Physico-chemical principles of cAMP-dependent protein phosphorylation

Catalysis of phosphoryl group transfer to nucleophilic agents

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The specificity of pig brain protein kinase towards high- and low-M_r 'analogs' of protein substrate was studied. Solvolysis of the phosphoenzyme intermediate by various nucleophilic agents is shown. The transition state structure of the phosphoryl transfer reaction is discussed.

Protein kinase *Phosphoryl transfer* *Brönsted equation* *Nucleophilic agent*

1. INTRODUCTION

cAMP-dependent protein kinase is one of the key enzymes of cellular metabolism regulation. There is a large body of literature on the physiological aspects of reversible phosphorylation of various proteins [1,2]. Some researchers have approached this as a physicochemical problem [3]. Pig brain protein kinase (cAMP-dependent, EC 2.7.1.37) has been studied by our group for a number of years. In [4–8] we showed that phosphotransferase and ATPase reactions catalysed by the enzyme proceed via phosphoenzyme intermediate formation (phosphohistidine form of the enzyme's catalytic subunit). A prominent feature of protein kinases is the polymeric character of the second substrate – the phosphoryl residue acceptor. In our opinion, elucidation of the enzyme's specificity towards protein acceptor is the most exciting problem in mechanistic studies of protein kinases.

We present here some details of the molecular mechanism of phosphoryl group acceptance by substrates of different nature.

2. MATERIALS AND METHODS

The catalytic subunit of pig brain protein kinase and histone H1 were isolated as in [9] and [10], respectively. Isolation of histone H1 fragment 1 (residues 33–121) was carried out as in [11] with modifications. A nuclear fraction was suspended in 10 mM Tris–HCl (pH 8.0) up to $A_{260} \sim 100$. This suspension was digested with trypsin (Calbiochem) at a ratio of 1:100 (w/w of DNA) for 1 h at 4°C. $HClO_4$ was added to 5% and the reaction mixture was incubated with stirring for 12 h at 4°C. After centrifugation the supernatant fraction was precipitated with trichloroacetic acid. The precipitate was collected, washed 3 times with acetone, dissolved in water and applied to a Sephadex G-100 column (1.5 × 100), equilibrated with 0.01 M HCl (flow rate 8 ml/h, 2-ml fractions being collected). The purity of the fragment was checked by SDS gel electrophoresis (20%). Fragment II of histone H1 (residues 1–72) was obtained after bromosuccinimide cleavage and Sephadex G-100 gel filtration as in [12].

Phosphotransferase and ATPase activities of the

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protein kinase were assayed as in [9] and [7], respectively. Measurement of kinetic parameters in the presence of nucleophilic agents was carried out at final concentrations of the latter of 50, 100, 200, 300 and 500 mM. Each kinetic experiment was repeated 3 times. Statistical error did not exceed 15%. Identification of solvolysis products was done by PEI–cellulose thin-layer chromatography (Schleicher & Schull plates) in 1.4 M LiCl. For autoradiography an RT-1 film and intensifying screen were used. ^{32}P-labeled 1,3-butanediol 1(3)-phosphate was synthesized using the carbodiimide method [13].

3. RESULTS AND DISCUSSION

Histone H1 is widely used as substrate in the protein kinase-catalysed phosphorylation reaction. This protein is not the best substrate for this enzyme, the rate of its phosphorylation being estimated as 8% from phosphorylation of the kinase phosphorylase β-subunit [14]. Nevertheless, due to the known primary [15] and secondary [16] structures, the simple method of isolation and well developed chemical modification techniques, it is a convenient model protein substrate. Our enzyme phosphorylates only one of its serine residues (Ser-37) in the sequence Lys-Arg-Lys-Ala-Ser-Gly-Pro-Pro, located at the boundary of the globular part and the 'tailed' N-terminal sequence.

To elucidate the influence of changes in substrate structures on the specificity and effectiveness of the phosphotransferase reaction, two polypeptide fragments of histone were obtained. These fragments, I (residues 33–121) and II (1–72), were shown to retain the structure of the globular part of the molecule [17]. Kinetic parameters of the phosphorylation reaction of these fragments are listed in table 1. As follows from table 1 both fragments are characterized by a decrease in the affinity for enzyme and the rate of catalysis. The amino acid sequences of these fragments overlapped only partially, however, the corresponding K_m values were similar. These results suggest the deterioration of the substrate parameters of acceptor protein upon changes in the spatial structure and insignificance of the terminal part of the histone molecule for recognition by the kinase.

The reaction of phosphoryl transfer from the enzyme's active site consists of at least two stages, namely, specific binding of the protein substrate followed by nucleophilic attack of the phosphorus atom by the serine group. The study of the second stage of this process required a suitable model for the 'nucleophilic' step using nonspecific analogues of the acceptor group. We previously demonstrated that increased concentrations of Tris caused inhibition of the ATPase activity of the enzyme [7]. One could propose alternate hydrolysis and solvolysis of the phosphoenzyme by Tris and other nucleophilic agents. This suggestion requires further experimental proof.

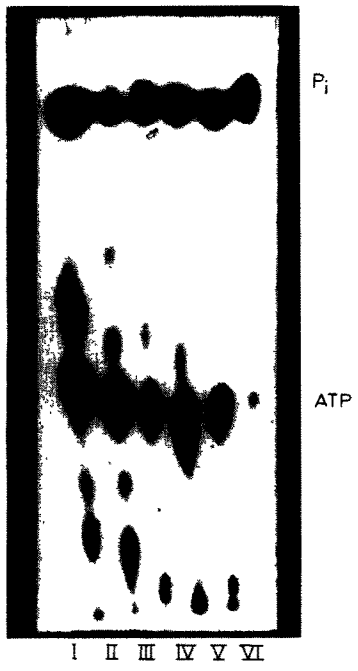
Incubation of the catalytic subunit of protein kinase with increasing concentrations of various nucleophiles (alcohols, phenols, amines, etc.) resulted in inhibition of ATPase activity. Fig.1 shows the autoradiography of a TLC plate of the ATPase reaction mixture in the presence of a number of added nucleophiles. Incubation of the

Table 1

Kinetic parameters of the protein kinase-catalysed phosphorylation of different substrates

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Histone H1	0.4 ± 0.08	10.4 ± 1.1	26 × 10^4
Fragment I (residues 33–121)	0.15 ± 0.03	5.2 ± 1.0	3.4 × 10^4
Fragment II (residues 1–72)	0.19 ± 0.04	2.5 ± 0.3	1.3 × 10^4
Nonspecific nucleophile			100[a]

[a] k_4



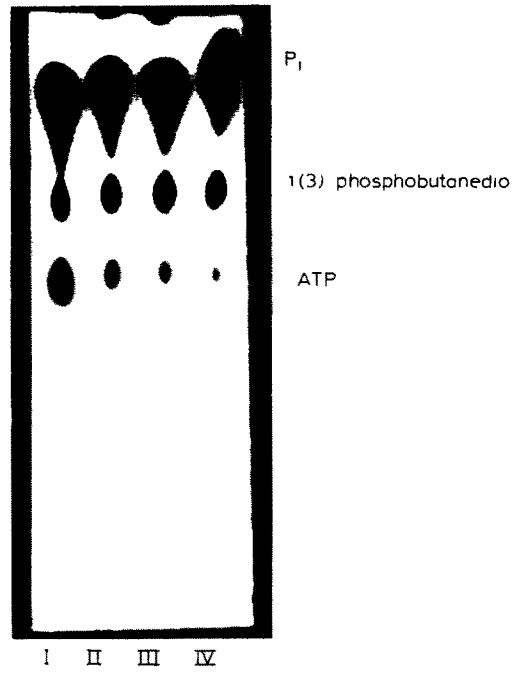


Fig.1. Autoradiograph (TLC) of the ATPase reaction in the presence of various nucleophiles. [E], 25 nM; [γ-^{32}P]ATP (0.1 Ci/nmol), 10^{-4} M; nucleophiles, 500 mM; I, methylamine; II, hydroxylamine; III, GlyGlyOEt; IV, imidazole; V, pyridine; VI, control (without nucleophile). Incubation time, 2 h; exposure time, 12 h.

catalytic subunit with MgATP and various nucleophiles resulted in the appearance of new spots. These were suggested to be the products of interaction between the phosphoenzyme and the added nucleophile. In the case of 1,3-butanediol the product was identified unambiguously. The chromatographic mobility of the product coincided with the mobility of chemically synthesized 1(3)-phosphobutanediol (fig.2a).

Quantitative estimation of reactant concentrations was carried out after elution of the corresponding spots and measurement of the radioactivity (fig.2b).

It was concluded that inhibition of the ATPase reaction was connected with the solvolysis reaction, alternating with hydrolysis of the substrate. These results lend support to our previous kinetic scheme [7] (eq.1):

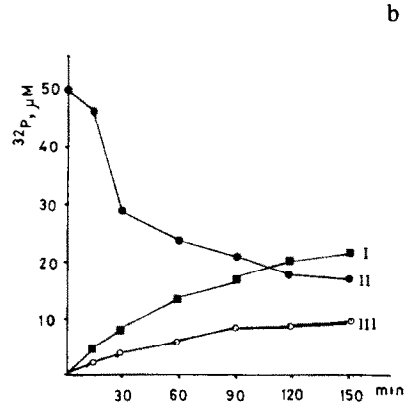


Fig.2. ATPase reaction in the presence of 200 mM 1,3-butanediol. [E], 25 nM. (a) Autoradiograph of the reaction mixture (12 h exposure). I, 15 min; II, 60 min; III, 120 min; IV, control (synthetic 1,3-butanediol 1(3)-phosphate, ATP, P_i, without enzyme). (b) Progress of the reaction. I, P_i; II, ATP; III, 1,3-butanediol 1(3)-phosphate.

$$E + MgATP \overset{K_S}{\rightleftharpoons} E\cdot MgATP \xrightarrow{k_2} E\sim P \begin{matrix} \xrightarrow{k_3} E + P_i \\ \xrightarrow[k_4]{[N]} E + P\text{-}N \end{matrix} \qquad (1)$$

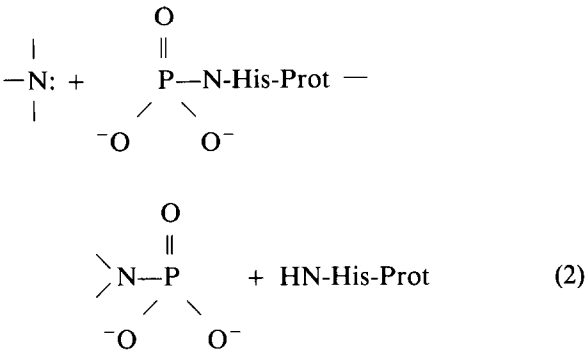
$$\downarrow$$

$$MgADP$$

where E·MgATP is the Michaelis complex of the reaction, E-P the phosphoenzyme intermediate and N the nucleophile.

Strict proof of the solvolysis reaction allowed sstudy of the reactivity of a wide number of nucleophiles towards the phosphoenzyme intermediate. This approach appeared to be fruitful in investigation of the transition states of non-enzymic [18] and enzymic [19] reactions.

Stage 4 of eq.1 can be represented by the following molecular scheme (eq.2) where N-His-Prot is a 'leaving group', i.e., the His residue in the active site:



(2)

To estimate some features of the transition state at this stage an empirical approach based on the principle of free energy linearity [18] was applied. The correlation equation of Brönsted (eq.3) relating the reaction rate and basicity of the attacking species is most widely used in such studies [18]:

$$\log k = A + \beta pK_\alpha \quad (3)$$

where k is the rate constant, β the Brönsted coefficient and A a constant.

Table 2 shows the series of nitrogen bases used as nucleophiles. The selection of such compounds was based on the ability to form a continuous range of pK values from 4.5 to 12.0 (table 2). In the case of the solvolysis reaction the best parameter reflecting the reactivity is k_4. The rate equation corresponding to scheme 1 can be written as:

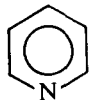
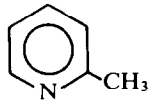
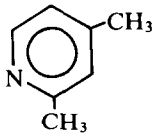
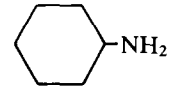
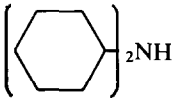
$$\frac{v_o}{v_N} - 1 = k_4[N] \cdot \frac{k_3 + [S]_o}{k_3K_S + (k_2 + k_3)[S]_o} \quad (4)$$

The values of the constants k_2, k_3 and K_S in the absence of nucleophile have been determined [7,8]. Thus the plotting of kinetic data in coordinates $[(v_o/v_N) - 1][k_3K_S + (k_2 + k_3)[S]_o]/(k_3 + [S]_o)[N]$ at constant ATP concentration allowed us to estimate k_4 values. The corresponding results on different nucleophiles are summarized in table 2. (The concentration of the non-ionized form at pH 8.0 was used to calculate the k_4 values.)

The plot of ln k_4 *vs* pK_α is linear and corresponds to a β value (the slope) equal to 0.23 (see fig.3). The increased reactivity of compounds 10–15 appears to be due to high hydrophobicity. Consequently, the solvolysis reaction has a similar transition state for all the nucleophiles tested. One of the widespread interpretations of β is the characterisation of 'a degree of bound formation' in the transition state [19]. In our case β is rather low. Thus the bond between the nucleophile and the attacked group (i.e., phosphoryl group) is loosened whereas that with the leaving group is nearly broken. Hence, the transition state is rare with electrons, and their pumping from the negatively charged oxygens of the phosphoryl group appears to be a driving force of the reaction. This type of transition state remotely resembles the metaphosphate monoanion [20]. It should be noted that metaphosphate was suggested to be an intermediate in the protein kinase reaction [21]. A similar situation also takes place in non-enzymatic nucleophilic catalysis of monosubstituted phosphate hydrolysis [20]. The above-mentioned viewpoints can be applied with some caution to the phosphotransferase reaction of enzyme. In [7,8] we have demonstrated that the ATPase reaction of enzyme appears to be a satisfactory model of the initial stages of the phosphotransferase reaction, i.e., ATP binding and phosphoenzyme formation. In the case of solvolysis, namely, in the presence of added nucleophile, phosphoryl transfer from the phosphoenzyme takes place. Thus this scheme can be considered as a model for a full phosphotransferase reaction and the corresponding nucleophile as some 'low molecular mass analogue' of substrate acceptor. In connection with this, one could suggest that transition state structures of phosphoryl transfer reactions for both types of analogues should bear some resemblance. This speculation raises the question as to the specificity and effectiveness of catalysis. In the case of H1 histone,

Table 2

Second-order rate constants (k_4) of the phosphoenzyme solvolysis by nucleophiles

Nucleophile	pK	k_4 ($M^{-1} \cdot s^{-1}$)
(1) CH_3ONH_2	4.5	32 ± 5
(2) 	5.2	61 ± 7
(3) 	5.2	85 ± 10
(4) 	5.3	77 ± 10
(5) NH_2OH	6.2	101 ± 20
(6) 	7.0	138 ± 30
(7) GlyOMe	8.0	180 ± 23
(8) GlyGlyOEt	8.5	450 ± 50
(9) NH_4OH	9.3	1362 ± 150
(10) CH_3NH_2	10.7	(50 ± 7) × 10^3
(11) $(C_2H_5)NH$	11.04	(93 ± 10) × 10^3
(12) $(C_2H_5)_3N$	11.09	(70 ± 9) × 10^3
(13) $CH_3CH_2CH(CH_3)\text{-}NH_2$	11.01	(84 ± 11) × 10^3
(14) 	11.09	(209 ± 18) × 10^3
(15) 	11.0	(100 ± 18) × 10^3
(16) Histone H1	–	4 × 10^5 (k_{cat}/K_m)

full specificity brought about both nucleophilic properties of serine to be phosphorylated and specific protein–protein, enzyme–substrate interaction. When nucleophile is used we observe only a pure nucleophilic contribution in this process, so the difference in reactivity of phosphoenzyme in both cases can be caused by this specific interaction. From the results presented one can attempt to estimate the difference in standard activation free energies of the reaction transition state in the case of specific and nonspecific phosphoryl acceptors:

$$\Delta\Delta G^{\neq} = RT\ln \frac{k_{II}}{k_N} \quad (5)$$

where k_{II} is the limiting second-order rate constant of protein substrate equal to k_{cat}/K_m and k_N the mean value of the rate constant of solvolysis k_4 (~100 $M^{-1} \cdot s^{-1}$). For histone H1 this difference is

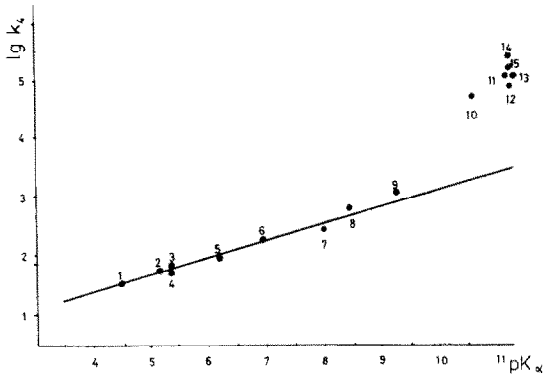


Fig.3. Brönsted dependence of the phosphoenzyme solvolysis by nucleophiles. Numbers correspond to those in table 2.

about 5.5 kcal/mol, for fragments I and II 3.46 and 2.89 kcal/mol, respectively. Thus, the specific protein–protein interaction even in the case of alteration of substrate structure leads to a considerable decrease in the activation energy of the phosphotransferase reaction.

We have shown (unpublished) that the enzyme under study effectively catalyses the phosphorylation of synthetic peptide analogues of histone. This result correlates with other data [3,14]. We hope that a detailed investigation of protein kinase specificity towards acceptors in the order: histone H1; its fragments; synthetic peptides; nonspecific nucleophiles; will help to elucidate some quantitative characteristics of catalysis by this enzyme.

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