proteinases, intralysosomal proteolysis is retarded, either leading to an intralysosomal accumulation of protein or perhaps to a feedback inhibition of uptake. Either of these events may occur also when lysosomal proteinases are genetically defective; that this has not been detected in man[19] implies they are lethal in early embryonic life. The mechanisms of uptake, and their control and specificity, now deserve considerable further attention[6].

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Synthetic substrate for cyclic AMP-dependent protein kinase

MOST hormones and neurotransmitters are thought to stimulate the synthesis of adenosine 3′,5′-cyclic monophosphate (cyclic AMP) which in turn activates protein kinases[1]. On activation, the protein kinases transfer the terminal phosphate group of ATP to serine or threonine residues in enzymic or membrane proteins involved in metabolic regulation, thereby either activating or inactivating them. Only a few key proteins are phosphorylated[1,2] which raises the question of the molecular basis for the recognition of a particular serine by the cyclic AMP-dependent protein kinases. Cohen *et al.*[2] considered this problem with respect to the phosphorylation of phosphorylase kinase and glycogen synthetase and concluded that the cyclic AMP-dependent protein kinase recognises some specific three-dimensional configuration formed by a particular amino acid sequence at the site of phosphorylation. The features of this structure, however, remain to be defined. It has been shown[3] that small peptides from myelin basic protein could act as substrates, thus reducing the number of parameters for consideration.

A major site of phosphorylation by cyclic AMP-dependent protein kinase in intact myelin[4], isolated myelin basic protein[5], and in peptic peptides[6] was serine 110 in the sequence Gly–Arg–Gly–Leu–Ser–Leu. Here we examine the phosphorylation of a synthetic peptide equivalent to amino acid residues 106–113 in the basic protein of human myelin[7]. The peptide is thus the first synthetic substrate for the cyclic AMP-dependent protein kinases which seem to be so important in regulatory processes.

The peptide Gly–Arg Gly–Leu–Ser–Leu–Ser–Arg was synthesised by the solid phase technique[8], as modified for automatic synthesis (M. D. Geier, F. S. Geier, and J.D.Y., unpublished). The double coupling programme included chloroform as solvent, dicyclohexylcarbodiimide as coupling agent, 25% trifluoroacetic acid to remove *t*-butyloxycarbonyl protecting groups and anhydrous HF at 0 °C for 30 min to cleave the peptide from the resin and to remove the O-benzyl and nitro groups from serine and arginine respectively. Initial purification was on Sephadex G-15 and the yield was 20%. The peptide was stained with ninhydrin and had a mobility of +0.59 relative to aspartic acid[9] on high-voltage electrophoresis using Whatman 3MM paper in pyridine–acetate buffer, *p*H 6.5. Two minor contaminants with relative mobilities of +0.34 and +0.65 were removed by this technique. (These contaminants, which also acted as substrates for the protein kinase, presumably arose from the incomplete removal of the nitro-protecting group on the C-terminal arginine[10].) The purified peptide on analysis contained equimolar ratios of its four constituent amino acids.

Protein kinases were prepared from bovine cardiac muscle and brain[3,11] and further purified by gel filtration on Sepharose 4B in 5 mM potassium phosphate buffer, *p*H 7.0, containing 2 mM EDTA. The kinases were assayed with histone and with myelin basic protein as substrates and cyclic AMP produced a stimulation between four- and sevenfold.

In the experiments with synthetic peptide, the electrophoretic assay for protein kinase activity was used[3]. The actual incubation conditions are described in Fig. 1 and below. The peptide (250 nmol) was incubated with γ-^{32}P-ATP (1 μmol, 8.7×10^6 c.p.m.), cardiac protein kinase (8.5 μg), 4 nmol cyclic AMP, 1.4 μmol magnesium acetate, 120 nmol EGTA, in 30 mM sodium acetate buffer, *p*H 6.5, in a total volume of 350 μl at 30 °C for 2 h. The mixture was fractionated by preparative paper electrophoresis as described above and the paper sheet

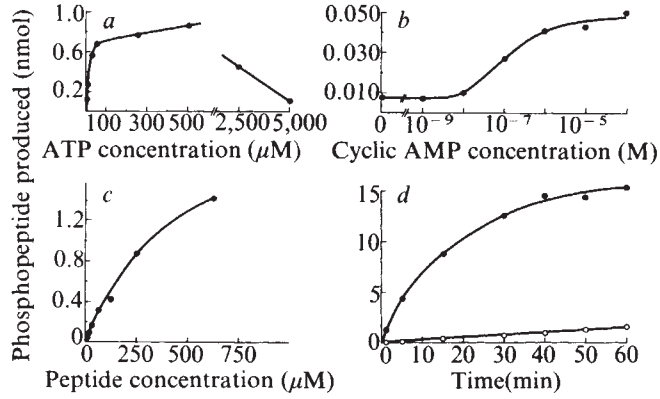

Fig. 1 Production of phosphorylated peptide Gly–Arg–Gly–Leu–Ser(P)–Leu–Ser–Arg; ordinate refers to the total nmol of phosphopeptide produced at 30 °C in each incubation tube in 5 min or, in *d*, for the time indicated. In *a*, *b* and *c* 10 μl of each of the solutions (i), (ii) and (iii) were incubated in separate tubes with 10 μl of cardiac protein kinase (85 μg ml^{-1}). Incorporation of ^{32}P into the peptide was assayed on 25 μl samples by the electrophoretic method[3]. *a*, Effect of ATP: (i) peptide in water (10 nmol per 10 μl); (ii) 14 mM magnesium acetate, 1.2 mM EGTA and 40 μM cyclic AMP, adjusted to *p*H 6.5, and (iii) γ-^{32}P-ATP (3.0×10^5 c.p.m. per 10 μl) mixed with ATP to give the final concentration indicated in 100 mM sodium acetate buffer, *p*H 6.5. *b*, Effect of cyclic AMP: (i) peptide in water (3.1 nmol per 10 μl); (ii) as in *a* but incorporating cyclic AMP to give the final concentration indicated; and (iii) 277 μM γ-^{32}P-ATP (2.5×10^5 c.p.m. per 10 μl) in buffer as in *a*. *c*, Effect of peptide: (i) peptide in water to give the final concentration indicated; (ii) as in *a*; and (iii) 2 mM γ-^{32}P-ATP (3.0×10^5 c.p.m. per 10 μl) in buffer as in *a*. *d*, Effect of time and cyclic AMP. Two tubes were prepared with the following solutions (i) 5 μl peptide in water (50 nmol per 10 μl); (ii) 50 μl as in *a* or in the second tube as in *a* but without cyclic AMP present; (iii) 50 μl 2 mM γ-^{32}P-ATP (1.4×10^6 c.p.m. 50 μl) in buffer as in *a*; and (iv) 50 μl cardiac protein kinase. At times indicated 20 μl were removed for assay.

Table 1 Sites of phosphorylation by cyclic AMP-dependant protein kinases

Substrate		Sequence	Sources of kinase	Ref.
Non-globular substrates				
Histone F1		Arg–Arg–Lys–Ala–Ser*–Gly–Pro	Liver	22, 23‡
Histone F2b		Ser–Arg–Lys–Glu–Ser*–Tyr–Ser	Lymphocytes	24
Myelin basic protein	Site 1	Gly–Arg–Gly–Leu–Ser*–Leu–Ser	Muscle	
	Site 2	Gln–Arg–His–Gly–Ser*–Lys–Tyr	and	
	Site 3	Arg–His–Arg–Asp–Thr*–Gly–Ile	Brain	4, 5, 6
RCMM Lysozyme†	Site 1	Tyr–Arg–Gly–Tyr–Ser*–Leu–Gly		
	Site 2	Arg–Asn–Thr–Asp–Gly–Ser*–Thr–Asp	Muscle	21
Protamine	Site 1	Arg–Arg–Arg–Ser*–Ser*–Ser*–Arg		
	Site 2	Ala–Arg–Arg–Val–Ser*–Arg–Arg	Testis	25, 26
Globular substrates				
Phosphorylase kinase	α subunit	Arg–Leu–Ser*–Ile–Ser		
	β subunit	Lys or Arg–Gln–Ser–Gly–Ser*– Val or Tyr Ile	Muscle	2
Troponin I	Site 1	Val–Arg–Met–Ser*–Ala–Asp		
	Site 2	Ser*–Val–Met	Muscle	18, 19
Pyruvate kinase (Liver)		Leu–Arg–Arg–Ala–Ser*–Leu	Liver	17
Glycogen synthetase		Lys or Arg–Gln–Ile–Ser*–Val	Muscle	27

*Amino acid residue phosphorylated.
†RCMM Lysozyme, reduced carboxymethylated maleylated lysozyme.
‡S. C. Rall and R. D. Cole, in ref. 23.

subjected to autoradiography. A zone of radioactivity with a relative mobility of +0.19 was detected. A sample of this zone stained the typical yellow colour given by glycyl peptides. The zone was eluted with 0.01 M NH_4OH and from the incorporated radioactivity and amino acid analysis 1 mol of phosphate per mol of peptide was calculated; the total yield of phosphopeptide was 71 nmol. Successive Edman degradations of the phosphopeptide and electrophoresis of the products at *p*H 6.5 showed that the phosphate was present as shown in the sequence Gly–Arg–Gly–Leu–Ser(P)–Leu–Ser–Arg. This result was supported by studies with aminopeptidase and thermolysin. An identical labelling pattern was obtained with bovine brain protein kinase.

In these experiments Offord's formula[9] was used to follow the change in electrophoretic mobility on the addition or removal of charged groups. We found that the average value for the change in charge on the addition of a phosphate group to a peptide was −1.35 at *p*H 6.5 in pyridine acetate buffer. When the phosphoserine was N-terminal, however, the phosphate group carried a more negative charge (approximately −1.6).

In kinetic experiments the cardiac kinase was used. Human myelin basic protein was used at the same molar concentration as the peptide to enable a comparison to be made. The effect of increasing ATP concentration on the production of phosphopeptide is shown in Fig. 1*a*. Michaelis–Menten kinetics were exhibited and from a double reciprocal plot a K_m value of 2.8×10^{-5} M was determined for ATP, similar to values reported for various protein kinases with proteins as substrates[12,13]. The K_m found with myelin basic protein was 6.8×10^{-6} M. At high concentrations of ATP, in excess of Mg^{2+}, there was a marked inhibition of the production of phosphopeptide. A similar effect observed with phosphorylase kinase[14,15] was attributed to the complexing of Mg^{2+} by ATP.

Half-maximal stimulation by cyclic AMP was produced at 10^{-7} M for both the synthetic peptide (Fig. 1*b*) and myelin basic protein as substrate. Values ranging from 0.4×10^{-7} to 3×10^{-7} M have been reported for a bovine cardiac protein kinase with histone (calf thymus type IIA) as substrate[12,13].

The effect of increasing concentration of synthetic peptide is shown in Fig. 1*c*. Again Michaelis–Menten kinetics were exhibited and from the linear double reciprocal plot a K_m of 2.4×10^{-4} M for the synthetic peptide with the cardiac enzyme was found. This value is similar to that reported for a peptic peptide substrate[3]. With myelin basic protein as substrate the K_m was lower (5.3×10^{-5} M). Miyamoto and Kakiuchi[16] found a K_m of 2×10^{-5} M for the protein with a brain kinase. The V_{max} for the synthetic peptide was 1.47 nmol ^{32}P incorporated per 5 min and for the protein 0.36 nmol ^{32}P incorporated per 5 min. It should be emphasised that in the protein both threonine and serine sites are subject to phosphorylation[5] (Table 1), whereas with the synthetic peptide only a single site was phosphorylated, thus the comparison of K_m and V_{max} is of limited value.

The effect of increasing time of incubation on the production of phosphopeptide in the absence and presence of cyclic AMP is shown in Fig. 1*d*. In 1 h very little phosphorylation of the peptide was observed in the absence of cyclic AMP. Thus it seems that the peptide is not able to dissociate the catalytic and regulatory subunits. A similar result was obtained with intact myelin basic protein whereas other studies[16] have reported activation of protein kinase by both this protein and histone. But another sample of the same cardiac kinase as used above that had been frozen and thawed repeatedly showed less cyclic

AMP dependence and much greater activation with time when incubated without cyclic AMP (compare ref. 12).

The synthetic peptide was digested with proteolytic enzymes[7] or subjected to Edman degradation[7] and the products examined for substrate activity. Removal of the C-terminal arginine with carboxypeptidase B yielded a peptide, with relative mobility +0.38, which was readily converted to a phosphopeptide with relative mobility −0.06. From the N-terminal end glycine could be removed by Edman degradation without apparent effect on substrate activity, whereas the arginine was essential for activity. This requirement for arginine was confirmed by tryptic digestion, which also destroyed the capacity of the peptide to be phosphorylated. It could be argued that the loss of activity was simply due to the resultant shortening of the peptide but the need for arginine, rather than a neutral amino acid, is supported by the following observations. Although there are numerous serine and threonine residues within quite large tryptic peptides from myelin basic protein, only a few peptides in the tryptic digest were phosphorylated. All the peptides which were not phosphorylated had lysine or arginine to the C-terminal side of the serines whereas those that were phosphorylated contained arginine to the N-terminal side in trypsin resistant bonds[6]. One of these was the peptide Gly-N^G-monomethyl Arg–Gly–Leu–Ser–Leu–Ser–Arg. Preliminary evidence indicated that when the arginine was in the N^G,N'^G-dimethyl form phosphorylation of the serine was prevented[5]. Moreover an examination of amino acid sequences around the site phosphorylated by cyclic AMP-dependent protein kinases in a number of non-globular and globular proteins (Table 1) shows that arginine is frequently found close to the serine that is phosphorylated.

Table 1 summarises the amino acid sequences in proteins phosphorylated by cyclic AMP-dependent protein kinases from a variety of tissues. Although in several cases the sequence Arg–X–Y–Ser–Z can be identified, there are also some instances where the arginine is only one residue removed from the serine or one further away. Hjelmquist *et al.*[17] suggested a similar pattern and also that the serine should be surrounded by hydrophobic residues. The latter concept is not fully supported by the data in Table 1. It is puzzling that phosphorylase kinase phosphorylates the same type of site in phosphorylase b and in troponin I yet these sites are not those phosphorylated by the cyclic AMP-dependent protein kinases[18,19], which act on other serines in these proteins (Table 1).

Using probability factors listed by Chou and Fasman[20] for each amino acid at each position, 1–4, in a β bend, the probability of the sequences listed in Table 1 taking up a β-bend conformation was calculated. Although in several, a high probability emerged for the serine being in position 4 in a β bend, such high probability values were also obtained for sequences around serines in the basic protein which are known not to be phosphorylated.

Sites of phosphorylation in myelin basic protein were independent of the source of the kinase; purified kinases from rabbit skeletal muscle and bovine brain and cardiac muscle all produced the same labelling pattern (Table 1). In some substrates more than one site is phosphorylated at variable rates[2,4,5,21]; however, no feature is apparent in the more readily phosphorylated sequences. This would suggest that once some minimum requirement is met, the rate of phosphorylation is governed by other factors.

Substrates in Table 1 have been categorised as non-globular and globular but no consistent differences are apparent in the sequences around the site of phosphorylation. Bylund and Krebs[21] have shown that lysozyme, phosphorylase b and bovine serum albumin contain certain serine residues which are quite inactive as substrates in the native form, but which are readily phosphorylated when the proteins are denatured. This implies that in the native structure the sequence around these particular serines is constrained from taking up the conformation necessary for interaction with the active site of the enzyme. The sequence Arg–X–X–Ser is not a rare sequence in proteins which do not act as substrates in their native form[2]. Whether the failure of the kinase to phosphorylate these serines is due to the lack of accessibility or to the arginine being too remote spatially from the serine remains to be determined. Whatever the three-dimensional arrangement of the enzyme–substrate complex, however, we predict the need for a basic amino acid in close proximity to the serine to be phosphorylated. Chemical and physical studies with other synthetic peptides could help solve this problem.

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Tubulin requires an accessory protein for self assembly into microtubules

Borisy and Taylor have demonstrated a correlation between the presence of the colchicine-binding protein, tubulin, and the microtubular system of eukaryotic cells[1]. Tubulin assembles *in vitro* into microtubules when a brain homogenate is warmed to 37 °C and is supplied with a cofactor, GTP (refs 2 and 3). These microtubules dissociate into tubulin subunits when the solution is chilled to 0 °C (refs 3 and 4), or Ca^{2+} is introduced[2,4]. Tubulin can thus be purified by repeated cycles of assembly and disassembly[4,5]. We have found, and others report similarly[6–10], that some very high molecular weight polypeptides copurify with the tubulin in these conditions.

We have examined the role of these polypeptides in the assembly process and found that separation of the high molecular weight fraction from tubulin prevents self assembly of the tubulin; the colchicine-binding property of the tubulin is un-