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ADP-Ribosylation of an ~70-Kilodalton Protein of *Klebsiella pneumoniae*

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An ~70-kDa protein in the culture supernatant of a human pathogenic strain of *Klebsiella pneumoniae* was labeled in the presence of [^{32}P-adenylate]NAD. Labeling was significantly increased by the addition of dithiothreitol (>1 mM) but prevented by treatment of the culture supernatant for 3 min at 56°C. The addition of unlabeled NAD, but not of ADP-ribose, blocked labeling of the ~70-kDa protein. The radioactive label was released by formic acid but not by $HgCl_2$ (1 mM) or neutral hydroxylamine (0.5 M). The addition of homogenates of human platelets, human neutrophils, rat brain, rat lung, or rat spleen tissues to the culture supernatant did not induce labeling of eukaryotic proteins. The data indicate that the *K. pneumoniae* strain produces ADP-ribosyltransferase which modifies an endogenous protein.

Several bacterial toxins cause dramatic changes in eukaryotic cell function by ADP-ribosylation of regulatory proteins (for a review see reference 2). Among these toxins are cholera toxin (11) and pertussis toxin (12, 25), which modify G proteins. Diphtheria toxin (8) and *Pseudomonas aeruginosa* exotoxin A (26) ADP-ribosylate elongation factor 2, and another group of ADP-ribosylating toxins (*Clostridium botulinum* C2 toxin, *Clostridium perfringens* iota toxin, *Clostridium spiroforme* toxin) modifies actin (1, 5, 9, 18). Moreover, several bacterial exoenzymes which ADP-ribosylate small GTP-binding proteins of the rho family have been identified (4, 6, 13, 23). However, mono-ADP-ribosylation is not restricted to the action of toxins. Recently, it has been shown that bacterial ADP-ribosyltransferases play a role in endogenous regulation. In *Rhodospirillum rubrum*, reversible ADP-ribosylation regulates dinitrogenase activity, which is responsible for reduction of N_2 to ammonium (15, 27). ADP-ribosylation of dinitrogenase reductase inhibits activity. Conversely, the reductase is activated by the removal of the ADP-ribose group by glycohydrolase. Moreover, some bacterial glutamine synthetases appear to be targets for endogenous ADP-ribosylation (14, 20, 27). Finally, various not-well-characterized proteins in *Streptomyces griseus* (17, 19, 21) and *Pseudomonas maltophilia* (*Stenotrophomonas maltophilia*) (10) have been shown to be modified by ADP-ribosylation. Here we report the identification and characterization of ADP-ribosyltransferase activity in the culture supernatant from a clinical isolate of *Klebsiella pneumoniae.*

MATERIALS AND METHODS

Materials. [^{32}P]NAD and [^{32}P]ATP were obtained from NEN-Du Pont (Dreieich, Germany). All other reagents were analytical grade and were purchased from various commercial sources.

Cultures. Strains of different bacteria were grown in tryptic soy broth, Mueller-Hinton broth, brain heart infusion broth, Brewer modified thioglycolate broth, gram-negative broth, selenite broth, and Schaedler broth for 1 to 13 days at 35°C with 5% CO_2. Thereafter, the cultures were centrifuged to remove cells, and the supernatants were sterilized by filtration (0.22-μm-pore-size filters); the sterile filtrates were subsequently used in the ADP-ribosylation assay. *K. pneumoniae* was identified by the presence of gram-negative rods and typical growth on blood and MacConkey agar and by typical biochemical analysis (API 20E; bioMérieux, Nürtingen, Germany).

ADP-ribosylation assay. The filter-sterilized culture supernatants were incubated in a medium containing 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.01 μM NAD (~3,000 Bq), and 50 mM triethanolamine-HCl (pH 7.5) in a total volume of 50 to 100 μl for 20 min at 30°C. The reaction was stopped by precipitation with methanol-chloroform. Chloroform (100 μl) was added to the reaction mixture (100 μl). After mixing, 300 μl of H_2O was added, and the suspension was centrifuged for 1 min at 15,000 × *g*. The supernatant was removed, 300 μl of methanol was added and mixed, and the mixture was centrifuged for 1 min at 15,000 × *g*. The supernatant was removed, and the pellet was dried at room temperature. Alternatively, the ADP-ribosylation reaction was stopped by the addition of Laemmli sample buffer (with 20 mM DTT, final concentration). ADP-ribosylation was analyzed by sodium dodecyl sulfate (SDS)–11% polyacrylamide gel electrophoresis (SDS–11% PAGE) and subsequent autoradiography with Kodak X-Omat-AR film or with a PhosphorImager (Molecular Dynamics).

Chemical stability of ADP-ribose-protein bond. The chemical stability of the ADP-ribose-protein bond was determined as described previously (3, 7, 16). The ADP-ribosylation reaction was terminated by the addition of 2% SDS and 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.5]). Aliquots (50 μl) were added to an equal volume of either 1 M hydroxylamine (pH 7.5) or 2 mM $HgCl_2$ or formic acid (at concentrations as indicated). Incubation was carried out for 30 or 180 min, respectively, at 37°C. Thereafter, proteins were precipitated by the addition of methanol-chloroform as described above. Pellets were dissolved in Laemmli sample buffer (with 20 mM DTT), and proteins were subjected to (SDS–11% PAGE).

Immunoblotting and immunoprecipitation. Immunoblotting was performed according to the method of Towbin et al. (24), with polyclonal rabbit anti-glutamine synthetase antiserum (1:500, a gift from P. W. Ludden, Madison, Wisconsin) and peroxidase-coupled swine immunoglobulin G to rabbit immunoglobulin G as the second antibody. Visualization was carried out with a chemiluminescence (ECL) Western blotting detection system (Amersham). For immunoprecipitation, 435 μl of culture supernatant was ADP-ribosylated in a total volume of 500 μl for 25 min at 30°C. Fifteen microliters of the anti-glutamine synthetase antibody was added. After 1 h at 4°C with mixing, 200 μl of 20% protein A-Sepharose was added and upside-down mixed for 1 h at 4°C. The mixture was centrifuged for 5 min at 5,000 × *g*. After two washes with phosphate-buffered saline, 50 μl of Laemmli sample buffer (with 20 mM DTT) was added, and the proteins were analyzed by SDS–11% PAGE and subsequent autoradiography or with a PhosphorImager.

RESULTS

Different bacterial strains were tested for ADP-ribosyltransferase activity after growth in tryptic soy broth, Mueller-Hinton broth, brain heart infusion broth, Brewer modified thioglycolate broth, gram-negative broth, selenite broth, and Schaedler broth for 1 to 13 days at 35°C with 5% CO_2. Incubation of the filter-sterilized culture supernatant of the *K. pneumoniae* strain

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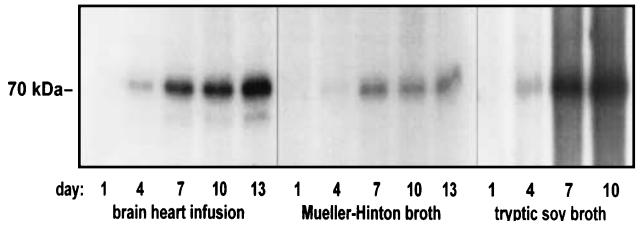

FIG. 1. Influence of different culture media and duration of growth on production of ADP-ribosyltransferase from *K. pneumoniae*. *K. pneumoniae* was grown in different culture media for 1 to 13 days at 35°C in the presence of 5% CO_2. After the indicated times, 30 μl of the culture supernatant was incubated with 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.01 μM NAD (~5,000 Bq), and 50 mM Tris-HCl (pH 7.5) in a total volume of 100 μl for 15 min at 30°C. The autoradiograph of the [^{32}P]ADP-ribosylated protein after SDS-PAGE is shown.

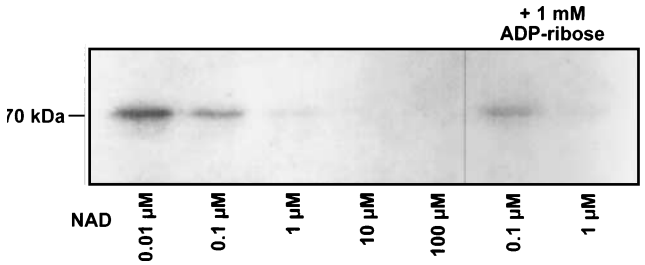

FIG. 3. Influence of NAD and ADP-ribose on ADP-ribosylation. The culture supernatant (5 μl) of *K. pneumoniae* was [^{32}P]ADP-ribosylated in the presence of the indicated concentrations of unlabeled NAD or ADP-ribose. After 30 min at 30°C, the reaction was stopped, and the labeled proteins were analyzed by SDS-PAGE and autoradiography.

isolated from a patient with a urinary tract infection induced the labeling of an ~70-kDa protein in the presence [^{32}P]NAD. In contrast, no labeling was observed with eight other *K. pneumoniae* strains isolated from different patients. No major differences in the protein patterns of the culture supernatants of the *K. pneumoniae* strains were observed. The highest levels of incorporation of ADP-ribose were detected after growth of the *K. pneumoniae* strain in brain heart infusion broth, tryptic soy broth and Mueller-Hinton broth (Fig. 1). No ADP-ribosylation was observed after growth in gram-negative broth, selenite broth, and Schaedler broth. Whereas the addition of fresh culture broth (brain heart infusion broth or tryptic soy broth) to the reaction mixture had no effect, labeling of the ~70-kDa protein increased proportionally with increasing amounts of the culture supernatant of *K. pneumoniae* (Fig. 2). However, we were not able to assign the labeled 70-kDa protein to any specific protein band by SDS-PAGE.

The addition of homogenates of human platelets, human neutrophils, rat brain, rat lung, or rat spleen tissues neither changed the labeling of the 70-kDa protein nor induced labeling of eukaryotic proteins (data not shown). Thus, these results suggest ADP-ribosylation of a *K. pneumoniae* protein. In line with this notion was the finding that unlabeled NAD decreased the modification of the ~70-kDa protein in a concentration-dependent manner, whereas the addition of ADP-ribose at a concentration up to 1 mM had no effect (Fig. 3). Furthermore, no labeling of culture supernatant proteins was observed in the presence of [α-^{32}P]ATP (~1,500 Bq) (data not shown).

In the time course of the reaction, maximal ADP-ribosylation was observed within 1 min of incubation at 30°C (data not shown). When the incubation was carried out in an ice bath, the reaction rate was decreased and maximal ADP-ribosylation occurred after 5 min (Fig. 4). To study the temperature stability of the transferase, the *Klebsiella* culture supernatant was heated for 3 min at 56 and 95°C, respectively, and thereafter, the ADP-ribosylation was initiated by the addition of [^{32}P]NAD at 30°C. Figure 5a shows that preincubation at 56 and 95°C largely decreased and completely inhibited, respectively, ADP-ribosylation. Inhibition of ADP-ribosylation was also observed when fresh broth was added to the reaction mixture containing the heated culture supernatant. ADP-ribosylation of the ~70-kDa protein was increased in the presence of DTT with a maximal effect at 10 mM DTT (Fig. 5b). The various nucleotides studied (GTP, GDP, GTPγS, ATP, and ADP) had no effect on ADP-ribosylation (data not shown).

Chemical stability of ADP-ribose-protein bond. Next we studied the stability of the ADP-ribose-protein bond. Incubation of the denatured ADP-ribosylated protein with either 0.5 M neutral hydroxylamine or 1 mM $HgCl_2$ for 30 or 180 min, respectively, had no effect. In contrast, treatment with formic acid (up to 50%) decreased the label in a concentration-dependent manner (Fig. 6).

We attempted to clarify whether the ADP-ribosylated protein is somehow related to glutamine synthetase. The anti-glutamine synthetase antibody cross-reacted with an ~55-kDa protein in the culture supernatant of *K. pneumoniae* (Fig. 7, panel II). However, the labeled ~70-kDa protein (Fig. 7, panel I) was clearly distinct from the cross-reacting protein band in the immunoblot. In line with this result, the glutamine synthetase antibody was not able to precipitate the labeled protein (data not shown).

To separate the ADP-ribosylating enzyme from its substrate, gel filtration (Superdex-75 26/60 or Superose 12 10/30; Pharmacia) and affinity chromatography (Dyematrex; Amicon, Witten, Germany) were performed. The fractions eluted were subsequently used in the ADP-ribosylation assay. ADP-ribosylation of the ~70-kDa protein was observed in some fractions.

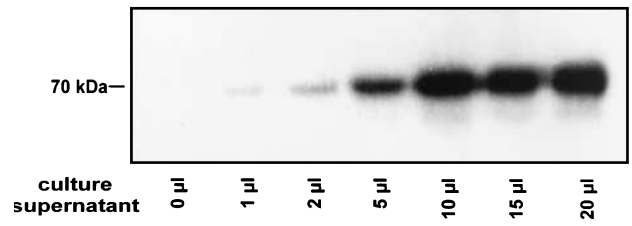

FIG. 2. Influence of increasing amounts of culture supernatant on ADP-ribosylation. Increasing amounts of the culture supernatant of *K. pneumoniae* (culture medium was tryptic soy broth) were [^{32}P]ADP-ribosylated in a total volume of 100 μl for 30 min at 30°C. Thereafter, labeled proteins were analyzed by SDS-PAGE and subsequent autoradiography.

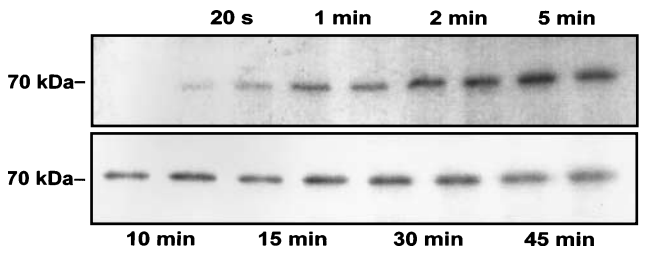

FIG. 4. Time course of ADP-ribosylation of culture supernatant of *K. pneumoniae*. The culture supernatant of *K. pneumoniae* (10 μl) was [^{32}P]ADP-ribosylated in a total volume of 100 μl at 0°C. The reaction was stopped at the indicated times, and the labeled proteins were analyzed by SDS-PAGE and autoradiography.

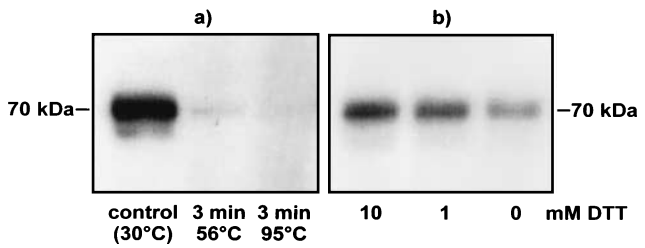

FIG. 5. Effects of heat treatment and dithiothreitol on ADP-ribosylation. (a) *K. pneumoniae* culture supernatant (37.5 μl) was incubated for 3 min at the indicated temperatures. Subsequently, the supernatant was [^{32}P]ADP-ribosylated for 15 min at 30°C. (b) The culture supernatant of *K. pneumoniae* (30 μl) was incubated with the indicated concentrations of dithiothreitol for 10 min at 30°C. Thereafter, ADP-ribosylation was initiated and incubation was carried out for another 15 min at 30°C. The PhosphorImaging data of the [^{32}P]ADP-ribosylated proteins analyzed by SDS-PAGE are shown.

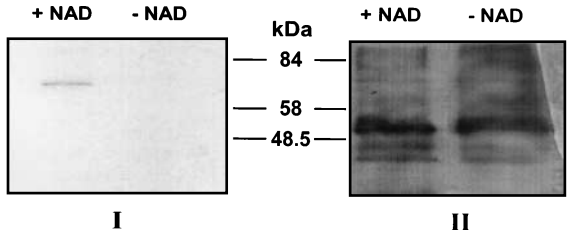

FIG. 7. Immunoblotting by anti-glutamine synthetase antibody. The culture supernatant of *K. pneumoniae* (32 μl) was incubated with 2 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, and 50 mM Tris-HCl (pH 7.5) with or without 0.01 μM NAD (~3,000 Bq) in a total volume of 40 μl for 30 min at 35°C. After SDS-PAGE, immunoblot analysis was performed with glutamine synthetase antibody. (I) Autoradiograph of the [^{32}P]ADP-ribosylated protein; (II) immunoblot.

However, separation of the enzyme activity from the protein substrate fraction was not possible.

DISCUSSION

Here we report on the labeling of an ~70-kDa protein in the culture supernatant of a clinical isolate of *K. pneumoniae*. Most likely, the modification is caused by mono-ADP-ribosylation. The reaction was dependent on NAD but not on ATP or ADP-ribose, indicating enzymatic ADP-ribosylation, but not adenylylation, and thus excluding a nonenzymatic modification by ADP-ribose. The transferase and the substrate were *Klebsiella* proteins and not contaminants from the culture medium. The *Klebsiella* transferase did not modify any eukaryotic proteins of preparations from various mammalian tissues. Therefore, we suggest that the activity is due to endogenous ADP-ribosylation. The initial characterization of the ADP-ribosylation showed that the reaction is heat labile but occurs even at 0°C. A rather unusual stability of the ADP-ribose-protein bond was observed. Treatment with hydroxylamine and $HgCl_2$, which are known to cleave arginine and cysteine-ADP-ribose bonds, respectively, had no effect on stability. In contrast, formic acid released the label. According to Cervantes-Laurean et al. (7), this finding suggests that ADP-ribose is bound to a serine or threonine residue. Studies to separate the transferase activity from the 70-kDa protein were performed using gel permeation chromatography. Because these studies did not result in inhibition or separation of the enzyme activity, it seems possible that modification of the 70-kDa protein is caused by auto-ADP-ribosylation of the transferase.

One of the best-studied prokaryotic systems that is regulated by ADP-ribosylation is the nitrogenase of *Rhodospirillum rubrum* that catalyzes the reduction of N_2 to ammonium (15). The enzyme is composed of two proteins, dinitrogenase reductase and dinitrogenase. Dinitrogenase is regulated by ADP-ribosylation at arginine 101 catalyzed by dinitrogenase reductase ADP-ribosyltransferase, an ~30-kDa protein (15). Genes encoding dinitrogenase reductase ADP-ribosyltransferase have been identified in other organisms such as *Azospirillum lipoferum*, *Azospirillum brasilense*, and *Rhodobacter capsulatus* (15). Although *K. pneumoniae* is a nitrogen-fixing organism, no regulation of nitrogenase by ADP-ribosylation has been observed so far (15). It has been shown that some bacterial glutamine synthetases are targets for endogenous ADP-ribosylation (14, 20, 22, 27). For example, glutamine synthetase from *Rhodospirillum rubrum* is modified by ADP-ribosylation, a reaction that is apparently not caused by dinitrogenase reductase ADP-ribosyltransferase (27). We studied whether the labeled *Klebsiella* protein is related to glutamine synthetase. However, the antibody against glutamine synthetase cross-reacted with a protein of ~55 kDa, which clearly differed from the labeled ~70-kDa *Klebsiella* protein. Many glutamine synthetases are regulated also by adenylylation (22, 27). However, we did not detect any modification of the ~70-kDa protein in the presence of [^{32}P]ATP. ADP-ribosylation of prokaryotic proteins with M_rs of 32 and 20 kDa have been reported for *P. maltophilia* (*Stenotrophomonas maltophilia*) (10). Moreover, several proteins were endogenously ADP-ribosylated in *Streptomyces griseus*; the major ADP-ribosylated proteins reported had M_rs of ~46 and 61 kDa (21). Additionally, an ~70-kDa protein which is growth dependently ADP-ribosylated has been detected previously (21). So far we do not know whether this protein is somehow related to the *Klebsiella* protein described herein. Further studies are underway to identify the ADP-ribosyltransferase target protein from the *K. pneumoniae* culture supernatant.

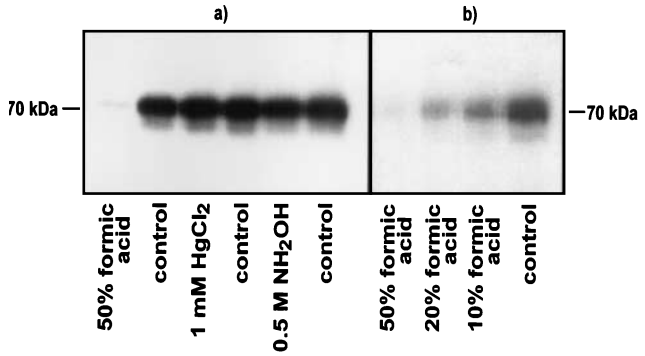

FIG. 6. Chemical stability of ADP-ribose-protein bond. The *K. pneumoniae* culture supernatant (32.5 μl) was [^{32}P]ADP-ribosylated for 15 min at 30°C. Thereafter, SDS (2%, final concentration) and HEPES (100 mM [pH 7.5], final concentration) were added. (a) Samples of the reaction mixture were incubated either with 50% formic acid, 0.5 M NaCl (control) 1 mM $HgCl_2$, or 1 mM NaCl (control) for 30 min or with 0.5 M NH_2OH or 1 M NaCl (control) for 180 min at 37°C. (b) Samples of the reaction mixture were incubated with 50%, 20%, or 10% formic acid or 0.5 M NaCl (control) for 30 min at 37°C. After methanol-chloroform precipitation, labeled proteins were separated by SDS-PAGE and autoradiography.

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