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Amino Acid Sequence of Retinal Transducin at the Site ADP-ribosylated by Cholera Toxin*

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Transducin was [^{32}P]ADP-ribosylated by cholera toxin in bovine retinal rod outer segments and then partially purified on ω-amino octyl agarose to remove other ADP-ribosylated proteins. Trypsin digestion of the ADP-ribosylated transducin and further purification using boronate-polyacrylamide beads and high performance liquid chromatography yielded a single radiolabeled tetrapeptide, Ser-Arg-Val-Lys. The ADP-ribose is linked to the guanidinium group of arginine.

Cholera toxin, an exotoxin produced by *Vibrio cholerae*, activates adenylate cyclase in many vertebrate cells by catalyzing ADP-ribosylation of N_s[1] (1–3). ADP-ribosylation by cholera toxin inhibits the GTPase associated with N_s and prolongs the regulatory protein's activated state (4). Cholera toxin also ADP-ribosylates transducin, a protein of retinal ROS which is structurally (5) and functionally (6–8) homologous to N_s. This covalent modification inhibits transducin's GTPase activity. Guanosine triphosphate analogs (8–10) and either hormones (9) or light (8) markedly enhance cholera toxin-catalyzed ADP-ribosylation of N_s or transducin, respectively. Thus, the two proteins exhibit remarkable similarities with respect both to the functional effects of toxin-catalyzed ADP-ribosylation and also to the conditions under which ADP-ribosylation can occur.

Here, we report the primary structure of the site on transducin that is ADP-ribosylated by cholera toxin. It is likely that the toxin ADP-ribosylates a closely homologous site on N_s.

EXPERIMENTAL PROCEDURES

Materials—Frozen, dark-adapted bovine retinas were obtained from American Stores, Lincoln, NE. Other materials were obtained commercially as follows: [α-^{32}P]NAD$^+$ (New England Nuclear), ω-amino octyl agarose (Miles), boronate-polyacrylamide beads and polyacrylamide gel electrophoresis materials (Bio-Rad), trypsin (EC 3.4.21.4; treated with L-1-*p*-tosylamino-2-phenylethyl chloromethyl ketone, Millipore), chemicals for sequence analysis (Pierce), and dansyl chloride (Eastman). Cholera toxin (Schwarz/Mann) was activated just prior to use (3).

Preparation of ADP-ribosylated T_α—ROS were isolated from 500 retinas in the dark as described by Fung and Stryer (11). The ROS, which contained 150–250 mg of rhodopsin, were then suspended in 50 ml of 200 mM potassium phosphate, pH 6.8, 10 mM thymidine, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 10 μM guanosine-5′-(β,γ-imino)triphosphate, 1 mM [^{32}P]NAD$^+$ (20 Ci/mol), and 50 μg/ml of cholera toxin. After incubating in the light for 3 h at 20 °C, the ROS were extracted four times with 120 ml of 5 mM Tris-Cl, pH 7.5, 0.5 mM $MgCl_2$, 100 μM GTP, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride. After the extracts were pooled, chromatography on ω-amino octyl agarose was performed as previously described (12). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis of fraction aliquots on 10% gels (13), gels were dried on tissue paper and exposed Kodak XAR-5 film. Fractions containing [^{32}P]ADP-ribosylated T_α (identified by autoradiography) were pooled and concentrated using Amicon PM-10 filters. The yield of [^{32}P]ADP-ribosylated T_α was 10–25 nmol.

Isolation of ADP-ribosylated Tryptic Fragment—[^{32}P]ADP-ribosylated T_α was exposed to trypsin (1:100, w/w) in 0.2 M ammonium acetate, pH 8.5, 2 M urea, and 1 mM thioglycollate for 1 h at 37 °C. ADP-ribosylated peptides were isolated using boronate-polyacrylamide chromatography as previously described (14) and then further purified by reverse phase HPLC (Fig. 1). In these two chromatographic steps, approximately 80% of the ^{32}P co-purified with the peptide.

Amino Acid Sequencing—Amino acid composition was determined in an automatic amino acid analyzer (Beckman Model 119C) (15) and the peptide was sequenced using a micro dansyl-Edman procedure (16). For NH_2-terminal determination and sequencing, a portion of the intact peptide (0.3 nmol) or an aliquot of the aqueous phase of each Edman degradation step were dansylated (16) and hydrolyzed in 6 N HCl for 6–10 h at 110 °C. The dns-amino acids were identified on polyamide thin layer chromatography (Schleicher & Schuell). A second aliquot of the aqueous phase was hydrolyzed before dansylation for analysis of amino acid composition using polyamide thin layer chromatography.

Intact peptide (0.3 nmol) was also digested with carboxypeptidases A and B (1:100, mol/mol) in 0.1 M $NaHCO_3$, pH 9.0, for 2 h at 37 °C. Free amino acids were dansylated and identified as dns-amino acids using polyamide thin layer chromatography.

The mass of the ADP-ribosylated peptide was determined by liquid secondary ion mass spectrometry (17) at the Bio-organic Biomedical Mass Spectrometry Resource of the University of California, San Francisco.

ADP-ribosyl arginine was synthesized (18) and then purified using boronate-polyacrylamide beads (14) or high voltage paper electrophoresis as described in Fig. 1. The purified ADP-ribosyl arginine was reacted with Edman reagent under the same conditions that were used for the ADP-ribosylated peptide and detected after high voltage paper electrophoresis using ultraviolet fluorescence or a silver nitrate-ammonia reagent (19).

RESULTS

The ADP-ribosylated peptide isolated by reverse phase HPLC was homogeneous by high voltage paper electrophoresis. The homogeneity of this peptide was further confirmed by NH_2-terminal analysis which showed serine as the sole NH_2-terminal amino acid. The yield of the final product was 8.28 nmol by amino acid analysis and 7.73 nmol by measurement of ^{32}P radioactivity, demonstrating the presence of one ADP-ribosyl moiety/peptide molecule.

The amino acid sequence was established as Ser-Arg-Val-

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[1] The abbreviations used are: N_s, stimulatory guanine nucleotide-binding regulatory protein of adenylate cyclase; ROS, rod outer segments; T_α, α-subunit of transducin, HPLC, high performance liquid chromatography; dns or dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

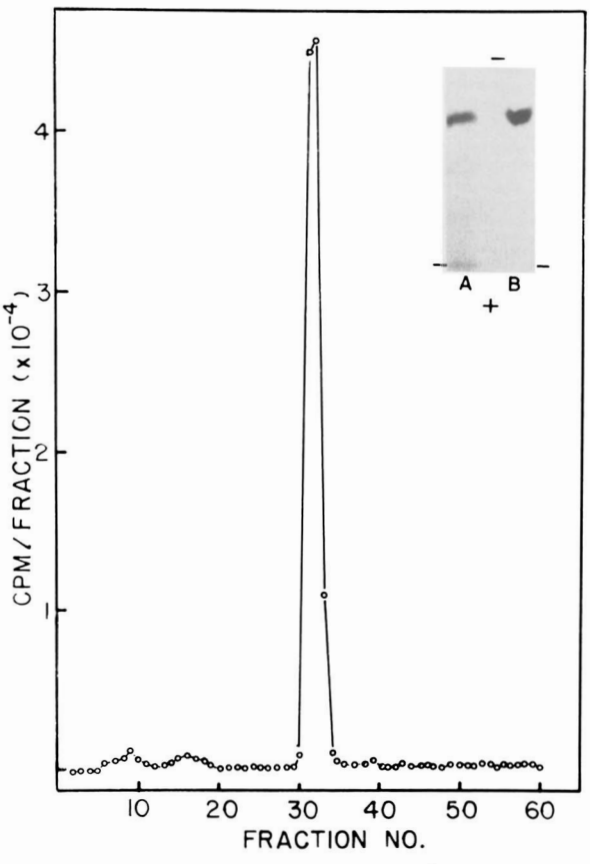

FIG. 1. **Reverse phase HPLC of [^{32}P]ADP-ribosylated tryptic peptide.** The boronate-polyacrylamide eluate was lyophilized and dissolved in 500 μl of 50 mM pyridine, 25 mM acetic acid (pH 5.5). The material was fractionated by HPLC on a C_{18} reverse phase column (Alltech, VYDAC 201-TP, 10 μm; 4.6 × 250 mm) by isocratic elution with 50 mM pyridine, 25 mM acetic acid (pH 5.5). Flow rate was 0.5 ml/min. The column effluent (0.5 ml/fraction) was monitored for ^{32}P using scintillation counting of Cerenkov radiation. The *inset* shows the electrophoretic and autoradiographic analysis of the [^{32}P] ADP-ribosylated tryptic peptide before (*A*) and after (*B*) reverse phase HPLC. High voltage paper electrophoresis was performed on Whatman 3MM paper in pH 2.1 buffer (HCOOH:CH_3COOH:H_2O, 218:63:2219 by volume) at 2kV for 30 min. Subsequent autoradiography was carried out on Kodak XAR-5 film.

Lys by a micro dansyl-Edman procedure (15) in which each newly exposed NH_2-terminal was determined following dansylation. This sequence was confirmed using a subtractive method by determining the amino acid composition of the residual peptide after each Edman cycle. Digestion of the intact peptide with carboxypeptidases A and B released lysine and valine, confirming the COOH-terminal sequence. However, determination of the amino acid composition by acid hydrolysis revealed the following five amino acids: Ser 0.85, Gly 0.45, Val 1.10, Lys 0.91, and Arg 1.13. The presence of glycine in acid hydrolysates of peptides that contain ADP-ribose has been previously reported (20); it appears to result from degradation of adenine during acid treatment. Mass spectrometry of the ADP-ribosylated peptide revealed a mass of 1030 Da, which corresponds to the mass predicted from the sequence data.

To determine which amino acid is ADP-ribosylated by cholera toxin, aliquots of the residual water phase (15), after each of the sequential Edman reaction cycles, were subjected to high voltage paper electrophoresis and autoradiography (Fig. 2). Following the first Edman reaction cycle, the water phase contained three radiolabeled species with a net positive

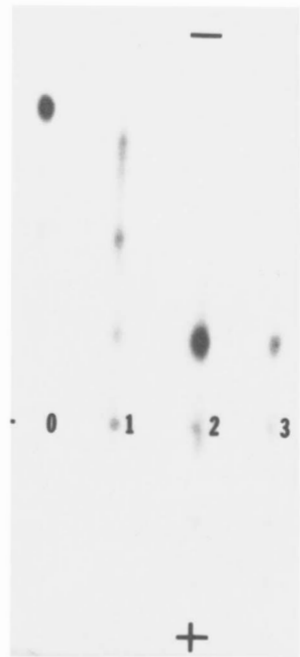

FIG. 2. **Electrophoretic and autoradiographic analysis of Edman-degraded materials.** After each cycle of the sequential Edman degradation, aliquots (3 μl) of the aqueous phase (30 μl) were subjected to high voltage paper electrophoresis and autoradiography, as described in Fig. 1. *Numbers at the origin* indicate the number of Edman degradation cycles: *0*, untreated, ADP-ribosylated peptide after lyophilization; *1*, ADP-ribosylated peptide following removal of the NH_2-terminal serine; *2* and *3*, sequential treatments of ADP-ribosylated peptide to remove successive NH_2-terminal amino acids.

charge at pH 2.1. Although we have not defined the chemical structures of the three species,[2] their conversion to a uniformly migrating species during the next Edman reaction cycle suggests that the ADP-ribose is linked to the second amino acid, arginine. This was confirmed by the finding that the electrophoretic mobility of the Edman derivative of authentic ADP-ribosyl arginine was identical to that of the major ^{32}P-labeled species observed after the second Edman reaction cycle. The resistance of the arginine-valine bond in the ADP-ribosylated peptide to tryptic hydrolysis also suggests that arginine's guanidinium group was modified. Furthermore, attachment of the ADP-ribose to one of the other amino acids is unlikely since the ϵ-amino group of lysine can be dansylated in the intact peptide (result not shown) and valine has no reactive side group. We propose that for two reasons serine is also not the acceptor for the ADP-ribose. The radiolabeled product of the first Edman degradation cycle is altered in the next cycle and the ADP-ribosylated Edman derivative of serine should not have a net positive charge at pH 2.1. All the evidence thus indicates that an arginine on transducin is the acceptor for cholera toxin-transferred ADP-ribose.

[2] We suspect that the electrophoretic heterogeneity of the radiolabeled species after the first Edman degradation cycle is partly due to incomplete reaction of the ϵ-amino group on lysine with Edman reagent. In addition, lyophilization alone (before the first Edman cycle) introduced small amounts of slower migrating ^{32}P-labeled material (Fig. 2, *lane 0*). The first Edman degradation cycle, which included four lyophilization steps (16), markedly increased the amounts of these slower migrating species. Appearance of a homogeneously migrating species after the next cycle suggests that the altered mobility was due to changes in amino acid residues distal to the second amino acid, arginine. Such changes could have resulted from radiation damage or from pH changes during lyophilization.

DISCUSSION

This is the first identification of an ADP-ribosylated arginine in a protein that is specifically ADP-ribosylated by cholera toxin. Incubations of cholera toxin with NAD^+ and various amino acids previously showed that the guanidinium group of arginine can serve as an acceptor for ADP-ribose (21). Like cholera toxin, two other bacterial toxins affect cellular metabolism by mono-ADP-ribosylating specific cellular proteins (22, 23). Diphtheria toxin ADP-ribosylates diphthamide on elongation factor 2 (24) and pertussis toxin ADP-ribosylates an asparagine on retinal transducin.[3] In addition, ADP-ribosylation of an arginine on RNA polymerase has been reported during T4 phage infection of *Escherichia coli* (20). Thus, each toxin appears to ADP-ribosylate a unique amino acid on its specific protein substrate.

Because of the close structural and functional similarities between N_s and transducin, we predict that the amino acid sequence around the cholera toxin substrate site on N_s will closely resemble that of transducin, and that the ADP-ribosylated amino acid will be arginine.

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