A Conserved Glu–Arg Salt Bridge Connects Coevolved Motifs That Define the Eukaryotic Protein Kinase Fold

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Eukaryotic protein kinases (EPKs) feature two coevolved structural segments, the Activation segment, which starts with the Asp-Phe-Gly (DFG) and ends with the Ala-Pro-Glu (APE) motifs, and the helical GHI subdomain that comprises αG–αH–αI helices. Eukaryotic-like kinases have a much shorter Activation segment and lack the GHI subdomain. They thus lack the conserved salt bridge interaction between the APE Glu and an Arg from the GHI subdomain, a hallmark signature of EPKs. Although the conservation of this salt bridge in EPKs is well known and its implication in diseases has been illustrated by polymorphism analysis, its function has not been carefully studied. In this work, we use murine cAMP-dependent protein kinase (protein kinase A) as the model enzyme (Glu208 and Arg280) to examine the role of these two residues. We showed that Ala replacement of either residue caused a 40- to 120-fold decrease in catalytic efficiency of the enzyme due to an increase in $K_m$(ATP) and a decrease in $k_{cat}$. Crystal structures, as well as solution studies, also demonstrate that this ion pair contributes to the hydrophobic network and stability of the enzyme. We show that mutation of either Glu or Arg to Ala renders both mutant proteins less effective substrates for upstream phosphoinositide-dependent kinase 1. We propose that the Glu208–Arg280 pair serves as a center hub of connectivity between these two structurally conserved elements in EPKs. Mutations of either residue disrupt communication not only between the two segments but also within the rest of the molecule, leading to altered catalytic activity and enzyme regulation.

Introduction

Protein kinases are a large family of enzymes that perform posttranslational phosphorylation of proteins and thus regulate a broad range of cellular events. All protein kinases share a conserved kinase domain with significant sequence and structure similarities. This domain has a distinctive bilobal tertiary fold with a smaller N-terminal lobe (N-lobe),
Conserved Glu–Arg Salt Bridge

which contains mostly β-strands, and a larger C-terminal lobe (C-lobe), which is predominantly α-helical. ATP binds in the deep cleft between these lobes, and the substrate binds mostly to the C-lobe. The latter includes a large regulatory Activation segment that usually contains a phosphorylation site, allowing the protein kinases to be regulated by phosphorylation. This regulatory mechanism, however, is specific only for eukaryotic protein kinases (EPKs). Eukaryote-like kinases (ELKs), which are abundant in bacteria and archaea, instead of the Activation segment have a short loop and are not regulated by phosphorylation.

The Activation segments of different EPKs are very well studied. They contain two highly conserved sequence motifs: Asp-Phe-Gly (DFG) at the N-terminus and Ala-Pro-Glu (APE) at the C-terminus. The DFG motif is a part of the magnesium positioning loop, allowing the DFG-aspartate to coordinate magnesium ions in the active site. The APE motif is a central point of interaction between the Activation segment and the GHI subdomain. The APE-glutamate binds to another conserved residue from the GHI subdomain: Arg280, which is located in the loop between the αH and αI helices (αH–αI loop). This loop is also a docking site for regulatory proteins and substrates. Although conservation of the Glu208–Arg280 salt bridge in EPKs is well recognized, little is known about rationale or function of such conservation. Nevertheless, bioinformatic analysis of single-nucleotide polymorphisms in EPKs demonstrated that multiple diseases are correlated with mutations of both Glu208 and Arg280.

To elucidate the structural and biochemical role of the Glu208–Arg280 salt bridge, we studied Glu208Ala and Arg280Ala mutants of cAMP-dependent protein kinase [protein kinase A (PKA)]. PKA is the best-studied protein kinase in terms of structure and function and has served as a model system and paradigm for the whole EPK family. Here, we present crystal structures of these mutants, their kinetic analysis and dynamic properties through hydrogen/deuterium (H/D) exchange studies. We discovered that mutation of either residue causes over a 10-fold decrease in kcat and a 4- to 15-fold increase in Km (ATP), leading to a 40- to 120-fold decrease in catalytic efficiency (kcat/Km) and destabilizes the entire molecule. Furthermore, destabilization of the kinase after Glu208Ala and Arg280Ala mutations impaired recognition of PKA by the upstream kinase phosphoinositide-dependent kinase 1 (PDK1). Significantly, and unrecognized previously, we found that the aliphatic part of Glu208 and Arg280 side chains contributed to the continuity of the hydrophobic network in the C-lobe. Our results suggest that the conserved Glu–Arg pair plays a critical role bridging the Activation segment and the GHI subdomain, the two coevolved signature motifs of EPKs. We demonstrate that this connection between the two motifs in EPKs is critical for protein kinase function and mutations at their interface can cause global destabilization of the molecule.

Results

Decreased catalytic efficiency of the enzyme by Ala replacement of Arg280 or Glu208

To understand the function of the conserved Arg280–Glu208 interaction, we mutated each of the residues to Ala in the catalytic subunit (C) of PKA and examined the kinetic properties of both mutants (referred to as CEE208A and CRC280A for mutant proteins). For the kinetic assay, besides the conventional substrate peptide Kemptide, we also used two established protein substrates for PKA, the type 1 Ser/Thr phosphatase (PP1) inhibitors DARPP-32 and PP1_I-1.

As shown in Table 1, when using Kemptide as a substrate, the wild-type enzyme had a Kcat for Kemptide of 26 μM, a Km for ATP of 23 μM and a kcat of 23/s; these values were similar to previously published data. When using protein substrates, the wild-type enzyme showed marginal kinetic variations in the 2-fold range in Kcat and Km comparing with the peptide substrate. Significantly, whether using peptide or protein substrate, both CEE208A and CRC280A mutants exhibited similar Kms for substrates but ~10-fold decrease in kcat when compared with the wild-type enzyme with corresponding substrates. Furthermore, CEE208A showed a 13-fold increase in Km for ATP (309 μM), whereas CRC280A had a modest 3.7-fold increase (85 μM). As a result, catalytic efficiency (kcat/Km) of CEE208A and CRC280A decreased by over 100-fold and 40-fold, respectively, as compared to the wild-type enzyme (Table 1).

Crystal structure of CRC280A and CEE208A

To capture any possible structural changes caused by the mutations and to understand the molecular basis for how the mutations, especially the distal Arg280Ala mutation, affect kinetics, we crystallized both CEE208A and CRC280A. For both mutants, we were only able to get crystals under the condition where both MgATP and inhibitory peptide IP20 were present. The ternary complexes were thus solved.
with resolutions of 1.9 Å and 1.7 Å for CE208A and CR280A, respectively (Table 2). Although overall structures of the mutants were very similar to that of the ternary structure of wild-type C-subunit complexed with MgATP and IP20 [Protein Data Bank (PDB) ID 1ATP], specific localized differences were observed. For both mutants, no significant structural differences were observed for the Activation segments or the three helices in the GHI subdomain. However, for CR280A, the loop...
connect the αH and αI helices, also where R280A resides, was disordered.

**Destabilized αH–αI loop in C^R280A**

Compared to the structure of the wild-type C-subunit or C^E208A, the αH–αI loop region in C^R280A exhibited significantly increased flexibility. Residues around Arg/Ala280 were disordered. Side chains of a 12-residue stretch (275VDLTKR/AFGNLKN286) in the loop were not traceable in C^R280A (Fig. 2, bottom), except for Phe281 with a weaker density for its aromatic side chain. Correspondingly, the temperature B-factor of this region was 2.5-fold (250%) compared to the average B-factor of the whole molecule (Fig. 2, top). By comparison, in C^E208A, the electron density map in this region was clearly resolved (Fig. 2, bottom), and the B-factor of the region was only slightly higher than the average B-factor of the whole molecule (Fig. 2, top).

For C^E208A, the electron density and B-factor of the αH–αI loop region are similar to those of the wild-type protein (Fig. 2, bottom). Notably, the glycine-rich loop in C^E208A exhibited higher temperature B-factors compared to the wild-type protein or C^R280A (Fig. 2, top). When comparing B-factors of different segments within the same molecule, the glycine-rich loop has been shown to be one of the low-B-factor regions in the wild-type C-subunit ternary complex. As shown in Fig. 2 (top), in C^E208A as well as in the wild-type C-subunit ternary structures, the B-factor for the glycine-rich loop was ~140% of the average B-factor of the whole molecule. Thus, the B-factor for the glycine-rich loop in C^E208A had a nearly 2-fold increase when comparing with the wild-type protein. This is consistent with the enzyme kinetic data where it showed that C^E208A had a 13-fold higher K_m for ATP than that of the wild-type protein.

### Table 1. Enzyme kinetics of C^E208A and C^R280A in comparison with the wild-type enzyme

<table>
<thead>
<tr>
<th>Substrate</th>
<th>wt-C</th>
<th>C^R280A</th>
<th>C^E208A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kemptide</td>
<td>K_m (μM)</td>
<td>26 ± 3</td>
<td>31 ± 3^a</td>
</tr>
<tr>
<td></td>
<td>k_cat (s⁻¹)</td>
<td>24 ± 1</td>
<td>33.1 ± 3^a</td>
</tr>
<tr>
<td></td>
<td>K_m(ADP)</td>
<td>23 ± 5</td>
<td>21.2 ± 4^a</td>
</tr>
<tr>
<td></td>
<td>k_cat/K_m(ADP) (μM⁻¹ s⁻¹)</td>
<td>1.0</td>
<td>1.07^a</td>
</tr>
<tr>
<td>DARPP-32</td>
<td>K_m (μM)</td>
<td>13 ± 2</td>
<td>16.1 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>k_cat (s⁻¹)</td>
<td>48 ± 3</td>
<td>44 ± 0.9</td>
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<tr>
<td></td>
<td>K_m(ADP)</td>
<td>2.1</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>k_cat/K_m(ADP) (μM⁻¹ s⁻¹)</td>
<td>9.2</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>PPI I-1</td>
<td>K_m (μM)</td>
<td>8.7 ± 0.7</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>k_cat (s⁻¹)</td>
<td>0.378</td>
<td>0.011</td>
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Kinetic assay was carried out as described in Materials and Methods. Data shown were analyzed from three to five replicated experiments. wt-C refers to the wild-type C-subunit. Sequence alignment of PKA recognition site in DARPP32 and PPI I-1 with Kemptide is shown below. The number indicates the residue numbering for the protein. Phosphorylation consensus of PKA substrate is: RRxS/TΦ (Φ represents a hydrophobic residue).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Sequence Alignment</th>
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<tbody>
<tr>
<td>Kemptide</td>
<td>LRRASLG</td>
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<tr>
<td>DARPP-32</td>
<td>28IRRRRPTPA36</td>
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<tr>
<td>PPI I-1</td>
<td>29IRRRRPTPA37</td>
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^a From Ref. 13.

### Table 2. Data collection and refinement statistics

<table>
<thead>
<tr>
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<th>C^R280A</th>
<th>C^E208A</th>
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<tr>
<td><strong>Data collection</strong></td>
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<tr>
<td>Space group</td>
<td>P2₁;2₁;2₁</td>
<td>P2₁</td>
</tr>
<tr>
<td>Cell dimensions</td>
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<td>80.1 79.6</td>
</tr>
<tr>
<td></td>
<td>97.7 60.8</td>
<td>113.3</td>
</tr>
<tr>
<td></td>
<td>17.4</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>0.080 (0.42)</td>
<td>0.051 (0.44)</td>
</tr>
<tr>
<td></td>
<td>97.8 (82.0)</td>
<td>88.8 (65.5)</td>
</tr>
<tr>
<td></td>
<td>21.2 (2.4)</td>
<td>17.0 (2.2)</td>
</tr>
<tr>
<td></td>
<td>48,566</td>
<td>32,026</td>
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<tr>
<td><strong>Refinement</strong></td>
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<tr>
<td>Resolution (Å)</td>
<td>50.0–1.7</td>
<td>50.0–1.9</td>
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<tr>
<td>Rmerge (%)</td>
<td>17.6/19.4</td>
<td>19.5/22.8</td>
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<tr>
<td>No. of protein residues</td>
<td>250</td>
<td>350</td>
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<tr>
<td>No. of ligand/ION</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>No. of water</td>
<td>371</td>
<td>282</td>
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<tr>
<td>RMSDs Bond lengths (Å)</td>
<td>0.007</td>
<td>0.008</td>
</tr>
<tr>
<td>Bond angles (%)</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Ramachandran angles (%)</td>
<td>91.7</td>
<td>90.7</td>
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</table>

Values in parentheses are for highest-resolution shell: 1.71–1.77 Å for C^R280A and 1.92–1.99 Å for C^E208A.
Disruption of the hydrophobic network that bridges the Activation segment and the GHI subdomain

By careful examination of the Arg280 and Glu208 environment in the wild type, we found that the aliphatic side chains of Glu208 and Arg280 contribute to extend the hydrophobic core network in the large lobe centered by the two highly conserved and buried $\alpha$E and $\alpha$F helices. This network connects the coevolved Activation segment and the GHI subdomain in EPKs. Arg280 packs immediately against Leu277 from the $\alpha$H–$\alpha$I loop, and Leu277 in turn...
packs against the aliphatic part of Glu208, Ala218 and Trp222 from the αF helix. The interaction directly connects the αH–αI loop to the Activation segment (Fig. 3a, center).

In the C^{R280A} mutant, loss of the Arg280 side chain broke the continuity of this hydrophobic network (Fig. 3a, left). Similarly, in the C^{E208A} mutant, loss of the Glu208 side chain also broke the hydrophobic continuity from the αH–αI loop to the Activation segment. However, the hydrophobic interactions within the αH–αI loop and the connection between the αH–αI loop and the αF helix were maintained (Fig. 3a, right).

**Increased solvation and maintaining of polar interactions at the mutation sites**

In C^{E208A}, two water molecules W565 and W566 occupied the void created by the Ala replacement of Glu208 and made hydrogen bond interactions with the guanidine group of Arg280 as seen in the wild-type protein. W565 also made a hydrogen bond interaction with the amide nitrogen of Ala208, similarly as seen in wild-type where Glu208:OE2 hydrogen bonds with its own amide nitrogen atom (Fig. 3a, right).

In C^{R280A}, three water molecules occupied the void space left by NH1 (W931), NH2 (W934) and NE (W973). The four hydrogen bond interactions made through NH1, NH2 and NE atoms of Arg280 in the wild-type protein were all replaced by interactions with the three water molecules (Fig. 3a, left). These interactions are Arg280:NH1–Glu208:OE1; Arg280: NH1–Ala218:O, Arg280:NH2–Glu208:OE2 and Arg280:NE–Gln274:O.

Interestingly, a fourth water molecule (W935) was found in close proximity to the aliphatic part of the Arg280 side chain and formed a water network with the three water molecules mentioned above (Fig. 3a, left). W935 was not present in other PKA C-subunit structures where Arg280 and Glu208 are intact. Thus, the mutations, especially that of Arg280 to Ala, brought a solvent network in place of the originally hydrophobic environment.

**Buried Glu208–Arg280 salt bridge in eukaryotic kinases**

Studies have shown that a buried salt bridge usually contributes to stabilization of a protein. The large decrease in energy resulting from the perfect

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**Fig. 3.** (a) Comparison of the Glu/Ala208 and Arg/Ala280 sites in C^{E208A} (right), C^{R280A} (left) and the wild-type C-subunit (center). The turquoise spheres represent the water molecules that occupy void space caused by the mutations. The color scheme for the stick representation of residues is by atom types (N, blue; O, red; and C, gray). Connolly surface was calculated from Leu277, Ala218 and the aliphatic parts of Glu208, Asp283, Glu274 and Arg280 and shown in 40% transparency colored light gray. (b) Hydrophobic environment of Glu208–Arg280 pair is highly conserved. Left, PKA (PDB ID 1L3R); right, CDK2 (PDB ID 1FIN). In PKA, Leu277 from the αH–αI loop and Ala218 from the αF helix cap the ion pair of Glu208–Arg280. In CDK2, Pro271 and Ala183 achieve the same function to shield its Glu–Arg ion pair from solvent. (c) ASA of Glu208 and Arg280 compared to selected surface-exposed Glu or Arg residues in PKA. ASA was calculated on the PISA server, using a probe of 1.6 Å. ASA values of two fully solvent-exposed arginines, Arg194 and Arg256, were averaged as comparison. Similarly, ASA values of two fully solvent-exposed glutamic acids, Glu248 and Glu331, were also averaged for comparison.

<table>
<thead>
<tr>
<th>Residue</th>
<th>ASA (Å²)</th>
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<tbody>
<tr>
<td>R194</td>
<td>165.6</td>
</tr>
<tr>
<td>R256</td>
<td>181.9</td>
</tr>
<tr>
<td>R280</td>
<td>0</td>
</tr>
<tr>
<td>E248</td>
<td>119.6</td>
</tr>
<tr>
<td>E331</td>
<td>133.8</td>
</tr>
<tr>
<td>E208</td>
<td>3.9</td>
</tr>
</tbody>
</table>
geometry of the salt bridge interaction in the hydrophobic environment often exceeds the desolvation penalty.\textsuperscript{16} The wild type and the two mutant structures clearly indicated that, despite the polar interactions, the residues were in a hydrophobic environment. The structure showed that Leu277 from the αH–αI loop and Ala218 from the αF helix form a hydrophobic cap, shielding the ion pair from the solvent (Fig. 3b, left). Interestingly, at the position of Leu277, most protein kinases have a Pro. We examined several kinases from different subfamilies and found that Pro functions similarly to Leu277 to shield the Glu–Arg pair from the solvent, as exemplified by cyclin-dependent kinase 2 (CDK2) (Fig. 3b, right). Solvent-accessible surface area (ASA) calculation of wild-type PKA confirmed that both Glu208 and Arg280 residues were excluded from the solvent (Fig. 3c). By comparison, ASA values of two surface-exposed Glu and two surface-exposed Arg residues were also calculated. The average ASA values for exposed Glu and Arg were 127 Å\textsuperscript{2} and 174 Å\textsuperscript{2}, respectively. The ASA for Glu208 was 3.9 Å\textsuperscript{2}, and for Arg280, the value was 0.0 Å\textsuperscript{2}.

Fig. 4. Comparison of hydrogen/deuterium exchange profiles of CE208A, CR280A and the wild-type C-subunit. Crystal structure of the wild-type C-subunit is rendered in cartoon representation with the N-lobe colored white and the C-lobe colored tan. Regions that exhibit increased H/D exchange in the mutants are colored red. Comparison of the time course of H/D exchange profile for four peptides in CE208A (triangle), CR280A (reversed triangle) and wild-type C-subunit (circle) is shown. Time points are indicated.
Increased dynamics of the αH–αI loop and the catalytic loop in apo-CE<sup>208A</sup> and CR<sup>280A</sup> monitored by hydrogen/deuteron exchange

It has been shown from previous studies that binding of MgATP and IP20 peptide helps to stabilize the C-subunit and shields away some dynamic features of the protein. Unfortunately, efforts on crystallizing the apo-form of the mutants without MgATP and IP20 did not yield crystals. In order to examine the dynamic effects of the mutants without the influence of the MgATP and IP20, we carried out solution hydrogen/deuteron exchange studies<sup>17,18</sup> of the mutants in their apo-forms. For both C<sup>E208A</sup> and C<sup>R280A</sup>, increased H/D exchange rate was observed in at least three regions (Fig. 4). Representative peptides include peptide 163–172 from the catalytic loop, 247–267 from the αG and αG–αH loop and 279–297 from the αH–αI loop containing Arg280Ala. Increased exchange was also observed for peptide 212–221 in C<sup>E208A</sup>; this peptide was at the loop connecting the APE motif and the αF helix. We thus term it APE-F linker. Unfortunately, similar peptides were not recovered in C<sup>R280A</sup> samples. The difference in H/D exchange reflected on the total number of deuterons incorporated to the peptides and also on the kinetics of the exchange. During the course of 10 s to 50 min, peptides 163–172 from both mutants and 212–221 from C<sup>E208A</sup> gradually picked up two and four more deuterons, respectively from the wild-type C-subunit. Peptides 247–267 and 279–297 each showed faster kinetics in the mutant enzymes by picking up almost the maximum number of deuterons at the first 10-s time points, whereas in the wild-type protein, the peptides gradually reached the maximum deuteron exchanges during the course of 50 min (Fig. 4). It is interesting to note that, from the crystal structure of C<sup>E208A</sup> ternary complex, all four of these regions did not show significant relative increase in temperature B-factors compared to the wild-type ternary complex structure. It is likely that, indeed, the presence of MgATP and IP20 stabilized the structure and, thus, H/D exchange of the apo enzyme did help to reveal some of the dynamic regions of the protein that resulted from the mutations and were masked in the crystal structures. However, for C<sup>R280A</sup>, the effect on the destabilization of the αH–αI loop was quite substantial that even the presence of MgATP and IP20 could not stabilize it as observed for C<sup>E208A</sup>.

Decreased folding stability of C<sup>R280A</sup> and C<sup>E208A</sup>

The above data showed that, in the absence of ligands, such as MgATP and IP20, both C<sup>R280A</sup> and C<sup>E208A</sup> exhibited increased H/D exchange rates in regions mainly in the large lobe of the protein molecule. The large lobe is composed of a stable hydrophobic core centered by the αE and αF helices; most loop regions outside the core showed increased H/D exchange rates. We reasoned that either Glu208Ala or Arg280Ala mutation would likely decrease the stability of the protein. To assess the effect by each mutation on the destabilization of the protein, we examined the protein unfolding in the presence of urea and monitored the fraction of the unfolded protein through internal fluorescent signal. As shown in Fig. 5, the concentrations of urea required to unfold 50% of the protein for C<sup>R280A</sup> and C<sup>E208A</sup> were both 2.2 M, lower as compared to 3.2 M required for the wild-type protein.

Reduced efficiency for C<sup>E208A</sup> and C<sup>R280A</sup> phosphorylation by upstream kinase PDK1

PDK1 has been shown to phosphorylate the C-subunit at the Thr197 site, the key residue of which phosphorylation is necessary to render the kinase fully active. The detailed interactions between PDK1 and PKA are not clear. Earlier work showed that residues from the Activation segment including Glu208 affected the phosphorylation of C-subunit by PDK1. We therefore examined whether Arg280Ala mutation also affected PDK1 phosphorylation. It has been shown that when expressed in *Escherichia coli*, wild-type C-subunit gets autophosphorylated at Thr197 due to the consensus PKA recognition sequence at the region: 190RKGKTWLT. This hinders the study of phosphorylation by PDK1. An Arg194Ala mutation has been introduced to eliminate the autophosphorylation at Thr197 to overcome the problem, and it has been shown that the effect of Arg194Ala itself on other properties of the kinase.
is not significant, since after phosphorylation at Thr197 by PDK1, the Arg194Ala mutant behaved very much like the wild-type enzyme in many ways.21

Like the wild-type C-subunit, both C<sup>R280A</sup> and C<sup>E208A</sup> were autophosphorylated at Thr197 when expressed in <i>E. coli</i> (Fig. 6a). Taking advantage of the known fact that Arg194Ala mutation prevented the autophosphorylation at Thr197 by destroying the PKA recognition site, we generated two double mutants, C<sup>R194A/R280A</sup> and C<sup>R194A/E208A</sup>, to study the effects of the ion pair mutation on phosphorylation by PDK1. As shown in Fig. 6, Arg194Ala mutation indeed prevented autophosphorylation as shown earlier. Under similar level of protein expression for all the mutants and the wild-type protein, while CR194A was readily phosphorylated by PDK1, phosphorylation of either CR194A/R280A or CR194A/E208A by PDK1 was greatly reduced (Fig. 6a). Results were reproduced with <i>in vitro</i> phosphorylation carried out with purified proteins. As shown in Fig. 6b, purified PDK1 can phosphorylate purified C<sup>R194A</sup>, but not C<sup>R194A/E208A</sup> or C<sup>R194A/R280A</sup> proteins.

**Discussion**

Because the EPKs represent one of the largest gene families and have enormous impact on the regulation of biological events, they have been intensely studied over the past few decades. Although considerable attention has focused on the conserved residues that cluster around the active-site cleft, little attention has been given to the conserved salt bridge in the C-lobe between Glu208 and Arg280 that lies distal to the active site (Fig. 1b). Glu208 is part of the Activation segment, which is inserted into the C-lobe of the kinase core and is quite large and typically regulated by phosphorylation in the EPKs. Arg280 is part of the GHI subdomain, which serves as a protein docking site and is part of the EPK-specific allosteric network. The Activation segment and the GHI subdomain emerge as structural elements that can play a critical role not only for docking of substrates and regulatory proteins but also for contributing to allosteric regulatory mechanisms.

By replacing Glu208 and Arg280 with Ala, we have elucidated the importance of this salt bridge for the activity and structural stability of the enzyme. The Glu208Ala and Arg280Ala mutants are each severely defective in their catalytic efficiency due primarily to an increased <i>K<sub>m</sub></i> for ATP and a reduced <i>k<sub>cat</sub></i>, resulting in a <i>k<sub>cat</sub>/K<sub>m</sub>(ATP)</i> that is reduced by over 2 orders of magnitude (Table 1). Hydrogen/deuterium exchange experiments showed that the destabilizing effect of these mutations can be observed in the entire kinase structure and is not restricted to the immediate residues that surround the mutated site (Fig. 4). The catalytic loop, the APE-F linker, the αH–αI loop and the αG–αH loop are all significantly more exposed to solvent in each mutant. This global destabilizing effect of the mutations is also supported by the urea unfolding assays (Fig. 5) and by the inability of PDK1 to phosphorylate the Activation segments of both Glu208Ala and Arg280Ala mutants (Fig. 6).

Crystal structures of the Glu208Ala and Arg280Ala mutants could be solved only in the presence of MgATP and peptide inhibitor protein kinase inhibitor (PKI). They did not show any global structural differences between the mutants and the wild-type molecule, and that can be explained by the stabilizing effect of MgATP and PKI. There was, however, a significant difference in the Arg280Ala mutant, which showed significant destabilization of the αH–αI loop that contains the mutated Arg280. This mutation caused more than a 2-fold increase in the temperature factors for residues in this loop compared to the wild-type structure or the Glu208Ala mutant (Fig. 2). Side
chains of almost all residues inside the αH-αI loop also were not resolved. Such destabilization of the loop in the Arg280Ala, but not in the Glu208Ala mutant, is consistent with the distinctive properties of the arginine side chain.

Neutron diffraction of guanidinium ions showed that their interaction with water molecules is very weak. In fact, no recognizable hydration shell was detected around the guanidinium ions. This can be explained by incompatible geometric properties of these flat, rigid ions and tetrahedral molecules of water. This makes arginine a unique amino acid residue. On the one hand, it carries a positive charge on its guanidinium group and is capable of polar and charged interactions; on the other hand, it behaves almost as a hydrophobic residue, not only due to the aliphatic part of its side chain but also due to the poor ability of the guanidinium group to interact with water molecules. Such excessive hydrophobicity explains the fact that arginines in protein structures are usually more buried than can be expected from experimentally measured hydrophobicity. It is known that buried salt bridges often contribute to protein stability. Typically, a large desolvation penalty is thought to be related to these buried salt bridges. In the case of arginine, however, such penalty may be overestimated due to the unique properties of this residue.

In accordance with these properties of arginine, the Glu208–Arg280 salt bridge has a hydrophobic environment that is conserved in different protein kinases. Toward the protein interior side, Arg280 packs against Trp222 from the αF helix at the center of the hydrophobic core. To the surface side, Arg280 packs against Leu277, which provides a hydrophobic cap separating the Glu208–Arg280 salt bridge from solvent. These connections are reinforced by favorable geometry of hydrogen bonding to the Glu280 as well as interactions with nearby charged residues. In addition, the side chain of Arg280 is in hydrogen-bonding distance to the main-chain carbonyl oxygens of Gln274 and Ala218, and Glu208 makes hydrogen bonds to its own main-chain nitrogen atom (Fig. 3) as well as two water molecules in the wild-type structures of PKA (PDB IDs 1L3R and 3FJQ).

Similar interactions can be observed in the structure of CDK2 (PDB ID 1FIN); besides the Glu–Arg interaction, each residue makes hydrogen bonds to several main-chain atoms. This may contribute to the stability of the salt bridge in the otherwise hydrophobic environment. In the Glu208Ala or Arg280Ala mutants, water molecules enter the hydrophobic pocket usually occupied by the Glu208–Arg280 salt bridge and, thus, destabilize this structurally important junction between the Activation segment and the GHI subdomain.

It is intriguing to note that, besides mediating signal relay within the molecule, both Glu208 and Arg280 may also contribute to the interaction with other proteins. Here, we show that, in contrast to the wild-type protein, neither C–E208A nor C–R280A is phosphorylated by PDK1. It has been reported in several protein kinase structures that the highly conserved Glu208–Arg280 pair within one molecule swaps with the same pair in an adjacent molecule in either a naturally occurring dimer or a crystal-packing-induced dimer. This observation provides an attractive hypothesis that when one kinase acts on another kinase, the switch/swapping of the conserved Glu208–Arg280 pair might serve as a mechanism for interaction. Whether this mechanism is true for PKA:PDK1 interaction awaits more experiments.

Several features emerge from this study that have relevance for the entire family. These include the unique importance of an arginine residue and the extended definition of the Activation segment to include the APE-αF helix (APE-F) linker that is located between the APE motif and the αF helix. In the past, we have considered the Activation segment to extend from the DFG motif at the N-terminus to the APE motif at the C-terminus. Included within this segment (Fig. 1d) is the Mg positioning motif (DFG motif), β-strand 9, the Activation Loop, the P+1 Loop and the APE motif. Almost every residue in this segment plays a specific role, and often, that role is different in the active versus inactive conformation of the enzyme. The DFG motif through the Activation Loop can be ordered very differently when the kinase is not phosphorylated on the Activation Loop, and under these conditions, the Regulatory Spine is broken, which is typical for most inactive kinases. Both the Activation segment and the GHI subdomain are firmly anchored to the hydrophobic αF helix and are thus part of the core architecture that defines this family and distinguishes the EPKs from the larger family of eukaryotic-like kinases. Three residues that were found to be conserved in all GHI subdomains (Pro237, Phe238 and Ile250) form a hydrophobic cluster that binds to the APE-alanine and proline (Fig. 1c). This cluster docks to the conserved Trp222 in the αF helix, which serves as a centerpiece of the conserved protein kinase core.

From this study, we see clearly that the segment extending from the APE motif to the αF helix is also an important part of the Activation segment (Fig. 1d). Regulatory subunits, substrates, phosphatases and other protein kinase molecules dock to the junction between the N-terminal part of the APE-F linker and the αG helix (Fig. 1c). In some kinases, such as Csk, there are key phosphorylation sites in the C-terminal part of APE-F linker and these sites can now be considered more carefully in terms of their potential to have importance for regulation. While further studies will be required to demonstrate the full importance of this buried salt bridge in different kinases, we
already have indications that this region has biological significance. It was demonstrated previously that the αH–α1 loop is not only a distal tethering site for certain PKA substrates but also a key allosteric site for PKA and that this region has the capacity to feed back to the active site.26 The mutations of Glu208 and Arg280 confirm this mechanism for feedback to the site of catalysis. Other kinases such as the mitogen-activated protein kinases have an insert in this region that feeds back to the Activation segment and allows for further integration of the Activation segment and GHI subdomains. Finally, it was found that germ line mutations in Glu208 and Arg280 are a hot spot for disease association, suggesting that mutations in the region may prime an individual for subsequent disease phenotypes.27 Our analyses of this conserved salt bridge and its importance for the function and stability of PKA suggest that other kinases should be revisited and examined for the functional relevance of this region that has coevolved to be such a unique and important feature of the EPKs.

Materials and Methods

Materials

Inhibitory peptide IP20 (TTYADFIASGRGTGR-NAIHD, residues 5–24 from the heat-stable PKA inhibitor PKI) was synthesized on a Milligen peptide synthesizer and purified by high-performance liquid chromatography. Pre-packed Mono S 10/10 ion-exchange column and Superdex 75 gel-filtration column were purchased from GE Healthcare. Crystallization reagents polyethylene glycol 6000, methyl-2,4-pentanediol, N,N-bis(2-hydroxyethyl)glycine (Bicine), glycerol and ammonium acetate were obtained from Sigma-Aldrich. Other reagents were from Qiagen, Life Technology and EMD Biosciences. Polycolonal phospho-Thr197 antibody was raised in rabbits against the epitope peptide 556, was KH2PO4 (pH 7.5), 100 mM NaCl, plus protease inhibitors [5 mM benzamidine, 0.5 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 30 μM 1-tosylamido-2-phenylethyl chloromethyl ketone and 30 μM Nε-tosyl-L-lysine chloromethylketone]. The cells were lysed in a microfluidizer at 18,000 psi and spun down by centrifugation at 30,000 g for 45 min at 4 °C. The lysate was incubated with Probindung resin for 1 h at 4 °C. The resin was washed with lysis buffer containing 20 mM NaCl followed by 20 mM imidazole. The protein was then eluted with lysis buffer containing 100–300 mM imidazole.

Enzyme kinetic assay

Kinetic analysis of the mutant and the wild-type C-subunits was performed by radioisotope [γ-32P]ATP labeling as described previously,38 using peptide substrate Kemptide (LRRASLG) or protein substrates DARPP-32 or PPI_I-1. For measurement of the K_m for ATP, ATP concentration was varied, while the concentration of Kemptide was fixed at 250 μM. For determination of the K_m for Kemptide, the ATP concentration was fixed at 250 μM for the wild type and the C_E208A mutant and at 500 μM for C_R280A, with Kemptide concentration varied from 2 μM to 500 μM. To determine K_m for protein substrates, we varied concentration of DARPP-32 or PPI_I-1 from 0.5 μM to 40 μM. Enzyme was diluted in 20 mM Hepes (pH 7.0), 1 mg/ml bovine serum albumin, plus 0.2% β-mercaptoethanol. The assay was carried out in 20 mM Hepes, pH 7.0, and 10 mM MgCl2 with varied enzyme and substrates. Bovine serum albumin of 0.2 mg/ml was also included in the assay to prevent nonspecific loss of diluted enzyme to the assay tube. Final concentrations of the enzyme in the assay were 0.5–1 nM for the wild type and 5–10 nM for C_E208A or C_R280A. Reaction mix of 20 μl was incubated at 30 °C for 10 min and then quenched with 20 μl of 40% acetic acid. Paper chromatography on phosphocellulose paper (Whatman P81) was used to separate unreacted [γ-32P]ATP from the protein-bound radioactivity. Radioactivity was measured by Cerenkov counting on a Beckman LS 6000SC liquid scintillation system.39

Crystallization of C_E208A and C_R280A

For crystallization, the protein from a Mono S column was further purified on a Superdex 75 gel-filtration column (GE Healthcare) and eluted with 50 mM Bicine,
200 mM ammonium acetate, (pH 8.0), plus 2 mM dithiothreitol (DTT). The protein was concentrated to ~7–10 mg/ml and mixed with MgCl₂, ATP and IP20 at a molar ratio of 1:10:10:10 before setting up the crystallization trials. Hanging-drop vapor diffusion was used; each drop consisted of 1 μl of the protein mixture and 1 μl of well solution. Crystals appeared after 1–2 week at 4 °C. For C²E₂⁰⁸Å, the well conditions consisted of 10–12% (w/v) polyethylene glycol 6000, 100 mM Tris–HCl (pH 7.5), plus 10 mM DTT. For C²R₂⁸⁰Å, crystals appeared at well conditions of 13–15% (v/v) methyl-2,4-pentanediol, 100 mM Bicine (pH 8.0), plus 10 mM DTT.

**Data collection and structure refinement**

A 1.7-Å data set for C²R₂⁸⁰Å and a 1.9-Å data set for C²E₂⁰⁸Å were collected at Advanced Light Source (Berkeley, CA) beamline 8.2.2 or 8.2.1 at synchrotron source. Data collection was performed under a liquid nitrogen stream. The cryoprotectant consisted of 1–2% higher precipitant concentration, plus 15% glycerol. The data set was processed and scaled using HKL2000. Molecular replacement was performed using wild-type C-subunit ternary structure (PDB ID 1ATP) as the searching model. Structure refinement was carried out using CNS Solve 1.2. The statistics are shown in Table 1. C²R₂⁸⁰Å was crystallized in a “canonical” PKA C-subunit ternary complex space group P²₁₂₁₂₁, with cell dimensions similar to those for most C-subunit crystals, and one molecule in each asymmetric unit (e.g., PDB IDs 1ATP, 1L3R and 3FJQ). However, the space group for C²E₂⁰⁸Å was P₂₁ (Table 1). It also has only one molecule in each asymmetric unit. For most of the C-subunit structures, including C²R₂⁸⁰Å solved in this study, the first 10–14 residues of the protein are often disordered, making the N-terminal α helix usually starting from residue 11 or 15. In C²E₂⁰⁸Å structure, however, the full N-terminal α helix was well resolved. It formed a straight helix as seen in two other C-subunit mutant structures reported previously. One was a mutant where four residues at the ATP binding site were mutated (Gln84Glu, Val113Ala, Leu173Met and Phe187Leu) (PDB ID 1SMH), and the other was the structure of a Glu230Gln mutant (PDB ID 1SYK). The significance of the ordering of the full α helix was not clear.

**Hydrogen/deuterium exchange analysis**

Preparation of deuterated samples and subsequent DXMS analysis were carried out as previously described. Briefly, the deuterium exchange reaction was started by combining 5 μl of protein sample (5 mg/ml, or 0.125 mM, mutant or wild-type C-subunit) with 15 μl of 20 mM Mops (pH 7.4), 50 mM NaCl, 1 mM DTT in 2H₂O (deuterium buffer). After incubation at room temperature for various amount of time, 10, 30, 100, 300, 1000 or 3000 s, the reaction was quenched with 30 μl of 0.8% (v/v) formic acid and 1.6 M guanidine-HCl in 2H₂O, pH 2.3–2.5 at 0 °C. Samples were immediately passed through a solid-phase pepsin column followed by a V₈ protease column and eluted with 0.5% trifluoroacetic acid at 0.2 ml/min for 2 min. Proteolytic peptides were collected by a C₁₈ column (Vydac), which was subsequently developed with a linear gradient of 10 ml of 8–40% (v/v) acetonitrile in 0.05% trifluoroacetic acid, at 0.2 ml/min. Mass spectrometric analysis was carried out with a Finnigan LCQ mass spectrometer as previously described. Recovered peptide identification and analysis were carried out using in-house software specialized in processing DXMS data.

**Urea unfolding and fluorescence measurements**

Amberlite MB-150 (Sigma, St. Louis, MO) mixed-bed exchanger was added to 8.0 M urea solution and stirred for 1 h to remove ionic urea degradation products and then filtered. Proteins (0.12 mg/ml) were unfolded in various concentrations of urea ranging from 0 to 4 M for 0.5 h at room temperature and monitored by steady-state fluorescence. Fractional unfolding curves were constructed assuming a two-state model and using Fₚ = 1 − [(Rₚ − R₀)/(Rₘ − R₀)], where Fₚ is the fraction of the unfolded protein, Rₚ is the fluorescence measurement and R₀ and Rₘ represent the values of R for the folded and unfolded states, respectively. For unfolding monitored by fluorescence, R is the observed ratio of intensity at 356/334 nm with excitation at 295 nm.

**Phosphorylation of His(6)-C¹⁹⁴₄Å/R²⁸⁰Å and His(6)-C¹⁹⁴₄Å/R²⁸⁰Å mutants by PDK1**

PDK1 phosphorylation of the C-mutants was examined by coexpression of His(6)-C¹⁹⁴₄Å/E₂⁰⁸Å or His(6)-C¹⁹⁴₄Å/R₂⁸⁰Å with PDK1 in E. coli cells and by in vitro phosphorylation using purified proteins. His(6)-C¹⁹⁴₄Å/E₂⁰⁸Å, His(6)-C¹⁹⁴₄Å/R₂⁸⁰Å or His(6)-C¹⁹⁴₄Å was co-expressed with PDK1 in pGEX vector in E. coli BL21(DE3) cells overnight at 16 °C. Cells were lysed by sonication in 50 mM Tris–HCl and 100 mM NaCl, pH 7.0, and the insoluble material was removed by centrifugation at 15,000 g for 30 min. The soluble fraction was subjected to SDS-PAGE, transferred to a nitrocellulose membrane and blotted for phospho-Thr197, C-subunit and PDK1. For the in vitro assay, 0.1 mg/ml of purified His(6)-C¹⁹⁴₄Å/E₂⁰⁸Å, His(6)-C¹⁹⁴₄Å/R₂⁸⁰Å or His(6)-C¹⁹⁴₄Å proteins was incubated with 0.002 mg/ml purified PDK1 in 50 mM Hepes (pH 7.4), 100 mM NaCl, 10 mM DTT, 10 mM MgCl₂ and 1 mM ATP, at room temperature for 90 min. The reaction was quenched in SDS sample buffer and probed for phospho-Thr197 by Western blot analysis.

**Accession numbers**

Coordinates have been deposited at the PDB with accession codes 3QAM (C²E₂⁰⁸Å structure) and 3QAL (C²R₂⁸⁰Å structure).

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