ADP-Ribosyltransferase Type A from Turkey Erythrocytes Modifies Actin at Arg-95 and Arg-372

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ADP-Ribosyltransferase Type A from Turkey Erythrocytes Modifies Actin at Arg-95 and Arg-372†

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ABSTRACT: Turkey erythrocyte ADP-ribosyltransferase A catalyzes the transfer of ADP-ribose from NAD to both monomeric and polymeric skeletal muscle α-actin with the incorporation of 2 mol of ADP-ribose per mol of actin. In contrast, *Clostridium perfringens* iota toxin ADP-ribosylates only G-actin, with modification at arginine-177 [Vandekerckhove, J., et al. (1987) *FEBS Lett. 255*, 48−42]. Transferase A-catalyzed modifications are sensitive to 0.5 M neutral hydroxylamine, consistent with the arginine side chain modification. Radiolabeled peptides ADP-ribosylated by transferase A were generated by tryptic digestion and purified by reversed phase high-performance liquid chromatography. Amino acid sequence and molecular mass analysis identified the ADP-ribosylation sites as Arg-95 and Arg-372 of actin; both residues are located within subdomain-1 of the actin 3D structure [Kabsch, W., et al. (1990) *Nature 347*, 37−44]. ADP-ribosylation did not affect cytochalasin D-stimulated G-actin ATPase, the binding of actin to DNase I or to gelsolin, or the ability of actin to polymerize. Following ADP-ribosylation, however, a prolonged delay in polymerization was observed, consistent with a decreased rate of nucleation.

Bacterial ADP-ribosylating toxins are characterized by highly efficient amino acid-specific mono-ADP-ribosylation of their eukaryotic protein substrates. Diphtheria toxin ADP-ribosylates diphthamide, a posttranslationally modified histidine residue, in elongation factor-2 (Van Ness et al., 1980). Small GTP-binding proteins of the Rho family are selectively ADP-ribosylated at asparagine-41 (Sekine et al., 1989) by *Clostridium botulinum* C3 ADP-ribosyltransferase (Aktories et al., 1987) and other C3-like ADP-ribosyltransferases (Just et al., 1992; Sugai et al., 1990). Cholera toxin and the related *Escherichia coli* heat-labile toxins specifically ADP-ribosylate arginine residues of the α-subunits of various heterotrimeric G-proteins (Moss & Vaughan, 1988; Gierschik, 1992).

Arginine is the acceptor amino acid for ADP-ribosylation by a family of clostridial toxins that selectively modify actin (Aktories et al., 1986, 1992). Members of this family are *Clostridium perfringens* iota toxin (Schering et al., 1988; Simpson et al., 1987), *Clostridium botulinum* C2 toxin (Aktories et al., 1986), and *Clostridium spiroforme* toxins (Popoff & Boquet, 1988; Simpson et al., 1989). All of these toxins ADP-ribosylate actin at arginine-177 (Vandekerckhove et al., 1987, 1988). They differ, however, in their substrate specificities. Whereas iota toxin[1] ADP-ribosylates all actin isoforms, *Clostridium botulinum* C2 toxin modifies nonmuscle actin and smooth muscle γ-actin but not α-actin isoforms (Aktories et al., 1986; Schering et al., 1988; Mauss et al., 1990). The toxin-catalyzed ADP-ribosylation impairs the ability of actin to polymerize (Aktories et al., 1986). Furthermore, the ADP-ribosylation of actin inhibits its ATPase activity (Geipel et al., 1989, 1990) and turns actin into a capping protein that binds to the barbed ends of actin filaments and inhibits the further addition of unmodified actin monomers onto the filaments (Weigt et al., 1989; Wegner & Aktories, 1988). The toxins also ADP-ribosylate actin−gelsolin complexes, and the modified complex is unable to nucleate the polymerization of unmodified actin (Wille et al., 1992).

Arginine is also the acceptor amino acid for several eukaryotic mono-ADP-ribosyltransferases. At least four ADP-ribosyltransferases from turkey erythrocytes have been described (Moss et al., 1985). One type, designated transferase A, is a 28 kDa enzyme that was purified from the cytosolic fraction (Moss et al., 1980; Yost & Moss, 1983). Transferase A modifies simple guanidino compounds, like agmatine, a simple arginine derivative, which is also a cholera toxin substrate (Moss et al., 1985) but not a substrate for actin−ADP-ribosylating toxins. Glutamine synthetases from ovine brain (Moss et al., 1984) and from *Escherichia coli* (Moss et al., 1990) are ADP-ribosylated by transferase A, a modification inhibiting synthetase activity (Moss et al., 1984, 1990).

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[1] Abbreviations: iota toxin, *Clostridium perfringens* iota toxin; NBD-actin, 7-chloro-4-nitro-2-oxa-1,3-benzdiazole-labeled actin at Lys-373; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; transferase A, ADP-ribosyltransferase type A from turkey erythrocytes.

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We studied whether actin, which is the target for several bacterial ADP-ribosylating toxins, could also serve as a substrate for transferase A. In these studies, we characterized the site and functional effects of modification.

EXPERIMENTAL PROCEDURES

Materials. [^{32}P]NAD was obtained from NEN (Bad Homburg, Germany). All other reagents were analytical grade and came from commercial sources. Skeletal muscle α-actin was purified as described (Spudich & Watt, 1971). For some studies, it was modified first with *N*-ethylmaleimide at cysteine-374 and subsequently with 7-chloro-4-nitro-2-oxa-1,3-benzdiazole at lysine-373 to produce the fluorescently labeled protein (Detmers et al., 1981). The concentration of actin was determined photometrically at 290 nm using an absorption coefficient of 24 900 M^{-1} cm^{-1} (Wegner, 1976). The gelsolin−actin complex was prepared as described (Wille et al., 1992). *Clostridium perfringens* iota toxin (Stiles & Wilkens, 1986) and turkey erythrocyte ADP-ribosyltransferase type A (Moss et al., 1980) were purified as described.

ADP-Ribosylation Reaction. ADP-ribosylation reactions were carried out essentially as described (Just et al., 1990). Briefly, ADP-ribosylation was performed by turkey erythrocyte ADP-ribosyltransferase A (6−10 μg/mL) or *C. perfringens* iota toxin (3 μg/mL) in a medium containing 50 mM sodium phosphate (pH 7.5), 1 mM dithiothreitol, 50 μM $MgCl_2$, 30 μM [^{32}P]NAD (0.5 μCi), and 5−10 μM skeletal muscle α-actin for 60 min at 30 °C, or as indicated.

Electrophoresis. ADP-ribosylated proteins were precipitated by trichloroacetic acid (20%), dissolved in sample buffer, and separated by SDS−PAGE according to Laemmli (1970).

2D gel electrophoresis was performed in a Mini-Protean II 2D cell (Bio-Rad, Munich, Germany) according to O'Farrell (1975). Unmodified actin (1.0 μg) was combined with 1.0 μg of [^{32}P]ADP-ribosylated actin and loaded onto the rod gels for isoelectric focusing in a pH gradient of 5−7. The second dimension was run on an 11% SDS slab gel.

Nondenaturing gel electrophoresis was performed in a Mini-Protean II gel system (Bio-Rad Munich, Germany) according to Safer (1989). Sample buffer (4 μL) (0.5 mM $CaCl_2$, 1.0 mM ATP, 0.5 mM dithiothreitol, 10 mM Tris-HCl (pH 8.0), and 50% glycerol (v/v)) was added to the ADP-ribosylation reaction mixture (20 μL). After incubation for 10 min at 21 °C, the sample was centrifuged (10 min at 13000*g*), 6 μL of the supernatant was loaded onto the gel, and electrophoresis was performed for 50 min at 140 V.

Filter Assay. For the determination of the amount of ADP-ribose incorporated in actin, the ADP-ribosylation reaction was terminated by the addition of 1 mL of 20% trichloroacetic acid (w/v). Precipitated proteins were collected on nitrocellulose (0.45 μm pore size, Schleicher & Schuell), and filters were washed with 15 mL of 6% (w/v) trichloroacetic acid before the radioactivity was determined with liquid scintillation counting. As observed previously, the maximal iota toxin-catalyzed ADP-ribosylation of actin was 0.7−0.8 mol of ADP-ribose per mol of actin, as determined by filter assay (Wille et al., 1992). However, using 2D or nondenaturing gel electrophoresis, incorporation of 1 mol of ADP-ribose per mol of actin was determined. Therefore, the maximal incorporation of ADP-ribose induced by iota toxin was set to 1 mol of ADP-ribose per mol of actin for the filter assay method. All other data were adjusted to this reference value (see Figure 2).

Chemical Stability of the ADP-Ribose−Actin Bond. The chemical stability of the ADP-ribose−protein bond was determined as described (Mayer et al., 1988; Bredehorst et al., 1978). After termination of the ADP-ribosylation reaction by the addition of 2% sodium dodecyl sulfate and 100 mM Hepes (pH 7.5), 50 μL samples of the reaction mixture were added to 50 μL of 1 M NaCl and 1 M NH_2-OH (pH 7.5) or 2 mM $HgCl_2$ and incubated for 90 or 30 min, respectively, at 37 °C. Thereafter, proteins were precipitated with 20% trichloroacetic acid. The pellets were dissolved in sample buffer and analyzed by SDS−PAGE followed by autoradiography.

Sequential ADP-Ribosylation of Actin by Both Transferases. Actin was ADP-ribosylated with 30 mM unlabeled NAD and transferase A (10 μg/mL) or iota toxin (3 μg/mL) for 30 min at 30 °C. Thereafter, 1 μM [^{32}P]NAD (0.5 μCi) and iota toxin or transferase A were added, followed by incubation for 30 min at 30 °C and analysis by SDS−PAGE and autoradiography.

Actin-Catalyzed ATP Hydrolysis. Actin (7 μM) was preincubated with DNase I (10 μM) for 30 min on ice to form the actin−DNase I complex, which was then incubated with 50 μM NAD and transferase A (10 μg/mL) or iota toxin (3 μg/mL) for 60 min at 30 °C. Control actin−DNase I was incubated with transferase A without NAD. The blank contained only DNase I. ATPase activity was determined as described (Geipel et al., 1990). After ADP-ribosylation (100 μL assay volume), free nucleotides were removed by the addition of 20 μL Dowex 1 × 8 (50%, v/v), mixing, and centrifugation (1 min at 13800*g*). The supernatant was incubated with 50 μM $MgCl_2$, 50 μM [γ-^{32}P]ATP (0.5 μCi), and 1 μM cytochalasin D for the indicated times at 37 °C before the addition of 450 μL of ice-cold 20 mM sodium phosphate buffer (pH 2.0) containing 5% activated charcoal to 50 μL samples of the reaction mixture. After centrifugation (15 min at 13800*g*), radioactivity was measured in 300 μL of the supernatant.

Identification of the Sites of ADP-Ribosylation in Actin. Skeletal muscle F-actin was ADP-ribosylated in the presence of [^{14}C]NAD with transferase A under conditions where 2 mol of ADP-ribose were incorporated per mol of actin (60 min at 37 °C). Complete ADP-ribosylation was verified by nondenaturing gel electrophoresis after depolymerization. ADP-ribosylated actin was sedimented by centrifugation (60 min at 150000*g*). The pellet (300 μg) was resuspended in 30 μL of 8 M urea and then diluted with 100 mM Tris-HCl (pH 8.0) to a final concentration of 1 M urea. Actin was digested with 25 μg of trypsin for 16 h at 37 °C. Peptides were separated first by reversed phase HPLC on a Sephasil RP-C18 5 μm SC 2.1 × 10 column (Pharmacia) using a linear gradient starting from 0.1% trifluoroacetic acid in water and ending with 70% acetonitrile in 0.1% trifluoroacetic acid in water. The gradient was applied over a period of 120 min. Peptide elution was followed by absorbance at 214 nm and by liquid scintillation counting. ^{14}C-labeled peptides were rechromatographed on a Vydac C18 column (2.1 mm i.d. × 250 mm) using an Applied Biosystem Inc. Model 1490A solvent delivery system. The gradient elution and detection of the peptides were performed as described earlier. One of the labeled peptides was subdigested further with

staphylococcal V8 protease in an aliquot for 18 h at 37 °C with an enzyme−substrate ratio of 1/5. The secondary fragments were separated and detected as described earlier.

Labeled peptides were analyzed by matrix-assisted laser-desorption mass spectrometry using a Vestec instrument. The rest was used for amino acid sequence analysis using an Applied Biosystem Inc. 177A protein sequenator equipped with an on-line 120A phenylthiohydantoin amino acid analyzer.

Determination of the Arginine Residue That Was First Modified. To study the time course of modification more exactly, the rate of modification was decreased by performing ADP-ribosylation at 0 °C. In preliminary experiments, the incorporation of ADP-ribose was checked by nondenaturing gel electrophoresis. Each ADP-ribose caused a shift in migration behavior. Mono-ADP-ribosylation with traces of double ADP-ribosylation was detected after 5 h, and the incorporation of about 2 mol of ADP-ribose was found after incubation for more than 24 h. At these two times, [^{32}P]-ADP-ribosylated actin was subjected to tryptic proteolysis, and peptides were separated by reversed phase HPLC on a Sephasil RP-C18 5 μm SC 2.1 × 10 column as described earlier. The radioactive peptides that correspond to P1 (elution time, 23 min) and P2 (36 min) were counted for radioactivity.

Polymerization of Actin. Skeletal muscle G-actin (5 μM) was ADP-ribosylated overnight at 4 °C with 25 μM NAD in the absence and presence of transferase A (10 μg/mL) or iota toxin (3 μg/mL). Similar results were obtained with incubation for 1 h at 37 °C. Afterward, all solutions were prewarmed for 5 min at 37 °C. ADP-ribosylated actin was supplemented with 5% NBD−actin, and polymerization was initiated by the addition of 2 mM $MgCl_2$, 100 mM KCl, and 0.5 mM ATP (all final concentrations) to the actin solution. Since polymeric actin has approximately 2-fold greater fluorescence intensity than monomeric actin (Detmers et al., 1981), polymerization was followed by the increase in fluorescence intensity. The excitation wavelength was 480 nm, and the fluorescence intensity was measured at 540 nm.

For determination of the critical concentration of actin, polymerization was studied in the presence 0.5 μM F-actin at various concentrations of G-actin (0.4, 0.8, 1.6, and 3.2 μM) previously ADP-ribosylated without and with NAD and transferase A. Changes in the fluorescence intensity were plotted against the concentration of monomeric actin, and the critical actin concentration was extrapolated.

RESULTS

Figure 1A shows the ADP-ribosylation of skeletal muscle α-actin by erythrocyte transferase A and by iota toxin. Whereas iota toxin exclusively modified monomeric G-actin, transferase A catalyzed the incorporation of ADP-ribose into both G-actin and F-actin. The same amount of incorporation was observed with F-actin in the presence of phalloidin (10 μM). Phalloidin decreases the critical concentration for actin polymerization and stabilizes the filaments (Cooper, 1987). The labeling of actin by transferase A or iota toxin was inhibited by nicotinamide (1 mM) but not by ADP-ribose (1 mM), which is consistent with a typical ADP-ribosylation reaction (data not shown). [^{32}P]ADP-ribosylated actin

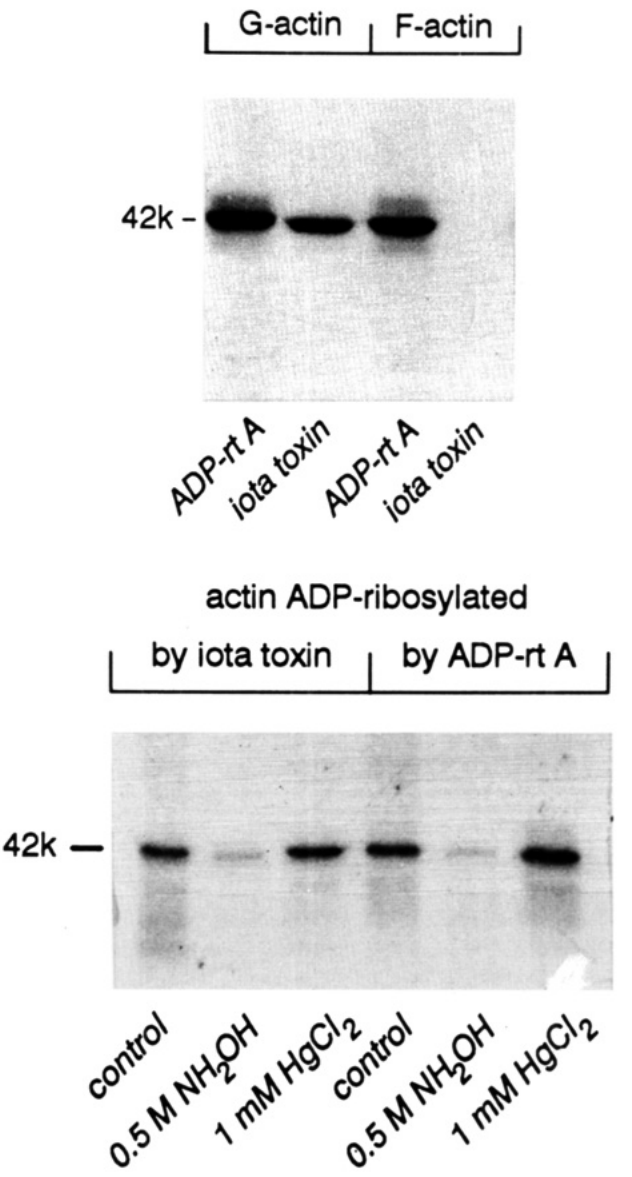

FIGURE 1: (A, top) ADP-ribosylation of skeletal muscle α-actin by turkey erythrocyte ADP-ribosyltransferase A or *C. perfringens* iota toxin. Skeletal muscle actin (5 μM) was incubated without (G-actin) or with 10 μM phalloidin (F-actin) for 30 min at 21 °C, followed by [^{32}P]ADP-ribosylation with 10 μg/mL transferase A (ADP-rt A) or 3 μg/mL iota toxin, as indicated, for 30 min at 30 °C. Proteins were subjected to SDS−PAGE, followed by autoradiography (shown). (B, bottom) Chemical stability of the ADP-ribose−actin bond formed by ADP-ribosyltransferase A or iota toxin. Skeletal muscle actin (5 μM) was [^{32}P]ADP-ribosylated by ADP-ribosyltransferase A (10 μg/mL) or iota toxin (3 μg/mL) for 60 min at 30 °C, as described in the Experimental Procedures. After the addition of 2% SDS and 100 mM Hepes (pH 7.5), mixtures were incubated in the presence of 0.5 M NaCl (control), 0.5 M NH_2OH (pH 7.5), or 1 mM $HgCl_2$ for 90 min at 37 °C. The proteins were analyzed by SDS−PAGE and autoradiography (shown).

synthesized by transferase A or iota toxin was cleaved by incubation with neutral 0.5 M hydroxylamine for 90 min at 37 °C (Figure 1B). In contrast, 1 mM $HgCl_2$, which selectively cleaves ADP-ribose−cysteine bonds, was without effect, as expected since both transferase A and iota toxin catalyzed the formation of ADP-ribose−arginine bonds (Moss et al., 1980; Vandekerckhove et al., 1987).

Iota toxin catalyzes the incorporation of 1 mol of ADP-ribose per mol of actin, and the reaction proceeds to completion in 60 min (Figure 2). In contrast, transferase A

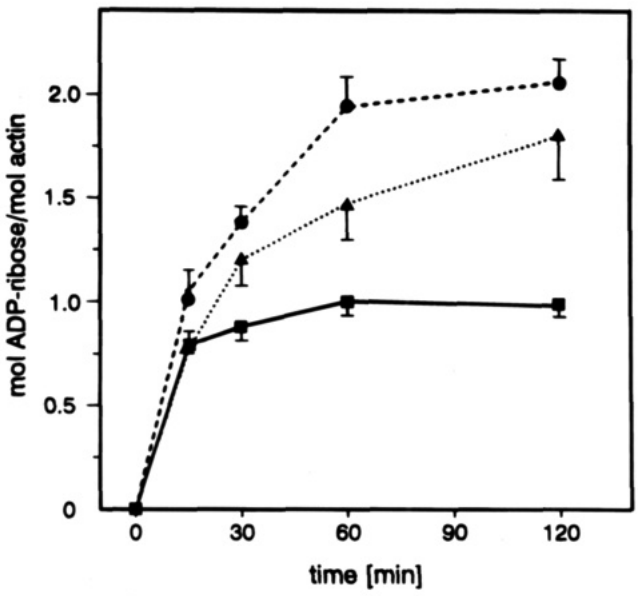

FIGURE 2: Time course of the ADP-ribosylation of actin by ADP-ribosyltransferase A and iota toxin. Skeletal muscle actin (5 μM) was incubated without (■, ●) or with (▲) 10 μM phalloidin for 30 min at 4 °C, followed by incubation at 30 °C with transferase A (6 μg/mL; ●, ▲) or iota toxin (3 μg/mL; ■). At the indicated times, the incorporated [^{32}P]ADP-ribose was determined by the filter assay. Data are the means ± SEM of triplicate determinations.

catalyzes the incorporation of 2 mol of ADP-ribose per mol of G-actin after an incubation period of 120 min. Incubation for an additional 24 h did not further increase the extent of ADP-ribosylation. The rate of ADP-ribosylation of F-actin by transferase A was slower than that of G-actin, although almost 2 mol of ADP-ribose per mol of actin were finally incorporated (Figure 2). Iota toxin-catalyzed ADP-ribosylation shifts the isoelectric point of actin to more acidic values (Figure 3). This shift was also observed after the ADP-ribosylation of actin by transferase A, but as expected, the extent of the shift was almost doubled. These findings indicate that transferase A modifies actin at more than one and possibly two positions. Furthermore, two labeled proteins were detected on the autoradiogram, possibly indicating that part of the actin was modified by transferase A at only one site.

To determine whether transferase A uses the same acceptor site for ADP-ribose in the actin molecule as iota toxin, actin was modified by either transferase A or iota toxin in the presence of unlabeled NAD. Thereafter, a second ADP-ribosylation was performed in the presence of [^{32}P]NAD. When the same transferase was used for the first and second ADP-ribosylations, no major labeling occurs, indicating that actin was completely modified in the first incubation with unlabeled NAD (Figure 4). However, when the second ADP-ribosylation was performed with an enzyme different from that of the first incubation, actin was additionally labeled, indicating that transferase A and iota toxin modified different arginines.

To locate the sites of modification, actin was ADP-ribosylated at both sites (for conditions, see Figure 2) in the presence of [^{14}C]NAD and subsequently digested with trypsin. Peptides were first separated by reversed phase HPLC (Figure 5A) and each radioactive peak was additionally purified over a narrow-bore reversed phase column (Figure 5B,C). Peptide P1 displayed a single sequence overlapping with residues 360−373 of actin. No assignment could be made at position 372, corresponding to an arginine residue in the unmodified protein. The peptide mass (2148.6 mass units) (Figure 5E) corresponds with residues 360−373 containing an additional single ADP-ribosyl group. P2 was not pure at this stage, and one peptide (P3) was subdigested further with the staphylococcal V8 protease and the fragments were repurified by narrow-bore HPLC (Figure 5D). The radioactive peak showed a sequence corresponding to residues 94−107, with blank identification corresponding to arginine-95. The peptide mass (2171.3 mass units) (Figure 5F) is consistent with a singly ADP-ribosylated peptide 94−107, indicating that ADP-ribosylation was at arginine-95.

Next we determined which of the two arginine acceptor amino acids is modified at first in the time course of ADP-ribosylation. Therefore, the rate of modification was decreased by performing ADP-ribosylation at 0 °C. In preliminary experiments, we found incorporation of 1 mol of ADP-ribose per mol of actin after 5 h and incorporation of 2 mol of ADP-ribose after incubation for more than 24 h. At these two times, [^{32}P]ADP-ribosylated actin was subjected to tryptic proteolysis, and peptides were separated by reversed phase HPLC as described in legend to Figure 5. The radioactive peptides that correspond to P1 (Arg-372) and P2 (Arg-95) (see Figure 5A) were counted for radioactivity. After 5 h, most radioactivity was found in peptide P2 (43 657

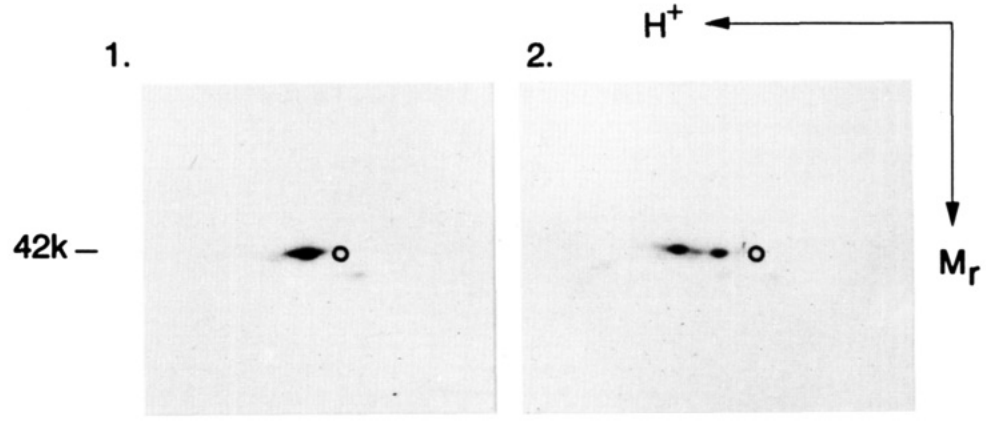

FIGURE 3: 2D gel electrophoresis of actin ADP-ribosylated by ADP-ribosyltransferase A or iota toxin. Actin was [^{32}P]ADP-ribosylated by iota toxin (3 μg/mL, panel 1) or transferase A (10 μg/mL, panel 2) for 45 min at 30 °C. ADP-ribosylated actin (1 μg) plus 1 μg of unmodified actin was applied to 2D gel electrophoresis, which was analyzed subsequently by autoradiography (shown). Open circles indicate the position of unmodified α-actin.

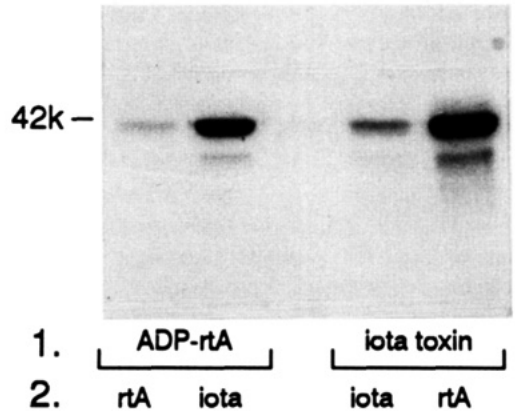

FIGURE 4: Effect of ADP-ribosylation of actin by transferase A or iota toxin on subsequent [^{32}P]ADP-ribosylation by either enzyme. Skeletal muscle α-actin (5 μM) was ADP-ribosylated in the presence of unlabeled NAD (30 μM) by ADP-ribosyltransferase A (10 μg/mL; 1, ADP-rt A) or by iota toxin (3 μg/mL; 1, iota toxin). The second ADP-ribosylation (2) was performed in the presence of 1 μM [^{32}P]NAD by transferase A (rtA) or iota toxin (iota). Autoradiography of SDS–polyacrylamide gel electrophoresis shown.

cpm) and much less was in P1 (8430 cpm). After 24 h, ^{32}P-incorporation into peptide P1 increased (27 811 cpm), whereas incorporation into peptide P2 was almost constant (48 160 cpm). These findings indicate that Arg-95 (P2) is ADP-ribosylated first followed by Arg-372 (P1) after a delay and that unmodified actin as a substrate for transferase A is superior to mono-ADP-ribosylated actin.

It has been shown that iota toxin is capable of ADP-ribosylating actin in a complex with gelsolin and DNase I (Geipel et al., 1990; Wille et al., 1992). The same experiments were carried out here, and the ADP-ribosylated complexes were separated under nondenaturing conditions (Figure 6). Iota toxin ADP-ribosylated actin or gelsolin–actin (1:1) complexes showed a single radioactive band. Transferase A-modified proteins showed two bands, consistent with the incorporation of one and two ADP-ribose molecules, respectively. Similar results were obtained for the actin–DNase I complex (data not shown).

Iota toxin inhibits actin ATPase activity under conditions that prevent actin polymerization, i.e., at an actin concentration below its critical concentration with <50 μM Mg^{2+} and even in the complex with DNase I (Geipel et al., 1989, 1990). We investigated the influence of transferase A-catalyzed ADP-ribosylation on actin ATPase activity in the actin-DNase I complex, which stabilizes a quasi-monomeric G-actin form (Lazarides & Lindberg, 1974). Because G-actin ATPase activity is rather low, ATP hydrolysis was stimulated by cytochalasin D (1 μM) (Geipel et al., 1990). ADP-ribosylation of the actin–DNase I complex by transferase A did not affect the ATPase activity (Figure 7). In contrast, iota toxin-catalyzed ADP-ribosylation inhibited the ATPase activity, as recently reported (Geipel et al., 1990).

Next we studied whether transferase A-catalyzed ADP-ribosylation affects the polymerization of actin. Actin polymerization was determined by the increase in fluorescence of NBD-labeled actin after the addition of 2 mM $MgCl_2$ and 100 mM KCl. At first we tested whether NBD–actin is modified in the same way as control G-actin. Using nondenaturing gel electrophoresis, the incorporation of 2 mol of ADP-ribose per mol of NBD–actin was detected. Polymerization of the control actin caused doubling in fluorescence with a half-time of 1250 s (Figure 8). In the presence of iota toxin and NAD, polymerization was completely blocked. Transferase A did not significantly alter the final extent of polymerization. However, the kinetic behavior of the polymerization reaction changed. There was a delay in filament formation with doubling of the half-time (2600 s). The delay was highly reproducible, but the increase in half-time varied with the actin preparation used. The retarded polymerization was actually caused by transferase-catalyzed ADP-ribosylation because neither the enzyme nor NAD alone induced this change. When polymerization was determined in the presence of preformed F-actin (0.5 μM) (nucleated polymerization), no change in kinetic behavior was detected, indicating an alteration of the initial phase of polymerization. The maximal extent of fluorescence intensity was decreased to a minor extent by transferase A (by about 20%). To test whether this observation correlates with a reduced formation of filaments or with interference between the NBD label and the closely located ADP-ribose moiety, we checked the amount of filamentous actin formed via ultracentrifugation. Therefore, control actin and actin ADP-ribosylated by transferase A were polymerized, and the F-actin was pelleted by centrifugation (60 min at 150000*g*). The amount of actin in the supernatant and pellet was determined photometrically (290 nm) and by densitometric analysis of the SDS–PAGE, respectively. There was no difference detected in the amount of G- and F-actins from controls and transferase A-treated actin. Thus, the difference in the fluorescence of maximally polymerized actin is most likely caused by an interaction of the NBD fluorescence label attached to Lys-373 with the ADP-ribose at Arg-372.

The delay in polymerization of actin ADP-ribosylated by transferase A could be caused by an increase in the critical concentration of actin. However, determination of this parameter showed that control actin and actin that was previously ADP-ribosylated by transferase A exhibited the same critical concentration of 0.24 μM. Thus, the incorporation of 2 mol of ADP-ribose into actin did not alter the critical concentration, but it did retard polymerization, which is most likely caused by deceleration of the nucleation phase.

DISCUSSION

Turkey erythrocyte ADP-ribosyltransferase type A modifies several proteins, such as glutamine synthetase (Moss et al., 1984), tubulin (Scaife et al., 1992), microtubule-associated proteins (Scaife et al., 1992), and skeletal muscle α-actin. Because ADP-ribose had no effect on the rate and extent of actin ADP-ribosylation, a nonenzymatic mechanism could be excluded. The ADP-ribosyl–actin bonds are sensitive to neutral hydroxylamine treatment, in accordance with a guanidinyl ribose type linkage (Aktories et al., 1988). This finding is in agreement with the reported specificity of transferase A for arginine side chains (Yost & Moss, 1983; Moss et al., 1980, 1984, 1985, 1990). It seems that transferase A rather unspecifically catalyzes ADP-ribosylation because of the various substrate proteins. With the exception of microtubule-associated proteins (MAPs), the stoichiometry of modification varies from 1 to 2 mol of ADP-ribose per mol of substrate protein. Actin contains 17 arginine residues, 6 of which are located at the surface of the molecule, and exhibits a free guanidinyl group, but only two arginines are selectively modified.

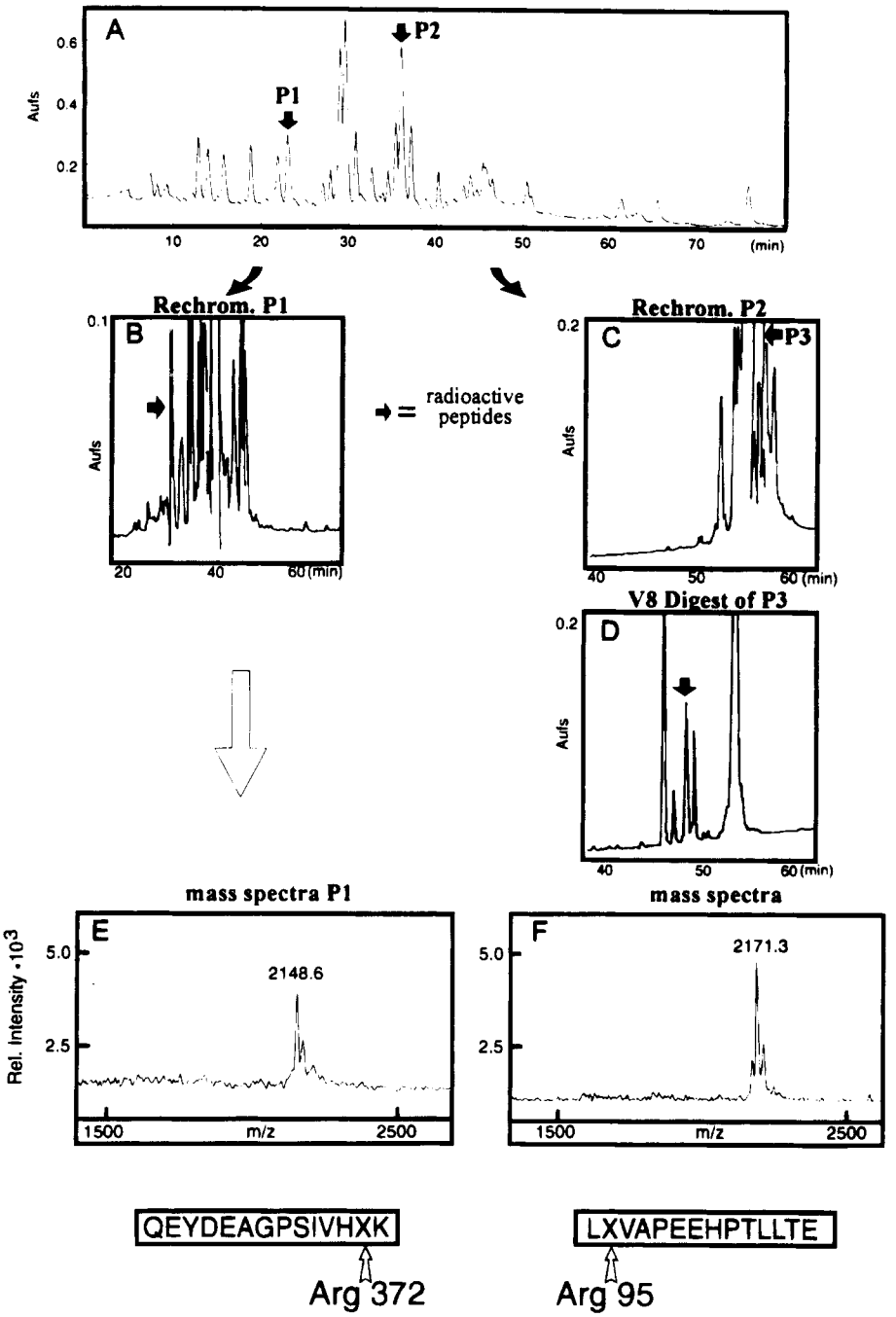

FIGURE 5: Location of the ADP-ribosylated residues in the actin sequence. Skeletal muscle α-actin was ADP-ribosylated with transferase A in the presence of [^{14}C]NAD. The labeled protein was digested with trypsin, and the peptides were separated over a Sephasil RP-C18 5 μm SC 2.1/10 column (panel A). Detection was by absorbance at 214 nm and by radioactivity of the collected peaks (bars). AUFS: absorption units full scale. Each labeled peptide was rechromatographed over a Vydac 2.1 × 20 cm C18 column and detected as before (panels B and C). The labeled peptide from panel C was subdigested with V8 protease, and the fragments again were separated as in B and C. The purified peptides (panels B and C) were subsequently analyzed by matrix-assisted laser desorption mass spectrometry, and their molecular masses were determined (panels E and F). The sequences of the peptides are shown in one-letter notation in boxes under panels E and F, respectively. X refers to the ADP-ribosylarginine residue.

Arginine is also the acceptor amino acid for the actin–ADP-ribosylating toxins *Clostridium perfringens* iota toxin and *Clostridium botulinum* C2 toxin (Vandekerckhove et al., 1987, 1988). However, whereas transferase A modifies actin at Arg-95 and Arg-372, the bacterial transferases select Arg-177 as the target side chain (Vandekerckhove et al., 1987). The different sites of modification explain not only the difference in G- and F-actin modification but also the different properties of the modified actin. Arg-177, the target of iota and C2 toxins, is located on subdomain-3 of actin. This residue is located at the surface of the monomeric actin molecule but is buried in the double-stranded helix of F-actin (Holmes et al., 1990). This explains at the molecular level why G-actin, but not F-actin, is the toxin substrate (Schering et al., 1988) and why Arg-177 ADP-ribosylated actin has lost the ability to form polymers (Aktories et al., 1986; Schering et al., 1988) and behaves as an F-actin capping protein (Wegner & Aktories, 1988; Weigt et al., 1989).

Transferase A-catalyzed ADP-ribosylation of actin occurs at Arg-95 and Arg-372. These residues are located on

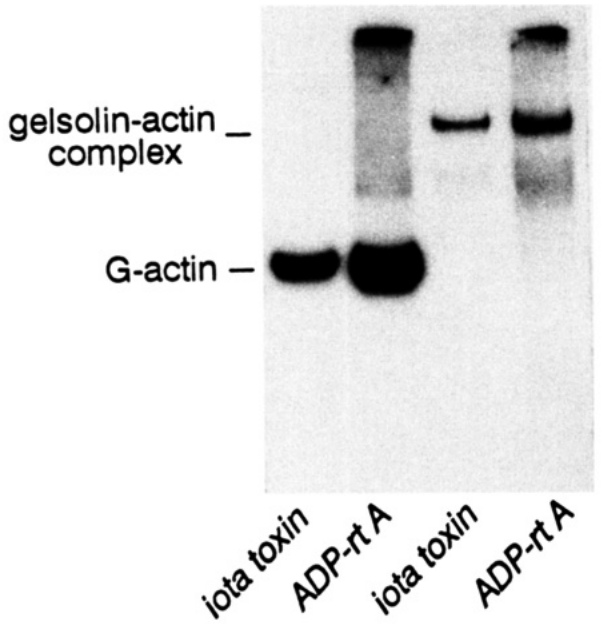

FIGURE 6: Nondenaturing gel electrophoresis of actin and gelsolin–actin ADP-ribosylated by ADP-ribosyltransferase A or iota toxin. Skeletal muscle actin (0.5 μM) and gelsolin-actin (0.35 μM) were ADP-ribosylated in the presence of 10 μM [^{32}P]NAD by transferase A or iota toxin for 30 min at 30 °C, in a total volume of 20 μL. After the addition of 4 μL of sample buffer, samples (6 μL) were run on nondenaturing gels. An autoradiogram of the gel is shown.

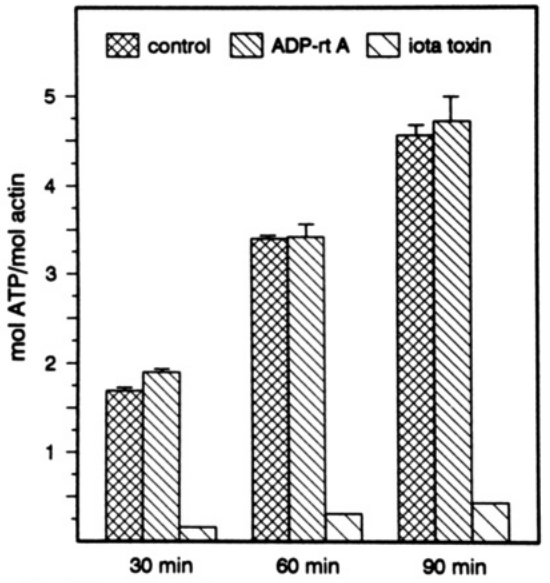

FIGURE 7: Effect of transferase A- and iota toxin-catalyzed ADP-ribosylation on the ATPase activity of actin. The DNase I–actin complex, which was formed by the incubation of skeletal muscle actin (7 μM) with DNase I (10 μM) for 30 min on ice, was ADP-ribosylated by transferase A (10 μg/mL) or iota toxin (3 μg/mL) in the presence of 50 μM NAD for 60 min at 30 °C. Free nucleotides were removed by treatment with Dowex. ATPase activity was determined after the addition of 50 μM [γ-^{32}P]ATP (0.5 μCi), 1 μM cytochalasin D, and 50 μM $MgCl_2$ for the indicated times. Data are means ± SEM of three independent determinations.

subdomain-1 of actin (Kabsch et al., 1990) and, according to the F-actin model (Holmes et al., 1990), also at the surface of the filaments. Therefore, transferase A is capable of modifying both G- and F-actins; consequently, transferase A-modified actin is able to form polymers. In contrast, according to the model of F-actin by Schutt et al. (1993), Arg-95 is part of the actin–actin contact site. The finding

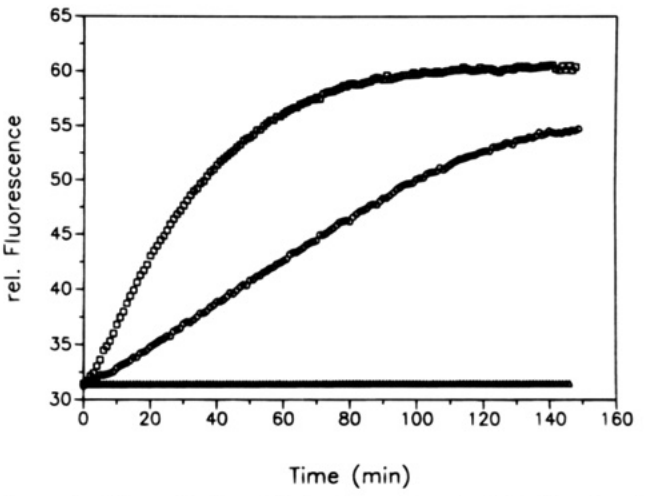

FIGURE 8: Polymerization of skeletal muscle actin ADP-ribosylated by transferase A. Skeletal muscle α-actin (5 μM) was ADP-ribosylated in the presence of 25 μM NAD without enzyme (□), with transferase A (10 μg/mL; ○), or with iota toxin (3 μg/mL, △) before polymerization was initiated by the addition of 2 mM $MgCl_2$, 100 mM KCl, and 0.5 mM ATP (all final concentrations) to the actin solution containing 5% NBD–actin.

that a bulky ADP-ribose group is located in Arg-95 of F-actin argues against this model. This discrepancy may be based on the different actin isoforms used for the models: β-actin in the case of Schutt et al. (1993) and skeletal muscle α-actin in the case of Holmes et al. (1990).

Although transferase A treatment does not change the amount of polymers formed, the polymerization kinetics seems to be altered in the nucleation step, as seen from a decreased rate of polymerization. The variability in the amount of delay may be caused by traces of F-actin formed because transferase A induces only a decreased rate of polymerization in non-nucleated (absence of preformed F-actin) polymer formation. The process of actin nucleation still is not completely understood. It appears that the binding of divalent cations to high- and low-affinity binding sites in actin is crucial for the initiation of polymer formation (Estes et al., 1992; Pollard & Cooper, 1986; Zimmerle et al., 1987; Zimmerle & Frieden, 1988). Thus, it is possible that ADP-ribosylation may have altered the surface charge of actin, affecting cation binding and therefore altering the nucleation process.

Other parameters of actin polymerization, such as the critical concentration for polymerization and the extent of polymer formation, are not affected by the ADP-ribosylation of transferase A. It should be noted, however, that the relative fluorescence of NBD-labeled actin at steady state is only 80% of that of non-ADP-ribosylated actin. Because NBD–actin is double ADP-ribosylated, the decrease in fluorescence is probably due to the quenching effect of the neighboring ADP-ribosyl group on the Lys-373–NBD moiety.

Neither DNase I nor gelsolin (in the EGTA-resistant complex) inhibits Arg-95 and Arg-372 ADP-ribosylation. This is in agreement with the 3D structure derived from the DNase I complex (Kabsch et al., 1990) and the gelsolin segment-1–actin complex (McLaughlin et al., 1993). The former interacts with loop structures in subdomain-2 and subdomain-4 (Kabsch et al., 1990), whereas the latter binds at the interface of subdomain-1 and subdomain-3 (McLaughlin et al., 1993).

Iota toxin-catalyzed ADP-ribosylation inhibits actin ATPase activity (Geipel et al., 1989, 1990). Using cytochalasin D as a stimulator of actin ATPase, the inhibition of ATP hydrolysis by toxin-catalyzed ADP-ribosylation was observed even with actin bound to DNase I. In contrast, transferase A had no effect on actin-catalyzed ATP hydrolysis, neither under conditions that allow actin polymerization nor in the pseudomonomeric actin−DNase complex. Because the ATPase assay measures steady state ATP hydrolysis, it appears that the transferase A-catalyzed ADP-ribosylation of actin has no effect on the binding, hydrolysis, or exchange of the actin-bound nucleotide. Furthermore, the unchanged ATPase activity is consistent with the finding that actin that is ADP-ribosylated by transferase A is able to polymerize. However, we cannot exclude that binding, hydrolysis, and exchange of the nucleotide are affected by ADP-ribosylation without a net change in the overall rate of the reaction.

In summary, we report that the arginine-specific turkey erythrocyte ADP-ribosyltransferase A is capable of modifying actin at Arg-95 and Arg-372 in a stoichiometric manner. The transferase A-catalyzed ADP-ribosylation of actin had no effect on actin ATPase activity or on the ability of actin to polymerize, but it delayed the nucleation of actin polymerization. The findings indicate that the ADP-ribosylations of actin by toxins and by the turkey erythrocyte ADP-ribosyltransferase A have completely different structural requirements and different functional effects.

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