Effect of Temperature and Host Factors on the Activities of Pertussis Toxin and *Bordetella* Adenylate Cyclase†

Toshihiko Murayama,‡ Erik L. Hewlett,§ Nancy J. Maloney,§ John M. Justice,‡ and Joel Moss*,‡

Laboratory of Cellular Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892, and Division of Clinical Pharmacology, Departments of Medicine and Pharmacology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

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ABSTRACT: Pertussis toxin and adenylate cyclase toxin both contribute to the pathogenesis of whooping cough. Production of these proteins is controlled by the *bvg* locus, which is inactive at 25 °C, but at 37 °C produces a Vir^+ phenotype. In view of the temperature dependence of virulence factor synthesis, the effects of temperature and host factors on their action were examined. The NAD glycohydrolase activity of the S1 subunit of pertussis toxin was enhanced by CHAPS, a zwitterionic detergent, with a temperature optimum of ~35 °C. Similar temperature optima for the ADP-ribosylation by pertussis toxin of transducin and recombinant $G_{o\alpha}$ were observed. Since the temperature−activity relationship of S1 differed from that of S1 in activated holotoxin, and since S1 in activated holotoxin was more stable at 42 °C than was S1, it appears that S1 associated with the B oligomer components may, in fact, be an active species. *Bordetella pertussis* adenylate cyclase is activated by a host factor, calmodulin. In the absence of calmodulin, the temperature optimum for enzymatic activity was ~25 °C, whereas in its presence it was ~35 °C. Thus, the temperature optima for pertussis and adenylate cyclase toxins, virulence factors whose production is increased through the *bvg* locus at physiological temperatures, are either at or near these temperatures when stimulated by host factors.

Several classes of bacterial toxins produce their effects on mammalian cells via their enzymatic activity (e.g., ADP-ribosyltransferase, adenylate cyclase) [for a review, see Moss and Vaughan (1988)]. Cholera toxin, a secretory product of *Vibrio cholerae*, ADP-ribosylates a guanine nucleotide-binding (G^1) protein G_s, which is involved in the regulation of adenylate cyclase and ion flux in mammalian cells (Moss & Vaughan, 1988; Birnbaumer et al., 1990). Pertussis toxin, a secretory product of *Bordetella pertussis*, ADP-ribosylates G_i, the inhibitory GTP-binding protein of adenylate cyclase, as well as some other members of the G protein family (Moss & Vaughan, 1988; Birnbaumer et al., 1990; Ui, 1990). It is believed that alterations of G protein function contribute to the pathogenesis of whooping cough (Weiss & Hewlett, 1986; Ui, 1990). *Bordetella pertussis* also produces an adenylate cyclase that enters mammalian cells (e.g., neutrophils) and elevates the intracellular cAMP content, thereby interfering with their function (Confer & Eaton, 1982; Pearson et al., 1987; Hewlett & Gordon, 1988).

It might be expected that these enzymatic activities would be especially sensitive to environmental and host conditions. For example, *in vitro*, cholera toxin NAD glycohydrolase and ADP-ribosyltransferase activities were significantly less at 37 °C than at 25−30 °C (Murayama et al., 1993). The temperature optimum was increased by the addition of host cell components, phospholipids, and ~20-kDa guanine nucleotide-binding proteins termed ADP-ribosylation factors or ARFs, which markedly enhanced activity at 37 °C (Murayama et al., 1993).

Bordetella pertussis is an exclusively human pathogen that resides in association with the ciliated epithelium of the respiratory tract (Weiss & Hewlett, 1986), but it can enter and survive within eukaryotic cells (Ewanowich et al., 1989). The organism produces several virulence factors, including pertussis toxin and adenylate cyclase toxin, that are responsible in part for the pathogenesis of disease (Weiss & Hewlett, 1986). The synthesis of pertussis toxin and adenylate cyclase toxin is under the control of the *bvg* locus, which is inactive at 25 °C (Rappuoli et al., 1992). Activation of the *bvg* locus at 37 °C, with the appearance of the Vir^+ phenotype, as might occur within the host, results in the production of filamentous hemagglutinin and, several hours later, pertussis toxin, adenylate cyclase toxin, and other components (Rappuoli et al., 1992). Additional levels of control might reside in the temperature dependence of the toxins and the effects of host factors on their activities. We report here the effects of temperature and host factors on the activities of these two toxins. Pertussis toxin has full activity at physiological temperature. In contrast, the basal adenylate cyclase activity apparently is maximal at ~25 °C, but like cholera toxin (Murayama et al., 1993), it exhibits maximum activity at a higher temperature when activated by a host factor, in this case calmodulin.

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* Please address correspondence to Joel Moss, National Institutes of Health, Room 5N-307, Building 10, Bethesda, MD 20892. Telephone: 301-402-1787. FAX: 301-402-1610.

‡ National Institutes of Health.

§ University of Virginia School of Medicine.

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[1] Abbreviations: PT, pertussis toxin; S1, subunit of pertussis toxin containing ADP-ribosyltransferase activity; G protein, guanine nucleotide-binding protein; G_s, stimulatory G protein of the adenylate cyclase system; G_i, inhibitory G protein of the adenylate cyclase system; G_o, a G protein from brain; $G_{o\alpha}$, α-subunit of G_o; $rG_{o\alpha}$, recombinant $G_{o\alpha}$; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol.

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EXPERIMENTAL PROCEDURES

Materials

[*carbonyl*-^{14}C]NAD (53 mCi/mmol) was purchased from Amersham Corp. (Arlington Heights, IL); [^{32}P]NAD (20−40 Ci/mmol) was from New England Nuclear (Boston, MA). Pertussis toxin and S1 subunit were from List Biological Laboratories (Campbell, CA) or were prepared as described previously (Cronin et al., 1984). CHAPS was from Calbiochem-Behring (San Diego, CA), and ATP, thymidine, and NAD were from Sigma Chemical Co. (St. Louis, MO). Transducin was purified from bovine retina as described (Kühn, 1980). Sources of other materials have been published (Tsai et al., 1988; Noda et al., 1990; Murayama et al., 1993).

Methods

NAD Glycohydrolase Assay. Pertussis toxin (PT) and S1 subunits were activated with dithiothreitol as described in the legends to the figures. The NAD glycohydrolase activities of PT and S1 subunits were assayed (Moss et al., 1976, 1983, 1986) in 50 mM potassium phosphate (pH 7.5), 20 mM thymidine, 40 μM [*carbonyl*-^{14}C]NAD ((1−1.5) × 10^5 cpm/tube), 30 mM dithiothreitol, 0.5 mg/mL ovalbumin, and other additions as indicated. After incubation for 40 or 60 min at the indicated temperature, assays were terminated by transfer to 4 °C, and [*carbonyl*-^{14}C]nicotinamide was isolated for radioassay using AG 1-X2.

[^{32}P]ADP-Ribosylation of Transducin by Pertussis Toxin. Reactions were incubated for 20 min at the indicated temperature in 50 mM potassium phosphate (pH 7.5), 5 mM $MgCl_2$, 20 mM thymidine, 10 μM [^{32}P]NAD (1−2 μCi/tube), 20 mM dithiothreitol, purified bovine transducin (4.4 μg), and activated PT (7 μg) (total volume, 100 μL). After termination of the reaction with 2 mL of cold 10% trichloroacetic acid and bovine serum albumin, 10 μg, samples were kept at 4 °C for 2 h. Precipitated proteins were dissolved in 1% SDS and 5% 2-mercaptoethanol (65 °C, 10 min) and subjected to electrophoresis in 14% SDS−polyacrylamide gels by the method of Laemmli (1970). Gels were exposed to Kodak X-Omat AR film.

Preparation of Recombinant $G_{o\alpha}$ ($rG_{o\alpha}$). The coding region of bovine $G_{o\alpha}$ cDNA was inserted into the pT7/Nde expression vector (Hong et al., 1994). This was transfected into BL21 (DE3) *Escherichia coli* (Novagen), which had been previously transfected with the pACYC177/ET3d/yNMT vector (Haun et al., 1993). These bacteria overexpressed yeast *N*-myristoyltransferase and $G_{o\alpha}$, with the resultant production of myristoylated $rG_{o\alpha}$ (Duronio et al., 1990).

An overnight culture of the cotransfected bacteria was diluted 1:10 into LB medium containing the appropriate antibiotics and grown at 37 °C for 1 h, followed by induction with 0.2 mM IPTG in the presence of 100 μM myristic acid. Growth was continued for an additional 3 h before 50 mL of medium was pelleted by centrifugation. The bacteria were resuspended in 3 mL of cold phosphate-buffered saline (pH 7.4), lysed by sonication, and centrifuged (15000*g*, 45 min). The supernatant contained crude $rG_{o\alpha}$, and this material was used for the ADP-ribosylation reactions.

ADP-Ribosylation of $rG_{o\alpha}$ by Pertussis Toxin. To 100 μL of the crude $rG_{o\alpha}$ fractions containing approximately 2 μg of $rG_{o\alpha}$, were added 50 μL of reaction buffer (200 mM KPO_4 (pH 7.5)/20 mM $MgCl_2$/100 mM thymidine/40 μM [^{32}P]-NAD (1 μCi)/80 mM DTT) and 50 μL of activated pertussis toxin mix (0.5 μg of pertussis toxin/50 mM glycine (pH 7.5)/80 mM DTT). All reactions were mixed on ice, and following the addition of pertussis toxin, were incubated at the indicated temperatures for 20 min before the reactions were stopped by the addition of 40 μL of 50% TCA. Proteins were incubated for 2 h on ice and then pelleted and resuspended in SDS−PAGE sample buffer. Following electrophoresis in 12% minigels (NOVEX), the proteins were stained with Coomassie Blue, dried to filter paper, quantified on a Molecular Dynamics phosphorimager, and exposed to Kodak X-Omat AR film for photographic purposes.

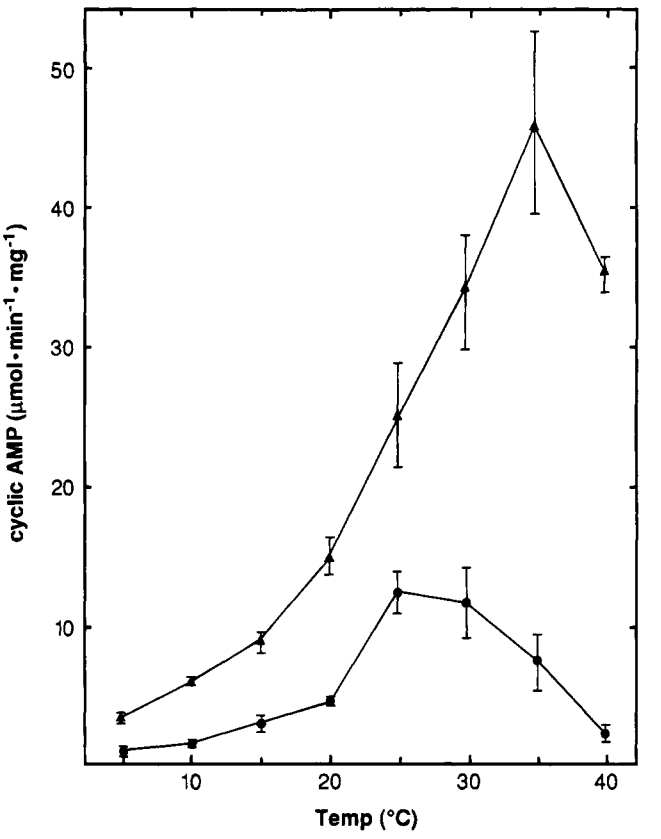

FIGURE 1: Effect of temperature and calmodulin on the activity of pertussis adenylate cyclase. Adenylate cyclase toxin purified through preparative sucrose gradient centrifugation and stored in 60 mM Tricine was assayed for adenylate cyclase activity without (●) or with (▲) 1 μM calmodulin, as described in Experimental Procedures. Results are expressed as micromoles of cAMP formed/minute/milligram of protein (mean ± SEM).

Pertussis Adenylate Cyclase Assay. Samples of adenylate cyclase (purified through preparative sucrose gradient centrifugation) were assayed without or with 1 μM calmodulin as described (Hewlett et al., 1989). Samples were incubated at the indicated temperature for 5 min before initiation of the reaction. Conversion of [^{32}P]ATP to [^{32}P]cAMP during incubation at the indicated temperatures for 4, 8, and 12 min in a final volume of 60 μL was measured. Each assay contained 60 mM Tricine (pH 8.0), 10 mM $MgCl_2$, and 1 mM ATP (with (2−5) × 10^3 cpm of [α-^{32}P]ATP). The reaction was terminated by the addition of 100 μL of a solution containing 1% sodium dodecyl sulfate (SDS), 20 mM ATP, and 6.25 mM cAMP (including 15 000−20 000 cpm of [^{3}H]cAMP per tube for recovery calculation). cAMP

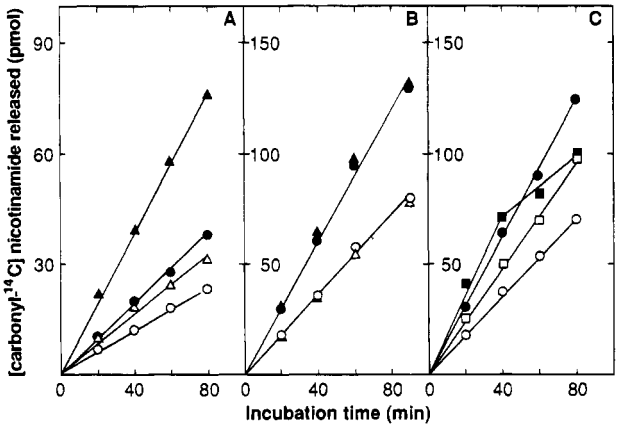

FIGURE 2: Release of [*carbonyl*-^{14}C]nicotinamide from [*carbonyl*-^{14}C]NAD catalyzed by pertussis toxin. Lyophilized toxin (A), reconstituted to 500 μL with sterile water, contains 10 mM sodium phosphate (pH 7.0) with 50 mM sodium chloride. Lyophilized S1 subunit (B and C), reconstituted with 500 μL of sterile water, contains 10 mM Tris (pH 8.0), 0.1 mM Na_2EDTA, and 0.04% CHAPS. To 100 μL of these solutions (10 μg of PTX or 2 μg of S1 subunit) were added 100 μL of 400 mM dithiothreitol, 50 μL of ovalbumin (10 mg/mL), and 150 μL of 50 mM potassium phosphate (pH 7.5). After incubation for 10 min at 30 °C, 200 μL of 5 mM ATP (△, ▲) and/or 200 μL of 5% CHAPS (●, ▲) or water (○), all in 50 mM potassium phosphate buffer, were added in A and B. Reaction at 30 °C was initiated by the addition of 200 μL of a mixture containing [*carbonyl*-^{14}C]NAD and thymidine in a potassium phosphate buffer (total volume, 1 mL). Final concentrations were 50 mM potassium phosphate (pH 7.5), 20 mM thymidine, 40 μM [*carbonyl*-^{14}C]NAD, 40 mM dithiothreitol, 1 mM ATP, and 1% CHAPS, with ovalbumin (0.5 mg/mL). At the indicated times, 100-μL samples (containing 1 μg of PTX or 0.2 μg of S1 subunit) were transferred to columns of AG 1-X2. In C, assays of S1 were incubated at 30 °C (○, ●) or 37 °C (□, ■) without (○, □) or with 1% CHAPS (●, ■). The experiment was done in triplicate with duplicate tubes.

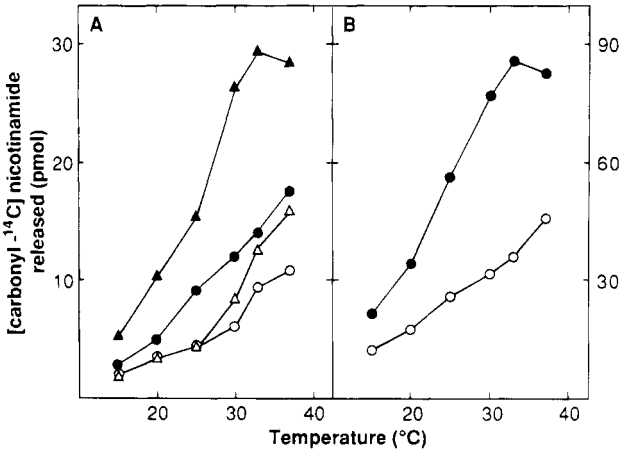

FIGURE 3: Effects of ATP and CHAPS on the temperature dependence of pertussis toxin NAD glycohydrolase activity. Lyophilized PT (50 μg in 50 mM potassium phosphate (pH 7.5), 25 mM NaCl, and 5 mM sodium phosphate) or S1 subunit (10 μg in 50 mM potassium phosphate (pH 7.5), 5 mM Tris, 0.05 mM Na EDTA, and 0.02% CHAPS) was incubated with 100 mM dithiothreitol for 10 min at 30 °C in a total volume of 1 mL. Samples (20 μL) were taken for the assay of NAD glycohydrolase activity, as described in the legend for Figure 2. Assays with PTX (1 μg) (A) or S1 subunit (0.2 μg) (B) were incubated at the indicated temperature for 40 min in a total volume of 100 μL, without (○) or with 2 mM ATP (△), 1% CHAPS (●), or 1 mM ATP and 1% CHAPS (▲). The experiment was done in duplicate with duplicate tubes.

was isolated by the method of Salomon et al. (1974). Activity was calculated as a mean rate using the three time points in each of two experiments and is expressed as mean ± standard error of the mean from assays at indicated temperatures from 5 to 40 °C.

RESULTS AND DISCUSSION

The temperature optima for the enzymatic activities of adenylate cyclase toxin were strikingly different in the presence and absence of the host activator, calmodulin. As shown in Figure 1, the modest level of adenylate cyclase activity observed without added calmodulin was highest at 25−30 °C and declined as the temperature was increased to 40 °C. In contrast, enzymatic activity in the presence of 1 μM calmodulin increased with increasing temperature to a maximum at 30−40 °C and was slightly lower at 40 °C. These data indicate that the enzyme operates more efficiently at lower temperatures in the absence of calmodulin and more efficiently at higher temperatures with bound calmodulin, the conditions within the host cell.

We next examined the conditions required for the activation of pertussis toxin and its S1 subunit as a function of toxin preparation, activators, and temperature. Both PT at 30 °C (Figure 2A) and S1 subunit at 30 °C (Figure 2B) or 37 °C (Figure 2C), after incubation with dithiothreitol, showed a constant rate of [*carbonyl*-^{14}C]nicotinamide formation from [^{14}C]NAD with no initial delay. ATP enhanced the NAD glycohydrolase activity of holotoxin, but had no effect on the activity of the S1 subunit, as previously reported (Lim et al., 1985; Mattera et al., 1986; Moss et al., 1986) (Figure 2). The NAD glycohydrolase activities of pertussis toxin in the presence or absence of ATP or of the S1 subunit were enhanced by CHAPS, a zwitterionic detergent (Moss et al., 1986) (Figure 2). The NAD glycohydrolase activities of holotoxin and S1 subunit were constant for at least 80 min at 30 °C; however, the activity of the S1 subunit at 37 °C declined significantly after 40 min in the presence, but not in the absence, of CHAPS (Figure 2).

Pertussis toxin can be used after reconstitution from a lyophilized preparation or following purification and storage in buffer (nonlyophilized). Preliminary studies indicated that its responsiveness to CHAPS and ATP was dependent on the preparation (data not shown). CHAPS increased the NAD glycohydrolase activity of lyophilized toxin by ~100% (Figure 3), although it did not affect the activity of nonlyophilized PT in the absence of ATP (Figure 4). In the presence of ATP and CHAPS, the optimal temperature for lyophilized PT was 33−37 °C (Figure 3). For S1, the temperature dependence was similar with and without CHAPS. Cholate (0.4%) did not stimulate the NAD glycohydrolase activity of nonlyophilized PT in the absence of ATP, although it did stimulate the activity of lyophilized PT, perhaps consistent with the presence of some free S1 in this preparation (data not shown). Like its NAD glycohydrolase activity, the ADP-ribosylation by PT of transducin (Figure 5) or recombinant $G_{o\alpha}$ (Figure 6) was also optimal at 30−37 °C; of note, these assays were performed in the absence of phospholipids or detergents.

Adenine nucleotides such as ATP stimulated the activity of holotoxin, but not that of the S1 subunit (Lim et al., 1985; Moss et al., 1986), which is consistent with the observation that the binding site for ATP is located on the B oligomer (Hausman et al., 1990). Burns and Manclark (1986)

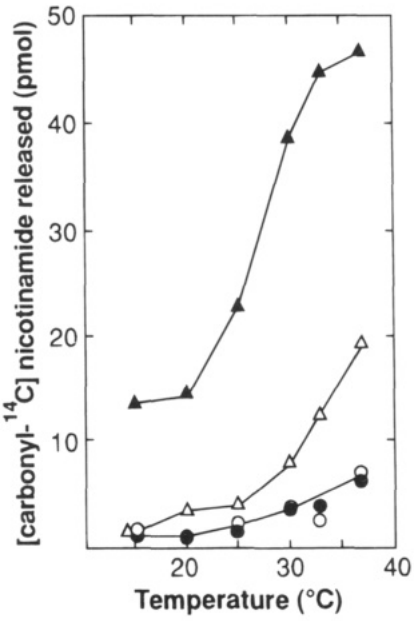

FIGURE 4: Effects of ATP and CHAPS on the NAD glycohydrolase activity of nonlyophilized PT. 210 μL of nonlyophilized PT (52.5 μg in 100 mM potassium phosphate (pH 7.0) and 0.5 M NaCl) plus 2.1 mL of 50 mM potassium phosphate (pH 7.5) were incubated with 100 mM dithiothreitol for 10 min at 30 °C. Samples (40 μL, 1 μg of PT) were assayed for NAD glycohydrolase activity as described in the legend to Figure 2. Symbols are the same as for Figure 3. The experiment was done once in duplicate.

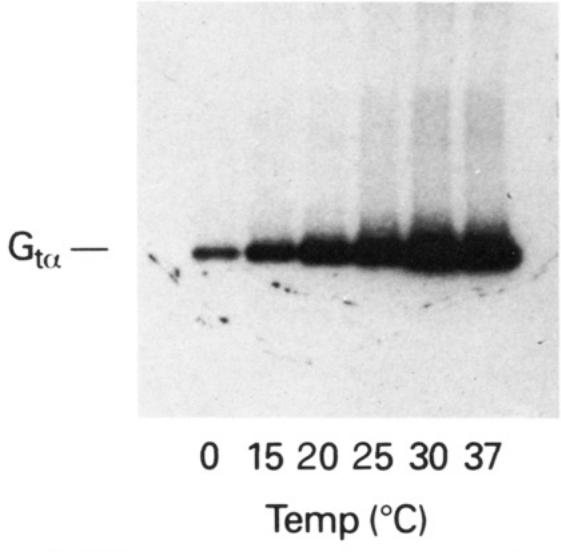

FIGURE 5: Effect of temperature on the ADP-ribosylation of transducin by pertussis toxin. Lyophilized PT (50 μg in 50 mM potassium phosphate (pH 7.5)/70 mM sodium phosphate/350 mM NaCl) was incubated with 100 mM dithiothreitol in a total volume of 70 μL for 10 min at 30 °C. Samples (7.1 μg, 10 μL) plus purified bovine transducin (4.4 μg, 20 μL) were then incubated in a total volume of 100 μL for 20 min at the indicated temperatures in the [^{32}P]ADP-ribosylation assay described in Methods. The experiment was repeated twice.

demonstrated that the combination of ATP and CHAPS enhanced the release of S1 from the B oligomer. However, at least half of the PT behaved as a holotoxin in the presence of ATP and CHAPS. In the present study, there was no delay before the appearance of NAD glycohydrolase activity in PT treated with ATP plus CHAPS (Figure 2), which is consistent with the hypothesis that ATP-treated PT consisted of an enzymatically active S1 that remained associated with B components. To provide further evidence that there was

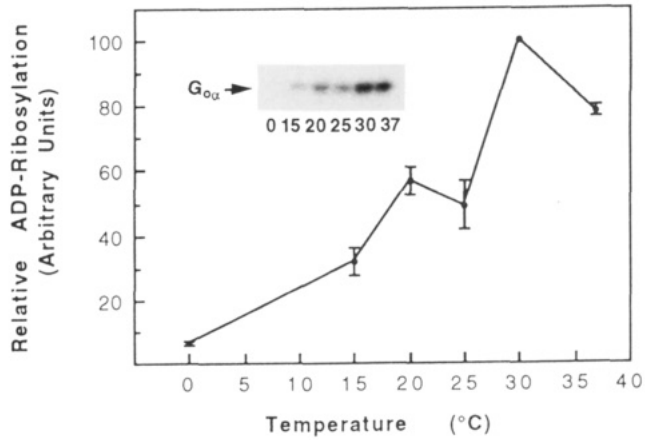

FIGURE 6: Effect of temperature on ADP-ribosylation of $rG_{o\alpha}$ by pertussis toxin. Pertussis toxin-catalyzed ADP-ribosylation of $rG_{o\alpha}$ was performed as described in Methods at 0, 15, 20, 25, 30, and 37 °C. The graph depicts relative percentages of ADP-ribosylation of $rG_{o\alpha} \pm$ SD obtained by phosphoimaging after first arbitrarily setting the 30 °C reaction to 100% ($N = 3$). The experiment was performed three times, with similar results. The inset shows the results of a typical experiment.

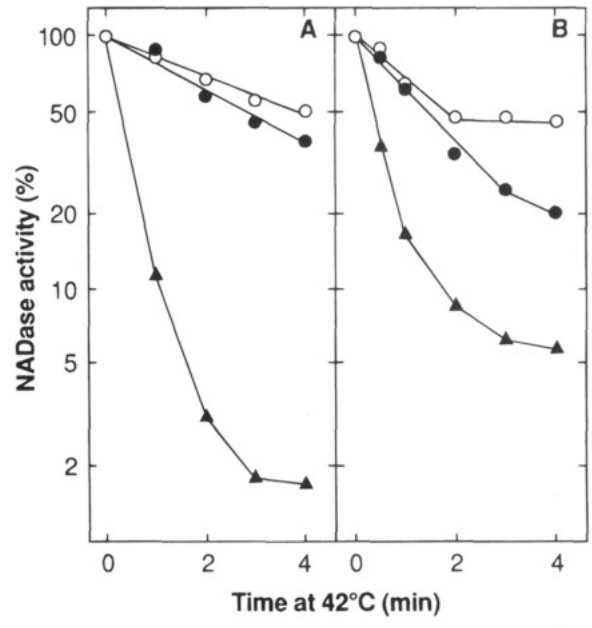

FIGURE 7: Thermal stability of pertussis toxin. Lyophilized PT (50 μg) was dissolved in 50 mM potassium phosphate (pH 7.5)/10 mM sodium phosphate/50 mM NaCl/1% CHAPS/20 mM dithiothreitol with 0.4 mg/mL ovalbumin (total volume, 500 μL). Lyophilized S1 subunit (10 μg) was dissolved in 50 mM potassium phosphate (pH 7.5)/10 mM Tris/0.1 mM EDTA/1% CHAPS/20 mM dithiothreitol with 0.4 mg/mL ovalbumin in a total volume of 500 μL. Samples [40 μL, 4 μg of PT (○, ●) or 0.8 μg of S1 (▲)] were incubated for the indicated times at 42 °C with (●, ▲) or without (○) 10 μL of 10 mM ATP solution (A) or were incubated for 5 min at 30 °C with or without 10 μL of ATP, and then further incubated for the indicated times at 42 °C (B). Assay of NAD glycohydrolase activity was started by the addition of 100 μL of reaction mixture. Assays were incubated at 30 °C for 60 min, and experiments were repeated three times.

an S1−B oligomer complex, thermal stability was examined (Figure 7). In the presence of 30 mM dithiothreitol and 1% CHAPS, PT with or without ATP and S1 subunit with ATP were incubated for the indicated times at 42 °C before assay (Figure 7A). Other samples were incubated first for 5 min at 30 °C and then for the indicated times at 42 °C, before assay for 60 min at 30 °C in the presence of ATP and

CHAPS (Figure 7B). PT, with or without ATP, was relatively stable at 42 °C for 4 min. In contrast, the NAD glycohydrolase activity of the S1 subunit was rapidly lost. The thermal stability of PT in the presence of ATP and CHAPS was clearly much greater than that of the S1 subunit under identical conditions, which is consistent with the possibility that, even in ATP and CHAPS, the S1 subunit continues to interact with the B oligomer. These data are consistent with those of Krueger and Barbieri (1993), who showed that activation by ATP does not require dissociation of S1 from the B oligomer.

In summary, two major toxins from *B. pertussis*, pertussis toxin and adenylate cyclase toxin, behave differently in response to reaction temperatures. PT exhibits a broad temperature optimum, from 30 to 37 °C, whereas adenylate cyclase toxin has an apparent optimum at 25 °C in the absence of its activator, calmodulin, which is provided by the host cell. In the presence of the host factor, the temperature optimum is shifted to that encountered within the host. Thus, similar to cholera toxin, the two major *B. pertussis* toxins exhibit the maximal activity temperatures found in the host cell when other host factors, e.g., calmodulin for the adenylate cyclase toxin, are present, optimizing their abilities to serve as virulence factors in disease.

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