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Pseudomonas aeruginosa exoenzyme S: An adenosine diphosphate ribosyltransferase distinct from toxin A

(ADP-ribose/NAD$^+$/bacterial exoenzymes/elongation factors 1 and 2/diphtheria toxin)

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ABSTRACT *Pseudomonas aeruginosa* exoenzyme S is an adenosine diphosphate ribosyltransferase distinct from *Pseudomonas* toxin A. Exoenzyme S catalyzes the transfer of radioactivity from all portions of radiolabeled NAD$^+$ except nicotinamide. Digestion of the radiolabeled product(s) formed in the presence of [*adenine*-^{14}C]NAD$^+$ and exoenzyme S with snake venom phosphodiesterase yields only AMP, suggesting that ADP-ribose is present as monomers and not as poly(ADP-ribose). Exoenzyme S does not catalyze the transfer of ADP-ribose from NAD$^+$ to elongation factor 2, as do toxin A and diphtheria toxin, but to one or more other proteins present in crude extracts of wheat germ or rabbit reticulocytes and in partially purified preparations of elongation factor 1. The ADP-ribosyltransferase activity of exoenzyme S is distinct from toxin A by several tests: it is not neutralized by toxin A antibody, it is destroyed rather than potentiated by pretreatment with urea, and it is more heat stable. These latter observations and the substrate specificity suggest that exoenzyme S is different from any previously described prokaryotic ADP-ribosyltransferase.

Diphtheria toxin and *Pseudomonas* toxin A inhibit protein synthesis in eukaryotic cells by catalyzing the transfer of the ADP-ribose (ADP-Rib) moiety of NAD$^+$ to elongation factor 2 (EF-2) (1–3). The only eukaryotic protein known to be modified by these two toxins is EF-2, and all existing information supports the conclusion that the ADP-ribosylation of EF-2 is responsible for the lethality of these two toxins (2, 4). Diphtheria toxin is encoded by a phage gene (2, 5), but the location of the structural gene for *Pseudomonas* toxin A is unknown. Approximately 90% of all isolates of *Pseudomonas aeruginosa* tested produce toxin A (6, 7).

In this report we describe an ADP-ribosyltransferase (exoenzyme S) that is present in the culture supernatant fluid of a strain of *P. aeruginosa* (Ps 388). Ps 388 was consistently negative in an immune precipitation assay (7) using specific toxin A antibody. In the presence of limiting amounts of EF-2, exoenzyme S catalyzed the transfer of far more ADP-Rib from NAD$^+$ than could be accounted for by the production of ADP-ribosylated EF-2. Data are presented to show that exoenzyme S, unlike diphtheria or *Pseudomonas* A toxins, does not modify EF-2 but some other eukaryotic protein(s). ADP-Rib appears to be present in the modified proteins(s) as monomeric units rather than as poly(ADP-Rib). We also show that exoenzyme S is distinct from *Pseudomonas* toxin A by several other tests. It is not neutralized by toxin A antibody, it is destroyed rather than potentiated by pretreatment with urea, and it is more heat stable.

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MATERIALS AND METHODS

Exoenzyme S and Toxins. Ps 388, kindly provided by B. Minshew (Seattle, WA), was grown in a liquid medium adjusted to pH 7.0 consisting of the dialysate from trypticase soy broth (Baltimore Biological Lab.) (8) supplemented with 0.1 M monosodium glutamate, 1% glycerol, and 0.01 M nitrilotriacetic acid (Sigma Chem. Co.). A 25-ml amount of this medium in a 500-ml erlenmeyer flask was inoculated with an overnight culture of Ps 388 to an initial cell density of approximately 5 $\times 10^7$ cells per ml. The culture was incubated at 32° on a reciprocating shaker (200 linear excursions/min) (Lab.-Line Inst.) for 22 hr. The culture supernatant fluid was obtained by centrifugation at 10,000 $\times$ *g* for 20 min at 4°; it was filter sterilized and stored at −70° in small aliquots. Crude exoenzyme S, present in the culture supernatant fluid prepared and stored in this manner, retains enzymatic activity for several months.

P. aeruginosa (PA103) (8) was used as a source of toxin A, which was produced and purified as described (9). The purified toxin A had a mouse median lethal dose (LD_{50}) of 0.2 μg/22-g mouse when injected irtraperitoneally. Fragment A was obtained by treating purified diphtheria toxin with trypsin and dithiothreitol (10). The fragment A was then chromatographed on Sephadex G-100 with 1 mM EDTA/1 mM dithiothreitol/50 mM Tris·HCl, pH 7.8.

Enzymatic Activity. Crude extracts containing aminoacyl transferase factors were prepared from wheat germ as described by Chung and Collier (11). ADP-ribosyltransferase activity was measured by the incorporation of radioactivity from [*adenine*-^{14}C]NAD$^+$ into trichloroacetic acid-precipitable material in the presence of crude wheat germ extracts as previously described (9). Unless otherwise noted, the reaction was performed at 25° for 5 min in 0.1 ml of 50 mM Tris·HCl (pH 7.0), 1 mM EDTA, 50 mM dithiothreitol, 0.12 mM [*adenine*-^{14}C]-NAD$^+$ (10.6 Ci/mol) (Amersham/Searle), wheat germ extract containing 150–160 μg of protein, and various amounts of exoenzyme S, diphtheria toxin fragment A, or *Pseudomonas* toxin A. The reaction was stopped by the addition of 0.1 ml of 10% trichloroacetic acid. The precipitates were collected and washed, and radioactivity was measured as described (9). The enzymatic activity of *Pseudomonas* toxin A was potentiated by first incubating the toxin A in 4 M urea/1% dithiothreitol for 15 min at 25° (12). Where noted, [*nicotinamide*-^{14}C]NAD$^+$ (59 Ci/mol) (Amersham/Searle) or [*Rib*(*NMN*)-^{14}C]NAD$^+$ (75 Ci/mol), prepared as described (13), was substituted for the [*adenine*-^{14}C]NAD$^+$. The following proteins were used in experiments designed to elucidate the substrate specificity of

Abbreviations: ADP-ribose, ADP-Rib; EF, elongation factor.

exoenzyme S: crude extract of rabbit reticulocytes prepared as described by Allen and Schweet (14) as modified by Collier and Kandel (10), purified EF-2 (15) and elongation factor-1 (EF-1) (16) from rabbit reticulocytes (kindly provided by W. Merrick, National Institute of Health), rat liver EF-1 partially purified as described (17), poly(L-lysine) (Sigma), bovine serum albumin (Sigma), egg white lysozyme (Calbiochemicals), and RNase and DNase (Worthington Biochemicals).

Enzyme Neutralization. Specific toxin A antiserum was produced in rabbits as described (7). Enzyme inactivation by antibody was determined by assaying the ADP-ribosyltransferase activity after incubation of 0.1 μg of toxin A or 0.15 μg of crude exoenzyme S with antiserum or normal rabbit serum for 5 min at 37° as described (3). The antiserum was diluted in saline containing 0.1 mg of bovine serum albumin per ml.

Analysis of Reaction Product. Radiolabeled products formed in the ADP-ribosyltransferase assays were electrophoresed in sodium dodecyl sulfate/polyacrylamide gels by a described (12) modification of the method of Weber and Osborn (18). Gel slices (1 mm thick) were incubated 12 hr at 25° in 1 ml of buffer (0.1 M Tris·HCl, pH 7.5/10 mM EDTA/0.1 mg of bovine serum albumin). The eluted protein was precipitated with trichloroacetic acid (5%), collected on Millipore filters, and washed. Radioactivity was measured as described (3).

In order to determine if ADP-Rib was present in the acceptor protein(s) as monomeric units or as poly(ADP-Rib), we incubated a 0.3-ml reaction mixture containing 50 mM Tris·HCl (pH 7.0), 0.03 mM EDTA, 50 mM dithiothreitol, 0.14 mM [*adenine*-^{14}C]NAD$^+$ (26 Ci/mol), rat liver EF-1 (60 μg of protein), and 50 μl of exoenzyme S for 150 min at 25°. At 60, 90, 120, and 150 min, 10-μl aliquots were removed and trichloroacetic acid-precipitable radioactivity was determined. No further incorporation of radioactivity occurred after 120 min. The reaction mixture remaining (260 μl) was chromatographed on a column of Sephadex G-25 (bed volume, 4.0 ml) in 0.2 M Tris·HCl (pH 7.0) to separate the labeled product(s) from unreacted [^{14}C]NAD$^+$. Fractions containing high-molecular-weight radioactive material were pooled (total volume, 0.56 ml) and an aliquot of 250 μl was incubated for 60 min with 240 μg of snake venom phosphodiesterase (Sigma) in a total volume of 0.5 ml containing 0.1 M Tris·HCl (pH 7.0) and 20 mM $MgCl_2$. The products of this digestion were chromatographed on Sephadex G-25 (equilibrated and eluted with water), and the included low-molecular-weight radioactive material was concentrated by lyophilization and resuspended in water. The digested products were then chromatographed on thin-layer polyethyleneimine-cellulose plates (J. T. Baker), with 0.3 M lithium chloride as the solvent (19). AMP and ADP-Rib (Calbiochemicals) and NAD$^+$ (Sigma) were cochromatographed as markers. The chromatogram was cut into small pieces and eluted with 0.3 ml of 1.6 M lithium chloride. After addition of 5 ml of scintillation fluid (16.5 g of diphenyloxazole dissolved in 2 liters of toluene and 1 liter of Triton X-100) radioactivity of the samples was determined in a Nuclear Chicago scintillation counter.

RESULTS

The culture supernatant fluid of *P. aeruginosa* strain 388 (Ps 388) contained activity which, like *Pseudomonas* toxin A (9), transferred radioactivity [*adenine*-^{14}C]NAD$^+$ into acid-insoluble material in the presence of crude wheat germ extracts. Nevertheless, Ps 388 was consistently negative in immune precipitation assays (7) with rabbit toxin A antiserum. Similarly, preparations of culture supernatant fluid from Ps 388 failed to react with toxin A antiserum in agar gel diffusion assays, even when the preparations were concentrated 50-fold and a wide range of antiserum dilutions was tested. We then compared the ability of the toxin A antiserum to neutralize the ADP-ribosyltransferase activity of toxin A and the activity present in the culture supernatant fluid of Ps 388 (exoenzyme S). As shown in Fig. 1, the toxin A antiserum neutralized 80–100% of the enzymatic activity of pure toxin A at dilutions of 1:16 or less. Similar results were obtained with crude toxin A preparations or a purified enzymatically active peptide (12) derived from toxin A (data not shown). In contrast, even at the highest concentration of toxin A antiserum used (dilution 1:2), the enzymatic activity of exoenzyme S was not decreased (Fig. 1).

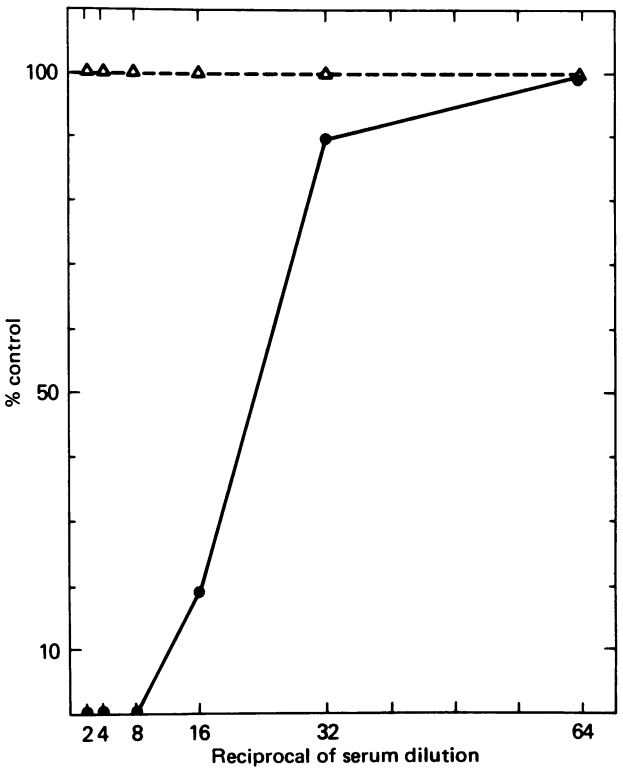

FIG. 1. Neutralization of the enzymatic activity of exoenzyme S and *Pseudomonas* toxin A by toxin A antiserum. △, Exoenzyme S; ●, *Pseudomonas* toxin A. Inactivation is expressed as the percentage of activity obtained when the toxin or enzyme was incubated with normal rabbit serum (control).

To determine which portions of NAD$^+$ are transferred by exoenzyme S, we incubated the enzyme with wheat germ extracts and NAD$^+$ radioactively labeled at different positions. All portions of NAD$^+$ except nicotinamide were incorporated into trichloroacetic acid-insoluble reaction product(s) in the presence of exoenzyme S or toxin A (Table 1). Thus, exoenzyme S is an ADP-ribosyltransferase.

Pseudomonas toxin A is produced as a toxic proenzyme that is virtually devoid of ADP-ribosyltransferase activity (12, 20). The transferase activity of toxin A is expressed when the molecule is cleaved by proteolysis to yield an enzymatically active fragment (11, 12) or when it is denatured and reduced (12, 20). In contrast, we found that treatment of exoenzyme S with 4 M urea markedly reduced its enzymatic activity. The presence of dithiothreitol slightly modified the effect of urea on exoenzyme S activity (Table 2).

The enzymatic activity of toxin A and that of the enzymatically active peptide derived from toxin A was heat sensitive (refs. 3 and 11 and Table 3). The enzymatic activity of exoenzyme S was relatively heat stable. As shown in Table 3, at least

Table 1. Incorporation of label from NAD^+ preparations into protein by exoenzyme S

NAD^+ used	Radioactivity incorporated, cpm	
	No enzyme	Enzyme
[*Adenine*-^{14}C]NAD^+	180	26,000
[*Rib(NMN)*-^{14}C]NAD^+	220	3,560
[*Nicotinamide*-^{14}C]NAD^+	110	118

[*Adenine*-^{14}C]NAD, 5.0 μM (266 Ci/mol), [*Rib(NMN)*-^{14}C]NAD^+, 3.0 μM (75 Ci/mol), or [*nicotinamide*-^{14}C]NAD^+, 5.0 μM (59 Ci/mol) was added to the reaction mixtures containing 10 μl of H_2O or 10 μl of exoenzyme S, 150 μg of wheat germ extract, and reaction buffer.

90% of the enzymatic activity of either crude or purified toxin A was destroyed by incubation at 100° for 2 min, whereas only 30% of the activity of exoenzyme S was lost after 10 min at 100°. The data in Fig. 1 and Tables 2 and 3 suggest that exoenzyme S and toxin A are structurally different.

The data in Table 4 show that in the presence of excess NAD^+ and limiting amounts of wheat germ extract, exoenzyme S catalyzed the transfer of far more ADP-Rib than could be accounted for by the formation of an ADP-ribosylated EF-2 product such as has been described (1–4). When excess toxin A or fragment A was incubated with limiting amounts of wheat germ extract and excess NAD^+, approximately 32 pmol of ADP-Rib was transferred from [*adenine*-^{14}C]NAD^+ to acid-insoluble material. Doubling the amount of toxin A or fragment A did not increase the amount of ADP-Rib transferred. Under the same conditions, 10 μl of exoenzyme S catalyzed the transfer of 3670 pmol of ADP-Rib.

An investigation of the product formed in the presence of exoenzyme S revealed it to be stable to treatment with DNase or RNase but digestible with trypsin or Pronase. When this radiolabeled product was subjected to electrophoresis on sodium dodecyl sulfate/polyacrylamide gels, the acid-precipitable material obtained after elution of the individual gel slices indicated that the product was heterogeneous in size (Fig. 2). No significant amount of radioactivity was associated with material larger than 50,000 daltons. In agreement with previous reports (3, 4), the product formed in the presence of toxin A was homogeneous and had a molecular weight of 100,000 (the known molecular weight of EF-2) (Fig. 2).

These data (Table 4 and Fig. 2) suggested that EF-2 was not the acceptor for ADP-Rib in the reaction catalyzed by exoenzyme S. This was confirmed in experiments with highly purified reticulocyte EF-2. As shown in Table 5, exoenzyme S failed to transfer [*adenine*-^{14}C]ADP-Rib from [*adenine*-^{14}C]NAD^+ to pure EF-2. Under the same conditions, 0.2 μg of toxin A catalyzed the transfer of 2050 pmol of ADP-Rib to this preparation of EF-2 (data not shown).

Table 2. Effect of urea and dithiothreitol on ADP-ribosyltransferase activity of exoenzyme S and *Pseudomonas* toxin A

Treatment	ADP-Rib incorporated, pmol	
	Toxin A	Exoenzyme S
H_2O	0.72	1200
1% Dithiothreitol	0.67	1250
4 M Urea	10.9	760
4 M Urea + dithiothreitol	26.1	960

Pseudomonas toxin A (0.02 μg) or exoenzyme S (5 μl) was mixed with 5 μl of the solution to be tested and incubated at 25° for 15 min, then immediately assayed for ADP-ribosyltransferase activity.

Table 3. Comparison of thermal stability of *Pseudomonas* toxin A and exoenzyme S

Time, min	% unheated enzymic activity		
	Exoenzyme S	Crude toxin A	Purified toxin A
0	100	100	100
2	111	6	10
5	97	3	6
10	71	1	4
20	11	3	2
30	0.3	1	1

Samples (exoenzyme S, 10 μl; crude toxin A, 10 μl; purified toxin A, 0.2 μg) were heated at 100° for the indicated times. After the samples were diluted 1:10 in ice-cold mM Tris·HCl (pH 7.4), they were assayed for ADP-ribosyltransferase activity as described in *Materials and Methods*, except that 5 μM [*adenine*-^{14}C]NAD^+ (266 Ci/mol) was used.

Various other proteins were tested for acceptor activity in the reaction catalyzed by exoenzyme S (Table 5). Poly(L-lysine), bovine serum albumin, egg white lysozyme, and RNase were inactive. The two EF-1 preparations tested contained acceptor activity. The rat liver EF-1 and reticulocyte EF-1 preparations, purified by different methods, were reported to be 50 and 95% pure, respectively (16, 17). The acceptor(s) could well be contaminating proteins and not EF-1. However, a substantial amount of the radioactive product in wheat germ extract was associated with material of about 50,000 molecular weight (Fig. 2), a result consistent with ADP-ribosylation of EF-1. The smaller labeled material (Fig. 2) might be the result of proteolysis since the exoenzyme S preparation contains contaminating proteolytic enzymes.

Previously described prokaryotic ADP-ribosyltransferases have been shown to transfer single molecules of ADP-Rib from NAD^+ directly to proteins and not to each other in a repetitive fashion to yield poly(ADP-Rib) (21). In order to determine if the protein(s) modified by exoenzyme S contained monomers or polymers of ADP-Rib, we synthesized the product(s) formed in the presence of exoenzyme S, [*adenine*-^{14}C]NAD^+, and rat liver EF-1 and treated then as described in *Materials and Methods*. Eighty-two percent (2.0 nmol) of the acid-precipitable radioactivity in the original reaction mixture was recovered from Sephadex G-25 as high-molecular-weight material. After incubation with venom phosphodiesterase only 62% of the applied radioactive material was recovered, but all the recovered activity was in low-molecular-weight fractions. Ninety-three percent of this low-molecular-weight material

Table 4. Comparison of ADP-ribosyltransferase activity of exoenzyme S, *Pseudomonas* toxin A, and diphtheria toxin fragment A

Enzyme	ADP-Rib incorporated, pmol
Diphtheria toxin fragment A: 0.1 μg	29
0.2 μg	33
Pseudomonas toxin A: 0.1 μg	33
0.2 μg	32
Exoenzyme S: 10 μl	3670

Activity was determined as described in *Materials and Methods* with incubation for 15 min.

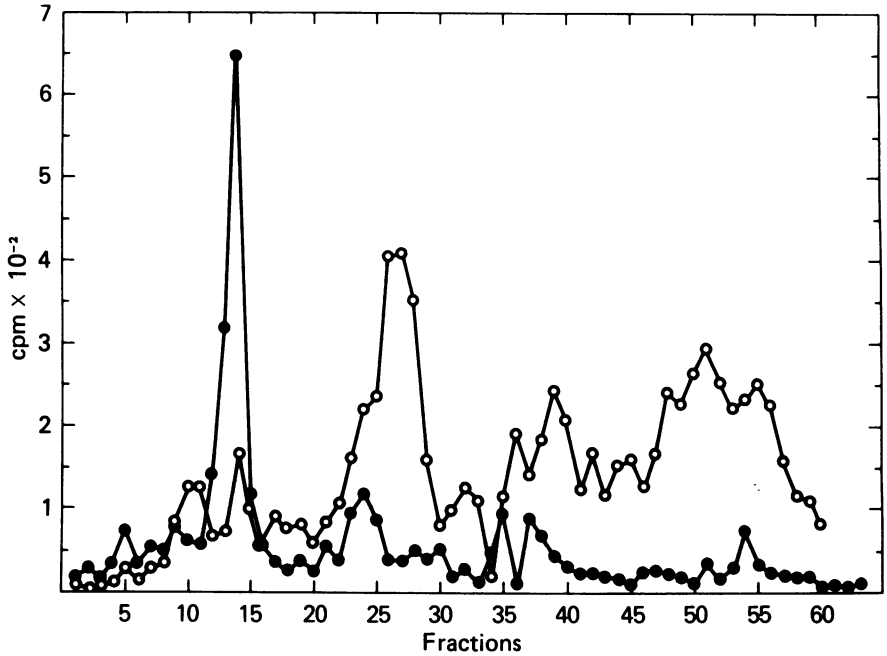

FIG. 2. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of acid-precipitable radiolabeled products formed in the presence of wheat germ extracts, 0.5 μg of *Pseudomonas* toxin A (●) or 1.0 μg of crude exoenzyme S (○), and 5 μM [*adenine*-^{14}C]NAD$^+$ (266 Ci/mol). Incubation was for 15 min.

chromatographed with AMP on polyethyleneimine-cellulose plates. No radioactivity migrated with marker ADP-Rib [which comigrates in the system used with *iso*ADP-Rib, the product of venom phosphodiesterase action on poly(ADP-Rib)] (21). When the product formed with wheat germ extract was analyzed similarly, the only radioactive product recovered after snake venom phosphodiesterase digestion was AMP. This strongly suggests that ADP-Rib is present on the acceptor(s) in both wheat germ extracts and in the rat liver EF-1 preparation as monomeric units, rather than as poly(ADP-Rib).

DISCUSSION

Five prokaryotic ADP-ribosyltransferases have been described: diphtheria toxin (1, 2), *Pseudomonas* toxin A (3, 4), T4 phage "alteration" enzyme (22), T4 phage "modification" enzyme (23), and an N4 phage enzyme (24). Existing evidence indicates that these enzymes play major roles in infection (i.e., diphtheria toxin) or in the regulation of various cellular activities (i.e., T4 modification enzyme) (2, 21). In addition, preliminary evidence has been presented that choleragen may be an ADP-ribosyltransferase (25).

Table 5. Incorporation of [*adenine*-^{14}C]ADP-Rib into acid-insoluble material with exoenzyme S and various substrates

Substrate	ADP-Rib incorporated, pmol
Wheat germ extract (160 μg)	3890
Reticulocyte extract (150 μg)	358
Reticulocyte EF-2 (76 μg)	0
Poly(L-lysine)(50 μg)	0
Bovine serum albumin (50 μg)	0
Lysozyme (50 μg)	0
RNase (85 μg)	0
Rat liver EF-1 (13 μg)	220
Reticulocyte EF-1 (20 μg)	45
H_2O (25 μl)	0

The reaction was carried out as described in *Materials and Methods* with 10 μl of exoenzyme S and an incubation time of 15 min, with the following exceptions: both preparations of EF-1 were incubated for 60 min and the NAD$^+$ concentration was 0.14 mM (16.5 Ci/mol).

The present results demonstrate the existence of a sixth prokaryotic ADP-ribosyltransferase, exoenzyme S, which is produced by some strains of *P. aeruginosa*. Exoenzyme S differs immunologically from *Pseudomonas* toxin A, is heat stable relative to toxin A, and is partially inactivated by conditions that potentiated the enzymatic activity of toxin A (Fig. 1 and Tables 2 and 3). Exoenzyme S catalyzed the transfer of ADP-Rib from NAD$^+$ to protein(s) present in wheat germ extracts, rabbit reticulocyte extracts, and partially purified EF-1 preparations (Tables 4 and 5). EF-2 failed to serve as an acceptor in the reaction catalyzed by exoenzyme S, indicating a substrate specificity different from that of *Pseudomonas* toxin A and diphtheria toxin. Furthermore, lysozyme, which serves as an acceptor in the *in vitro* reaction catalyzed by the T4 phage alteration enzyme (22) and the N4 virion-associated enzyme (24), was not ADP-ribosylated in the presence of exoenzyme S. The T4 modification enzyme reportedly does not ADP-ribosylate proteins present in rabbit reticulocyte extracts (2) as did exoenzyme S (Table 5). Thus, exoenzyme S differs in its substrate specificity from previously described prokaryotic ADP-ribosyltransferases.

Our results raised the possibility that exoenzyme S ADP-ribosylates EF-1. As seen in Table 5, in the presence of exoenzyme S [^{14}C]ADP-Rib was transferred from [*adenine*-^{14}C]-NAD$^+$ to trichloroacetic acid-insoluble material when EF-1 preparations were used as the substrate. The rat liver EF-1 preparation was approximately 50% pure and contained four or five proteins other than EF-1 (17). The reticulocyte EF-1 preparation was approximately 95% pure (16). These proteins were purified by different procedures (16, 17). Considerably more ADP-Rib per μg of protein was transferred to the rat liver preparation than to the reticulocyte preparation. Thus, if exoenzyme S is ADP-ribosylating EF-1, then either EF-1 from different sources differs in the extent to which it can be modi-

fied by exoenzyme S or it may somehow be altered during purification. A third, perhaps more likely, possibility is that some other protein(s) contaminating the EF-1 preparations is being ADP-ribosylated by exoenzyme S and this protein is in a smaller concentration in the more highly purified reticulocyte EF-1 preparation than in the rat liver EF-1 preparation. Purification of exoenzyme S to remove proteolytic contaminants, together with the use of recently developed methods for purifying catalytically active EF-1 and EF-1 α and β factors (26), should provide the necessary reagents to distinguish among these possibilities.

The role of diphtheria toxin in *Corynebacterium diphtheriae* infections is clear (2). There is increasing evidence that toxin A is also important in the pathogenesis of disease caused by *P. aeruginosa* (6, 7, 27). *P. aeruginosa* strain 388 produces exoenzyme S but no detectable amounts of toxin A. More recently we have identified other strains of *P. aeruginosa* that produce exoenzyme S and several that produce both toxin A and exoenzyme S.

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