THE SUBSTRATE SPECIFICITY OF CYCLIC AMP-DEPENDENT PROTEIN KINASE:

Amino acid sequences at the phosphorylation sites of herring protamine (clupeine)

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1. Introduction

Cyclic AMP-dependent protein kinase probably mediates most, if not all, of the intracellular actions of hormones that work through cyclic AMP, according to the scheme:

hormone ⟶ increased cyclic AMP ⟶ activated protein kinase ⟶ phosphorylated protein ⟶ physiological event [1].

In support of this hypothesis, a number of proteins that are likely to be physiological substrates for the enzyme have been identified (reviewed [2]).

This concept has raised the question of why cyclic AMP-dependent protein kinase is able to phosphorylate just one or two residues in relatively few proteins. Recently, the amino acid sequences at the phosphorylation sites of several of the physiological substrates of the enzyme have been determined [3], and in conjunction with studies using synthetic peptides that are almost as good substrates as the native proteins [4,5] it has become apparent that the presence of two adjacent basic amino acids, at least one of which is arginine, just N-terminal to the phosphorylation sites, may be critical for specific substrate recognition in vivo [2].

However the molecular specificity of the enzyme is likely to be more complex. Firstly the distance of the phosphorylatable residue from the pair of adjacent basic amino acids has been found to be variable [2,3]. Secondly, the synthetic peptide studies have shown that the nature of the amino acids C-terminal to the phosphorylatable residue can profoundly affect the rate of phosphorylation of a peptide [4,5]. Thirdly, the substitution of a serine by a threonine residue in synthetic peptides was found to drastically reduce the rate of phosphorylation [4,5] and yet the protein, termed inhibitor-1, which is a powerful inhibitor of phosphorylase phosphatase only after it has been phosphorylated by cyclic AMP-dependent protein kinase, has been found to be phosphorylated on a unique threonine residue at rates comparable to other physiological substrates of the enzyme [6].

To obtain further information about the specificity of cyclic AMP-dependent protein kinase, we have investigated the sites of phosphorylation in herring protamines. These proteins, which are phosphorylated in fish testes in vivo [7] and which were one of the first proteins to be recognised as substrates for cyclic AMP-dependent protein kinase in vitro [8], contain both serine and threonine residues in close proximity to sets of arginine residues, while the distances of the serine residues from the arginine residues are variable (fig.1).

2. Methods

Clupeine sulphate (Sigma, type III) was dissolved in water and its concentration was measured by amino acid analysis, using the known amino acid sequences of these proteins (fig.1) and norleucine as an internal standard. The valine/isoleucine ratio was 5.1, indicating that clupeine Y1 comprised 28% of the material while the threonine/isoleucine ratio indicated that clupeine Y2 and Z were present in almost equal amounts (Y2 35%, Z 37%).

Protamine	Sequence
	1 5 10 15 20 25 30 31
Clupeine Y1	A R R R R S S S R P I R R R R P R R R T T R R R R A G R R R R
Clupeine Y2	P R R R T R R A S R P V R R R R P R R V S R R R R A R R R R
Clupeine Z	A R R R R S R R A S R P V R R R R P R R V S R R R R A R R R R

Fig.1. Amino acid sequences. Sites of herring protamines [9]. Serine (S) and threonine (T) residues are underlined.

Protamine was phosphorylated at pH 7.0 and 30°C using the partially-purified peak-1 isoenzyme of cyclic AMP-dependent protein kinase [10], in the following incubation: clupeine sulphate (0.03 mg/ml or 6.7 μM), sodium glycerophosphate 10 mM, EDTA 0.4 mM, cyclic AMP 0.01 mM, cyclic AMP-dependent protein kinase, $MgCl_2$, 2.0 mM and [γ-^{32}P]ATP 0.2 mM. The reaction was initiated by the addition of ATP–Mg. After approx. 1 molecule phosphate had been incorporated/molecule protamine (ca. 30 min), the reactions were terminated by the addition of NaF and EDTA to final concentrations of 50 mM and 10 mM, respectively. The excess [γ-^{32}P]ATP was then removed by absorbing the protamine to CM-cellulose (Whatman CM-52) equilibrated in water. The column was washed with water until ^{32}P-radioactivity could no longer be detected in the eluate, and the phosphorylated protamine was eluted with 1.0 M HCl and lyophilised [11].

Electrophoresis at pH 1.9, to test for the presence of phosphoserine and phosphothreonine [6], was on Whatman 3 MM chromatography paper in acetic acid/formic acid/water, (2:1:25), at 50 V/cm for 60 min.

Two dimensional peptide mapping was carried out on thin-layer cellulose sheets (Whatman 13255) using electrophoresis at pH 3.6 (pyridine/acetic acid/water; 1:10:190) or pH 6.5 (pyridine/acetic acid/water; 50:5:450) for 90 min at 15 V/cm, and ascending chromatography (butanol/pyridine/acetic acid/water; 90:60:18:72) for 120–240 min. Radioactive peptides were detected by autoradiography, sometimes also by fluorescamine staining, and were eluted with 10% acetic acid. The amino acid sequences were determined by the micro-dansyl Edman procedure [12].

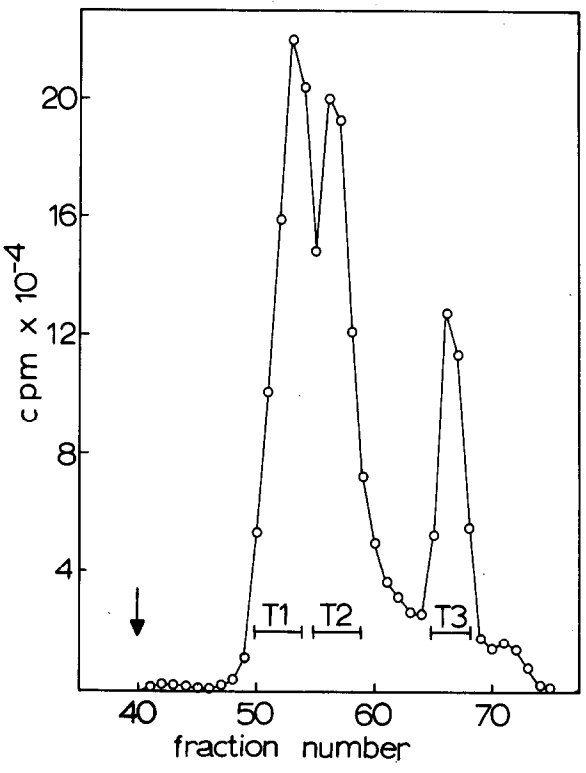

Fig.2. Gel filtration of tryptic phosphopeptides from herring protamine on Sephadex G25 superfine (145 × 1.5 cm). The flow rate was 15 ml/h and the fractions (3 ml) were analysed by Cerenkov counting. The horizontal bars indicate the fractions pooled after elution. The void volume V_o is denoted by an arrow. Further details are given under section 3.

The position of phosphorylated residues was determined from peptide net charge at pH 6.5 after each cycle of Edman degradation [13,14] and by the appearance of inorganic phosphate, as described under section 3. Electrophoretic mobilities (e) were expressed relative to aspartic acid at pH 6.5, and chromatographic mobilities (c) were expressed relative to the solvent front.

3. Results

3.1. *The phosphorylation sites of herring protamines*

An aliquot of the phosphorylated protamine was hydrolysed for 2 h at 110°C in 6 N HCl and subjected to electrophoresis at pH 1.9 to test for the presence of phosphoserine and phosphothreonine. Only phosphoserine was detected (not illustrated), showing that none of the threonine residues in herring protamine (fig.1) had been phosphorylated.

The remainder of the phosphorylated protamine (0.5 mg/ml) containing 1.1 molecules phosphate/molecule mixed protamine was then digested with trypsin (0.005 mg/ml) in 4.0 ml 0.1 M ammonium bicarbonate at 37°C for 12 h, and lyophilised. The material was taken up in 1.0 ml of 1.0 M acetic acid and subjected to gel filtration on Sephadex G-100 superfine equilibrated in 1.0 M acetic acid, and the elution profile is shown in fig.2. Three peaks of radioactivity, termed T1, T2 and T3, were resolved, and these fractions were pooled, lyophilised and analysed as described below.

Fraction T1 was further purified by peptide mapping using electrophoresis at pH 6.5 followed by chromatography. A single major radioactive spot was detected by autoradiography ($e = -0.35, c = 0.08$), which coincided with a fluorescamine positive spot. This component termed site-1 was eluted, and its composition (table 1) suggested that it was approx. 70–80% pure. Its sequence was established unambiguously by the dansyl-Edman procedure (table 2) which showed that it corresponded to residues 5–12 of clupeine Y1 (fig.1).

The amino acid composition and net charge of

Table 1
Amino acid compositions of the tryptic phosphopeptides isolated from herring protamines

Amino acid	Peptide		
	T1	T2	T3
Aspartic acid	0.26		
Threonine	0.19		
Serine	2.50 (3)	1.18 (1)	0.87 (1)
Glutamic acid	0.30		
Proline	0.97 (1)	0.96 (1)	
Glycine	0.40		0.19
Alanine	0.32	1.00 (1)	
Valine	0.28	0.85 (1)	
Isoleucine	1.00 (1)		
Leucine	0.31		
Arginine	2.75 (3)	3.3 (3)	1.05 (1)
^{32}P-radioactivity	0.91 (1)	0.83 (1)	1.0 (1)
Total	8	7	2
Net charge at pH 6.5 [13]	+2	+2	0

Hydrolyses were carried out in 6 N HCl + 0.01 M phenol for 20 h at 110°C. Compositions were determined on a Beckman Multichrom Analyser using a single column separation system. Serine and threonine were corrected for 10% and 5% destruction, respectively. Impurities below 0.1 molecules are omitted

Cycle number	0	1	2	3	4	5	6	7	8
^{32}P-inorganic phosphate (%)	0	1.3	3.3	12	67	68	74		
Net charge on peptide	+2	+1	+1	+1	+2*	+1	+1		
Sequence of peptide		Arg	Ser	Ser	Ser(P)	Arg	Pro	Ile	Arg

Fig.3. Amino acid sequence of phosphopeptide T1. The amino acid sequence of the peptide was established by the micro-dansyl Edman procedure [12]. Aliquots of the material were subjected to electrophoresis at pH 6.5 before the first cycle and after each of the subsequent six cycles, and examined by autoradiography and after staining with fluorescamine. The net charge was calculated by standard procedure [13,14] and the proportion of ^{32}P-radioactivity migrating in a position corresponding to inorganic phosphate was calculated by elution of the radioactive components and Cerenkov counting. (*) After this cycle, the major fluorescamine positive spot was no longer radioactive.

peptide T1 (table 1) indicated that not more than one of the three serine residues was phosphorylated. The phenylthiohydantoin derivative of phosphoserine is unstable and rapidly undergoes hydrolysis to inorganic phosphate [3]. To determine which serine was phosphorylated, aliquots of the material obtained after each cycle of Edman degradation were subjected to electrophoresis at pH 6.5. The results (fig.3) showed that little radioactivity migrated with inorganic phosphate after the first three cycles and that the major fluorescamine-positive spot was still radioactive. However, after the fourth cycle, the majority of the radioactivity migrated with inorganic phosphate (e = 1.0) and the major fluorescamine positive spot was no longer radioactive (fig.3). This establishes the site of phosphorylation in peptide T1 as the serine at residue 8 of clupeine Y1 (fig.1).

Since inorganic phosphate, unlike the phenylthiohydantoin amino acids, is not removed from the peptide by extraction with butyl acetate, the proportion of radioactivity migrating as phosphate after each cycle is cumulative. The appearance of small amounts of inorganic phosphate after the first and second cycles (fig.3) is probably caused by trace hydrolysis of phosphoserine during each cycle of the Edman degradation. The slightly longer rise after the third cycle is explained partly by the accumulation of inorganic phosphate, and probably also by trace contamination with a peptide missing the N-terminal arginine residue of tryptic peptide T1.

Fraction T2 was further purified by peptide mapping using electrophoresis at pH 3.6 followed by chromatography. A single major radioactive spot was again detected by autoradiography (e = –0.43 (pH 6.5), c = 0.11) which coincided with a fluorescamine positive spot. The composition of this peptide suggested that it had a purity of at least 90% (table 1), and its amino acid sequence was established as:

arg–ala–ser(P)–arg–pro–ile–arg

The location of the phosphate group was established by the method above for peptide T1. This sequence (termed site-2) is found in both clupeine Y2 and Z (fig.1).

Fraction T3 was purified by ascending chromatography, followed by electrophoresis at pH 3.6. A single radioactive component was again observed (e = 0 (pH 6.5), c = 0.31) which coincided with a fluorescamine-positive spot. The composition showed that this dipeptide was essentially pure (table 1) and its sequence was found to be:

ser(P)–arg

Based on the specificity of trypsin, this peptide, termed site-3, can only be derived from the serine at residue 6 of clupeine Z. This was confirmed in another tryptic digest in which the tripeptide arg–ser(P)–arg rather than the dipeptide ser(P)–arg was obtained (not illustrated).

3.2. *Relative rates of phosphorylation of sites-1, -2 and -3*

^{32}P-Labelled protamines were prepared which were phosphorylated to 0.13, 0.15, 0.40 and 0.43 molecules phosphate/molecule mixed protamine. When these preparations were digested with trypsin and subjected to gel filtration on Sephadex G-25, the proportion of site-1 was found to be much higher than in more highly phosphorylated preparations containing about 1.0 molecules phosphate/molecule protamine (not illustrated). To estimate the relative rates of phosphorylation of the three sites, the tryptic digests of these preparations were subjected to electrophoresis at pH 3.6. The relative mobilities of the peptides T1, T2 and T3 under these conditions were 1.0 : 0.87 : 0.67. Assuming that clupeines Y1, Y2 and Z represent 28%, 35% and 37% of the total protamine, respectively (see section 2) and allowing for the fact that site 2 is present in both clupeines Y2 and Z, the relative rates of phosphorylation site-1 : site-2 : site-3 were estimated to be 1.0 : 0.17 : 0.1.

The initial rate of phosphorylation of herring protamines relative to two other substrates of cyclic AMP-dependent protein kinase was also investigated at a constant substrate concentration of 6 μM. It was found that site-1 in herring protamine was phosphorylated at 8% of the rate at which the β-subunit of phosphorylase kinase was phosphorylated, and it was phosphorylated 5–6-fold more slowly than histone H2B (table 2).

Table 2
Amino acid sequences at the phosphorylation sites of substrates for cyclic AMP-dependent protein kinase

Substrate	Sequence	Ref.
Phosphorylase kinase (β-subunit)	Ala Arg Thr Lys — Arg Ser Gly $\overset{100}{\text{Ser(P)}}$ $\overset{\text{Val}}{\underset{\text{Ile}}{}}$ Tyr Glu Pro Leu Lys	[15]
Glycogen synthetase (site-2)	$\overset{\text{Lys}}{\underset{\text{Arg}}{}}$ Arg Ala $\overset{80}{\text{Ser(P)}}$	[3]
Histone H2B (calf thymus)	Lys Lys Arg Lys Arg $\overset{8}{\text{Ser(P)}}$ — Arg Lys Glu $\overset{50}{\text{Ser(P)}}$ Tyr Ser Val Tyr Val Tyr Lys	[15]
Pyruvate kinase (rat liver)	Gly Val Leu — Arg Arg Ala $\overset{35}{\text{Ser(P)}}$ Val Ala Glx Leu	[15,16]
(pig liver)	Leu — Arg Arg Ala $\overset{?}{\text{Ser(P)}}$ Leu Gly	
Inhibitor-1	Ile Arg Arg — Arg Arg Pro $\overset{30}{\text{Thr(P)}}$ Pro Ala Thr	[6]
Phosphorylase kinase (α-subunit)	Phe — Arg Arg Leu $\overset{20}{\text{Ser(P)}}$ Ile Ser Thr Glu Ser Glx Pro	[15]
Glycogen synthetase (site-1)[a]	$\overset{\text{Lys}}{\underset{\text{Arg}}{}}$ $\overset{10}{\text{Ser}}$ Asn Ser(P) Val Asp Thr Ser Ser Leu Ser	[3]
Histone H1 (calf thymus)	Ala Lys — Arg Lys Ala $\overset{8}{\text{Ser(P)}}$ Gly Pro Pro Val Ser	[17]
Troponin I (rabbit heart)	— Val Arg Arg $\overset{?}{\text{Ser(P)}}$ Asp Arg Ala Tyr Ala	[18]
Protamine (herring) site-1 (peptide T1)	Ala Arg Arg Arg — Arg Ser Ser $\overset{8}{\text{Ser(P)}}$ Arg Pro Ile Arg	this work
Protamine (herring) site-2 (peptide T2)	— Arg Arg Ala $\overset{1.5}{\text{Ser(P)}}$ Arg Pro Val Arg	this work
Protamine (herring) site-3 (peptide T3)	Arg — Arg Arg Arg $\overset{0.8}{\text{Ser(P)}}$ Arg Arg Ala	this work

[a] This sequence also contains two adjacent basic amino acids N-terminal to the phosphoserine (Krebs, E. G., personal communication)

The numbers above the phosphorylated residues refer to the rates at which the sites are phosphorylated relative to the β-subunit of phosphorylase kinase (100%) at 6 μM substrate concentration under a defined set of assay conditions [6]. Each protein was from rabbit skeletal muscle unless otherwise stated

4. Discussion

Inspection of the amino acid sequences in table 2 shows that the residue phosphorylated by cyclic AMP-dependent protein kinase can be either one residue (position A), two residues (position B) or three residues (position C) C-terminal to the set of adjacent basic amino acids. However, only in relatively few of the sequences was a choice of serine residues presented to the enzyme. With the β-subunit of phosphorylase kinase and site-1 of glycogen synthetase, the serine at position C was phosphorylated in preference to the serine at position A, and no significant phosphorylation at position A was detected [3,15]. Site-1 of herring protamine was therefore of particular interest, since cyclic AMP-dependent protein kinase was presented with the choice of phosphorylating either positions A, B or C (table 2). Only position C was found to be phosphorylated at a significant rate (fig.3, table 2), and the preference for position C over position B was also supported by the finding that site-1 of herring protamine was phosphorylated more rapidly than site-2, although the primary structures at these two phosphorylation sites are otherwise very similar (table 2). On the other hand, position B appears to be phosphorylated in preference to a serine located four residues C-terminal to the set of adjacent basic amino acids (position D) as shown by the sequence of the α-subunit of phosphorylase kinase and histone H2B (table 2) and phosphorylation at position D has yet to be observed [2]. The preferred position for phosphorylation therefore appears to follow the order position C > position B > position A > position D; and in addition, phosphorylation at position C strongly inhibits further phosphorylation at any other position. In a synthetic peptide corresponding to the phosphorylation site of pig liver pyruvate kinase [5], two adjacent arginine residues were essential for the peptide to be phosphorylated with a K_m in the μM range. If either arginine was substituted by alanine, the K_m for the peptide was increased over 100-fold. Whether the preference for position C over position B and A is related to binding or catalysis is not yet known. It should, however, be possible to answer this question by the use of appropriate synthetic peptides.

Sites-1, -2 and -3 of herring protamine and histone H1 are the poorest substrates for cyclic AMP-dependent protein kinase of the proteins shown in table 2. The relatively slow phosphorylation of these sites is likely to be related to the nature of the amino acids C-terminal to the phosphoserine residues. Using synthetic peptides corresponding to the phosphorylation site of rat liver pyruvate kinase [4], it was shown that substitution of the valine residue by either a basic amino acid or glycine reduced the rate of phosphorylation of the peptide by 90–95%, while it was shown [5] that substitution of an arginine residue two amino acids C-terminal to a phosphoserine, by alanine, increased the rate of phosphorylation of a synthetic peptide more than 100-fold. The very poor rate of phosphorylation of site-3 in herring protamine (table 2) may either be related to the fact that the phosphoserine is sited at position A or to the presence of two adjacent arginine residues C-terminal to the phosphoserine. Similarly, the failure to phosphorylate the serine at residue 23 of clupeine Y2, or serine 24 of clupeine Z, may be related to the presence of four consecutive arginine residues C-terminal to these serines.

Although site-3 of herring protamine was phosphorylated very slowly, it is of interest that the corresponding threonine in clupeine Y2 was not, although the amino acid sequences surrounding these residues are otherwise identical (fig.1). This supports the work with synthetic peptides corresponding to pyruvate kinase, in which the substitution of the serine by a threonine residue drastically reduced the rate of phosphorylation [4]. Since the three threonine residues in clupeine Y1 were also not phosphorylated, the present work has not helped to explain why inhibitor-1 is an effective substrate for cyclic AMP-dependent protein kinase (table 2, [6]).

When rainbow trout testis cell suspensions undergoing spermatogenesis were incubated with ^{32}P-inorganic phosphate, the protamines became phosphorylated in vivo [19]. Two phosphopeptides were isolated, in a region corresponding to site-1 of herring protamine, which had the sequences:

ser(P)–ser(P)–ser(P)–arg–pro–$\begin{matrix}\text{val}\\\text{ile}\end{matrix}$–arg

ser(P)–ser–ser –arg–pro–$\begin{matrix}\text{val}\\\text{ile}\end{matrix}$–arg

The serine residues phosphorylated in this region of protamines in vivo therefore differ from that phos-

phorylated by cyclic AMP-dependent protein kinase in vitro (table 2). Since cyclic AMP-dependent protein kinases from such diverse species as the silkworm and the rabbit appear to have identical specificities [2,20], the results suggest that the phosphorylation of protamines in vivo may be catalysed by protein kinases other than cyclic AMP-dependent protein kinase. This idea is also supported by the additional finding that the sequence arg–arg–val–ser–$(arg)_5$ was phosphorylated in trout testis in vivo [19], whereas no phosphorylation of the corresponding residues in herring protamine (residues 23 and 24 of clupeines Y2 and Z, respectively) by cyclic AMP-dependent protein kinase in vitro could be detected.

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