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INFLUENCE OF ENZYME INACTIVATION ON THE KINETICS OF PEPTIDE PHOSPHORYLATION BY PROTEIN KINASE C

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Abstract. The influence of inactivation of protein kinase C on the kinetics of the catalytic phosphorylation of stereoisomeric peptides Lys-Arg-Pro-Ser-Glu-Arg-Ala Lys-Tyr, where the L-amino acids were successively replaced by the corresponding D-isomers, was analysed by means of an integrated rate equation. It was shown that the completeness of peptide phosphorylation in these reactions was governed by the ratio of the rates of substrate phosphorylation and enzyme inactivation. As the rate of the phosphorylation of the substrates depended remarkably on the position of the D-amino acid in the peptide structure, the yields of the phosphorylated products were rather different. Besides that the degree of substrate conversion was also dependent on the enzyme concentration in the reaction mixture, which also determined the rate of catalysis.

Key words: protein kinase C, peptide, phosphorylation, integrated rate equation, kinetics of enzyme inactivation.

INTRODUCTION

Protein kinase C catalyzes the transfer of γ-phosphate from ATP to phosphorylatable protein or peptide substrates [1]. This reaction needs the presence of enzyme-activating components, Ca^{2+}, phospholipids, and diacylglycerols [2]. The initial rates of the phosphorylation reaction follow well the Michaelis–Menten rate equation, at least at substrate concentrations comparable with the appropriate K_m values [3]. On the other hand, the enzyme activation obviously destabilizes the protein, as our attempts to use an integrated form of the Michaelis–Menten rate equation instead of the initial rate measurements revealed

a rather unusual kinetic behaviour of this process. The phenomena observed were explained by the influence of enzyme inactivation in the course of the catalytic process. Formally this influence can be described by the following kinetic scheme:

$$E + S \overset{K_S}{\rightleftharpoons} ES \overset{k_{cat}}{\longrightarrow} E + \text{products}, \tag{1}$$

$$E \overset{k_{in}}{\longrightarrow} E_{\text{inactivated.}} \tag{2}$$

The appropriate rate equations for these two processes can be presented as follows:

$$v = -\frac{d[S]}{dt} = \frac{k_{cat}[E][S]}{K_m + [S]} \tag{3}$$

and

$$[E] = [E]_0 \exp(-k_{in}t) . \tag{4}$$

Combination of these equations gives:

$$v = -\frac{d[S]}{dt} = \frac{k_{cat}[E]_0[S]\exp(-k_{in}t)}{K_m + [S]} . \tag{5}$$

Integration of this rate equation from $[S] = S_0$ to $[S] = [S]$, and $t = 0$ to $t = t$, and taking into consideration that $[P] = S_0 - [S]$, gives:

$$[P] = \left(\frac{k_{cat}[E]_0}{k_{in}}\right)(1 - \exp(-k_{in}t)) + K_m \ln\left(\frac{[S]_0 - [P]}{[S]_0}\right). \tag{6}$$

Under the conditions $[S] << K_m$, the integration of the rate equation for the same reaction scheme (1–2) yields a somewhat different expression:

$$\ln\left(\frac{[S]_0}{[S]_0 - [P]}\right) = \frac{V}{K_m k_{in}}(1 - \exp(-k_{in}t)), \tag{7}$$

where $V = k_{cat}[E]_0$.

In both integrated rate equations (6) and (7) the product formation depends on the time-course of the enzyme inactivation reaction (k_{in}). Secondly, it can be seen that the ratio of the rates of product formation (k_{cat}) and enzyme inactivation (k_{in})

determines the maximal level of product formation. Therefore, for the evaluation of the validity of the reaction mechanism (1, 2) it would be convenient to use congeneric substrates that react with the enzyme at quite different rates.

The kinetic experiments with protein kinase C were performed using peptides Lys-Arg-Pro-Ser-Glu-Arg-Ala Lys-Tyr, where each L-amino acid was successively replaced by the corresponding D-amino acid. Because of this these peptides were phosphorylated at different rates [3]. On the other hand, the similar chemical composition of these peptides should provide their equal sorption onto phosphocellulose paper, used for the quantification of the phosphorylated product, and thus exclude the possibility of pitfalls in the assay of phosphopeptides due to their unequal binding with the ion exchange paper [4].

MATERIALS AND METHODS

Chemicals. γ-[^{32}P] ATP was obtained from Amersham (UK). 1,2-Diolein and L-α-phosphatidyl-α-serine were products of Sigma (USA). Phosphocellulose paper P81 was from Whatman (UK). All the other chemicals used were of the highest commercially available grade. Buffers and washing solutions were made using Seralpur UP-50 deionized water.

Synthesis and purification of peptides. The peptides were prepared by the solid phase method of Merrifield [5] using Boc-amino acids. Experimental details and description of the compounds are given elsewhere [3].

Enzyme purification. Protein kinase C was prepared from pig spleen by the method of Parker et al. [6], except the final chromatography on Phenyl-Sepharose, which was excluded because of low recovery. The purified enzyme consisted mainly of the β-isozyme. The enzyme stock solution contained 2.6 mg protein per ml and had a specific activity of 80 U per mg protein, where 1 unit was defined as the amount of enzyme transferring 1 nmol of phosphate from ATP to histone H1 (1 mg/ml in incubation mixture) in 1 min.

Assay of peptide phosphorylation. Peptide phosphorylation by protein kinase C was carried out at 25 °C in a reaction mixture composed as follows: 115 μl of 15 μM substrate solution in 50 mM Tris-HCl buffer, pH 7.5; 30 μl 150 mM Tris-HCl buffer, pH 7.5, containing 5 mM calcium acetate; 30 μl 20 mM Tris-HCl buffer, pH 7.5, containing 450 μg ml^{-1} phosphatidylserine and 8 μg ml^{-1} diolein; 30 μl 0.75 mM γ[^{32}P]ATP in 37.5 mM $MgCl_2$, with a specific radioactivity of about 100 cpm/pmol and 20 μl of protein kinase C solution in 20 mM Tris-HCl buffer, pH 7.5, containing 2 mM EDTA and 0.5 mM dithiothreitol. This enzyme solution was made immediately before the experiments by a 20-fold dilution of protein kinase C stock solution.

The phosphorylation reaction was started by the addition of the enzyme into the reaction mixture and peptide phosphorylation was followed at 25 °C by taking 15–30 μl aliquots of this mixture onto pieces of phosphocellulose paper. Then the

reaction was immediately stopped by immersing the pieces of paper into ice-cold 75 mM H_3PO_4. The pieces of paper were washed four times with ice-cold 75 mM H_3PO_4 (10 min each time) and dried at 80 °C for 30 min. The radioactivity bound to the paper was measured as Cerenkov radiation using a Beckman LS 7500 scintillation counter. Proceeding from these data the product concentration vs. time plots were constructed (Fig. 1).

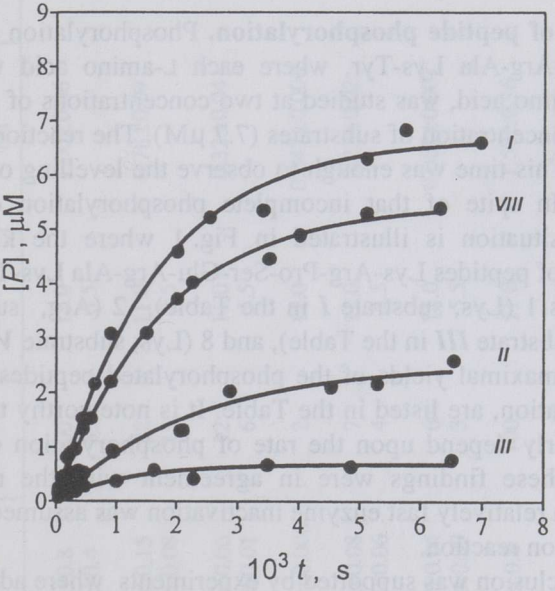

Fig. 1. Phosphorylation of peptides Lys-Arg-Pro-Ser-Glu-Arg-Ala Lys-Tyr with D-amino acids in positions 1 (peptide ***I***, contained D-Lys), 2 (peptide ***II***, contained D-Arg), 3 (peptide ***III***, contained D-Pro), and 8 (peptide ***VIII***, contained D-Lys) by protein kinase C. Substrate concentration 7.7 μM, enzyme concentration 11 μg/ml.

Spontaneous enzyme inactivation. The time course of spontaneous inactivation of protein kinase C was followed by measuring the enzyme activity during its incubation in the reaction buffer used for the assay of peptide phosphorylation, but where both peptide and ATP were omitted. In these experiments the enzyme stock solution was diluted in the reaction mixture and incubated for predetermined time intervals t to follow the time course of the decrease of enzyme activity. Thereafter both substrates were added into the reaction mixture and the initial velocity of peptide phosphorylation (v) was measured during 1–2 min as described above. The results were analysed in coordinates $\ln(v/v_0)$ vs. t, according to the reaction mechanism (2):

$$\ln(v/v_0) = -k_{in}t. \qquad (8)$$

Data processing. Data processing was performed using the StatMost version 2.50 software (Data Most Corporation, USA) and GraphPad Prism version 1.03 (GraphPad Software Inc., USA). The values reported are given with standard errors.

RESULTS

Time course of peptide phosphorylation. Phosphorylation of peptides Lys-Arg-Pro-Ser-Glu-Arg-Ala Lys-Tyr, where each L-amino acid was successively replaced by D-amino acid, was studied at two concentrations of protein kinase C and at a single concentration of substrates (7.7 μM). The reactions were followed during 1.5–2 h. This time was enough to observe the levelling off of the product vs. time plots. In spite of that incomplete phosphorylation of peptides was observed. This situation is illustrated in Fig. 1 where the kinetic curves of phosphorylation of peptides Lys-Arg-Pro-Ser-Glu-Arg-Ala Lys-Tyr with D-amino acids in positions 1 (Lys, substrate ***I*** in the Table), 2 (Arg, substrate ***II*** in the Table), 3 (Pro, substrate ***III*** in the Table), and 8 (Lys, substrate ***VIII*** in the Table) are shown. The maximal yields of the phosphorylated peptides, measured after 1.5–2 h of incubation, are listed in the Table. It is noteworthy that the yields of the reaction clearly depend upon the rate of phosphorylation of the particular substrates. All these findings were in agreement with the reaction scheme proposed where a relatively fast enzyme inactivation was assumed in parallel with the phosphorylation reaction.

The same conclusion was supported by experiments where additional amounts of enzyme and substrate were added into the assay medium after the phosphorylation of peptide KrPSQRAKY had levelled off. In the former experiment additional phosphorylation of the peptide was observed, while no increase in the product concentration took place in the latter case.

Kinetic analysis of peptide phosphorylation. Kinetic data were analysed by means of the rate equation (7), as the substrate concentration (7.7 μM) was below the K_m values for all the peptides studied (see the Table). These calculations yielded the rate constants k_{in} for enzyme inactivation and the values of the combined kinetic parameter V/K_m, related to the second-order rate constant k_{cat}/K_m of the phosphorylation reaction:

$$\frac{V}{K_m} = \frac{k_{cat}}{K_m}[E]_0 \,. \qquad (9)$$

The results of the processing of data are listed in the Table.

Kinetics of phosphorylation of diastereomeric peptides, related to Lys-Arg-Pro-Ser-Glu-Arg-Ala Lys-Tyr, taking into account spontaneous inactivation of protein kinase C. Kinetic parameters k_{in} and V/K_m were calculated from Eq. (7). Concentration of substrates 7.7 μM. D-Amino acids are denoted with lowercase letters

No.	Peptide	$10^4\ k_{in}$, s^{-1}	$10^4\ V/K_m$, s^{-1}	Yield, %	[E], μg ml^{-1}	$10^9\ V$, mole $s^{-1}\ mg^{-1}$ [3]	K_m, μM [3]	* $10^5\ k'_{II}$, 1 $s^{-1}\ mg^{-1}$
I	kRPSQRAKY	4.2±2.8 3.5±1.0	4.3±0.8 1.8±0.4	100 65	11.0 5.5	1.00±0.06	24±4	4.23
II	KrPSQRAKY	7.6±6.1 5.1±1.1	1.03±0.15 0.43±0.08	22 13	11.0 5.5	0.78±0.09	60±17	1.30
III	KRpSQRAKY	6.4±1.7 5.2±2.3	0.43±0.06 0.26±0.01	12 6	11.0 5.5	0.32±0.04	66±18	0.48
IV	KRPsQRAKY	14.0±7.1	0.0023±0.006	2	11.0	0.047±0.006	64±19	0.073
V	KRPSqRAKY	6.4±5.4 7.5±3.9	0.32±0.08 0.13±0.06	7 4	11.0 5.5	0.22±0.02	104±26	0.22
VI	KRPSQrAKY	7.1±2.4 6.4±5.5	0.23±0.04 0.15±0.09	6 3	11.0 5.5	0.068±0.007	85±28	0.080
VII	KRPSQRaKY	9.0±7.0	5.0±0.8	100	11.0	1.58±0.16	38±8	4.17
VIII	KRPSQRAkY	5.0±1.1 4.0±1.7	1.56±0.18 0.72±0.18	55 30	11.0 5.5	1.82±0.2	124±24	1.47

* Calculated from the V and K_m values listed in [3].

The peptide phosphorylation rates V/K_m depended on enzyme concentration as well as on substrate structure. This fact is illustrated in Fig. 2, where the kinetic constants, calculated by means of the integrated rate equation (7), were compared with the results of initial rate measurements [3]. It can be seen that a good linear relationship between these kinetic parameters was obtained, pointing to the fact that both sets of data are governed by the same structure–activity relationship.

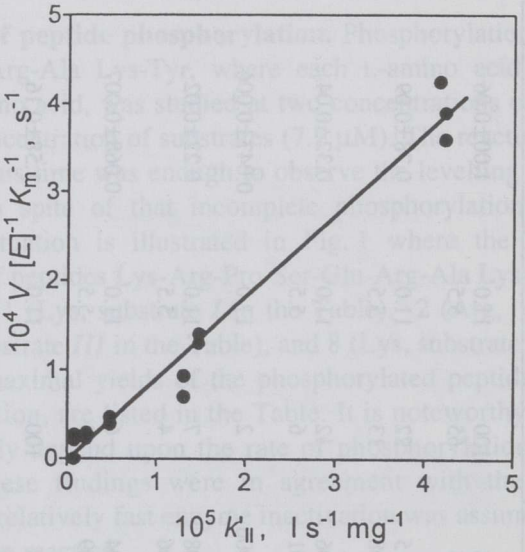

Fig. 2. Comparison of kinetic data, calculated by means of the integrated rate equation (7), and the second order rate constants k'_{II} obtained from the initial rate measurements in [3]. Data were taken from the Table.

The rate constants k_{in} did not depend on substrate structure and thus should characterize the spontaneous inactivation of the enzyme during the peptide phosphorylation reaction. The mean k_{in} value $(6.5\pm3.5)\ 10^{-4}\ s^{-1}$, calculated from the appropriate data in the Table, corresponded to a half-life of the inactivation process of approximately 18 min. This means that the inactivation of protein kinase C at 25 °C is a relatively fast process and should hamper the application of the integrated rate equation if the enzyme inactivation reaction is not taken into consideration. On the other hand, the inactivation process had negligible influence on the initial rate measurements made during 1–2 min.

Enzyme inactivation. The kinetics of the inactivation of protein kinase C was measured separately in the reaction medium resembling that used for substrate reaction but omitting both ATP and peptide (Fig. 3). The enzyme inactivation

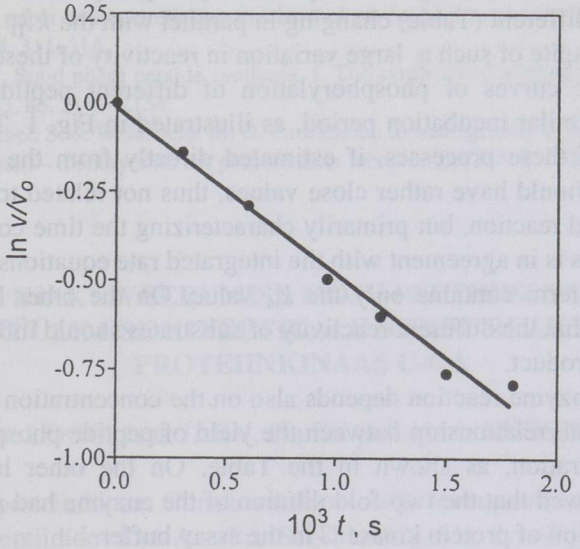

Fig. 3. Inactivation of protein kinase C at 25 °C (see Eq. (8) in text).

process followed the first order rate equation (8) for at least 0.5 h and allowed for the calculation of the rate constant $k_{in} = (4.7 \pm 0.3)10^{-4}$ s^{-1}. At longer incubation times scattering of the experimentally determined v values was observed. In spite of that this k_{in} value was close to the appropriate rate constant $6.5\ 10^{-4}$ s^{-1} calculated from the integrated rate equation (7).

DISCUSSION

The influence of enzyme inactivation on the time course of peptide phosphorylation was dependent on the ratio of the rates of the inactivation process and the enzyme-catalyzed reaction. When the rate of the former process was comparable with the rate of the catalytic reaction, the formation of the reaction product ended before all the substrate was consumed. Therefore the phenomenon of uncomplete peptide phosphorylation may rise not only from the shortcomings in the assay procedure, as was proposed in [4], but also from the kinetic behaviour of the enzymatic reaction itself.

The rate of enzyme reaction is determined by the structure of the particular substrate. The Table shows that the second-order rate constants of phosphorylation of the stereoisomeric peptides Lys-Arg-Pro-Ser-Glu-Arg-Ala Lys-Tyr varied more than 50 times, depending on the position of the D-amino acid in this sequence.

Therefore it was not surprising that the yields of phosphorylation of these peptides were also quite different (Table) changing in parallel with the k'_{II} values.

However, in spite of such a large variation in reactivity of these substrates, the observed kinetic curves of phosphorylation of different peptides levelled off during quite a similar incubation period, as illustrated in Fig. 1. This means that the half-lives of these processes, if estimated directly from the shape of these kinetic curves, should have rather close values, thus not related to the rate of the enzyme-catalyzed reaction, but primarily characterizing the time course of enzyme inactivation. This is in agreement with the integrated rate equations (6, 7) in which the exponential term contains only the k_{in} value. On the other hand, the same equations show that the different reactivity of substrates should influence the yield of the reaction product.

The rate of enzyme reaction depends also on the concentration of the enzyme, explaining the interrelationship between the yield of peptide phosphorylation and enzyme concentration, as shown in the Table. On the other hand, the same experiments showed that the two-fold dilution of the enzyme had no effect on the rate of inactivation of protein kinase C in the assay buffer.

It is important to emphasize that the enzyme inactivation rate constants calculated from different kinetic experiments with substrates of different structure and reactivity were in agreement with the k_{in} values determined directly from the time course of the enzyme inactivation. This also supports the validity of the kinetic analysis made. On the other hand, the same results show that saturation of protein kinase C with ATP, which was one of the substrates of the phosphorylation reaction, did not stabilize the enzyme, at least not in the presence of the relatively low peptide concentration.

In summary, the present data show that inactivation of enzyme during the substrate reaction might considerably influence the shape of the observed kinetic curves and alter the degree of substrate conversion into reaction product. Therefore the physical meaning of the calculated parameters could depend on the reaction scheme used for the derivation of the appropriate integrated rate equations. On the other hand, the knowledge of the true kinetic mechanism of enzyme reaction is also important for a meaningful interpretation of the results of initial rate measurements.

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ENSÜÜMI INAKTIVEERUMISE MÕJU PEPTIIDSUBSTRAATIDE FOSFORÜÜLIMISE KINEETIKALE NENDE REAKTSIOONIL PROTEIINKINAAS C-GA

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On uuritud proteiinkinaas C inaktivatsiooni mõju tema poolt katalüüsitud stereoisomeersete peptiidide Lys-Arg-Pro-Ser-Glu-Arg-Ala Lys-Tyr fosforüülimise kineetikale, kasutades integreeritud reaktsioonikiiruse võrrandit. Nimetatud peptiidides on L-aminohapped järjestikku asendatud vastavate D-aminohapetega. On leitud, et peptiidide fosforüülimisreaktsiooni sügavust (täielikkust) määrab selle reaktsiooni ja ensüümi inaktivatsioonireaktsiooni kiiruse suhe. Kuivõrd substraatide fosforüülimisreaktsiooni kiirus oleneb D-aminohappe asukohast peptiidahelas, olid fosforüülimisreaktsiooni produktide saagised seeria piires väga erinevad. Substraatide konversatsiooniaste sõltus oluliselt ka ensüümi kontsentratsioonist reaktsioonisegus, kusjuures kontsentratsioon omakorda mõjutas katalüüsi kiirust.