Inhibition of the cAMP-Dependent Protein Kinase by Synthetic A-Helix Peptides†

Stéphane Gamboni,‡ Catherine Chaperon,‡ Kirstin Friedrich,§ Pascal J. Baehler,‡ and Christophe D. Reymond*,‡

Institut de biologie cellulaire et de morphologie, Rue du Bugnon 9, CH-1005 Lausanne, Switzerland, and EPFL, Laboratoire de chimie physique des polymères et membranes, CH-1015 Lausanne, Switzerland

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ABSTRACT: The catalytic subunit of the cAMP-dependent protein kinase from *Dictyostelium discoideum*, PkaC, displays the same properties as its mammalian counterpart, except for being about twice as large in size. Sequence comparisons indicated the presence of a conserved α-helix (A-helix) within the N-terminal region of PkaC which could potentially establish close contacts with the catalytic core [Véron, M., et al. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10618−10622]. We show in this report that a synthetic peptide with the A-helix sequence inhibits PKA activity, whereas unrelated peptides display no inhibitory activity. The inhibition seems competitive with respect to the kemptide substrate rather than due to binding to a secondary site. We further show by amino acid replacements that the last lysine of the A-helix sequence is involved in this specific inhibition. A model is proposed for the possible role of the A-helix.

The cAMP-dependent protein kinase (PKA)[1] is composed of a heterotetramer of catalytic and regulatory subunits (C and R, respectively). Binding of cAMP to the R subunits dissociates the holoenzyme, releasing C subunits which can phosphorylate protein substrates. In addition to two cAMP binding sites, the R subunits contain a pseudosubstrate sequence able to bind the C subunit. In mammals, two types of biochemically distinct R subunits encoded by four genes (RIα, RIβ, RIIα, and RIIβ) have been characterized, whereas three highly homologous C subunits (Cα, Cβ, and Cγ) are encoded by different genes. In lower eukaryotes such as yeast, different isozymes have been found (for review, see *2*).

Besides its essential role during development (for review, see *3*), the cAMP-dependent protein kinase from *Dictyostelium discoideum* shows the particularity of being composed of a heterodimer with a single regulatory and a single catalytic subunit (*4*). The R subunit gene encodes a 41 kDa protein very similar to the mammalian subunits of RI type. The catalytic subunit has a size of 73 kDa, about twice the size of its mammalian counterpart (*5*). Based on sequence conservation, a catalytic core can be defined which corresponds to the C-terminal half of the protein. In addition to the catalytic core, an α-helix motif (residues 77−98) resembling the A-helix of mammalian PKA was identified within the 332 amino acid long N-terminal half of PkaC (*1*). We thus wanted to determine the possible function of this A-helix.

The crystal structure of mammalian C subunits revealed that the conserved catalytic core consists of two lobes. The small lobe, beginning at residue 43, displays an antiparallel β-sheet conformation, whereas the large lobe, beginning at residue 128, is dominated by α-helices with a small β-sheet located near the cleft that is essential for catalysis. The A-helix, located within the first 40 residues of the mouse C subunit, lies outside of the catalytic core. According to the crystal structure, this amphipathic N-terminal helix interacts with the surface of the core opposite to the catalytic site (*6*). We hypothesized that A-helix synthetic peptides could possibly displace the original helix and thus modulate PKA activity.

In the present work, we tested the effect of an oligopeptide with the A-helix sequence on PKA activity. We further verified the specificity of the observed inhibition by using related peptides, taking into account their potential to form helical secondary structures.

MATERIALS AND METHODS

Strains and Culture Conditions. *Dictyostelium discoideum* cells K-P (*7*) overexpressing PkaC were grown under shaking at 260 rpm at 22 °C in HL-5 broth containing 20 mg/mL G418. For PKA activity tests, cells were grown to (3−6) × 10^6/mL and harvested by centrifugation at 1000*g*. The supernatant was removed and the cells washed twice in PDF buffer (20 mM KCl, 1.2 mM $MgSO_4$, 6.7 mM K_2HPO_4, 13.3 mM KH_2PO_4) prior to lysis.

Synthetic Peptides. Synthetic peptides were obtained from the Institute of Biochemistry, University of Lausanne (Switzerland).

PKA Activity Tests. Vegetative cells were lysed at 4 °C in 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 0.1% Triton X-100, and protease inhibitors (0.2 mM AEBSF,

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* Corresponding author: IBCM, Rue du Bugnon 9, CH-1005 Lausanne, Switzerland. Phone: 41-21-692 5271. FAX: 41-21-692 5255. Email: Christophe.Reymond@ibcm.unil.ch.

‡ Institut de biologie cellulaire et de morphologie.

§ EPFL, Laboratoire de chimie physique des polymères et membranes.

[1] Abbreviations: cAMP, cyclic adenosine 3′,5′-monophosphate; DEAE, diethylaminoethyl; *E. coli*, *Escherichia coli*; ^{125}I, iodine isotope 125; IC_{50}, concentration of inhibitor at which 50% of the total activity is measured; K_i, inhibition constant; PKA, cAMP-dependent protein kinase; PkaC, PKA catalytic subunit; PKI, PKA inhibitor; SDS, sodium dodecyl sulfate; C309, truncated C-subunit of *Dictyostelium* PKA, in which residues 1−308 were deleted.

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1 mM benzamidine, 10 mg/mL leupeptin, and 0.1 mM TLCK). DEAE-Sephacel (Pharmacia) was added, and binding was performed at 4 °C for 30 min. After centrifugation, PKA was eluted in high-salt buffer (Tris-HCl, pH 7.5, 100 mM NaCl) by incubation for 5 min at 4 °C.

PKA activity was measured using the PepTag kit from Promega with the fluorescently labeled peptide L-R-R-A-S-L-G (kemptide) as a substrate according to the manufacturer's instructions. Phosphorylation by PKA alters the net charge of the kemptide from +1 to −1, so that the phosphorylated and unphosphorylated peptides can be separated on buffered agarose gels. Enzymatic reactions were carried out at 30 °C for 30 min. Phosphokemptide was eluted from gel slices after migration, and the extent of phosphorylation was measured in a fluorimeter. In some experiments, we used purified bovine PKA (1.4 mg/mL) from the PepTag kit diluted to a concentration of 0.01 mg/mL in 20 mM Tris, pH 7.5, 10 mM $MgCl_2$ with 0.1 mg/mL labeled kemptide. A truncated version of the *Dictyostelium* C subunit, C309, expressed in *E. coli* (gift from Dr. M. Véron, Institut Pasteur, Paris), was also used at a concentration of 0.2 mg/mL. C309 was stored at a 10-fold higher concentration and diluted immediately before use, due to its tendency to precipitate. A high-affinity inhibitor of PKA, the PKI peptide fragment encompassing amino acids 5−24, was used at a concentration of 1 mM to test the specificity of kemptide phosphorylation by PKA.

Binding of ^{125}I-Labeled H2 Peptide. H2 peptide (Figure 1) was labeled according to the Bolton−Hunter method as previously described (*8*). Binding experiments were performed in the presence of increasing concentrations of PKI (from 0 mM to 2.5 mM) mixed with constant amounts of labeled H2 (200 mM) and bovine PKA C subunit (1.4 mM) in reaction buffer (20 mM Tris-HCl, 5 mM $MgCl_2$). Bound and free peptides were separated by gel filtration on Sephadex G-50 columns, and radioactivity in the flow-trough was measured in a gamma counter.

Circular Dichroism. Circular dichroism spectra were obtained using an AVIV circular dichroism spectrometer (Model 62 DS; Lakewood, NJ) coupled with a data processor (IgorPro for MacIntosh). A sample cell of 1 mm path length was used to determine the secondary structures. Synthetic peptides were diluted at concentrations of 50 and 250 mM in the reaction mixture used for PKA tests, however, omitting compounds known or likely to interfere with CD measurements (i.e., Triton X-100, ATP, and protease inhibitors). Measurements for secondary structure determinations were performed at wavelengths ranging from 186 to 250 nm.

RESULTS

Sequence comparison had shown the presence of an α-helix (A-helix) conserved throughout evolution, which is located close to the N-terminus of the *Dictyostelium* PKA (*1*). To determine how the A-helix modulates PKA activity *in vitro*, we used a PKA activity test based on synthetic fluorescent kemptide as substrate (see Materials and Methods). Assays with whole cell extracts failed due to the presence of a trypsin-like activity which cleaved the peptide between the two arginines (data not shown). To overcome this problem, PKA present in extracts of vegetatively growing *Dictyostelium* cells was prepurified on DEAE-Sephacel; 100 mM NaCl eluates were then assayed for PKA activity, leading to reproducible measurements (Figure 1). To ensure that we dealt with PKA and not any phosphotransferase activity, PKI(5−24), a known specific inhibitor of PKA (*9*), was added to the reaction mixture, resulting in the inhibition of about 90% of the activity.

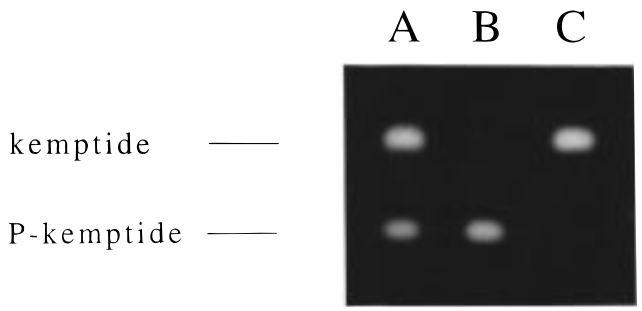

FIGURE 1: (Lane A) PKA activity of DEAE-prepurified cell extracts of *Dictyostelium*. Phosphorylated kemptide and unphosphorylated kemptide are separated on a buffered agarose gel. (Lane B) Phosphorylation of kemptide by purified bovine catalytic subunit as a positive control. (Lane C) Unphosphorylated kemptide as a negative control.

H2	S	A	T	D	R	L	T	K	M	D	I	E	E	K	W	D	N	K
H5	–	–	–	–	–	–	–	–	–	–	**A**	–	–	–	–	–	–	–
H4	–	–	–	–	–	–	–	–	–	–	–	–	–	**A**	–	–	–	–
H3	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**A**	–	–	–
H6	–	**E**	–	–	–	**E**	–	–	**E**	–	–	–	–	–	–	–	–	–
H7	–	**K**	–	–	–	**K**	–	–	**K**	–	–	–	–	–	–	–	–	–
H8	–	–	–	–	–	–	–	–	–	–	**E**	–	–	–	**E**	–	–	–
H9	–	–	–	–	–	–	–	–	–	–	–	–	–	**A**	–	–	–	**A**
P10	K	V	K	T	A	K	K	S	S	K	K	S	S	A	K	K		
P2	S	R	S	S	P	K	R	A	A	V	G	K	K	T	G	A	K	K	V	A
P6	S	A	A	T	T	S	P	Q	K	S	S	R	S	S	P	K	R	A	A	V

FIGURE 2: Synthetic A-helix oligopeptides. H2 peptide is identical to the A-helix sequence from residues 77 to 98 of Dd PkaC. Amino acid replacements are shown in boldface type. The peptides 2, 6, and 10 display A-helix unrelated sequences.

We first synthesized an α-helix peptide with a sequence identical to that of the Dd PkaC A-helix (H2 in Figure 2) and tested its effect on PKA activity. Increasing concentrations of H2 added to prepurified cell extracts reduced PKA activity with an apparent IC_{50} of 75 μM (Figure 3A). The level of residual PKA activity of around 10%, reached upon addition of 200 μM H2, stayed constant with higher concentrations of H2 (data not shown) and corresponded to a phosphotransferase activity unrelated to PKA (see above). A synthetic peptide, P10, with a sequence unrelated to H2 (Figure 2) did not affect PKA activity (Figure 3A). P10 is not of the same size as H2 and its derivatives. To ascertain that the absence of inhibition was not linked to peptide size, we tested further H2-unrelated peptides, P2 and P6 (Figure 2). Both P2 and P6 were unable to inhibit PKA activity (Figure 7).

To further investigate the role of the A-helix, we measured the activity of a truncated version of the *Dictyostelium* PKA C subunit expressed in *E. coli* (*10*). C309 contains essentially only the catalytic core of PKA and is missing the A-helix. H2 shows the same inhibitory capacity on both crude *Dictyostelium* extracts and purified C309 (data not shown), indicating that displacement of the A-helix is probably not responsible for the observed inhibition.

We then asked whether inhibition by H2 was restricted to *Dictyostelium* PKA. Kemptide phosphorylation by the catalytic subunit purified from bovine was measured in the

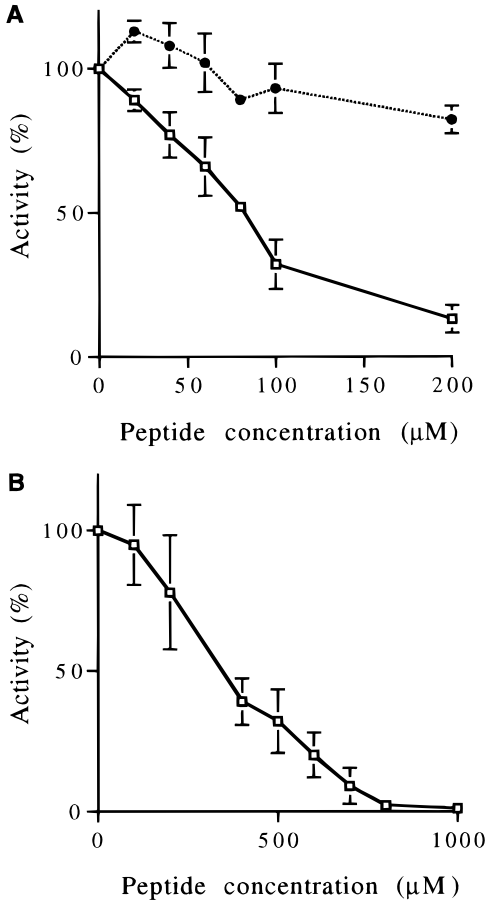

FIGURE 3: (A) Inhibition by the synthetic oligopeptides H2 (□) and P10 (●). The peptides were added to partially purified *Dictyostelium* cell lysates, and PKA activity was measured by using fluorescent kemptide. Activity without peptide was taken as 100%. (B) Inhibition of purified bovine catalytic subunit by H2. The enzyme and the fluorescent kemptide was diluted as indicated under Materials and Methods prior to the assays. As for tests performed with cell extracts, activity without peptide was taken as 100%. Mean values of three independent experiments are shown in panels A and B.

presence of increasing concentrations of H2 (Figure 3B). H2 inhibits purified mammalian PKA to completion.

From the crystal structure of the mouse catalytic subunit, the corresponding A-helix is predicted to bind to the "back" of the core, being in contact with both lobes (*1*). We would thus predict from this model that H2 should bind to a site remote from the active phosphorylation site, thus resulting in noncompetitive inhibition. To address this question, we performed experiments with purified bovine PKA in which we added increasing amounts of kemptide, keeping the H2 concentration constant. A modified Eadie−Hofstee plot of the data obtained with three different concentrations of H2 indicates competitive instead of noncompetitive inhibition, with a K_i of about 0.65 mM (Figure 4A). However, based on these kinetic results, partial competitive inhibition, that is, H2 reducing the affinity of PKA for its substrate while binding to a remote site, cannot be clearly distinguished from competitive inhibition due to the binding of both H2 and kemptide to the same site.

We then measured directly the binding of ^{125}I-labeled H2 (200 μM) to PKA in the presence of increasing concentrations of PKI, which is known to bind at the active site of the enzyme with affinity in the nanomolar range (Figure 4B).

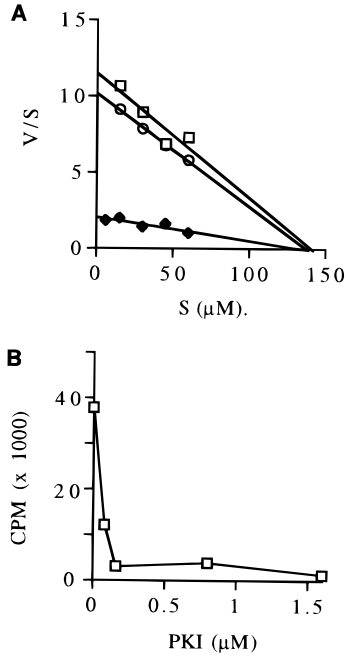

FIGURE 4: (A) Eadie−Hofstee plot of inhibition kinetics. Speed (V) of kemptide phosphorylation by purified bovine PkaC over substrate concentration (S) was plotted versus kemptide concentration. The concentration of peptide H2 varied from 0 (□) or 100 μM (○) to 250 μM (◆). (B) Binding of labeled A-helix peptide to the catalytic subunit: H2 peptide labeled with ^{125}I (200 μM) was incubated in the presence of increasing concentrations of PKI with constant amounts of bovine PKA C subunit.

H2 binding was fully prevented by a concentration of PKI (0.2 μM) roughly equivalent to that of the enzyme. Thus, either the H2 peptide competes with PKI at the catalytic site or the binding of PKI dramatically reduces the affinity of PKA for H2 binding at a remote site and vice versa.

To determine how specific the inhibition by H2 was, we replaced amino acids within the A-helix by alanines (Figure 2). In peptides H3, H4, and H5, we substituted each amino acid predicted to bind to the core according to the model based on the crystal structure of the catalytic subunit (*1*, *12*). As seen in Figure 5, these peptides showed inhibition kinetics close to the original H2.

We then searched for amino acids potentially participating in the inhibition by checking the relative position of hydrophobic and charged residues. The amino acids can be projected on one plane down the helix axis to obtain a helical wheel representation (Figure 6). Hydrophobic amino acids are clustered on opposite sides of the helix, namely, Ile11 and Trp15 on one side and Ala2, Leu6, and Met9 on the other. Ile11 and Trp15 were already substituted in H5 and H3, respectively, without changing the inhibitory potency. We further substituted both of these residues with charged amino acids in peptide H8. The capacity to inhibit the *Dictyostelium* catalytic subunit was unchanged, however (Figure 5). We then replaced Met9, Ala2, and Leu6 with either negatively or positively charged amino acids (H6 and H7, respectively, in Figures 2 and 6). Again no difference was observed with these peptides compared with the original H2 (Figure 5). Thus, substitution of hydrophobic residues on both sides of the A-helix does not interfere with the inhibitory activity.

Since the A-helix peptides possibly bind to the active site of the catalytic subunit, we compared the sequences of H2

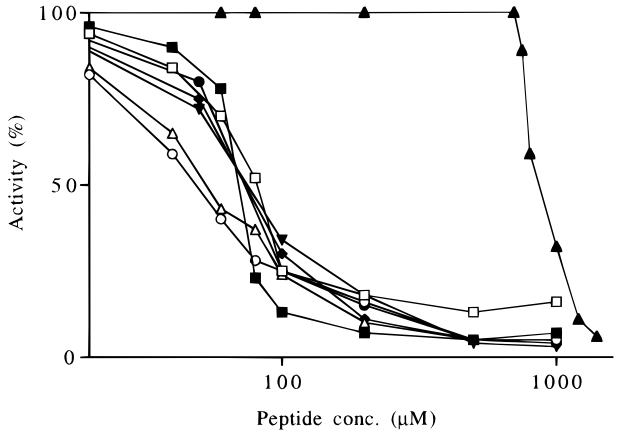

FIGURE 5: Inhibition by synthetic oligopeptides. The peptides were added to partially purified *Dictyostelium* cell lysates, and PKA activity was measured using fluorescent kemptide. Activity without peptide was taken as 100%. Amino acid changes in the H2 sequence have no significant effects on inhibition kinetics, except the replacement of lysines 14 and 18 by alanines which leads to a marked loss of inhibitory potency. The logarithmic scale starts at 20 μM. Each point represents the mean value of three independent experiments. Legend: H2 (□); H3 (■); H4 (○); H5 (△); H6 (●); H7 (◆); H8 (▼) H9 (▲).

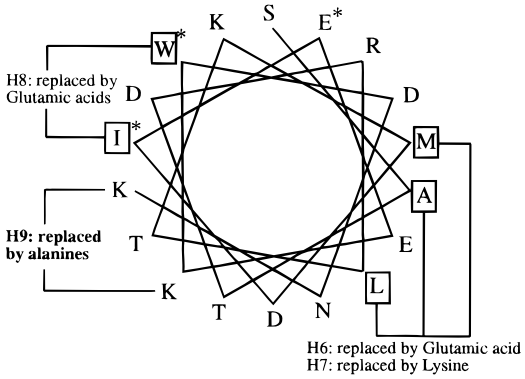

FIGURE 6: Helical wheel model of A-oligopeptides. The amino acids are projected on one plane down the α-helix axis. The H2 sequence comprises hydrophobic (boxed) residues which are clustered on opposite sides. Amino acids proposed to interact with the core are labeled by asterisks (*1*). Residue replacements are shown with the indication of the corresponding peptides.

and PKI(5−24), a peptide known as a potent inhibitor of PKA. Although the linear amino acid sequences differed, we detected a superimposable localization of basic and hydrophobic residues by comparing their helical wheel diagrams. In particular, Lys14 and -18 in H2 and Arg15 and -19 in PKI can be superimposed, when Ile11, Leu 6, Ala2, and Met9 in H2 are fitted with Tyr7, Phe10, Ala12, and Ala21 in PKI(5−24). Lys14 had already been substituted in H4 without any effect. We thus replaced both Lys14 and -18 by alanines and measured the inhibitory potential of the H9 peptide (Figure 5). A decrease in the inhibitory potency was observed, increasing the K_i by at least 10-fold. This result indicates that either the last basic amino acid or the last two lysines of the A-helix are required for potent inhibition of the catalytic subunit.

In all experiments, we devised the modified peptides so that they should theoretically adopt an α-helical conformation according to secondary structure predictions using both Chou−Fasman and Robson−Garnier models (*13*). To verify that such short peptides were able to form α-helices in solution, we performed circular dichroism. All peptides predicted to form α-helices showed between 7 and 15% α-helical conformation (4% for H9), indicating that at least a portion of the peptides adopted proper conformation in solution.

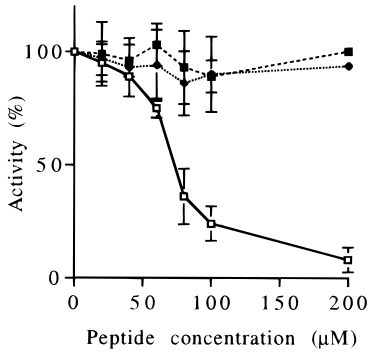

FIGURE 7: Effect of the synthetic oligopeptides P6 and P10 on *Dictyostelium* PKA activity measured from partially purified cell lysates compared to that obtained with H3 (□). Neither P6 (●) nor P10 (■) are inhibitors of the enzymatic activity. Mean values of three independent experiments are shown.

To further test the role of conformation on inhibitory properties, we tested A-helix-unrelated sequences able to form α-helices. The oligopeptides P2 and P6 form up to 6 and 7% α-helices in solution, respectively, as seen by circular dichroism; however, both were unable to inhibit PKA (Figure 7). These results indicate that the ability of the peptides to form α-helices in solution is not sufficient to confer an inhibitory potential. The inhibition is due to the presence of specific amino acids within the A-helix related peptides, in particular the last lysine.

DISCUSSION

We show in this report that a synthetic oligopeptide (H2), with a sequence identical to that of the A-helix found in *Dictyostelium* PkaC, inhibits activity from both *Dictyostelium* and mammalian PKA catalytic subunits. Our experiments are thus dealing with a fundamental property of all PKAs conserved throughout evolution (*14*, *15*). The A-helix had been proposed to bind to the back of the core based on the crystal structure of the mouse catalytic subunit (*1*). Recent experiments both *in vivo* and *in vitro* indicated a minor role if any for the A-helix (*10*, *16*). However, in these experiments, it was observed that removal of the N-terminal half of the *Dictyostelium* PKA results in a destabilization of the protein, possibly reducing the steady-state catalytic activity. Thus, the increase in PKA activity resulting from the absence of the A-helix may have been masked by enzyme instability.

The specificity of H2 interaction with the catalytic core is indicated by the fact that H2-unrelated peptides 2, 6, and 10 show basically no PKA inhibitory activity. Furthermore, the ability to form α-helices per se is not sufficient to confer an inhibitory potential, as seen with H2-unrelated peptides able to form an α-helix in solution. Specific replacement of lysines 14 and 18 within the A-helix leads to a clear decrease in inhibitory potency, whereas other replacements, including hydrophobic and charged residues, had no measurable effects. Thus, the interaction of A-helix related peptides with the catalytic core at the active site seems specific.

The A-helix peptide can be compared with PKI (*9*), since biochemically both peptides seem to compete for the same

site on the core. PKI, a potent inhibitor of PKA, presents a pseudosubstrate site composed of the five-residue consensus recognition sequence (R-R-X-S-I) with the actual phosphorylatable Ser (position P) replaced by an Ala (for review, see *9*). The H2 peptide contains the sequence RXXKXXI which is very similar to the alternative substrate sequence RXXRXXSI. However, a negatively charged aspartic acid (D), rather than Ser, Thr, or Ala, is present at the putative phosphorylation site just before the Ile. Furthermore, the Ile (I) was replaced by Ala and Glu in peptides H5 and H8, respectively, without changing the inhibitory activity. We thus conclude that this potential pseudosubstrate site is probably not responsible for the inhibition of PKA by H2. Interestingly, the affinities of H2 and kemptide for the bovine C subunit of PKA are in a similar range (K_i and K_d of 650 and 200 μM, respectively; *17*), largely above that of PKI (K_i of 2.3 nM).

In addition, the high inhibitory potential of PKI(5−24) relies on the presence of an amphiphilic N-terminal α-helix. The synthetic A-helix peptide H2 shows some resemblance with the α-helix moiety of PKI, particularly in the relative localization of basic and hydrophobic residues. Replacement of Lys14 and -18 by Ala in H2 (peptide H9) resulted in about a 10-fold decrease in inhibitory potency. Likewise, mutations of Arg P^{-6} and P^{-2} to Ala reduced the inhibitory ability of PKI(5−24) (*18*). Both Arg P^{-6} and P^{-2} are implicated in the binding of PKI to the core as depicted by the cocrystal of PKI with recombinant mouse catalytic subunit (*12*). In addition, the amphiphilic α-helix region of PKI from P^{-16} to P^{-8} (TTYADFIAS) binds to the core, in particular via the Phe at position P^{-11}. In peptides H6 and H7, substitution of Met9, present at a position equivalent to Phe P^{-11} in PKI, with charged amino acids did not result in a change in inhibitory activity. Furthermore, replacement of all hydrophobic residues on either side of the α-helix did not modify inhibition kinetics. Thus, the binding of the A-helix in solution relies mostly on the interaction of Lys18, or possibly both Lys14 and -18, with the core enzyme.

To pursue the potential role of secondary structures in substrate recognition, we searched for the presence of α-helices in the vicinity of phosphorylation sites by the method of Kyte and Doolittle (*19*). Some PKA substrates, such as phosphorylase kinase α and β subunits, protein phosphatase inhibitor-1, and tyrosine hydroxylase, showed α-helices of over 10 amino acids in close proximity to the P site, ending between P^{-4} and P^{-11} like PKI (P-6). However, this trend was not common to all substrates. Pyruvate kinase, CREB δ and ϵ, and 6-phosphofructo-2-kinase showed no potential α-helical structure close to their phosphorylation sites. Furthermore, hormone-sensitive lipase showed such structure further apart (P^{-16} and P^{-19} in mouse), whereas phospholamban possesses a substrate site within an α-helix. From such comparison, α-helices within substrates seem thus not necessarily associated with binding to the catalytic site of the PKA core.

In summary, our results indicate that competitive inhibition of PKA activity by A-helix related oligopeptides depends on the last basic amino acid at position 18, as well as possibly on a partial α-helical conformation. Even though we were unable to prove that the inhibition is due to the binding of the A-helix peptides to the catalytic site, where PKI, kemptide, and other substrates bind, our experiments point to such a possibility for the following reasons: (1) The inhibitory peptide H2 cannot bind when PKI is present in roughly equimolar amounts with PKA; (2) the kinetics of PKA inhibition by H2 are compatible with competitive inhibition; (3) the inhibition is seen even in the presence of the A-helix attached to the core of the PKA enzyme. Cocrystallization of PKA C-subunit with H2 peptide will be required to obtain a detailed understanding of the interaction between this inhibitory peptide and the catalytic core.

According to the model proposed in (*1*), the N-terminal A-helix both in *Dictyostelium* and in mammalian catalytic subunits is probably anchored to the core at the opposite side of the substrate binding site. If the A-helix really binds to the catalytic site, we can put forward the following hypothesis. The A-helix, which is linked to the core via a relatively flexible stretch of amino acids, could rotate so that it would interact with residues close to the active site. A competition between bona fide substrates and A-helix would result in the displacement of the A-helix. The potential role of this mechanism could be to prevent binding of unspecific substrates to the active site. Further studies on the substrate specificity of catalytic subunits missing the A-helix will possibly allow to test such a hypothesis.

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