The Substrate Specificity of Adenosine 3':5'-Cyclic Monophosphate-Dependent Protein Kinase of Rabbit Skeletal Muscle

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The known amino acid sequences at the two sites on phosphorylase kinase that are phosphorylated by cyclic AMP-dependent protein kinase were extended. The sequences of 42 amino acids around the phosphorylation site on the α -subunit and of 14 amino acids around the phosphorylation site on the β -subunit were shown to be: α -subunit Phe-Arg-Arg-Leu-Ser(P)-Ile-Ser-Thr-Glu-Ser-Glx-Pro-Asx-Gly-His-Ser-Leu-Gly-Ala-Asp-Leu-Met-Ser-Pro-Ser-Phe-Leu-Ser-Pro-Gly-Thr-Ser-Val-Phe(Ser,Pro,Gly)His-Thr-Ser-Lys; β -subunit, Ala-Arg-Thr-Lys-Arg-Ser-Gly-Ser(P)-^{Val}_{Ile}-Tyr-Glu-Pro-Leu-Lys. The sites on histone H2B which are phosphorylated by cyclic AMP-dependent protein kinase *in vitro* were identified as serine-36 and serine-32. The amino acid sequence

in this region is: ³² Lys-Lys-Arg-Lys-Arg-Ser(P)-Arg-Lys-Glu-Ser(P)-Tyr-Ser-Val-Tyr-Val- [Iwai, K., Ishikawa, K. & Hayashi, H. (1970) *Nature (London)* **226**, 1056-1058]. Serine-36 was phosphorylated at 50% of the rate at which the β -subunit of phosphorylase kinase was phosphorylated, and it was phosphorylated 6-7-fold more rapidly than was serine-32. The amino acid sequences when compared with those at the phosphorylation sites of other physiological substrates suggest that the presence of two adjacent basic amino acids on the *N*-terminal side of the susceptible serine residue may be critical for specific substrate recognition *in vivo*.

Cyclic AMP-dependent protein kinase is thought to mediate the intracellular actions of hormones that work through cyclic AMP, according to the following scheme:

Hormone $\rightarrow$ increased cyclic AMP concentration $\rightarrow$
 activated protein kinase $\rightarrow$
 phosphorylated protein $\rightarrow$ physiological response

It is implicit in this hypothesis that many intracellular substrates for the enzyme exist, and proteins that are thought to be physiological substrates include phosphorylase kinase, glycogen synthase, triacylglycerol lipase, cholesterol esterase, pyruvate kinase (L-type) and histone H1 [see Cohen (1976) and Nimmo & Cohen (1977) for reviews]. Nevertheless, this enzyme is very specific in that it phosphorylates just one or two serine residues at significant rates in relatively few proteins (Cohen *et al.*, 1975; Nimmo & Cohen, 1977). This raises the question of what structural features determine whether any given protein will be phosphorylated.

Bylund & Krebs (1975) observed that several

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proteins which could be phosphorylated by the enzyme *in vitro*, such as histones, protamine and casein, had little tertiary structure, and this led them to test the effect of denaturation of some proteins on their susceptibility to phosphorylation. They found that lysozyme, serum albumin, creatine kinase and phosphorylase *b* were not substrates when they were in their native states, but that they became substrates when they had been subjected to reduction, carboxymethylation and maleylation. Similarly, Humble *et al.* (1975) showed that L-type pyruvate kinase became a better substrate for cyclic AMP-dependent protein kinase after it had been denatured in alkali. These results suggested that some feature of the substrate's primary structure rather than its tertiary structure must be important in determining whether it can be phosphorylated, and that the native conformation of the substrate may only be important in a negative sense in that it may prevent the phosphorylation of an otherwise suitable residue. These ideas were supported by the work of Daile & Carnegie (1974), who showed that several basic peptides produced by thermolytic or peptic digestion of myelin basic protein were almost as good substrates

as was the undigested protein. A similar result was obtained by Humble *et al.* (1975), who found that a peptide produced by digestion of L-type pyruvate kinase with CNBr was a better substrate than the native enzyme.

However, in our earlier work we reported that the amino acid sequences of two tryptic peptides around the two phosphorylation sites on phosphorylase kinase phosphorylated *in vivo* resembled neither each other nor the sequence round the histone H1 site (Langan, 1969, 1971; Cohen *et al.*, 1975; Yeaman & Cohen, 1975). Thus *in vivo* cyclic AMP-dependent protein kinase clearly does not recognize a specific linear sequence of amino acids, and this conclusion has been reached by a number of other workers (Kemp *et al.*, 1975; Bylund & Krebs, 1975; Daile *et al.*, 1975), who have compared the amino acid sequences at the phosphorylation sites of other proteins phosphorylated *in vitro*. However, in 1974 and 1975 several groups noticed that the amino acid sequences around a number of phosphorylation sites all contained at least one basic amino acid (usually arginine) in close proximity to and on the *N*-terminal side of the susceptible serine (Hjelmqvist *et al.*, 1974; Edlund *et al.*, 1975; Kemp *et al.*, 1975; Bylund & Krebs, 1975; Daile *et al.*, 1975).

We therefore decided to re-examine the amino acid sequences around the phosphorylated sites in rabbit skeletal-muscle phosphorylase kinase, using a proteinase that would not cleave on the C-terminal side of lysine or arginine. As a result we have been able to sequence regions beyond the *N*-terminal sides of the tryptic peptides that were originally studied (Cohen *et al.*, 1975). We have also identified the sites on histone H2B that are phosphorylated by cyclic AMP-dependent protein kinase: this protein is phosphorylated *in vitro* at rates comparable with those of physiological substrates. The results suggest that two adjacent basic amino acids on the *N*-terminal side of the susceptible serine residue may be critical for specific substrate recognition *in vivo*.

Experimental

Materials

Sephadex G-25 and G-50 (superfine grade) were obtained from Pharmacia (G.B.) Ltd. (London W.5, U.K.); Whatman 3MM chromatography paper (57cm×46cm) was from Whatman Biochemicals (Maidstone, Kent, U.K.); DX80 X-ray film was from Kodak (Glasgow, Scotland, U.K.); [γ - 32 P]ATP was from The Radiochemical Centre (Amersham, Bucks., U.K.); bovine serum albumin (fraction V) was from British Drug Houses (Poole, Dorset, U.K.). Histones H1 (rat liver) and H2B (calf thymus) [see Bradbury (1975) for nomenclature] were generous gifts from Professor T. A. Langan (University of Denver, Denver, CO, U.S.A.) and Dr. E. W. Johns

(Chester Beatty Research Institute, London S.W.3, U.K.) respectively. L-Type pyruvate kinase (rat liver) was a generous gift from Dr. O. Zetterqvist (University of Uppsala, Sweden).

Enzymes

Phosphorylase kinase was purified from the skeletal muscle of New Zealand White rabbits as described previously (Cohen, 1973). The absorbance index $A_{280}^{1\%}$ was taken as 12.4 and the minimum binding weight ($\alpha\beta\gamma$) as 318000g.

The peak-I isoenzyme of rabbit muscle cyclic AMP-dependent protein kinase was partially purified (Cohen, 1973), freed of phosphorylase (Nimmo *et al.*, 1976) and stored at -15°C .

The protein inhibitor of cyclic AMP-dependent protein kinase was partially purified up to the 15% (w/v) trichloroacetic acid-precipitate step (Walsh *et al.*, 1971).

Trypsin (Worthington), treated with 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one, and three-times recrystallized chymotrypsin (Worthington) were obtained from Cambrian Chemicals (Croydon, Surrey, U.K.); papaya latex papain, porcine pepsin, *Escherichia coli* alkaline phosphatase and di-isopropyl phosphorofluoridate-treated carboxypeptidases A and B were obtained from Sigma Chemical Co. (London S.W.6, U.K.); thermolysin was from Calbiochem (London W.1, U.K.).

Protein phosphorylation by cyclic AMP-dependent protein kinase

The incubations were carried out at pH 6.8, 20°C , and contained the substrate protein ($5\mu\text{M}$), sodium glycerol 2-phosphate (10mM), EDTA (0.4mM), EGTA (0.1mM), cyclic AMP (0.01mM), MgCl_2 (2.0mM), [γ - 32 P]ATP (0.2mM) and cyclic AMP-dependent protein kinase. Reactions were started by the addition of MgATP and incorporation of radioactivity into substrate protein was analysed as described previously (Nimmo *et al.*, 1976).

Large-scale phosphorylation of phosphorylase kinase

Phosphorylase kinase (520mg; $1.63\mu\text{mol}$) was phosphorylated for 30min under the conditions described above. The phosphorylation reaction was terminated by the addition of EDTA (final concentration 10mM) to chelate Mg^{2+} ions, and NaF (final concentration 50mM) to inhibit dephosphorylation (Yeaman & Cohen, 1975). The phosphorylated protein was concentrated to 30mg/ml by ultrafiltration at 4°C and excess of [γ - 32 P]ATP removed by gel filtration on a column (25cm×3cm) of Sephadex G-25 (superfine) equilibrated in sodium glycerol 2-phosphate (50mM)/EDTA (2mM)/2-mercaptoethanol (14mM)/NaF (50mM), pH 6.8. The incorporation of ^{32}P radioactivity reached a maximum of

1.95 mol of phosphate per mol of phosphorylase kinase ($\alpha\beta\gamma$). Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate showed that, as expected, equal amounts were incorporated into the α - and β -subunits of the enzyme with no incorporation into the γ -subunit (Cohen, 1973).

Large-scale phosphorylation of histone H2B

Histone H2B (21 mg; 1.4 μ mol) was phosphorylated for 60 min as described above. The reaction was terminated by the addition of 0.1 vol. of 100% (w/v) trichloroacetic acid. The solution was centrifuged at 10000g for 10 min, the precipitate containing the 32 P-labelled histone H2B redissolved in 3.0 ml of water, and insoluble material (derived from the partially purified cyclic AMP-dependent protein kinase) was removed by centrifugation at 10000g for 10 min. The histone solution was then extracted with ether to remove residual trichloroacetic acid and adjusted to pH 8.5 by the addition of 0.1 vol. of 2M-N-ethylmorpholine adjusted to pH 8.5 with HCl. In this particular experiment incorporation of 32 P reached a maximum of 1.35 mol per mol of histone.

Isolation of a tryptic peptide containing the phosphorylation site on the α -subunit of phosphorylase kinase

The dephosphorylated form of phosphorylase kinase was purified through the 30% $(\text{NH}_4)_2\text{SO}_4$ precipitation step (Cohen, 1973), at which stage the enzyme was approx. 60% pure as judged by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. To this material (containing approx. 1 g of phosphorylase kinase) was added 0.1 vol. of 0.5M-NaF and 10 mg of 32 P-labelled phosphorylase kinase, which had been fully phosphorylated by incubation with cyclic AMP-dependent protein kinase and [γ - 32 P]ATP. This material was then digested with trypsin and the α -subunit tryptic peptide purified by trichloroacetic acid fractionation and gel filtration on Sephadex G-50 (superfine) (Cohen *et al.*, 1975). The trace of radioactively labelled phosphorylase kinase served as a marker during the isolation of the tryptic peptide (Yeaman & Cohen, 1975). The amount of peptide obtained was 1 μ mol, corresponding to an overall yield of 35%. Peptic and chymotryptic subfragments of this peptide were isolated by peptide 'mapping'. C-Terminal analysis of the subfragments was carried out by digestion with carboxypeptidase A or B (Ambler, 1967) and N-terminal analysis by the subtractive Edman procedure (Konigsberg, 1967).

Analysis of peptides

Peptide 'mapping', detection and elution of peptides and subdigestion of peptides with proteinases were carried out as described previously (Cohen *et al.*, 1975). Electrophoretic mobilities (R_E) are

expressed relative to alanine = 1.0 and chromatographic mobilities (R_C) relative to Phenol Red = 1.0.

Amino acid analysis

This was carried out with either a Beckman Multichrome or Beckman 121M amino acid analyser. The latter was used in conjunction with automated sequence analysis. Peptides were hydrolysed for 20 h at 110°C in 6M-HCl/10mM-phenol *in vacuo*. Values of serine and threonine were corrected for 10% and 5% destruction respectively. Analysis for tryptophan was carried out after hydrolysis at 125°C in 4M-methanesulphonic acid (Liu & Chang, 1971), and cysteine was measured as cysteic acid after performic acid oxidation (Moore, 1963). None of the peptides analysed in this paper was found to contain either of these two amino acids.

Automatic sequencer analysis

This was carried out with a Beckman 890C Sequencer. The methods were as described previously (Cohen *et al.*, 1975), except that each solvent contained 1.0mM-dithioerythritol to stabilize the thiazoline derivatives, and the pH of the dimethylallylamine/trifluoroacetic acid buffer was 9.8 (instead of 9.5). The thiazolinone derivatives were first analysed for 32 P radioactivity by Čerenkov counting and then hydrolysed in 57% HI and analysed as described by Smithies *et al.* (1971), to yield free amino acids. The phenyl isothiocyanate derivative of norleucine (15.0 nmol) was added to each tube before hydrolysis as a semi-internal standard and recoveries of amino acids were standardized to the yield of norleucine. Alanine, serine and cysteine are all recovered as alanine by this procedure.

Results

Isolation of 32 P-labelled thermolytic phosphopeptides from the α - and β -subunits of phosphorylase kinase

32 P-labelled phosphorylase kinase (28 ml at 18.5 mg/ml) was incubated with an equimolar quantity of thermolysin (2.8 ml at 20 mg/ml) at 25°C for 30 min. The reaction was terminated by the addition of 0.1 vol. of 50% (w/v) trichloroacetic acid and, after cooling to 0°C in ice, the solution was centrifuged at 30000g for 10 min. This procedure released 86% of the 32 P radioactivity as trichloroacetic acid-soluble phosphopeptides, whereas less than 10% of the A_{280} material was released. This supernatant was extracted with 5 $\times$ 1 vol. of ether and freeze-dried. The freeze-dried material was dissolved in 3.0 ml of 1.0M-acetic acid, clarified by brief centrifugation at 15000g and then fractionated by gel filtration on a column (150 cm $\times$ 1.5 cm) of Sephadex G-25 (superfine) equilibrated in 1.0M-acetic acid. An elution profile is shown in Fig. 1. Three major peaks of radioactivity, termed Th1, Th2 and Th3, were resolved,

Fig. 1. Gel filtration on a column (1.50 cm $\times$ 15 cm) of Sephadex G-25 (superfine) of a thermolytic digest of ^{32}P -labelled phosphorylase kinase

●, ^{32}P radioactivity; ○, A_{280} . Fraction size was 3.0 ml, and the arrow denotes the excluded volume (V_0) of the column. The phosphopeptides Th1, Th2 and Th3 are defined in the text.

Table 1. Amino acid analyses of thermolytic phosphopeptides from phosphorylase kinase

Peptide Th1 is peptide Th1a + Th1b. As described in the text, peptides Th1a and Th1b are identical, except that peptide Th1a contains valine whereas peptide Th1b contains isoleucine. Valine and isoleucine therefore together add up to one residue. Values below 0.1 residue are omitted.

Amino acid	Content (residues/100 residues)		
	Th1	Th2	Th3
Aspartic acid	0.16	0.29	0.21
Threonine	1.04 (1)	0.17	
Serine	1.81 (2)	0.99 (1)	1.03 (1)
Glutamic acid	1.08 (1)	0.40	0.21
Proline	1.04 (1)		
Glycine	1.01 (1)	0.53	0.20
Alanine	0.95 (1)		
Valine	0.41	0.29	
Isoleucine	0.50 (1)		
Leucine		1.00 (1)	1.00 (1)
Tyrosine	0.67 (1)		
Phenylalanine		0.86 (1)	
Histidine		0.27	
Lysine	1.00 (1)		
Arginine	2.07 (2)	2.10 (2)	
^{32}P	0.86	0.97	0.83
Total	12	5	2

which were eluted at V_e/V_0 values of 1.35, 1.62 and 1.74 respectively.

Peptide Th1 was further purified by high-voltage electrophoresis at pH 3.6 (Cohen *et al.*, 1975). A single major radioactive spot (R_F 2.4) was detected. This was eluted in 20 mM-acetic acid, and then dried. The material was subjected to peptide 'mapping', by electrophoresis at pH 6.5 and descending chromatography (40 h) (Cohen *et al.*, 1975). The prolonged chromatography resolved the phosphopeptide into two components, Th1a (R_C 0.19) and Th1b (R_C 0.21), which were present in almost equal amounts. These were eluted together and freeze-dried. The amount of peptide obtained was 230 nmol, corresponding to an overall yield of 15%.

The amino acid composition of this peptide (12 residues) (Table 1), when compared with the sequences of the tryptic phosphopeptides (Cohen *et al.*, 1975), showed that peptide Th1 is derived from the phosphorylation site on the β -subunit of phosphorylase kinase. The characteristic 'doublet' observed on chromatography is due to the presence of either valine or isoleucine in the position immediately C-terminal to the phosphoserine. This results from the presence of two alleles for the β -subunit of phosphorylase kinase in the New Zealand White rabbit population (Cohen *et al.*, 1976).

Peptide Th2 was purified in a manner identical with

that for peptide Th1, except that the descending chromatography was run for only 24h. It behaved as a single main component on peptide 'mapping' [R_E (pH 6.5) 3.0, R_C 0.54), and 115nmol was obtained finally (8% yield). However, ninhydrin staining of the final peptide 'map' indicated that peptide Th2 was still contaminated with another peptide. It was therefore further purified by repeating the electrophoresis at pH 6.5.

Amino acid analysis showed that peptide Th2 (five residues) was still only approx. 75% pure (Table 1), but comparison with the known sequence of the tryptic phosphopeptides (Cohen *et al.*, 1975) showed that it must be derived from the phosphorylation site on the α -subunit.

Peptide Th3 was completely purified by peptide 'mapping', by electrophoresis at pH 3.6 (R_E -0.86) and descending chromatography for 16h (R_C 0.44). One radioactive spot was detected; its amino acid composition (Table 1) showed it to be a dipeptide (Leu,Ser) from the α -subunit phosphorylation site (Cohen *et al.*, 1975). This peptide was not studied further.

Determination of the amino acid sequences of the peptides

Peptide Th1. Some 90nmol of this peptide was analysed on the Beckman 890C Sequencer. The amino acid analyses at each cycle after hydrolysis with HI are shown in Table 2. These data, taken together with the amino acid composition of peptide Th1 and sequence information obtained previously from the tryptic phosphopeptide (Cohen *et al.*, 1975),

allow the unequivocal determination of the complete sequence.

Since the tryptic phosphopeptide contains two serine residues and is devoid of alanine, the residue at positions 6 and 8 of peptide Th1 must be serine and not alanine. The *N*-terminal residue of peptide Th1 must therefore be alanine. The residue at position 11 is assigned as glutamic acid and not glutamine on the basis of two pieces of evidence. Firstly, there was no significant rise in the NH_3 concentration at the cycle (Table 2). Secondly, when peptide Th1 was subdigested with trypsin and then treated with alkaline phosphatase, the peptide Ser-Gly-Ser-^{Val}Ile-Tyr-Glx-Pro migrated on electrophoresis at pH 6.5 with a net charge of -1 (not illustrated).

The sequence of the *C*-terminal segment of the tryptic phosphopeptide Pro-Leu-Lys (Cohen *et al.*, 1975) provides an overlap with peptide Th1 and therefore gives a sequences of 14 residues around the phosphorylation site on the β -subunit. The sequence is: Ala-Arg-Thr-Lys-Arg-Ser-Gly-Ser(P)-^{Val}Ile-Tyr-Glu-Leu-Lys.

In the earlier sequence analysis of the tryptic phosphopeptide (Cohen *et al.*, 1975), the Glx residue was erroneously assigned to the *N*-terminus of that peptide (i.e. preceding the first serine residue). This positioning was based on two pieces of negative evidence: (a) failure to detect an *N*-terminal amino acid by the Edman procedure which was consistent with cyclization of an *N*-terminal glutamine to pyrrolidonecarboxylic acid; (b) failure of chymo-

Table 2. Automated sequencer analysis of peptide Th1
Quantities below 3 nmol are omitted.

Amino acid	Amino acid recovered (nmol)														
	Cycle number	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Threonine	—	4.0	<u>28.4</u>	9.3	3.5	—	—	—	—	—	—	—	—	—	—
Glutamic acid	—	—	—	—	—	3.7	—	—	—	—	—	<u>18.1</u>	10.6	5.0	—
Proline	—	—	—	—	—	—	—	—	—	—	—	—	<u>12.0</u>	7.3	—
Glycine	3.5	—	—	3.9	—	7.1	<u>27.1</u>	15.3	8.3	5.2	3.1	—	—	—	—
Alanine	<u>65.6</u>	6.7	—	3.0	—	<u>21.1</u>	9.3	<u>16.8</u>	10.9	3.9	—	—	—	—	—
Valine	—	—	—	—	—	—	—	—	<u>5.6</u>	—	—	—	—	—	—
Isoleucine	—	—	—	—	—	—	—	—	<u>8.1</u>	3.7	—	—	—	—	—
Leucine	3.0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Tyrosine	—	—	—	—	—	—	—	—	—	—	<u>11.6</u>	6.5	—	—	—
Lysine	—	—	—	38.0	6.7	3.4	—	—	—	—	—	—	—	—	—
Ammonia	102.8	73.4	76.7	79.9	71.9	80.0	70.2	62.7	65.2	75.4	75.5	71.7	69.1	66.5	—
Arginine	—	<u>27.8</u>	6.71	—	<u>21.1</u>	10.5	3.3	—	—	—	—	—	—	—	—
³² P (c.p.m.)	14	21	37	31	46	61	219	4419	3899	2724	2279	1412	1289	1036	—
Sequence	Ala Ser	Arg	Thr	Lys	Arg	Ser Ala	Gly	Ser(P)	Val Ile	Tyr	Glx	Pro	—	—	—

Table 3. *Automated sequence analysis of peptide Th2*
Quantities below 0.5 nmol are omitted.

Amino acid Cycle number ...	Amino acid recovered (nmol)						
	1	2	3	4	5	6	7
Aspartic acid	—	—	0.8	0.6	1.0	—	—
Glutamic acid	0.5	—	1.4	1.8	0.9	0.5	—
Proline	—	0.6	0.5	0.7	—	—	—
Glycine	1.8	1.0	1.0	1.8	1.6	1.1	0.7
Alanine	0.9	1.2	0.8	0.5	1.2	1.0	0.6
Valine	0.9	—	1.3	—	—	—	—
Isoleucine	0.9	—	—	—	—	—	—
Leucine	3.2	0.8	0.8	8.2	2.7	1.1	0.5
Phenylalanine	12.5	2.0	0.5	—	—	—	—
Histidine	—	1.1	0.6	—	—	—	—
Lysine	—	—	—	0.6	—	—	—
Ammonia	64.4	56.4	70.0	63.9	43.9	45.7	47.6
Arginine	—	10.5	12.2	4.1	1.1	1.1	—
³² P (c.p.m.)	143	133	191	248	298	298	306
Sequence	Phe	Arg	Arg	Leu	Ser(P)	—	—

trypsin to cleave a tyrosine-X bond, which would be consistent with a Tyr-Pro bond (Cohen *et al.*, 1975).

Peptide Th2. Some 35 nmol was analysed on the Beckman Sequencer and the results are shown in Table 3. Although amino acid analysis of the peptide indicated that it was only 75% pure (Table 1), the sequence analysis was unambiguous: Phe-Arg-Arg-Leu-Ser(P).

Although only a slight rise in alanine concentration (derived from serine) was detected at cycle 5, this residue can be assigned as phosphoserine on the basis of the amino acid composition of the peptide and previous knowledge of the sequence of the tryptic phosphopeptide that commences Arg-Leu-Ser(P) (Cohen *et al.*, 1975).

α -Subunit tryptic peptide. This peptide consists of 39 residues, as shown by amino acid analysis of the tryptic peptide (38 residues) and the sum of chymotryptic subfragments (39 residues) (C1 to C6 in Table 4).

Analysis on the automatic sequencer yielded the sequence shown in Fig. 2. Positive identification could be made only as far as residue 31. This peptide is devoid of cysteine and contains only one alanine (in subfragment C3). Inspection of the sequencer data and Table 4 shows that the alanine residue is at position 17. Alanine detected at all the other positions is therefore derived from serine (see the Experimental section). Analysis of residues 1–11 is consistent with previous work (Cohen *et al.*, 1975), and results for residues 12–31 are entirely consistent with the amino acid composition and end-group analysis of the chymotryptic subfragments (Table 4). The amino acid at position 20 was not identified by the sequencer analysis but the data of Table 4 show that position 20

1	2	3	4	5	6	7	8	9	10	11	12
Leu-Ser	-Ile	-Ser	-Thr	-Glx	-Ser	-Glx	-Pro	-Asx	-Gly	-Gly	
13	14	15	16	17	18	19	20	21	22	23	24
His-Ser	-Leu	-Gly	-Ser	-Asx	-Leu	?	-Ser	-Pro	-Ser	-Phe	
25	26	27	28	29	30	31					
Leu-Ser	-Pro	-Gly	-Thr	?	-Val						

Fig. 2. *Analysis of the α -subunit tryptic peptide on the automatic sequencer*

Detailed quantitative data can be supplied by the authors on request. It is also presented in Yeaman (1976).

is a methionine residue. Hydrolysis with HI is known to destroy methionine (Smithies *et al.*, 1971). The amino acid at position 30 was not identified, but, as position 31 was valine, residue 30 must be serine as judged from the composition of peptide C5A (Table 4). The electrophoretic mobilities of peptides C3 and C3A (Table 4) showed that residue 18 is aspartic acid and not asparagine, and it is also known that position 6 is glutamic acid and not glutamine (Cohen *et al.*, 1975). Since the C-terminal residue of the tryptic peptide is lysine, peptide C6 is the C-terminal chymotryptic peptide. The N-terminal amino acid of this tripeptide C6 is threonine (Table 4), showing that the sequence of residues 37–39 is Thr-Ser-Lys. Peptide C5B consists of residues 32–36, and peptide P1 is 33–39. Since peptide P1 is devoid of phenylalanine, this indicates that the phenylalanine is present at position 32. Since the C-terminal residue of peptide C5B was histidine, this gives the sequence of the

Table 4. *Amino acid analyses of subfragments of α -subunit tryptic peptide*

Abbreviations: T, tryptic peptide; C, chymotryptic peptide; P, peptic peptide. Impurities below 0.1 residue are omitted.

Amino acid Peptide ...	Content (residues/100 residues)										
	T	C1	C2	C3	C3A	C4	C5	C5A	C5B	C6	P1
Aspartic acid	1.96 (2)	1.00 (1)	—	1.00 (1)	1.10 (1)	—	—	—	—	—	—
Threonine	3.14 (3)	0.92 (1)	—	—	—	—	0.95 (1)	1.05 (1)	—	0.84 (1)	0.98 (1)
Serine	8.53 (9)	2.87 (3)	0.89 (1)	—	—	1.36 (2)	3.03 (3)	1.60 (2)	0.99 (1)	1.02 (1)	1.96 (2)
Glutamic acid	2.00 (2)	2.43 (2)	—	—	—	—	—	—	—	—	—
Proline	4.04 (4)	1.50 (1)	—	—	—	1.09 (1)	2.20 (2)	1.14 (1)	1.10 (1)	—	1.18 (1)
Glycine	5.25 (5)	2.25 (2)	—	0.80 (1)	0.94 (1)	0.20	2.18 (2)	0.90 (1)	1.29 (1)	0.18	1.00 (1)
Alanine	1.20 (1)	0.20	—	0.95 (1)	1.23 (1)	—	—	—	—	—	—
Valine	1.09 (1)	—	—	—	—	—	0.80 (1)	0.70 (1)	—	—	0.14
Methionine	0.69 (1)	—	—	0.98 (1)	—	—	—	—	—	—	—
Isoleucine	0.98 (1)	0.79 (1)	—	—	—	—	—	—	—	—	—
Leucine	3.60 (4)	0.82 (1)	1.00 (1)	1.07 (1)	1.00 (1)	1.11 (1)	—	—	—	—	0.13
Phenylalanine	1.68 (2)	—	—	—	—	1.00 (1)	0.77 (1)	—	0.80 (1)	—	0.27
Histidine	2.23 (2)	0.77 (1)	—	—	—	—	1.17 (1)	—	1.00 (1)	—	0.92 (1)
Lysine	0.84 (1)	—	—	—	—	—	—	—	—	1.0 (1)	0.55 (1)
Total	38	13	2	5	4	5	11	6	5	3	7
N-Terminus	Leu	—	Ser	—	—	—	—	—	—	Thr	—
C-Terminus	Lys	—	—	Met	Leu	Leu	His	—	His	Lys	—
Net charge at pH 6.5	—	—	0	-1	-1	—	—	—	—	—	—

α -subunit tryptic peptide, apart from ambiguity at residues 33–35. Taken with the sequence analysis of peptide Th2, this gives the sequence of 42 amino acids at the phosphorylation site on the α -subunit of phosphorylase kinase. This sequence is: Phe-Arg-Arg-Leu-Ser(P)-Ile-Ser-Thr-Glu-Ser-Glx-Pro-Asx-Gly-Gly-His-Ser-Leu-Gly-Ala-Asp-Leu-Met-Ser-Pro-Ser-Phe-Leu-Ser-Pro-Gly-Thr-Ser-Val-Phe(Ser,Pro,Gly)His-Thr-Ser-Lys.

Phosphorylation of histone H2B by cyclic AMP-dependent protein kinase

Phosphorylation of histone H2B by cyclic AMP-dependent protein kinase reached a plateau at 2 mol of phosphate per mol of histone H2B in pilot-scale experiments, implying that at least two sites on the protein had been phosphorylated. The phosphorylation was dependent on the addition of the protein kinase and was blocked by addition of an excess of the protein inhibitor of the cyclic AMP-dependent protein kinase (Fig. 3). In large-scale phosphorylations the plateau was often reached at a rather lower extent of phosphorylation (see the Experimental section).

Isolation of tryptic phosphopeptides from histone H2B

32 P-labelled histone H2B (21 mg or 1.4 μ mol) was incubated with trypsin (0.3 mg) at 37°C for 20 h in a total volume of 3.0 ml. The solution was clarified by centrifugation, at 15000g for 2 min made 1.0 M in acetic acid and fractionated by gel filtration on a

Fig. 3. *Phosphorylation of histones by cyclic AMP-dependent protein kinase*

□, Histone H1; ○, histone H2B; ■, histone H2B in the presence of an excess of the protein inhibitor of cyclic AMP-dependent protein kinase. The exact phosphorylation conditions were as described in the Experimental section and carried out at a histone concentration of 5 μ M.

column (150 cm $\times$ 1.5 cm) of Sephadex G-25 (superfine). One broad peak was eluted (V_e/V_0 1.75), which contained 95% of the initial radioactivity. This was

pooled, dried and subjected to peptide 'mapping' by electrophoresis at pH 6.5 and descending chromatography. Two phosphopeptides, T1 (R_E -2.18; R_C 0.52) and T2 (R_E -1.08; R_C 0.16) were obtained in approximately equal amounts and together they accounted for over 90% of the radioactivity.

The amino acid compositions of peptides T1 and T2 are shown in Table 5. The complete amino acid sequence of histone H2B is known (Iwai *et al.*, 1970) and inspection of the sequence allows the conclusion that the tryptic dipeptide T2 (Ser, Arg) is derived from the residues serine-32 and arginine-33. The amino acid composition of peptide T1 shows that this corresponds to residues 34-40, which have the sequence: Lys-Glu-Ser-Tyr-Ser-Val-Tyr.

This peptide contains two serine residues, but the stoichiometry of ^{32}P in this peptide is consistent with phosphorylation of only one serine (Table 5). To decide which serine was phosphorylated, 60 nmol of peptide T1 was digested with papain and then subjected to peptide 'mapping'. One radioactive spot, T1 Pap (R_E -2.38; R_C 0.16), was detected after radioautography, and its composition is shown in Table 5. The composition of this peptide [Lys-Glu, Ser(P)] showed conclusively that only serine-36 and not serine-38 was phosphorylated in the peptide. It is noteworthy that phosphorylation at position 36 prevented trypsin cleaving the Lys-Glu bond between positions 34 and 35. This phenomenon has also been observed in the isolation of the α -subunit tryptic phosphopeptide from phosphorylase kinase (Yeaman & Cohen, 1975) and a tryptic phosphopeptide from histone H1 (Langan, 1969). The cleavage of a Tyr-Val bond between residues 40 and 41 is presumably a pseudotryptic cleavage.

Table 5. Amino acid analyses of phosphopeptides from histone H2B

Abbreviations: T, tryptic peptide; T Pap, papain subdigest of tryptic peptide. Impurities below 0.1 residue are omitted.

Amino acid	Content (residues/100 residues)		
	T1	T1 Pap	T2
Aspartic acid	0.12	—	—
Serine	2.05 (2)	0.91 (1)	0.96 (1)
Glutamic acid	1.30 (1)	1.05 (1)	—
Glycine	0.13	0.13	—
Alanine	—	0.12	—
Valine	1.27 (1)	—	—
Isoleucine	0.12	—	—
Tyrosine	1.77 (2)	—	—
Lysine	0.81 (1)	1.08 (1)	0.17
Arginine	—	—	1.00
^{32}P	1.00	1.00	0.92
Total	7	3	2

Phosphorylation of the same two residues on histone H2B, serine-32 and serine-36, was also reported by Hashimoto *et al.* (1975) using cyclic AMP-dependent protein kinase from silkworm. The additional phosphorylation of serine-14 reported by Shlyapnikov *et al.* (1975) using pig brain protein kinase was not detected either by ourselves or by Hashimoto *et al.* (1975).

Relative rates of phosphorylation of serine-32 and serine-36 on histone H2B

The initial rate of phosphorylation of histone H2B by cyclic AMP-dependent protein kinase carried out in the standard phosphorylation assay described in the Experimental section was found to be 55% that of the β -subunit of phosphorylase kinase and was six times the rate of phosphorylation of histone H1.

To investigate the relative rates of phosphorylation of each of the two sites on histone H2B, two portions were removed at different time-intervals from a standard phosphorylation reaction. One sample was assayed for incorporation of ^{32}P radioactivity into the protein. The second sample was precipitated with trichloroacetic acid, redissolved in NaOH, reprecipitated in trichloroacetic acid and then redissolved in 0.2M-N-ethylmorpholine, pH 8.5. This material was then incubated with trypsin (histone H2B/trypsin, 50:1) at 37°C for 20 h. The digestion was terminated by freeze-drying, and the products were subjected to high-voltage electrophoresis at pH 6.5. Radioautography showed only two phosphopeptides corresponding to T1 and T2. The radioactive spots were cut out and counted for ^{32}P radioactivity. The

Fig. 4. Phosphorylation of serine-32 and serine-36 on histone H2B by cyclic AMP-dependent protein kinase ●, Serine-32; ○, serine-36. Details of the experiment are given in the Results section. The relative rates of phosphorylation were calculated from the initial slopes of the graph.

measurements enabled the degree of phosphorylation at each site to be calculated for each time-point. The results showed that serine-36 was phosphorylated six times faster than was serine-32 (Fig. 4).

Discussion

The amino acid sequences at the sites phosphorylated on phosphorylase kinase and histone H2B are given in Table 6, together with the corresponding sequences for histone H1 and L-type pyruvate kinase. These proteins are the best substrates for cyclic AMP-dependent protein kinase *in vitro* that have been identified, and it is a striking feature that each of the sequences contains two adjacent basic amino acids, of which at least one is arginine, just *N*-terminal to the target serine.

While this work was in progress, the importance of one arginine residue *N*-terminal to the susceptible serine as a minimum specificity determinant of cyclic AMP-dependent protein kinase had been emphasized by the work of several other laboratories.

Kemp *et al.* (1975) studied the phosphorylation of genetic variants of β -casein and found that the rate of phosphorylation of β -casein B was 100-fold greater than for the variant termed A₁. β -Casein B differs from A₁ in only one position, serine-122 being replaced by arginine. Since the only significant site of phosphorylation in β -casein B was serine-124, this suggested that replacement of serine-122 by arginine was responsible for the enhanced rate of phosphorylation.

Daile *et al.* (1975) synthesized the peptide Gly-Arg-Gly-Leu-Ser-Leu-Ser-Arg, which corresponds to the major site of phosphorylation in myelin basic protein. The peptide was phosphorylated by cyclic AMP-dependent protein kinase on the first serine

residue of the sequence, as occurred when the intact protein was used as a substrate. Removal of the *N*-terminal glycine or the *C*-terminal arginine residue (or both) did not affect the ability of the peptide to act as a substrate, but the subsequent removal of the arginine on the *N*-terminal side of the serine residues removed this ability.

Kemp *et al.* (1976a) synthesized the peptide Arg-Gly-Tyr-Ser-Leu-Gly, which corresponds to the sequence around serine-24 of lysozyme, the major site that was phosphorylated after this protein was denatured (Bylund & Krebs, 1975). If the *N*-terminal arginine was replaced by glycine, histidine or lysine, the $V_{\max}$ was decreased 20-fold and the K_m was increased by a factor of 3.

However, the above substrates, in which only one arginine was present, were still very poor substrates compared with phosphorylase kinase, glycogen synthase and pyruvate kinase. In particular, the synthetic peptide corresponding to serine-24 of lysozyme had a K_m of 4mM, about 1000-fold higher than the K_m for native pyruvate kinase (Kemp *et al.*, 1976a,b; Zetterqvist *et al.*, 1976). Nevertheless, the $V_{\max}$ of this peptide was comparable with pyruvate kinase.

The results of Zetterqvist *et al.* (1976) and Kemp *et al.* (1976b) suggest that it is the presence of two adjacent basic amino acids which is necessary for phosphorylation to occur with K_m values in the micromolar range. Zetterqvist *et al.* (1976) found that the heptapeptide Leu-Arg-Arg-Ala-Ser-Val-Ala, corresponding to the phosphorylation site of rat liver pyruvate kinase, was phosphorylated with kinetic constants (K_m and $V_{\max}$) comparable with the native protein: the K_m for this peptide was 10 μ M. If either arginine residue in the pentapeptide Arg-Arg-Ala-Ser-Val was replaced by leucine, the rate of phosphorylation of the peptide fell by more than 100-fold.

Table 6. *Substrate specificity of cyclic AMP-dependent protein kinase*

The numbers above each phosphoserine refer to rates of phosphorylation relative to the β -subunit of phosphorylase kinase (100). Assays were carried out at 5 μ M-substrate concentration (Cohen *et al.*, 1975). The amino acids underlined are the phosphorylation site and the basic amino acids *N*-terminal to it.

Substrate	Amino acid sequence at phosphorylation site													
Phosphorylase kinase (β -subunit)	Ala	<u>Arg</u>	Thr	<u>Lys</u>	<u>Arg</u>	Ser	Gly	¹⁰⁰ <u>Ser(P)</u>	<u>Val</u> Ile	Tyr	Glu	Pro	Leu	Lys
				8				50						
Histone H2B	<u>Lys</u>	<u>Lys</u>	<u>Arg</u>	<u>Lys</u>	<u>Arg</u>	<u>Ser(P)</u>	<u>Arg</u>	<u>Lys</u>	Glu	<u>Ser(P)</u>	Tyr	Ser	Val	Tyr
Pyruvate kinase* (L-type)														
Phosphorylase kinase (α -subunit)														
Histone H1†														

* Edlund *et al.* (1975).

† Langan (1971).

Table 7. *Phosphorylation sites in vivo in histones and protamines*

The values above the phosphoserine residues refer to the residue position in the amino acid sequence of each protein. The amino acids underlined are the basic amino acids *N*-terminal to the phosphorylation site.

Protein		Sequence	Reference
Histone H2B		6 Ala- <u>Lys</u> -Ser(P)-Ala-Pro-	Dixon <i>et al.</i> (1975)
Histone H3	(1)	10 - <u>Arg</u> - <u>Lys</u> -Ser(P)-Thr-Gly-	Marzluff & McCarty (1972); Dixon <i>et al.</i> (1975)
	(2)	28 - <u>Arg</u> - <u>Lys</u> -Ser(P)-Ala-Pro	Marzluff & McCarty (1972); Dixon <i>et al.</i> (1975)
Histone H6 (histone T)		5 - <u>Arg</u> - <u>Lys</u> -Ser(P)-Ala-Thr	Dixon <i>et al.</i> (1975)
Histone H1	(1)	145 - <u>Lys</u> - <u>Lys</u> -Ser(P)-Pro-Lys	Dixon <i>et al.</i> (1975)
	(2)	161 -Ala- <u>Lys</u> -Ser(P)-Pro-Lys	Dixon <i>et al.</i> (1975)
	(3)	182 -Ala- <u>Lys</u> -Ser(P)-Pro-Lys	Dixon <i>et al.</i> (1975)
Protamine (<i>Salmo gairdnerii</i>)	(1)	- <u>Arg</u> - <u>Arg</u> - <u>Val</u> -Ser(P)-Arg-Arg-	Ingles & Dixon (1967); Sanders & Dixon (1972)
	(2)	- <u>Arg</u> - <u>Arg</u> -Ser(P)-Ser(P)-Ser(P)-Arg-	

Similar results were obtained by Kemp *et al.* (1976b) using the peptide Leu-Arg-Arg-Ala-Ser-Leu-Gly (K_m 15 μM), corresponding to part of the phosphorylation site in pig liver pyruvate kinase. These workers stated that the results of making substitutions in synthetic peptides indicated that two basic amino acids had to be present on the *N*-terminal side of the serine in order that the peptides be phosphorylated with kinetic constants comparable with those of physiological substrates.

Since two adjacent basic amino acids seem to be necessary in order that a peptide be phosphorylated with a K_m in the micromolar range, it might be expected that substrate proteins present in such concentrations *in vivo* (i.e. most substrate proteins) would all have this common feature. This is, of course, strongly supported by the results shown in Table 6, and it is also notable that one of the major sites on muscle glycogen synthase phosphorylated by cyclic AMP-dependent protein kinase appears to be preceded by two adjacent basic amino acids (C. G. Proud, S. J. Yeaman, H. G. Nimmo & P. Cohen, unpublished work).

Zetterqvist *et al.* (1976) also showed that the rate at which a peptide was phosphorylated was dependent on the nature of the amino acid *C*-terminal to the phosphoserine. In addition, replacement of the serine by a threonine residue also destroyed the ability of the pentapeptide to act as a substrate. It is therefore clear that, once the requirement for basic amino acids has been met, other features of the primary sequence can influence the rate of phosphorylation.

Although histone H2B is a very good substrate *in vitro*, there is currently no evidence that it can be phosphorylated by cyclic AMP-dependent protein kinase *in vivo*. It is possible that the phosphorylation sites are inaccessible in native chromatin, and that this is another example where the native conformation prevents the phosphorylation of an otherwise suitable residue.

Histone H2B in trout testis, in common with some other histones and protamine, is, however, phosphorylated *in vivo* during the cell cycle at sites distinct from those phosphorylated by cyclic AMP-dependent protein kinase *in vitro*. These are presumably catalysed by another class or classes of protein kinase. However, a notable similarity in the sequence of these sites phosphorylated by undefined protein kinases *in vivo* is apparent from an inspection of Table 7. In almost every case a pair of basic amino acids (underlined) precedes the phosphorylation site. In protamines the two sites are preceded by a pair of arginine residues, in the two sites in histone H3 and one in H6 by an Arg-Lys sequence, and one site in histone H1 (residue 145) by a Lys-Lys pair. At one site in histone H2B (residue 6) and two in histone H1 (residues 161 and 182) there is only one lysine preceding the phosphorylation site. The fact that *in vivo* the phosphorylation sites conform so closely to the specificity requirements observed for cyclic AMP-dependent protein kinase suggest that enzymes with very similar specificity requirements are responsible for some of the phosphorylation observed *in vivo* in histones and protamines.

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