ADP-ribosylation of membrane proteins of *Streptomyces griseus* strain 52-1

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1. SUMMARY

Membranes purified from cells of *Streptomyces griseus* strain 52-1 possess an ADP-ribosyltransferase activity. The enzyme transfers the ADP-ribose moiety of NAD to one major membrane protein of $M_r$ 32,000 and 2–3 minor proteins of larger molecular weights. The effects of inhibitors on the ADP-ribosyltransferase activity proves that the reaction is enzymatic and suggests that the enzyme ADP-ribosylates the guanidine group of arginine. The kinetics of liberation of ADP-ribose during alkaline hydrolysis of the modified proteins is consistent with the arginine-ADP-ribose bond. This is the first report of ADP-ribosylation of proteins in a Gram-positive bacterium.

2. INTRODUCTION

Post-translational modification of proteins by ADP-ribosylation is an important mechanism for control of physiological processes in both eukaryotic and prokaryotic cells (for reviews [1,2]). ADP-ribosylation of proteins is widespread in eukaryotic cells and plays a role in regulating cell proliferation and differentiation, modification of chromatin structure and DNA repair [3–5]. Virulence of several pathogenic bacteria is attributable to production of exotoxins that catalyze ADP-ribosylation of specific host proteins (for reviews [6–8]). Only few reports have been made of ADP-ribosylation of proteins in prokaryotic cells [9]. Infection of *Escherichia coli* by T4 and N4 phages results in ADP-ribosylation of a ribonucleic acid polymerase molecule [10]. ADP-ribosylation of proteins was also reported to occur in uninfected *E. coli* cells [11]. Nitrogenase activity in *Rhodospirillum rubrum* [12] and *Azospirillum lipoferum* [13] is repressed by ADP-ribosylation of the iron-protein subunit of the enzyme. Two cytoplasmic proteins of *Pseudomonas maltophilia* were reported to be ADP-ribosylated [14]. The physiological consequence of modification of these proteins is not known. In this communication we present evidence that several membrane-bound proteins of *Streptomyces griseus* are ADP-ribosylated by a transferase activity present in the membrane preparations.

3. MATERIALS AND METHODS

3.1. Membrane preparation

Cells of *S. griseus* strain 52-1 obtained from the Institute of Biology, Debrecen, Hungary were grown at 30°C for 48 h in the synthetic medium...
described previously [15]. Cytoplasmic membranes were isolated and purified using the procedure described by Barabas et al. [16].

3.2. ADP-ribosylation assays

A 50 μl sample of membranes (2.8 mg ml⁻¹ protein) was suspended in 50 μl of a solution containing 0.025 M potassium phosphate buffer pH 7.0, 10 mM dithiothreitol, 0.5 mM MgCl₂, 29 mM KF, 0.1 mM CaCl₂ and 30 μM ³²P-NAD (2 × 10⁶ dpm) (I.C.N.). Following incubation at 37°C for 20 min, 100 μl of a mixture of 0.4% (w/v) SDS and 4 M urea was added. Next, 200 μl of 20% (w/v) trichloroacetic acid (TCA) was added. Following incubation in ice for 30 min the samples were centrifuged at 10 000 x g at 4°C for 10 min. The protein precipitates were washed twice with cold diethylether to remove TCA and then suspended in 50 μl of 10% β-mercaptoethanol. Proteins were analyzed by SDS–polyacrylamide gel electrophoresis using the procedure of Laemmli [17]. The stacking and running gels contained 4% and 12% acrylamide, respectively. Following electrophoresis the gels were fixed, dried and placed on Kodak X-Omat film using an intensifying screen (DuPont). Exposure was 3–8 days at −80°C.

The effects of inhibitors on ADP-ribosylation of membrane proteins was determined by incubating 100 μl of membrane preparation (0.28 mg protein) with 50 μM ³H-NAD (0.5 × 10⁶ dpm) (Amersham) for 30 min at 30°C. A 10 μl sample of 20% TCA was then added. The precipitated proteins were transferred to glass fiber filters and washed with cold 5% TCA, 70% ethanol and ether. Radioactivity was counted using a Beckman liquid scintillation counter.

3.3. Alkaline hydrolysis of ADP-ribose-protein

Susceptibility of the ADP-ribose-protein bond to alkaline hydrolysis was determined. Membrane samples labeled with ³H-NAD as described above were incubated with 0.1 N NaOH at 37°C. At various times, samples were removed and proteins were precipitated with TCA (5% final concentration). After 2 min centrifugation in a minifuge, the supernatant fluids were analyzed by high performance liquid chromatography using the method of Leoncini et al. [18]. Column effluent fractions of 1.0 ml were collected and their radioactive content was measured with a scintillation counter.

3.4. ADP-ribosylation of agmatine

ADP-ribosyltransferase activity of membranes was tested using agmatine as acceptor using a modification of the procedure of Moss et al. [19]. A 5.0 μl sample of membrane protein (0.16 mg protein) was incubated in mixture (300 μl) containing 25 mM potassium phosphate buffer pH 7.0, 30 mM agmatine, 5 mM dithiothreitol, 20 mM KF, 20 mM phenylmethylsulfanyl fluoride and 0.5 mM ³²P-NAD (2 × 10⁶ dpm). As a control for identification of ADPR-agmatine, the reaction mixture above was incubated with 2.0 μg of cholera toxin instead of membranes. The cholera toxin had been activated with 20 mM dithiothreitol [20].

Incubation was for 2 h at 37°C. At that time 10 μl samples were applied to a Silica Gel 60F₂₅₄ thin layer plate (Merck). The plate was developed with n-butanol–acetone–glacial acetic acid–5% (v/v) NH₄OH–H₂O (9 : 7 : 5 : 5 : 2) for 6 h. After drying, the plate was placed on Kodak X-Omat film with intensifying screen and incubated at −80°C for 2 h.

4. RESULTS

The results of SDS–PAGE analyses of labeling of membrane proteins by ADP-ribosylation are shown in Fig. 1. Short term exposure of the gel to X-ray film revealed that one distinct protein of molecular weight 32 000 Da was labeled with ³²P-NAD. Exposure of the gel for 8 days revealed a less intensely labeled protein with a molecular weight of 48–50 000 Da and two large proteins of approximately 84 000 and 90 000 Da.

These data show clearly that several membrane proteins are labeled during incubation with ³²P-NAD but do not prove that this is due to ADP-ribosylation. Non-enzymatic attachment of ADP-ribose, generated by NAD-glycohydrolase activity to NH₂ groups of proteins is known to occur [21]. Extracellular and intracellular (both soluble and membrane bound) NAD-glycohydrolase activity occurs in many strains of S. griseus [22,23] including...
Fig. 1. Autoradiogram of SDS-PAGE separation of ADP-ribosylated membrane proteins of S. griseus 52-1. Purified membranes were incubated with $^{32}$P-NAD for 20 min, then analyzed by SDS-PAGE. The gel was dried and placed on X-ray film. Lane 1 shows the results of 3 days exposure. Lane two is 8 days exposure. Positions of standard molecular weight proteins are shown at the right side.

In order to prove that the labeling of the membrane proteins was due to ADP-ribose-transferase activity, the nature of the transfer reaction and modified proteins were further investigated. Four inhibitors known to interfere with enzymatic ADP-ribosylation reactions were tested for their affect on incorporation of $^3$H-NAD into proteins (Table 1). Each of the inhibitors partially blocked incorporation of ADP-ribose from NAD into the membrane proteins. The molecules at the concentrations tested had essentially no effect on the NAD-glycosylase activity of the membrane preparations (data not shown). These results indicate that the incorporation of adenine labeled NAD into membrane proteins is a result of ADP-ribose transferase activity. Phenylglyoxal does not inhibit ADP-ribose transferase directly but instead blocks guanidine groups of the acceptor protein molecules [24]. The inhibition by phenylglyoxal transferase activity in the membranes involves modification of proteins at arginine residues.

Analyses of the products of alkaline hydrolysis of membranes that had been labeled with $^3$H-NAD showed only one compound, ADP-ribose, to be radiolabeled (Fig. 2). Kinetics of hydrolysis of the ADP-ribose-protein bond showed a half-life...
ADP-ribose that is released by the NAD-glycohydrolase activity. Lane 5 shows that activated cholera toxin hydrolyses NAD to a small amount of ADP-ribose and forms the two isomeric forms of ADP-ribose-agmatine. These data show clearly that the ADP-ribosylation activity of the membrane preparation of *S. griseus* 52-1, like cholera toxin, contains enzymatic activity that transfers ADP-ribose from NAD to the guanidine group of agmatine. The protein ADP-ribosylating activity of the membrane can thus be assumed to be due to an ADP-ribosyltransferase that modified arginine residues of proteins.

**5. DISCUSSION**

Several membrane-associated proteins of *S. griseus* 52-1 are ADP-ribosylated by an ADP-ribosyltransferase which is also a membrane protein. The membranes also contain NAD-glycohydrolase activity. We do not yet know whether the same or separate enzymes catalyze the two reactions. There is a precedent for two separate activities [9]. ADP-ribosylating bacterial toxins show NAD-glycohydrolase activity in the absence of acceptor molecules [8].

Continuing studies are directed to the question of the function of the ADP-ribosylating activity. Some preliminary results with *S. griseus* NRRL 2682, an organism that sporulates somewhat synchronously in submerged culture [25], show changes in the patterns of ADP-ribosylation of both soluble and membrane proteins. These changes correlate with timing of sporulation of the cells (unpublished observations).

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REFERENCES