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Aza-β^3-amino acid containing peptidomimetics as cAMP-dependent protein kinase substrates

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ABSTRACT

Peptidomimetic analogs of the peptide RRASVA, known as the "minimal substrate" of the catalytic subunit of the cAMP-dependent protein kinase (PKA), were synthesized by consecutive replacement of natural amino acids by their aza-β^3 analogs. The peptidomimetics were tested as PKA substrates and the kinetic parameters of the phosphorylation reaction were determined. It was found that the interaction of these peptidomimetics with the enzyme active center was sensitive to the location of the backbone modification, while the maximal rate of the reaction was practically not affected by the structure of substrates. The pattern of molecular recognition of peptidomimetics was in agreement with the results of structure modeling and also with the results of computational docking study of peptide and peptidomimetic substrates with the active center of PKA. It was concluded that the specificity determining factors which govern substrate recognition by the enzyme should be grouped along the phosphorylatable substrate, and such clustering might open new perspectives for pharmacophore design of peptides and peptide-like ligands.

1. Introduction

Protein kinases, which constitute one of the largest gene families encoded by human genome, control the majority of physiological phenomena through regulatory phosphorylation of functionally important proteins in eukaryotic cells as well as in plants and bacteria [1–3]. In this phosphorylation reaction, the γ-phosphate group of ATP is transferred to the phosphorylatable residue of the protein or peptide substrate and therefore, the accuracy of recognition of the phosphorylation sites, implemented through high substrate specificity of these enzymes, has a crucial impact on the effectiveness of this signaling mechanism. On the other hand, however, it has been found that all members of the protein kinase superfamily have a structurally conserved catalytic core comprising the nucleotide binding N-terminal lobe and the larger C-terminal lobe responsible for binding of the phosphorylatable site of the protein/peptide substrate [4–7]. These structural features have been first discovered for the catalytic subunit of the cAMP-dependent protein kinase (EC 2.7.11.11) [8], further denoted as PKA, which is the simplest and a well-studied enzyme among protein kinases and therefore recognized as a "model" of this kinase superfamily.

Molecular recognition of substrates by PKA can be presented on three structural levels. Firstly, the peptide sequence around the phosphorylatable serine is recognized and this fit seems to be obligatory for the following phosphorylation step. For PKA, this sequence should contain two arginine residues in positions −3 and −2 relatively to the phosphorylatable serine residue at position 0 [9,10], and the peptide RRASV, derived from the phosphorylation site of L-type pyruvate kinase and named a "minimum substrate" for this enzyme [9], represents this recognition pattern. The idea about the "primary substrate specificity" was confirmed by the results of the structural analysis of PKA complexes with peptides [11,12], and also by the results of the kinetic study of phosphorylation of L-pyruvate kinase mutants with various peptide structures flanking the phosphorylatable serine [13]. Secondly, substrate binding may occur apart from the primary binding site of the catalytic center [14,15]. These interactions are probably important in phosphorylation of proteins or long peptides [16,17], and much less information is available about specificity of these sites. Thirdly, some anchoring proteins may support the fine targeting of the kinase to its protein substrates [18] *in vivo*.

As summarized, the most thorough understanding of substrate specificity concerns the recognition of the peptide primary

Abbreviations: Boc, tert-butyloxycarbonyl; BSA, bovine serum albumin; DiPEA, N,N′-diisopropylethylamine; DMF, N,N-dimethylformamide; Fmoc, 9H-fluoren-9-ylmethoxycarbonyl; HOTB, 1-hydroxybenzotriazole; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PKA, cAMP dependent protein kinase catalytic subunit, EC 2.7.11.11; SPPS, solid phase peptide synthesis; TBTU, O-(1H-benzotriazole-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TRIS, 2-amino-2-hydroxymethyl-propane-1,3-diol.

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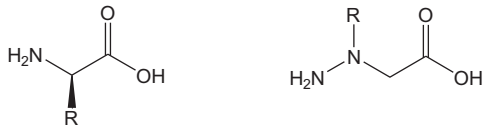
structure around the phosphorylation site, since many peptides have been involved in these studies. Moreover, an attempt has been made to rationalize these data by quantitative structure–activity relationships [19]. This analysis has revealed that the primary specificity of PKA for short peptides cannot be expressed by a single consensus sequence, while reactivity of these substrates is determined by a sum of various structural effects of amino acid residues depending on their position in the peptide. The same conclusion was reached through statistical analysis of the phosphorylation sites in natural substrates [14].

All these investigations, including QSAR analysis, have been concentrated on the study of α-peptides and do not concern the structure of the peptide backbone. Until recently, the most radical alteration of the backbone structure was made by gradual replacement of L-amino acids with their D-isomers [20]. This analysis revealed that all these chimerical peptides were still phosphorylated by PKA, except the substrate with D-serine whose OH-group was obviously wrongly orientated for the phosphoryl transfer reaction.

In summary, all the results quoted above agree that the rules for amino acid side group recognition in the peptide binding pocket of PKA are rather "loose". Therefore, it was interesting to analyze the influence of alterations in the structure of the substrate backbone upon their recognition by PKA. As no data on backbone-modified peptidomimetic substrates phosphorylation have been reported previously, we synthesized and tested a series of minimum substrate RRASVA analogs, where each amino acid, except serine, was sequentially replaced by an appropriate aza-β^3-amino acid.



L-amino acid aza-β^3-amino acid

This replacement introduced additional degrees of conformational flexibility with the rotation around the Cα and Cβ bonds that has made the aza-β^3-amino acid containing peptides as attractive tools for design of peptide-like drugs of improved biological profile and pharmacokinetic properties [21]. Secondly, introduction of aza-β^3-amino acids shifted the arginine side chains relatively to each other. Therefore this replacement provided a good possibility to analyze the role of mutual placement of these side groups in protein kinase specificity and test tolerance of PKA against modification of backbone of peptide-like ligands. As proteolytic stability [22] and antigenic and immunogenic properties [23] of this type of peptidomimetic compounds have been considered before, the present study has opened further perspectives for use of these compounds also for design of drugs acting on protein kinases, which are generally recognized as the second most important group of drug targets after G-protein-coupled receptors [24].

2. Experimental

2.1. Chemicals

γ-[^{32}P] ATP was obtained from Amersham (UK). The catalytic subunit C_{alpha} of mouse cAMP-dependent protein kinase (PKA), recombinantly expressed in *E. coli*, 30 U/mg, 0.1 mg/mL, lot 040916, was obtained from Biaffin GmbH & Co. KG (Germany). ATP, TRIS/HCl, BSA and H_3PO_4 were purchased from Sigma–Aldrich (USA). Phosphocellulose paper P81 was acquired from Whatman (UK). $MgCl_2$ was purchased from Acros (Germany). Peptide substrate RRASVA of purity above 95% was purchased from GL Biochem Ltd. (Shanghai, China) and was characterized by MS spectrum and HPLC. Peptidomimetic substrates RRASVazaβ^{3}A, RRASazaβ^{3}VA, RRazaβ^{3}ASVA, RazaβRASVA, and azaβ^{3}RRASVA were synthesized and characterized in this work as described below. Chemicals for peptide synthesis were obtained from commercial suppliers (Senn Chemicals, Iris Biotech GmbH, Fluka, and Novabiochem) and were used without further purification. Analytical thin-layer chromatography was carried out on pre-coated silica gel plates (60 F_{254}, Merck). Flash column chromatography was carried out using silica gel 60 (230–400 mesh ASTM) from Merck & Co., Germany.

2.2. Analytical methods

NMR spectra were measured on a 300 MHz instrument (Bruker, Germany) in DMSO-d_6 or $CDCl_3$ as solvent and internal reference. A Waters HPLC system was used for purification and analysis of synthetic products. Purification of aza-β^3-peptides was performed on a C18 XTerra® RP18 (19 × 300 mm, 10 μm) column using water/acetonitrile linear gradient (5–100% B in 40 min, 8 mL/min, rt, 215 nm). The following buffers were used: (eluent A) water containing 0.08% TFA by volume; (eluent B) acetonitrile containing 1% TFA by volume. Characterization of purified aza-β^3-peptide hybrids were performed on a C18 XTerra® (4.6 × 250 mm, 5 μm) column using water/acetonitrile linear gradient (5–100% B in 30 min, 1 mL/min, 30 °C, 215 nm). High resolution mass spectra of the products were measured on a LTQ Orbitrap (Thermo Electron) spectrometer (positive ionization, static nanospray, boron silicate emitters Proxeon, resolution 100,000 at *m*/*z* 400, external calibration).

2.3. Synthesis of Fmoc protected aza-β^3-amino acids

Fmoc-aza-β^3-Ala-OH was prepared from methyl hydrazine, and Fmoc-aza-β^3-Val-OH and Fmoc-aza-β^3-Arg(Boc)-OH were obtained from hydrazine hydrate as previously described [25,26]. All products were purified chromatographically and their structure was verified by NMR spectra. The details of these syntheses as well as the results of product analysis are given in Supplement.

2.4. Synthesis of aza-β^3-amino acid containing peptidomimetics

Peptide analogs were prepared on a 0.1 mmol scale by employing the Fmoc/*t*Bu SPPS strategy on a Liberty Microwave-Enhanced Peptide Synthesizer (CEM GmbH, Germany), using HOBt and TBTU as activators, and DiPEA as base in DMF. The preloaded Fmoc-α-Ala-OH Wang resin (0.40 or 0.81 mmol/g) was used. A coupling time of 10 min was used for the introduction of Fmoc-aza-β^3-amino acids into peptides. For introduction of Fmoc-Arg(Pbf)-OH, double couplings were performed. Cleavage was performed with TFA-TIS-H_2O 95:2.5:2.5 cocktail over 4 h. The yield of peptides with aza-β^3-amino acids was lower than the yield of the common peptide, indicating that coupling of aza-derivatives was not so effective as natural amino acids, and was dependent upon the particular amino acid derivative. The lowest yield was obtained with aza-β^3-arginine (Table 1). Crude peptides were purified chromatographically [27] and the purity of the products pooled was above 95%, as found from the analytical HPLC runs and MS analysis. The yields of these products were listed together with their experimental HR molecular masses in Table 1.

2.5. Kinetic measurements

The initial rate of peptide phosphorylation was measured at 30 °C as described previously [28,29]. Briefly, the reaction mixture (final volume 100 μL, 50 mM TRIS/HCl, pH 7.5) contained 100 μM γ-[^{32}P] ATP, 3–750 μM peptide or peptidomimetic substrate,

Table 1
Characterization of aza-β³-amino acid containing peptidomimetics, derived from the PKA "minimum substrate" RRASVA.

Peptidomimetics	*M* observed[a]	Overall yield of synthesis (%)
RRASVazaβ³A	674.4054	49
RRASazaβ³VA	674.4051	39
RRazaβ³ASVA	674.4054	69
Razaβ³RASVA	674.4052	22
azaβ³RRASVA	674.4053	21

[a] The calculated *M* is 674.4056.

Table 2
Phosphorylation of aza-β³-amino acid containing peptidomimetics by PKA at ATP concentration 100 μM, 30 °C, 50 mM TRIS/HCl, pH 7.5.

Substrate	K_m, μM	$10^2 \cdot V$, (mg/ml) s^{-1}	$10^2 \cdot k_{II}$, (mg/ml) s^{-1} μM^{-1}	$\frac{k_{II}^{aza\beta 3}}{k_{II}^{RRASVA}}$
RRASVazaβ³A	43.7 ± 8.8	26 ± 5	0.6 ± 0.2	0.18
RRASazaβ³VA	122.1 ± 21.6	25 ± 4	0.3 ± 0.04	0.09
RRazaβ³ASVA	59.8 ± 9.4	27 ± 1	0.5 ± 0.1	0.16
Razaβ³RASVA	19.1 ± 2.9	32 ± 2	1.7 ± 0.2	0.52
azaβ³RRASVA	273.5 ± 43.8	28 ± 5	0.1 ± 0.02	0.03
RRASVA	11.1 ± 3.5	36 ± 3	3.2 ± 0.1	1.0

10 mM $MgCl_2$, and 0.3 μg/mL of the enzyme. The stock solution of PKA was diluted 500-fold in 50 mM TRIS/HCl buffer (pH 7.5) containing 1 mg/mL BSA, and 15 μL of this solution was added into the reaction mixture to initiate the phosphorylation reaction. At known time intervals after starting the reaction, 10 μL aliquots were taken from the reaction mixture and spotted onto pieces of phosphocellulose paper, which were subsequently immersed into ice-cold 75 mM phosphoric acid to stop the reaction. The pieces were then washed four times with cold 75 mM H_3PO_4 (10 min each time) to remove excess γ-[^{32}P] ATP and were dried at 120 °C for 25 min. The radioactivity bound onto the paper was measured as Cherenkov radiation using a Beckman LS 7500 scintillation counter. The values of the initial rate of the phosphorylation reaction (v) were calculated from the slopes of the product concentration *vs* time plots.

2.6. Computational methods

Peptidomimetic structure modeling was performed using the Spartan 4.0 software suite (Wavefunction, Inc., USA) and the minimum-energy conformations of the compounds were obtained. Conformational searches were made by using molecular mechanics with additional conditions of the aqueous medium for finding stable geometry. All peptides were represented as zwitterions for these calculations. The peptidomimetic docking modeling with PKA was carried out using the AutoDock Vina software (ver. 1.0.3) [30]. The initial structures of peptide analogs were calculated as described above. The docking compatible structure formats of the protein were prepared by AutoDockTools (ver. 1.5.4) [31]. The fitting box with 0.3 Å of grid spacing was defined once and used for all docking calculations. The fitting area covers the peptide binding site of PKA and the best docking pose with the lowest energy for each peptidomimetic within this area was obtained.

2.7. Data processing

Calculations and statistical analysis of the data were carried out using the GraphPad Prism (ver. 4.0, GraphPad Software Inc., USA) software packages. The results were reported with standard errors.

3. Results and discussion

The methods of synthesis of Fmoc protected azaβ³-amino acids and their application for preparation of peptidomimetics have significantly developed in recent years [25,26,32,33] and were used in this study to prepare a series of analogs of the peptide RRASVA known as the "minimum substrate" for PKA. These synthetic procedures were based on the SPPS common strategy, with necessary modifications of the coupling step of aza-β³-amino acids to increase the overall yield of the synthesis (see Section 2). In this way, a series of peptide RRASVA analogs with a successive replacement of natural amino acids with their aza-β³-analogs was obtained with reasonable yields (Table 1) which, however, still remain below the results of the common peptide synthesis.

The sequence containing azaβ³-serine was not included into this series of peptidomimetic substrates. As it was seen from RRASVA analogs, where the L-amino acids were replaced with D-amino acids, the modification at the serine residue yielded a non-phosphorylatable substrate [20]. Therefore, we decided not to modify this part of the molecule, as this might similarly hinder the transfer of the phosphate group from ATP to the substrate molecule in the catalytic step.

It was found that all peptidomimetic compounds synthesized were relatively good substrates of PKA and the results of kinetic analysis, obtained at ATP concentration 100 μM, were listed in Table 2. Additionally, the kinetic constants for RRASVA phosphorylation were determined. The initial velocities of phosphorylation of all these substrates were well described by the common Michaelis–Menten equation:

$$v = \frac{k_{cat}[S][E]_0}{K_m + [S]}, \quad (1)$$

as illustrated in Fig. 1, and were used to calculate the V ($=k_{cat}[E]_0$) and K_m values listed in Table 2.

Additionally, we used the initial linear part of the Michaelis–Menten plots, characterizing the phosphorylation reaction under the second-order rate conditions, where $[S] < K_m$, and calculated the second-order rate constants k_{II}:

$$v = k_{II}[S][E]_0. \quad (2)$$

The kinetic parameters k_{II} have the same meaning as the ratio k_{cat}/K_m, as $k_{cat} = \frac{V}{[E]_0}$, but since their values ware determined from different sets of experimental data, this approach can be used as an internal test of applicability of the conventional reaction scheme. It can be seen from Table 2 that the constants k_{II} were, indeed, in agreement with the k_{cat}/K_m values, confirming that there was no substrate or product inhibition of the phosphorylation reaction.

As can be seen from Table 2, such variation in structure had practically no effect on the maximal velocity of the phosphorylation reaction of peptidomimetics. Noteworthily, a similar trend has been previously observed for an extensive series of short peptide substrates, as summarized in [19]. On the other hand, however, and again similarly with normal peptide substrates, variation in the structure of peptidomimetics had a significant influence on the K_m values which varied more than 20 times within the studied series of substrates. The same specificity pattern was also observed for the second-order rate constants (Table 2). Therefore, the K_m values should characterize the effectiveness of substrate interaction with this enzyme. Then again, these data should be comparable for peptidomimetic and peptide substrates, as the catalytic step controls the positioning of the serine residue of all these compounds in the enzyme active center.

The results listed in Table 2 revealed that PKA was, indeed, sensitive to the alteration of the backbone structure of substrates, where –CHR– group of natural amino acids was replaced by –NR–CH_2–, but substituents R were the same. This sequence-dependent effect of backbone modification is illustrated in Fig. 2,

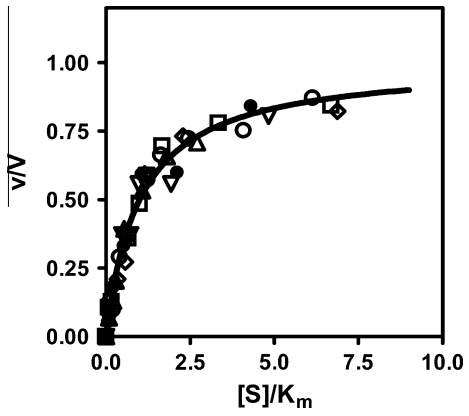


Fig. 1. Consolidated Michaels–Menten plot for phosphorylation of aza-β^3-amino acid containing peptidomimetic substrates RRASVazaβ^{3}A (◇), RRASazaβ^{3}VA (○), RRazaβ^{3}ASVA (□), Razaβ^{3}RASVA (▽), azaβ^{3}RRASVA (△), and RRASVA (●) by PKA (30 °C, 50 mM TRIS/HCl, pH 7.5).

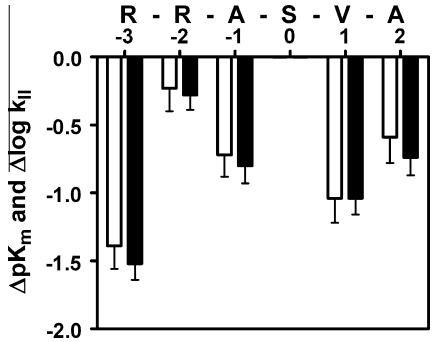


Fig. 2. Sequence-dependent effect of replacement of L-amino acids by their azaβ^3-analogs in RRASVA upon the PKA-catalyzed phosphorylation of these substrates. The effects on K_m and k_{II} were characterized by the following values: $\Delta pK_m = p\left(\frac{K_m^{RRASVA}}{K_m^{azaβ3}}\right)$ and $\Delta \log k_{II} = \log\left(\frac{k_{II}^{azaβ3}}{k_{II}^{RRASVA}}\right)$.

where the reactivity of the aza-analogs and RRASVA is compared by using the relative ΔpK_m and $\Delta \log k_{II}$ values, defined as $\Delta pK_m = p\left(\frac{K_m^{RRASVA}}{K_m^{azaβ3}}\right)$ and $\Delta \log k_{II} = \log\left(\frac{k_{II}^{azaβ3}}{k_{II}^{RRASVA}}\right)$ for different positions, labeled as −3, −2, −1, 0, +1, +2 relatively to the serine residue, denoted as position zero.

It can be seen from Fig. 2 that the most significant effect was observed if arginine was replaced by its azaβ^3-analog in position −3, while a relatively small effect was observed if this replacement was made in position −2. This result might be confusing, as the presence of two arginine residues in positions −3 and −2 has always been recognized as the most important substrate specificity factor for PKA. Here, however, the observed results could be explained if we view the two arginine side groups as a single specificity cluster and consider that in Razaβ^{3}RASVA these groups are still close, while this localization is ruled out in azaβ^{3}RRASVA where the additional methylene group of the peptidomimetic backbone separates these two residues.

The influence of the backbone shift on the peptide structure was studied by computer modeling of Razaβ^{3}RASVA, azaβ^{3}RRASVA and RRASVA, and the results were shown in Fig. 3. Indeed, the backbones of Razaβ^{3}RASVA and RRASVA have rather similar structures in their lowest energy state. At the same time, clear differences can be observed in the case of the third compound, azaβ^{3}RRASVA. Moreover, this backbone alteration has also changed the spatial orientation of the arginine side chains in azaβ^{3}RRASVA, if compared with Razaβ^{3}RASVA and RRASVA, and most probably has impact on the binding effectiveness of these substrates (Table 2). Thus, an azaβ^3-amino acid can efficiently replace a common amino acid within a peptide sequence, providing that this replacement does not hamper interaction of side chains with their recognition site.

For modeling the substrate docking in the peptide binding cleft of PKA, we proceeded from the structure of the enzyme complex with AMPPNP-Mn [4], suggesting that this nucleotide inhibitor is simulating the presence of ATP which is the second substrate of the phosphorylation reaction. This docking analysis revealed that peptidomimetic and peptide substrates had a somewhat different orientation when bound in the enzyme active center (Fig. 4) and were characterized by the following values of the docking energy: −7.0 kcal/mol for Razaβ^{3}RASVA, −6.3 kcal/mol for azaβ^{3}RRASVA, and −6.8 kcal/mol for RRASVA. At the same time, the distances between the oxygen atom of the substrate serine residue and the γ-phosphorus atom of the ATP derivative were all rather close for these compounds: 3.6 Å, 4.0 Å and 3.2 Å, respectively. This result was in agreement with the similar maximal rate values for substrates (Table 2).

The same explanation seems to be valid also for RRASazaβ^{3}VA where the azaβ^3-group was located in position +1 and the mutual positioning of two amino acids in the C-terminus of the substrate was affected. This change produced more than a 10-fold decrease in the binding effectiveness of the substrate as compared with RRASVA (Fig. 2). At the same time, the backbone modifications made in other places had smaller effects.

To sum up, the effect of backbone modification clearly depended upon its location along the substrate molecule. As the location of the most significant effects seems to be in correlation with

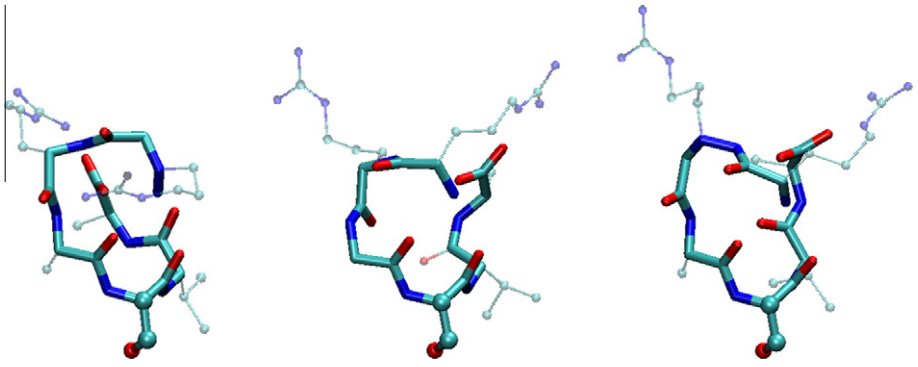


Fig. 3. Comparison of the calculated backbone structures of azaβ^{3}RRASVA (left), RRASVA (center) and Raza β^{3}RASVA (right). The backbones were shown as stick models, three atoms of the serine residue, which were similarly fixed in all three molecules were shown as ball and stick models. The side groups of both arginine residues of the first compound were directed "backwards", while the same residues in the two following compounds were oriented "upwards".

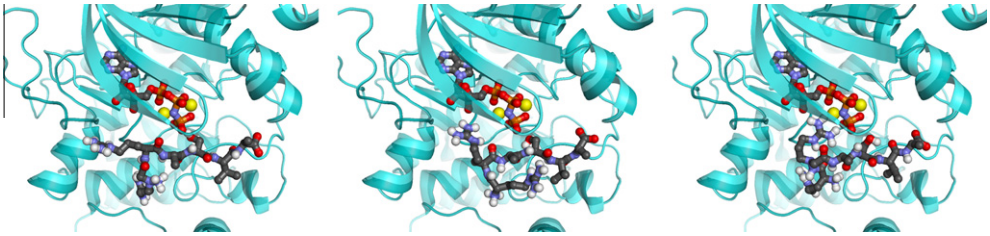


Fig. 4. Docking of azaβ[3]RRASVA (left), RRASVA (center) and Razaβ[3]RASVA (right) with the substrate-binding cleft of the PKA complex with AMPPNP-Mn, simulating the second substrate ATP of the phosphorylation reaction. The following color code was used for ligands: black – carbon, light gray – hydrogen, if shown, red – oxygen, blue – nitrogen, orange – phosphorus, and yellow – Mn.

the specificity pattern of the binding site for peptide side chains, it could be suggested that these phenomena are both governed by interaction of the amino acid side chains with their recognition sites on the protein molecule. These interactions can be changed either by variation of the side chain structure, as has been shown before, or by the backbone modification in the proper place. Therefore, instead of recognition of a single side chain, the interaction of the enzyme with clusters consisting of at least two amino acids should be considered. Outside of these recognition clusters, the backbone modifications are more tolerated and have smaller effects on ligand binding effectiveness. Therefore, the backbone modification can be used to define the clusters and even to rank them according to their stake into the overall specificity. On the other hand, the regions between these clusters may have importance as the sites of substrate modification with minimal effect on its activity. This might also be an important aspect to be considered in the design of peptidomimetic ligands.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bioorg.2010.05.004.

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