PEPTIDES FROM MYELIN BASIC PROTEIN AS SUBSTRATES FOR ADENOSINE 3', 5'-CYCLIC MONOPHOSPHATE-DEPENDENT PROTEIN KINASES

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Summary - A simple electrophoretic assay demonstrated that peptides from enzymic digests of the basic protein of human myelin were effective substrates for adenosine 3', 5'-cyclic monophosphate-dependent protein kinases from bovine cardiac muscle and brain. From a peptic digest a peptide of 17 amino acid residues was isolated and when used as a substrate a K_m of 1.9×10^{-4}M was found for the cardiac kinase.

A major problem in understanding the biological role of cAMP-dependent protein kinases is how they recognise specific serine or threonine residues within substrate proteins. Langan (1) suggested that the tertiary structure could be of importance whereas others (2,3) considered that the enzyme may recognise some feature of the primary structure of the substrate. In attempts to resolve this problem we are studying the phosphorylation of the basic protein of myelin from the central nervous system. This protein is a good substrate for cAMP-dependent protein kinases (4,5,6,7) and its amino acid sequence is well established for a number of species (8,9,10).

MATERIALS

Human myelin basic protein was prepared and enzymically digested as described previously (8,9,10). Cyclic AMP-dependent protein kinase was prepared from bovine cardiac muscle and purified on DEAE-cellulose (11). The fraction eluting between 160 and 180mM potassium phosphate buffer, pH 7.0, was used after dialysis against 5mM potassium phosphate buffer, 2mM Na_2EDTA,

pH 7.0. It contained 0.33 mg protein/ml and with histone (Sigma Type IIA) or myelin basic protein as substrates it transferred 4.2 and 3.4 nmoles of P_i/mg substrate protein respectively, when incubated for 5 min at 30°C with the solutions specified in Table 1. [γ-^{32}P]ATP was prepared by the method of Glynn and Chappell (12) and had a specific activity of 2-2.5 Ci/mmole. It was diluted with ATP as described in the text.

METHODS

Electrophoretic assay for peptide substrates. A sample (e.g. 25 µl) of the incubation mixture (Table 1) was rapidly dried onto 1 cm bands on a sheet of Whatman 3MM paper (57 x 34 cm) along a line 37 cm from the anode end (Fig. 1). The paper was carefully saturated with pH 6.5 buffer (pyridine:acetic acid:water, 100:3:897, by vol.), and the excess moisture throughly removed with blotting paper. Electrophoresis (1800V, 60-90mA at 1°C) was carried out in a "Pherograph" apparatus (Frankfurt) converted to a liquid cooled system by adding a 1 cm depth of "Freon" TF (Du Pont, Delaware) to the cooling plate and applying a heavy glass plate onto the paper. Electrophoresis in a tank cooled with petroleum spirit was equally effective but there was more diffusion of the spots at the higher temperature (25°C). The paper was autoradiographed for 18-48 hr with Kodak RP/S X-Omat X-ray film. All the radioactive spots were cut out and counted in a Packard scintillation counter with a Triton X-100-toluene scintillation cocktail. Results for each spot were expressed as percent of total radioactivity recovered and in selected cases as pmoles P_i incorporated per mole of peptide.

RESULTS

Electrophoretic assay for protein kinase. Paper electrophoresis at pH 6.5 provided a simple method for following the phosphory-

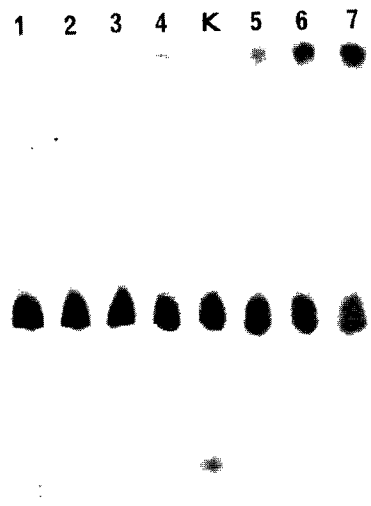

Figure 1. Electrophoretic assay
Autoradiography of electrophoretogram of phosphorylation of increasing amounts of peptic peptide P1 from myelin basic protein as described in Fig. 2B. 1, 0.5 nmoles P1; 2, 1.0 nmoles; 3, 1.5 nmoles; 4, 2.5 nmoles; 5, 6.25 nmoles; 6, 12.5 nmoles; 7, 25 nmoles; and K, no peptide present. Spots in ascending order represent P_i, ATP, protein kinase or a protein in the kinase preparation and peptide Q, the phosphorylated form of peptide P1.

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lation of protein and peptide substrates (Fig. 1, Table 1). It gave similar results to the usual precipitation assays for protein substrates and had the added advantage that changes in ATP concentration could be monitored (Fig. 2A) and thus any contaminating ATPase or phosphoprotein phosphatase activity readily detected. With a series of six duplicate samples of enzymic digests of the myelin basic protein, results were reproducible to within ± 2.3%.

Peptides as substrates for protein kinase. Tryptic, thermolytic and peptic digests of myelin basic protein functioned as substrates for protein kinase (Table 1). Under identical incubation conditions for phosphorylation the peptic and

TABLE 1

Phosphorylation of myelin basic protein and enzymic digests by cAMP-dependent protein kinase from cardiac muscle.

Incubation	Substrate	Percent ^{32}P incorporated	pmoles P_i incorporated	moles P_i/mole
1	Endogenous substrate			
	Precipitation assay	7.0	109	-
	Electrophoretic assay	5.4	84	-
2	Human myelin basic protein			
	Precipitation assay	82.5	1279	0.1
	Electrophoretic assay	82.4	1277	0.1
3	Tryptic digest of basic protein	40.3	625	0.05
4	Thermolytic digest of basic protein	74.8	1159	0.09
5	Peptic digest of basic protein	72.5	1124	0.09
5	Main ^{32}P-peptide, Q, in peptic digest	33.1	513	0.04

To incubations 2, 3, 4 and 5 were added 50µl of basic protein of enzymic digests, each 5 mg/ml in 100mM sodium acetate buffer, pH 6.5; 50µl of buffer was added to incubation 1. Incubations also contained 50µl of each of solutions (b), (c) and (d) as specified in Fig. 2A and were incubated for 1 hr at 30°C. For the precipitation assay 25µl of the incubation mixture was treated as described by Li and Felmly (13). For the electrophoretic assay 40µl of incubation mixture was applied to the electrophoresis paper.

thermolytic digests were almost as good substrates as the intact protein. The phosphorylated peptic digest was examined by paper electrophoresis at pH 6.5 and chromatography in butan-1-ol: pyridine:acetic acid:water (30:20:6:24, by vol.). From this the distribution of ^{32}P in the peptides was determined. One peptide,

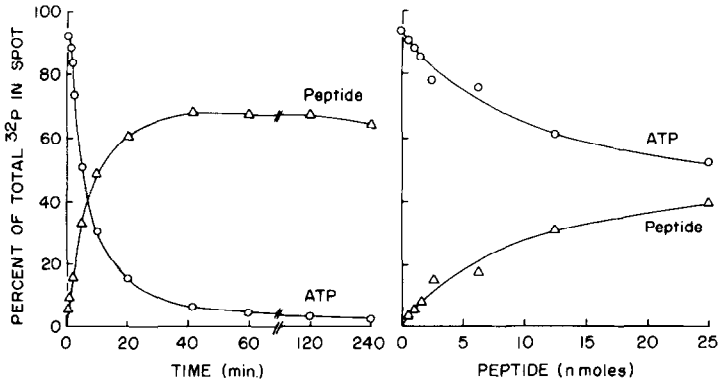

Figure 2. Phosphorylation of peptide P1

A) Time: Separate tubes were incubated for each time interval. The incubation mixture contained in each tube 10µl of each of the following solutions:

(a) peptide P1 in H_2O (25 nmoles/100µl);
(b) 14mM magnesium acetate, 1.2mM EGTA (ethylene glycol bis (β-aminoethyl ether)-N,N' tetraacetic acid) and 40µM cAMP;
(c) 31µM [γ-^{32}P]ATP (1.5 x 10^5 cpm per 10µl) in 100mM sodium acetate buffer, pH 6.5;
(d) cardiac protein kinase (0.33 mg/ml).

At appropriate times tubes were removed from the constant temperature bath (30°C) and 25µl applied to the electrophoresis paper.

B) Concentration: The incubation mixture in each tube contained varying amounts of peptide P1 (in 10µl) and the [γ-^{32}P]ATP solution added at 111µM (1.4 x 10^5 cpm per 10µl). Incubation time was 5 min. Other conditions were as for 2A.

Q, contained 33% of the total radioactivity incorporated into the digest.

Peptic peptides were isolated from the unphosphorylated protein (8,10) and tested for substrate activity with the protein kinase. Five basic peptides showed some substrate activity and the most readily phosphorylated corresponded, after phosphorylation, to peptide Q. This peptide had electrophoretic and chromatographic properties, and amino acid composition which were identical to those of peptide P1 from myelin basic protein (10). The amino acid sequence of peptide P1 is Acetyl-

Ala-Ser-Gln-Lys-Arg-Pro-Ser-Gln-Arg-His-Gly-Ser-Lys-Tyr-Leu-Ala-Thr.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

Phosphorylation of peptide P1. Peptide P1 was selected for detailed study. In Fig. 2A the transfer of ^{32}P to peptide P1 is demonstrated as a function of time. It is obvious that under the conditions used the concentration of ATP rapidly became limiting. In Fig. 2B where more ATP was available for the phosphorylation of P1, the enzyme was shown to exhibit Michaelis-Menten kinetics with a K_m of 1.9×10^{-4}M for peptide P1. This compared with a K_m of 2×10^{-5}M for the whole protein determined by the electrophoretic assay, and also reported by Miyamoto and Kakiuchi (6) using a precipitation assay. Under the conditions used in Fig. 2B a maximum of 0.09 mole P_i was incorporated per mole of peptide. When 12.5 nmole P1 was incubated for 1½ hr with 14 nmole ATP a total of 0.35 mole P_i per mole was incorporated.

A preparation of protein kinase from bovine brain, which eluted from DEAE cellulose between 180 and 205mM potassium phosphate buffer, pH 7.0, also phosphorylated several peptic peptides, including peptide P1, in the presence of cAMP but not in its absence.

Mild acid hydrolysis (4) showed the presence of phosphoserine in peptide Q. With the intact protein serine-12 was a major site of phosphorylation by rabbit muscle protein kinase (4); whether the cardiac and brain kinases also phosphorylated this site remains to be determined.

DISCUSSION

The use of the electrophoretic assay for protein kinase clearly demonstrated that cAMP-dependent protein kinases could phosphorylate quite small peptides. It is noteworthy that no

isolated acidic or neutral peptides were phosphorylated although they contained several serine and threonine residues. The ability of peptides to act as substrates indicated that the kinase recognises some feature of the primary structure of proteins around the serine to be phosphorylated. However, some specific conformation of the peptide chain close to the site of phosphorylation may also be necessary.

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