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ARTICLE

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Poisson-Boltzmann model studies of molecular electrostatic properties of the cAMP-dependent protein kinase

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Abstract Protonation equilibria of residues important in the catalytic mechanism of a protein kinase were analyzed on the basis of the Poisson-Boltzmann electrostatic model along with a *cluster*-based treatment of the multiple titration state problem. Calculations were based upon crystallographic structures of the mammalian cAMP-dependent protein kinase, one representing the so called *closed* form of the enzyme and the other representing an *open* conformation. It was predicted that at pH 7 the preferred form of the phosphate group at the catalytically essential threonine 197 (P-Thr197) in the closed form is dianionic, whereas in the open form a monoanionic ionization state is preferred. This dianionic state of P-Thr197, in the closed form, is stabilized by interactions with ionizable residues His87, Arg165, and Lys189. Our calculations predict that the hydroxyl of the Ser residue in the peptide substrate is very difficult to ionize, both in the closed and open structures of the complex. Also, the supposed catalytic base, Asp166, does not seem to have a pK_a appropriate to remove the hydroxyl group proton of the peptide substrate. However, when Ser of the peptide substrate is forced to remain ionized, the predicted pK_a of Asp166 increases strongly, which suggests that the Asp residue is a likely candidate to attract the proton if the Ser residue becomes deprotonated, possibly during some structural change preceding formation of the transition state. Finally, in accord with suggestions made on the basis of the pH-dependence of kinase kinetics, our calculations predict that Glu230 and His87 are the residues responsible for the molecular pK_a values of 6.2 and 8.5, observed in the experiment.

Key words Poisson-Boltzmann model · Protein kinases

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Introduction

Protein kinases represent a large family of very diverse enzymes that are poised to receive and transmit signals (Hanks et al. 1988). In eukaryotes, the protein kinases most frequently phosphorylate their protein substrates on serine, threonine, or tyrosine residues. The catalytic (C) subunit of the mammalian cAMP-dependent protein kinase (PKA) is one of the smallest active protein kinases. All kinetic evidence is consistent with a preferred ordered mechanism of catalysis with ATP binding preceding peptide binding (Taylor and Radzio-Andzelm 1994). The catalytic subunit then catalyzes the direct transfer of the γ-phosphate of ATP to the appropriate residue at the phosphorylation (P) position on its peptide substrate (see Fig. 1).

The eukaryotic protein kinases share a conserved catalytic core consisting of a small nucleotide-binding domain and a large domain that includes most of the residues that participate directly in peptide recognition and catalysis. Catalysis takes place in the cleft between the two domains. The relative orientation and position of these two lobes can be classified as either *closed* or *open*; for a review of the structures and function, see for example, Taylor and Radzio-Andzelm (1994).

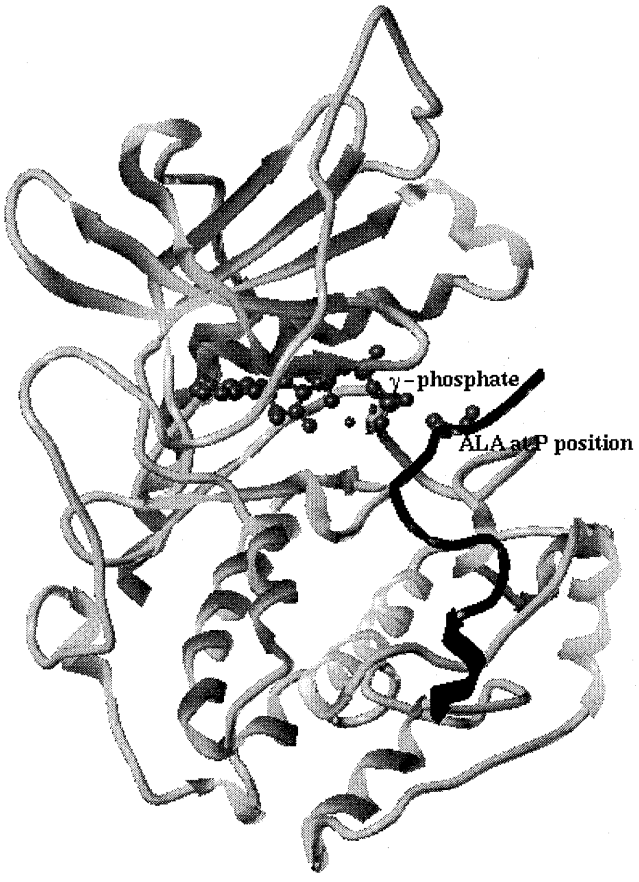

Fig. 1 Ribbon diagram of the mouse recombinant catalytic subunit cocrystallized with an inhibitor and Mn_2ATP. Based on the PDB (Bernstein et al. 1977) structural file with entry code 1ATP (Zheng et al. 1993b)

The first crystal structure of the C-subunit was a binary complex between an inhibitor peptide from the heat-stable protein kinase inhibitor PKI(5-24) and the recombinant mouse C-subunit (Knighton et al. 1991, 1993). It represents a closed conformation and was subsequently found to be superimposable upon the ternary complex containing the C-subunit, PKI(5-24), and Mg_2ATP (Zheng et al. 1993a) or Mn_2ATP (Zheng et al. 1993b). Both the apoenzyme and the binary complex of the porcine C-subunit with a di-iodinated inhibitor peptide represent the crystal structure in an open conformation (Karlsson et al. 1993).

In this work, preliminary results of the analyses of electrostatic properties of protein kinases in the closed and open conformations are presented, which are based on a computer simulation methodology developed for the prediction of pH-dependent properties of proteins (Antosiewicz et al. 1996a). Simulations were performed for the isolated C-subunits in both conformations and for their complexes with peptide inhibitors and/or Mn_2ATP. Titration properties of the residues which are important for ATP and peptide binding as well as for catalysis are considered in this study.

Methods

Electrostatic calculations and titration procedures

The prediction of ionization constants of titratable residues in proteins is based on the Poisson-Boltzmann (PB) model and the assumption that the difference in protonation behavior of a given group in the isolated amino acid in water (pK_{model}) and in the protein environment ($pK_{apparent}$) results exclusively from differences in electrostatic interactions occurring in the two states. The electrostatic free energy in a given ionization state of the protein is used to calculate average fractional protonations of the residues as functions of pH by solving the so-called multiple titration state problem (Gilson 1993). The $pK_{apparent}$ of each residue is defined as the pH corresponding to its half-protonation. The entire methodology is described in detail elsewhere (Antosiewicz et al. 1996a, b, 1999).

For selected residues, the multiple titration state problem was treated by a modified cluster treatment (Gilson 1993), as described in a recent paper (Trylska et al. 1999). The modification was introduced in order to treat groups with two possible neutral forms and one ionized form, like acidic amino acids and histidine, thus establishing an equilibrium between three forms of each group.

The investigated systems each contain more than 120 ionizable sites, of which all titratable sites were included in the calculations. However, the presentation of the results is limited to those residues which are known to be important for binding and catalysis, particularly those residues which were discussed in the experimental works summarized below. Those residues are listed in Table 1, which also provides information on their role in the protein, and Fig. 2 identifies the location of these residues in the protein.

Structures

Three protein kinase structures were used in this work. For the closed form of the C-subunit we used 1apm (Knighton et al. 1991, 1993), and 1atp (Zheng et al. 1993b), available from the Protein Data Bank (PDB) (Bernstein et al. 1977). Both structures are for the recombinant protein from mouse. In the 1apm structure, the Ser139 residue is mutated into Ala, and the Ser10, Thr197, Ser338 residues are phosphorylated. In the 1atp structure, the Thr197 and Ser338 residues are phosphorylated. Both structures contain coordinates for a peptide inhibitor [PKI(5-24)] and additionally the 1atp structure contains coordinates of bound Mn_2ATP. For the open form of the C-subunit we used a refined 1ctp structure of the porcine C-subunit (Karlsson et al. 1993). It also contains coordinates of the same bound peptide inhibitor. In this structure, residue Thr197 is phosphorylated, and residue 338 is modeled as glycine. The iodine

Table 1 List of titratable residues in PKA discussed in the present work and a description of their role in the protein. The data were collected from recent publications describing crystal structures of PKA (Knighton et al. 1991, 1993; Karlsson et al. 1993; Zheng et al. 1993a,b; Taylor and Radzio-Andzelm 1994)

Residue	Function in PKA
Lys72	Forms ion pairs with α- and β-PO_4 Forms ion pair with Glu91
His87	Forms ion pair with Thr197 phosphate
Glu91	Forms ion pair with Lys72
Glu121	Its α-carbonyl is hydrogen bonded to N^6 amino group on the adenine
Glu127	Binds to the P−3 Arg
Asp166	Is presumed catalytic base
Lys168	Forms ion pair with γ-PO_4 Hydrogen bonded to the α-carbonyl of the P−2 Arg in the peptide
Glu170	Binds to the P−3 Arg Ligand to inhibitory Mg^{2+}
Asp184	Chelates the essential activating Mg^{2+} ion that bridges the β- and γ-phosphates of ATP
Lys189	Forms ion pair with Thr197 phosphate
Thr197	Is essential phosphorylation site in PKA
Glu203	Binds to the P−6 Arg
Glu230	Binds to the P−2 Arg
Asp328	Binds to the P−3 Arg

atoms attached to the Tyr-I7 residue of the inhibitor were removed and the residue was simply treated as an ordinary tyrosine.

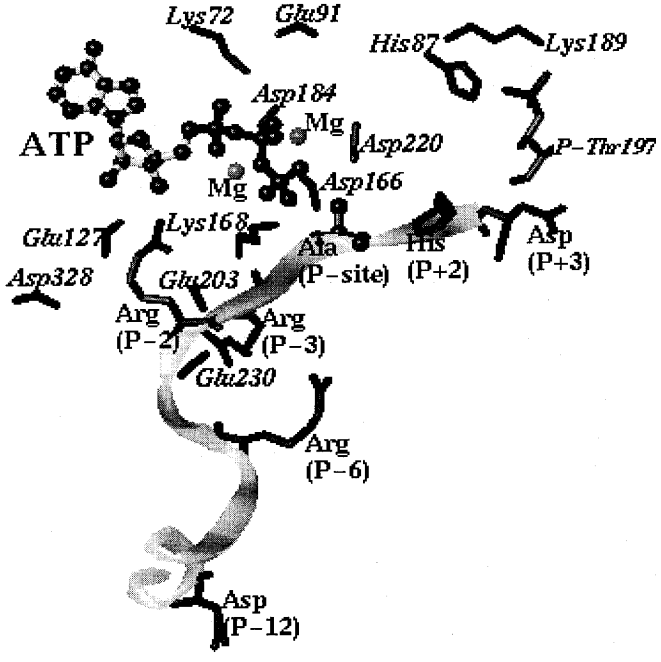

Fig. 2 Diagram of the essential residues that contribute to ATP binding, peptide binding, and catalysis in the ternary complex of the C-subunit of protein kinase with Mn_2ATP and the inhibitor peptide PKI(5-24)

From the above structures, only that representing the open form contains all heavy atoms. In the two first structures some residues are unobserved, presumably because of high mobility in the crystal. The complete closed structure of the recombinant mouse C-subunit with the protein inhibitor and the Mn_2ATP was modeled based on the 1apm file to which the lacking 10 N-terminal residues in the form of an α-helix and lacking side chains of 34 residues were added using the Biopolymer module of the InsightII software (Molecular Simulations 1992). The Mn_2ATP structure was taken from the 1atp file and built with the same relative orientation with respect to the surrounding residues. Similarly, InsightII was used to replace the Ala-I21 residue of the inhibitor with a Ser to generate the model of the substrate.

Polar and aromatic hydrogens, required for the calculations, were added with the HBUILD option (Brunger and Karplus 1988) of CHARMM (Brooks et al. 1983). The CHARMM forcefields allow two possible models for participation of hydrogen atoms in the molecular model: the first includes only polar hydrogens and the second one all hydrogens. For the purpose of the PB approach for predicting ionization equilibria in proteins, an intermediate hydrogen model was introduced; this includes all polar and all aromatic ring hydrogens. This allows for inclusion of potentially important interactions of ionizable groups with aromatic rings of such amino acids as phenylalanine and tyrosine. Hydrogens were added in such a way that the resulting structures were fully protonated, i.e., they corresponded to low pH conditions. The positions of the hydrogens were optimized by 500 steps of steepest descent energy minimization. In one test run all atoms of the side chains, except the CB atoms, were free to move during the minimization procedure.

In the case of several residues, i.e., P-Thr197, Asp166, Glu170, Glu203, and Glu230, we analyzed various combinations of the ionizable sites (OD2 and OD1 atoms for Asp, OE2 and OE1 for Glu, and OT, OC1, and OC2 for P-Thr; the names follow the PDB nomenclature).

Parameters

All simulations were performed at 293 K (RT at this temperature is equal to 0.58 kcal/mol; 1 kcal/mol corresponds to a pK_a shift of 0.75 pH unit), an ionic strength equivalent to 150 mM monovalent salt, and a solvent dielectric constant of 80.

The electrostatic calculations reported here were performed using a CHARMm22 parameter data set (Molecular Simulations Library, San Diego, Calif., USA). One should note that earlier full group calculations, e.g. Antosiewicz et al. (1996b), were performed using the PARSE parameter set (Sitkoff et al. 1994). However, this data set does not contain all required parameters for this study (no data for ATP, nor phosphoamino acids).

The calculations use 4 for the protein (i.e., interior) dielectric constant. It should be noted, however, that the issue of the protein dielectric constant is still an open question (Gilson and Honig 1986; King et al. 1991; Demchuk and Wade 1996; Loeffler et al. 1997; Sham et al. 1997).

As indicated above, phosphate groups of P-Ser and P-Thr were considered to be ionizable. We considered only the change in their charge accompanying proton dissociation from −1 to −2, i.e., the *reference* state for these residues has a single negative charge. This is because our current methodology allows only for a one-step proton dissociation from titratable groups, and it seems that because of the low value of the model pK_{model} for the first dissociation of the phosphate group, single and double ionized states of this group are the most probable ones. The ionizable proton of the phosphate group is assigned $pK_{model} = 7.2$ (Atkins 1994). Values of pK_{model} for standard amino acids are listed elsewhere (Antosiewicz et al. 1994).

In this preliminary study we are not treating ATP as being ionizable, and we consider the Mn_2ATP system to have a total net charge of 0. The partial charges of the ATP atoms were taken from the CHARMm22 parameter set in all cases. The Mn ions each had a charge of +2e.

In calculations where Ala-I21 (P-position) of the inhibitor was replaced by a Ser residue, to model the peptide substrate, the Ser-I21 was considered to be a titratable residue with $pK_{model} = 12.8$. Some other authors estimate this pK_a as approximately 14 (Zhou and Adams 1997). However, because our results indicate that the serine hydroxyl is very hard to ionize (see below), any higher value would make the ionization even harder, and it has no influence on the general conclusions of the present work.

Summary of the experimental data

All available experimental evaluations of the pK_a values of titratable residues in protein kinases (Yoon and Cook 1987; Adams and Taylor 1993; Cox and Taylor 1995; Zhou and Adams 1997) are based on measurements of pH-dependent enzyme kinetics (Tipton and Dixon 1979; Hammes 1982). We are not aware of any NMR spectroscopic studies of protein kinases in this respect, although the technique is particularly well established as a successful tool in pK_a determinations (Jardetzky and Roberts 1981; Wüthrich 1986). Kinetic methods for pK_a determinations are based on the interpretation of the fact that the variation of the initial velocity of enzymatic reactions with pH often gives a "bell-shaped" curve. Such behavior is interpreted in terms of a model in which the enzyme contains only two ionizable groups that are essential for activity (catalysis and/or binding), with only one of the ionized forms of this pair being catalytically active (Tipton and Dixon 1979; Hammes 1982). It should be noted that interpretation of kinetic data on its own is not trivial, having many pitfalls as described elsewhere (Knowles 1976; Brocklehurst 1994). One of the consequences is that it is not easy to indicate which titratable residues are responsible for the bell-shaped dependence of kinetic parameters on pH. However, there are examples in the literature that pK_a values derived from both NMR and kinetic measurements remain in a good agreement with each other (McIntosh et al. 1996; Stivers et al. 1996).

The initial velocity of the noninhibited reaction is (Hammes 1982)

$$v = -\frac{d[S]}{dt} = \frac{V_S[S]}{[S] + K_S} \tag{1}$$

with [S] being concentration of the substrate, V_S the maximal velocity of the reaction, and K_S the Michaelis constant. Kinetic experiments with the protein kinase consisted of initial velocity measurements performed as a function of pH under conditions in which either Mg_2ATP was saturating and the Ser peptide concentration was varied, or the Ser-peptide was saturating and the Mg_2ATP concentration was varied. These two types of measurements lead to two corresponding Michaelis constants, $K_{peptide}$ and K_{ATP}. In the case of the presence of a competitive inhibitor, the initial velocity equation changes into (Hammes 1982)

$$v = \frac{V_S[S]}{[S] + K_S(1 + [I]/K_I)} \tag{2}$$

where [I] is the concentration of the inhibitor which binds to the enzyme with an equilibrium dissociation constant of K_I. The maximal velocity V_S is converted to k_{cat} by dividing V_S by the total enzyme concentration.

The following equations explicitly show what pK_a values are involved in the pH dependencies of particular kinetic parameters. For uninhibited reactions we have (Tipton and Dixon 1979; Hammes 1982)

$$V_S = \frac{\tilde{V}_S}{1 + [H^+]/K_A^{ES} + K_B^{ES}/[H^+]} \tag{3}$$

and

$$\frac{k_{cat}}{K_S} = \frac{\tilde{k}_{cat}/\tilde{K}_S}{1 + [H^+]/K_A^{E} + K_B^{E}/[H^+]} \tag{4}$$

where K_A and K_B are the lower and higher acid dissociation constants, respectively, superscripts E and ES refer to the free enzyme and enzyme-substrate complex, respectively, and $\tilde{V}_S$ and $\tilde{k}_{cat}/\tilde{K}_S$ represent maximal values of V_S and k_{cat}/K_S. Note that the ratio k_{cat}/K_S is dependent only on the ionization constants of the free enzyme, whereas V_S is dependent only on the ionization constants of the enzyme-substrate complex. These functions exhibit a maximum at a definite pH. However, when the pK_a values are well below and well above the pH range of the studies, the observed kinetic parameters can be pH independent. For inhibition studies one can use (Trylska et al. 1999)

$$K_I = \tilde{K}_I \frac{1 + [H^+]/K_A^E + K_B^E/[H^+]}{1 + [H^+]/K_A^{EI} + K_B^{EI}/[H^+]} \quad (5)$$

According to this model, variation of the apparent inhibition constant, K_I, with pH is determined by the pK_a values of the free enzyme and of the enzyme-inhibitor complex. A group whose pK is seen in a k_{cat}/K_S or K_I pH profile may or may not act as an acid-base catalyst in the reaction, but its state of protonation clearly affects binding (Cleland 1982). pK_a values observed in kinetic experiments, i.e., $pK_A \equiv -\log K_A$ and $pK_B \equiv -\log K_B$, are usually molecular pK_a values, i.e., they cannot be attributed to individual residues unless they are well separated by more than 2–3 pH units. Molecular pK_a values obtained in experiments are summarized in Table 2. Based on Eqs. (3)–(5), these data can be interpreted as follows:

1. There are two residues in the catalytic subunit responsible for binding substrates, which have pK_a values of 6.2–6.5 and 8.3–9.3 in the complex of the catalytic subunit with Mg_2ATP.
2. pK_a values of these residues should be shifted significantly below pH 6 and above pH 10, respectively, for the complex of the catalytic subunit with the substrate or inhibitor.
3. The residue responsible for the higher molecular pK_a is probably His87, and is important for recognition of the P + 2 (where P is the number of the phosphorylated amino acid) site of peptide substrates.
4. The residue responsible for the lower molecular pK_a is the one interacting strongly with the P−2 arginine in the substrate, and is probably Glu230.

Zhou and Adams (1997) measured the steady and the pre-steady-state (the ''burst'' phase) kinetics of the protein kinase. Their work was undertaken to verify a hypothesis that the Asp166 residue acts as a general-base catalyst in the reaction catalyzed by the protein kinases. The authors proposed that Asp166 does not have the basicity to catalyze proton abstraction in the ground state but rather is a good hydrogen-bond acceptor.

Results

This section presents pK_a values predicted for inhibited protein kinases in the closed and open forms. In the light of experimental data and their interpretation presented in the previous section, it seems that besides the ternary complex of the closed form of kinase with the Mn_2ATP and the peptide inhibitor, and the binary complex of the open form of kinase with the peptide inhibitor, directly available as structural PDB files, two binary complexes are also of interest. One is the complex of the closed form with the Mn_2ATP and the other is the complex of the closed form with the peptide inhibitor. They were obtained from the ternary complex PDB file by extracting data for the inhibitor or the Mn_2ATP, respectively.

Computed pK_a values for the closed and open forms of PKA

Tables 3 and 4 contain the computed pK_a values and average charges at pH 7 for 14 selected residues of the closed form of the cAMP-dependent protein kinase complexed with Mn_2ATP, as well as the peptide inhibitor [PKI(5-24), with the sequence TTYADFIASGRTGRRNAIHD], and for two possible binary complexes, and for the open form complexed with the same inhibitor, respectively. Data for several choices of the protonation sites on Asp166, Glu170, P-Thr197, and Glu230 are presented. It should be noted that many values outside the usual range of pH in protein studies, i.e., 2–12, are obtained. These values result from very low solvent accessibility of the groups (see Tables 3 and 4) and strong interactions between titratable groups. Such extreme results should be understood as indicating large energetic costs of protonation or deprotonation, depending on the pK_a and the type of site. It should also be kept in mind that the results were obtained for rigid molecular structures.

Table 2 Summary of available experimental results

Substrate or inhibitor	Observed pH-dependent parameter	Molecular pK_a values		Ref
		pK_A	pK_B	
LRRASLG	$k_{cat}/K_{peptide}$	6.2	8.5	Yoon and Cook (1987)
	k_{cat}/K_{ATP}	< 6	> 10	
LRRAALG	$1/K_I$	6.2	8.5	
LRRASLG	$k_{cat}/K_{peptide}$	6.5	9.3	Adams and Taylor (1993)
LRRNSI	$k_{cat}/K_{peptide}$	6.3	9.4	
LARNSI	$k_{cat}/K_{peptide}$	6.3	8.5	
LRANSI	$k_{cat}/K_{peptide}$	–	8.3	
LRRNALG	$1/K_I$	6.5	8.4	
LRRASLG	$k_{cat}/K_{peptide}$	6.5[a]	–[a]	Cox and Taylor (1995)
	k_{cat}	< 5.8[a]	> 10.5[a]	

[a] Results for the mutant H87A

Table 3 Computed apparent pK_a values and average charges at pH 7 of selected residues in PKA in the closed form complexed with the inhibitor and/or Mn_2ATP. In parentheses, the percentage solvent accessibities of the residues for the ternary complex are given

Protonation site on	with inhibitor and Mn_2ATP						with inhibitor		with ATP	
Asp166	OD1	OD1	OD1	OD2	OD2	OD1	OD2	OD1	OD1	OD1
Glu170	OE2	OE2	OE2	OE2	OE2	OE1	OE2	OE2	OE2	OE1
P-Thr197	OT	OC1	OC2	OT	OC2	OT	OC2	OT	OT	OT
Glu230	OE2	OE2	OE2	OE2	OE2	OE1	OE2	OE2	OE2	OE1
residue										
Lys72	25.8	25.8	25.8	25.8	25.8	25.8	21.7	21.7	24.2	24.2
(5)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
His87	7.2	7.1	7.2	7.2	7.2	7.2	8.0	7.8	6.9	6.9
(14) (ND1)	0.6	0.5	0.6	0.6	0.6	0.6	0.8	0.8	0.5	0.5
Glu 91	−3.5	−3.4	−3.5	−3.5	−3.5	−3.5	−3.9	−5.5	−2.6	−2.7
(7) (OE1)	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0
Glu121	5.3	5.3	5.2	5.3	5.3	5.3	5.6	5.5	5.6	5.6
(22) (OE1)	−0.9	−0.9	−0.9	−0.9	−0.9	−0.9	−0.9	−0.9	−0.9	−0.9
Glu127	12.7	12.7	12.7	12.7	12.7	12.7	1.9	1.5	19.0	19.0
(4) (OE2)	0.0	0.0	0.0	0.0	0.0	0.0	−1.0	−1.0	0.0	0.0
Asp166	−17.1	−9.5	−9.6	−14.9	−15.0	−9.5	4.7	−15.1	−3.2	−2.5
(4)	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0
Lys168	14.2	14.0	14.0	13.9	13.9	13.8	18.4	18.6	10.8	10.4
(2)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Glu170	−10.5	−12.5	−12.5	−10.8	−10.8	−19.9	−12.7	−11.3	4.8	1.3
(3)	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−0.9	−1.0
Asp184	−25.1	−25.0	−25.1	−26.1	−26.1	−25.2	−8.3	3.9	−19.7	−19.8
(5) (OD1)	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0
Lys189	11.1	10.9	8.1	11.1	8.1	11.1	8.1	11.2	11.0	11.0
(19)	1.0	1.0	0.8	1.0	0.8	0.8	0.9	1.0	1.0	1.0
Thr197	1.1	3.6	−0.7	1.3	−0.7	1.3	−0.7	1.1	1.5	1.5
(33)	−2.0	−2.0	−2.0	−2.0	−2.0	−2.0	−2.0	−2.0	−2.0	−2.0
Glu203	−2.6	−2.9	−2.9	−2.6	−2.8	−2.3	−3.5	−3.2	5.8	5.2
(4) (OE2)	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−0.8	−0.8
Glu230	−20.4	−20.5	−20.5	−20.3	−20.4	−12.1	−20.4	−20.6	2.7	7.8
(1)	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−0.9	−0.2
Asp328	3.6	3.7	3.7	3.7	3.7	3.7	4.6	4.6	4.2	4.2
(61) (OD2)	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0	−1.0

One of the most significant predictions is that the important residue P-Thr197 has a markedly decreased pK_a for dissociation of the second proton in the closed form of the kinase, leading to the dianionic form of the phosphate group at pH 7. The computed pK_a depends on the choice of the protonation site, but in all cases the predicted charge of the group at pH 7 is −2 units of the elementary charge. This is also the predicted result for the kinase Mn_2ATP complex and for the kinase-inhibitor complex in the closed form, as well as for the apoenzyme (data not shown). Neither the inhibitor nor the Mn_2ATP have an influence on the pK_a of the P-Thr197 phosphate. On the other hand, for the open form, P-Thr197 is expected to have a less negative charge (between −1.6 and −1.1) at pH 7. Surprisingly, the pK_a values of His87 in the closed and open forms seem to be different than expected. In the closed form, the His87 residue has only a moderately elevated pK_a value over the pK_{model} (i.e., 6.3). This might be surprising in light of the anticipated role of this residue in the electrostatic interaction with P-Thr197, stabilizing the closed form (Cox et al. 1994). However, it should be noted that accuracy of predicted pK_a values is not better than approximately 1 unit of pH (Antosiewicz et al. 1996a, b). On the other hand, in the open form the pK_a of His87 is markedly increased in the complex and this increase is due to its interaction with the inhibitor. The shift in the pK_a of P-Thr197, in comparison to its model compound value, results from interactions with surrounding titratable groups. The most pronounced electrostatic interactions are with His87, Arg165, and Lys189. In the open form, interactions with His87 and Arg165 are significantly reduced, and interaction with Lys189 remains almost the same.

A surprising prediction for the open form is the very high pK_a of Asp166 and the relatively low pK_a of Lys168. These two residues interact strongly with each other, the result of which is that the total charge of this pair is zero, similar to the case of the closed form of the enzyme. This prediction can also be viewed as resulting

Table 4 Computed apparent pK_a values and average charges at pH 7 of selected residues in PKA in the open form complexed with the inhibitor

Protonation site on					
Asp166	OD2	OD2	OD2	OD1	OD2
Glu170	OE1	OE1	OE1	OE1	OE2
P-Thr197	OT	OC1	OC2	OC2	OT
Glu230: residue	OE1	OE1	OE1	OE1	OE2
Lys72	8.0	8.0	8.0	8.2	8.1
(22)	0.7	0.7	0.7	0.7	0.7
His87	10.5	10.5	10.5	10.6	10.5
(27) (NE2)	0.9	0.9	0.9	0.9	0.9
Glu91	−2.6	−2.6	−2.6	−2.4	−2.6
(17) (OE2)	−0.9	−0.9	−0.9	−0.9	−0.9
Glu121	6.3	6.3	6.3	6.3	6.3
(34) (OE2)	−0.8	−0.8	−0.8	−0.8	−0.8
Glu127	14.4	14.4	14.4	14.5	14.3
(4) (OE2)	0.0	0.0	0.0	0.0	0.0
Asp166	17.5	17.5	17.5	18.4	17.4
(10)	0.0	0.0	0.0	0.0	0.0
Lys168	6.3	6.3	6.3	0.9	6.4
(6)	0.3	0.3	0.3	0.0	0.3
Glu170	−14.2	−14.2	−14.2	−14.5	−10.6
(3)	−1.0	−1.0	−1.0	−1.0	−1.0
Asp184	2.0	2.0	2.0	4.0	2.0
(49) (OD2)	−1.0	−1.0	−1.0	−0.9	−1.0
Lys189	13.7	13.9	13.0	12.8	13.7
(24)	1.0	0.9	1.0	1.0	1.0
Thr197	6.7	7.3	8.1	8.0	6.7
(37)	−1.6	−1.4	−1.1	−1.1	−1.6
Glu203	−6.4	−6.4	−6.4	−6.6	−6.4
(3) (OE2)	−1.0	−1.0	−1.0	−1.0	−1.0
Glu230	−19.8	−19.8	−19.8	−20.0	−19.0
(1)	−1.0	−1.0	−1.0	−1.0	−1.0
Asp328	3.5	3.5	3.5	3.5	3.5
(40) (OD2)	−1.0	−1.0	−1.0	−1.0	−1.0

from the particular features of our modeling, described in the Methods section. During preparation of the structural data for calculations, the amino group of the Lys residue and the carboxyl group of the Asp residue were fully protonated and apparently subsequent optimization of the proton positions yielded a configuration such that the proton on Asp166 is quite stable, resulting in its high pK_a value.

Electrostatic interactions important for peptide recognition and binding

Tables 5 and 6 show all interaction energies that are at least kT in absolute value, between kinase residues and the inhibitor residues, for the closed and open forms of the kinase, respectively. It is interesting that Asp-I9 of the inhibitor is not involved in any electrostatic interactions with the enzyme exceeding the kT level. The

Table 5 Interaction energies (kcal/mol) between titratable residues, in their fully ionized states, in the closed form of PKA and the inhibitor. The presented data are for OD2 as the protonation site on Asp166 and OT as the protonation site on P-Thr197. Empty fields mean that the absolute value of the interaction energy is smaller than kT

Residue	Residue						
	Asp I9	Arg I15	Arg I18	Arg I19	His I23	Asp I24	Ter-C I24
Lys72			1.0				
His87					0.9		
Glu91			−0.7		−0.6		
Glu127			−11.5	−1.3			
His131			0.6	0.6			
Arg133		1.0	0.8	0.6			
Tyr146				0.8			
Tyr164			1.0	1.6			
Arg165							
Asp166		−0.7	−1.4	−2.2	−0.6		
Lys168		1.0	2.7	4.2			
Glu170		−1.5	−3.4	−18.1			
Asp184			−1.5	−1.0			
Lys189							
Thr197							
Glu203		−14.0		−6.9			
Tyr204		−1.2	−0.8	−8.5			
Asp220				−1.1			
Glu230		−1.4	−0.8	−14.6			
Tyr247		−0.9					0.8
Asp328			−1.2				
Tyr330			−3.7				

Table 6 Interaction energies (kcal/mol) between titratable residues, in the fully ionized state, in the open form of PKA and the inhibitor. The presented data are for OD2 as the protonation site on Asp166 and OC2 as the protonation site on P-Thr197. Empty fields mean that absolute value of the interaction energy are smaller than kT

Residue	Residue						
	Asp I9	Arg I15	Arg I18	Arg I19	His I23	Asp I24	Ter-C I24
Lys72			1.0		0.7	−1.0	
His87					0.8	−2.6	−3.7
Glu91			−1.0		−0.7	1.1	
Glu127			−6.3	−1.1			
Arg133		1.1		7.0			
Tyr146				−0.7			
Tyr164			−0.9	−1.2	−1.0	0.6	
Arg165					1.2		−1.2
Asp166			−1.1	−1.3	−3.7	1.2	
Lys168		1.0	1.5	3.4	1.9	−0.8	
Glu170		−1.1	−2.5	−10.0	−0.7		
Asp184			−1.4		−1.0	1.1	
Lys189							
Thr197							
Glu203		−13.8		−9.4	−0.6		
Tyr204		−1.3	−0.6	−9.7	−0.8		
Tyr215					−1.2		0.8
Asp220				−1.1	−1.1		
Tyr229				−0.8			
Glu230		−1.5		−13.1			
Asp241		−0.6					
Tyr247		−1.0			−0.7		

most important interactions for binding the three Arg residues of the inhibitor are those with Glu230, Glu203, Glu170, and Glu127, for both the closed and open forms. The interaction energies listed in Tables 5 and 6 are responsible for dramatic downward shifts of pK_a values of the three first acidic residues. In the case of Glu127, the large upward shift in its p$K_{intrinsic}$ is sufficient to keep the pK_a high, although the interactions with Arg residues of the inhibitor are also large.

It can be seen that in addition to the Arg residues at positions P−2, P−3, and P−6, the residues on the C-terminal side of the P-position in the inhibitor are also involved in substantial electrostatic interactions with the enzyme. These interactions are particularly numerous and significant for the open form. The interactions between His87 and Asp-I24 with the C-terminus of the inhibitor are responsible for the large upward shift of the pK_a of His87 in the complex of the open form with the inhibitor. The differences in the interactions with this side of the inhibitor, between the open and closed forms of the kinase, seem quite interesting.

Protonation equilibria and interactions of the Ser residue in the peptide substrate

Phosphorylation of the Ser residue in the peptide substrate by PKA requires dissociation of the hydroxyl proton from the substrate. Therefore, a question arises about the pK_a of the Ser residue in the peptide substrate, and some calculations were performed for the ternary complex with the Ala residue at the P-position in the inhibitor replaced by the Ser residue. Calculations were performed for two possible neutral forms of the carboxyl group of the Asp166 residue, which is situated in proximity to the hydroxyl of the peptide substrate. Calculations result in extremely high predicted pK_a values of Ser-I21 of the substrate, exceeding a value of 40, so that there is a large upward shift from the model pK_a. Part of this shift results from desolvation effects, but a more substantial shift results from the interactions with other titratable groups, particularly with Asp166 and Asp184, preventing ionization of Ser. However, interaction with positively charged Lys168 would also be substantial for the charged form of Ser-I21. Neutralization of the Asp166 side chain leads to a substantial decrease in the pK_a value of Ser-I21 of the peptide substrate (by 7 pK_a units), but this is not enough to allow the Ser residue to be ionized at around pH 7. We also computed the pK_a shifts for the Ser-I21 following neutralization of some other acidic residues, and compared our results with those reported elsewhere (Tsigelny et al. 1996). We obtain similar shifts but it does not clear up the problem because the pK_a of the Ser-I21 residue remains still very high. On the other hand, forcing Ser-I21 to be ionized causes large upward shifts of pK_a values of surrounding acidic residues, although all are well below pH 2. However, some structural changes could lead to a decrease in the pK_a of the Ser residue of the substrate with a simultaneous significant increase in the pK_a of Asp166. Investigation of such a possibility is beyond the scope of the present work.

Computed pK_a values for the closed form of the enzyme complexed with Mn_2ATP

Because the pH dependence of $k_{cat}/K_{peptide}$ for the C-subunit should correspond to the complex of kinase with ATP, a more detailed investigation of the kinase-Mn_2ATP structure, obtained from the ternary complex, was carried out.

Acidic residues Glu230, Glu203, and Glu170, which interact strongly with the Arg residue at the P-2 position (see Table 5), were included in a series of calculations where different protonation sites on the carboxylic groups were chosen. Table 7 shows all individual predicted pK_a values and the result of the merge procedure developed by Gilson and co-workers (Trylska et al. 1999), which produces an averaged result for cases with several possible protonation states for the three chosen titratable residues in the protein. From this table it can be seen that the pK_a value obtained for Glu230 by using the merge procedure is close to the pK_A values obtained

Table 7 Computed apparent pK_a values and average charges at pH 7 of selected residues in PKA in the closed form with Mn_2ATP. OC2 on P-Thr197 and OD2 on Asp166 are the protonation sites

Protonation site on Glu170 Glu203 Glu230 residue									All data merged
	OE2 OE2 OE2	OE2 OE2 OE1	OE1 OE2 OE1	OE1 OE2 OE2	OE2 OE1 OE2	OE2 OE1 OE1	OE1 OE1 OE1	OE1 OE1 OE2	
His87 (14) (NE2)	7.7 0.7	7.6 0.7	7.6 0.7	7.7 0.7	7.7 0.7	7.6 0.7	7.6 0.7	7.7 0.7	7.6 0.7
Glu170 (34)	5.0 −0.9	3.1 −1.0	1.6 −1.0	2.2 −1.0	5.1 −0.9	3.1 −1.0	1.6 −1.0	2.0 −1.0	2.7 −1.0
Glu203 (32)	5.8 −0.8	5.2 −1.0	5.2 −0.9	6.1 −0.8	5.2 −0.9	4.6 −1.0	4.7 −1.0	5.5 −0.9	5.0 −1.0
Glu230 (5)	2.8 −0.9	8.2 −0.1	7.7 −0.2	4.2 −1.0	2.8 −0.9	7.8 −0.2	7.8 −0.2	4.5 −1.0	7.5 −0.3

from kinetic studies (Yoon and Cook 1987; Adams and Taylor 1993; Cox and Taylor 1995; Zhou and Adams 1997), whereas the pK_a values obtained for Glu203 and Glu170 are too low. For His87, the calculated value is close to the observed pK_B values. Therefore, one can expect that microscopic pK_a values calculated for Glu230 and His87 can result in reasonable molecular pK_A and pK_B values when both are considered together as residues responsible for binding and/or catalysis of the kinase. Figure 3 shows the total charge on His87 and Glu230 as a function of pH. The molecular pK values resulting from this dependence are pH points at which the charge is 0.5 and −0.5 units of the elementary charge. These points correspond to pK_A = 6.9 and pK_B = 8.3, close to the experimental values. Also, representation of the population of the monoprotonated His87-Glu230 pair as a function of pH would lead to the same pK_a values.

Discussion

Assessment of the pK_a calculations

The present work uses the so-called full-group titration methodology (Antosiewicz et al. 1996a) and calculations are performed assuming a low dielectric constant of the solute. Another titration methodology, called the single-site model of titration, which uses a solute dielectric constant of 20, was shown to give slightly more accurate results than the full method with a protein dielectric constant of 4 (Antosiewicz et al. 1996b). However, the full-group method uses more a realistic charge distribution for ionized groups. Also, in a recent study of HIV-1 protease (Trylska et al. 1999) it was shown that the full-charge/4 calculations apparently agree better with experiment than the single-site/20 calculations for residues inaccessible to bulk solvent and conformationally restrained.

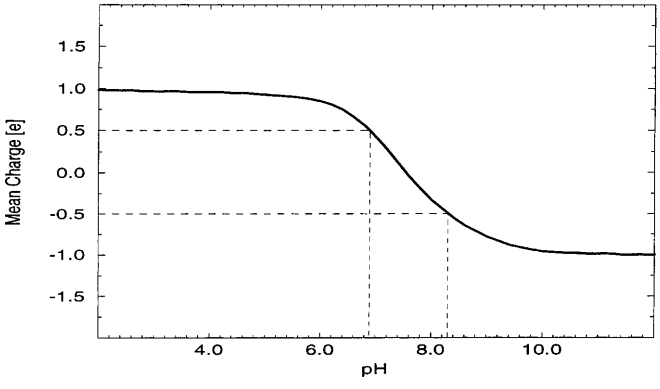

Fig. 3 Mean total charges of His87 and Glu230 residues in the complex of protein kinase with Mn_2ATP as a function of pH. The values of 0.5 (pH = 6.9) and −0.5 (pH = 8.3) units of the elementary charge correspond to molecular pK_a values visible in kinetic experiments

The pK_a values which show exaggerated shifts from those characteristic for isolated groups in aqueous solvent should be interpreted as an indication that the group remains fixed in a single ionization state at any pH for which the complex is actually stable in solution, provided that this structure is close to that found in the crystal. These fixed protonation states can be in principle verified by NMR spectroscopy methods. However, some large shifts in the pK_a values can result from problems with the structure. The method is run in such a way that, at the beginning, all polar and aromatic hydrogens, including the titratable ones, are added to the structure. Therefore, the number of added hydrogens is quite substantial and some conflicting pairs can appear, resulting in orientations of titratable hydrogens which render them too acidic or basic. In order to remove at least some of such problems and to check how sensitive the computed pK_a values are to local details of the structure, we ran the titration procedure in such a way that during the addition of protons, minimization of their orientations was performed with all side chain atoms beyond C_β allowed to move. Only main chain and C_β atoms were fixed during minimization. The difference in comparison to minimization including only hydrogens was not large – the titration curves for the whole protein differed by 1 unit of the elementary charge at the most (at pH 8), which is not much when one considers that there are 131 titratable sites in the protein. However, there were some significant changes in individual pK_a values. The pair of interacting residues Asp166 and Lys168 changed from both charged groups to both neutral in the pH range 2–12. The total charge of the pair remained unchanged, which is rather the expected result. The other significant shift in pK_a was 3.5 for Asp184 instead of −25.1, which is also the expected stabilization of the neutral state. However, the pK_a of Glu127 was predicted to be 7.9 instead of the original value of 12.7; therefore, stabilization of the neutral state is decreased. All this shows that manipulations of the structures used for pK_a calculations should be done with a great care. It will be always necessary to have some independent confirmation of the validity of structures used in the calculations, e.g. from NMR spectroscopy.

Electrostatic properties of PKA derived from the predicted pK_a values

Comparison between the pK_a of P-Thr197 in Tables 3 and 4 shows that there is a difference in the predicted ionization state of the phosphate group attached to threonine 197 in the closed and open conformations of the kinase. In the closed form, the pK_a of the second deprotonation of the phosphate group is shifted to low pH values and at pH 7 the predicted charge of the phosphate group is −2. In the open form, the predicted pK_a is several pH units higher and the predicted charge

at pH 7 is close to −1. This difference seems interesting because interactions between P-Thr197 and His87 were claimed to be the most significant electrostatic interactions between the two lobes (Cox and Taylor 1995). This prediction is confirmed for several choices of the protonation sites on the phosphate group and the carboxyl groups of the surrounding acidic residues; therefore it can be considered as reasonably plausible.

An interesting observation is that His87 has more significant electrostatic contacts with titratable residues on the C-terminus of the peptide in the open form of the kinase than in the closed form (see Tables 5 and 6). It should be remembered that the interaction energies listed in Tables 5 and 6 refer only to the interaction energy gain or loss when both interacting residues are ionized in comparison to the situation when they are neutral. There are obviously other contributions to the binding energy between the enzyme and its peptide substrate. However, the numbers provided in Tables 5 and 6 are also useful regarding analysis of the binding affinity in protein kinases. In the closed form, most of the electrostatic interactions with the peptide are due to three Arg residues of the latter (at P−6, P−3, and P−2 positions). In the open form the number of interactions greater than kT is significantly increased on the P+ side of the peptide in comparison to the closed form. On the other hand, the total interaction energy involving the Arg residues in the peptide is −82.5 kcal/mol in the closed form and −66.5 kcal/mol in the open form. Net values of the interaction energies on the P+ side are much smaller: for the closed form this is −0.3 kcal/mol and for the open form it is −11.4 kcal/mol. This balance does not include a contribution to the interaction energy due to phosphorylated serine in the peptide substrate. However, these results might indicate that the part of the inhibitor between its P-position and C-terminus is important for the recognition process, as was already suggested by experimentalists (Cox and Taylor 1995).

Another interesting problem arises concerning protonation equilibria of the hydroxyl group of the Ser-I21 of the peptide substrate and the function of Asp166 in protein kinases. A hypothesis was presented which invokes the participation of Asp166 as a general-base catalyst (Zhou and Adams 1997). Although the presented calculations predict extreme pK_a values for Ser-I21 of the peptide substrate and Asp166 residues, some interesting features can be seen. These are huge changes in pK_a values of Asp166 when going from the closed structure to the open structure (see Tables 3 and 4), a large upward shift in the pK_a of Asp166 when Ser-I21 ionizes, and a large downward shift in the pK_a of Ser-I21 when Asp166 is neutralized. These results make plausible that during some structural change accompanying formation of a transition state, both residues can shift their pK_a values to the level allowing for exchange of the proton between them at the pH of action of the enzyme.

Finally, the results of calculations presented in this work qualitatively correlate with suggestions made by experimentalists that Glu230 (Adams and Taylor 1993) and His87 (Cox and Taylor 1995) are responsible for the pK_A and pK_B observed in the kinetic experiments. In a previous section (see above) it was argued that the dependence of $k_{cat}/K_{peptide}$ on pH should be confronted with predictions made for the binary complex of the closed form of kinase with Mn_2ATP. Molecular pK_a values derived from microscopic pK_a values for these two residues are in reasonable agreement with the experimental data. Further evidence in favor of these two residues comes from experimental data of k_{cat}/K_{ATP}, which should be compared to predictions for the binary complexes of the closed and open forms with the inhibitor. In the binary complex of both closed and open forms with the inhibitor, the pK_a of Glu230 is shifted downward, well below pH 6. This explains why there is no pK_A in the pH dependence of V_S [see Eq. (3)] and why inhibition studies reveal the same pK_A values as those of $k_{cat}/K_{peptide}$ [see Eq. (5)]. For His87, the predictions are not so successful since the corresponding shifts are much smaller. It should be noted, however, that in the experimental work the sequence of peptides and inhibitors was different from the sequence of the inhibitor used in the calculations. This can partially explain why the agreement is not fully satisfactory. For Glu230, the differences were probably not important because the predicted effect is huge. For His87, details of the environment and conformation are apparently more important. From Table 4 it can be seen that in the binary complex the pK_a of His87 is already elevated to the upper limit of the experimental pH range. For the closed form of the kinase (see Table 3), the pK_a shift is much smaller. Differences in the interactions of His87 with peptides used in experiments and with the inhibitor used in the calculations are probably responsible for less successful agreement.

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References

Adams JA, Taylor SS (1993) Phosphorylation of peptide substrates for the catalytic subunit of cAMP-dependent protein kinase. J Biol Chem 268: 7747–7752

Antosiewicz J, McCammon JA, Gilson MK (1994) Prediction of pH-dependent properties of proteins. J Mol Biol 238: 415–436

Antosiewicz J, Briggs JM, Elcock AE, Gilson MK, McCammon JA (1996a) Computing the ionization states of proteins with a detailed charge model. J Comput Chem 17: 1633–1644

Antosiewicz J, McCammon JA, Gilson MK (1996b) The determinants of pK_as in proteins. Biochemistry 35: 7819–7833

Antosiewicz J, Bachut-Okrasińska E, Grycuk T, Briggs JM, Włodek ST, Lesyng B, McCammon JA (1999) Prediction of pK_as of titratable residues in proteins using a Poisson-Boltzmann model of the solute-solvent system. In: Deuflhard P,

Hermans J, Leimkuehler B, Mark A, Skeel R, Reich S (eds) Lecture Notes on Computer Science and Engineering, vol 4. Springer, Berlin Heidelberg New York, pp 176–196

Atkins PW (1994) Physical chemistry. Freeman, New York

Bernstein FC, Koettzle TF, Williams GJB, Meyer EF, Brice MD, Rodgers JR, Kennard O, Shimanouchi T, Tasumi MJ (1977) The protein data bank: a computer-based archival file for molecular structures. J Mol Biol 123: 557–594

Brocklehurst K (1994) A sound basis for pH-dependent kinetic studies on enzymes. Protein Eng 7: 291–299

Brooks BR, Bruccoleri RE, Olafson BD, States DJ, Swaminathan S, Karplus M (1983) CHARMM: a program for macromolecular energy, minimization, and dynamics calculations. J Comput Chem 4: 187–217

Brunger AT, Karplus M (1988) Polar hydrogen positions in proteins: empirical energy placement and neutron diffraction comparison. Proteins Struct Funct Genet 4: 148–156

Cleland WW (1982) The use of pH studies to determine chemical mechanisms of enzyme-catalyzed reactions. Methods Enzymol 87: 390–405

Cox S, Radzio-Andzelm E, Taylor SS (1994) Domain movements in protein kinases. Curr Opin Struct Biol 4: 893–901

Cox S, Taylor SS (1995) Kinetic analysis of cAMP-dependent protein kinase: mutations at histidine 87 affect peptide binding and pH dependence. Biochemistry 34: 16203–16209

Demchuk E, Wade RC (1996) Improving the continuum dielectric approach to calculating pK_as of ionizable groups in proteins. J Phys Chem 100: 17373–17387

Gilson MK, Honig BH (1986) The dielectric constant of a folded protein. Biopolymers 25: 2097–2119

Gilson MK (1993) Multiple-site titration and molecular modeling: two rapid methods for computing energies and forces for ionizable groups in proteins. Proteins Struct Funct Genet 15: 266–282

Hammes GG (1982) Enzyme catalysis and regulation. Academic Press, New York

Hanks SK, Quinn AM, Hunter T (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science 241: 42–52

Jardetzky O, Roberts GCK (1981) NMR in molecular biology. Academic Press, New York

Karlsson R, Zheng J, Zheng N, Taylor SS, Sowadski JM (1993) Structure of the mammalian catalytic subunit of cAMP-dependent protein kinase and an inhibitor peptide displays an open conformation. Acta Crystallogr Sect D 49: 381–388

King G, Lee FS, Warshel A (1991) Microscopic simulations of macroscopic dielectric constants of solvated proteins. J Chem Phys 95: 4366–4377

Knighton DR, Zheng J, Eyck LFT, Ashford VA, Xuong N, Taylor SS, Sowadski JM (1991) Crystal structure of the catalytic subunit of cAMP-dependent protein kinase. Science 253: 407–420

Knighton DR, Bell SM, Zheng J, Eyck LFT, Xuong N, Taylor SS, Sowadski JM (1993) 2.0 Å refined crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with a peptide inhibitor and detergent. Acta Crystallogr Sect D 49: 357–361

Knowles JR (1976) The intrinsic pKa-values of functional groups in enzymes: improper deductions from the pH-dependence of steady-state parameters. CRC Crit Rev Biochem 4: 165–173

Loeffler G, Schreiber H, Steinhauser O (1997) Calculation of the dielectric properties of a protein and its solvent: theory and a case study. J Mol Biol 270: 520–534

McIntosh LP, Hand G, Johnson PE, Joshi MD, Körner M, Plesniak LA, Ziser L, Wakarchuk WW, Withers SG (1996) The pK_a of the general acid/base carboxyl group of a glycosidase cycles during catalysis: a ^{13}C-NMR study of *Bacillus circulans* xylanase. Biochemistry 35: 9958–9966

Molecular Simulations (1992) InsightII. Molecular Simulations, Waltham, Mass

Sham YY, Chu ZT, Warshel A (1997) Consistent calculations of pK(a)'s of ionizable residues in proteins: semi-microscopic and microscopic approaches. J Phys Chem 101: 4458–4472

Sitkoff D, Sharp KA, Honig B (1994) Accurate calculation of hydration free energies using macroscopic solvent models. J Phys Chem 98: 1978–1988

Stivers JT, Abeygunawardana C, Mildvan AS (1996) 4-Oxalocrotonate tautomerase: pH dependence of catalysis and pK_a values of active site residues. Biochemistry 35: 814–823

Taylor SS, Radzio-Andzelm E (1994) Cyclic AMP-dependent protein kinase. In: Woodgett JR (ed) Protein kinases. IRL Press, Oxford

Tipton KF, Dixon HBF (1979) Effects of pH on enzymes. Methods Enzymol 63: 183–234

Trylska J, Antosiewicz J, Geller M, Hodge CN, Klabe RM, Head MS, Gilson MK (1999) Thermodynamic linkage between the binding of protons and inhibitors to HIV-1 protease. Protein Sci 8: 180–195

Tsigelny I, Grant BD, Taylor SS, Ten Eyck LF (1996) Catalytic subunit of cAMP-dependent protein kinase: electrostatic features and peptide recognition. Biopolymers 39: 353–365

Wüthrich K (1986) NMR of proteins and nucleic acids. Wiley, New York

Yoon M-Y, Cook PF (1987) Chemical mechanism of the adenosine cyclic 3′,5′-monophosphate dependent protein kinase from pH studies. Biochemistry 26: 4118–4125

Zheng J, Knighton DR, Eyck LFT, Karlsson R, Xuong N, Taylor SS, Sowadski JM (1993a) Crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with mgatp and peptide inhibitor. Biochemistry 32: 2154–2161

Zheng J, Trafny EA, Knighton DR, Xuong N, Taylor SS, Eyck LFT, Sowadski JM (1993b) 2.2 Å refined crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MnATP and a peptide inhibitor. Acta Crystallogr Sect D 49: 362–365

Zhou J, Adams JA (1997) Is there a catalytic base in the active site of cAMP-dependent protein kinase? Biochemistry 36: 2977–2984