Snapping of the Carboxyl Terminal Tail of the Catalytic Subunit of PKA onto Its Core: Characterization of the Sites by Mutagenesis†

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ABSTRACT: A set of 45 mutants of the carboxyl terminal tail of the PKA catalytic subunit was prepared and used to assess the contribution of this tail to the structure and function of the kinase. Ala substitutions of Asp 323, Phe 327, Glu 333, and Phe 350 resulted in a complete loss of enzymatic activity. Other replacements by Ala (Phe 314, Tyr 330, Glu 332, and Phe 347) brought about either a drop in activity to less than 10% of the wild-type enzyme or a reduction of affinity toward ATP (Lys 317, Lys 319, Tyr 330, and Glu 332) or toward Kemptide (Ile 315, Tyr 330, Val 337, Ile 339, Lys 345, and Glu 346). Mutations of Ser 338, a major autophosphorylation site of PKA, by Ala, Glu, Asp, Gln, and Asn showed that the kinetic parameters of these mutants are similar to those of the wild-type. The contribution of each of these tail mutations to the structure and stability of the kinase was assessed by monitoring its effect on the heat stability (when measurable) or by determining the susceptibility of the mutant kinase to cleavage by the Kinase Splitting Membranal Proteinase/Meprin β. Here we show that the tail of PKA has a key role in creating the active conformation of the kinase. It does so by means of specific amino acid residues, which act as "snapping points" to embrace the two lobes of the kinase and orient them in the correct juxtaposition for substrate docking, biorecognition, and catalysis.

The catalytic subunit of Protein Kinase A (PKA)[1] (*1*, *2*) is composed of a conserved catalytic core (residues 40−300), a "head" (residues 1−39), and a "tail" (residues 301−350) (*3*−*5*). The head and the tail are distinctly different in the various kinases (*6*) and could thus be involved in determining their structure, their specificity, and their cellular localization. The tail in PKA embraces the two lobes of the core and keeps them together (*4*, *7*, *8*).

We have previously shown that PKA has a malleable structure, which is reflected inter alia in its tail (*9*−*11*). This finding was subsequently confirmed and extended by X-ray crystallography, by small-angle X-ray scattering, and by molecular dynamics simulations, that demonstrated the occurrence of a loose ("open") conformation and a tightly packed ("closed") conformation (*6*, *7*, *12*−*15*). We have also shown that, in the nonligated kinase, the loose tail of PKA is susceptible to a specific proteolytic cleavage by a kinase splitting membranal proteinase (KSMP) discovered in our laboratory (*16*−*18*). The identification of the specific cleavage site in PKA (*19*) further confirmed the predictions regarding the conformational flexibility of this kinase (*11*, *20*, *21*), which was also demonstrated biochemically and physicochemically by low-angle neutron scattering (*22*), by circular dichroism (*23*, *24*), and by chemical modification with a water-soluble carbodiimide (*25*). The importance of the carboxyl terminal tail for the activity of the kinase was also shown with truncated mutants of the enzyme (*26*).

Upon binding of substrates, the two lobes of the core close the cleft between them (*7*, *27*). This structural change (*21*) involves also a translocation of the tail, which moves the phenolic hydroxyl of Tyr 330 from a distance of ∼10 Å (in the open conformation) down to a distance of ∼3 Å (in the closed conformation) from the Arg residue at position P-3 of the PKA consensus sequence (*28*, *29*).

Here we report a systematic analysis of a series of mutations-to-Ala in the tail of PKA. This analysis sheds light on the contribution of the individual amino acids to the structure, stability, and catalytic activity of the kinase.

MATERIALS AND METHODS

Preparation of Single Site Mutants. The wild-type murine catalytic subunit of PKA, cloned in the pRSET-B vector, served as a template for site-directed mutagenesis. Mutations were introduced using the Polymerase Chain Reaction (PCR) (see Ausbel, F. M., et al. *Curr. Protoc. Mol. Biol.*). The wild-type- or the mutant enzyme-carrying vector was used for transformation of the *Escherichia coli* BL21(DE3) strain. The conditions for induction and expression of the protein were described elsewhere (*30*, *31*). Expression was allowed to proceed for 4 h after induction, and then the bacteria were collected by centrifugation and lysed in an ultrasound disintegrator using 20 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 1 mM dithiotreitol, and 0.1% octyl-β-D-glucopyranoside. The insoluble particles were removed by centrifugation, and the supernatant was collected. The enzyme content was measured following SDS−PAGE (*32*) by immunoblotting with antibodies specific to the catalytic subunit of PKA (anti-P3) (*33*), using an ECL detection system (Amersham,

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[1] Abreviations: PKA, protein kinase A; C, catalyic subunit of protein kinase A; KSMP, kinase splitting membranal proteinase.

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U.K.). The bands in the X-ray films were quantitated by densitometer scanning.

Kinetic Parameters of the Mutant Kinases. The kinase assays were carried out as described earlier (*21*) using Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) as a substrate. K_m and V_{max} values were determined from Lineweaver−Burk plots of inverted initial velocity vs inverted substrate concentrations, according to the following equation (*34*):

$$1/v = 1/V_{max} + K_m/V_{max} \times [S]$$

where V_{max} is the maximal velocity, v is the initial velocity, [S] is the concentration of the substrate, and K_m is the Michaelis constant for that substrate. The results presented are the mean ± S.D. of at least three measurements.

Thermal Stability of the Enzyme and Its Mutants. Aliquots (40 μL) of the recombinant catalytic subunit (1−40 ng/mL, depending on the specific activity of the expressed kinase mutant) were incubated in the presence or in the absence of Kemptide (120 μM), at different temperatures for 2 min, in a buffer containing 20 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$, 1 mM dithiotreitol, and 0.1% octyl-β-D-glucopyranoside, and then the residual activity was measured in the kinase assay. The results presented are the mean ± S.D. of at least three measurements.

Assay of the KSMP/Meprin β Cleavage of Recombinant PKAs. The wild-type catalytic subunit of PKA and its mutants were radiolabeled with [^{35}S]methionine using the TNT Coupled Reticulocyte Lysate System (Promega). The KSMP/meprin β cleavage was allowed to proceed at 23 °C for 0, 3, 5, 10, and 20 min using a recombinant meprin β expressed in 293 cells by Dr. A. Chestukhin and L. Litovchick (*35*). The cleavage was arrested by adding Laemmli sample buffer (*32*), and boiling (5 min at 95 °C). The cleavage products were separated by SDS−PAGE (*32*). The protein bands were visualized by autoradiography, then quantitated by densitometer scanning. The initial rates of cleavage were calculated in comparison to the initial rate of cleavage of the wild-type C-subunit (taken as 100%).

Visualization and Analysis of the X-ray Structures. The following 3-D structures were used in this study. (i) The structure of the closed conformation was obtained from the ternary complex of the murine recombinant C-subunit with the inhibitor peptide PKI_{5-24} and MnATP (*12*) (refined to 2.2 Å) (Brookhaven National Laboratory Protein Data Bank, code 1ATP, R1ATPSF). (ii) The structure of the open conformation was obtained from the binary complex of the porcine heart C-subunit with the di-iodinated PKI_{5-24} (*36*), (structure refined to 2.9 Å) (Brookhaven National Laboratory Protein Data Bank, code 1CTP, R1CTPSF). (iii) The structure of the "intermediate" conformation was obtained from the binary complex of the C-subunit with adenosine (*37*) (refined to 2.6 Å) (Brookhaven National Laboratory Protein Data Bank, code 1BKX). The analysis of the 3-D structures was performed using the RasMol program (Version 2.6).

RESULTS

Role of the Carboxyl Terminal Tail of PKA by Mutagenesis—Approach and Goals. The interpretation of structure−function studies of proteins on the basis of mutagenesis experiments is sometimes not conclusive. It is now known that even single site mutations can sometimes bring about local conformational changes in the protein which affect its function. Eriksson et al. (*38*) showed that "cavity-creating mutations" significantly affect the stability of a protein. They showed that the removal of a native side chain in a wild-type enzyme results in a rearrangement of the surrounding amino acid side chains, in their attempt to cope with the new thermodynamic set of conditions created by the vacated space. Therefore, in studies involving mutagenesis, it is important to monitor the structural change of the mutants and to establish whether their change in function is due to the chemical alteration of the amino acid side chain itself or to a local or gross denaturation process in the protein.

This study was designed to get an insight into the possible structural and functional role(s) of the carboxyl terminal tail (residues 301−350) of PKA. This tail was shown earlier to be indispensable for PKA activity and its regulation (*16*−*19*).

Our approach was to prepare a set of single site mutants of the tail segment 314−350, in which the charged and the hydrophobic amino acid residues were replaced by Ala, and then to monitor the effect of the mutation on (i) the kinetic parameters (K_m and V_{max}) of the enzyme; (ii) the resulting conformational changes in the enzyme as reflected in the thermal stability of the mutant; and (iii) the tail-localized conformational changes as determined by the KSMP/meprin β cleavage of PKA (*16*, *17*, *21*, *35*).

Specifically, we address here the following questions. (a) What is the role played by the individual amino acids in the tail of PKA? Are they involved in substrate recognition? (if so, of the ATP or of the peptide/protein substrate?). (b) Which are the amino acids involved in stabilizing the active structure of PKA and which interactions are involved? (c) Is it possible to minimize (by mutation) the change in conformation yet to modulate the function of the kinase? In other words, is it possible to design a mutant enzyme that will have an essentially native conformation yet be catalytically inactive? Such mutant proteins, which may still be under the control of cAMP, could be very useful as competitive inhibitors of the kinase.

Effect of Single Site Mutations to Ala on the Affinity of PKA for ATP. To assess the role of individual amino acid residues in the carboxyl terminal tail of the catalytic subunit of PKA, we prepared the set of single-site mutations (substitution by Ala) depicted in Figure 1. Most of the single site substitutions by Ala did not alter significantly the affinity of PKA for ATP (Figure 2). However, Ala substitution of Lys 317 and Lys 319 altered it. Substitution of either one of these Lys residues increased the K_m for ATP by >18-fold (cf. Figure 2, panel I). It should be noted that each of the two mutations did not significantly alter the affinity of the enzyme for Kemptide and essentially did not alter the V_{max} of the kinase (Figure 2, panels II and III). It should also be noted that the heat stability of these mutants (Figure 3, panels 3 and 5) was not significantly different from that of the wild-type enzyme. However, an increased exposure of the KSMP cleavage site in the Lys 317 mutant is implicated by an increased rate (~30%) in its cleavage by KSMP (Figure 4). This exposure did not occur in the case of the Lys 319 mutant, for which the initial rate of the KSMP cleavage was ~14% lower (Figure 4).

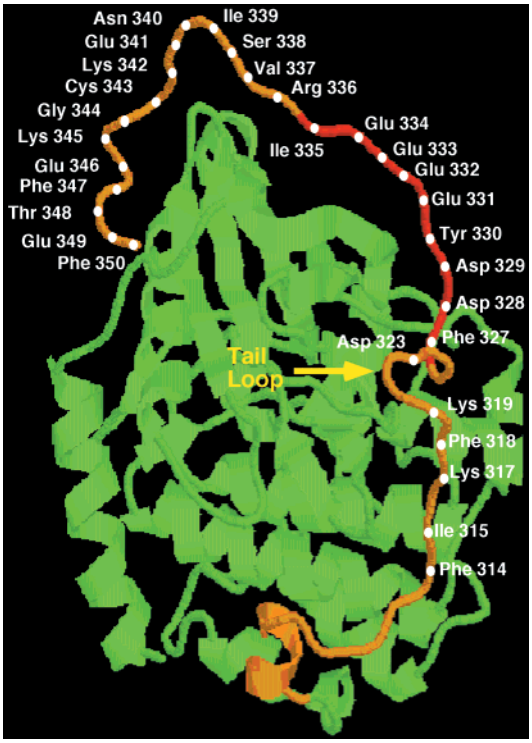

FIGURE 1: Ala scanning of the carboxyl terminal tail (residues 301−350) of the C-subunit of PKA. The carboxyl terminal tail (301−350) is shown in yellow (except the segment 327−335 containing the cluster of acidic amino acids, which is highlighted with red). The rest of the molecule (i.e., the catalytic core and the amino terminal "head") is shown in green. For each of the amino acid residues in the tail, marked by white circles, a single-site Ala substitution was prepared. The site of the tail loop (residues 319−323) is shown by a yellow arrow. The figure was prepared using RasMol program.

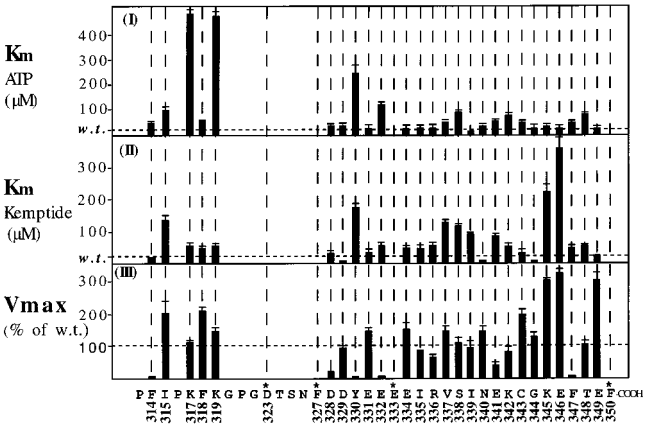

FIGURE 2: Effect of single-site Ala substitutions on the kinetic parameters of PKA. The wild-type enzyme and the mutants were expressed in *E. coli*. The K_m values were measured for both of the cosubstrates: ATP (I) and Kemptide (II). The specific activities (V_{max} values) were measured with Kemptide and ATP (III). For experimental details see Materials and Methods. Please note that the mutants indicated by an asterisk (Asp 323/Ala, Phe 327/Ala, Glu 333/Ala, and Phe 350/Ala) are devoid of any measurable enzymatic activity. Therefore, no K_m and V_{max} values could be given for them.

A decrease in affinity toward ATP as well as Kemptide was observed following the substitution of Tyr 330 by Ala (∼10-fold in each case) (Figure 2, panels I, and II) (*21*). Interestingly, we have recently shown that Tyr 330 is localized at a site, which accommodates a conserved water molecule (*39*). This water molecule is "locked" there by virtue of its interactions with ATP, with the peptide substrate/inhibitor of the kinase, and with the small and the large lobes of the kinase core (*39*). This reduced affinity was not reflected in the heat stability of the enzyme (Figure 3, panel 8).

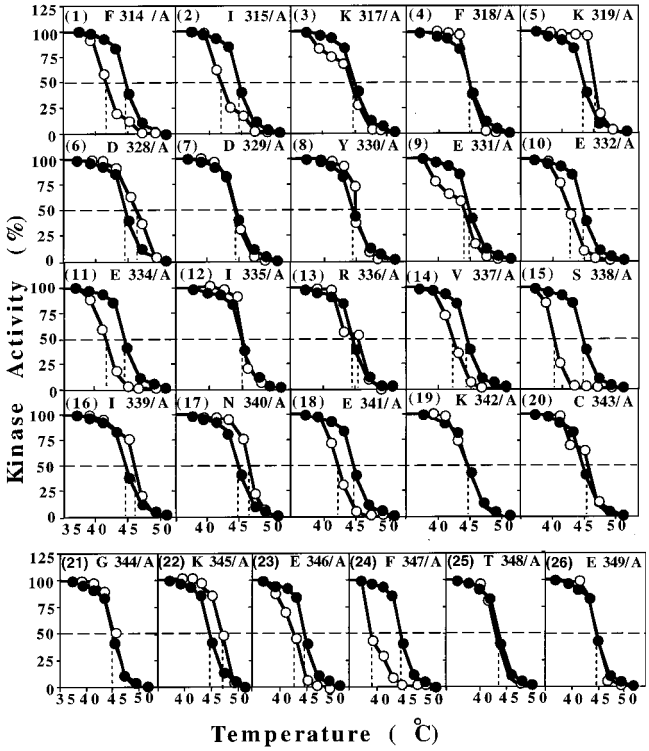

FIGURE 3: Comparison between the thermal stability of the wild-type recombinant catalytic subunit and its single-site mutants. Wild-type (filled circles) or a single-site mutant (open circles) were preincubated in the absence of substrates at the indicated temperature for 2 min before assay, and the residual activity was determined as described under the Materials and Methods. S.D. of the mean is not given for each of the measured values because of space limitations in the graph; however, these deviations were determined to be less than ±10%.

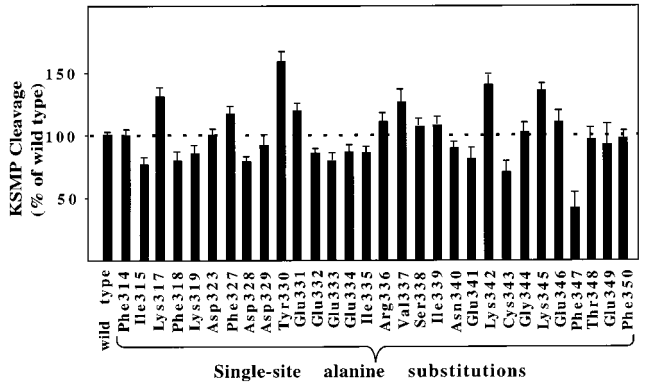

FIGURE 4: Effect of single Ala substitutions on the cleavability of the catalytic subunit of PKA by KSMP. The ^{35}S-radiolabeled wild-type enzyme and its indicated mutants were translated in a rabbit reticulocyte system and cleaved by KSMP as described under the Materials and Methods. The products were separated by SDS−PAGE, visualized by autoradiography, and quantitated by densitometry. The initial rates of cleavage were calculated as a percentage of the initial rate of cleavage of the wild-type enzyme which was taken as 100%.

Effect of Single Site Mutations to Ala on the Affinity of PKA for Its Peptide Substrate Kemptide. Two substitutions

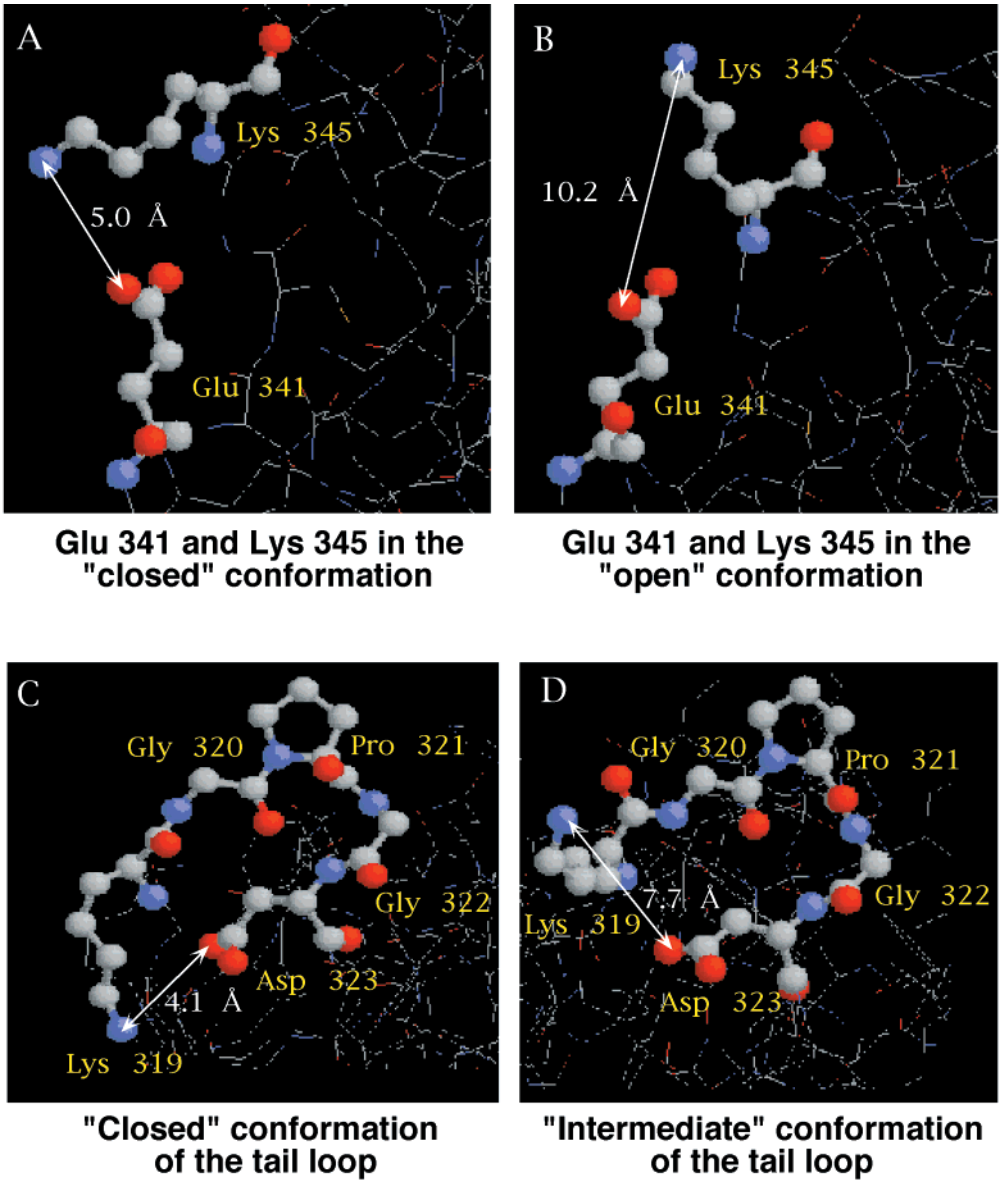

FIGURE 5: Ionic interactions in the tail of PKA stabilizing its closed conformation. (A) The interaction between Glu 341 and Lys 345 in the closed conformation. (B) The detachment of Glu 341 and Lys 345 in the "intermediate" conformation of the enzyme. (C) The tail loop (residues 319−323) in the closed conformation of PKA. (D) Loosening of the loop in the "intermediate" conformation of the enzyme. The distances for the closed conformation were measured from the rC:MnATP:PKI (5−24) ternary complex and for the "intermediate" conformation from the rC:adenosine binary complex. The Brookhaven National Laboratory Protein Data Bank codes are 1ATP and 1BKX, respectively. The figure was prepared using RasMol program.

by Ala significantly affected the affinity of PKA for Kemptide, but not its affinity for ATP (Figure 2, panel II): these were a replacement by Ala of Lys 345 and of Glu 346. In both cases, the K_m for Kemptide was found to be increased by ~17-fold and by ~25-fold, respectively (cf. Figure 2, panel II). The ϵ-amino group of Lys 345, which resides at a distance of 10.2 Å in the "intermediate" conformation, comes close (~5 Å) to the side chain of Glu 341 in the closed conformation (Figure 5, panels A and B). In the closed conformation, these two residues can interact electrostatically and may thus be involved in the "bending" of the tail and allowing Phe 347 and Phe 350 to "snap" the tip of the tail into its hydrophobic pocket (Figure 6A). This snapping may be needed to secure a correct juxtaposition of the two lobes that form the catalytic core of PKA.

The exact role of Glu 346 is not clear yet. Its side chain faces the exterior of the molecule, and its carboxylate does not seem to interact with any functional group of the protein. Several possibilities can be visualized to explain this observation. An intriguing possibility is that Glu 346 serves as a primary docking site for the positively charged peptide substrate of the kinase. This would implicate an interaction between the side chain of Glu 346 and the peptide substrate of the kinase. In support of this possibility, the heat stability curve of the Glu 346 mutant, which in the absence of Kemptide has a lower $T_{1/2}$ than the wild-type [(Figure 3, panel 23) becomes essentially identical to that of the wild-type in the presence of Kemptide (Figure 7, panel 8).

Amino Acid Residues Whose Mutation Affects the Heat Stability of the Kinase. Of the 30 Ala mutants we prepared and tested, only 13 had a significantly lower $T_{1/2}$ (a difference of >1 °C) (see Figure 3). In the open conformation, these include Phe 314, Ile 315, Glu 332, Glu 334, Val 337, Ser 338, Glu 341, Glu 346, and Phe 347 and the mutants Asp

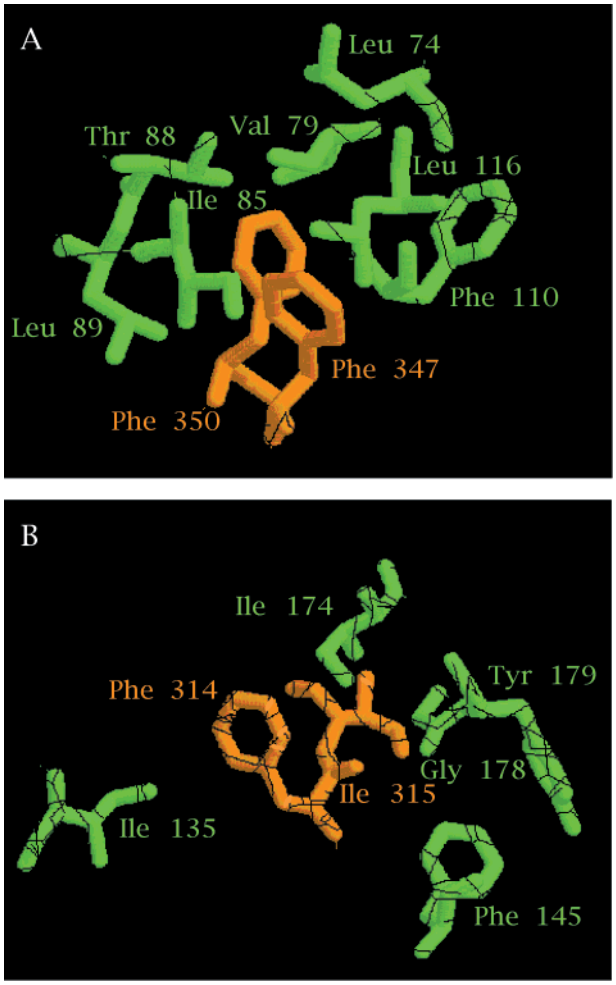

FIGURE 6: (A) Phe 347 and Phe 350 (shown in yellow) embedded in their hydrophobic pocket, formed by the residues of the small lobe (Leu 74, Val 79, Ile 85, Thr 88, Leu 89, Phe 110, and Leu 116), which are shown in green. (B) Phe 314 and Ile 315 (shown in yellow) embedded in their hydrophobic pocket, formed by the residues of the big lobe (Ile 135, Phe 145, Ile 174, Gly 178, and Tyr 179), which are shown in green. The figure was made using the RasMol program.

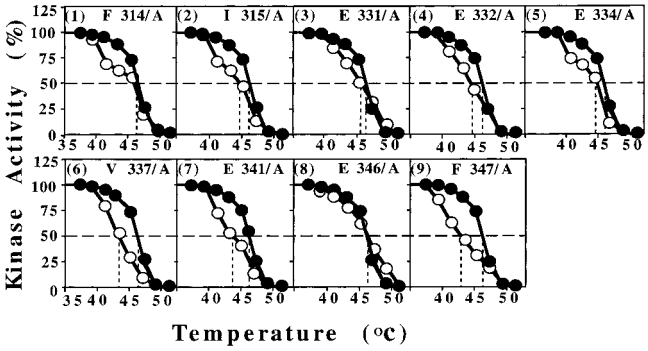

FIGURE 7: Comparison between the thermal stability of the wild-type recombinant catalytic subunit, and its single-site mutants in the Kemptide-ligated form. Wild-type (filled circles) or a single-site mutant (open circles) were preincubated in the presence of Kemptide (120 μM) at the indicated temperature for 2 min before assay, and the residual activity was determined as described under the Materials and Methods. S.D. of the mean is not given for each of the measured values because of space limitations in the graph; however, these deviations were determined to be less than ±10%.

323, Phe 327, Glu 333, and Phe 350, which were found to be totally inactive. It should be noted that the wild-type enzyme, as well as most of the active mutants, were more stable in the presence of the peptide substrate Kemptide (compare $T_{1/2}$ values in Figures 3 and 7). The fact that these sites are located all along the tail of the kinase leads us to propose that they provide a series of snapping points for anchoring the tail onto the core of the enzyme, and thus to enable the tail to stabilize the active conformation of the kinase by keeping together its two lobes.

Amino Acid Residues Whose Mutation Is Reflected in the KSMP Cleavage of the Kinase. Fifteen of the mutants had a significantly different rate of cleavage [a difference of >15% higher (↑) or lower (↓)] compared to the wild-type enzyme. In the open conformation, these were Ile 315 (↓); Lys 317 (↑); Phe 318 (↓); Lys 319 (↓); Phe 327 (↑); Asp 328 (↓); Tyr 330 (↑); Glu 331 (↑); Glu 333 (↓); Val 337 (↑); Glu 341 (↓); Lys 342 (↑); Cys 343 (↓); Lys 345 (↑); and Phe 347 (↓) (Figure 4). An increased rate of cleavage is probably due to a change in structure that exposes the KSMP cleavage site. However, such a change may also "bury" the cleavage site. Indeed, this is probably the case in the Phe 347/Ala mutant, where the KSMP cleavage site becomes less accessible to the proteinase (Figures 4 and 6A).

Contribution of Phe 314−Ile 315 to the Snapping of the PKA Tail onto the Conserved Kinase Core. It was previously shown that residues 301−316 do not move much in the course of the transition from the open to the closed conformation of PKA (*7*). However, the segment that follows (residues 317−326) adheres onto the large lobe in the closed conformation, but becomes detached from it in the open conformation (*39*). Looking at the surrounding of Phe 314 and Ile 315 in the 3-D structure of the enzyme (*12*), it can be seen that these two residues actually "snap" the tail onto the large lobe of the kinase. This snapping is achieved by hydrophobic interactions between these two residues and a hydrophobic pocket formed by the following residues in the core: Ile 135, Phe 145, Ile 174, Gly 178, and Tyr 179 (Figure 6B). In agreement with this suggestion, one would expect that replacement of Ile 315 by Ala would not be able to provide a hydrophobic anchor for snapping that segment onto the hydrophobic core and thus yield a less stable and possibly a less active enzyme. Indeed, the heat stability curves of the Phe 314 and Ile 315 mutants, which in the absence of Kemptide have a lower $T_{1/2}$ than the wild-type (Figure 3, panels 1 and 2) become quite similar to those of the wild-type in the presence of Kemptide (Figure 7, panels 1 and 2). Furthermore, the replacement of Phe 314 by Ala lowered the V_{max} of the enzyme by ~20-fold, and the replacement of Ile 315 by Ala increased the K_m of the enzyme for both ATP (~4-fold) and Kemptide (~7-fold) (Figure 2).

Interaction between Lys 319 and Asp 323 Closes and Stabilizes a Functionally Important Loop at the Tail of the Kinase. Further down in the tail of the kinase is Lys 319, which can interact electrostatically with Asp 323 (Figure 5). These residues form a loop in the tail, which can be regarded as a hinge for its movement. This is supported by the fact that the distance between these two functional groups decreases from 7.7 Å in the partly open ("intermediate") conformation of the kinase (Figure 5D) to 4.1 Å in the closed conformation (Figure 5C). Thus, the segment 314−323 in the tail can be involved in the formation of the ternary complex with the peptide and the nucleotide substrates.

Table 1: Properties of Multiple Site Mutations at Selected Loci in the Tail of PKA[a]

mutation	K_m (ATP)	K_m (kemptide)	V_{max} (% of wild-type)	$T_{1/2}$	KSMP cleavage (% of wild-type)
wild-type*	25	17	100	44.3 ± 0.4	100
Tyr 330/Ala	230 ± 15	170 ± 20	1.0 ± 0.2	45.0 ± 0.2	156 ± 17
Phe	51 ± 5	31 ± 4	14.5 ± 3.0	44.8 ± 0.3	112 ± 7
Ser	62 ± 7	45 ± 3	3.8 ± 0.9	44.6 ± 0.2	102 ± 8
Cys	98 ± 8	55 ± 15	2.7 ± 0.4	44.6 ± 0.2	87 ± 8
Glu	25 ± 2	261 ± 50	0.7 ± 0.2	**	105 ± 13
Gln	44 ± 9	507 ± 20	0.6 ± 0.05	**	103 ± 9
Asp	45 ± 4	350 ± 50	0.8 ± 0.1	**	98 ± 12
Asn	23 ± 2	453 ± 40	0.9 ± 0.1	**	107 ± 10
Lys	32 ± 2	512 ± 10	2.2 ± 0.7	44.1 ± 0.1	91 ± 11
Arg	96 ± 20	58 ± 5	8.0 ± 2.0	44.3 ± 0.3	100 ± 6
Ser 338/Ala	70 ± 19	105 ± 13	98 ± 11	39.3 ± 0.4	110 ± 7
Glu	37 ± 11	57 ± 10	65 ± 8	39.6 ± 0.2	98 ± 8
Gln	77 ± 12	75 ± 12	25 ± 4	40.1 ± 0.20	109 ± 10
Asp	45 ± 7	145 ± 35	22 ± 4	40.7 ± 0.20	107 ± 8
Asn	72 ± 18	250 ± 60	78 ± 10	40.7 ± 0.3	111 ± 12
Phe 350/Ala	**	**	**	**	100 ± 8
Trp	15 ± 5	12 ± 4	106 ± 17	42.0 ± 0.40	not measured
Tyr	24 ± 9	29 ± 8	107 ± 11	44.9 ± 0.10	not measured

[a] The wild-type enzyme and several mutants, in which Tyr 330, Ser 338, and Phe 350 had been replaced, were expressed in *E. coli*. The K_m values were measured for both substrates of the kinase: ATP and Kemptide. The specific activities (V_{max} values) were measured using Kemptide as a substrate. For experimental details see the Materials and Methods. The thermal stability of the single-site mutants was measured as described under the Materials and Methods, and the $T_{1/2}$ values were determined. The initial rate of cleavage by KSMP was calculated for each mutant (see under the Materials and Methods). Please note that the mutant Phe 350/Ala is devoid of enzymatic activity. Therefore, no K_m and V_{max} values could be given for it (**). Enzymatic activities of the mutants Tyr 330/Glu, Tyr 330/Asp, Tyr 330/Gln, and Tyr 330/Asn are too low to calculate the values of $T_{1/2}$ (*).

Importance of Phe 327. The cluster of acidic amino acids in the segment Asp328-Asp-Tyr-Glu-Glu-Glu-Glu334 is flanked by two hydrophobic residues: Phe 327 and Ile 335. There are a few pieces of evidence suggesting Phe 327 is part of the adenosine-binding pocket. These include the distance between the phenyl ring of Phe 327 and adenine [~3.5 Å in the closed conformation (*12*) and a distance of ~3.65 Å in the binary complex of the C-subunit with adenosine (*37*)]. In addition, when the C-subunit of PKA was cocrystallized with staurosporine (an inhibitor of ATP) (*40*), Phe 327 was found to be expelled from its original position by the indole carbasole ring. In the present study, we provide evidence confirming this suggestion, since the Phe 327/Ala mutant that lacks the ability to bind to adenine is enzymatically inactive (Figure 2). In line with these findings, Phe 327 is conserved not only in all the PKAs obtained from various species, but also in the sequences of some other protein kinases (*41*, *42*).

Cluster of Acidic Amino Acids. The recognition of the cluster of acidic amino acids as the target for KSMP was shown in our laboratory in the eighties (*18*, *43*, *44*). Subsequently, this cluster was suggested to serve as an initial docking site for basic peptides on the basis of circular dichroism studies (*24*). More recently, it was shown that the KSMP cleavage of PKA occurs at the Glu 332−Glu 333 bond within this cluster (*19*). The translocation of this segment upon the transition from the open to the closed conformation was also indicated from the following findings: (a) by a comparison of the 3-D structures of these enzyme forms (*7*); (b) by the ability of KSMP to discriminate between the open (cleavable) and the closed (noncleavable) conformations of the C-subunit (*21*); and (c) by the reaction of these residues with a soluble carbodiimide in the open but not in the closed conformation (*25*).

The role of Glu 331 has been debated in the literature (*5*, *13*). To get further insight into the function of this residue, we prepared the Glu 331/Ala mutant and found that its enzymatic parameters are similar to those of the wild-type enzyme (Figure 2). It should be noted that the side chain of this residue faces the exterior of the molecule in both the open and the closed conformations (*12*), and thus is not likely to participate directly in the anchorage of the tail onto the core of the enzyme. This conclusion is in line with the fact that Glu 331 is the only member of the cluster of acidic residues which was chemically labeled in the ternary complex with both ATP and the peptide inhibitor (*25*). The glutamic acid residues 332−334 were previously reported to interact (respectively) with Thr 48, Arg 56, and Arg 45 in the small lobe of the kinase core. The distances between these residues are 6−8 Å in the open conformation, and become 3−4 Å in the closed conformation (*21*). In line with these structural features we show here that the Glu 332/Ala and Glu 334/Ala mutants are less heat stable than the wild-type enzyme (Figure 3, panels 10 and 11), and the heat stability of these mutants is increased in the presence of the peptide substrate of the kinase (Figure 7, panels 4 and 5).

Role of Tyr 330. Upon binding of substrates, the two lobes of the core of PKA change their mutual orientation (*7*) closing the cleft between them. This structural change involves inter alia a translocation of its carboxyl terminal tail, which moves the phenolic hydroxyl of Tyr 330 from a distance of ~10 Å to a distance of ~3 Å from the nitrogen atoms of the P-3 Arg residue in the substrate (*21*).

Substitution of Tyr 330 by a polar residue (e.g., Glu or Asp, or their amide analogues Gln or Asn) yields mutant enzymes with a very low activity (less than 1% of that of the wild-type), and a very low affinity toward Kemptide (15−30-fold lower) (Table 1). The low activity of these mutants could be due (at least in part) to a detrimental effect of inserting a polar group at this locus. Indeed, among the series of Tyr 330 mutants characterized in this report, the mutant closest to the wild-type enzyme in its K_m (for ATP and for

Kemptide) and in its V_{max}, is Tyr 330/Phe (Table 1), i.e., a hydrophobic (non polar) mutant. If we assume that the catalytically active structure of the kinase has a preference for a hydrophobic side chain, then we could also account for the low activity of some other mutants we tested having a polar group at position 330. These include Tyr 330/Lys and Tyr 330/Arg (Table 1). In general, it seems that maintenance of the overall structure of the kinase is not the major role of Tyr 330, since the $T_{1/2}$ value of each of the Tyr 330 mutants (substitution by Ala, Phe, Ser, Cys, Lys, and Arg) was found to be identical to that of the wild-type enzyme (Table 1).

Role of Ser 338. It was previously shown that Ser 338 is an autophosphorylation site in PKA (*2*), which in principle, could have an important structural and functional role. To find out what this role may be, we prepared a set of mutants with replacements at this position (Table 1). We substituted Ser 338 by Ala (to simulate the nonphosphorylated state), by Asp and Glu (to simulate the negative charge that this site acquires in its phosphorylated state), and by Asn or Gln (close analogues of Asp and Glu that lack the negative charge of these residues). It was recently reported that Ala and Glu substitutions of Ser 338 result in very labile mutants of the kinase (*45*). Analysis of the kinetic parameters of the above-mentioned mutants showed that (a) these mutants are still active; (b) the affinity of Ser 338 mutants toward Kemptide is reduced (especially when Ser is replaced by Ala, by Asn, or even by the negatively charged Asp); and (c) all the replacements of this residue result in a decreased thermostability of the kinase (Table 1). The X-ray structure of PKA actually indicates that Ser 338 faces the solvent. Therefore, it is reasonable to assume that the phosphorylation of Ser 338 may not be involved directly in catalysis, but rather in an interaction with another protein such as a substrate or an inhibitor.

An ionic interaction of Ser 338 (in its phosphorylated state) with Lys 342 was originally proposed to play a role in stabilizing the conformation of PKA by forming a salt bridge (*7*). We show here that the mutation Lys 342/Ala does not alter markedly the kinetic parameters of the enzyme (Figure 2) and that the Lys 342/Ala mutant has essentially an identical thermal stability to that of the wild-type enzyme (Figure 3, panel 19).

Snapping of the Tip of the Carboxyl Terminal Tail (Phe 347 and Phe 350) onto the Small Lobe of the Core. Phe 347 and Phe 350 are "submerged" in a hydrophobic pocket formed by residues Leu 74, Val 79, Ile 85, Thr 88, Leu 89, Phe 110, and Leu 116 of the small lobe (Figure 6A). This hydrophobic pocket thus snaps the tail onto the core of the enzyme. Replacement of Phe 645 and Phe 648 by Ala in the C-subunit from *Dictyostelium*, in which these residues correspond (respectively) to Phe 347 and Phe 350 in the murine enzyme, was previously shown to bring about a dramatic decrease in the catalytic efficiency of the kinase (*42*). Furthermore, the carboxyl-terminal motif *-Phe-X-X-Phe* was found to be conserved in all the members of PKA family and in some other protein kinases (*41*, *42*). In this study, we measured the thermostability of the Phe 347/Ala mutant and clearly illustrated the structural contribution of this hydrophobic anchorage (Figure 3, panel 24). However, such thermostability measurements were not possible with Phe 350/Ala since this mutant was inactive (possibly because it denatures in the course of its preparation). The fact that the replacement of Phe 350 by Ala yields an inactive mutant (under our experimental conditions $<0.5\%$ of the wild-type), together with the fact that the Phe 347/Ala mutant was found to have only about 5% activity, support the suggestion that the interaction between these two Phe residues and the hydrophobic pocket shown in Figure 6A is essential for maintaining the catalytically active structure of the kinase.

DISCUSSION

The amino acid residues that have a structural or functional assignment in PKA can be divided into two classes: (a) those that are highly conserved in the protein kinase family and may thus have a direct role in the catalytic mechanism, and (b) those that are present in a given kinase and may thus be involved in its specific substrate recognition.

In this study, we systematically screen the interactions between specific amino acid residues in the carboxyl terminal tail of PKA and the sites that accommodate these residues in the core of the kinase. These results can be summarized as follows.

(a) *Hydrophobic interactions* between tail residues and the core serve as important snapping points in stabilizing the enzyme in its active conformation. Single site mutation of either Phe 314, Phe 347, or Phe 350 to Ala results in less active ($<5\%$) and more labile enzymes (Figures 2 and 3). However, this is not the case for all hydrophobic residues. For example, mutation of the hydrophobic residue Ile 315 to Ala results in an enzyme that has a V_{max} 2-fold higher than that of the wild-type, but this mutant enzyme is considerably more labile than the wild-type (Figure 3).

(b) *Electrostatic interactions* with companion residues in the core. These include Glu 332 with Thr 48; Glu 333 with Arg 56; and Glu 334 with Arg 45 (*21*). The movement of these residues toward each other upon forming the closed conformation supports the notion that the translocation of the tail vis-à-vis the core may well be involved in the substrate recognition of the kinase.

(c) *Tail Residues That Interact Directly with Substrates of the Kinase*. These include Phe 327, which participates in the formation of the ATP-binding pocket, and Tyr 330 which interacts with both the peptide and the ATP substrates.

(d) *Tail Residues That Shape Its Local Folding and Provide Flexibility for Its Translocation on the Core*. These include the interactions between Lys 319 and Asp 323, and between Glu 341 and Lys 345.

On the basis of the results reported here, we propose that the tail of PKA plays a key structural role in this enzyme. It does so by means of a set of amino acid residues (identified and characterized here), which act as snapping points that enable the tail to embrace the two lobes of the kinase and orient them in the correct juxtaposition for substrate recognition and binding. Furthermore, it seems reasonable to assume that the snapping points present all along the tail provide a molecular mechanism through which the tail of the enzyme can translocate (during the conversion of the open to the closed structure) for catalysis to occur.

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