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Thermodynamic Analysis of Protein Kinase A Iα Activation

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Abstract—Thermodynamic analysis of protein kinase A (PKA) Iα activation was performed using Quantum 3.3.0 docking software and a Gaussian 03W quantum mechanical computational package. Expected stacking interactions between adenine of 3′:5′-AMP and aromatic moieties of amino acids were taken into account by means of MP2/6-31G(d) IPCM (isodensity polarizable continuum model) computations (ε = 4.0). It is demonstrated that thermodynamically favorable agonist-induced PKA Iα activation is mediated by two processes. First, 3′:5′-AMP binding is accompanied by structural changes leading to a thermodynamically favorable regulatory subunit conformation, which is hardly realized in the absence of the ligand ($\Delta G^{\circ}_{R} = -23.9 \pm 8.2$ kJ/mol). Second, 3′:5′-AMP affinity to the regulatory subunit conformation observed after agonist-induced PKA Iα activation is higher than that to inactive holoenzyme complex ($\Delta G^{\circ}_{3':5'\text{-AMP}} = -28.1 \pm 9.7$ kJ/mol). ATP is capable of docking into the 3′:5′-AMP-binding site B of the regulatory subunit complexed with the catalytic one, resulting in inhibition of kinase activation. True constants of 3′:5′-AMP binding to PKA Iα holoenzyme were found to be 60 and 57 μM for the regulatory subunit domains A and B, respectively. These constants, unlike the binding equilibrium constant determined using established experimental techniques and ranging from 15 nM to 2.9 μM, are proved to be direct measures of 3′:5′-AMP–PKA Iα binding affinity. Their values are in a reasonable agreement with the changes in 3′:5′-AMP concentration in the cell (2-55 μM) and account for PKA Iα activation in response to adequate stimuli.

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Key words: protein kinase A Iα activation, 3′:5′-AMP (cyclic adenosine-3′,5′-monophosphate), ATP, standard Gibbs free energy change (ΔG°), stacking interaction

Protein kinase A (PKA) Iα is an important protein implicated in cell signal cascades, cell differentiation, immune response, and control of metabolic pathways [1-5]. Hence, the regulation of PKA Iα activation via specific agonists and antagonists is of great practical importance.

In its inactive state (incapable of phosphorylation), PKA Iα is a tetramer [5] in which the regulatory subunit dimer is interacting with two catalytic subunits. The protein kinase is activated when two 3′:5′-AMP molecules bind to each regulatory subunit resulting in detachment of catalytic subunits from the regulatory dimer [5]. The crystal structures have been reported for the complex between the regulatory and catalytic subunits (1U7E, 2QCS) [6, 7], as well as for the regulatory subunit in 3′:5′-AMP-bound form (1RGS) [8]. Various cyclic nucleotide derivatives are either agonists or antagonists of protein kinase [9, 10]. Kinetics of 3′:5′-AMP binding with the inactive PKA Iα-form [11] and with the free regulatory subunit [12] has been studied both for native enzymes and for proteins carrying point mutations chiefly within the 3′:5′-AMP binding site [13, 14]. The available data suggest that it is the regulatory subunit that determines enzyme activation [7]. According to the data of X-ray structural analysis, the conformation of the catalytic subunit remains virtually unchanged in the activation process, whereas the tertiary structure of the regulatory subunit undergoes substantial changes [6, 7, 15]. Even a visual comparison of tertiary structures of regulatory subunits demonstrates an apparent difference between the

Abbreviations: IPCM, Isodensity Polarizable Continuum Model; MP2, second-order Meller–Plesset perturbation theory; PKA Iα, protein kinase A Iα.

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# Deceased.

3′:5′-AMP-bound form and that comprising the inactive enzyme [7, 8]. One can suppose that the free regulatory subunit can alternately form one or another conformation in solution, and either 3′:5′-AMP or the catalytic subunit stabilizes one of these conformations, thus disabling spontaneous transition to the other [6]. Nevertheless, the regulatory subunit can also change its conformation when bound in a complex with the catalytic subunit. 3′:5′-AMP initiates the conformational rearrangement, and the catalytic subunit release is regarded as a concomitant process.

A model proposed for PKA Iα activation [7] assumes a strictly sequential attachment of 3′:5′-AMP to two 3′:5′-AMP-binding domains of the protein kinase regulatory subunit, first to its B-domain, and then to the A-domain. This model is based on data of X-ray structural analysis [7] suggesting the interaction between several amino acid residues of the 3′:5′-AMP-binding site of A-domain and the catalytic subunit resulting – in the authors' opinion – in inaccessibility of the A-domain for ligand. Thus, in accordance with the suppositions put forward in [7], 3′:5′-AMP binds first with the B-domain, which induces conformational rearrangements of the regulatory subunit and, finally, formation of the A-domain binding site accessible for 3′:5′-AMP. The interaction between 3′:5′-AMP and the A-domain and further conformational rearrangements of the regulatory subunit either follow dissociation of the subunit complex or favor the dissociation. The proposed model is completely confirmed by the data of kinetic studies [11], but it disagrees with the virtually existing possibility of PKA Iα activation with deleted B-domain [16]. Besides, it does not describe in detail all the transformations taking place in PKA Iα in the course of its activation and does not characterize the structure of the transition states and, therefore, does not completely represent the mechanism of PKA Iα activation.

Along with difficulties directly associated with determination of the protein kinase activation mechanism, several experimental facts remain without consistent explanation. In particular, in accord with various data, the experimentally determined constant of 3′:5′-AMP binding to inactive PKA Iα ranges from 15 nM to 2.9 μM [17, 18], whereas the local 3′:5′-AMP concentration in surroundings of functional adenylate cyclase varies within the range from 2 to 55 μM [19, 20]. An apparent consequence of the relation between these values would be a constitutively active PKA Iα, which is impossible in a living organism. Inhibition of PKA Iα activation by high concentrations of ATP (10 μM) should also be noted [21, 22]. The significance of this fact for PKA Iα functioning is still not evaluated, because it is known that 100 nM ATP is sufficient for saturation of all active sites of the catalytic subunit [21], and no other sites of ATP binding to PKA Iα have yet been found.

Thus, far from all evidence can be joint together into a holistic pattern, and apparently they require a new explanation. On the other hand, in spite of the studies devoted to thermodynamic analysis of 3′:5′-AMP binding to the free regulatory subunit [23-25], there is no explanation for the free energy changes in the course of PKA Iα activation, and, particularly, for the thermodynamic contribution of 3′:5′-AMP in this process. Apparently, appropriate analysis would contribute to solving many current contradictions. Besides, the drawing of free energy surface as a final stage of such studies is important for determination of intermediate structures for the complex between regulatory and catalytic subunits, and hence, for explanation of the enzyme activation mechanism. On this basis, the first task of the present study was to evaluate the free energy changes during PKA Iα activation and to determine the thermodynamic contribution of 3′:5′-AMP to this process.

The second task of the present study was to test for the possible role of ATP as a PKA Iα antagonist. The only natural agonist known for PKA Iα is 3′:5′-AMP; however, its sufficiency in the system of PKA Iα regulation is not well understood [26]. The experimentally demonstrated dependence of PKA Iα activation on ATP content in the medium [21, 22] suggests that ATP can be an additional natural regulator for PKA Iα, namely, its inhibitor. This suggestion is also based on the fact that 3′:5′-AMP is synthesized from ATP, thus the concentrations of these substances must be in inverse proportion to each other in the vicinity of an adenylate cyclase molecule.

We solved both tasks using docking methods and non-empirical quantum chemical calculations. An invaluable advantage of such approach is the possibility to analyze intermediates usually inaccessible during experiments and to visualize the resulting structures, which generally favors more comprehensive understanding of processes underlying PKA Iα activation.

MATERIALS AND METHODS

Software, crystal structures, and determined parameters. Crystal structures of bovine (*Bos taurus*) PKA Iα regulatory subunit in complexes with the catalytic subunit or 3′:5′-AMP were acquired from PDB under the numbers of PDB ID 2QCS (59,282 reflections, *R* factor 0.19) and 1RGS (10,254 reflections, *R* factor 0.22), respectively. Hereinafter, these structures are referred to as 2QCS and 1RGS conformations, respectively. All the studied PKA Iα protein complexes were visualized using VMD software [27].

The 2QCS structure representing the complex of regulatory and catalytic subunits contains the point mutation K333R within the 3′:5′-AMP-binding site of the regulatory subunit B-domain, which attenuates the interaction between 3′:5′-AMP and PKA Iα. In connection with this, we have substituted lysine residue for arginine using DeepView/Swiss-PdbViewer software (http://www.

expasy.org/spdbv/, [28]) before the calculations and optimized the resulting structure using the Gromacs 3.1.2 software package [29, 30][1].

The 3′:5′-AMP was docked into the binding sites of the regulatory subunit in 1RGS and 2QCS conformations using QUANTUM 3.3.0 (http://www.q-pharm.com) software, considering conformational flexibility of the ligand and the protein [31]. Relative error for calculations of free energy changes using this software is below 15% as stated by the software developers. As a result, the values of $\Delta\tilde{G}^{\circ}_{A}$ and $\Delta\tilde{G}^{\circ}_{B}$ parameters were determined, characterizing the free energy changes upon 3′:5′-AMP binding with A- and B-domains, respectively, of the regulatory subunit in its 2QCS conformation. The values of free energy changes ΔG°_{A} and ΔG°_{B} upon the binding of 3′:5′-AMP with the A- and B-domains, respectively, of the regulatory subunit in its 1RGS conformation were preliminarily evaluated directly in the course of the docking procedure. Consideration of stacking interaction energy is required for more exact values of ΔG°_{A} and ΔG°_{B}. In accord with the X-ray structure analysis of the 1RGS conformation, the aromatic rings of 3′:5′-AMP adenine and W260 or Y371 radicals (for A- and B-domains, respectively) are juxtaposed with each other at the distance of 3.5-4.5 Å [8] allowing the stacking interaction between them. The energy of the suggested stacking interaction was evaluated on the basis of Gaussian 03W quantum chemical software package [32] considering the effect of electron correlation in frames of the MP2 (second-order Meller– Plesset perturbation theory) method [33] using 6-31G(d) basis set [34-36]. The effect of solvent (protein with dielectric constant ε = 4.0) was considered using the IPCM (Isodensity Polarizable Continuum Model) solvation model [37]. As a result, the value of each parameter, ΔG°_{A} and ΔG°_{B}, was determined as a sum of the free energy change obtained using the docking software and the energy of stacking interaction calculated non-empirically.

In the present work, ATP was considered as a possible competitive inhibitor of PKA Iα. A conservative residue Y/F (Y321 by the sequence numbering of *Bos taurus* PKA Iα) is found in B-domains (but not in A-domains) of all known protein kinases A, as well as in all related proteins [38, 39]. The protein surface site containing both this tyrosine residue and the binding site for 3′:5′-AMP is able – to judge from its geometry – to interact with ATP in such a way that the adenine ring of ATP and the aromatic ring of tyrosine occupy positions favoring their stacking interaction. No similar position of amino acid residues is observed in the A-domain. So, it is the 3′:5′-AMP-binding site of the B-domain that was chosen for the role of possible ATP-binding site. The interaction between ATP^{4-} and PKA Iα was analyzed in accord with the above technique.

Note that in spite of PKA Iα tetrameric structure, the domain mediating the binding between regulatory subunits does not participate in the enzyme activation, and therefore the dimer composed of the regulatory and catalytic subunits is commonly used in experiments. Virtually all presently known PKA Iα activation parameters are obtained from experiments with this dimer. On this basis, in the present work we consider attachment of two 3′:5′-AMP molecules to the binary complex "regulatory subunit–catalytic subunit" rather than to the natural PKA Iα tetramer.

RESULTS

Peculiarities of PKA Iα activation. To obtain some characteristics of PKA Iα activation, we used experimental values of equilibrium constants for 3′:5′-AMP binding with free regulatory subunit (1.3-22 nM) [18, 40] and with inactive enzyme represented by the complex "regulatory subunit–catalytic subunit" (15 nM-2.9 μM) [17, 18]. Free energy changes ΔG°_{2} and ΔG°_{1}, respectively, were calculated from these constant values according to the following Eq. (1.1):

$$\Delta G^{\circ} = RT\ln K_{eq}, \qquad (1.1)$$

where R is the universal gas constant, T is experimental temperature specified in the corresponding literature report, and K_{eq} is the value determined as a square of the experimentally measured constants (because of the presence of two 3′:5′-AMP-binding sites in PKA Iα).

Thus, we have demonstrated that ΔG°_{1} value lies within the range from -82.9 ± 0.9 to -63.1 ± 0.9 kJ/mol, and ΔG°_{2} – within the range from -101.3 ± 0.9 to -87.3 ± 0.9 kJ/mol. The most preferable values for ΔG°_{1} and ΔG°_{2} are -63.1 ± 0.9 and -101.3 ± 0.9 kJ/mol, respectively, which is confirmed by combined results of the study in which the free energy change in the course of PKA Iα spontaneous activation has been determined by the surface plasmon resonance method [40] (~ +54 kJ/mol) and by the expression for this value (see below Eq. (2.11)) deduced in the present study.

[1] Optimization parameters: force field (gmx), electrostatic and Van der Waals interactions (coulombtype = PME, vdwtype = Cut-off, rcoulomb = 1.00 nm, rvdw = 1.40 nm, fourierspacing = 0.1 nm, pme_order = 6, ewald_rtol = 1e-5), neighbor searching (ns_type = grid, rlist = 1.00 nm, nstlist = 5), temperature coupling (Tcoupl = berendsen, T = 304 K, tau-t = 0.1 ps), pressure coupling (Pcoupl = berendsen, Pcoupltype = isotropic, P = 1 bar, tau_p = 0.5 ps), for energy optimization (steepest descent method), emstep = 0.01 nm, emtol = 100 $kJ\cdot mol^{-1}\cdot nm^{-1}$), molecular dynamics with constraints (LINCS algorithm, constraints = all-bonds, lincs order = 4, lincs warnangle = 30, morse = no, integrator = md, dt = 0.002 ps, nsteps = 40,000), molecular dynamics without constraints (integrator = md, dt = 0.002 ps, nsteps = 100,000).

Note that the temperature included as a parameter into QUANTUM 3.3.0 comprises 304 K and is not exactly equal to the values used in experimental practice. However, because of weak dependence of free energy change in the course of PKA Iα activation on temperature [24], this error is within the error range of docking software when the experimental data and the values calculated in our study are used together.

Evaluation of free energy change in the course of 3′:5′-AMP-induced PKA Iα activation. To evaluate correctly the free energy change in the course of 3′:5′-AMP-induced PKA Iα activation, we had to designate exactly which processes should be reasonably assigned directly to the activation of this enzyme. The binding of ligand, particularly 3′:5′-AMP, to the regulatory subunit is known either to favor protein kinase activation or make it impossible, for instance, when Rp-3′:5′-AMPS (cyclic adenosine-3′,5′-monophosphothioate Rp-isomer) is used [9]. So, the PKA Iα activation should be defined as the conformational change of the regulatory subunit accompanied by liberation of the catalytic subunit (Fig. 1a), and the binding of 3′:5′-AMP with the enzyme should be considered as an independent process.

Following this description of PKA Iα activation in the presence of 3′:5′-AMP, it is necessary to compare it with dissociation of the complex between the regulatory and catalytic subunits of the protein kinase occuring, although with extremely low probability, but without agonist, that occurs on spontaneous activation. It is reasonable to anticipate that the conformational change of the regulatory subunit during the spontaneous activation should not lead to the structure realized in the presence of 3′:5′-AMP. Indeed, free solubilized regulatory subunit is characterized by structural features different from those observed in its complexes with either the catalytic subunit or 3′:5′-AMP. This statement is based on the analysis of the crystal structure of the regulatory subunit (PDB ID 1RL3), whose A-site contains bound ligand, and B-site is free [41], as well as on the fact that the free regulatory subunit and PKA Iα antagonist participate in formation of the ligand–receptor complex following completely the structure of the 1RGS 3′:5′-AMP-bound form [9]. On the other hand, based on the structural features of the 2QCS inactive complex, the rearrangement of regulatory subunit into the conformation realized in solution in the absence of both catalytic subunit and 3′:5′-AMP seems to be a necessary and sufficient condition for the loss of most interactions between the enzyme subunits, and hence, for the protein kinase activation. Thus, the spontaneous PKA Iα activation should be described by the scheme given in Fig. 1b.

As a result, when the parameters $\Delta\tilde{G}^{\circ}_{act}$ and ΔG°_{act} are introduced characterizing free energy changes in the course of 3′:5′-AMP-induced and spontaneous PKA Iα activation, respectively, one can deduce:

$$\Delta G^{\circ}_{act} = \Delta G^{\circ}_{conf} + \Delta G^{\circ}_{diss}, \quad (2.1)$$

$$\Delta\tilde{G}^{\circ}_{act} = \Delta\tilde{G}^{\circ}_{conf} + \Delta G^{\circ}_{diss} + (\Delta G^{\circ}_{A} + \Delta G^{\circ}_{B} - \Delta\tilde{G}^{\circ}_{A} - \Delta\tilde{G}^{\circ}_{B}), \quad (2.2)$$

where ΔG°_{diss} is the free energy change in the course of complex dissociation between PKA Iα regulatory and catalytic subunits, and $\Delta\tilde{G}^{\circ}_{conf}$ and ΔG°_{conf} are the free energy changes when the regulatory subunit transits from its 2QCS conformation to the 1RGS conformation or to the conformation realized in solution in the absence of both ligand and catalytic subunit, respectively.

Subtraction (2.1) from (2.2) gives:

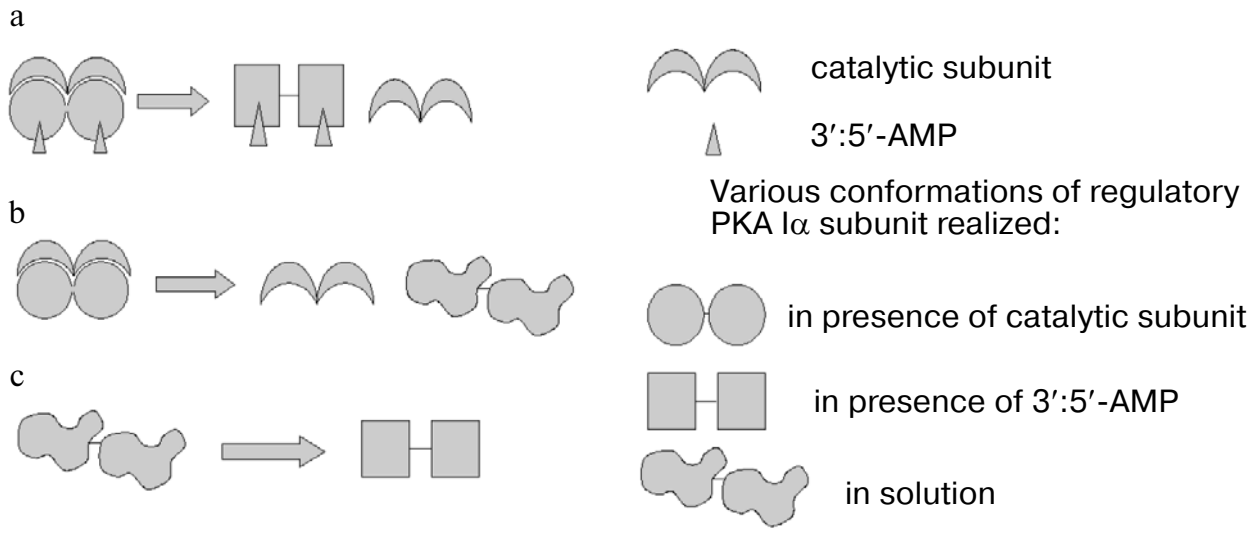

Fig. 1. Schemes describing: a) 3′:5′-AMP-induced PKA Iα activation; b) spontaneous PKA Iα activation; c) rearrangement of PKA Iα regulatory subunit from the conformation existing in solution into the conformation realized in the presence of 3′:5′-AMP.

$$\Delta\tilde{G}^{\circ}_{act} - \Delta G^{\circ}_{act} = (\Delta\tilde{G}^{\circ}_{conf} - \Delta G^{\circ}_{conf}) +$$

$$+ (\Delta G^{\circ}_{A} + \Delta G^{\circ}_{B} - \Delta\tilde{G}^{\circ}_{A} - \Delta\tilde{G}^{\circ}_{B}). \quad (2.3)$$

Finally, when the following designations are introduced:

$$\Delta G^{\circ}_{3':5'\text{-AMP}} = \Delta G^{\circ}_{A} + \Delta G^{\circ}_{B} - \Delta\tilde{G}^{\circ}_{A} - \Delta\tilde{G}^{\circ}_{B}, \quad (2.4)$$

$$\Delta G^{\circ}_{R} = \Delta\tilde{G}^{\circ}_{conf} - \Delta G^{\circ}_{conf}, \quad (2.5)$$

we can deduce:

$$\Delta\tilde{G}^{\circ}_{act} - \Delta G^{\circ}_{act} = \Delta G^{\circ}_{R} + \Delta G^{\circ}_{3':5'\text{-AMP}}. \quad (2.6)$$

Thus, in accordance with (2.6), the efficacy of PKA Iα activation in the presence of agonist (3′:5′-AMP in this case) in comparison with the spontaneous activation is determined by the thermodynamic contributions of $\Delta G^{\circ}_{3':5'\text{-AMP}}$ and ΔG°_{R}.

The thermodynamic contribution of 3′:5′-AMP ($\Delta G^{\circ}_{3':5'\text{-AMP}}$) is a result of change in affinity of 3′:5′-AMP to the binding sites of PKA Iα after the conformational rearrangement of the regulatory subunit (2.4) accompanying the protein kinase activation, and, as a result, depends only on the ligand under study. The thermodynamic contribution of the regulatory subunit (ΔG°_{R}) is a constant for the protein kinase activation by any agonist and formally characterizes its transition from the conformation existing in solution to the conformation realized in the presence of ligand (Fig. 1c). It is worth noting that the physical meaning of the ΔG°_{R} contribution is different free energy change under formation of the above conformations of the regulatory subunit directly from the complex "regulatory subunit–catalytic subunit" (2QCS).

In accordance with Eq. (2.4), the $\Delta G^{\circ}_{3':5'\text{-AMP}}$ value can be quantitatively evaluated directly from the ΔG°_{A}, ΔG°_{B}, $\Delta\tilde{G}^{\circ}_{A}$, and $\Delta\tilde{G}^{\circ}_{B}$ values obtained in the present work, whereas evaluation of ΔG°_{R} requires involvement of experimental data, namely the ΔG°_{2} value. As we have mentioned above, ΔG°_{2} characterizes the binding of 3′:5′-AMP with free regulatory subunit in solution, and, hence, is determined by the equation:

$$\Delta G^{\circ}_{2} = \Delta G^{\circ}_{R} + \Delta G^{\circ}_{A} + \Delta G^{\circ}_{B}. \quad (2.7)$$

Using the Eq. (2.7), we derive one for ΔG°_{R}:

$$\Delta G^{\circ}_{R} = \Delta G^{\circ}_{2} - (\Delta G^{\circ}_{A} + \Delta G^{\circ}_{B}). \quad (2.8)$$

Along with the described contributions of $\Delta G^{\circ}_{3':5'\text{-AMP}}$ and ΔG°_{R}, evaluation of $\Delta\tilde{G}^{\circ}_{act}$ and ΔG°_{act} values and their difference is also important. Because of its equilibrity, the above-introduced ΔG°_{1} parameter characterizes two processes: the initial 3′:5′-AMP binding to protein kinase and following activation of the latter. In accordance with the given definition, one can deduce:

$$\Delta G^{\circ}_{1} = \Delta\tilde{G}^{\circ}_{A} + \Delta\tilde{G}^{\circ}_{B} + \Delta\tilde{G}^{\circ}_{act}. \quad (2.9)$$

On the other hand, because Gibbs' free energy is a function of state, Eq. (2.10) is also true, although it does not reflect the real reaction mechanism:

$$\Delta G^{\circ}_{1} = \Delta G^{\circ}_{act} + \Delta G^{\circ}_{R} + \Delta G^{\circ}_{A} + \Delta G^{\circ}_{B}. \quad (2.10)$$

Thus, based on (2.9) and (2.10) and taking into consideration (2.7), one can deduce:

$$\Delta G^{\circ}_{act} = \Delta G^{\circ}_{1} - \Delta G^{\circ}_{2}, \quad (2.11)$$

$$\Delta\tilde{G}^{\circ}_{act} = \Delta G^{\circ}_{1} - (\Delta\tilde{G}^{\circ}_{A} + \Delta\tilde{G}^{\circ}_{B}), \quad (2.12)$$

$$\Delta\tilde{G}^{\circ}_{act} - \Delta G^{\circ}_{act} = \Delta G^{\circ}_{2} - (\Delta\tilde{G}^{\circ}_{A} + \Delta\tilde{G}^{\circ}_{B}). \quad (2.13)$$

It should be noted as a conclusion that validation of the described method for evaluation of free energy change during PKA Iα activation is based on the key possibility of interaction between 3′:5′-AMP and the A-site of the regulatory subunit bound in a complex with the catalytic subunit. In fact, the calculation for $\Delta\tilde{G}^{\circ}_{A}$ is only possible in the case of A-domain accessibility for 3′:5′-AMP or, at least, does not require conformational rearrangements for binding of ligand after complete or partial removal of the catalytic subunit.

Our calculation demonstrates the lack of steric hindrances for 3′:5′-AMP binding with the A-site of the regulatory subunit forming a complex with the catalytic subunit. In this complex, several native hydrogen bonds are formed between the regulatory subunit and 3′:5′-AMP (Fig. 2). These bonds are maintained after conformation-

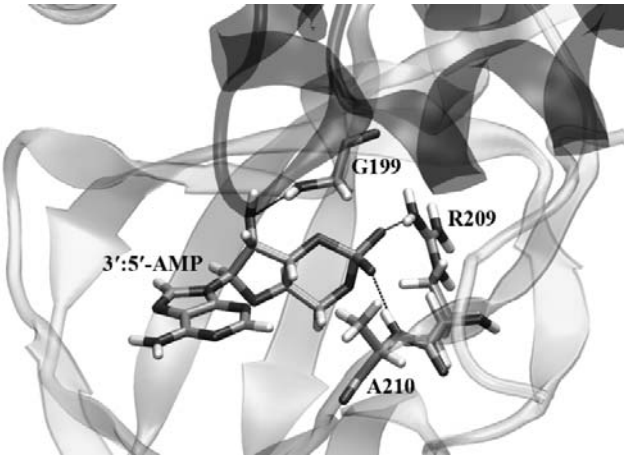

Fig. 2. Binding of 3′:5′-AMP with A-domain of the regulatory subunit which forms a complex with the catalytic subunit. Gray color designates the folding of regulatory subunit, and the catalytic subunit is given in black. Amino acid residues of the regulatory subunit which form native bonds with 3′:5′-AMP are shown.

al rearrangements of the protein kinase, thus suggesting productivity of the binding under consideration. The free energy change during the described interaction between 3′:5′-AMP and the A-domain ($\Delta\tilde{G}^{\circ}_{A}$) is −24.6 ± 3.7 kJ/mol.

When 3′:5′-AMP binds with the B-domain of the regulatory subunit, which forms a complex with the catalytic subunit, a pattern of native hydrogen bonds is formed similarly to the abovementioned one due to high homology between the A- and B-domains. Hence, the change of binding free energy ($\Delta\tilde{G}^{\circ}_{B}$) is close to $\Delta\tilde{G}^{\circ}_{A}$ and comprises −24.7 ± 3.7 kJ/mol.

The free energy change upon the binding of 3′:5′-AMP to the A- and B-domains of the regulatory subunit being in 1RGS conformation, without taking the stacking interaction into a consideration, were approximately equal and comprised −38.7 ± 5.8 and −37.9 ± 5.7 kJ/mol for A- and B-domains, respectively. However, the evaluation of stacking interaction energy revealed the following fact: the desirable interaction is only realized within the A-domain (−20.1 kJ/mol) at the final stage of the complex formation between the regulatory subunit and 3′:5′-AMP. The stacking interaction energy in the B-domain is +19.3 kJ/mol, in spite of the fact that the aromatic moieties are juxtaposed to each other at the distance of 3.5-4.5 Å. Thus, the resulting ΔG°_{A} and ΔG°_{B} values are −58.8 ± 5.8 and −18.6 ± 5.7 kJ/mol, respectively.

According to Eqs. (2.4), (2.8), and (2.11)-(2.13) and taking into account the obtained values of ΔG°_{A}, ΔG°_{B}, $\Delta\tilde{G}^{\circ}_{A}$, and $\Delta\tilde{G}^{\circ}_{B}$, we deduce the following equations:

$$\Delta G^{\circ}_{3':5'\text{-AMP}} = (\Delta G^{\circ}_{A} + \Delta G^{\circ}_{B}) - (\Delta\tilde{G}^{\circ}_{A} + \Delta\tilde{G}^{\circ}_{B}) =$$

$$= -28.1 \pm 9.7 \text{ kJ/mol},$$

$$\Delta G^{\circ}_{R} = \Delta G^{\circ}_{2} - (\Delta G^{\circ}_{A} + \Delta G^{\circ}_{B}) = -23.9 \pm 8.2 \text{ kJ/mol},$$

$$\Delta G^{\circ}_{act} = \Delta G^{\circ}_{1} - \Delta G^{\circ}_{2} = +38.1 \pm 1.3 \text{ kJ/mol},$$

$$\Delta\tilde{G}^{\circ}_{act} = \Delta G^{\circ}_{1} - (\Delta\tilde{G}^{\circ}_{A} + \Delta\tilde{G}^{\circ}_{B}) = -13.9 \pm 5.3 \text{ kJ/mol},$$

$$\Delta\tilde{G}^{\circ}_{act} - \Delta G^{\circ}_{act} = \Delta G^{\circ}_{2} - (\Delta\tilde{G}^{\circ}_{A} + \Delta\tilde{G}^{\circ}_{B}) =$$

$$= -52.0 \pm 5.3 \text{ kJ/mol}.$$

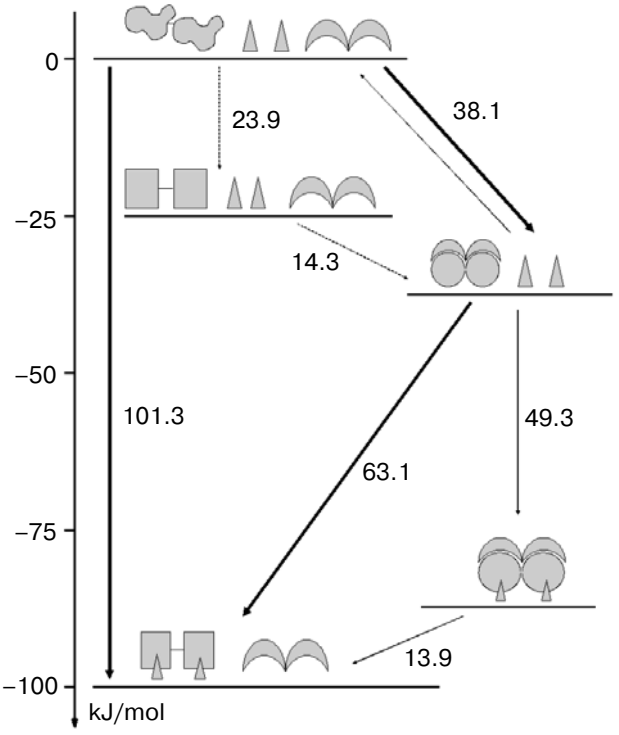

Fig. 3. General scheme of thermodynamic evaluation of PKA Iα activation. Bold arrows indicate processes demonstrated in experiments. Simple arrows indicate processes that take place naturally, but are not confirmed experimentally. Dashed lines indicate processes that are not realized under natural conditions. The other notations are the same as given in the legend to Fig. 1. The value characterizing the binding of regulatory subunit in conformation 1RGS with the catalytic subunit is evaluated according to the equation $\Delta G^{\circ} = \Delta G^{\circ}_{1} - (\Delta G^{\circ}_{A} + \Delta G^{\circ}_{B})$, resulting from the considerations given in the "Materials and Methods" section.

Figure 3 shows the ratios between the free energy changes for various natural and hypothetical PKA Iα conformational rearrangements. It should be noted in connection with this that the difference in ΔG°_{act} values obtained on the basis of experimentally measured values of ΔG°_{1} and ΔG°_{2} or by the method of surface plasmon resonance [40] (+38.1 and +54 kJ/mol, respectively) is not critical. One possible cause of the discrepancy might be the fact that the binding constant determined by surface plasmon resonance has been calculated indirectly, based on the rate constants for association and dissociation of protein kinase subunits. The values of these rate constants for the system "regulatory subunit–catalytic subunit" are near the limits of the instrumental range. The error of the surface plasmon resonance method is higher when the reaction rate constants are determined than when equilibrium binding constant is measured directly. These circumstances suggest for the calculation the scheme of PKA Iα thermodynamic activation parameters employed in our work.

ATP as PKA Iα antagonist. In the present work ATP has demonstrated its ability for binding with the 3′:5′-AMP binding site of the B-domain of the regulatory subunit when in a complex with the catalytic subunit. As this takes place, the position of ATP in the site completely coincides with the proposed one in the developed hypothesis: amino acid residues that are known to form hydrogen bonds with 3′:5′-AMP sugar phosphate form hydrogen bonds with ATP phosphates, and Y321 is implicated in stacking interaction with the adenine ring of the ligand (Fig. 4). Free energy of the described binding proved to be far below zero, and the stacking interaction energy was −11.3 kJ/mol. The given data suggest that ATP competes with 3′:5′-AMP for

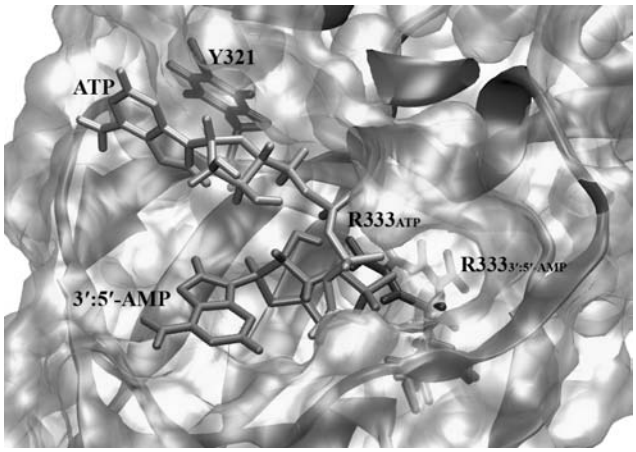

Fig. 4. ATP and 3′:5′-AMP compete for the same 3′:5′-AMP-binding site of the B-domain of the PKA Iα regulatory subunit. Tyrosine Y321 residue participating in the supposed stacking interaction with ATP adenine, as well as arginine R333 known to form a hydrogen bond with 3′:5′-AMP phosphate are shown. Two positions of R333 are shown: $R333_{3':5'\text{-AMP}}$ and $R333_{ATP}$ realized after the binding of 3′:5′-AMP and ATP, respectively.

the 3′:5′-AMP-binding site of the B-domain, thus playing the role of a PKA Iα antagonist in the cell.

DISCUSSION

An important result of our study is the demonstration of the ability of 3′:5′-AMP to bind with the A-site of the regulatory subunit associated in a complex with the catalytic subunit. This fact has not been considered by the current model of PKA Iα activation mechanism [7]; however, it does not conflict with experimental data [7, 11]. In fact, X-ray structural analysis has demonstrated that several amino acid residues of the 3′:5′-AMP-binding site of the A-domain interact with catalytic subunit [7], excluding the amino acid residues forming hydrogen bonds with 3′:5′-AMP. Moreover, a spatial localization of amino acid residues in the A-site does not differ from that in the homologous B-site and completely corresponds to the geometrical parameters of the ligand.

Possible interaction between 3′:5′-AMP and the A-site of the regulatory subunit forming a complex with the catalytic subunit can appear when regarding the unexplained fact of activation of mutants devoid of the B-domain [16]. Superposition of crystal structures of "regulatory subunit–catalytic subunit" complexes of this mutant (PDB ID 1U7E) [6] and wild-type protein (PDB ID 2QCS) [7] did not reveal any difference between positions of corresponding atoms in the two structures. Thus, the only possible mechanism of 3′:5′-AMP-induced activation of mutant PKA Iα with deleted B-domain is the binding of 3′:5′-AMP with the A-site of the regulatory subunit favoring subsequent conformational rearrangement. The activation constant of the considered mutants is only one order higher than the corresponding constant of the wild-type enzymes [16]. Obviously, the binding between 3′:5′-AMP and the A-domain must directly precede the protein kinase activation or occur at its very early stages to provide such a high efficacy of the process. The absence of calculated or experimental data makes impossible the choice between these suppositions, but in any case the accessibility of the regulatory subunit A-site for the ligand must be reevaluated in the light of the presented facts and the data of this study.

Nevertheless, it should be noted that the experimentally confirmed fact of 3′:5′-AMP binding with the A-site does not allow supposition that such a binding, unlike B-site binding, significantly relates to the activation of the wild-type (non-mutant) protein kinase. In fact, the A-domain structure is efficiently stabilized by numerous protein–protein contacts with the catalytic subunit, hindering motility of large groups of atoms. Contrariwise, the B-domain forms only a few bonds with the catalytic subunit, and hence the initiation of conformational rearrangements required for the enzyme activation should occur much more easily. Thus, one can conclude that it is this interaction between 3′:5′-AMP and the B-domain that results in PKA Iα activation unrelated to the presence of 3′:5′-AMP in the A-site. The results of kinetics studies confirm the crucial role of the B-domain in the wild-type PKA Iα activation initiation [11]. Possibly, as demonstrated in the present work, the key role of B-domain in PKA Iα activation is provided also by the presence of ATP-binding site overlapping with 3′:5′-AMP binding site in the B-domain, but not in A-domain. The role of ATP as PKA Iα competitive inhibitor seems more apparent in this context. Experiments for testing this assumption should be performed.

A series of important conclusions can be drawn from the thermodynamic evaluation of PKA Iα activation carried out in the present study.

First, the data suggest that the thermodynamically disadvantageous process of protein kinase activation ($+38.1 \pm 1.3$ kJ/mol) becomes easily realizable (-13.9 ± 5.3 kJ/mol) in the presence of 3′:5′-AMP. The more advantageous enzyme activation induced by any cyclic nucleotide or its derivative proves to be provided by two processes:

– by rearrangement of protein kinase regulatory subunit to the thermodynamically advantageous conformation realized only in the presence of ligand ($\Delta G^{\circ}_{R} = -23.9 \pm 8.2$ kJ/mol);

– by elevation of the ligand affinity to PKA Iα binding sites after the regulatory subunit conformational change accompanying the protein kinase activation ($\Delta G^{\circ}_{3':5'\text{-AMP}} = -28.1 \pm 9.7$ kJ/mol for 3′:5′-AMP).

The value of the latter contribution can be easily calculated according to Eq. (2.4) and used for evaluation of

thermodynamic possibility of various ligands to participate in PKA Iα activation.

The second important conclusion obtained from the analysis of the provided thermodynamic evidence relates to the conformation of protein kinase regulatory subunit realized in the presence of 3′:5′-AMP. From the ΔG°_{R} value, the rearrangement of regulatory subunit to this conformation (Fig. 1c) is thermodynamically advantageous, but it does not happen in the absence of 3′:5′-AMP or its analogs. Such behavior of the regulatory subunit can be a consequence of a compelling energy barrier. The role of 3′:5′-AMP as a factor decreasing the level of the energy barrier, that is, a catalyst of the regulatory subunit conformational rearrangement, is evident in this case. Aromatic rings of 3′:5′-AMP and Y371 are important participants of the catalysis process. Stacking interaction between them being not observable at the last stage of the complex formation between the regulatory subunit and 3′:5′-AMP can be more significant at an earlier stage of conformational rearrangements and favor the regulatory subunit rearrangement to its advantageous conformation.

Finally, the third, thermodynamic evaluation of PKA Iα activation has allowed determination of true constant values for 3′:5′-AMP binding with the inactive protein kinase form. As we noted above, a series of experimental studies has provided the values determined as the target binding constants within the range from 15 nM to 2.9 μM [17, 18]. However, the impossibility of protein kinase activity regulation by local changes of 3′:5′-AMP concentrations (from 2 to 55 μM) [19, 20], following from the data given above, induced the reevaluation of the physical meaning of the specified constants. In the course of the present study we have demonstrated that the experimentally measured constants characterize not only the binding of 3′:5′-AMP with protein kinase but directly PKA Iα activation, hence their values are incomparable with 3′:5′-AMP concentration in the cell. The values of true constants of 3′:5′-AMP binding with inactive PKA Iα form depend on $\Delta\tilde{G}^{\circ}_{A}$ and $\Delta\tilde{G}^{\circ}_{B}$ values and are determined according to the equation:

$$K = \exp\{\Delta\tilde{G}^{\circ}/RT\}, \qquad (3.1)$$

where R is the universal gas constant, $T = 304$ K (invariable parameter of QUANTUM 3.3.0 docking software), and $\Delta\tilde{G}^{\circ}$ is equal to $\Delta\tilde{G}^{\circ}_{A}$ or $\Delta\tilde{G}^{\circ}_{B}$. The resulting values for true constants of 3′:5′-AMP binding with PKA Iα inactive form comprised 60 and 57 μM for the A- and B-domains, respectively. Obviously, the rate of 3′:5′-AMP-induced PKA Iα activation (Fig. 1a) is directly proportional to the concentration of ternary complex "catalytic subunit–regulatory subunit–3′:5′-AMP", which, in turn, depends on the ratio between the local 3′:5′-AMP concentration and the value of binding constant of the ligand with inactive enzyme. The values of 60 and 57 μM obtained in this study are comparable with 3′:5′-AMP concentration observed during adenylate cyclase activation (55 μM) [18]. Thus, the evaluated 3′:5′-AMP binding constants for inactive PKA Iα form are in good agreement with 3′:5′-AMP concentration in the cell and explain the fact of protein kinase activation in response to adequate stimuli.

It is worth noting in conclusion that the signal transduction pathways involving 3′:5′-AMP are very ancient in evolution, and the ability to bind this messenger apparently originated many times. Thus, there is no apparent homology between 3′:5′-AMP-binding receptors of *Dictyostelium* and protein kinase A considered in the present study. The 3′:5′-AMP-binding domain of PKA Iα is highly conserved comprising many 3′:5′-AMP activated proteins, such as other protein kinase A isoforms, protein kinase G, catabolism activating protein (CAP), HCN-channels (hyperpolarization-activated cyclic nucleotide-gated channels), and EPACs (exchange proteins activated by 3′:5′-AMP) [39]. Considering structure–function linkage, these proteins are to be characterized by similar activation mechanisms and, hence, the approach proposed in the present study can be useful for understanding their functioning.

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