Preferential ADP-ribosylation of arginine-3 in synthetic heptapeptide Leu-Arg-Arg-Ala-Ser-Leu-Gly

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Hen liver nuclear ADP-ribosyltransferase modified the synthetic heptapeptide Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) at arginine-2 and/or arginine-3. Trypsin treatment of ADP-ribosyl-Kemptide revealed that the ADP-ribosylation of arginine-3 was constantly more abundant than that of arginine-2. ADP-ribosylation of Kemptide suppressed the subsequent phosphorylation by cyclic AMP-dependent protein kinase.

INTRODUCTION

Eukaryotic ADP-ribosyltransferase, which catalyses the transfer of mono-(ADP-ribose) moiety from NAD⁺ to arginine residue(s) in specific acceptor proteins or to small molecular guanidino compounds, forming the mono-(ADP-ribose)-acceptor conjugates, has been found in various tissues and cells (Ueda & Hayaishi, 1985). The enzymes from turkey erythrocytes (Moss *et al.*, 1980) and hen liver nuclei (Tanigawa *et al.*, 1984) have been purified and characterized.

We found that the ADP-ribosylation of whole histones from the calf thymus by hen liver nuclear ADP-ribosyltransferase suppressed the subsequent phosphorylation by cyclic AMP-dependent protein kinase (Tanigawa *et al.*, 1983). Similar results were obtained with both phosphorylase kinase (Tsuchiya *et al.*, 1985) and the L-type pyruvate kinase (Matsuura *et al.* 1988), the activities of which were regulated by phosphorylation-dephosphorylation reactions.

The synthetic heptapeptide Kemptide, corresponding to the pig liver pyruvate kinase phosphorylation site, has been generally used to study the kinetics and molecular bases of the phosphorylation by cyclic AMP-dependent protein kinase (Kemp *et al.*, 1977). Because the target amino acid for hen liver nuclear ADP-ribosyltransferase was identified to be arginine in Kemptide (Shimoyama *et al.*, 1985) and because two arginine residues present in the sequence at the N-terminal side of phosphorylation site in Kemptide were reported to play important roles in cyclic AMP-dependent phosphorylation (Kemp *et al.*, 1977), we used this peptide to further evaluate the possible relationship between the ADP-ribosylation and cyclic AMP-dependent phosphorylation. We now report that the ADP-ribosylation of either arginine residue in Kemptide completely loses its potential to serve as acceptor for the cyclic AMP-dependent protein kinase.

EXPERIMENTAL

Materials

Kemptide was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The amount of Kemptide was determined from the amino acid analysis of acid

hydrolysates, with respect to an amino acid standard (Wako standard type H).

Purification of enzymes

The ADP-ribosyltransferase purified from hen liver nuclei had a specific activity of 18.6 μ mol/h per mg under the conditions described previously (Tanigawa *et al.*, 1984). The catalytic subunit of cyclic AMP-dependent protein kinase (7500 units/mg) was purified from pig liver (Berglund *et al.*, 1977).

Reverse-phase h.p.l.c.

Reverse-phase h.p.l.c. was conducted in a Waters model 510 instrument with a Waters Nova-Pak C₁₈ column (8 mm $\times$ 100 mm, 5 μ m particle size). For identification of the ADP-ribosylated and/or phosphorylated Kemptide, the sample was injected into the column equilibrated with aq. 0.1% (v/v) trifluoroacetic acid and the chromatogram was developed with a linear 0–30% (v/v) gradient of acetonitrile in 0.1% trifluoroacetic acid over 30 min at a flow rate of 1 ml/min. The effluent was monitored at 214 nm with a Waters M-490 multi-wavelength detector and collected at the fraction size of 0.5 ml. The radioactivity in each fraction was measured with a liquid-scintillation counter. For analyses of the proteinase-digested peptides of ADP-ribosyl-Kemptide, the sample was injected into the reverse-phase column equilibrated with 20 mM-phosphate buffer, pH 7.2, and the elution was carried out with a linear 0–7.5% (v/v) gradient of acetonitrile in 20 mM-phosphate buffer, pH 7.2, over 15 min at a flow rate of 1 ml/min, followed by elution under isocratic conditions with 7.5% (v/v) acetonitrile in the same buffer over 15 min at a flow rate of 0.5 ml/min. The effluent was monitored at 214 nm and collected every 0.5 min.

ADP-ribosylation of Kemptide

Kemptide (6 nmol) was incubated with 1 mM-[adenylate-³²P]NAD⁺ (8500 c.p.m./nmol) and ADP-ribosyltransferase (0.1 μ g) in a mixture containing 50 mM-Tris/HCl buffer, pH 9.0, and 1 mM-dithiothreitol in a total volume of 0.05 ml at 25 °C. After incubation for

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10 min, a sample (0.04 ml) was analysed by reverse-phase h.p.l.c. as described above. Radioactive peaks eluted at 22.6 and 21.1 min were designated K_1 and K_2 respectively.

Proteinase digestion of K_1

K_1 (6.48 nmol as [32 P]ADP-ribose) was treated with L-tosylphenylalanylchloromethane-treated trypsin (0.1 μ g) in 100 mM-Tris/HCl buffer, pH 7.0, in a total volume of 0.05 ml at 37 °C for 2 h. A sample (0.04 ml) was separated by reverse-phase h.p.l.c. with the phosphate buffer system. Five peaks were designated T_1 to T_5 in the order of their elution from the column (17.0, 18.9, 24.1, 25.8 and 26.6 min respectively). T_3 (1.25 nmol as [32 P]ADP-ribose) was treated with carboxypeptidase B (0.1 μ g) in a mixture containing 12.5 mM-Tris/HCl buffer, pH 7.0, and 25 mM-NaCl in a total volume of 0.05 ml at 37 °C for 30 min. A sample (0.04 ml) was analysed by reverse-phase h.p.l.c. with the phosphate buffer system.

Phosphorylation of Kemptide

Kemptide (6 nmol) was preincubated with 1 mM unlabelled NAD^+ instead of [$adenylate$ - 32 P] NAD^+ and ADP-ribosyltransferase (0.1 μ g) in a total volume of 0.05 ml for the indicated time at 25 °C. A sample (0.04 ml) was separated by reverse-phase h.p.l.c. with the acetonitrile system, and the products eluted at 20–26 min were pooled, freeze-dried and dissolved in 0.1 ml of water. A sample (0.05 ml) was incubated with 20 units of cyclic AMP-dependent protein kinase and 0.25 mM- $[\gamma$ - 32 P]ATP (4980 c.p.m./nmol) in a mixture containing 50 mM-Mes/NaOH buffer, pH 6.8, 10 mM-magnesium acetate and 0.2 mM-EGTA for 3 min at 30 °C in a total volume of 0.1 ml. A sample (0.05 ml) was analysed by reverse-phase h.p.l.c. with the acetonitrile system. Phosphorylated Kemptide was eluted at 22.7 min and the radioactivity was measured.

RESULTS

ADP-ribosylation of Kemptide

A synthetic heptapeptide, Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly), was incubated with [$adenylate$ - 32 P] NAD^+ and hen liver nuclear ADP-ribosyltransferase for 10 min at 25 °C, and the products were analysed by reverse-phase h.p.l.c. Two new radioactive peaks were detected at the retention times of 22.6 min (K_1) and 21.1 min (K_2). The non-radioactive peak eluted at 7.2 min proved to be nicotinamide. Since two arginine residues at positions 2 and 3 are present in the sequence of Kemptide, arginine-2 and/or -3 may be modified. K_1 and K_2 contained 1.0 and 0.5 mol of Kemptide per mol of ADP-ribose, as estimated from the radioactivity measurements in K_1 and K_2 used for amino acid contents, respectively. These results support our proposal that K_1 and K_2 contained Kemptide modified with mono- and bis-ADP-ribose respectively.

Fig. 2 shows the time courses of ADP-ribosylation in K_1 and K_2 . ADP-ribosylation in K_2 increased in the incubation time and reached a plateau at 60 min of incubation. ADP-ribosylation in K_1 reached the maximum at incubation for 10 min and then waned. The question arises as to which of the two arginine residues in Kemptide is preferentially and initially modified with the mono-(ADP-ribose) moiety. The following experiments were then carried out.

Fig. 1. Reverse-phase h.p.l.c. of ADP-ribosyl-Kemptide

Kemptide (6 nmol) was incubated with 1 mM- $[adenylate$ - 32 P] NAD^+ (8500 c.p.m./nmol) and ADP-ribosyltransferase (0.1 μ g) in a total volume of 0.05 ml at 25 °C for 10 min. A sample (0.04 ml) was analysed by reverse-phase h.p.l.c. with a linear 0–30% (v/v) gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid over 30 min at a flow rate of 1 ml/min. The profile shows the absorbance at 214 nm as well as radioactivity in 0.5 ml fractions indicated by bars.

Fig. 2. Time course of ADP-ribosylation in K_1 and K_2

ADP-ribosylation in K_1 (●) and K_2 (○) was determined from radioactivity measurements in reverse-phase h.p.l.c. Data are the means for three representative experiments.

Determination of the sites of ADP-ribosylation in Kemptide

The radioactive K_1 obtained from incubation for 10 min under the standard ADP-ribosylation conditions was exhaustively treated with L-tosylphenylalanylchloromethane-treated trypsin and the products were analysed by reverse-phase h.p.l.c. with the phosphate buffer system. As shown in Fig. 3(a), T_3 , T_4 and T_5 were radioactive, indicating that these peaks contained trypsin-directed ADP-ribosyl-peptides or ADP-ribosyl-arginine. Each of T_1 – T_5 was isolated and hydrolysed to determine

Fig. 3. Reverse-phase h.p.l.c. of trypsin- (a) and/or carboxypeptidase B- (b) treated peptides of K_1

(a) K_1 was treated with trypsin and the products were analysed by reverse-phase h.p.l.c. as described in the Experimental section. (b) T_3 was treated with carboxypeptidase B and the products were analysed by reverse-phase h.p.l.c. as described in the Experimental section. The radioactive peak eluted at 18.0 min is designated T_6 . The profile shows the absorbance at 214 nm as well as radioactivity in fraction indicated by bars.

the amino acid composition. T_1 and T_2 were non-radioactive and contained Leu-Arg and Ala-Ser-Leu-Gly respectively. T_3 contained Leu-Arg-Arg. To determine which arginine residue was ADP-ribosylated, T_3 was then treated with carboxypeptidase B. As shown in Fig. 3(b), in addition to the unhydrolysed T_3 , another radioactive peak (T_6) was eluted at 18.0 min from the column and the amino acid sequence proved to be Leu-Arg. Thus we concluded that the site for the ADP-ribosylation in T_3 was arginine-2. T_4 and T_5 consisted of the same peptide, Arg-Ala-Ser-Leu-Gly, and they were interconvertible with each other. We reported previously that α - and β -anomers of ADP-ribosyl-arginine are interconvertible and can be separated by reverse-phase h.p.l.c. (Tsuchiya *et al.*, 1986). Therefore we assumed that T_4 and T_5 were anomers of ADP-ribosyl-Arg-Ala-Ser-Leu-Gly.

Using the procedure described above, by which the differentiation of ADP-ribosylation sites for arginine-2 or -3 in K_1 was made feasible, we attempted to determine which arginine site was preferentially modified in K_1 . The ADP-ribosylation of arginine-3 was constantly more abundant than that of arginine-2 during the incubation time for ADP-ribosylation (results not shown).

Suppression of the subsequent phosphorylation of ADP-ribosyl-Kemptide

K_1 containing Kemptide ADP-ribosylated at arginine-2 and -3 at a ratio of 1:1.98 was subsequently phosphorylated with cyclic AMP-dependent protein

Fig. 4. Suppression of phosphorylation of Kemptide by ADP-ribosylation

Average values for triplicate determinations are expressed as percentages of the phosphorylation without preincubation (0.246 nmol/3 min).

kinase and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. K_1 could not serve as an acceptor for the cyclic AMP-dependent phosphorylation. The same results were obtained with K_2 . Then Kemptide was preincubated with unlabelled NAD^+ and ADP-ribosyltransferase and the product was subsequently phosphorylated by cyclic AMP-dependent protein kinase. Fig. 4 shows the drastic suppression of phosphorylation of Kemptide with increasing ADP-ribosylation. A significant phosphorylation was not detected when ADP-ribosylation occurred during preincubation for 15 min.

DISCUSSION

Mono-(ADP-ribosylation) of Kemptide showed a different sensitivity for trypsin. Trypsin cleaved the arginyl-alanine bond alone when arginine-2 was ADP-ribosylated, but the proteinase hydrolysed only the arginyl-arginine bond when arginine-3 was modified. Kemp *et al.* (1977) noted the importance of arginine residues at the *N*-terminal side of the phosphorylation-site serine residue in the substrate-specificity of the cyclic AMP-dependent protein kinase, and that when either of the arginine residues was replaced with alanine the apparent K_m value for the respective peptide greatly increased. We found that ADP-ribosyl-Kemptide could not serve as acceptor for the cyclic AMP-dependent phosphorylation. We propose that when ADP-ribose is attached to the peptide a negative charge (e.g. phosphate group) may be introduced, and hence a marked alteration of accessibility of the peptide to trypsin or cyclic AMP-dependent protein kinase may occur.

A number of NAD^+ -dependent ADP-ribosylation systems, such as bacterial toxins, may affect cellular metabolism by modifying critical processes (Ui, 1984). Endogenous ADP-ribosylation reactions have been noted in eukaryotes (Ueda & Hayaishi, 1985); however, the biological function of the modification has yet to be determined. Further studies will be required to obtain support for our hypothesis that ADP-ribosylation and de-ADP-ribosylation may regulate an important pathway *in vivo*.

Since this paper was submitted a report has appeared

that indicates that cholera toxin preferentially ADP-ribosylates arginine-2 in Kemptide (Kharadia & Graves, 1987). These results show that the preferential ADP-ribosylation sites in Kemptide by hen liver nuclear ADP-ribosyltransferase and cholera toxin are not identical.

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