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BIOCHEMISTRY

Single-subunit allostery in the kinetics of peptide phosphorylation by protein kinase A

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Abstract. Allosteric cooperativity between peptide and ATP binding sites on cAMP-dependent protein kinase catalytic subunit was studied kinetically for the reaction of phosphorylation of seven peptide substrates. The allosteric effect was quantified in terms of the interaction factor α by comparing binding effectiveness of a substrate molecule with the free enzyme and with the enzyme complex with another substrate. It was discovered that the magnitude of the allosteric feedback between these binding sites was governed by the effectiveness of substrate binding, which was varied by using different peptides, and the principle 'better binding: stronger allostery' was formulated. This interrelationship was further formalized in terms of a linear-free-energy relationship $\mathrm{p}\alpha = \mathrm{C} + S\mathrm{p}K_\mathrm{b}$, holding between the free energy of the allosteric interaction, quantified by the negative logarithm of the interaction factor α ($\mathrm{p}\alpha$) and the effectiveness of substrate binding quantified by $\mathrm{p}K_\mathrm{b}$. For the peptide phosphorylation reaction $\mathrm{C} = -1.4$ and $S = 0.4$ were obtained. The negative intercept C indicated that the positive cooperativity between the binding sites, characterized by $\alpha < 1$ at sub-millimolar K_b values, changed into negative cooperativity with $\alpha > 1$ at millimolar K_b values. This means that inversion of the cooperative effect was induced by substrate structure, and allostery was used by this enzyme as an additional mechanism to discriminate between substrates, facilitating phosphorylation of good substrates and providing additional protection against phosphorylation of bad substrates. Some implications of this allosteric mechanism on substrate specificity of protein kinases were discussed.

Key words: allosteric cooperativity, single-subunit allostery, enzyme kinetics, protein kinase A, peptide phosphorylation, interaction factor, allostery inversion.

Abbreviations: Ala-kemptide – peptide inhibitor LRRAALG; AMPPNP – β,γ-imidoadenosine 5′-triphosphate; kemptide – peptide substrate LRRASLG; protein kinase A – catalytic subunit of cAMP dependent protein kinase.

Enzyme: cAMP dependent protein kinase – EC 2.7.11.1.

INTRODUCTION

Allosteric regulation of enzyme activity is widely used by living cells to control diverse physiological processes [1,2]. Although this phenomenon has been initially related to multi-subunit enzyme complexes, where ligand binding at one subunit affects binding site affinity on other subunit(s) [1–3], more recently attention has been paid to the possibility of allosteric regulation within monomeric proteins [3,4]. In this case, the enzyme should possess at least two binding sites with cooperative feedback between their binding properties. In general, this may happen with every enzyme, catalysing reaction between two substrates that have distinct binding sites and bind simultaneously to form a ternary enzyme–substrate complex. In this study we established cooperative interaction between substrate binding sites of the cAMP-dependent protein kinase catalytic subunit (protein kinase A, EC 2.7.11.1) and investigated dependence of this allosteric effect upon the structure of the phosphorylatable peptide. This enzyme is generally recognized as a 'model enzyme' of the protein kinase superfamily [5,6], whose members govern the activity and location of cell proteins via their

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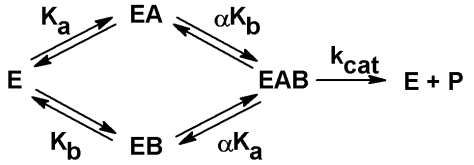
phosphorylation [7,8]. Therefore, similar allosteric regulation of substrate phosphorylation may have a more general meaning and play a significant role in various 'decision-making' steps of the cell cycle.

The catalytic mechanism of protein kinase A involves direct transfer of the γ-phosphate group of ATP to the phosphorylatable residue of peptide substrate [9], and occurs via formation of the ternary complex including the enzyme (E), ATP (A), and the phosphorylatable protein/peptide substrate (B). This situation is formalized by the following reaction scheme:



(1)

Formation of the ternary complex EAB requires the presence of separate binding sites for both substrates A and B on the enzyme molecule, and affinity of the free enzyme for these substrates can be characterized by the dissociation constants K_a and K_b, respectively. However, binding of one of these substrates with the enzyme may affect the affinity of the enzyme for the second substrate, and this interaction between two binding sites is taken into consideration by the 'interaction factor' α, as formulated in [10]. Depending on the α value, the binding of one substrate can favour ($\alpha < 1$) or hinder ($\alpha > 1$) the binding of the second substrate, leading to a positive or negative effect of allosteric cooperativity. The allosteric effect is absent if $\alpha = 1$.

Various indications can be found in earlier papers about the significant role of the allosteric cooperativity in ligand binding with protein kinase A. Most of these data have been still discussed as the influence of ATP (more precisely the ATP–Mg complex) on the binding effectiveness of peptide or protein inhibitors of this enzyme, like the regulatory subunit of protein kinase A and the heat-stable protein inhibitor [11], but also short peptide inhibitors like peptide inhibitor LRRAALG (Ala-kemptide) [12]. Recently it was shown that the binding effectiveness of ATP analogue β,γ-imidoadenosine 5′-triphosphate (AMPPNP) and peptide substrate LRRASLG (kemptide) with protein kinase A is effectively controlled allosterically [13]. A summary of these data revealed that the allosteric effect is dependent upon the binding effectiveness of reversible inhibitors of protein kinase A, and the principle 'better binding: stronger allostery' was formulated [14].

In the present study allostery was revealed in the protein kinase A catalysed reaction of peptide phosphorylation, and the effect was quantified in terms of the interaction factor α for seven substrates. As peptides of different binding effectiveness were selected for this study, the dependence of the allosteric effect upon substrate structure was revealed and some implications of this phenomenon on the specificity of the peptide phosphorylation reaction were discussed.

METHODS

Chemicals

γ-[^{32}P]ATP was obtained from Amersham (UK). Peptides RRYSV, RRASVA, LRRASLG (kemptide), RKRSRKE, LRKASLG, LARASLG, and LRAASLG of purity above 95% were purchased from GL Biochem Ltd. (Shanghai, China) and where characterized by MS spectra and HPLC. ATP, TRIS/HCl, BSA, and H_3PO_4 were obtained from Sigma-Aldrich (USA). Phosphocellulose paper P81 was acquired from Whatman (UK). $MgCl_2$ was purchased from Acros (Germany). The catalytic subunit C_{alpha} of mouse cAMP-dependent protein kinase (protein kinase A), recombinantly expressed in *E. coli*, 30 U/mg, 0.1 mg/mL, lot 040916, was obtained from Biaffin GmbH & Co KG (Germany) as stock solution.

Kinetic measurements

The initial rate of peptide phosphorylation was measured at 30 °C as described previously [15,16]. Briefly, the reaction mixture (final volume 100 µL, 50 mM TRIS/HCl, pH 7.5) contained γ-[^{32}P]ATP (concentrations between 2.5 and 150 µM), peptide (concentrations depended on the affinity of protein kinase A for the substrate), 10 mM of $MgCl_2$, and 0.015–0.03 µg/mL of the enzyme. The stock solution of protein kinase A was diluted 500–1000-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 different time moments 10 µL aliquots were removed 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. These 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.

Data processing algorithm

For data processing, we proceeded from the rate equation derived for reaction scheme (1).

$$v = \frac{V\dfrac{[A][B]}{\alpha K_a K_b}}{1+\dfrac{[A]}{K_a}+\dfrac{[B]}{K_b}+\dfrac{[A][B]}{\alpha K_a K_b}}, \qquad (2)$$

where $V = k_{cat}[E]_o$. For practical data analysis the algorithm described in [17] was used. Briefly, the initial velocities of substrate phosphorylation reaction (v) were measured at various ATP (A) and peptide (B) concentrations. The arrays of these kinetic data were processed in two subsets. In one subset, v vs ATP concentration plots were used to calculate the parameters of the Michaelis–Menten rate equation (V^A and K_m^A) at different constant peptide concentrations. Similarly, v vs peptide concentration plots were used for the calculation of the V^B and K_m^B values at different ATP concentrations.

In summary, this method is based on the experimental finding that the apparent Michaelis constant values depend on the concentration of the second substrate. This dependence can be easily observed if appropriate experiments are made, and these results do not depend upon the kinetic scheme and the mathematical algorithm applied for data processing. Moreover, similar plots should be revealed for both substrates. The ratio of the Michaelis constants obtained at zero and saturating substrate concentrations allow calculation of the interaction factor α. Initially this approach was suggested by Symcox and Reinhart, who successfully applied this analysis for a multimeric enzyme [18]. We have improved this approach by applying the second-order rate constants to characterize the enzyme affinity at zero substrate concentration.

Following the rate equation (2), the Michaelis constants for ATP should depend on the peptide (B) concentration:

$$K_m^A = K_a\frac{1+\dfrac{[B]}{K_b}}{1+\dfrac{[B]}{\alpha K_b}}. \qquad (3)$$

By analogy, the Michaelis constants for peptides should depend on the ATP (A) concentration:

$$K_m^B = K_b\frac{1+\dfrac{[A]}{K_a}}{1+\dfrac{[A]}{\alpha K_a}}. \qquad (4)$$

Further the plots of K_m^A vs [B] and K_m^B vs [A] were used for the calculation of the interaction factor α. Although the same plots (3) and (4) can be used for simultaneous calculation of the K_a and K_b values, these parameters were obtained from separate analysis, using the second-order rate constants of the enzymatic reaction, $k_{II}^A = \dfrac{V^A}{K_m^A}$ and $k_{II}^B = \dfrac{V^B}{K_m^B}$, respectively. These parameters have the following meanings:

$$k_{II}^A = \frac{\dfrac{V}{\alpha K_a}[B]}{K_b+[B]} \quad \text{and} \qquad (5)$$

$$k_{II}^B = \frac{\dfrac{V}{\alpha K_b}[A]}{K_a+[A]}. \qquad (6)$$

The plots of k_{II}^A vs [B] and k_{II}^B vs [A] were used for the calculation of the K_b and K_a values, which were thereafter used as constraints in the calculation of the α values from K_m^A vs [B] and K_m^B vs [A] plots. As the results of these calculations did not depend on the V value, the catalytic activity of the enzyme was estimated on milligram basis and was used for planning the experiments.

Calculations

Calculations and statistical analysis of the data were made using the GraphPad Prism (version 5.0, GraphPad Software Inc., USA) and SigmaPlot (version 8.0, SPSS Inc., USA) software packages. The results were reported with standard errors.

RESULTS

Affinity of the free protein kinase A for peptides and ATP

The equilibrium constants K_b and K_a were calculated from Eqs (5) and (6) using the second-order rate constants of the peptide phosphorylation reaction. The plots of k_{II}^B vs [A] and k_{II}^A vs [B] were hyperbolic, as illustrated for LRRASLG (kemptide) and ATP in Fig. 1. The hyperbolic plots allowed reliable calculation of the

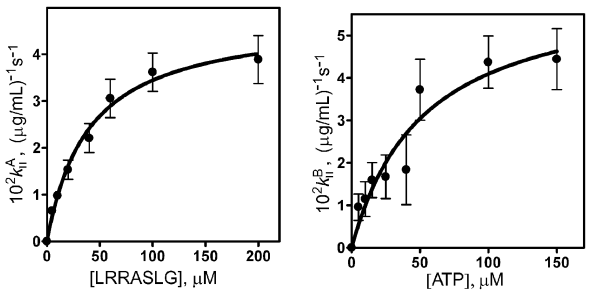


Fig. 1. Calculation of protein kinase A affinity for kemptide (left panel) and ATP (right panel) by using the second-order rate constants of kemptide phosphorylation reaction, catalysed by protein kinase A.

Table 1. Results of kinetic analysis of the phosphorylation of peptide substrates by protein kinase A (the catalytic subunit of cAMP-dependent protein kinase) in 50 mM TRIS/HCl, pH 7.5, 30 °C. The meaning of the kinetic parameters is given in Scheme (1). Parameters are listed with standard errors

	Peptide	K_b, μM	K_a, μM	α_b	α_a	pα (average)	pK_b
I	RRYSV	2.1 ± 0.5	48 ± 11	0.11 ± 0.02	0.08 ± 0.01	1.02 ± 0.10	5.68 ± 0.05
II	RRASVA	25 ± 8	53 ± 10	0.19 ± 0.03	0.19 ± 0.02	0.72 ± 0.10	4.60 ± 0.14
III	LRRASLG	40 ± 5	51 ± 14	0.36 ± 0.04	0.37 ± 0.03	0.44 ± 0.07	4.39 ± 0.05
IV	RKRSRKE	117 ± 14	49 ± 10	0.52 ± 0.06	0.46 ± 0.05	0.31 ± 0.05	3.92 ± 0.06
V	LRKASLG	231 ± 36	52 ± 17	0.60 ± 0.08	0.76 ± 0.09	0.17 ± 0.07	3.64 ± 0.07
VI	LARASLG	1880 ± 541	45 ± 13	1.2 ± 0.2	1.6 ± 0.3	–0.14 ± 0.07	2.72 ± 0.14
VII	LRAASLG	6454 ± 2328	49 ± 23	3.5 ± 0.6	2.5 ± 0.4	–0.48 ± 0.15	2.19 ± 0.15

K_a and K_b values. The same procedure was used for all peptides studied. The results of these calculations are listed in Table 1.

As the parameter K_a characterizes the affinity of the free enzyme for ATP, it was not surprising that all these values, calculated from phosphorylation data for different peptides, coincided well with one another. Therefore, the mean value $K_a = 49.7$ μM was calculated from these results. It was noteworthy that this K_a value was somewhat higher than the K_m values commonly reported for ATP in the protein kinase A catalysed reaction of peptide phosphorylation, most often ranging between 5 μM and 20 μM. However, this difference between K_a and K_m for ATP can be explained by Eq. (3). Following this equation, the K_m value for substrate A should depend on the concentration of the second substrate B, and $K_a > K_m^A$ if $\alpha < 1$. Obviously this was the case for LRRASLG, as seen from the K_m^A vs [B] plot for this peptide in Fig. 2. Thus, the dependences of K_m^A vs [B] were used for the calculation of the interaction factors α as set by Eq. (3).

Differently from the results for ATP, the affinity of the free protein kinase A for peptides was rather diverse, and the K_b values, ranging from 2 μM to 6 mM (see Table 1), were obtained for a series of selected substrates. This variation in substrate reactivity was not surprising, as the recognition of peptide primary structure by protein kinase A has been a well-known fact since phosphorylation of the model substrates has been studied [19]. Therefore, substrates of different primary structure and reactivity were specially selected for this study proceeding from their K_m values reported in the literature [20–23]. The diversity of the K_b values listed in Table 1 reveals that the selection was successful. Moreover, our general understanding of protein kinase A substrate specificity, developed on the basis of the Michaelis constants, seems to hold also for constants K_b. However, like with K_m^A, the parameters K_b cannot be directly compared with the appropriate K_m^B values, as $K_b = K_m^B$ only if $\alpha = 1$. In all other cases, i.e. if the interaction factor α is different from unity, the Michaelis constant K_m^B for peptide should depend upon the ATP concentration, as predicted by Eq. (4). Indeed, the appropriate dependences of the K_m^B values upon the ATP concentration were observed experimentally, as illustrated for kemptide phosphorylation reaction in Fig. 2. Therefore the K_m^B vs [ATP] plots were also used for the calculation of the α values as described below.

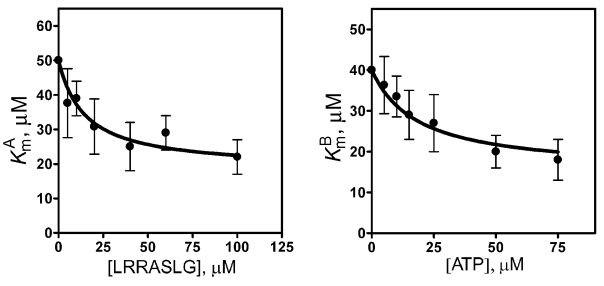


Fig. 2. Calculation of the interaction factor α from K_m^A vs peptide concentration plot (left panel) and K_m^B vs ATP concentration plot (right panel) for the protein kinase A catalysed reaction of kemptide phosphorylation.

Interaction factor α for protein kinase A substrates

As the next step of this study, the K_m^A values were determined for ATP at different peptide concentrations and similarly, the K_m^B values were determined for each peptide at different ATP concentrations, as described by Eqs (3) and (4), respectively. This analysis revealed that the conventional Michaelis constants were indeed dependent upon the concentration of the 'second' substrate. These plots were further used for the calculation of the α values listed in Table 1.

As the plots of K_m^B vs [A] and K_m^A vs [B] were separately analysed for each pair of substrates, two α values were obtained from these independent sets of experimental data. Therefore, two values of the interaction factor for each ATP–peptide pair were listed in Table 1 as α_a and α_b, respectively. It can be seen that there was a good agreement between these results.

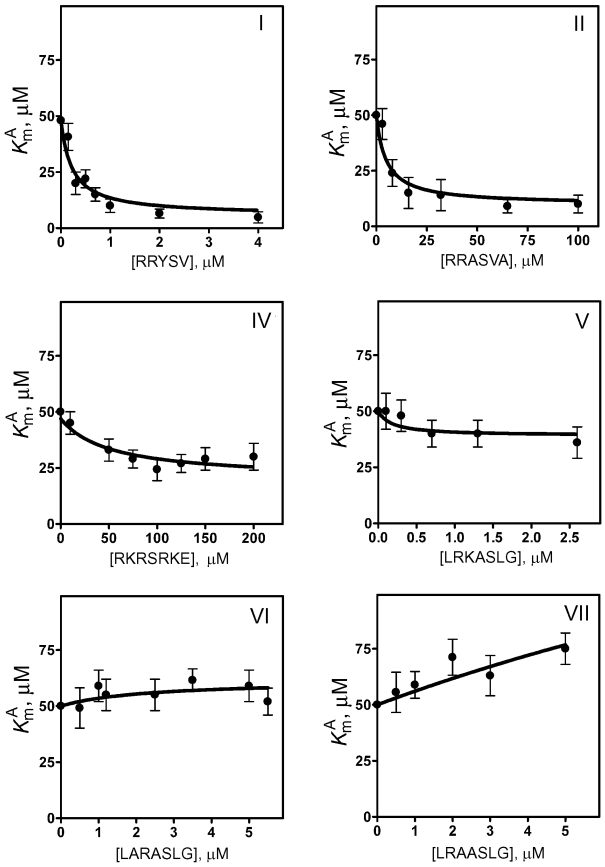


Fig. 3. Influence of peptide concentration upon the value of the Michaelis constant for ATP, determined for the protein kinase A catalysed phosphorylation reaction of these peptides. Numbers of panels correspond to peptide numbers in Table 1.

Therefore the mean value of the interaction factor α was calculated from α_a and α_b for further analysis.

It can be seen in Fig. 3 that K_m^A vs peptide concentration plots had rather different shapes when different peptides were used as substrates. This divergence manifested also in the α values, varying from 0.09 for RRYSV to approx. 3 for LRAASLG. Interestingly, the same peptides had the highest and the lowest binding effectiveness with the free enzyme, as seen from the appropriate K_b values in Table 1. Moreover, concurrent changes in the K_b and α values were also observed for other peptides (Table 1).

Linear-free-energy relationship for α

The systematic dependence of the α values upon the binding effectiveness of peptide substrates was presented in terms of a linear-free-energy (LFE) relationship between the free energy of the allosteric effect and the free energy of peptide binding with the enzyme, quantified by the $p\alpha$ and pK_b values, respectively.

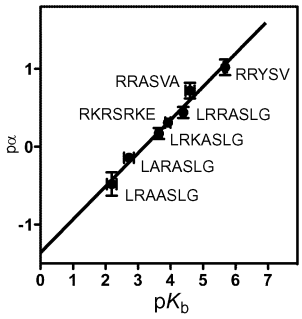


Fig. 4. Linear-free-energy relationship between the allosteric effect in the protein kinase A catalysed peptide phosphorylation reaction, quantified by the negative logarithm of the interaction factor α and affinity of the free protein kinase A for these peptides (pK_b).

This interrelationship was expressed by the following equation:

$$p\alpha = C + SpK_b, \qquad (7)$$

where C and S stand for the intercept and slope of the linear plot between the $p\alpha$ and pK_b values, as shown in Fig. 4. Analysis of experimental data listed in Table 1 yielded the following results: $C = -1.4 \pm 0.1$, $S = 0.43 \pm 0.03$, $r^2 = 0.98$.

It is important to mention that the negative logarithmic scale, used to quantify the allosteric effect by the $p\alpha$ values, was selected to keep analogy with the pK_b scale, characterizing the free energy of protein–substrate interaction.

DISCUSSION

The understanding of the term 'allostery' has been significantly widened during some recent years, and today this phenomenon can be defined as the coupling of binding properties of two separate ligand binding sites, independently whether the sites are located on the oligomeric or monomeric protein molecule [4]. This means that the allosteric interaction may be revealed in the case of any monomeric bi-substrate enzyme that simultaneously binds two substrates to form the ternary enzyme–substrate complex. Formally this situation can be presented by the reaction scheme (1), where the feedback between the binding properties of substrate binding sites is quantified by the interaction factor α.

It is noteworthy that in the presence of the allosteric interaction between two binding sites of substrates, the experimentally determined Michaelis constants should depend on the concentration of the second substrate. This situation is specified by Eqs (3) and (4). On the other hand, however, it is very important to understand that the presence of such interrelationship is in no way

connected with the kinetic scheme used for the interpretation of these dependences, and application of the second-order rate constants for the characterization of the interaction of substrates with the free enzyme removes also the question about the rate-limiting step of the catalytic mechanism.

In the present study, we used this very straightforward kinetic analysis for the protein kinase A catalysed reaction of phosphorylation of peptide substrates. The results listed in Table 1 demonstrate that binding properties of the ATP and peptide binding sites of protein kinase A were, indeed, allosterically coupled, and the effect of cooperativity was quantified by the interaction factor α. Besides the interaction factor, also the affinity of the free enzyme for ATP and peptides was characterized in terms of the appropriate dissociation constants, K_a and K_b, respectively.

As phosphorylation of different peptides was studied, the results provided a unique possibility of analysing the interrelationship between the substrate binding effectiveness and the allosteric behaviour of protein kinase A. This analysis revealed that a more efficient substrate binding was accompanied by a more significant allosteric effect. Considering this trend the principle 'better binding: stronger allostery' was formulated for protein kinase A catalysis. Proceeding from this observation the principle 'better binding: stronger allostery' was formulated also for the protein kinase A catalysed reaction of peptide phosphorylation. Previously we revealed the same trend for interactions of the same enzyme with its reversible inhibitors [14].

This formulation is similar to the principle 'better binding: better reaction', advanced by Knowles for the α-chymoptrypsin catalysed reactions in 1965 [24]. Later the same principle was validated for other hydrolytic enzymes, and was also quantified in terms of LFE relationships [25,26]. Proceeding from this analogy, we were able to quantify the principle 'better binding: stronger allostery' in terms of the LFE relationship, as defined by Eq. (7) and shown in Fig. 4.

In practice, the statement 'better binding: stronger allostery' compares two processes of ligand binding. Firstly, we consider the interaction of substrate molecule with the free enzyme (pK_b). Secondly, the interaction of the same substrate with the pre-formed enzyme complex, containing another substrate, is considered. At the same time the principle 'better binding: better reaction' links substrate binding effectiveness with the free energy of the transition state of the catalytic step. However, as the activation free energy of the catalytic step also includes the interaction of the substrate transition state with the protein, analogy can be found between these formulations. Summing up, this analogy consists in the enzyme ability to couple effectiveness of ligand binding with effectiveness of some following step of the catalytic process.

Intuitively, the interrelationship between the ligand binding effectiveness and the extent of the allosteric effect, triggered off by the binding of this ligand, was not very surprising. Indeed, stronger ligand binding may cause major perturbation in the protein molecule, either by inducing some new conformational state, or by shifting the equilibrium between pre-existing conformations, as suggested in [27]. More explicitly this situation can be described as energetic coupling of closely located amino acid residues, forming a sparse energetic network for transmission of the allosteric effect [28].

On the other hand, however, some supplementary conclusions can be drawn from the LFE relationship shown in Fig. 4. Firstly, the dependence of the allosteric effect upon ligand binding effectiveness (and structure) seems to be a continuous function. Therefore, the phenomenon of allosteric regulation can hardly be explained by a shift between two or more but a fixed number of conformational states of the enzyme. Preferably, this phenomenon agrees with the understanding that the dynamic protein molecule may continually change its conformation and through these changes modulate the binding properties of its binding sites. Similar changes can be observed in the case of non-specific solvation phenomena of molecules in different media. Certainly, this model of allostery presumes an 'extra-soft' and highly dynamic protein structure, and complicates the presentation of the ligand recognition mechanism in terms used by conventional structural biology, counting the presence or absence of distinct interactions between ligand and protein molecules. Perhaps protein kinase A is an example of such highly dynamic protein.

Secondly, as seen in Fig. 4, the linear plot between $p\alpha$ and pK_b has an intercept with the x-axis at $pK_b \approx 3$. Formally this means that at this point $\alpha = 1$, and no allosteric feedback between the substrate binding sites should occur if the substrate is characterized by the K_b value around 1 mM. It can be seen in Table 1 that peptide LARASLG had its K_b value rather close to this critical threshold. Indeed, as seen in Fig. 3, there was only a minor dependence of the K_m^A values upon the concentration of this peptide. Simultaneously, the α value was also rather close to unity (Table 1).

Moreover, when the effectiveness of peptide binding was below the critical K_b value, $\alpha > 1$, substrate binding with the enzyme and formation of the ternary complex were hindered. In other words, negative cooperativity between the binding sites should appear for these substrates. Interestingly, this inversion of allostery was observed experimentally for peptide LRAASLG, as the affinity of protein kinase A for this substrate was quite significantly below the critical limit. Accordingly, the K_m^A values for ATP, determined at various concentrations of this peptide, were slightly increasing when more peptide was added into the

reaction medium (Fig. 3). A similar result was obtained for the K_m^B vs [ATP] plot, confirming the standpoint that the allosteric effect has no 'direction' and affects similarly the binding of both substrates.

Thirdly, this mechanism of allosteric control over enzyme specificity also pointed out that the structural factors that govern substrate recognition by the enzyme active centre could not be presented by simple additive models. This means that the contribution of a certain structural fragment of substrate molecule to its binding effectiveness might be governed by the binding properties of the second substrate. This should certainly complicate theoretical analysis of the substrate specificity of protein kinase A and the specificity of bi-substrate enzymes in general.

Finally, the LFE relationship between the $p\alpha$ and pK_b values suggested that the same specificity determining factors governed peptide binding effectiveness and the allosteric effect. This means that at least this part of the substrate specificity of protein kinase A that is based on the recognition of the primary structure of phosphorylatable peptides is amplified by allostery. On the other hand, however, as the α value depends on the enzyme affinity for the particular substrate, the effect of amplification is governed by substrate structure. This means that the enzyme affinity for good substrates can be additionally enhanced by allostery, while this enhancement should be moderate for less good substrates. For bad substrates the enzyme affinity is even diminished as $\alpha > 1$, and the increment of $p\alpha$ becomes negative. Using series of peptides, which were all phosphorylated by protein kinase A, we were able to demonstrate these possibilities. This additional mechanism of specificity control may have significant biological implications and can be used to prevent occasional phosphorylation of 'wrong' substrates. Indeed, as the physiological ATP concentration is around 2 mM in cells, the phosphorylation processes occur at the saturating concentration of this substrate and therefore should reveal maximal allosteric 'tuning' effects.

CONCLUSIONS

An allosteric effect was found in the peptide phosphorylation reaction catalysed by the catalytic subunit of cAMP dependent protein kinase (protein kinase A), which is a monomeric bi-substrate enzyme. Variation of the structure of the phosphorylatable peptides was used to reveal that the allosteric effect depended upon the effectiveness of substrate binding with the enzyme. The principle 'better binding: stronger allostery' was formulated. Further this principle was formalized in terms of a linear-free-energy relationship (7). This relationship had a significant negative intercept at the *y*-axis, revealing inversion of the allosteric effect: the positive allostery for good substrates changed to negative allostery for bad substrates in this model reaction of regulatory phosphorylation. This implies that allostery could be used as an additional efficient specificity determining factor in enzyme catalysis. This new extrathermodynamic aspect of allostery seems to be important to be considered in parallel with commonly discussed structural and thermodynamic aspects of this phenomenon.

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Proteiinkinaasi A katalüütilise alaühiku allosteeria ilmnemine peptiidide fosforüleerimise kineetikas

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Uuriti cAMP-sõltuva proteiinkinaasi katalüütilise alaühiku allosteeria ilmnemist seitsme peptiidi fosforüleerimise kineetikas. Allosteerilise efekti kirjeldamiseks määrati nende reaktsioonide jaoks interaktsioonifaktori α väärtused. Selleks võrreldi substraatide sidumise efektiivsust vaba ensüümiga ja ensüüm-substraadi kompleksiga, kus teine substraat oli juba eelnevalt seotud. Leiti, et allosteerilise efekti suurus muutub koos substraadi sidumise efektiivsusega, mida oli võimalik muuta substraadi struktuuri varieerides. Nende andmete põhjal sõnastati uuritud katalüütilise reaktsiooni jaoks reegel: "parem sidumine – tugevam allosteeria". Selle seose kvantitatiivseks kirjeldamiseks kasutati vabaenergia sõltuvust $\mathrm{p}\alpha = \mathrm{C} + S\mathrm{p}K_\mathrm{b}$, kus allosteerilise efekti suurust iseloomustab interaktsioonifaktori α negatiivne logaritm ($\mathrm{p}\alpha$) ja substraadi sidumise efektiivsust ensüüm-peptiidi kompleksi dissotsiatsioonikonstandi negatiivne logaritm $\mathrm{p}K_\mathrm{b}$. Uuritud peptiidide fosforüleerimise reaktsiooni jaoks leiti selle sõltuvuse parameetrid $\mathrm{C} = -1,4$ ja $S = 0,4$. Negatiivne telglõigu väärtus näitab, et substraatide sidumiskohtade vaheline positiivne kooperatiivsus ($\alpha < 1$) muutub negatiivseks ($\alpha > 1$), kui K_b väärtused suurenevad. Seega ilmneb allosteerilise efekti inversioon, mille tekitab substraadi struktuuri muutus. Niisiis avaldub allosteeria kui substraatspetsiifilisuse täiendav mehhanism. See mehhanism lubab efektiivselt eristada häid ja halbu substraate ning takistada valede peptiidide ja valkude fosforüleerimist. Sellisel täiendaval kaitsemehhanismil võib olla suur bioloogiline tähtsus olukorras, kus regulatoorse fosforüleerimise reaktsioonid toimuvad suhteliselt kõrgel ATP kontsentratsioonil, mis on lähedane selle aine füsioloogilisele kontsentratsioonile rakkudes.