Expression of FAS-Independent ADP-Ribosyltransferase Activity by a Catalytic Deletion Peptide of *Pseudomonas aeruginosa* Exoenzyme S[†]

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ABSTRACT: Earlier studies reported that *Pseudomonas aeruginosa* exoenzyme S (ExoS) possessed an absolute requirement for the eukaryotic protein factor activating exoenzyme S (FAS) for expressing ADP-ribosyltransferase activity. During the characterization of a serum-derived FAS-like activity, we observed the ability of a catalytic deletion peptide of ExoS (ΔN222) to ADP-ribosylate target proteins in the absence of FAS. Characterization of the activation of ΔN222 by FAS provided an opportunity to gain insight into the mechanism of ExoS activation by FAS. Under standard enzyme assay conditions, the initial rate of FAS-independent ADP-ribosyltransferase activity of ΔN222 was not linear with time and rapidly approached zero. Dilution into high-ionic strength buffers stabilized ΔN222 so it could express FAS-independent ADP-ribosyltransferase activity at a linear rate. This stabilization was a general salt effect, since dilution into a 1.0 M solution of either $NaCH_3COOH$, NaCl, or KCl stabilized the ADP-ribosyltransferase activity of ΔN222. Kinetic analysis in a high-ionic strength buffer showed that FAS enhanced the catalytic activity of ΔN222 by increasing the affinity for NAD and stimulating the turnover rate. Velocity experiments indicated that the stabilization of ΔN222 by high salt was not functionally identical to stabilization by FAS. Together, these data implicate a dual role for FAS in the allosteric activation of ExoS, involving both substrate binding and catalysis.

Neutropenia, cystic fibrosis, and burn wounds predispose individuals to infection by *Pseudomonas aeruginosa* (*1*). *P. aeruginosa* produces numerous virulence factors (*2*), including exotoxin A and exoenzyme S, which are members of the family of bacterial ADP-ribosyltransferases (*3*). Exoenzyme S was purified as an aggregate which was composed primarily of two proteins with molecular masses of 53 and 49 kDa (*4*), which were encoded by separate genes, *exoT* and *exoS*, respectively (*5*, *6*). Although ExoT (457 amino acids) and ExoS (453 amino acids) exhibited 76% primary amino acid homology, ExoT ADP-ribosylated target proteins at only 0.2% of the specific activity of ExoS. Functional mapping localized the ADP-ribosyltransferase domain of ExoS to its 222 carboxyl-terminal amino acids, termed ΔN222. Kinetic studies showed that ΔN222 and ExoS possessed similar affinities for NAD and target proteins and catalyzed the ADP-ribosylation reaction with similar V_{max} values (*7*). ΔN222 has been subsequently used to characterize the ADP-ribosyltransferase domain of ExoS (*8*).

Relative to other members of the family of bacterial ADP-ribosylating exotoxins, ExoS possesses several unique biochemical properties. First, most bacterial ADP-ribosyltransferases modify specific host proteins, and ExoS ADP-ribosylates several host proteins in vitro, including intracellular proteins such as vimentin (*9*) and several low-molecular mass GTP-binding proteins (e.g., including $p21^{c-Hras}$) (*10*) and extracellular proteins such as human immunoglobulin 3 and apolipoprotein A1 (*11*). Recently, ExoS has been shown to ADP-ribosylate Ras in vivo (*12*), which appears to uncouple a Ras-mediated signal transduction pathway (*13*). Second, ExoS possesses an absolute requirement for a eukaryotic protein, termed FAS (factor activating exoenzyme S), for catalyzing the ADP-ribosylation reaction (*7*, *14*). Finally, while most bacterial ADP-ribosyltransferases possess "A:B" structure−function organization (*3*), ExoS is secreted into the eukaryotic cell cytoplasm by a type III secretion apparatus, following direct contact between the bacterium and the eukaryotic cell (*15*).

The requirement of ExoS for FAS (*7*, *14*) for expressing ADP-ribosyltransferase activity appears to be analogous to the requirement of cholera toxin (CT) and heat-labile enterotoxin (LT) for the ADP-ribosylation factor (ARF) (*16*). Like FAS, ARF is ubiquitous in eukaryotes, but absent from prokaryotes. These proteins differ in that ARF activates CT in a GTP-bound state (*17*, *18*), whereas FAS is not a GTP binding protein. Fu et al. (*19*) cloned the gene encoding FAS from a bovine brain cDNA library and showed it to be a 14-3-3 protein. The 14-3-3 proteins are involved in a variety of regulatory functions, including the modulation of cell proliferation and intracellular signaling (*20*). The 14-3-3 proteins have been isolated from organisms throughout the eukaryotic kingdom, including yeast and mammals. The 14-3-3 proteins from numerous species have been shown to activate the expression of the ADP-ribosyltransferase activity of ExoS (*19*).

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In this study, we describe a serendipitous observation, which showed that a deletion peptide of ExoS, ΔN222, possessed FAS-independent ADP-ribosyltransferase activity. Subsequent studies showed that FAS activated ΔN222 by increasing both its affinity for NAD and turnover rate. This indicates that ExoS possesses intrinsic FAS-independent ADP-ribosyltransferase activity and implicates FAS in the activation of ExoS via an allosteric mechanism, but does not contribute directly to the ADP-ribosyltransferase reaction mechanism.

MATERIALS AND METHODS

Materials

Reagents were purchased from Sigma unless otherwise stated. [*adenylate phosphate*-^{32}P]NAD was purchased from Dupont-New England Nuclear. Ni^{2+} affinity resin and pET vectors were purchased from Novagen. Bovine serum albumin (BSA) was purchased from Pierce Biochemicals.

Methods

Purification of Histidine Fusion Proteins. Histidine fusion proteins (FAS and ΔN222) were expressed in *Escherichia coli* and purified as previously described (*7*). Briefly, proteins were purified in the presence of a mixture of protease inhibitors, which were added to the cell suspension prior to breakage with a French press. The lysate was subjected to centrifugation. The soluble fraction was passed through a 0.45 μm filter and then subjected to Ni^{2+} affinity chromatography. Affinity-purified proteins were subjected to gel-exclusion chromatography [Sephacryl S200HR equilibrated in 10 mM Tris (pH 7.6), containing 20 mM NaCl]. FAS was stored in buffer at −80 °C, while ΔN222 was stored in buffer with 40% glycerol at −20 °C. ExoS was purified from the culture supernatant of *P. aeruginosa* PA103 (pUCPExoS) as previously described.

ADP-Ribosylation of Soybean Trypsin Inhibitor (SBTI) or Ras. Standard assay conditions were as follows (25 μL): 0.2 M sodium acetate (pH 6.0), 150 μM [*adenylate phosphate*-^{32}P]NAD (specific activity of 2×10^5 cpm/3 nmol), 150 μM SBTI or 5 μM Ras, and an aliquot of ΔN222 with or without an equivalent amount of FAS relative to ΔN222. In these velocity reactions, the final concentration of ΔN222 is indicated in the individual figure legend. Incubation times were varied between 2.5 and 240 min at room temperature. Reactions were stopped by the addition of 0.5 volume of gel loading buffer and β-mercaptoethanol followed by SDS−PAGE and autoradiography. The radiolabel was quantitated by scintillation counting. The molar concentrations of purified proteins (e.g., ΔN222) were extrapolated by densitometry following SDS−PAGE and staining with Coomassie blue, using an AMBIS optical imaging system. SDS−PAGE was performed as described by Laemmli (*21*). Proteins were normalized to a known concentration of bovine serum albumin with the assumption that these proteins exhibited an equal molar staining for Coomassie blue. ΔN222 was diluted into 25 mM Tris-HCl (pH 7.6), containing 0.1 mg of egg albumin/mL or the indicated buffer. In the reactions in which the FAS-independent ADP-ribosyltransferase activity of ΔN222 was measured, <10% of the available NAD or target protein was utilized prior to the addition of FAS. The velocity experiments were performed at 150 μM NAD and SBTI, concentrations that are approximately 5-fold above their determined K_m values when assayed in low salt (*7*).

Kinetic Analysis of the ADP-Ribosylation of SBTI by ΔN222. (i) Variable SBTI. Reaction mixtures contained (25 μL) 25 mM Tris-HCl (pH 7.6), 1.0 M NaCl, 150 μM [*adenylate phosphate*-^{32}P]NAD (specific activity of 2×10^5 cpm/3 nmol), SBTI, and ΔN222 (final concentration of 7.4×10^{-9} M) with or without an equivalent amount of FAS relative to ΔN222. The final concentration of SBTI varied between 19 and 300 μM. After incubation for 5−20 min at room temperature, reactions were stopped by the addition of 0.5 volume of gel loading buffer and β-mercaptoethanol followed by SDS−PAGE and autoradiography. The radiolabel was quantitated by scintillation counting. The amount of SBTI utilized was approximately <10%. Data were transformed to the Lineweaver−Burk equation with the assistance of Enzfitter (Elsevier, Cambridge, U.K.).

(ii) Variable NAD. Assays were performed as described above, with the following exceptions. The concentration of SBTI was 150 μM, while the concentration of NAD was varied between 19 and 300 μM (specific activities were adjusted by adding nonradiolabeled NAD to 2×10^5 cpm of [*adenylate phosphate*-^{32}P]NAD). After incubation for 5−20 min at room temperature, reactions were stopped by the addition of 0.5 volume of gel loading buffer and β-mercaptoethanol followed by SDS−PAGE and autoradiography. The radiolabel was quantitated by scintillation counting as described above. Experimental conditions were adjusted so that approximately <10% of the available NAD was utilized.

RESULTS

FAS-Independent ADP-Ribosyltransferase Activity of ExoS. Previous studies, including those from our laboratory, reported that ExoS possessed an absolute requirement for FAS for expressing ADP-ribosyltransferase activity (*7*, *14*). The mechanism for FAS activation of ExoS has not been determined, but could involve the binding of FAS to ExoS, which could result in an allosteric activation of ExoS. Alternatively, FAS could directly contribute to the ADP-ribosylation reaction mechanism of ExoS. During the characterization of an extracellular FAS-like activity in serum, we observed that high concentrations of ΔN222 catalyzed the ADP-ribosylation of soybean trypsin inhibitor (SBTI) in the absence of exogenous FAS. ΔN222 is a recombinant deletion peptide, which consists of the 222 carboxyl-terminal amino acid residues of ExoS and possesses FAS-dependent ADP-ribosyltransferase activity (*7*). While ΔN222 ADP-ribosylated SBTI in the absence of FAS, the amount of SBTI that was ADP-ribosylated was only a fraction of the amount of SBTI that was ADP-ribosylated in the presence of FAS (data not shown). In addition, the amount of ΔN222 required to detect FAS-independent ADP-ribosyltransferase activity was approximately 250−1000-fold greater than was typically used to generate linear velocity rates in the presence of FAS. This, along with the unique kinetic properties of the FAS-independent ADP-ribosyltransferase activity of ΔN222, which are described below, is probably responsible for the previous failure to detect and/or recognize the intrinsic ADP-ribosyltransferase activity of ΔN222. This observation sug-

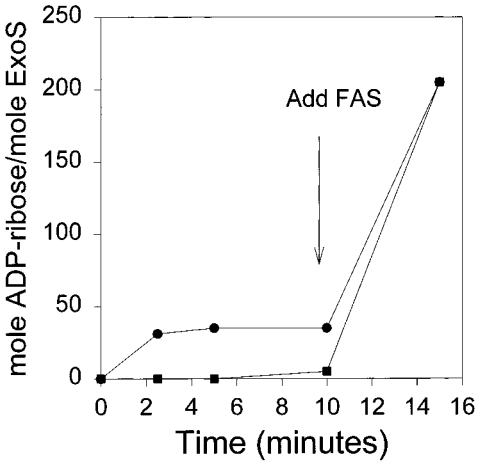

FIGURE 1: FAS-independent ADP-ribosyltransferase activity of ΔN222. ΔN222 (0.5 μM final concentration) was diluted into a standard assay reaction mixture and assayed for ADP-ribosyltransferase activity in the absence of FAS (●) or diluted and incubated for 15 min prior to the assay for ADP-ribosyltransferase activity in the absence of FAS (■). At the indicated time (arrow), FAS was added to each reaction mixture. At the indicated time point, an aliquot was removed from the reaction mixture and subjected to SDS−PAGE and treatment with β-mercaptoethanol. The radiolabel incorporated into SBTI was quantitated by scintillation counting. A background control (a reaction mixture without ExoS) was subtracted from each sample. This is a representative experiment that was repeated three times.

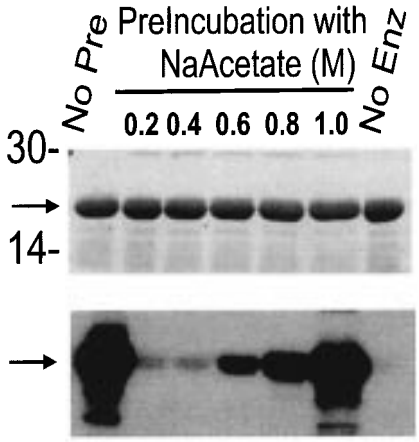

FIGURE 2: Stabilization of the FAS-independent ADP-ribosyltransferase activity of ΔN222 by dilution into a high-ionic strength buffer. ΔN222 (0.5 μM final concentration) was immediately assayed for ADP-ribosyltransferase activity in the absence of FAS (No Pre) or diluted into the indicated concentration of sodium acetate, as indicated above the appropriate lane. After a 15 min preincubation, ΔN222 was assayed for ADP-ribosyltransferase activity in the absence of FAS. No Enz depicts the ADP-ribosyltransferase reaction carried out in the absence of ΔN222. Reaction mixtures were subjected to SDS−PAGE and treatment with β-mercaptoethanol. The amount of radiolabel incorporated into SBTI was determined by autoradiography (lower panel) of the Coomassie-stained gel (upper panel). Molecular mass markers (kilodaltons) are indicated to the left of the No Pre lane. Arrows indicate the migration of SBTI. Results that are shown are from a single representative experiment repeated three times.

gested that ExoS possessed the intrinsic ability to ADP-ribosylate target proteins and prompted the subsequent characterization of the FAS-independent ADP-ribosyltransferase activity of ΔN222.

Characterization of the FAS-Independent ADP-Ribosyltransferase Activity of ΔN222. In the presence of FAS, ΔN222 catalyzes the ADP-ribosylation of SBTI at a linear rate (*7*, *8*). Although ΔN222 catalyzed a detectable amount of ADP-ribosylation in the absence of FAS, the rate was not linear at even the earliest time points and approached zero over time (Figure 1). Ten minutes after the initiation of the assay, in the absence of FAS, ΔN222 had ADP-ribosylated only 5% of the available NAD and SBTI, whereas in the presence of FAS, ΔN222 had ADP-ribosylated essentially all of the available SBTI. Thus, the nonlinear rate of ADP-ribosylation catalyzed by ΔN222 in the absence of FAS was not due to substrate or target protein limitation. When this slow velocity rate was achieved, addition of FAS stimulated the ADP-ribosyltransferase activity of ΔN222 (Figure 1), which indicated that the inactive state of ΔN222 was reversible. These data indicate that ΔN222 possesses intrinsic ADP-ribosyltransferase activity and that FAS is an allosteric activator, which stimulates the turnover rate of ΔN222.

The FAS-Independent ADP-Ribosyltransferase Activity of ΔN222 Is Modulated by Ionic Strength. Several mechanisms were considered to be responsible for the observed attenuation of the ADP-ribosyltransferase activity of ΔN222 in the absence of FAS, including the possibility that the dilution of ΔN222 into the standard assay buffer (0.2 M sodium acetate at pH 6.0) inhibited the ADP-ribosyltransferase activity. Consistent with this hypothesis was the observation that preincubation of ΔN222 for 15 min in standard assay buffer eliminated the intrinsic FAS-independent ADP-ribosyltransferase activity of ΔN222 (Figure 1). Again, this inhibition was reversible, since the addition of FAS restored the ADP-ribosyltransferase activity of ΔN222 (Figure 1).

An evaluation of incubation conditions showed that ionic strength was responsible for the inactivation of ΔN222 upon dilution into the assay buffer. The preincubation control shown in Figure 2 represents the expression of the FAS-independent activity of ΔN222 upon immediate dilution into an assay mix. ΔN222 that had been diluted into 0.2 or 0.4 M sodium acetate and incubated for 15 min did not express substantial amounts of FAS-independent ADP-ribosyltransferase activity. In contrast, ΔN222 that had been diluted into higher-ionic strength (0.6−1.0 M) sodium acetate and incubated for 15 min expressed increasing amounts of FAS-independent ADP-ribosyltransferase activity (Figure 2). The autoradiogram presented was overexposed to show the lack of ADP-ribosyltransferase activity of ΔN222 upon dilution into either 0.2 or 0.4 M sodium acetate buffer. This stabilization of ΔN222 was a general ionic effect, since ΔN222 that had been diluted into 25 mM Tris-HCl (pH 7.6) containing either 1.0 M NaCl or 1.0 M KCl also expressed FAS-independent ADP-ribosyltransferase activity (data not shown). Thus, it appeared that dilution into high-ionic strength buffers stabilized ΔN222 in a FAS-independent enzymatically active conformation.

The specific activity of the FAS-independent ADP-ribosyltransferase activity of ΔN222 that had been diluted and assayed at high ionic strengths was similar to the ADP-ribosyltransferase activity of ΔN222 assayed in the presence of FAS, under standard assay conditions (Figure 3). In the presence of high ionic strengths and FAS, the rate of ADP-ribosyltransferase activity of ΔN222 was greater than when assayed with either reagent. The additive effect of salt and FAS on the expression of ADP-ribosyltransferase activity

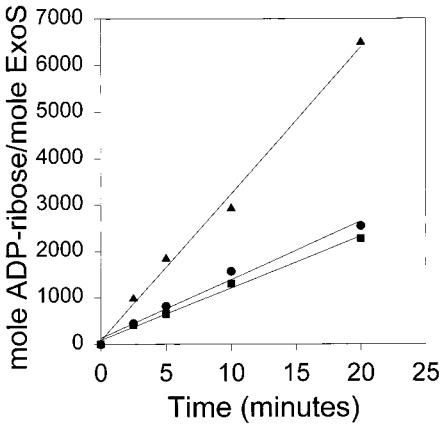

FIGURE 3: Additive effect of 1.0 M NaCl and FAS on the ADP-ribosyltransferase activity of ΔN222. The ADP-ribosylation of SBTI by ΔN222 (12 nM final concentration) was performed in the presence of FAS under standard assay conditions (●), in 1 M NaCl in the absence of FAS (■), or in 1 M NaCl in the presence of FAS (▲). At the indicated times, an aliquot was removed and subjected to SDS−PAGE. The amount of radiolabel incorporated into SBTI was determined by scintillation counting. A background control (a reaction mixture without ΔN222) was subtracted from each sample. A representative experiment is shown. Specific activities (moles of ADP-ribose per minute per mole of ΔN222) calculated from several experiments are (●) 137 ± 41 (n = 8), (■), 90 ± 41 (n = 4), and (▲) 255 ± 81 (n = 4).

Table 1: Kinetic Constants for ΔN222 in the ADP-Ribosylation of SBTI at 1.0 M NaCl in the Presence and Absence of FAS[a]

	K_m (μM)	V_{max} (mol min^{-1} mol^{-1})	V_{max}/K_m
varying SBTI with FAS	96.0 ± 29.6	183.5 ± 30.4	1.91
varying SBTI without FAS	76.5 ± 27.6	49.0 ± 15.6	0.64
varying NAD with FAS	185.3 ± 32.5	218.0 ± 78.0	1.18
varying NAD without FAS	457.3 ± 77.9	78.0 ± 31.2	0.17

[a] Reaction mixtures contained 25 mM Tris-HCl (pH 7.6), 1.0 M NaCl, [*adenylate phosphate*-^{32}P]NAD, SBTI, and ΔN222 (final concentration of 7.4×10^{-9} M) with or without an equivalent amount of FAS relative to ΔN222. The final concentration of SBTI or NAD varied between 19 and 300 μM, with a fixed 150 μM concentration of the reciprocal reagent. Reactions were stopped by the addition of 0.5 volume of gel loading buffer and β-mercaptoethanol, followed by SDS−PAGE and autoradiography. The radiolabel was quantitated by scintillation counting. The amount of SBTI or NAD that was utilized was approximately <10%. Data were transformed to the Lineweaver−Burk equation with the assistance of Enzfitter (Elsevier).

of ΔN222 suggested that these two agents stabilized via different mechanisms.

Kinetic Constants for FAS-Dependent and FAS-Independent ADP-Ribosyltransferase Activity of ΔN222 at High Ionic Strengths. The determination that high-ionic strength buffers stabilized the expression of FAS-independent ADP-ribosyltransferase activity provided an opportunity to investigate how FAS activated the kinetic properties of ΔN222 (Table 1). Kinetic parameters were determined in 25 mM Tris-HCl, containing 1.0 M NaCl, alone, or in the presence of FAS. ΔN222 possessed similar affinities for SBTI in the presence and absence of FAS, with K_m values of 96 and 77 μM, respectively. With variable SBTI levels, ΔN222 catalyzed the ADP-ribosylation of SBTI with a 3.8-fold greater V_{max} in the presence of FAS than in the absence of FAS (Table 1) with determined catalytic efficiencies (V_{max}/K_m) of 1.9 in the presence of FAS and 0.6 in the absence of FAS. ΔN222 possessed a lower K_m for NAD in the presence of FAS than

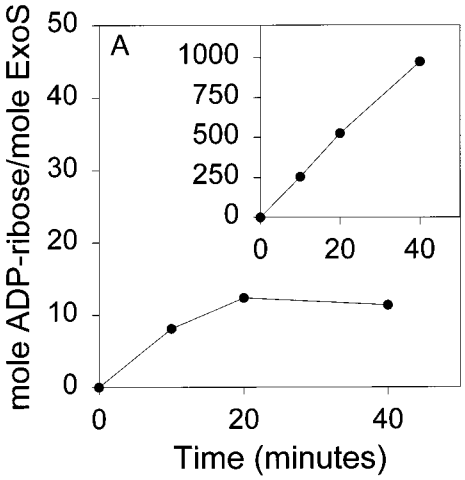

FIGURE 4: FAS-independent ADP-ribosylation of p21^{c-Hras} by ΔN222. p21^{c-Hras} was ADP-ribosylated under standard assay conditions by ΔN222, at a final concentration of 20 nM in the presence of FAS (inset) and 200 nM in the absence of FAS. At the indicated times, an aliquot was removed and subjected to SDS−PAGE. The amount of radiolabel incorporated into Ras was determined by scintillation counting. A background control (a reaction mixture without ΔN222) was subtracted from each sample. This experiment was performed three times with similar results, and results from a representative experiment are shown.

in the absence of FAS. With variable NAD levels, ΔN222 catalyzed the ADP-ribosylation of SBTI with a 2.8-fold greater V_{max} in the presence of FAS than in the absence of FAS with determined catalytic efficiencies of 1.2 in the presence of FAS and 0.2 in the absence of FAS. Thus, FAS enhances the affinity for NAD and increases the turnover rate of ΔN222.

ADP-Ribosylation of p21^{c-Hras} by ΔN222. ΔN222 was also observed to ADP-ribosylate p21^{c-Hras} in the presence and absence of FAS, both under standard assay conditions and in high-ionic strength buffers. In the presence of FAS under standard assay conditions, ΔN222 ADP-ribosylated Ras at a linear rate [16 ± 9 (n = 3) mol of ADP-ribose incorporated per minute per mole of ΔN222] until about 50% of the Ras had been ADP-ribosylated (Figure 4, inset). In the absence of FAS under standard assay conditions, the rate of ADP-ribosylation of Ras by ΔN222 was not linear with time and approached zero over time (Figure 4). In contrast, in 1 M NaCl, but in the absence of FAS, ΔN222 ADP-ribosylated Ras at a linear rate of 2 ± 1 (n = 6) mol of ADP-ribose incorporated per minute per mole of ΔN222. This was about 25% of the rate catalyzed by ΔN222 in 1 M NaCl in the presence of FAS, which occurred at 8 ± 4 (n = 6) mol of ADP-ribose incorporated per minute per mole of ΔN222. Although the limited availability of purified Ras prevented rate constants from being determined, these data showed that ΔN222 could ADP-ribosylate a physiological target by a FAS-independent mechanism.

Stabilization of ΔN222 by High-Ionic Strength Buffers or FAS Is Functionally Different. An experiment was performed to determine if FAS and high-ionic strength buffers activated ΔN222 via a similar mechanism (Figure 5). ΔN222 was incubated in the absence of FAS until its capacity to ADP-ribosylate the target protein had been attenuated. At this time, either FAS or NaCl (0.5 or 1.0 M) was added with the subsequent measurement of ADP-ribosyltransferase activity. Unlike FAS, which restored the ability of ΔN222 to ADP-

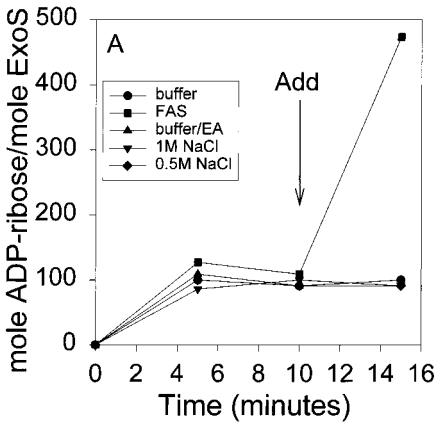

FIGURE 5: Characterization of the reversibility of ΔN222 stabilization. ΔN222 (0.5 μM final concentration) was added to a reaction mixture containing [^{32}P]NAD and SBTI under standard assay conditions as described in Methods. Either sodium chloride (final concentrations of 0.5 and 1.0 M, as indicated), saturating levels of FAS or egg albumin (EA), or storage buffer [10 mM sodium chloride/5 mM Tris-HCl (pH 7.6)] was added to the reaction mixtures as indicated by the arrow. At the indicated times, an aliquot was removed and mixed with 0.5 volume of gel loading buffer containing β-mercaptoethanol and boiled for 5 min. Samples were subjected to SDS−PAGE, and radioactive bands were determined by scintillation counting. A background control (a reaction mixture without ExoS) was subtracted from each sample. This experiment was performed in duplicate with results from one of the independent experiments shown.

ribosylate SBTI, neither 0.5 M NaCl nor 1.0 M NaCl restored the ADP-ribosyltransferase activity of ΔN222 (Figure 5). Therefore, although the dilution of ΔN222 into the high-ionic strength buffer placed ΔN222 in a functionally stable conformation (Figure 2), it appears that the high-ionic strength buffer does not convert ΔN222 from an inactive to a catalytically active form. The reversible activation of ΔN222 by FAS was not a general property of protein concentration, since egg albumin did not restore the ADP-ribosyltransferase activity of attenuated ΔN222.

DISCUSSION

The observation that ΔN222 possessed FAS-independent ADP-ribosyltransferase activity was serendipitous, occurring during the purification of a FAS-like activity in serum. In these studies, the FAS-like activity was measured in the ADP-ribosyltransferase reaction, using chromatographic fractions as the source of FAS. The sensitivity of the assay was increased by using concentrations of ΔN222 (approximately 1000-fold higher) that were unusually higher than those typically used under standard assay conditions. During the purification of the FAS-like activity, we observed a baseline amount of ADP-ribosyltransferase activity in all chromatographic fractions that were tested. This suggested that ΔN222 possessed the intrinsic ability to ADP-ribosylate target protein in the absence of FAS. Other experiments have elucidated FAS-independent ADP-ribosyltransferase activity of full-length ExoS; however, the amount of FAS-independent activity was modest compared to that observed for ΔN222 (data not shown). Thus, the efficient expression of FAS-independent ADP-ribosyltransferase activity appears to be primarily a property of the deletion peptide, ΔN222. It is possible that the ionic conditions required for expressing efficient FAS-independent ADP-ribosyltransferase activity are different for full-length ExoS, relative to ΔN222. This suggests that FAS-dependent ADP-ribosyltransferase activity of ExoS is physiologically relevant for ExoS. The significance of the characterization of the FAS-independent ADP-ribosyltransferase activity of ΔN222 should be emphasized, since it allowed the identification of FAS as an allosteric activator of ExoS and provided insight into the mechanism of activation of ExoS by FAS.

Coburn et al. (*14*) identified the eukaryotic protein, FAS, as an activator of ExoS. The data presented in this study have shown that FAS modifies the capacity of ΔN222 to ADP-ribosylate target proteins by increasing its affinity for NAD and increasing its turnover rate. These data are consistent with the earlier proposal of Coburn et al. (*14*), where FAS was proposed to modify ExoS directly, and not to modify target proteins. This earlier proposal was based upon the observation that FAS activated ExoS to ADP-ribosylate numerous proteins, not specific subsets of proteins.

The behavior of ΔN222 under the various conditions employed during this study indicates that ΔN222 can assume an inactive or active form. ΔN222 is in an enzymatically active conformation in storage buffer. Dilution into a low-ionic strength buffer destabilizes the active conformation of ΔN222, while dilution into a high-ionic strength buffer ΔN222 stabilizes the active form. The destabilization of ΔN222 in a low-ionic strength buffer is reversible by the addition of FAS, but not with NaCl. The salts used in this study are structure-stabilizing salts (*22*), which suggests that salt serves only to stabilize the current conformation of ΔN222, and not to facilitate conformational change between the inactive and active forms. The physiological relevance of the stabilization of ΔN222 by high salt concentrations is unclear. Coburn et al. (*9*) reported that a low-salt buffer reduced native exoenzyme S ADP-ribosyltransferase activity by a factor of 9, relative to that of a physiological buffer, which suggested that ionic strength might modulate the physiologically relevant rate of ADP-ribosylation by ExoS in vivo.

Others have shown that salts can stimulate and/or attenuate enzyme activity (*22*). One example is the activation of the RecA protein ATPase, where salt functionally mimics DNA to activate the RecA protein ATPase (*22*). Pugh and Cox (*22*) developed a model system to explore the mechanism of ATP hydrolysis by RecA and showed that the activation of RecA was due to a structural change which was induced by DNA binding or salt. Activation of RecA ATPase induced the structural transition from a compact, inactive form to an open, active conformation. Generally, salts affect proteins through several different mechanisms, including the direct binding of ions to protein, or indirectly through solvent effects (*22*−*24*).

Exotoxins are typically produced as proenzymes, which are activated in vivo. Mechanisms of activation include proteolysis of the holotoxin for producing a catalytic domain (*25*) or incubation with nucleotides or detergents (*26*) for converting the toxin into a catalytically active conformation. In addition to ExoS, several bacterial exotoxins are activated by eukaryotic proteins. Calmodulin activates the adenylate cyclases of *Bordetella pertussis* and *Bacillus anthracis* (*27*, *28*), while ARF activates the enterotoxins of *Vibrio cholerae* and *E. coli*. Activation of cholera toxin (CT) by ARF was enhanced in a concentration-dependent manner by detergents

such as SDS and cholate (*29*). Low concentrations of other detergents (Triton X-100 or CHAPS) decreased the extent of the apparent activation of CT by ARF by increasing the ARF-independent activity of CT. ARF stimulated ADP-ribosyltransferase activity by increasing the affinity of CT for NAD, and increasing the rate of the reaction (*29*). ARF decreased the K_m values for NAD and the ADP-ribose acceptor, agmatine, approximately 2-fold. Thus, ARF and FAS appear to activate CT and ExoS, respectively, via functionally similar mechanisms.

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REFERENCES

1. Bodey, G. P., Bolivar, R., Fainstein, V., and Jadeja, L. (1983) Infections caused by *Pseudomonas aeruginosa*, *Rev. Infect. Dis. 5*, 279−313.
2. Nicas, T. I., and Iglewski, B. H. (1985) The contribution of exoproducts to the virulence of *Pseudomonas aeruginosa*, *Can. J. Microbiol. 31*, 387−392.
3. Krueger, K. M., and Barbieri, J. T. (1995) The family of bacterial ADP-ribosylating exotoxins, *Clin. Microbiol. Rev. 8*, 34−37.
4. Kulich, S. M., Frank, D. W., and Barbieri, J. T. (1993) Purification and characterization of exoenzyme S from *Pseudomonas aeruginosa* 388, *Infect. Immun. 61*, 307−313.
5. Yahr, T. L., Barbieri, J. T., and Frank, D. W. (1996) Genetic relationship between the 53- and 49-kilodalton forms of exoenzyme S from *Pseudomonas aeruginosa*, *J. Bacteriol. 178*, 1412−1419.
6. Kulich, S. M., Yahr, T. L., Mende-Mueller, L. M., Barbieri, J. T., and Frank, D. W. (1994) Cloning the structural gene for the 49-kDa form of exoenzyme S (*exoS*) from *Pseudomonas aeruginosa* strain 388, *J. Biol. Chem. 269*, 10431−10437.
7. Knight, D. A., Finck-Barbancon, V., Kulich, S. M., and Barbieri, J. T. (1995) Functional domains of *Pseudomonas aeruginosa* exoenzyme S, *Infect. Immun. 63*, 3182−3186.
8. Liu, S., Yahr, T., Frank, D. W., and Barbieri, J. T. (1997) Biochemical relationships between the 53-kilodalton (Exo53) and 49-kilodalton (ExoS) forms of Exoenzyme S, *J. Bacteriol. 179*, 1609−1613.
9. Coburn, J., Dillon, S. T., Iglewski, B. H., and Gill, D. M. (1989) Exoenzyme S of *Pseudomonas aeruginosa* ADP-ribosylates the intermediate filament protein vimentin, *Infect. Immun. 57*, 996−998.
10. Coburn, J., Wyatt, R. T., Iglewski, B. H., and Gill, D. M. (1989) Several GTP-binding proteins, including $p21^{c-H-ras}$, are preferred substrates of *Pseudomonas aeruginosa* exoenzyme S, *Infect. Immun. 254*, 9004−9008.
11. Knight, D. A., and Barbieri, J. T. (1997) Ecto-ADP-ribosyltransferase activity of *Pseudomonas aeruginosa* exoenzyme S, *Infect. Immun. 65*, 3304−3309.
12. McGuffie, E. M., Frank, D. W., Vincent, T. S., and Olson, J. C. (1998) Modification of Ras in eukaryotic cells by *Pseudomonas aeruginosa* Exoenzyme S, *Infect. Immun. 66*, 2607−2613.
13. Ganesan, A. K., Frank, D. W., Misra, R. P., Schmidt, G., and Barbieri, J. T. (1998) *Pseudomonas aeruginosa* Exoenzyme S ADP-ribosylates Ras at multiple sites, *J. Biol. Chem. 273*, 7332−7337.
14. Coburn, J., Kane, A. V., Feig, L., and Gill, D. M. (1991) *Pseudomonas aeruginosa* exoenzyme S requires a eukaryotic protein for ADP-ribosyltransferase activity, *J. Biol. Chem. 266*, 6438−6446.
15. Yahr, T. L., Goranson, J., and Frank, D. W. (1996) Exoenzyme S of *Pseudomonas aeruginosa* is secreted by a type III pathway, *Mol. Microbiol. 22*, 991−1003.
16. Serventi, I. M., Moss, J., and Vaughan, M. (1992) Enhancement of cholera toxin-catalyzed ADP-ribosylation by guanine nucleotide-binding proteins, *Curr. Top. Microbiol. Immunol. 175*, 43−67.
17. Kahn, R. A., and Gilman, A. G. (1986) The protein cofactor necessary for ADP-ribosylation of Gs by Cholera toxin is itself a GTP binding protein, *J. Biol. Chem. 261*, 7906−7911.
18. Gill, D. M., and Coburn, J. (1987) ADP-ribosylating by cholera toxin: functional analysis of a cellular system that stimulates the enzymatic activity of cholera toxin fragment A_1, *Biochemistry 26*, 6364−6371.
19. Fu, H., Coburn, J., and Collier, R. J. (1993) The eukaryotic host factor that activates exoenzyme S of *Pseudomonas aeruginosa* is a member of the 14-3-3 protein family, *Proc. Natl. Acad. Sci. U.S.A. 90*, 2320−2324.
20. Leffers, H., Madsen, P. L., Rasmussen, H. H., Honore, B., Andersen, A. H., Walbum, E., Vanderkerckhove, J., and Celis, J. E. (1993) Molecular cloning and expression of the transformation sensitive epithelial marker Stratifin. A member of a protein family that has been involved in the Protein Kinase C signaling pathway, *J. Mol. Biol. 231*, 982−998.
21. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−685.
22. Pugh, B. F., and Cox, M. M. (1988) High salt activation of RecA protein ATPase in the absence of DNA, *J. Biol. Chem. 263*, 76−83.
23. Warren, J. S., Stowring, L., and Morales, M. F. (1966) The effect of structure-disrupting ions on the activity of myosin and other enzymes, *J. Biol. Chem. 241*, 309−316.
24. Arakawa, T., and Timasheff, S. N. (1982) Preferential interactions of proteins with salts in concentrated solutions, *Biochemistry 21*, 6545−6552.
25. Rasheed, K. L., Guzman-Verduzco, M., and Kupersztoch, Y. M. (1990) Two precursors of heat-stable enterotoxin of *Escherichia coli*: Evidence of extracellular processing, *Mol. Microbiol. 4*, 265−274.
26. Kaslow, H. R., and Burns, D. L. (1992) Pertussis toxin and target eukaryotic cells: binding, entry, and activation, *FASEB J. 6*, 2684−2690.
27. Wolff, J., Cook, G. H., Goldhammer, A. R., and Berkowitz, S. A. (1980) Calmodulin activates prokaryotic adenylate cyclase, *Proc. Natl. Acad. Sci. U.S.A. 77*, 3841−3844.
28. Leppla, S. H., Ivins, E., and Ezel, J. W., Jr. (1985) in *Microbiology* (Leive, L., Ed.) pp 63−66, American Society for Microbiology, Washington, DC.
29. Bobak, M., Bliziotes, M., Noda, M., Tsai, S. C., Adamik, R., and Moss, J. (1990) Mechanism of activation of cholera toxin by ADP-ribosylation Factor (ARF): both low- and high-affinity interactions of ARF with guanine nucleotides promote toxin interaction, *Biochemistry 29*, 855−861.

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