

# The negative charge of Glu-127 in protein kinase A and its biorecognition

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Received 23 March 1999

**Abstract** A set of mutants of protein kinase A (PKA) in which Glu-127 was replaced by Gln, Asp, Asn, and Arg was prepared. Their  $K_m$  and  $V_{max}$  values show that the negative charge of Glu-127 (not merely its hydrogen bonding capacity) is indispensable for the kinase activity, since Glu-127/Gln is inactive, in spite of the fact that it can form hydrogen bonds and is very similar in bulkiness and conformation to wt-PKA. Glu-127 is involved in the biorecognition of PKA, interacting ionically with the positively charged guanido group of Arg P-3 (a major recognition element in the consensus sequence of PKA). In support of this conclusion, it is shown that a regression of the Glu-127 carboxylate by 1.54 Å (as in Glu-127/Asp) results in an active kinase with a similar thermal stability and susceptibility to conformation-dependent proteolysis, a similar  $V_{max}$ , an identical  $K_m$  for ATP, but a >20-fold higher  $K_m$  for kemptide. The two inactive mutants of PKA, Glu-127/Gln and Glu-127/Asn, are potentially useful for studying protein-protein interactions of PKA, e.g. for monitoring enzymatically the displacement of active PKA from its complexes.

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**Key words:** Protein kinase A; Site-directed mutagenesis; Active site; Biorecognition; Specificity; Ionic interactions

## 1. Introduction

The 3-D structure of the catalytic subunit of protein kinase A (PKA) [1,2] is composed of two lobes: one residing within residues 40–119, and the other within residues 128–300. A short strand (residues 120–127) links these two lobes. Together, these three segments constitute the conserved catalytic core of the protein kinase family [3–5]. PKA has in addition two segments that flank this core: a ‘head’ (residues 1–39) and a ‘tail’ (residues 301–350). The ‘head’ and the ‘tail’ are characteristic of each kinase in the family, and therefore may be involved in determining the individual specificity (and the cellular localization) of each kinase. In PKA, the ‘head’ provides a complementary scaffold on which the kinase core is docked while the ‘tail’ is an extended chain, which embraces the two lobes of the core to keep them together.

Based on the relative reactivity of its cysteine residues, it was shown that PKA has a malleable structure [6,7]. This finding was subsequently extended by X-ray crystallography, which demonstrated the occurrence of a loose (‘open’) conformation and a tightly packed (‘closed’) conformation [8,9]. In the non-liganded kinase, the loose tail of PKA is susceptible to distinct proteolysis by a kinase splitting membranal proteinase (KSMP) [10–12]. The identification of the specific cleavage site for KSMP further confirmed the earlier predic-

tions regarding the conformational flexibility of this kinase [10,11,13]. The occurrence of this flexibility was supported by low angle neutron scattering [14], by circular dichroism [15,16], and by chemical modification with a water-soluble carbodiimide [17]. Upon binding of substrates, the two lobes of the core change their mutual orientation [9], closing the cleft between them. This structural change [12] also involves a translocation of the carboxy-terminal tail, which moves the phenolic hydroxyl of Tyr-330 from a distance of ~10 Å (in the ‘open’ conformation) to reach (in the closed conformation) a distance of ~3 Å from the nitrogen atoms of the Arg residue at position P-3 of the PKA consensus sequence [12].

Charge-to-alanine scanning previously showed that Glu-171 in yeast (equivalent to Glu-127 in mammals) is essential for PKA to be active [18–20]. On the basis of spatial proximity findings [2,21] it was subsequently concluded that Glu-127 may take part in the recognition of both the ATP and the peptide co-substrates of PKA as it is located at a distance of ~2.9 Å from the side-chain guanido group of the Arg residue at position P-3 of the consensus sequence of this kinase, and at distances of ~3 Å from the 2'-OH and ~2.6 Å from the 3'-OH groups of the ribose ring of ATP (Fig. 1A) [22]. Interestingly, the carbonyl group of Leu-49 (from the small lobe), the carboxylate of Glu-127 (from the linker), and the phenolic hydroxyl of Tyr-330 (from the tail), together with the ribose hydroxyls of ATP (the nucleotide substrate), and the guanido group of Arg P-3 in PKI(5–24) (the peptide substrate/inhibitor), form a nest-like structure which accommodates a water molecule (Fig. 1B) conserved in the different crystal structures of PKA [22].

Here we report that the negative charge (not merely the hydrogen bonding capacity) of Glu-127 is indispensable for PKA to be active and that this charge is mainly involved in the specific biorecognition of the protein substrates of PKA. On the basis of the kinetic parameters of a set of single site mutants we show that the carboxylate of Glu-127 is mainly involved in a charge-charge interaction with the positively charged guanido group of Arg P-3, i.e. with a major recognition element in the consensus phosphorylation sequence of the kinase [23–25]. We also report here some catalytic and structural properties of three single site mutants of PKA (Glu-127/Gln, Glu-127/Asn, and Glu-127/Asp), which are conformationally similar to the wild type enzyme, yet are either inactive or possess a significantly lowered affinity for the peptide substrate(s) of the kinase, and are potentially useful in studying structure-function relationships of protein kinases in general.

## 2. Methods

### 2.1. Preparation of Glu-127 mutants

The wild type murine  $C\alpha$  subunit gene inserted into the pRSET-B

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vector served as a template for the site-directed mutagenesis. It was a gift from Susan S. Taylor (University of California, San Diego, CA, USA). Mutations were introduced using the polymerase chain reaction (PCR) [see Ausbel, F.M. et al., *Current Protocols in Molecular Biology* §8.5]. The wild type or the mutant enzyme-carrying vector was used for transformation of the *Escherichia coli* BL21(DE3) strain. The conditions used for growing, induction, and expression have been described elsewhere [26,27]. 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<sub>2</sub>, 1 mM dithiothreitol and 0.1% octyl-β-D-glucopyranoside. The insoluble particles were removed by centrifugation, the supernatants were collected, and the level of expression was measured by SDS-polyacrylamide gel electrophoresis [28]. The enzyme content was measured by quantitative immunoblotting with antibodies specific to the catalytic subunit of PKA (anti-P3) ([13] and references therein) using an ECL detection system (Amersham, UK) for developing. X-ray films were exposed to the ECL-developed blots, and quantitated by computing densitometer scanning.

## 2.2. Kinetic parameters of the mutant kinases

The kinase assays were carried out as described earlier [12] using kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) or histone II-A as substrates.  $K_m$  and  $V_{max}$  values were determined from the Lineweaver-Burk plots of inverted initial velocity vs. inverted substrate concentrations, according to the following equation [29]:

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

where  $V_{max}$  is the maximal velocity,  $v$  is the initial velocity,  $[S]$  is the concentration of the substrate used and  $K_m$  is the Michaelis constant of that substrate.

## 2.3. Thermal stability measurements

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 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<sub>2</sub>, 1 mM dithiothreitol and 0.1% octyl-β-D-glucopyranoside, and then the residual activity was measured in the kinase assay buffer.

## 2.4. Assay of the KSMP/meprin β cleavage of recombinant PKAs

The wild type (wt) catalytic subunit of PKA and the Glu-127 mutants were radiolabeled with [<sup>35</sup>S]methionine using the TnT coupled reticulocyte lysate system (Promega). The KSMP/meprin β cleavage [10–13] was allowed to proceed at 23°C for 0, 3, 5, 10, or 20 min using a recombinant meprin expressed in 293 cells by Dr. A Chestukhin and L. Litovchick from our laboratory [13]. The cleavage was arrested by adding Laemmli sample buffer used for SDS-PAGE [28], and boiling (5 min at 95°C), and the products were separated by SDS-PAGE [28]. The protein bands were visualized in autoradiograms, then scanned for quantitation. The initial rates of cleavage (arbitrary units) are reported in comparison to rate of cleavage of the wt catalytic subunit (taken as 100%).

## 3. Results and discussion

### 3.1. The role of Glu-127 in PKA studied by single site mutations

The interpretation of structure-function studies of proteins using mutagenesis is often not conclusive, due to the fact that such mutations bring about local conformational changes, whose contribution to the change in function is difficult to evaluate. This question was quantitatively addressed by Eriksson et al. [30] using six ‘cavity-creating mutations’ in T4 lysozyme to show that such substitutions decrease the stability of the protein, since the removal of the wild type side chain allows some of the surrounding atoms to move towards the vacated space.

Here we attempt to get an insight into the role of Glu-127 in PKA, since it is indispensable for the enzyme to be active,

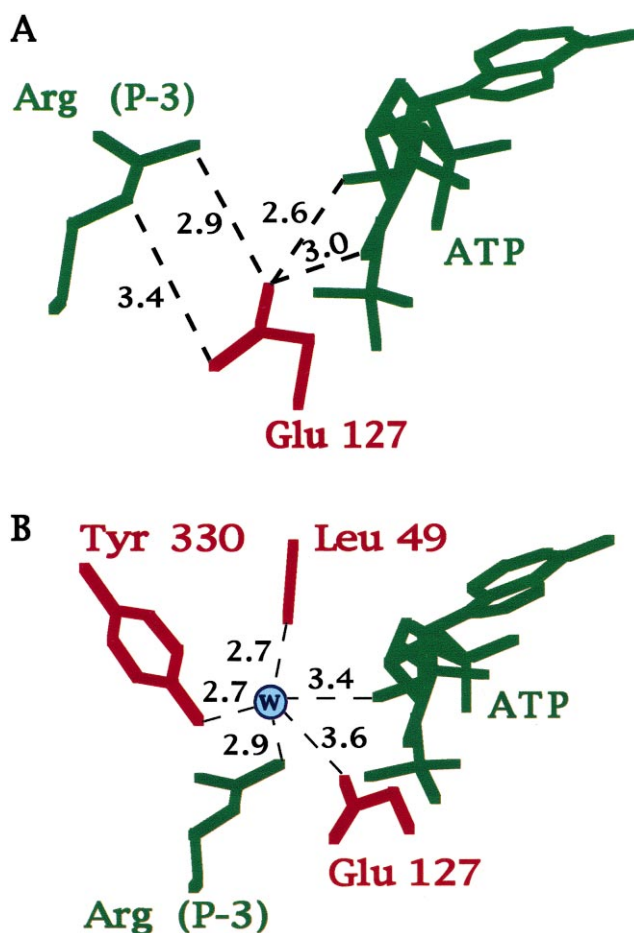


Fig. 1. A: Distances between the carboxylate group of Glu-127 (in red) and its vicinal functional groups in the 3-D structure of the catalytic subunit of PKA. The distances given are from this carboxylate and the guanido nitrogens of Arg (P-3), as well as the 2' and 3' hydroxyls of the ribose ring of ATP. B: Distances between the active site conserved water molecule 'W' [22] (marked 'W' here), and its surrounding functional groups which include Glu-127, Leu-49 and Tyr-330 (all in red and provided by the kinase), as well as the functional groups provided by the peptide substrate (Arg P-3), and by the ribose of (ATP) (both in green). The distances are measured from the rC:MnATP:PKI(5–24) ternary complex (Brookhaven National Laboratory Protein Data Bank code 1ATP) [36].

and since it is part of a conserved assembly of functional groups originating from distal amino acids in the PKA sequence. This nest-like assembly also includes the two co-substrates of the enzyme: ATP, and the peptide inhibitor analogous to the substrate, as well as a conserved water molecule (Fig. 1B) [22].

The questions addressed in this report include:

What is the major role played by the carboxylate of Glu-127? Is it involved in substrate recognition? If so, of which of the two co-substrates?

What is the nature of the interactions through which this carboxylate acts: ionic? hydrogen bonding?

Is it possible to minimize (by the use of an appropriate set of mutants) the change in functional effects which are due to local conformational changes? In other words, is it possible to design a mutant enzyme that will have a native conformation yet be catalytically inactive? (Such proteins

may yield, for example, very useful competitive inhibitors of the kinase.)

### 3.2. Choice of a set of substitutions for Glu-127

On the basis of distance considerations Glu-127 can be involved in multiple interactions with both ATP and the peptide substrate/inhibitor. To evaluate the relative contribution of these interactions, we prepared a set of mutants in which the changes in the size of the side chain were minimal, and thus decreased the probability of a functional change ensuing from a local conformational change, resulting from the mutation. In addition, we monitored two gross structural parameters to ascertain that this assumption is backed by experimental evidence.

The set of mutants contained the following members: (I) the recombinant wt enzyme as a reference; (II) a Glu-127/Asp mutant which has the negative charge of the carboxylate, at the tip of a chain shorter by one methylene group (1.54 Å); (III) a Glu-127/Gln mutant (has no negative charge but is essentially identical to the native enzyme in the size of the side chain at position 127 and can still form hydrogen bonds); (IV) a Glu-127/Asn mutant, which serves as a non-charged analog of the Glu-127/Asp mutant. In addition we prepared (V) a Glu-127/Ala mutant which was previously reported to have a drastically lowered catalytic efficiency ( $k_{\text{cat}}/K_m$ ), specifically 1200-fold smaller than the wild type enzyme [18]; and (VI) a Glu-127/Arg mutant (positively charged side chain).

### 3.3. The major role of Glu-127 is to identify (by ionic interactions) the Arg P-3 residue of the peptide substrates/inhibitors of PKA

The kinetic parameters obtained with the various mutants at position 127 are summarized in Table 1. It is clearly evident from these results that all the mutations at position 127 which do not preserve the negatively charged carboxylate group yield a catalytically inactive enzyme. This includes the replacement of Glu-127 by Gln, a residue in which the negative charge of the Glu-127 carboxylate is neutralized by its conversion into an amide. This replacement actually represents a minimal structural change from the point of view of size and bulkiness of the side chain. Therefore, the lack of activity in this mutant cannot be attributed to a local structural change, as could be the case in the Glu to Ala mutation. In line with this hypothesis, the replacement of Glu-127 by Asp, a residue in which the negative charge of the Glu-127 carboxylate is preserved, yielded a mutant which was still (yet less) catalytically active. The fact allowed us to get a deeper insight into the role of Glu-127. As seen in Table 1, the regression of the

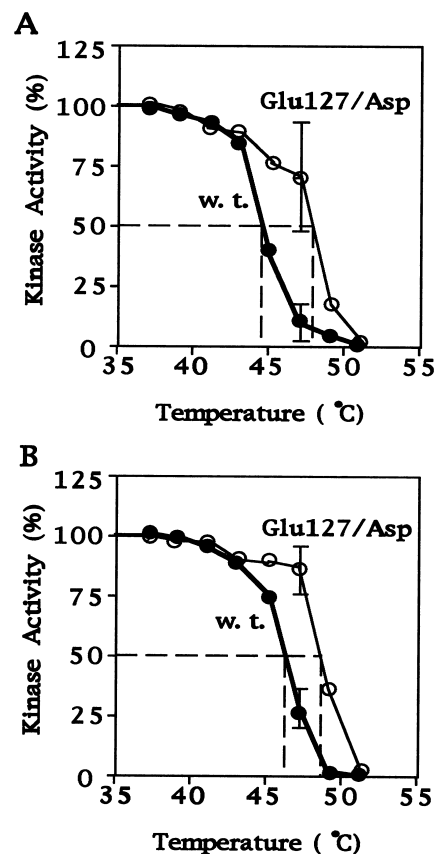


Fig. 2. A comparison between the thermal stability of the wild type (w.t.) recombinant catalytic subunit (filled circles), and its Glu-127/Asp mutant (open circles). A: Inactivation in the absence of substrates. B: Inactivation in the presence of kemptide (120  $\mu$ M). The enzymes were preincubated at the indicated temperature for 2 min before assay and the residual activity was determined as described in Section 2. For the values of half maximal inactivation ( $T_{1/2}$ ) see text.

carboxylate by 1.54 Å results in a 21-fold increase in the  $K_m$  for kemptide, without any significant change in the  $K_m$  for ATP. This finding clearly indicates that the main contribution of Glu-127 is to interact with (and thus to identify) the Arg P-3 residue in the specific peptide substrates or inhibitors of PKA.

### 3.4. Does the mutation of Glu-127 yield a labile, and thus an inactive protein?

Having the mutant Glu-127/Asp that was found to be still

Table 1

The effect of single site mutations at Glu-127 on the kinetic parameters ( $K_m$  and  $V_{\text{max}}$ ) of PKA, measured with kemptide or with histone II-A as substrates

Kinetic parameter	Wild type enzyme	Single site mutation at position 127				
		Asp	Gln	Asn	Arg	Ala
$K_m$ , $\mu$ M (for kemptide)	17 $\pm$ 5	360 $\pm$ 50	*	*	*	*
$K_m$ , $\mu$ M (for ATP)	41 $\pm$ 7	43 $\pm$ 9	*	*	*	*
$V_{\text{max}}$ , nmol/mg min (for kemptide)	515 $\pm$ 160	620 $\pm$ 130	< 0.5	< 0.5	< 0.5	< 0.5
$V_{\text{max}}$ , nmol/mg min (for histone II-A)	480 $\pm$ 60	430 $\pm$ 60	< 2.5	< 2.5	< 2.5	< 2.5

The wild type enzyme and the mutants Glu-127/Asp, Glu-127/Gln, Glu-127/Asn, Glu-127/Arg, and Glu-127/Ala were expressed in *E. coli*. The  $K_m$  values were measured for both of the co-substrates kemptide and ATP. The specific activities ( $V_{\text{max}}$  values) were measured for the protein histone II-A, and for the peptide substrate kemptide. For experimental details see Section 2. Please note that the mutants Glu-127/Gln, Glu-127/Asn, Glu-127/Arg, and Glu-127/Ala are devoid of enzymatic activity on both the peptide and protein substrates. Therefore no  $K_m$  and  $V_{\text{max}}$  values could be given for them (asterisks).

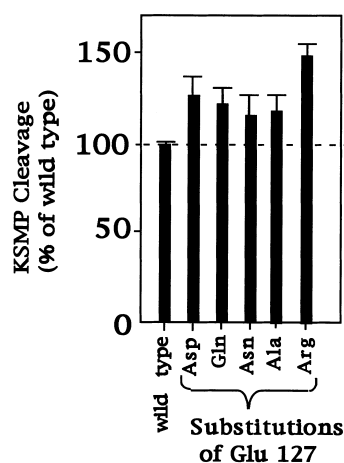


Fig. 3. The effect of Glu-127 single site mutations on the cleavability of the catalytic subunit of PKA by KSMP. The  $^{35}\text{S}$ -radiolabeled wild type kinase and its indicated mutants were translated in a rabbit reticulocyte system and cleaved by KSMP as described in Section 2. The products were 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%.

catalytically active (Table 1), we could attempt to find out whether a mutation at this position can, in and of itself, make the enzyme more labile, so that by the time we express it and measure its activity it becomes less active. As seen in Fig. 2, in the absence of the peptide substrate, the Glu-127/Asp mutant ( $T_{1/2} = 48.0^\circ\text{C}$ ) is in fact more stable than the wild type enzyme ( $T_{1/2} = 44.6^\circ\text{C}$ ), clearly showing that the absence of the Glu residue at position 127 is not critical by itself for the stability of the enzyme. Interestingly, the enhanced stability of the Glu-127/Asp mutant occurs also in the presence of the peptide substrate of PKA at the saturating concentrations used in the assay ( $T_{1/2} = 48.7^\circ\text{C}$  for the mutant, compared with  $T_{1/2} = 46.3^\circ\text{C}$  for the wild type enzyme, Fig. 2A,B). On the basis of these findings it seems reasonable to conclude that the major role of Glu-127 in this kinase is not structural but rather in the biorecognition of the Arg P-3 residue of its peptide substrates/inhibitors.

### 3.5. Assessing possible conformational changes of all the mutants

To obtain a deeper insight into the reason for the lack of activity of some of the mutants, we made use of its conformation-dependent cleavage by the kinase splitting membran

proteinase (KSMP/meprin  $\beta$ ) [10–13]. This proteinase was previously shown to act as a conformation-dependent probe for the catalytic subunit of PKA [31]. The cleavage occurs at the carboxy-terminal end of the kinase, between E332 and E333 [13], and it can be monitored not only by activity, but also by the change in molecular weight, i.e. for the active as well as the inactive mutants.

As seen in Fig. 3, the loosening of structure which brings about the exposure of E332–E333 is not dramatically altered by mutation of Glu-127 into either another negatively charged amino acid residue (Glu-127/Asp) or a neutral residue. The biggest change seems to occur with the Glu-127/Arg mutant, which introduces a positive charge at this position.

## 4. Concluding remarks

Glu-127 is indispensable for PKA to be active. Here we provide evidence showing that the role of this amino acid residue is not just to provide a hydrogen bonding device, but to be directly involved in the biorecognition of this kinase. On the basis of the kinetic parameters of a set of single site mutants, their heat denaturation curves (where applicable), and the susceptibility of these mutants to undergo conformation-dependent proteolysis [31], we show that the major role of Glu-127 is to provide a negative charge for ionic interaction with the Arg P-3 in the RRXS/T consensus sequence of PKA (for the specificity of this enzyme, see [34,35,37]). The contribution of Glu-127 to the binding of the nucleotide co-substrate ATP is relatively minor.

The two Arg residues in the above mentioned consensus sequence were previously shown to contribute dramatically to the efficacy of PKA substrates. For example the  $V_{\max}/K_m$  value of GGGGGGRRGSGG was found to be  $\sim 630$  and  $\sim 425$  fold bigger than for GGGGGGRRSGG and GGGGGRRRGGSGG respectively [23–25]. In fact, on the basis of an elegant study using an oriented degenerate peptide library, it was proposed that the specific occupancy at position P-3 strongly affects the substrate specificity of a wide variety of protein kinases [32]. As seen in Table 2, all the protein kinases which are known to have a preference for a positively charged amino acid residue (Arg or Lys) at position P-3 have a Glu or Asp residue at the position corresponding to Glu-127. However, the opposite is not true: there are protein kinases that have a Glu or Asp residue at the position corresponding to Glu-127, yet do not have a preference for a positively charged amino acid at position P-3. In fact, some of these kinases even have a preference for a negatively charged amino acid residue at this position of their consensus sequence

Table 2  
Multiple alignment of nine Ser/Thr protein kinases around the position corresponding to Glu-127 in PKA

Protein kinase	Sequence corresponding to Glu-127 in PKA (underlined)	Consensus sequence for phosphorylation (position P-3 underlined)	References
cAMP-dep. PK (PKA)	PGGE <sup>127</sup> MFS	XRRX(S/T)	[33]
cGMP-dep. PK	LGGE <sup>443</sup> LWT	(R/K)(R/K)X(S/T)	[34]
PKC- $\alpha$	NGGD <sup>423</sup> LMY	XRXX(S/T)XRX	[33]
Phosphorylase kinase- $\gamma$	KKGE <sup>109</sup> LFD	X(R/K)XX(S/T) or KRKQI(S/T)VR	[35,34]
Calcium-calmodulin-dep. PK- $\alpha$	TGGE <sup>95</sup> LFE	X(R/K)XX(S/T)	[33]
Myosin light chain kinase	EGGE <sup>142</sup> LFF	XKKRXXRXX(S/T)	[34]
MAPK (Erk 1)	IVQD <sup>123</sup> LME	XXPX(S/T)P	[35]
Casein kinase I (isoform 1)	PSLE <sup>126</sup> DLF	XXDXX(S/T)IXX or X(S/P)XX(S/T) or XEXX(S/T)	[35]
Casein kinase II	NNTD <sup>117</sup> FKQ	EDDE(S/T)EDEE or X(S/T)XXEX	[35,33]

For each kinase the consensus phosphorylation sequence is shown.

(e.g. casein kinase I and II, Table 2). In that respect, it is interesting to note that the biorecognition of the negative charge in the P-3 position of the consensus sequence may be transferred to the position of another amino acid residue, which resides in this subsite, such as Tyr-330 (Fig. 1B and [22]). In line with this suggestion is the findings of Songyang et al. [32,33], who prepared an oriented degenerate peptide library to study the substrate specificities of protein kinases. They showed that in the case of casein kinase I  $\gamma$  and  $\delta$ , which have a non-charged amino acid residue (Leu) at the position equivalent to Glu-127, the kinase does have an Arg residue at the position of Tyr-330 (Fig. 3 in Songyang et al. [33]).

Finally, we also report here some catalytic and structural properties of three single site mutants of PKA (Glu-127/Gln, Glu-127/Asn, and Glu-127/Asp), and show that they are conformationally similar to the wild type enzyme, yet are either inactive or possess a significantly lowered affinity for the peptide substrate(s) of the kinase. These mutants are potentially useful to gain an additional insight into structure-function relationships in PKA and in protein kinases in general.

**Acknowledgements:** We thank Dr. Iris Schwartz for her helpful discussions. This work was supported by the Minerva Foundation, Munich (Germany), by the German-Israeli Foundation for Scientific Research and Development (G.I.F.), and by the Kekst and the Marcus Sieff Foundations. S.Sh. is the incumbent of the Kleeman Professorial Chair in Biochemistry at the WIS, and M.B. is the recipient of a training fellowship from the Israeli Ministry of Science.

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