THE STRUCTURAL REQUIREMENTS OF SUBSTRATES OF CYCLIC AMP-DEPENDENT PROTEIN KINASE

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1. Introduction

The primary structure required by substrates of cyclic AMP-dependent protein kinase (EC 2.7.1.37) has been detailed by the aid of synthetic peptides, representing the phosphorylatable site of pyruvate kinase (EC 2.7.1.40) of rat and pig livers [1,2]. The minimum substrate emerging from these studies may be represented by the general sequence: Arg–Arg–X–Ser–X [3].

The substitution of either arginine residue with other amino acids considerably increases the app. K_m for cyclic AMP-dependent protein kinase [2]. The distance between the phosphorylatable serine residue and the block of 2 arginine residues is critical, as elucidated in [4] using synthetic dodecapeptides of the general sequence:
$(Gly)_x$–Arg–Arg–$(Gly)_y$–Ala–Ser–Leu–Gly
and the peptide:
$(Gly)_7$–Arg–Arg–Ser–Leu–Gly.
The optimal location of the block of 2 arginine residues was the same as that in the phosphorylatable site of liver pyruvate kinase (cf. [5,6]). The distance between the 2 arginine residues and the phosphorylatable serine residue being critical is also shown by our finding that the substitution of β-alanine for L-α-alanine in the peptide Arg–Arg–Ala–Ser–Val decreased the rate of phosphorylation by >10-fold at 100 μM substrate (unpublished).

The general structure Arg–Arg–X–Ser–X of the phosphorylatable site of liver pyruvate kinase is not met by all protein substrates of cyclic AMP-dependent protein kinase. For instance, in the β-subunit of rabbit skeletal muscle phosphorylase kinase (EC 2.7.1.38), the phosphorylated site contains the sequence: Ala–Arg–Thr–Lys–Arg–Ser–Gly–Ser(P)–Val [7]. It has been deduced from this and other examples that the required structure of substrates of cyclic AMP-dependent protein kinase is met also by the general structure Lys–Arg–X–X–Ser–X [3]. However, in experiments with synthetic peptides of the general structure $(Gly)_x$–Lys–Arg–$(Gly)_y$–Ala–Ser–Leu–Gly, the spacing of the Lys–Arg-block from the phosphorylatable serine residue by 2 neutral residues gave extremely high K_m-values [4]. Additional structural elements are apparently required to form the optimal environment of the phosphorylatable serine residue in the β-subunit of phosphorylase kinase.

The nature of these elements was suggested from experiments with cyclic AMP-dependent protein kinase of rat liver [8,9]. In these studies, Arg–Thr–Lys–Arg–Ser–Gly–Ser–Val was found to be the minimal peptide which gave a significant rate of phosphorylation. We now report data from experiments with the protein kinase of rabbit skeletal muscle, indicating the great importance of the arginine residue at position 6 on the N-terminal side of the phosphorylatable serine residue of the β-subunit of phosphorylase kinase. The lysine residue has less influence than the 2 arginine residues on the rate of phosphorylation. The implications of these findings are discussed.

2. Experimental

The catalytic subunit of cyclic AMP-dependent protein kinase (type I) from rabbit skeletal muscle was isolated according to method I in [10].

The peptides were synthesized by the solid phase method as in [11], using a Beckman model 990 peptide synthesizer, and purified as in [1]. The quality of the purified peptides was controlled by amino acid

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analysis. The amino acid composition deviated from the theoretical one by <2%.

The phosphorylation of synthetic peptides was performed in incubation mixtures (0.04 ml) composed essentially as in [2,12]. The mixtures contained 0.25 mM [γ-^{32}P]ATP, 62.5 mM 2-(*N*-morpholino)-ethanesulfonic acid (Mes) (pH 6.9), 12.5 mM magnesium acetate, 0.25 mM EGTA, bovine serum albumin (BSA) at 0.3 mg/ml, peptide substrate and protein kinase. [γ-^{32}P]ATP was purchased from New England Nuclear (Boston MA) and diluted with unlabelled ATP to give spec. radioact. 10–50 cpm/pmol. Each peptide was used over a 10-fold range of concentration around its K_m-value. The enzyme dilutions were made in 10 mM Mes (pH 6.9) and BSA, 0.5 mg/ml, and were chosen to give <10% phosphorylation. As the enzyme diluted 250–5000-fold from the stock solutions (1.4–1.9 mg/ml) was stable for only a short period, the diluted enzyme was used within 5 min. The reaction was started by the addition of 0.01 ml enzyme. Although linear with time for ≥5 min, the phosphorylation was carried out for 2 min, at 30°C and was terminated by 0.25 ml 35% acetic acid. The separation of the phosphorylated peptide from [γ-^{32}P]ATP was performed by applying 0.25 ml mixture to 1.4 ml anion exchange resin, AG1 X 8 (acetate form), packed in a Pasteur pipette. The [^{32}P]phosphopeptide was eluted directly into a scintillation vial using 30% acetic acid as in [12]. Radioactivity was measured as Cerenkov radiation.

For the determination of kinetic constants, phosphorylations as well as control experiments were performed in triplicate for each peptide concentration. Single net values of phosphorylation rate were fitted to the Lineweaver-Burk double reciprocal plot by the least squares method. The plots were linear for all peptide substrates. Kinetic constants in table 1 are the mean of 3 independent determinations, unless otherwise indicated. In each series of experiments performed in 1 day, a complete set of data for peptide M 147 was always collected to check for changes in the activity of the enzyme. The V_{max} thus obtained, was used as the norm for calculation of the relative V_{max} of all other peptides run on the same occasion. The absolute values (mean ± SD) of the app. V_{max} of peptide M 147 was 13.2 ± 3.9 μmol . min^{-1} . mg^{-1} for one preparation, and 7.8 ± 2.6 μmol . min^{-1} . mg^{-1} for the other enzyme preparation used.

3. Results and discussion

3.1. *Minimal peptide required for a significant rate of phosphorylation*

The results of the investigation of peptides representing the phosphorylatable site of the β-subunit of phosphorylase kinase, the peptides M 136 and M 147–149 are shown in table 1. It is confirmed that Arg–Thr–Lys–Arg–Ser–Gly–Ser–Val is the minimal peptide containing the information necessary for

Table 1

Kinetic constants of synthetic peptides representing the phosphorylatable site of the β-subunit of phosphorylase kinase and effects of substitutions, at the phosphorylation with 0.25 mM [γ-^{32}P]ATP and the catalytic subunit of cyclic AMP-dependent protein kinase

Peptide no.	Amino acid sequence	K_m (mM) (mean ± SD)	Relative V_{max}[a] (mean ± SD)
M 136	Ala–Arg–Thr–Lys–Arg–Ser–Gly–Ser–Val	0.010 ± 0.001	1.01 ± 0.09
M 147	Arg–Thr–Lys–Arg–Ser–Gly–Ser–Val	0.021 ± 0.003	1.00[a]
M148	Arg–Thr–Lys–Arg–Ser–Gly–Ser	1.2 ± 0.5	0.03 ± 0.01
M149	Thr–Lys–Arg–Ser–Gly–Ser–Val	2.1 ± 0.6	0.48 ± 0.17
M 179	Arg–Thr–Lys–Arg–Ser–Gly–*Thr*–Val	0.65 ± 0.19	0.05 ± 0.01
M 271	*Gly*–Thr–Lys–Arg–Ser–Gly–Ser–Val	1.3 ± 0.0	0.57 ± 0.08
M 212	Arg–Thr–*Gly*–Arg–Ser–Gly–Ser–Val	0.14 ± 0.02	0.74 ± 0.08
M 213	Arg–Thr–Lys–*Gly*–Ser–Gly–Ser–Val	2.7 ± 0.6[b]	0.09 ± 0.02[b]
M 180	Arg–*Ser*–Lys–Arg–Ser–Gly–Ser–Val	0.026 ± 0.005	0.92 ± 0.12
M 181	Arg–*Ala*–Lys–Arg–Ser–Gly–Ser–Val	0.033 ± 0.015	1.03 ± 0.22

[a] V_{max}-values are normalized to the V_{max} of peptide M 147 as in section 2
[b] Mean of 2 values ± half range

a significant rate of phosphorylation with the catalytic subunit of cyclic AMP-dependent protein kinase (cf. [8,9]). The apparent K_m-value of the minimum peptide approaches those of the protein substrates of the protein kinase [13] but is ~5-times higher than the calculated in vivo concentration of phosphorylase kinase [14]. However, in experiments with the catalytic subunit of cyclic AMP-dependent protein kinase of rat liver, the K_m was found to be considerably decreased if the peptide was elongated in the C-terminal direction [9]. This is also the case with peptides representing the phosphorylated site of liver pyruvate kinase [1,2].

3.2. *Site of phosphorylation*

The second serine residue of the peptides M 136 and M 147–149 corresponds to the residue phosphorylated in the intact β-subunit of phosphorylase kinase [7,15]. That this serine residue is also the one phosphorylated in the synthetic peptides used in this study is supported by the localization of most of the [^{32}P]-phosphate in the fragment Ser[^{32}P]–Val–Tyr, obtained by tryptic digestion and Edman degradation after phosphorylation of the peptide Ala–Arg–Thr–Lys–Arg–Ser–Gly–Ser–Val–Tyr by cyclic AMP-dependent protein kinase [16]. This conclusion is also consistent with the effect upon kinetic parameters of the exchange of the second serine residue with threonine, as demonstrated with peptide M 179 (table 1).

3.3. *Importance of each of the 3 basic residues*

The data on the peptides M 271, M 212 and M 213 (table 1), demonstrate that all 3 basic residues of the minimal peptide influence the properties of the substrate. This is shown by the substitution of glycine for either basic residue. Of special interest is the demonstration of the great importance of the arginine residue at position 6 on the N-terminal side of the phosphorylatable serine residue. Each of the arginine residues has a greater influence than the lysine residue on the kinetic parameters. These results appear to explain why the myosin light chain of gizzard smooth muscle is phosphorylated in vitro by cyclic AMP-dependent protein kinase [17]. They also explain why a peptide of the structure $(Gly)_5$–Lys–Arg–Gly–Ala–Ser–Leu–Gly is a poor substrate of the protein kinase [4].

3.4. *Importance of the threonine residue*

The insertion of serine or alanine at the position of the threonine residue did not make any significant changes to the kinetic parameters, as seen from the comparison of the data of the peptides M 180 and M 181 with those of peptide M 147 (table 1). Apparently, the threonine function is of little importance for the rate of phosphorylation.

4. Conclusions

This work gives new insight into the minimal structural requirements that must be fulfilled to make a protein a substrate of cyclic AMP-dependent protein kinase. We conclude that 2 arginine residues are essential and can be arranged in at least 2 ways in the phosphorylatable site. One way is represented by the general amino acid sequence Arg–Arg–X–Ser–X, found, e.g., in liver pyruvate kinase [5,6]. This could be the most compact arrangement possible of the arginine and serine residues. The other way is represented by the general sequence Arg–X–X–Arg–X–X–Ser–X, found, e.g., in the β-subunit of rabbit skeletal muscle phosphorylase kinase [7]. These data further support the view that the common denominator of different substrates of cyclic AMP-dependent protein kinase is mainly a property of the primary structure of the phosphorylated site. This view originated from [18–20] where phosphorylation of small peptides and denatured proteins was demonstrated.

Apparently, this view contradicts the view [15,21] that the common denominator is some property of the three-dimensional structure of the substrates of cyclic AMP-dependent protein kinase. However, the fact that the 2 essential arginine residues can be arranged in at least 2 ways in the primary structure of the phosphorylatable site suggests to us that, in some substrates, the arginine residues may be spaced even further from each other. The proper position of the 2 arginine residues would then depend on the three-dimensional structure of the protein. Therefore, the original hypothesis by Langan [21] may deserve further consideration, and the 2 apparently contradictory views may be joined into one, that considers alternative ways of obtaining the proper proximity of 2 arginine residues and the phosphorylatable serine residue.

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