are tightly bound via three hydrogen bonds (Scheme I and Figure 3). Two are formed with enzyme residues: from the sidechain of Glu127 to the 2'OH-group and from the carbonyl group of Glu170 to the 3'OH-group, while the third is from the sidechain of ArgI14, one of the two basic residues of the peptide substrate

recognition sequence, to the 3'OH. A number of residues come within 3−4 Å to provide van der Waals interactions with this part of the nucleotide.

The triphosphate group. Hydrogen bonds to the phosphate oxygen atoms are formed by the mainchain amides of Ser53, Phe54 and Gly55, the sidechains of Lys72 and Lys168, the backbone amide of AlaI17 and three metal chelating water molecules (see Scheme I and Figure 3). The invariant residues Gly50, Gly52 and Val57 of the highly conserved phosphate-binding flap do not interact directly with any of the phosphoryl groups.

Several findings have led to the belief that Lys72, an invariant and essential residue located on the third β strand, and the invariant residue Asp184 could play critical roles in the phosphotransfer mechanism of the protein kinase. Mutating this particular lysine in the EGF, insulin and PDGF receptor kinases completely abolished kinase activity (Ullrich and Schlessinger, 1990) although in yeast TPK1 some residual activity was found (Gibbs and Zoller, 1991a). Lys72 can be labelled with the ATP-derivative 5'-[*p*-(fluorosulfonyl)benzoyl]adenosine (Zoller and Taylor, 1979), and is protected from modification by acetic anhydride in the presence of MgATP (Buechler *et al.*, 1989). Substitution of Asp184 by an alanine does not yield a viable phenotype of TPK1 in yeast (Gibbs *et al.*, 1992). The proximity of Lys72 to Asp184 has been demonstrated by crosslinking with DCCD (Buechler and Taylor, 1989). MgATP prevents the covalent modification of both Asp184 and Glu91, another invariant residue (Buechler and Taylor, 1988). While Asp184 has been considered to participate in the nucleophilic attack of the γ phosphate acting as a catalytic base (Yoon and Cook, 1987; Benner and Gerloff; 1991), Lys72 has been proposed to be involved in protonation of MgATP, especially of the leaving β phosphoryl group (Yoon and Cook, 1987; Kamps and Sefton, 1986), or in stabilizing the extra negative charge of a phosphointermediate during transfer (Benner and Gerloff, 1991).

Lys72 forms hydrogen bonds to oxygen atoms of both α and β phosphoryl groups and a salt bridge to the carboxyl group of the invariant Glu91 (Scheme I). It is thus implausible that there is direct involvement of this residue in the phosphoryl transfer reaction, as will be discussed later. The role of Asp184 clearly is the chelation of the activating metal ion M^2, which in turn is bound to the β and γ phosphoryl group oxygens. Another invariant residue, Asn171, appears to be indirectly involved in interactions with phosphoryl oxygens. The sidechain carbonyl group of Asn171 is in close contact with the M^1 metal ion, which in turn bridges oxygens of the α and γ phosphoryl groups in a manner similar to Glu91, which in its turn hydrogen bonds to the oxygen-bridging Lys72 and to Asp184; this co-ordinates the oxygen bridging M^2 metal ion. Thus the triphosphate chain is tightly connected via hydrogen bonding, involving directly or indirectly most of the invariant amino acids of the protein kinases.

The metal ions. The manganese ion M^2 co-ordinates oxygens of the β and γ phosphoryl groups while M^1 co-ordinates oxygens of the α and γ phosphoryl groups of AMP-PNP (Figure 5). M^2 is octahedrally co-ordinated, with distortions arising from the two carboxylate oxygens of Asp184 occupying neighbouring positions. M^1 is co-ordinated with four ligands in an unusual trigonal bipyramidal geometry, if the proximity of one of the Asp184 carboxylate oxygens (2.6 Å) is considered a fifth weakly bound axial ligand. The incomplete co-ordination of M^1 is consistent with its designation as the more weakly bound inhibitory metal, as is the relative number of charged ligands. Binding of one metal ion is essential for kinase activity; at higher magnesium concentrations a second ion binds and reduces the activity 5-fold (Armstrong *et al.*, 1979a). Both magnesium sites can be replaced by Mn^{2+} whereby Mn^{2+} binding to the high affinity site (K_d 6−10 μM) leads to an activation 4-fold lower than with Mg^{2+}, and Mn^{2+} binding to the low affinity site (K_d 50−60 μM) decreases the activity by one or two orders of magnitude. The β γ co-ordinated metal ion has been postulated to be the high affinity, activating ion (Granot *et al.*, 1979b). This view is consistent with other enzyme metal−nucleotide complexes. (Shirakihara and Evans, 1988; Kabsch *et al.*, 1990; Pai *et al.*, 1990).

Peptide consensus interactions

Three glutamate residues of the yeast enzyme TPK1, homologous to Glu127, Glu170 and Glu230, are involved in recognition of the basic residues of the peptide substrate (Gibbs and Zoller, 1991b). Those enzyme residues which interact with the peptide are conserved among protein kinases of the Ser/Thr family, which characteristically recognize basic substrates (Pearson and Kemp, 1991). With the substrate Kemptide (Kemp *et al.*, 1977), exchange of either one of the two basic residues yields a poor substrate, with a 300- to 400-fold lower affinity. In the crystal structure of both the binary complex (Knighton *et al.*, 1991b) and the ternary complex described here, ArgI15 makes ion pairs with Glu230 and Glu170, both residues of the large lobe (Figure 3). The other of the two basic consensus residues, ArgI14, makes an ion pair with Glu127, which is located on the coil connecting both lobes. Knighton *et al.* (1991b) described a second ion pair of ArgI14−Glu331, but this interaction is not present in our model. In the ternary complex, the sidechain of ArgI14 forms hydrogen bonds to the 3'-hydroxyl group of the ribose and also to Thr51 of the glycine-rich flap, and thus interacts directly with the small lobe. The two arginines of the consensus sequence span the cleft like a clamp. The backbone carbonyl of ArgI15 is 2.8 Å from the sidechain of Lys168. Part of the consensus sequence motif is a large hydrophobic residue following the site of phosphotransfer which is IleI18 in PKI(5-24). Its sidechain lies in a hydrophobic environment provided by the sidechains of Pro202, Leu205 and Leu198, found within 4 Å. The backbone amide of IleI18 forms a hydrogen bond to the backbone carbonyl of Gly200. Thus the position of the peptide consensus sequence is firmly fixed and precisely oriented by ion pairs, hydrogen bonds and hydrophobic interactions.

Post-translational modifications

The phosphorylation site. The electron density clearly shows the phosphorylation of only one residue, Thr197, which is known to be subject to autophosphorylation, as also found with the recombinant mouse enzyme (Shoji *et al*, 1979; Knighton *et al.*, 1991a). This is in contrast to molar ratios of phosphate:enzyme of between 1.4 and 1.9 previously reported by Peters *et al.* (1977). We found that both C_A and C_B isoforms from porcine heart contained ~1.8 mol/mol phosphate (Bossemeyer *et al.*, in preparation) indicating

partial phosphorylation of amino acids besides Thr197, apparently disordered in the crystal structure.

Residue 197, a threonine or a tyrosine in most protein kinases (Hanks and Quinn, 1991), is thought to be phosphorylated for correct assembly of the structure (Knighton *et al.*, 1991b; Shoji *et al.* 1979) or for regulation of enzyme activity (Gibbs *et al.*, 1992; Levin and Zoller, 1990) and may interact with regulatory molecules or domains (Gibbs *et al.*, 1992). The phosphate oxygens of phosphothreonine 197 form hydrogen bonds with the charged sidechains of Arg165, Lys189 and the hydroxyl group of Thr195, and are in the vicinity of His87. Arg165 is invariant in the tyrosine kinase subfamily and almost invariant in Ser/Thr kinases. Mutating Arg165 to alanine yields a phenotype impaired in k_{cat} and K_m of peptide and MgATP (Gibbs *et al.*, 1992). An almost direct contact to the heart of the active site could be provided by Arg165, the residue interacting directly with the phosphoryl group via its sidechain and with the putative catalytic base Asp166 via the mainchain.

N-terminal modification. The native catalytic subunit carries an N-terminal myristylic acid. For many proteins a role of the myristylic acid residue in membrane binding has been demonstrated (Johnson *et al.*, 1990; Thelen *et al.*, 1991). For the catalytic subunit the role is unclear. Expression of a myristylation-deficient catalytic subunit in NIH3T3 cells did not lead to any recognizable effects (Clegg *et al.*, 1989). A general role in structural stabilization of the catalytic subunit has been proposed. In the crystal structure a hydrophobic groove on an outer region of the large lobe, covered by the N-terminal helix, contains very well defined electron density, which probably corresponds to six methyl groups of the tetradecanoic acid. The connection to the first resolved residue, Lys8, is not visible. The hydrophobic residues Val15, Phe18, Leu19, Phe106, Leu152, Trp302, Ile303 and Ile305 comprise this region.

Enzyme – substrate interactions

Open and closed conformation. The catalytic subunit has been postulated to exist in an open conformation when unbound and in a closed conformation with peptide or peptide and MgATP bound (Reed and Kinzel, 1984a). The free form is characterized by a higher content in α helix and random coil secondary structure as derived from circular dichroism (Reed and Kinzel, 1984a). The binding of peptide or protein substrates is a process of at least three steps with discrete structural changes and substrate trapping, indicating 'enclosure' of the substrate after initial electrostatic binding. This has been demonstrated by induced circular dichroism using blue dextran (Reed and Kinzel, 1984b; Reed *et al.*, 1985). An ordered reaction sequence has been postulated by several investigators indicating a steady state ordered or a steady state random mechanism with preferred MgATP binding first, and ordered release of products, MgADP being released last (Granot *et al.*, 1981; Cook *et al.*, 1982; Whitehouse and Walsh, 1983; Bhatnagar *et al.*, 1984; Kong and Cook, 1988).

The movement of the glycine flap and entering of the nucleotide. A least squares fit was made of the binary complex structure from Knighton *et al.* (1991a,b) to the ternary structure, using the conserved regions including parts of the packed helices (amino acids 138–161, 202–210 and 234–237) for fitting. The fit revealed the extensive coincidence of both structures, including the upper lobe which was not used for fitting (Figure 7). Excepting exposed or corrected regions, the greatest difference is at the glycine-rich flap, Leu49–Val57, which is shifted as a whole towards the triphosphate chain of the nucleotide (Figure 7), in accordance with its proposed task of anchoring the phosphate chain. The tip of the flap, at Cα of Ser53, moves over a distance of 1.3 Å. Lesser shifts are observed at Thr51 and Gly52. This provides an explanation for the complete enclosure of the nucleotide; the entry of the nucleotide may be facilitated by an opening of this flap-like structure, perhaps when the peptide is already bound, as suggested by kinetic data, and perhaps assisted by other mechanisms such as hinge bending to open the cleft (Reed and Kinzel, 1984a), which is observed in other enzymes (Bennett and Huber, 1984). However, no movement of the upper lobe or hinge bending is indicated by the comparison of binary and ternary complexes of the protein kinase. A crystal structure of the free enzyme would clarify this point.

Synergism of multiple substrate binding. The synergistic binding of ATP and substrate or inhibitor to the kinase is seen as an increase in the affinity of MgATP for the enzyme by two orders of magnitude upon addition of inhibitor protein, from a K_d of 5 μM (Armstrong *et al.*, 1979b) to 20–60 nM (Whitehouse and Walsh, 1983), and conversely as the binding of the inhibitor protein at low concentrations only to the enzyme–MgATP complex with a K_d of 0.49 nM, and not to the free enzyme (Whitehouse and Walsh, 1983). At higher concentrations, the inhibitor peptides or Kemptide can bind to the free enzyme (Reed and Kinzel, 1984b; Knighton *et al.*, 1991b). Also, the affinity of acetyl-Kemptide to the free enzyme is weak (K_d = 0.2 mM) compared with the K_m of 3 μM in the kinase reaction with MgATP (Whitehouse *et al.*, 1983). A similar synergism is observed in formation of the holoenzyme (Ringheim and Taylor, 1990). At least two reasons for the observed synergisms can be considered: mutual binding enhancement through direct interactions between the peptide and MgATP, or conformational changes of the enzyme induced by either component. The crystal structure of the ternary complex and comparison with the enzyme/peptide binary complex show both phenomena (Figures 3 and 7): a hydrogen bond from one of the nucleotide γ phosphoryl oxygens to the backbone amide of AlaI17 and hydrogen bonds from the sidechain of ArgI14 to the 3'OH of the ribose enhance the mutual binding directly (Scheme I); this verifies an earlier suggestion based on the 10^7-fold lowered reactivity of a substrate peptide with a chemically modified serine backbone amide (Thomas *et al.* 1987). The superposition of the binary and ternary structures (Figure 7) shows a movement of the glycine-rich flap toward the triphosphate chain upon nucleotide binding, which allows the formation of three additional hydrogen bonds between the flap and the peptide. These are between the mainchain carbonyl of Val51 and both Nη groups of the peptide ArgI14, and between the Oγ of Ser53 at the tip of the flap, and the mainchain carbonyl of AlaI17 (Figure 3), the critical residue of the consensus peptide. It is possible that the three hydrogen bonds from the flap to the β and γ phosphoryl groups of the nucleotide are only formed in the ternary complex, when the flap also shares hydrogen bonds with the peptide. Thus, eight hydrogen bonds, which are seen in the crystal structure of the ternary complex

catalytic subunit with MnAMP-PNP and inhibitor peptide (those between MnAMP-PNP and peptide, those between the phosphoryl oxygens and the flap and those between the peptide and the flap), may exist only in the presence of both nucleotide and inhibitor peptide. These interactions, together with the movement of the glycine-rich flap, may well have implications for the scheme of substrate binding order which probably does not have to be strictly ordered, since the nucleotide might be able to enter the cleft in a complex with the peptide already bound. This is supported by recent kinetic studies of Adams and Taylor (1992) who found in viscosity experiments under limiting amounts of peptide a formally random sequential scheme with a preferred binding order with ATP binding first.

Protein kinase mechanism

The conserved active site lysines. In adenylate kinase the sidechain of the consensus sequence lysine of the mononucleotide fold is not fully extended, and is proposed to follow the γ phosphoryl group during transfer (Schulz, 1992). It forms hydrogen bonds to both β and γ phosphoryl groups. In H-ras p21 with bound GppNp (Pai *et al.*, 1990), the critical lysine sidechain also bridges the β and γ phosphoryl group oxygens, somewhat closer to the γ phosphate. Protein kinases do not have a lysine in their glycine-rich consensus sequence, but do have an invariant lysine in the third of the anti-parallel β strands that comprise the protein kinase nucleotide fold. This residue, Lys72, is unlikely to fulfil a similar role as it is fully extended, hydrogen bonded to α and β phosphoryl oxygens, and cannot reach the γ phosphoryl group without large structural movements of its whole β strand. A better candidate for stabilizing the transition state of the γ phosphoryl group is Lys168. However, this residue is invariant only in the Ser/Thr and probably also in the newly discovered multi-specificity STY protein kinase subfamily (Hanks and Quinn, 1991; Letwin *et al.*, 1992). 'Charged-to-alanine' mutagenesis of the Lys168 homologue in TPK1 in yeast leads to a residual activity of only 0.3% of wildtype, and an interaction with MgATP in the kinase reaction has been postulated because of the high $\Delta\Delta G_R$ for Kemptide and MgATP (Gibbs and Zoller, 1991a). In the tyrosine kinase subfamily, two sequences found at this position, either **Arg**(168)-Ala-Ala, or as Ala(168)-Ala-**Arg** (Hanks and Quinn, 1991) thus probably conserving the position of a positive charge among protein kinases, since an extended residue at position 170 could place its charge in a position occupied by the Lys168 charge in PKA if the relative position 168 is small and hydrophobic. We suggest that in Ser/Thr kinases (and probably in multispecificity STY kinases) the residue Lys168, and in tyrosine kinases the conservative Lys168 homologue, arginine, or alternatively an Arg two residues further C-terminal are responsible for neutralizing the negative charge of the γ phosphate during transfer, thus lowering the free energy of the transition state.

The catalytic base. Schemes proposing an in-line mechanism for phosphoryl transfer require the presence of a catalytic base to abstract a proton from the attacking hydroxyl group (Bramson *et al.*, 1984; Yoon and Cook, 1987). Residue Asp184, an invariant residue that has been identified as absolutely required for kinase activity (Gibbs *et al.*, 1992), has attracted much attention in this respect. However, Asp184, while close to the γ phosphoryl group, is quite removed from any potential hydroxyl group at the position of AlaI17 and co-ordinates the activating high affinity metal cation. A role for this position in metal chelation rather than as the catalytic base has been suggested (Knighton *et al.*, 1991a) because of its distance from the peptide AlaI17. Asp184 apparently helps to orient the γ phosphoryl group via the co-ordinating metal ion. This may explain its invariance; however, a more direct role in catalysis is not excluded. Yoon and Cook (1987) demonstrated that catalysis involves a residue with a pK_a of 6.2. As there is no histidine in the neighbourhood, aspartates or glutamates are candidates if in a sufficiently negatively charged environment. The sidechain of Asp166 is within hydrogen bonding distance (2.8 Å) of the hydroxyl group of a serine modelled into the position of AlaI17 (Figure 5). Asp166 is one of the invariant amino acid residues found not only in all protein kinases, but also in bacterial phosphotransferases responsible for antibiotic resistance, suggesting a general role for binding of ATP and phosphorylation of substrates (Brenner, 1987). Substitution of the Asp166 homologue in yeast to alanine yields a residual activity of 0.4% compared with wildtype and a 370-fold decrease in k_{cat}, but only a 1.5-fold increase in the K_m for MgATP (Gibbs and Zoller, 1991a). Its carboxyl group may have a perturbed pK_a in the neighbourhood of the triphosphate group, what might be indicated by a movement away from it in the ternary complex, and thus is quite possibly the catalytic base in protein kinase action.

The asparagine 171. In the present complex, invariant Asn171 is co-ordinated to the inhibitory metal ion, but its invariance suggests a role in catalysis which is optimal in the absence of the inhibitory metal. However, since enzyme activity is not abolished in the presence of the inhibitory metal ion, any essential function of this residue must either be preserved upon inhibitor metal binding or be replaced by other groups. The amide sidechain of Asn171 comes within hydrogen bonding distance of the carboxyl oxygen of Asp166, which is close to the AlaI17 sidechain and which probably represents the proton acceptor from the hydroxyl group. The uncertainties of the role of Asn171 make it a good candidate for investigation by site-directed mutagenesis, particularly because of its likely role in the perturbation of catalysis upon binding of the inhibitory metal ion.

The phosphotransfer reaction. A great deal of work has been done to define the catalytic mechanism of protein kinases, i.e. the γ phosphoryl group transfer to the hydroxyl group of an amino acid on a substrate. A double displacement or ping-pong mechanism is not supported by most data or has been ruled out by stereochemical data indicating an inversion of the configuration of the γ phosphoryl group during transfer, consistent with an in-line mechanism (Ho *et al.*, 1988).

The structure of the ternary complex sets the stage for the scenario of the catalytic reaction. We assume that both the ATP analogue and the inhibitor peptide represent substrates in the stereochemistry of their binding mode. Most relevant is the short distance between AlaI17 and the γ phosphate with $C\beta$ $P\gamma$ of 3.7 Å. To elucidate the active site situation, we modelled a serine residue into the position of AlaI17 by assuming an ideal g− conformer, which occurs at a

frequency of ~30% in protein structures (Ponder and Richards, 1987; Figure 5). In kinetic experiments the exchange of this alanine to serine changes the inhibitor peptide into a substrate (Scott *et al.*, 1986; Glass *et al.*, 1989). A seryl Oγ could approach the γ phosphate by a sidechain rotation to generate a pentacoordinate Pγ. Granot *et al.* (1980) determined a distance of 5.3 ± 0.7 Å from the γ phosphate to the seryl Oγ, based on NMR measurements and model building, supporting an associative mechanism of phosphoryl transfer with an elongated transition state (Bramson *et al.*, 1984), but not completely ruling out a metaphosphate intermediate. It should be noted, however, that the distance along the reaction co-ordinate of 5.3 ± 0.7 Å was calculated under conditions where the substrate and nucleotide binding sites were not occupied simultaneously. In the present crystal structure the distance from the oxygen of the modelled serine hydroxyl group to the γ phosphorus atom is 2.7 Å and can be as short as 2.45 Å with only χ_1 angle adjustment. Nucleophilic addition to Pγ is assisted by charge dispersal by the metal ion and Lys168 bond to the γ phosphate. The geometry of their interaction supports a pentacoordinate geometry at the phosphorus. The nucleophile Ser117 Oγ requires a base for proton abstraction, which may be the carboxylate group of Asp166 at a distance of 2.8 Å. The ternary complex as described here shows that virtually no nuclear displacements are necessary to form a transition state consisting of a pentacoordinate Pγ. The structure is clearly poised towards this state.

The hydroxyl group of Thr201 comes very close to both the carboxyl group of Asp166 (2.7 Å) and the Ser117 Oγ. This residue is conserved in the subfamily of Ser/Thr kinases with very few exceptions. Product release is probably driven by the forces repelling the phosphoserine from the β phosphate of ADP and the catalytic base Asp166, which is reflected in a K_d substantially greater than 1 mM in the case of the product phospho-Kemptide (Whitehouse and Walsh, 1983).

It seems appropriate to review briefly the situation in phosphofructokinase (PFK). Two stages of the catalytic process have been trapped: a closed conformation of the enzyme immediately after phosphotransfer to the substrate fructose-6-phosphate, with the magnesium ion bridging the leaving Pβ and the 1′-phosphate and Arg72 hydrogen bound to the 1′-phosphate, and an open conformation after transfer with MgADP and Arg72 hydrogen bound to the Pα oxygen (Shirakihara and Evans, 1988). Asp127 in PFK is postulated as the catalytic base and has hydrogen bonding contact to the 1′-oxygen of fructose-1,6-diphosphate. The situation thus appears similar to the protein kinase, but the detailed distances differ.

The ATPase activity. The catalytic subunit is known to exhibit some ATPase activity, which is ~1300-fold lower than the kinase activity (Armstrong *et al.*, 1979b; Salerno *et al.*, 1990), both with Mg^{2+} or Mn^{2+}ATP. However, in the presence of inhibitor protein or regulatory subunit the ATPase activity is efficiently inhibited, while a 20-fold increase in ATPase activity during phosphorylation of a substrate protein has been reported (Moll and Kaiser, 1976). This could indicate a difference in the access of water molecules to the active site in the presence of a real substrate compared with a pseudosubstrate, or ATPase activity as a byway of the substrates nucleophilic attack. The present high resolution structure allows determination of water molecules in the active site. Three water molecules are present close to the phosphate chain: WAT644, WAT536 and WAT551. However, all are inner sphere ligands of the manganese ions. Not involved in metal coordination are WAT500, which is <2.8 Å from the ammonium group of Lys72 and <4 Å from one oxygen of the β phosphoryl group, and WAT538, which is 2.7 Å from one carboxylate oxygen of the putative catalytic base Asp166. However, the position of water molecules might be quite different after changes in the arrangement of the active site. None of these water molecules is in a position which makes participation in phosphotransferase activity apparent. A contribution of Asn116 to the reduction of ATPase activity by the inhibitor and interaction of this residue with important catalytic residues has been proposed (Salerno *et al.*, 1990). The crystal structure shows this not to be the case. At most a weak interaction of Asn116 with the glycine-rich flap could be envisioned, insufficient to block the catalytic machinery with respect to its ATPase activity.

Remarks

Justification of the relevance of the inhibitor peptide used as an analogue of a substrate and of MnAMP-PNP as an analogue of MgATP proceeds from the following arguments. Although the N-terminal high affinity binding region of the inhibitor peptide, which is not present in a substrate, could influence the enzyme catalysis, substitution of the critical alanine to the consensus serine in an inhibitor peptide renders it a substrate, with a K_m dependent on the peptide length [280 μM for PKI(11-30), Scott *et al.*, 1986, and more efficient for PKI(14-22), Glass *et al.*, 1989] showing some effects of inhibitor peptide binding. The enzyme retains kinase activity with substitution of Mg^{2+}ATP by Mn^{2+}ATP (Armstrong *et al.*, 1979b). It has been reported that the inhibitor protein does not bind to an enzyme−MgAMP-PNP complex, which led to the hypothesis that the inhibitor exclusively recognizes an enzyme−MgATP complex where ATP is already hydrolysed (Whitehouse and Walsh, 1983). In another report, binding of the inhibitor protein was restored when MnAMP-PNP was used instead of MgAMP-PNP (Van Patten *et al.*, 1986), arguing against hydrolysis of ATP as necessary for inhibitor protein binding. AMP-PNP is believed to be structurally almost indistinguishable from ATP (Taylor, 1981). The K_d of MgAMP-PNP (35 μM) for the enzyme is of the same magnitude as the K_d of MgADP or the estimated K_d of MgATP (Whitehouse *et al.*, 1983). In the crystal structure we do not observe any specific interactions with the nitrogen atom bridging the β and γ phosphoryl groups. A large number of tight interactions of the inhibitor peptide with the enzyme-bound nucleotide AMP-PNP and with functionally important, highly conserved or invariant residues occur in accord with the majority of the kinetic and biochemical data.

Materials and methods

Monoclinic crystals of the catalytic subunit were grown from a homogenous preparation of the minor isoform C_A (Hotz *et al.* 1989b) of porcine heart catalytic subunit (E.C.2.7.1.37, ATP:protein serine phosphotransferase) in methanol as a precipitating agent at pH 5.6 by hanging drop vapour diffusion. The isoform C_A differs from C_B at least at position two of the amino acid sequence where an aspartate replaces the asparagine, as demonstrated with enzyme from bovine heart (Hotz *et al.*, 1989a). A detailed description of

the purification of the isoforms and the crystallization procedure will be given elsewhere (Bossemeyer *et al.*, in preparation). As the cDNA sequence of the porcine catalytic subunit has not yet been determined completely (Adavani *et al.*, 1987), the structural refinement proceeded with the sequence of mouse Cα (Chrivia *et al.*, 1988). We conclude from the published data and our own preliminary experiments that there are probably not more than four differences between the mouse and pig sequences. Possible variations in the amino acid sequence are not expected to affect any of the conclusions presented here.

Data collection

Intensity data were collected from three crystals using the FAST (Enraf Nonius, Delft, The Netherlands) television area detector and nickel-filtered copper Kα radiation from a rotating anode generator (Rigaku Donki, Japan) operated at 5.4 kW for one crystal and an image plate (Hendrix-Lonther, Mar Research, Hamburg, Germany) and graphite monochromatized copper Kα radiation from a similar X-ray source for the other two crystals. The lattice was indexed on the basis of an unconventional monoclinic I-centred lattice instead of the conventional C-centred setting for the convenience of a near orthogonal cell. The asymmetric unit contains two molecules. The unit cell dimensions varied somewhat between the different crystals with values of $a = 107.6$, $b = 80.6$, $c = 110.1$, $\beta = 88.6°$ and $a = 107.5$, $b = 80.6$, $c = 109.4$, $\beta = 88.8°$, accounting perhaps for the unsatisfactory merging of the FAST and image plate data. With FAST there were 109 168 observations of 31 909 independent reflections which merged with an R value of 0.059 based on intensities. With the image plate 103 697 observations of 58 447 independent reflections were made from two crystals which merged with an R value of 0.090. Independently averaged Friedel pairs of the individual crystals had R = 0.042. As the latter data set was much more complete (84% 20–2.0 Å; 83% 2.1–2.05 Å; 80% 2.05–2.0 Å) it was used in the final refinement. The FAST data set was available earlier and used in the structure solution and initial refinement. No detailed comparison was made but we noted no systematic differences between the two refinements.

Structure solution

An initial solution of the crystal structure was found by Patterson search calculations (Huber, 1965) using the real space routines in PROTEIN (Steigemann, 1974) for the orientation determination and the reciprocal space translation function (Crowther and Blow, 1967) in the version of Lattman modified by Deisenhofer and Huber. The search model was the protein kinase (Knighton *et al.* 1991a) as deposited in the Brookhaven Protein Data Base under 1CPK.BRK. From this model placed into a cubic cell of 150 Å cell length, a Patterson synthesis was calculated for the resolution range 8–3 Å. The rotation search yielded an outstanding maximum of $\sim 5\sigma$ with the next highest maximum of $\sim 2\sigma$. As there was only one peak the two molecules in the asymmetric unit were seen to have similar orientations. The translation function calculated with data of 8–3.2 Å resolution showed a single outstanding peak of 26σ on the Harker section at $y = 0$, defining the translation and demonstrating the identical self vector of the two independent molecules. The search for the crossvector between them provided a solution with a peak of 36σ. The search model was transformed accordingly in molecules A and B respectively. Refinement was then carried out using XPLOR (Brünger, 1988) with Cambridge Data Base derived parameters (Engh and Huber, 1991). Initially, the two molecules were treated as rigid bodies whereby the R value in the resolution range 10–3 Å fell from 0.36 to 0.244. The resulting electron density map in the resolution range 8–2.5 Å was averaged between the two molecules using Main (Turk, 1992). Averaging was done in cycles whereby the R-value after back transformation of the electron density typically decreased from 0.24 to 0.14. This procedure helped in recognizing differences from the starting model regarding a few sets of alternative chain tracing and the localization of AMP-PNP and manganese. Later, the resolution was extended to include 8–2 Å data and cycles of model refinement were continued by minimizing crystallographic and energy terms with intermittent model corrections (FRODO, Jones, 1978). Very weak local symmetry restraints between the two independent molecules were retained throughout the refinement. The refinement was terminated at R = 0.194 (7–2.0 Å resolution) with deviations from target values of 0.037 Å for bond lengths, 5.1° for interbond angles and 4.2° for improper angles. The deviations between the molecules A and B are 0.51 Å for all atoms (0.26 after elimination of 442 atoms) 0.29 Å for Cα atoms and 0.18 Å for Cα atoms after elimination of 89 atoms deviating by $>1\sigma$. Molecules A and B differ in their orientation by a rotation of only −1.7°. The RMS deviations from the starting mouse model are 0.487 Å excluding two segments, A54–A64 (A66) and A310–A340, which differ by >5 Å. These are explained by single residue shifts between loop regions and may result from errors in the unrefined 1CPK model.

The atomic coordinates have been deposited in the Brookhaven Protein Data Bank

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