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# Characterization of the Enzymatic Component of *Clostridium perfringens* Iota-Toxin

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**The iota$_a$ component (i$_a$) of *Clostridium perfringens* ADP ribosylates nonmuscle β/γ actin and skeletal muscle α-actin. Replacement of Arg-295 in i$_a$ with alanine led to a complete loss of NAD$^+$-glycohydrolase (NADase) and ADP-ribosyltransferase (ARTase); that of the residue with lysine caused a drastic reduction in NADase and ARTase activities (<0.1% of the wild-type activities) but did not completely diminish them. Substitution of alanine for Glu-378 and Glu-380 caused a complete loss of NADase and ARTase. However, exchange of Glu-378 to aspartic acid or glutamine resulted in little effect on NADase activity but a drastic reduction in ARTase activity (<0.1% of the wild-type activity). Exchange of Glu-380 to aspartic acid caused a drastic reduction in NADase and ARTase activities (<0.1% of the wild-type activities) but did not completely diminish them; that of the residue to glutamine caused a complete loss of ARTase activity. Replacement of Ser-338 with alanine resulted in 0.7 to 2.3% wild-type activities, and that of Ser-340 and Thr-339 caused a reduction in these activities of 5 to 30% wild-type activities. The kinetic analysis showed that Arg-295 and Ser-338 also play an important role in the binding of NAD$^+$ to i$_a$, that Arg-295, Glu-380, and Ser-338 play a crucial role in the catalytic rate of NADase activity, and that these three amino acid residues and Glu-378 are essential for ARTase activity. The effect of amino acid replacement in i$_a$ on ARTase activity was similar to that on lethal and cytotoxic activities, suggesting that lethal and cytotoxic activities in i$_a$ are dependent on ARTase activity.**

*Clostridium perfringens* type E produces iota-toxin, which is lethal and dermonecrotic (35, 37). Iota-toxin is a binary toxin which is composed of an enzymatic component called iota$_a$ (i$_a$) and a binding component called iota$_b$ (i$_b$). i$_a$ causes ADP-ribosylation of skeletal muscle α-actin and nonmuscle β/γ actin, and i$_b$ is required for penetration of i$_a$ into the cytosol (1, 2, 3, 12, 34, 38, 40). It has been reported that iota-toxin may be associated with antibiotic-associated colitis caused by *C. perfringens* type E in rabbits (9, 26). In addition, *C. spiroforme* is known to cause antibiotic-associated enterotoxemia of rabbits and to produce iota-like-toxin (8), which shows partial antigenic identity with iota-toxin (32). Several workers have reported the presumptive involvement of iota-like-toxin in sudden outbreaks of enteritis in rabbit colonies (17, 20). It therefore appears that iota-toxin also is able to be a major agent in enterotoxemia (16, 37). Stiles and Wilkins (43) reported the purification of iota-toxin from cultures of *C. perfringens* type E. However, large amounts of iota-toxin were difficult to purify from the culture supernatant fluid of *C. perfringens* type E by to their method. To understand the mode of action of iota-toxin, large amounts of highly purified i$_a$ and i$_b$ are required. *Bacillus subtilis* is reported to produce and secrete secretory proteins of other gram-positive bacteria (39). We first tried to purify large amounts of i$_a$ from cultures of a *B. subtilis* transformant carrying the component gene.

Bacterial ADP-ribosylating toxins, such as diphtheria toxin (DT) (11), *Pseudomonas* exotoxin A (ETA) (45), cholera toxin (CT) (42), *Escherichia coli* heat-labile enterotoxin (LT) (42), pertussis toxin (22), *Pseudomonas* exoenzyme S (ExoS) (24), *Clostridium botulinum* C3 enzyme (C3) (5), *C. botulinum* C2 toxin (C2 toxin) (4), *C. spiroforme* toxin (2), and epidermal cell differentiation inhibitor (EDIN) from *Staphylococcus aureus* (44), have been studied as agents that contribute to the pathogenesis of bacteria. The Glu-14 and -226 residues in i$_a$ are included within the E-X-X-X-X-W sequence in the active sites of DT and ETA. ADP-ribosylating toxins such as CT, LT, and C3 are known to contain three conserved regions, aromatic residue-R/H, E-X-E, and hydrophobic residue-S-T-S-hydrophobic residue, in the cavity formed by the β/α motif (14). The analysis of the LT crystal suggested that the nicotinamide ring of NAD$^+$ docks into the cavity (14). The role of these regions is thought to be as follows. The polar side chains of E-X-E extend toward the catalytic cavity and the consensus sequence involved in forming the NAD$^+$ cleft; an aromatic residue-R/H located deep in the cavity binds NAD$^+$; and the S-T-S consensus sequence is folded in a β strand representing the floor of the cavity. The Glu-378 and -380 residues in i$_a$ are included within the E-X-E sequences essential for the enzymatic activities of CT, LT, and ExoS. The Arg-295 residue in the component is present in the aromatic residue-R/H sequence. Ser-338, Ser-340, and Thr-339 are present in the S-T-S consensus sequence.

Recently, Perelle et al. (31) reported that Glu-378, Glu-380, and Arg-295 are involved in the ADP-ribosylation of i$_a$. Damme et al. (13) reported the pivotal role of Glu-378 in i$_a$ which was photoaffinity labeled with [*carbonyl*-$^{14}$C]NAD$^+$. However, the reaction mechanism of i$_a$ has not been investigated by use of conservative substitutions and kinetic analysis. To study the functional roles of these conserved regions of i$_a$ in more detail, we replaced Arg-295, Glu-14, Glu-226, Glu-378, Glu-380, Ser-338, Ser-340, and Thr-339 with various amino acids by site-directed mutagenesis, determined the ADP-ribosyltransferase (ARTase) and NAD$^+$-glycohydrolase (NADase) activities of these variant components, determined the cytotoxic and lethal activities of these variant components in the

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presence of $i_b$, and analyzed the enzymatic properties of these components.

## MATERIALS AND METHODS

**Materials.** Restriction endonucleases and DNA-modifying enzymes were obtained from Takara Shuzo (Kyoto, Japan) and Toyobo (Osaka, Japan), respectively. pT-7 Blue was obtained from Novagen (Madison, Wis.). [*adenylate*-$^{32}$P]$NAD^+$ and [*carbonyl*-$^{14}$C]$NAD^+$ were obtained from New England Nuclear (Boston, Mass.) and Amersham (Tokyo, Japan), respectively. Purified rabbit muscle actin was purchased from Worthington Biochemical Corp., Lakewood, N.J. All other chemicals were of analytical grade.

**Bacterial strains and plasmids.** *C. perfringens* type E strain NCIB 10748, kindly donated by M. Popoff (Institut Pasteur, Paris, France), was grown in brain heart infusion broth under anaerobic conditions. Plasmid DNA was extracted and purified as described by Perelle et al. (30). *E. coli* JM109 or C600 was the host for the plasmid used. *B. subtilis* ISW1214 was used for production of the toxin (28).

**DNA cloning and expression of the $i_a$ gene.** *C. perfringens* type E strain NCIB 10748 carrying the entire iota-toxin gene was PCR amplified using a set of primers. A pair of primers for $i_a$ (5′-GAGAATTCAGAAAATACAATC-3′ and 5′-TCTTATCATAGCTGTAAGTG-3′) was designed from the published $i_a$ sequence (30). The PCR was carried out for 25 cycles under standard reaction conditions with a GeneAmp DNA amplification reagent kit (Perkin-Elmer Cetus, Norwalk, Conn.). After PCR amplification, the 1.7-kbp fragment obtained was gel purified and cloned into pT-7 Blue (pTIA). The 1.7-kbp *Eco*RI/*Xba*I fragment of the wild-type $i_a$ gene was subcloned from pTIA into the pHY300PLK (*E. coli-B. subtilis* shuttle vector) *Sma*I site and transformed into *B. subtilis* ISW1214.

**Site-directed mutagenesis.** The 1.7-kbp *Eco*RI/*Xba*I fragment of the $i_a$ gene, encoding the entire reading frame for the 454 amino acids of $i_a$, was introduced into the *Sma*I site of the pUC19 vector (pUIA) and used as a template for mutagenesis. Site-directed mutagenesis was carried out by the unique restriction enzyme site elimination technique with a Transformer mutagenesis kit (Clontech Laboratories, Inc., Palo Alto, Calif.) and synthetic oligonucleotide primers having mutations as described previously (28, 29). All mutants were obtained by this method and were identified by sequencing with a Dye Deoxy termination kit (Applied Biosystems), sequencing primers, and a DNA sequencer (374A; Applied Biosystems).

**Purification of $i_a$ and $i_b$ from *C. perfringens*.** Native $i_a$ and $i_b$ were purified from culture supernatant fluid of *C. perfringens* NCTC 8084 as described previously (43).

**Determination of lethal activity.** Serial twofold dilutions of $i_a$ and 5 μg of $i_b$ were mixed in 0.01 M phosphate buffer (pH 7.0) containing 0.9% NaCl (final volume, 1.0 ml). The mixture (0.1 ml) was injected intravenously into adult mice (about 25 g), and deaths occurring within 24 h were recorded.

**Determination of cytotoxic activity.** Vero cells were cultivated in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. For cytotoxicity assays, the cells were inoculated into 48-well tissue culture plates (Falcon, Oxnard, Calif.). Serial dilutions of various concentrations of $i_a$ and 200 ng of $i_b$ per ml were mixed in Dulbecco modified Eagle medium and inoculated onto a cell monolayer. The cells were observed for morphological alterations 8 h after inoculation.

**NADase assay.** $NAD^+$ glycohydrolysis due to $i_a$ was determined by the release of free radiolabeled nicotinamide into solution, resulting from the hydrolysis of [*carbonyl*-$^{14}$C]$NAD^+$ (41 mCi/mmol) (46). The assays were performed at various $NAD^+$ concentrations in the presence of $i_a$ in a final volume of 50 μl of 40 mM Tris-HCl (pH 7.5) containing 10 mM EDTA, 10 mM dithiothreitol, and 100 μg of bovine serum albumin/ml at 37°C for 6 h. After incubation, hydrolyzed nicotinamide was separated from $NAD^+$ by the addition of 200 μl of water-saturated ethyl acetate. The amount of nicotinamide in the ethyl acetate phase was determined by liquid scintillation counting. The data were corrected by subtraction of the radioactivity due to nonenzymatic hydrolysis of $NAD^+$.

**ARTase assay.** ARTase activity on globular (G)-actin due to $i_a$ was monitored by the incorporation of the radiolabeled ADP-ribose moiety of $NAD^+$ into the trichloroacetic acid (TCA)-precipitable protein fraction of the reaction mixture. [*adenylate*-$^{32}$P]$NAD^+$ (800 Ci/mmol) was diluted to a final specific activity of 16 Ci/mmol with unlabeled $NAD^+$ to give a final concentration of 5 μM. Reaction mixtures in a final volume of 0.1 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol, 40 μM ATP, 40 μM $CaCl_2$, 50 μM $MgCl_2$, and the specified concentrations of $NAD^+$ and actin for the kinetic experiments (see below) were incubated with $i_a$ samples at 37°C for 60 min. After incubation, 0.5 ml of cold 7.5% TCA and 10 μg of bovine serum albumin were added, and the reaction mixtures were allowed to stand for 30 min on ice. Proteins were precipitated by centrifugation at 10,000 × *g* for 20 min. The precipitate was washed twice by centrifugation in 1 ml of ice-cold 7.5% TCA. The precipitated proteins were dissolved in 10 μl of 0.02 M Tris-HCl buffer (pH 7.5) containing 3% sodium dodecyl sulfate (SDS), 2% 2-mercaptoethanol, 5% glycerol, and 0.001% bromophenol blue and then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (12% polyacrylamide gel). The gel was stained with Coomassie blue, destained, and dried in a gel dryer; labeled actin was analyzed with a Fuji BAS 2000 system (Fuji Photo Film Co., Ltd., Tokyo, Japan). The radioactive bands were excised from the gel, and the incorporated radioactivity was counted in a liquid scintillation counter (Aloka Co., Ltd., Tokyo, Japan).

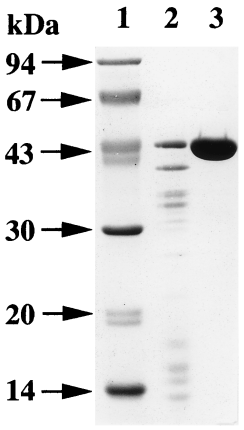


FIG. 1. SDS-PAGE analysis of purified $i_a$. Lanes: 1, molecular mass standards; 2, ammonium sulfate fraction; 3, purified r $i_a$.

**Kinetic experiments.** Initial rate data for the single-substrate NADase reaction were collected under conditions where $NAD^+$ concentrations were varied from 0.2 to 2.5 $K_m$. Initial rate data for the ARTase reaction were similarly determined for $NAD^+$ binding at a fixed actin concentration (0.23 μM) and various $NAD^+$ concentrations from 0.2 to 2.5 $K_m$ and for actin binding at a fixed $NAD^+$ concentration (5 μM) and various actin concentrations from 0.2 to 2.5 $K_m$. The kinetic parameters were obtained by analysis of a Hanes-Woolf plot (18).

**Other procedures.** Protein was assayed by the method of Lowry et al. (25). Double-gel immunodiffusion and SDS-PAGE were carried out as described previously (23, 27). Anti-$i_a$ antiserum was prepared by immunizing a rabbit with 50 μg of purified $i_a$ as described previously (36). Determination of the N-terminal amino acid sequence was performed as described previously (21).

## RESULTS

**Purification of $i_a$ from culture supernatant fluid of the *B. subtilis* transformant carrying the $i_a$ gene.** Growth of the *B. subtilis* transformant reached a maximum in Luria-Bertani broth after 7 h of incubation. The time course of $i_a$ production (ARTase activity) was roughly parallel to the growth of the transformant. ARTase activity remained constant in the culture within 5 h after growth stopped. Ammonium sulfate (313 g/liter) was added to the culture supernatant fluid and allowed to stand overnight at 4°C. SDS-PAGE of the ammonium sulfate fraction revealed that the corresponding band of $i_a$ (about 43 kDa) was dominant in the fraction (Fig. 1), suggesting that the *B. subtilis* transformant produces and secretes large amounts of the component. The ammonium sulfate fraction was dialyzed against 0.02 M Tris-HCl buffer (pH 7.5) and loaded onto a DEAE–Sepharose CL-6B column, previously equilibrated with the same buffer. Elution of the column was done with a 0 to 0.1 M NaCl linear gradient (300 ml total) in 0.02 M Tris-HCl buffer (pH 7.5). Figure 2 shows a typical elution profile of the ammonium sulfate fraction applied to the column. The eluted fractions were separated into two peaks, based on the absorbance at 280 nm. An immunodiffusion test of each fraction was performed with anti-$i_a$ antiserum. Only the second peak (fractions 61 to 68) reacted with the antiserum. Furthermore, the second peak showed ARTase activity, which was completely inhibited by anti-$i_a$ antiserum (data not shown). SDS-PAGE analysis of the preparation (50 μg of protein) revealed only one band of approximately 43 kDa, as shown in Fig. 1. An immunodiffusion test showed that the test preparation and the native component reacted with anti-$i_a$ antiserum, showing a single fused precipitin line (data not shown).

Furthermore, the N-terminal sequence of the purified pro-

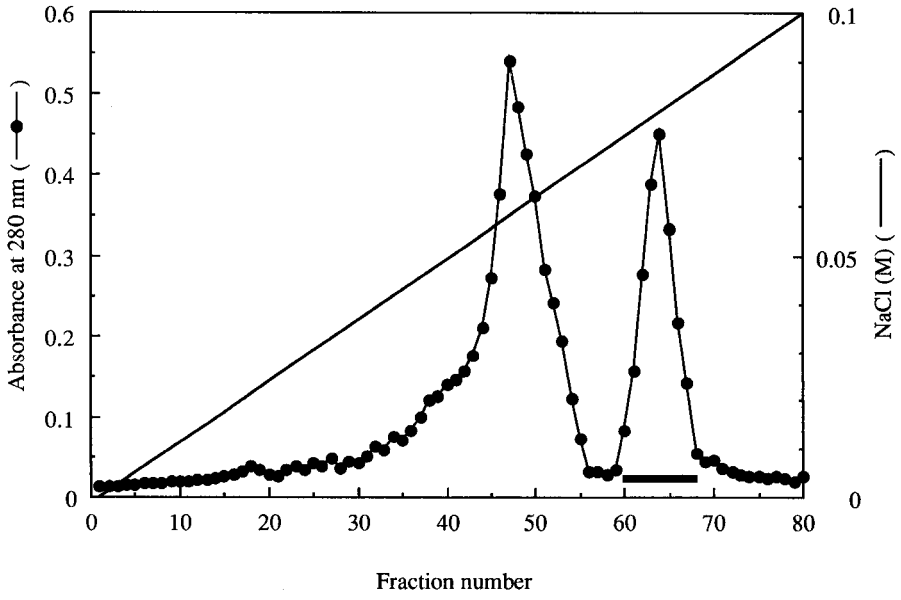


FIG. 2. DEAE–Sepharose CL-6B column chromatography of the ammonium sulfate fraction. The ammonium sulfate fraction obtained from cultures of the *B. subtilis* transformant carrying the $i_a$ gene was applied to a DEAE–Sepharose CL-6B column (2 by 25 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.5). After application, the column was washed with 150 ml of the same buffer and then eluted with 300 ml of a linear gradient of 0 to 0.1 M NaCl in the same buffer. The fractions were collected and assayed for $A_{280}$. The heavy horizontal bar indicates the peak containing $i_a$.

tein was identical to the amino acid sequence of $i_a$ expected from the gene and the native component. The purification steps for recombinant $i_a$ (r $i_a$) are summarized in Table 1. The final step resulted in a homogeneous product that was purified approximately ninefold, starting from the ammonium sulfate fraction, with a yield of about 75% with respect to ARTase activity. The amount of purified r $i_a$ obtained from 1,000 ml of culture was about 35 mg. Stiles and Wilkins (43) reported that the dose required to kill 50% of mice injected intraperitoneally was 0.62 μg of $i_a$ in the presence of 0.94 μg of $i_b$, but administration of 0.31 μg of $i_a$ and 0.47 μg of $i_b$ killed no mice. In the present work, approximately 2 ng of purified r $i_a$ was required to kill 50% of mice in the presence of approximately 500 ng of $i_b$. However, 500 μg of purified r $i_a$ alone showed no lethal activity.

**Biological activities of $i_a$ with variations at Arg-295, Glu-14, Glu-226, Glu-378, Glu-380, Ser-338, Ser-340, and Thr-339.** The Arg-295 in $i_a$ was replaced with alanine, lysine, and histidine by site-directed mutagenesis; Glu-14 and -226 were replaced with alanine; Glu-378 and -380 were replaced with alanine, aspartic acid, and glutamine; Ser-338 and -340 were replaced with alanine, cysteine, threonine, and phenylalanine; and Thr-339 was replaced with alanine and phenylalanine. These variant components were purified from cultures of *B. subtilis* transformants carrying the corresponding genes by use of the purification procedure described for r $i_a$. All of the purified variant components showed only one band of approximately 43 kDa on SDS-PAGE (data not shown).

R295A showed a complete loss of NADase, ARTase, cytotoxic, and lethal activities under our experimental conditions (Tables 2, 3, and 4). On the other hand, the NADase, ARTase, cytotoxic, and lethal activities of R295K were about 100- to 250-fold lower than those of the wild-type component (Tables 2, 3, and 4). The ARTase and cytotoxic activities of R295H were about 0.2% these activities in wild-type $i_a$, but the lethal activity of R295H was about 6% the wild-type component activity; these results show that replacement of Arg-295 with histidine did not cause a severe reduction in lethal activity, compared with the reduction in enzymatic and cytotoxic activities. These observations suggest that Arg-295 is able to be partially substituted by basic amino acids, judging from the effect of the replacements on NADase, ARTase, cytotoxic, and lethal activities.

Replacement of Glu-14 and -226 had little effect on the ARTase activity of $i_a$ (Table 2) and did not cause a significant reduction in the cytotoxic and lethal activities of $i_a$ in the presence of $i_b$ (Tables 3 and 4); these results indicate that these residues are not required for the biological activities of $i_a$. Replacement of Glu-378 with alanine resulted in a complete loss of NADase, ARTase, cytotoxic, and lethal activities (Tables 2, 3, and 4), suggesting that Glu-378 is important for these activities. Conservative substitution of aspartic acid for Glu-378 caused a drastic decrease in ARTase, lethal, and cytotoxic activities, below our detection limit. That of glutamine reduced ARTase, lethal, and cytotoxic activities to below our detection limit. However, replacement of the residue with aspartic acid and glutamine had little effect on NADase activity. These results suggest that the carboxyl group of Glu-378 is essential for

TABLE 1. Summary of purification of r $i_a$ from culture supernatant fluid of the *B. subtilis* transformant

| Step | Total activity (mU)[a] | Total protein (mg) | Sp act (mU/mg) | Purification (fold) | Recovery of activity (%) |
|---|---|---|---|---|---|
| 70% $(NH_4)_2SO_4$ precipitation | 790 | 183 | 4.32 | 1 | 100 |
| DEAE–Sepharose CL-6B | 598 | 35.2 | 39.6 | 9.2 | 74.6 |

[a] One unit of activity (milliunit) was defined as the activity that catalyzed the transfer of 1 mmol of ADP-ribose from $NAD^+$ to actin per min at 37°C.

TABLE 2. NADase and ARTase activities of wild-type $i_a$ and variant $i_a$[a]

| Wild type or variant | NADase activity (ng/ml) | ARTase activity (μg/ml) |
|---|---|---|
| Wild type | 2.8 | 5.0 |
| E14A | — | 5.6 |
| E226A | — | 5.9 |
| R295A | ND | ND |
| R295K | 764 | 906 |
| R295H | — | 2,520 |
| S338A | 420 | 220 |
| S338C | 196 | 181 |
| S338T | — | ND |
| S338F | — | ND |
| T339A | 60 | 30.5 |
| T339F | — | 192 |
| S340A | 10 | 61 |
| S340C | — | 31 |
| S340T | — | 154 |
| E378A | ND | ND |
| E378D | 4.0 | 2,050 |
| E378Q | 5.0 | ND |
| E380A | ND | ND |
| E380D | 720 | 1,110 |
| E380Q | — | ND |

[a] Wild-type $i_a$ or variant $i_a$ was incubated with 50 μM [*carbonyl*-$^{14}$C]$NAD^+$ at 37°C for 6 h, and NADase activity was determined by the release of radiolabeled nicotinamide. Wild-type $i_a$ or variant $i_a$ was incubated with 5 μM [$^{32}$P]$NAD^+$ in the presence of 2 μg of actin for 90 min at 37°C. Labeled actin was analyzed by SDS-PAGE and autoradiography. NADase and ARTase activities were expressed as the amount required to induce 50% maximal activity of each. ND, not detected (<5,000 μg/ml); —, not done.

ARTase, lethal, and cytotoxic activities but not for NADase activity. As shown in Table 2, changing Glu-380 to alanine caused a complete loss of NADase and ARTase activities, changing it to aspartic acid resulted in over a 200-fold reduction in these enzymatic activities, and changing it to glutamine reduced ARTase activity to below our detection limit. Furthermore, the cytotoxic and lethal activities of E380D were about 500 and 1,000 times lower than those of r $i_a$, respectively, but these activities of E380A and E380Q were below the detection limit (Tables 3 and 4). These observations show that Glu-380 is essential for any activities of $i_a$.

Replacement of Ser-338 with alanine and cysteine resulted in about a 30- to 150-fold reduction in the NADase, ARTase, cytotoxic, and lethal activities of wild-type $i_a$ (Tables 2, 3, and 4). It is likely that Ser-338 is important but not essential for these activities. Replacement of Ser-338 with threonine and phenylalanine caused ARTase, cytotoxic, and lethal activities to fall below the detection limit, implying that replacement of Ser-338 with amino acids containing large side chains reduced these activities more severely than did that with amino acids containing small side chains. Replacement of Ser-340 with alanine, cysteine, and threonine caused only a 3- to 30-fold reduction in the NADase, ARTase, cytotoxic, and lethal activities of wild-type $i_a$ (Tables 2, 3, and 4). Replacement of Thr-339 with alanine or phenylalanine led to about a 6- to 30-fold reduction.

**Kinetics of ADP-ribosylating activities of wild-type $i_a$ and variant $i_a$.** Kinetic analyses of the six variant components (R295K, S338A, T339A, S340A, E378D, and E380D) were performed to help determine the mechanistic basis for the events caused by NADase and ARTase in the presence of increasing $NAD^+$ or actin concentrations. The kinetic parameters were obtained by analysis of the Hanes-Woolf plots of initial velocities of the NADase reaction. Table 5 shows that the $K_m$ values for $NAD^+$ binding to E380D, S338A, S340A, and T339A were similar to that of the wild type. The $K_m$ value for binding to R295K was significantly higher than that of the wild type (2.5-fold higher $K_m$ value), and the *kcat* values associated with R295K, S338A, and E380D were considerably lower than that of the wild type (200-, 67- and 250-fold lower *kcat* values, respectively); these results show that the kinetic effects on these residues almost exclusively involved *kcat*. The $K_m$ and *kcat* values for E378D were similar to those for the wild-type component.

The kinetic parameters obtained by analysis of the Hanes-Woolf plots of initial velocities for the ARTase reaction are summarized in Table 6. The $K_m$ values for $NAD^+$ binding to the variant components for the ARTase reaction were determined from initial rate data obtained at a fixed actin concentration and various $NAD^+$ concentrations. The results showed that the $K_m$ values of R295K and S338A were significantly higher than that of the wild-type and that the $K_m$ values for the ARTase activity of T339A, S340A, E378D, and E380D were identical to that of the wild type (Table 6); these results show that replacement of Arg-295 and Ser-338 resulted in a significant alteration of the $K_m$ for $NAD^+$. Replacement of Arg-295, Glu-378, and Glu-380 drastically reduced *kcat* values. The *kcat*

TABLE 3. Cytotoxic activities of wild-type $i_a$ and variant $i_a$[a]

| Wild type or variant | Cytotoxic activity at the following dose of wild-type or variant $i_a$ (ng/ml): | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1,000 | 500 | 100 | 50 | 10 | 5 | 1 | 0.5 |
| Wild type | | | | +++ | +++ | ++ | + | − |
| E14A | | | | +++ | +++ | ++ | + | − |
| E226A | | | | +++ | +++ | + | − | − |
| R295A | − | − | − | | | | | |
| R295K | +++ | ++ | + | − | | | | |
| R265H | ++ | + | − | − | | | | |
| S338A | +++ | +++ | ++ | + | − | | | |
| S338C | +++ | ++ | + | − | | | | |
| S338T | − | − | − | | | | | |
| S338F | − | − | − | | | | | |
| T339A | | | | +++ | +++ | ++ | + | − |
| T339F | | | +++ | +++ | ++ | + | − | − |
| S340A | | +++ | +++ | +++ | ++ | − | − | |
| S340C | | | +++ | +++ | ++ | + | − | − |
| S340T | | +++ | +++ | ++ | + | − | − | |
| E378A | − | − | − | | | | | |
| E378D | − | − | − | | | | | |
| E378Q | − | − | − | | | | | |
| E380A | − | − | − | | | | | |
| E380D | +++ | ++ | − | − | | | | |
| E380Q | − | − | − | | | | | |

[a] Confluent monolayers of Vero cells ($2 \times 10^6$ cells) were exposed to various doses of wild-type or variant $i_a$ in the presence of 200 ng of $i_b$ per ml at 37°C. Cell rounding was recorded after 8 h. Cell rounding in a Vero cell assay (cytotoxic activity) was scored as follows: +++, 100% rounding; ++, 50 to 80% rounding; +, 20 to 40% rounding; −, no rounding.

TABLE 4. Lethal activities of wild-type $i_a$ and variant $i_a$[a]

| Wild type or variant | No. of deaths at the following dose of wild-type or variant $i_a$ (ng/mouse): | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2,048 | 1,024 | 512 | 256 | 128 | 64 | 32 | 16 | 8 | 4 | 2 | 1 |
| Wild type | | | | | | | | 4 | 4 | 4 | 2 | 0 |
| E14A | | | | | | | | 4 | 4 | 4 | 1 | 0 |
| E226A | | | | | | | | 4 | 4 | 4 | 1 | 1 |
| R295A | 0 | 0 | 0 | | | | | | | | | |
| R295K | | 4 | 4 | 0 | 0 | | | | | | | |
| R295H | | | | 4 | 4 | 4 | 2 | 0 | | | | |
| S338A | | | 4 | 4 | 4 | 4 | 0 | | | | | |
| S338C | | | 4 | 4 | 4 | 4 | 0 | | | | | |
| S338T | 0 | 0 | 0 | | | | | | | | | |
| S338F | 0 | 0 | 0 | | | | | | | | | |
| T339A | | | | | | | | 4 | 4 | 4 | 2 | 0 |
| T339F | | | | | | | | 4 | 4 | 2 | 0 | 0 |
| S340A | | | | | 4 | 4 | 4 | 2 | 0 | | | |
| S340C | | | | | 4 | 4 | 3 | 2 | 0 | | | |
| S340T | | | | | 4 | 4 | 4 | 2 | 0 | | | |
| E378A | 0 | 0 | 0 | | | | | | | | | |
| E378D | 0 | 0 | 0 | | | | | | | | | |
| E378Q | 0 | 0 | 0 | | | | | | | | | |
| E380A | 0 | 0 | 0 | | | | | | | | | |
| E380D | 2 | 0 | 0 | | | | | | | | | |
| E380Q | 0 | 0 | 0 | | | | | | | | | |

[a] Various doses of wild-type or variant $i_a$ and $i_b$ (500 ng) were intravenously injected into 4 mice, and deaths occurring within 24 h were recorded.

values associated with Arg-295, Glu-378, and Glu-380 were markedly reduced, by 1,000-, 250-, and 1,000-fold, respectively, compared to that of the wild type, as shown in Table 6. The *kcat* values associated with S338A, T339A, and S340A were about 5, 20, and 26% that of the wild type, respectively.

Next, the $K_m$ values for actin binding to the variant $i_a$ components for the ARTase reaction were determined from initial rate data obtained at a fixed $NAD^+$ concentration and various actin concentrations. The $K_m$ values for actin binding to any variant components were in agreement with that determined for the wild type, within experimental error; none of the variants showed altered affinity for actin (Table 6). Replacement of Arg-295, Ser-338, Glu-378, and Glu-380 resulted in drastic reductions in *kcat* values. The *kcat* values associated with Arg-295, Ser-338, Glu-378, and Glu-380 were markedly reduced, by

TABLE 5. Kinetic analysis of NADase activities of wild-type $i_a$ and variant $i_a$[a]

| Wild type or variant | $K_m$ (μM) | Relative $K_m$ | *kcat* ($min^{-1}$) | Relative *kcat* | *kcat*/$K_m$ | Relative *kcat*/$K_m$ |
|---|---|---|---|---|---|---|
| Wild type | 23 | 1 | 0.2 | 1 | 0.009 | 1 |
| R295K | 57 | 2.5 | 0.001 | 0.005 | 0.00002 | 0.002 |
| S338A | 24 | 1.0 | 0.003 | 0.015 | 0.0001 | 0.01 |
| T339A | 17.1 | 0.74 | 0.009 | 0.045 | 0.0005 | 0.06 |
| S340A | 13.1 | 0.57 | 0.034 | 0.17 | 0.0026 | 0.29 |
| E378D | 19 | 0.8 | 0.074 | 0.37 | 0.004 | 0.44 |
| E380D | 18.5 | 0.8 | 0.0008 | 0.004 | 0.00004 | 0.005 |

[a] NADase activity was assayed with various amounts (5 to 25 μM) of $NAD^+$ as described in Materials and Methods. The standard deviation was not greater than ±15% ($n = 4$).

TABLE 6. Kinetic analysis of ARTase activities of wild-type $i_a$ and variant $i_a$[a]

| Varied ingredient | Wild type or variant | $K_m$[b] | Relative $K_m$ | *kcat* ($min^{-1}$) | Relative *kcat* | *kcat*/$K_m$ | Relative *kcat*/$K_m$ |
|---|---|---|---|---|---|---|---|
| $NAD^+$ | Wild type | 6.0 | 1 | 2,024 | 1 | 337 | 1 |
| | R295K | 23 | 3.8 | 2.9 | 0.001 | 0.74 | 0.002 |
| | S338A | 20 | 3.3 | 104 | 0.05 | 5.1 | 0.015 |
| | T339A | 4.9 | 0.8 | 405 | 0.20 | 83 | 0.25 |
| | S340A | 11 | 1.8 | 518 | 0.26 | 48 | 0.14 |
| | E378D | 4.9 | 0.8 | 8.4 | 0.004 | 1.7 | 0.005 |
| | E380D | 5.5 | 0.9 | 2.0 | 0.001 | 0.4 | 0.001 |
| Actin | Wild type | 152 | 1 | 441 | 1 | 2.9 | 1 |
| | R295K | 96 | 0.6 | 3.6 | 0.008 | 0.04 | 0.014 |
| | S338A | 223 | 1.5 | 12 | 0.027 | 0.06 | 0.021 |
| | T339A | 265 | 1.7 | 344 | 0.78 | 1.3 | 0.45 |
| | S340A | 140 | 0.9 | 81 | 0.18 | 0.58 | 0.20 |
| | E378D | 126 | 0.8 | 5.1 | 0.012 | 0.04 | 0.014 |
| | E380D | 176 | 1.2 | 2.3 | 0.005 | 0.01 | 0.003 |

[a] ARTase activity was measured with various $NAD^+$ or actin concentrations as described in Materials and Methods. The standard deviation was not greater than ±15% ($n = 4$).

[b] The $K_m$ concentration is given as micromolar for $NAD^+$ and as picomolar for actin.

about 125-, 37-, 83-, and 200-fold, respectively, compared to that of the wild type as shown in Table 6. However, replacement of Thr-339 and Ser-340 reduced by about 78 and 18%, respectively, the *kcat* value associated with the wild type.

## DISCUSSION

The yield of purified r $i_a$ was approximately 35 mg/liter from cultures of the *B. subtilis* transformant carrying the $i_a$ gene. The N-terminal sequence of r $i_a$ was identical to that of native $i_a$ and that deduced from the $i_a$ gene. The molecular mass and antigenicity of r $i_a$ also were coincident with those of native $i_a$. Based upon the lethal activities of r $i_a$ purified from cultures of the *B. subtilis* transformant and of $i_a$ purified from *C. perfringens* cultures by Stiles and Wilkins (43), the specific activity of the former was calculated to be about 300 times higher than that of the latter; this result shows that our purification procedure is very useful for the isolation of large amounts of $i_a$ with high specific activity.

Replacement of Glu-14 and -226, which are included within the E-X-X-X-X-W sequence involved in the $NAD^+$ binding sites of DT and ETA (15), had no effect on the activities of $i_a$, confirming that $i_a$ belongs to the CT group of in the ADP-ribosylating enzyme family (14).

Replacement of Arg-295 with alanine in $i_a$ led to a complete loss of NADase, ARTase, lethal, and cytotoxic activities. Perelle et al. (31) also reported that this residue is essential for ARTase activity. Thus, it appears that Arg-295 of $i_a$ is equivalent to Arg-7 of LT and Arg-299 of C2 toxin, which are required for ARTase activity (6, 15). However, replacement of Arg-295 with histidine or lysine caused a drastic reduction in NADase and/or ARTase activities, although the activities were still detectable, showing that the charge of the basic side chain of Arg-295 is essential for the NADase and ARTase activities of $i_a$. Substitution of lysine for Arg-295 in $i_a$ resulted in a significant reduction in the $K_m$ for $NAD^+$, markedly reduced the *kcat* values for NADase and ARTase, but had little effect on the $K_m$ for actin. Accordingly, these observations suggest that Arg-295 plays an essential role in both the binding of $i_a$ to $NAD^+$ and the catalytic actions of NADase and ARTase (Fig. 3).

Substitution of Glu-378 with alanine in $i_a$ resulted in a com-

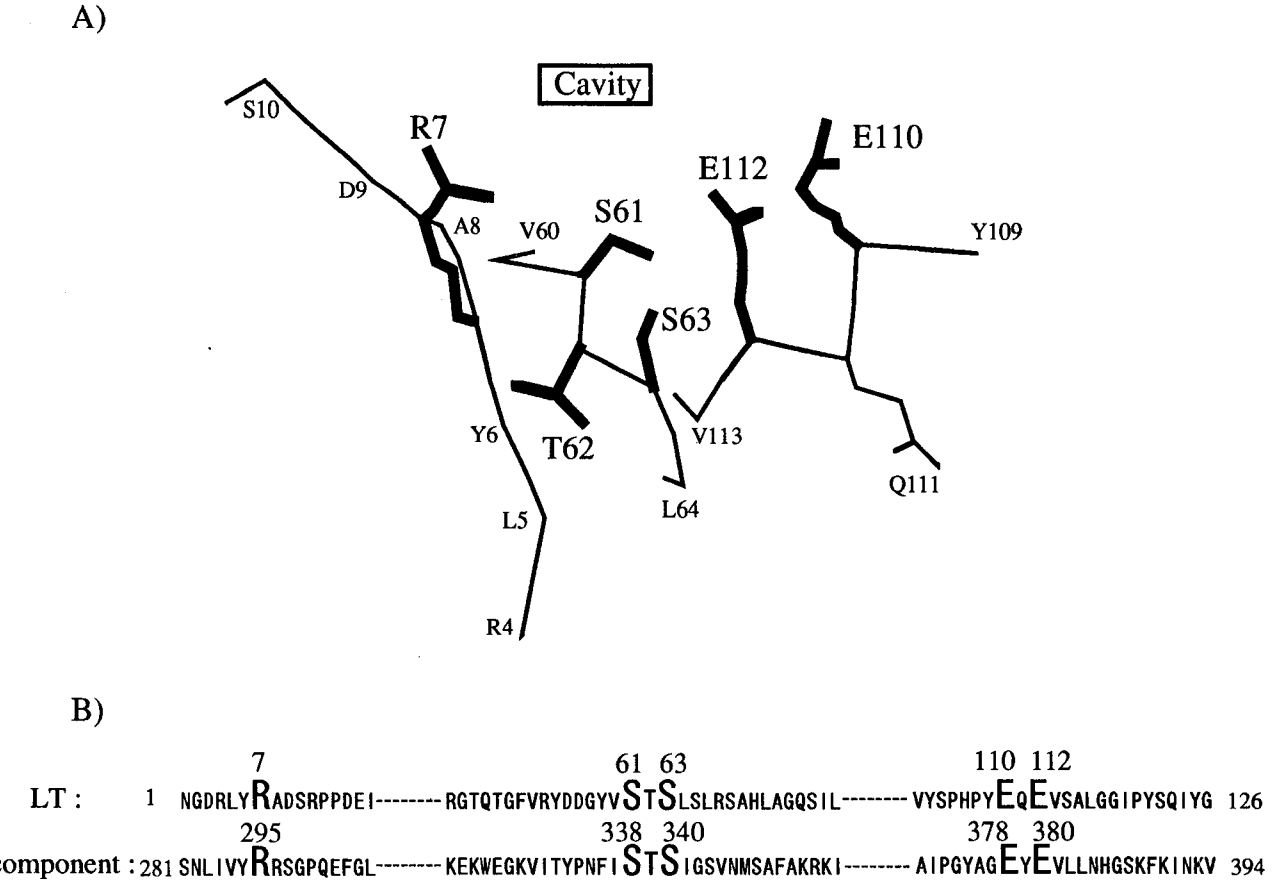


FIG. 3. (A) Active-site structure of LT. This active-site configuration was drawn on the basis of the refined coordinates of LT (41). The Arg-7 (R7), Ser-61 (S61), Thr-62 (T62), Ser-63 (S63), Glu-110 (E110), and Glu-112 (E112) side chains are shown in bold. (B) Sequence alignments of LT and the $i_a$ component. Sequences were taken from porcine LT (SWISSProt accession no., P06717) and component $i_a$ (GenBank accession no., X73562). The residues in bold are those that are conserved in ADP-ribosylating enzymes.

plete loss of NADase and ARTase activities, as reported by Perelle et al. (31); however, conservative substitution of Glu-378 with aspartic acid or glutamine in $i_a$ had little effect on NADase activity and drastically reduced but did not diminish ARTase activity. These results indicate that Glu-378 plays an important role in ARTase activity but not in NADase activity. It therefore is likely that the side chain of the amino acid at position 378 is important for maintenance of the active-site integrity in NADase activity and that the carboxyl group of the amino acid at position 378 is essential for ARTase activity. Glu-378 within the E-X-E motif ($Glu^{378}$-X-$Glu^{379}$) of C2 toxin and Glu-379 within the motif $Glu^{379}$-X-$Glu^{381}$ of ExoS were essential for ARTase activity but were not required for NADase activity (6, 33). Our results coincided with these results.

The kinetic analysis showed that replacement of Glu-378 with aspartic acid resulted in a severe reduction in the *kcat* values for ARTase activity but had little effect on the $K_m$ values for $NAD^+$ and actin, suggesting that the residue plays an important role in catalytic mechanism but is not required for binding to $NAD^+$ and actin. The glutamic acid at position 380 could be replaced with aspartic acid, although the replacement was not wholly effective, but could not be replaced with alanine and glutamine; these results show that conservative substitution, such as reduction of the carboxyl group at position 380 by one methylene unit or replacement of the carboxyl group by an uncharged amide, resulted in a significant reduction and a complete loss, respectively, of ARTase activity. Thus, the role of Glu-380 in $i_a$ appears to be equivalent to that of the corresponding residues in C2 toxin and LT.

Replacement of Glu-380 with aspartic acid markedly reduced the *kcat* values for ARTase and had little effect on the $K_m$ values for $NAD^+$ and actin, suggesting that Glu-380 is essential for the catalytic mechanism of ARTase but not for binding to $NAD^+$ and actin. Damme et al. (13) reported the Glu-378 in $i_a$ was photolabeled by $NAD^+$ but that Glu-380 was not. Therefore, the Glu-378 and -380 residues seem to play different roles in the ARTase activity of $i_a$.

Cieplak et al. (10) reported that substitution of Glu-112 ($E^{110}$-X-$E^{112}$) in LT resulted in a marked reduction in ARTase activity, suggesting that the residue plays a specific role in the mechanism of ADP-ribosylation and represents an essential catalytic residue. In addition, they suggested that Glu-110 is unlikely to play a specific role in the reaction mechanism. Hara et al. (19) reported that rat T-cell antigen RT 6.1 ($Q^{207}$-X-$E^{209}$) catalyzes $NAD^+$ glycohydrolysis but not $NAD^+$ ribosyltransfer and that mutant RT 6.1 in which Gln-207 was replaced with glutamic acid exhibited ARTase activity. Our result is coincident with their findings in that the first glutamic acid residue in the E-X-E motif is essential for ARTase activity but not for NADase activity. However, C3 and EDIN in the ADP-ribosyltransferase family, which ADP-ribosylate small GTP-binding proteins of the rho family, have a glutamine residue in the motif, suggesting that the residue in C3 and EDIN which

corresponds to the residue at position 380 in $i_a$ is not required to be glutamic acid for ARTase activity. Thus, the residue in the motif may depend on the substrate.

Ser-338 could be replaced with alanine and cysteine, although the replacements were not wholly effective, but could not be replaced with threonine and phenylalanine; these results suggest that the hydroxyl group of Ser-338 is not essential for these activities and that the large side chain of the amino acid at position 338 completely disturbed ARTase activity. Thus, Ser-338 may be extremely close to the catalytic site, as shown in Fig. 3. Replacement of Ser-338 with alanine caused a significant reduction in the $K_m$ value of $NAD^+$ for ARTase and drastically reduced the *kcat* values for NADase and ARTase activities. These observations imply that the side chain of the amino acid position at 338 is required for maintenance of the $NAD^+$-binding site and catalytic site in these activities.

Bell and Eisenberg (7) reported that the S-T-S motif in DT binds to either the ribose or the phosphate of the AMP moiety of $NAD^+$. Furthermore, Barth et al. (6) reported that Ser-348 in the motif $S^{348}$-T-$S^{350}$ in C2 toxin may play an essential role in $NAD^+$ binding or catalysis. There is no contradiction between our results and their model. Replacement of Thr-339 or Ser-340 with alanine resulted in a significant but not severe reduction in NADase and ARTase activities. Replacement of Thr-339 and Ser-340 did not result in a drastic reduction in the $K_m$ and *kcat* values for NADase or ARTase, compared with that of Ser-338, suggesting that these residues do not play an important role in binding and catalytic reactions, as shown in Fig. 3.

NADase and ARTase activities catalyze the cleavage of the N-glycosidic bond of $NAD^+$; the former transfers the ADP-ribose moiety to water, and the latter transfers the moiety to actin. Thus, it seems that Arg-298, Ser-338 and Glu-380, required for these activities in $i_a$, play an important role in the cleavage of the N-glycosidic bond of $NAD^+$ and that Glu-378, required for ARTase activity but not for NADase activity, is essential for the transfer of the ADP-ribose moiety to actin.

From these observations, changes in ARTase activity as a result of the substitution of various amino acid residues in $i_a$ were on the whole similar to those in cytotoxic and lethal activities, except for changes in these activities in R295H. It therefore is likely that the cytotoxicity and lethality of iota-toxin are closely related to the ARTase activity of $i_a$.

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