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Linear Free Energy Relationships in cAMP-Dependent Protein Kinase Reactions with Synthetic Substrates

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The quantitative structure–activity relationships for cAMP-dependent protein kinase reactions with synthetic substrates were used to analyze the mechanism of peptide phosphorylation and specificity of the enzyme. It was shown that in most cases the variation in peptide structure affected the binding affinity of substrate but had only a weak effect on the observed rate of the phosphorylation reaction. Moreover, the intrinsic reactivity of the phosphorylatable OH-group has no effect on the apparent rate of the catalytic step, pointing to the fact that the chemical act of peptide phosphorylation is not the rate-limiting step of the overall process. Introduction of a proline residue after the phosphorylatable amino acid changes the specificity pattern of the enzyme. Variation in the structure of the latter type of substrates alters the observed rate of phosphorylation of peptides while it has almost no effect on their binding affinity. The influence of structure of amino acids on the binding effectiveness of peptides was analyzed by taking into account hydrophobicity and steric properties of their sidechains, quantified by the π-constants and steric constants E_S^o. © 1991 Academic Press, Inc.

INTRODUCTION

The effectiveness of protein phosphorylation by cAMP-dependent protein kinase is mostly determined by the local structure of the polypeptide chain around the phosphorylatable amino acid residue (*1–5*). Therefore it became possible to use short synthetic peptides as substrates for further investigation into the kinetics and specificity of this protein kinase (*6, 7*).

The studies made with synthetic substrates have revealed that the minimum substrate for cAMP-dependent protein kinase can be defined as RRXSX (*2, 5, 8*), having a block of two arginines preceding the phosphorylatable serine residue. This structure was derived from the amino acid sequence around the phosphorylated site of pyruvate kinase (*9*). More recently an alternate structure RXKRXXSX for the minimum substrate has been proposed on the basis of the amino acid sequence of the phosphorylated site of the β-subunit of phosphorylase kinase (*10*). It is noteworthy that both these sequences involve positively charged residues and it is now generally accepted that the positive charges in the peptide structure are fundamental for recognition of substrates by the active center of cAMP-dependent protein kinase (*7*).

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TABLE I

Phosphorylation of Synthetic Peptides by cAMP-Dependent Protein Kinases, Isolated from Beef Skeletal Muscle [Refs. (*8, 11*)], Rabbit Skeletal Muscle [Refs. (*10, 12*)], and Rat Liver [Refs. (*2, 13, 14*)]. The Phosphorylatable Sites Are in Italics

No.	Peptide	pK_m	log V	log(V/K_m)	Ref.
5.1	RRL*S*I	5.40	—	—	(*7*)
5.2	RRP*S*V	4.45	0(rel)	—	(*12*)
5.3	RRF*S*V	5.30	—	—	(*7*)
5.4	RRD*S*V	4.25	—	—	(*7*)
5.5	RRV*S*V	4.02	—	—	(*7*)
5.6	RRG*S*V	5.30	—	—	(*7*)
5.7	RRA*S*R	3.76	—	—	(*7*)
5.8	RRA*S*S	3.74	—	—	(*7*)
5.9	RRA*S*I	5.15	—	—	(*7*)
5.10	RRA*S*L	4.58	—	—	(*7*)
5.11	RRA*S*F	4.88	—	—	(*7*)
5.12	RRA*S*V	4.10	—	—	(*2*)
5.12	RRA*S*V	4.62	0(rel)	—	(*12*)
5.13	RA*S*LG	2.36	1.00(μmol/min mg)	3.36	(*8*)
6.1	LRRA*S*L	4.24	1.26(μmol/min mg)	5.50	(*8*)
6.2	RRA*S*LG	4.58	1.25(μmol/min mg)	5.83	(*8*)
6.3	RRA*S*VA	5.49	−0.05(rel)	—	(*12*)
6.4	RRP*S*PA	2.22	2.32(pmol/min)	4.54	(*13*)
6.5	RRA*S*VA	4.86	2.89(pmol/min)	7.75	(*13*)
6.6	RRP*T*PA	2.73	1.18(pmol/min)	3.91	(*13*)
6.7	RRA*T*PA	2.92	0.69(pmol/min)	3.61	(*13*)
6.8	RRP*T*VA	2.67	2.52(pmol/min)	5.19	(*13*)
6.9	RRA*T*VA	2.70	2.33(pmol/min)	5.03	(*13*)
7.1	LRHA*S*LG	2.87	0.81(μmol/min mg)	3.68	(*8*)
7.2	LHRA*S*LG	3.38	1.10(μmol/min mg)	4.48	(*8*)
7.3	LRKA*S*LG	3.58	1.23(μmol/min mg)	4.81	(*8*)
7.4	LKRA*S*LG	2.85	1.23(μmol/min mg)	4.08	(*8*)
7.5	LRAA*S*LG	2.20	0.72(μmol/min mg)	2.92	(*8*)
7.6	LARA*S*LG	2.31	0.94(μmol/min mg)	3.25	(*8*)
7.7	LRRA*S*LG	4.80	1.31(μmol/min mg)	6.11	(*8*)
7.8	LRRP*S*LG	5.22	1.3 (μmol/min mg)	6.52	(*11*)
7.9	RTKRSG*S*	2.96	—	—	(*10*)
7.10	LRRA*S*VA	5.64	0(rel)	—	(*12*)
7.11	TKRSG*S*V	2.68	—	—	(*10*)
7.12	LRRA*T*LG	3.23	0.78(μmol/min mg)	4.01	(*8*)
7.13	RTKRSG*S*	2.92	−0.50(μmol/min mg)	2.42	(*10*)
7.14	TKRSG*S*V	2.68	0.70(μmol/min mg)	3.38	(*10*)
8.1	RSKRSG*S*V	4.58	0.98(μmol/min mg)	5.56	(*10*)
8.2	RAKRSG*S*V	4.48	1.03(μmol/min mg)	5.51	(*10*)
8.3	RTKGSG*S*V	2.57	−0.05(μmol/min mg)	2.52	(*10*)
8.4	RTGRSG*S*V	3.85	0.89(μmol/min mg)	4.74	(*10*)
8.5	GTKRSG*S*V	2.88	0.78(μmol/min mg)	3.66	(*10*)
8.6	RTKRSG*S*V	4.68	1.02(μmol/min mg)	5.70	(*10*)
8.7	GRGLSL*S*R	3.62	—	—	(*6*)
8.8	VLRRA*S*VA	5.60	0(rel)	—	(*12*)
8.9	RRRRP*T*PA	2.9	1.2 (pmol/min)	4.1	(*13*)
9.1	ARTKRSG*S*V	5.00	1.02(μmol/min mg)	6.02	(*10*)

TABLE 1–*Continued*

No.	Peptide	pK_m	log V	log(V/K_m)	Ref.
9.2	GVLRRA*S*VA	5.88	−0.02(rel)	—	(*12*)
9.3	RRP*T*PATVA	3.0	0.9 (pmol/min)	3.9	(*13*)
12.1	VLQRRRP*T*SIPQ	3.93	0.82(μmol/min mg)	4.75	(*14*)
12.2	VLQARRG*T*SIPQ	3.86	0.83(μmol/min mg)	4.69	(*14*)
12.3	VLQRRRG*T*SIPQ	4.41	0.66(μmol/min mg)	5.07	(*14*)
12.4	VLQRRRP*S*SIPQ	4.90	1.16(μmol/min mg)	6.06	(*14*)
12.5	VLQARRG*S*SIPQ	4.95	0.92(μmol/min mg)	5.87	(*14*)
12.6	VLQRRRG*S*SIPQ	5.42	1.13(μmol/min mg)	6.55	(*14*)

Moreover, it is well documented that the effectiveness of peptide phosphorylation depends also upon the structure of other nonionic amino acids in the substrate molecule. However, until now no attempts have been undertaken to quantify all these effects, although this is an important step to discuss more thoroughly the specificity of cAMP-dependent protein kinase. In the present report we try to fill this gap and analyze the physicochemical background of substrate recognition by the active center of cAMP-dependent protein kinase by using quantitative structure–activity relationships for model peptide substrates.

KINETIC DATA

The reaction of peptide phosphorylation by cAMP-dependent protein kinase follows satisfactorily the Michaelis–Menten rate equation, allowing determination of the K_m and V values (see in Table 1). As the first approximation, these constants can be treated as parameters which characterize the binding affinity and phosphorylation velocity of peptides, although the exact physical meaning of these parameters depends on the rate-limiting step of the enzyme reaction. Therefore, if possible, the ratio V/K_m was used in structure–activity relationships, as the latter values should have the same meaning as the second-order rate constants of enzyme reaction. This procedure is hampered by the fact that the V-values of the phosphorylation reaction are given only for a limited set of substrates and often these data are presented in different units that do not allow their direct comparison. We did not use the method of normalization of these different data and therefore only the constants given in the same units were used in each correlation.

In the case of peptides reported on in Ref. (*10*) the absolute values of V were calculated from the average V-value 10.5 μmol/min · mg for peptide RTKRSGSV (No. 8.6 in Table 1) and the relative V-values.

The list of peptide substrates and the kinetic data of their phosphorylation by cAMP-dependent protein kinase(s) compiled from literature (*2, 8, 10–14*) are presented in Table 1. These kinetic parameters do not reveal systematic dependence on the source of the enzyme used in the kinetic studies. Therefore all these data were used together in the following correlation analysis.

The positions of amino acids in the peptides are numbered starting from the residue phosphorylated, the position of which is given the number 0. The positions from 1 to n and from -1 to $-n$ tend toward the carboxyl terminus and the amino terminus of the peptide, respectively.

All the structural parameters used to characterize the properties of sidechains of amino acids are listed in Table 2. The hydrophobicity of amino acids was compared by using the π-constants for their sidechains as proposed by Fauchere and Pliska (*15*). Steric constants E_s^o for alkyl groups and several radicals containing heteroatoms were taken from (*16*). For some other groups the steric constants were selected according to the principle of isostericity (*16*) and these values are marked by an asterisk in Table 2. The "bulkiness" of the sidechains of amino acids was characterized by molecular refractivity constants MR (*17*).

BINDING–ACTIVITY RELATIONSHIPS

Particular features of substrate structure can be recognized by the enzyme active center on the noncovalent binding step as well as on the following bond-breaking steps of the catalytic process (*18–20*). Such distribution and cooperation of the specificity-determining factors on these separate steps of the enzyme reaction can be analyzed by studying the binding–activity relationships (*18*). In the

TABLE 2

Substituent Constants for Amino Acid Side Chains, Used in the Correlation Analysis

Amino Acid	π(*15*)	E_s^o(*16*)	MR(*17*)
Alanine	0.31	0	5.65
Arginine	−1.01	0.60*	30.05
Asparagine	−0.60	1.08*	14.46
Aspartic acid	−0.77	1.08*	11.58
Glutamine	−0.22	1.00*	19.11
Glutamic acid	−0.64	1.08	16.23
Glycine	0	−0.25	1.03
Histidine	0.13	0.72	23.79
Isoleucine	1.80	1.53	19.59
Leucine	1.70	1.13	19.59
Lysine	−0.99	0.60*	25.05
Methionine	1.23	0.97*	23.12
Phenylalanine	1.79	0.72	30.01
Proline	0.72	0.56*	13.95
Serine	−0.04	0.40	11.82
Threonine	0.26	0.64	11.82
Tryptophan	2.25	0.72*	39.81
Tyrosine	0.96	0.72	31.83
Valine	1.22	0.85	14.96

* Isosteric constants.

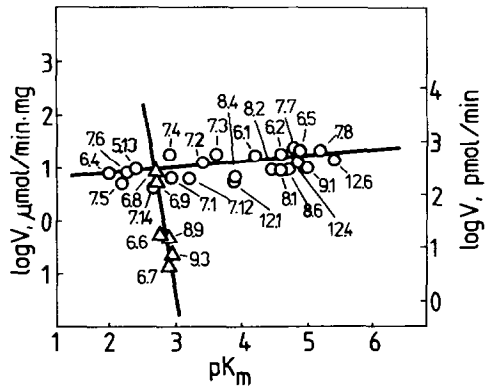

FIG. 1. Plot of log V vs pK_m for phosphorylation of synthetic peptides by cAMP-dependent protein kinase. The numbers of the points correspond to the peptide numbers in Table 1. Phosphorylation of serine (○) and threonine (△).

present case this is done by plotting the pK_m and log V values for substrates of cAMP-dependent protein kinase.

It can be seen in Fig. 1 that in the majority of substrates the variation of structure affects mostly their noncovalent binding while the rates of phosphorylation of these peptides are quite similar. This situation can be quantified by a small value of the intensity factor of the linear free-energy relationship between pK_m and log V,

$$\log V = (0.69 \pm 0.18) + (0.09 \pm 0.05)\ \mathrm{p}K_m,$$
$$n = 18,\ s = 0.21,\ r = 0.415,\ F = 3.3. \quad [1]$$

In this and the following correlation equations n is the number of data points, s is the standard error of estimation, r is the correlation coefficient, F is the ratio of variance between calculated and observed values. The 95% confidence intervals of the parameters are given.

It can be concluded that the mechanism of enzyme–substrate interaction on both the binding and the reaction steps remains similar within the series of substrates analyzed in Fig. 1. In this connection it should be emphasized that the apparent rate of peptide phosphorylation is not affected even by changing the structure of the phosphorylatable amino acid by replacement of a serine residue by threonine or in one case by hydroxyproline, although the reactivity of these primary and secondary alcohols should be different due to their different steric hindrances for the nucleophilic displacement reaction at the tetrahedral phosphorus atom (*21*).

Concerning the catalytic mechanism of cAMP-dependent protein kinase, the latter fact points to the possibility that the chemical act of peptide phosphorylation is not the rate-limiting step of this process.

On the other hand, however, it can also be seen in Fig. 1 that a group of threonine-containing peptides yield a qualitatively different log V vs pK_m plot. In

the latter case, primarily the rate of peptide phosphorylation depends upon the peptide structure, yielding the intercept and slope values 14 ± 4 and −(4.6 ± 1.5) for this plot, respectively. It is noteworthy that all the latter substrates have a proline residue following the phosphorylatable threonine. Therefore it can be assumed that this factor is responsible for the different behavior of these two groups of substrates, most probably due to some stereochemical pecularities of the threonine–proline combination in the peptide chain, putatively making some hindrances for the reaction step of threonine phosphorylation, while having no effect on the accessibility of the serine residue if this is followed by a proline residue in position +1 of the peptide structure. Thus, it appears that there are two different groups of synthetic substrates for cAMP-dependent protein kinase which cannot be treated within a framework of a common reaction series.

AMINO ACIDS IN POSITIONS +1 AND +2

The phosphorylatable serine in the synthetic substrates of cAMP-dependent protein kinase is followed by different amino acids with aliphatic sidechains (see Table 1) and these variations are sensitively recognized by the enzyme active center on the noncovalent binding step. As these sidechains are "chemically inert" and can not be involved in electrostatic or donor–acceptor interactions, the variations in binding affinity of these substrates clearly point to the possibility of a hydrophobic interaction between the amino acids in positions +1 and +2 and the enzyme active center.

It can be seen in Table 1 that two subseries of substrates can be compiled with a variable structure of the amino acids in positions +1 and +2: RRASX and RRASXX. In both cases the pK_m values are well correlated with hydrophobicity constants π. For the peptides RRASX this correlation yielded the equation

$$pK_m = (4.0 \pm 0.1) + (0.46 \pm 0.09)\pi_{+1},$$
$$n = 6,\ s = 0.25,\ r = 0.923,\ F = 23. \quad [2]$$

While treating together the peptides RRASX and RRASXX, Eq. [3] was obtained:

$$pK_m = (4.0 \pm 0.1) + (0.44 \pm 0.10)\pi_{+1} + (1.9 \pm 0.7)\pi_{+2},$$
$$n = 9,\ s = 0.28,\ r = 0.902,\ F = 13. \quad [3]$$

Addition of parameters E_S^o and MR into these equations did not improve the quality of correlation. The same is valid for introduction of an indicator variable I_{+2} to take into account the presence ($I = 1$) or absence ($I = 0$) of the amino acid in this position. Hence, the simplest model of hydrophobic interaction seems to be effective for the region of the active center of cAMP-dependent protein kinase responsible for binding of amino acids in positions +1 and +2.

It should be emphasized that the value of the intensity factor of the hydrophobic interaction, calculated for the amino acid in position +1, is similar in Eqs. [2] and [3]. This is consistent with the general idea that the mechanism of interaction of the constituent parts of the peptide with the appropriate binding site does not depend upon the peptide length and its affinity can be calculated in additivity

terms of the particular effects of the component amino acids. Other results discussed below agree with this conclusion.

The intensity factor of the hydrophobic interaction in Eq. [3], calculated for the amino acid in position +2, is less precise due to a narrow selection of substituents. However, within the error limits it can be concluded that the hydrophobic effect is different in positions +1 and +2 and is remarkably large in the latter case as follows from the value of the appropriate coefficient 1.9 in Eq. [3]. The latter fact seems to be promising for synthesis of novel highly effective substrates for cAMP-dependent protein kinase.

AMINO ACIDS IN POSITION −1

The variations in the structure of aliphatic sidechains of the amino acid in position −1, intervening between the phosphorylatable serine and the block of arginines in model substrates of cAMP-dependent protein kinase, change the binding affinity of peptides. The factors governing these effects were analyzed in the case of minimum substrates RRXSV listed in Table 1. It can be seen from these data that the sidechains of low (glycine) or high (phenylalanine) hydrophobicity in position −1 lead to the highest binding affinity of the peptide, while other substrates with moderately large groups in this position are all less effective. The latter phenomenon cannot be explained on the basis of a single interaction between the enzyme and the substrate molecules. This qualitative conclusion is supported by the absence of significant correlations if only a single parameter π, E_s° or MR was used to correlate the pK_m values.

It has been found, however, that the affinity of substrates RRXSV can be described by the simultaneous influence of two factors, hydrophobicity and steric effect of the sidechain of the variable amino acid,

$$pK_m = (4.5 \pm 0.3) + (1.3 \pm 0.5)\pi + (2.2 \pm 0.8)E_s^\circ$$
$$n = 5,\ s = 0.34,\ r = 0.900,\ F = 4.25. \quad [4]$$

No other combinations of the π, E_s°, and MR constants yielded better correlation for this set of data.

It should be emphasized that the steric effect, quantified by the corrected steric parameter E_s°, governs together with hydrophobicity also the partition of tripeptides between water and octanol (*22*). This phenomenon has been explained by the steric effect of the amino acid sidechains on the solvation of peptide with partitioning solvents. In our case the partitioning takes place between water and the enzyme active center, which seems to involve several hydrophobic regions. Therefore, the close analogy of the partitioning of peptides between water and octanol and water and enzyme active site is not surprising.

It can be added that the steric effect of the amino acid sidechains on the partition coefficients depends upon their location in the peptide molecule. The appropriate sensitivity factor for an N-terminal sidechain is considerably larger than that for a C-terminal sidechain and the effect of the central moiety lies in between (*22*). Thus, if this analogy can be applied for substrates of cAMP-depen-

dent protein kinase, the steric effect in positions +1 and +2 should be weaker than in the case of position −1, which agrees well with the present results.

AMINO ACIDS AROUND THE "CORE" OF THE MINIMUM UNIT RRAS

The data collected in Table 1 clearly show that the effectiveness of substrate binding to cAMP-dependent protein kinase is mainly determined by the structure of the pentapeptide fragment containing the phosphorylatable serine residue and longer peptides do not reveal drastically higher affinity. However, the addition of amino acids into positions +2 or −4 and −5 or variation of the peptide structure in these positions also has some effect on the K_m values. These effects may arise through the direct interaction between these residues and the enzyme active center, as discussed above. In addition the possibility that the conformation of these longer peptides plays some role in their interaction with the enzyme active center should be taken into account.

In the present report we have also made an attempt to analyze whether the influence of the structure of these "peripheral" amino acids upon the reactivity of peptides is additive in terms of the hydrophobic interaction. As above, the peptide series XXRRASXX, containing the core unit RRAS, was compiled on the basis of data in Table 1. For all of these substrates the correlation of pK_m with hydrophobicity parameters π yielded the multilinear equation,

$$pK_m = (3.8 \pm 0.3) + (0.6 \pm 0.2)\pi_{+1} + (2.8 \pm 0.7)\pi_{+2} + (0.0 \pm 0.1)\pi_{-4} + (0.4 \pm 0.3)\pi_{-5} \quad n = 14,\ s = 0.32,\ r = 0.901,\ F = 8.72. \quad [5]$$

It can be seen that there are no clear hydrophobic binding sites for the amino acids in positions −4 and −5 of the peptide XXRRASXX. Thus, the putative binding sites for the cationic arginine residues in positions −2 and −3 are not immediately followed by new hydrophobic sites. On the other hand, the properties of the hydrophobic binding sites for the amino acids in positions +1 and +2 are characterized by intensity factors which agree within the error limits with the results given by Eq. [3]. Thus, the specificity factors seem to be additive also in the case of these longer peptides, pointing to the fact that the elements of secondary structure, which may reveal in the case of longer peptides, play no remarkable role in the phosphorylation reaction.

AMINO ACIDS IN POSITIONS −2 AND −3

The two arginines, located in positions −2 and −3 of the peptide substrates for cAMP-dependent protein kinase, can be replaced by lysine, histidine, or even by alanine without drastic loss in the maximal velocity of peptide phosphorylation (see Table 1). At the same time this substitution leads to a remarkable decrease in the binding affinity of these substrates. Therefore it can be concluded that the arginine residues are somehow important for the noncovalent binding step of the substrates. In the present report an attempt is also made to analyze the role of

these amino acids by using the reaction series LXXASLG (see Table 1). In this case besides the pK_m values the combined parameters log (V/K_m) were also used in the correlation analysis.

It can be seen in Table 1 that the replacement of the arginines by lysine also remarkably reduces the pK_m value of the peptides LXXASLG, although the sidechains of both these amino acids are positively charged and their hydrophobicity is characterized by nearly identical values of the π-constants (Table 2). This indicates that some additional factors should govern the binding effectiveness of these peptides. This conclusion is supported also by the results of correlation analysis, showing that the influence of hydrophobicity cannot be considered statistically significant, even if the indicator variables are added to take into account the presence ($I = 1$) or absence ($I = 0$) of ionic charges in the positions -2 and -3 of the peptide.

Better correlations were obtained in this case if the "bulkiness" of the sidechains was taken into account instead of the hydrophobicity by using the MR-constants,

$$pK_m = (-0.5 \pm 1.3) + (0.07 \pm 0.03)MR_{-2} + (0.07 \pm 0.03)MR_{-3}$$
$$n = 7;\ s = 0.64,\ r = 0.807,\ F = 3.74. \quad [6]$$

The quality of this correlation can be improved by using the combined parameters V/K_m instead of K_m,

$$\log(V/K_m) = (-0.3 \pm 1.5) + (0.10 \pm 0.04)MR_{-2} + (0.08 \pm 0.04)MR_{-3}$$
$$n = 7,\ s = 0.73,\ r = 0.829,\ F = 4.41. \quad [7]$$

Introduction of indicator variables to characterize the presence ($I = 1$) or absence ($I = 0$) of the ionic charges of the amino acid sidechains in positions -2 and -3 had no significant effect on the quality of the correlation. Formally speaking, this means that the presence of cationic groups in positions -2 and -3 of the peptide substrates is not crucial for their interaction with the enzyme active center. If so, it can be postulated that the presence of arginines in the vicinity of the phosphorylatable site is important to provide the access of this site in protein tertiary structure for protein kinases, as these positive groups can hardly be "buried" in the hydrophobic interior of the protein. The same model explains the different location of arginine residues in minimum substrates, as two different peptide sequences were designed for cAMP-dependent protein kinase. However, the final conclusion about the importance of cationic residues for binding of substrates can be made only when data for a wider selection of peptides becomes available.

CONCLUSIONS

cAMP-dependent protein kinase is until now the only known enzyme among other protein kinases which effectively phosphorylates a large variety of synthetic peptides. The latter fact provides a unique possibility to analyze the catalytic mechanism and specificity of protein phosphorylation, as well as to discuss the

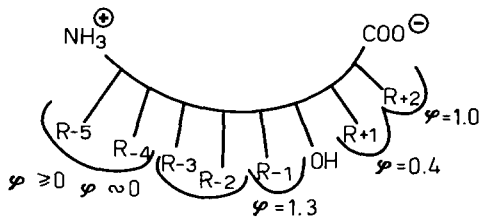

FIG. 2. Schematic representation of "topography" of the hydrophobic binding sites on the active surface of cAMP-dependent protein kinase. The constants φ characterize the intensity of hydrophobic interaction between individual residues of the peptides and the enzyme.

physicochemical principles of molecular recognition of peptide structure by the enzyme. In the present paper a first attempt has been made to use quantitative structure–activity relationships for this enzyme. This analysis of the available kinetic data allowed the systematization of the reaction series of peptides. It provided evidence for an alternative mechanism of the peptide phosphorylation by introducing the threonine–proline sequence into the substrate molecule.

Second, the important role of hydrophobic interactions follows from the results of the analysis, pointing to the fact that several hydrophobic binding sites exist around the catalytic site of the enzyme. The hydrophobicity of these binding sites is quantified by appropriate intensity factors calculated from the pK_m vs π plots. In summary, the "topography" of the hydrophobic regions can be schematically presented, as shown in Fig. 2.

Third, it should be mentioned that the binding effectiveness of peptide substrates seems to depend entirely on properties of amino acid sidechains as the introduction of indicator variables into the correlation equations revealed no effects connected with interaction of the peptide backbone with the enzyme. Thus it can be proposed that the loop of peptide interacts with its binding area according to the "plug–socket" model as illustrated in Fig. 2.

In summary, we expect that the results obtained can be used for further design of peptide substrates which possess high binding effectiveness and therefore are even more highly specific substrates for cAMP-dependent protein kinase.

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