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Sulfhydryl-Alkylating Reagents Inactivate the NAD Glycohydrolase Activity of Pertussis Toxin†

Harvey R. Kaslow* and David D. Lesikar
Department of Physiology and Biophysics, University of Southern California School of Medicine, Los Angeles, California 90033

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ABSTRACT: The combination of ATP, CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), and DTT (dithiothreitol) is known to promote the expression of the NAD glycohydrolase activity of pertussis toxin, which resides in the toxin's S1 subunit. By monitoring changes in electrophoretic mobility, we have found that ATP and CHAPS act by promoting the reduction of the disulfide bond of the S1 subunit. In addition, ATP, CHAPS, and DTT allowed sulfhydryl-alkylating reagents to inactivate the NAD glycohydrolase activity. In the presence of iodo[^{14}C]acetate, the combination of ATP, CHAPS, and DTT increased ^{14}C incorporation into only the S1 subunit of the toxin, indicating that alkylation of this subunit was responsible for the loss of activity. If iodoacetate is used as the alkylating reagent, alkylation can be monitored by an acidic shift in the isolectric point of the S1 peptide. Including NAD in alkylation reactions promoted the accumulation of a form of the S1 peptide with an isoelectric point intermediate between that of native S1 and that of S1 alkylated in the absence of NAD. This result suggests that NAD interacts with one of the two cysteines of the S1 subunit. In addition, we found the pH optimum for the NAD glycohydrolase activity of pertussis toxin is 8, which may reflect the participation of a cysteine in the catalytic mechanism of the toxin.

The bacterium *Bordetella pertussis* causes the disease whooping cough [for review, see Weiss and Hewlett (1986)]. Since the introduction of a "whole-cell" vaccine, consisting of killed *B. pertussis*, the incidence of the disease has declined dramatically. Unfortunately, this vaccine may cause severe adverse reactions (Fulginiti, 1984; Hinman & Koplan, 1984; Cody et al., 1981; Miller et al., 1984). *B. pertussis* produces several toxins, including one termed pertussis toxin, which is considered to be an important protective component of the vaccine (Munoz et al., 1981; Oda et al., 1983; Sato et al. 1981; Sato & Sato, 1984). Thus, in an attempt to minimize adverse

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* Address correspondence to this author.

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reactions, an "acellular" vaccine has been developed, containing purified pertussis toxin treated with formalin. Unfortunately, native pertussis toxin may cause the adverse reactions (Steinman et al., 1985), and formalin treatment may not produce a completely detoxified toxin that is protective (Garcia-Sainz et al., 1985). This problem suggests a need for a defined detoxification of pertussis toxin, on the basis of its structural and functional characteristics. In addition, pertussis toxin is a valuable tool for studying mechanisms of cellular regulation (Weiss & Hewlett, 1986). These uses of pertussis toxin suggest a need for a better understanding of its molecular mechanisms.

Pertussis toxin consists of five types of peptides, organized as an A protomer, containing the S1 peptide, and a B oligomer, consisting of one S2, one S3, two S4, and one S5 peptides. The B oligomer binds to target cells, delivering the S1 peptide (Katada et al., 1983; Tamura et al., 1982, Sekura et al., 1983). The S1 peptide catalyzes the transfer of ADP-ribose from NAD to specific guanine nucleotide binding proteins, thereby disrupting their functions and producing some of the characteristics of the disease (Katada & Ui, 1982a,b; Weiss & Lewlett, 1986). The activity of the S1 peptide, also detected as an NAD glycohydrolase activity, is not expressed by the native toxin but can be revealed in vitro by incubating the toxin with sulfhydryl-reducing agents such as DTT[1] (Tamura et al., 1982; Katada et al., 1983; Moss et al., 1983). ATP and the detergent CHAPS promote the activation of the toxin by DTT (Lim et al., 1985; Moss et al., 1986). A structure–activity analysis (Kaslow et al., 1987) has better defined the three classes of activating compounds represented by ATP, DTT, and CHAPS.

These discoveries suggested that activation by ATP, DTT, and CHAPS, under mild conditions, might expose key amino acids of the toxin, allowing them to be covalently modified, yielding an inactivated toxin with defined and stabile structural differences. If such a preparation could confer protective immunity, it might be a useful component of a defined pertussis vaccine. If it did not, it might help define structural characteristics important for immunoprotection. Such data should also aid in the use of the toxin as a biochemical tool. This paper describes the inactivation of pertussis toxin by a stabile covalent modification amenable to precise definition.

Experimental Procedures

NAD Glycohydrolase Assay. The assay measures the hydrolysis of NAD labeled in the nicotinamide moiety, with ion-exchange column chromatography to resolve product from substrate. We modified a described method (Moss et al., 1983). First, we substituted QAE-Sephadex to avoid variable recovery of nicotinamide noted with different lots of Dowex resins. Second, because [*nicotinamide*-^{14}C]NAD is expensive, we used [*nicotinamide*-4-^{3}H]NAD. Because this [^{3}H]NAD gave a blank of >5%, we purified it on a QAE-Sephadex column: A 2-mL QAE-Sephadex column was first washed with 10 mL of 1 N HCl, followed by 10 mL of H_2O, 20 mL of 0.5 M Tris-HCl, pH 7.0, and 40 mL of H_2O. [^{3}H]NAD was applied and the column washed with H_2O (20 mL) to elute impurities. Purified [^{3}H]NAD was eluted with 0.25 M NaCl and stored at −70 °C.

After NAD glycohydrolase reactions, [^{3}H]nicotinamide was isolated from [^{3}H]NAD as follows: 2-mL QAE-Sephadex columns were treated with 10 mL of 0.1 M imidazole hydrochloride, pH 7.0, with 1.0 M NaCl, followed by 40 mL of H_2O. The NAD glycohydrolase reaction (in 100 μL) was terminated by adding 0.85 mL of ice-cold water, and the entire 0.95 mL was pipetted onto a treated QAE column. The [^{3}H]nicotinamide was then eluted with 3 mL of H_2O into a scintillation vial and counted. The adsorbed [^{3}H]NAD was then eluted to radioactive waste with 10 mL of 0.1 M imidazole hydrochloride, pH 7.0, with 1.0 M NaCl, and the column was stored partially immersed in water. We have used the same columns many times. Before its first use, each column was first treated with 10 mL of 1 N HCl followed by 20 mL of H_2O. At pH 8 the blank is due in large part to nonenzymatic hydrolysis of NAD during the assay, not a failure to resolve product from substrate (Figure 1).[2] After this paper was submitted, we learned that a similar method has been previously described (Tait & Nassau, 1984).

Electrophoresis. One-dimensional sodium dodecyl sulfate electrophoresis employed a modification (Fling & Gregerson, 1986) of established procedures (Laemmli, 1970). Two-dimensional electrophoresis employed a modification (Kaslow et al., 1981) of an established procedure (O'Farrell, 1975) except that ampholytes (LKB) with a pH range of 5–8 were used and the second dimension was modified (Fling & Gregerson, 1986). Proteins were identified by staining with Serva Blue R (Serva). The electrophoretic mobility of the S1 peptide was established for all types of experiments with purified S1, essentially free of B oligomer.

Purification of the S1 Peptide of Pertussis Toxin. The method is based upon the observation that ATP and CHAPS promote the release of the S1 peptide from the B oligomer (Burns& Manclark, 1986). The toxin (66 μg) was incubated for 15 min at 37 °C in buffer C (125 mM Tris-HCl, pH 8.0 at 30 °C, 1 mM EDTA, 1% CHAPS, and 1 mM ATP). This solution was then mixed with packed fetuin–Sepharose beads previously equilibrated in buffer C (packed volume 240 μL) and then incubated with occasional vortexing at 30 °C for 15 min. The beads were pelleted, and the supernatant, containing purified S1 peptide, was recovered. Fetuin–Sepharose was used instead of an ion-exchange resin [as described by Burns and Manclark (1986)] to allow for binding of the B oligomer to beads in the high ionic strength solutions described herein. Fetuin–Sepharose was prepared by exposing 100 mg of fetuin (Sigma, type IV) to 5 g of CNBr-activated Sepharose 4B according to the instructions of the manufacturer (Pharmacia).

Results

Under the assay conditions described herein, the pH optimum of the NAD glycohydrolase reaction was near pH 8 and was substantially independent of the type of buffer used (Figure 1). The significance of this pH optimum is considered under Discussion.

Table I shows that native (not activated) pertussis toxin resisted inactivation by iodoacetate, a sulfhydryl-alkylating reagent. However, a 15-min exposure of the toxin to ATP, DTT, and CHAPS allowed a subsequent, 15-min exposure to iodoacetate to reduce the activity of the toxin to 14% of the control. Omitting either ATP, DTT, or CHAPS prevented iodoacetate from markedly reducing activity. Table II shows

[1] Abbreviations: DTT, dithiothreitol; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

[2] The increased blank associated with increasing pH in Figure 1 is seen only after an incubation. In a separate experiment (data not shown), [^{3}H]NAD was added to assay mixtures containing various buffers with concentrations as high as 400 mM at pH 9. If the combination of [^{3}H]NAD and assay mixture was prepared and immediately applied to the QAE column, the ^{3}H not binding to the column was equivalent to that seen for [^{3}H]NAD diluted with only water.

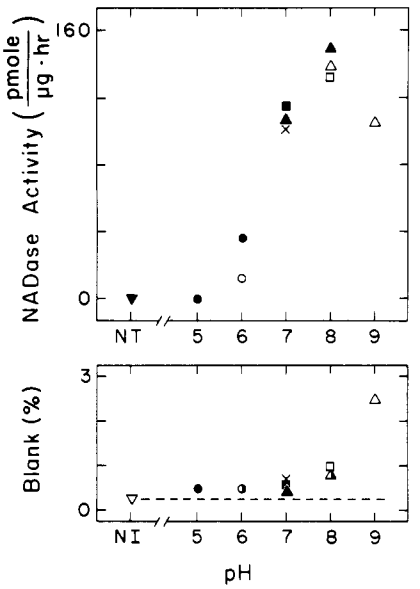

FIGURE 1: Assay of pertussis toxin NAD glycohydrolase activity. Release of nicotinamide from NAD during 90 min at 37 °C was measured in the presence (top panel) or absence (bottom panel) of pertussis toxin. The assay mixture contained 1 mM ATP, 1% w/w CHAPS, and 10 mM DTT, 50 mM NaCl, and 25 μM [^{3}H]NAD in different buffers (100 mM) at the indicated pHs. (●) Sodium citrate; (○) sodium 2-(*N*-morpholino)ethanesulfonate (MES); (×) imidazole hydrochloride; (■) Na_xPO_4; (▲) sodium *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate (HEPES); (□) tris(hydroxymethyl)aminomethane (Tris) hydrochloride; (△) sodium 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonate (TAPS). In the top panel, nonenzymatic hydrolysis of NAD was subtracted before the NAD glycohydrolase activity was calculated; (▼) NT = no toxin. In the bottom panel the blank value (amount of ^{3}H detected in the column eluate, see Experimental Procedures) is expressed as the percent of the total amount of [^{3}H]NAD in the assay tube; (▽) NI = no incubation. [^{3}H]NAD was kept on ice during the incubation of the other tubes, diluted with water without the addition of a buffered assay mixture, and applied to a QAE column.

that increasing the time of exposure to iodoacetate further reduced activity to 3% of control and that other sulfhydryl-alkylating reagents were effective with a rank order of potencies correlating with their generally accepted reactivities (Lundblad & Noyes, 1984). These results support the hypothesis that the residual activity is a function of the number of S1 peptides that escaped alkylation, as opposed to the hypothesis that alkylated toxin retains a fraction of its original activity. The data in Table II also indicate that the decrease in activity is the result of alkylation and not the result of some other side reaction (e.g., iodination by iodide released from iodoacetic acid).

Figure 2 shows that in the presence of iodo[^{14}C]acetate the combination of ATP, DTT, and CHAPS increased the incorporation of ^{14}C into only the S1 peptide of the toxin, indicating that alkylation of the S1 peptide caused the decrease in NAD glycohydrolase activity. Similar results obtained with *N*-[^{14}C]ethylmaleimide (data not shown) support this hypothesis.

Table I also shows that when CHAPS was absent, ATP and DTT, either alone or in combination, caused a loss of NAD glycohydrolase activity *not* dependent upon the subsequent addition of iodoacetate. In contrast, CHAPS, which interacts directly with at least the S1 peptide (Moss et al., 1986), stabilized the activity, either in the presence or in the absence of ATP and/or DTT. In addition, Figure 2 shows that in the presence of iodo[^{14}C]acetate CHAPS *decreased* ^{14}C incorporation into the B oligomer of the toxin. Similar results have been obtained with *N*-[^{14}C]ethylmaleimide (data not shown).

Table I: Inactivation of Pertussis Toxin[a]

additions		% NAD glycohydrolase activity relative to	
first incubation	second incubation	iced control	no addition to second incubation
none	none	49 ± 3	100
	iodoacetate		87 ± 11
DTT	none	38 ± 2	100
	iodoacetate		123 ± 5
ATP	none	13 ± 2	100
	iodoacetate		153 ± 8
ATP, DTT	none	16 ± 2	100
	iodoacetate		107 ± 6
CHAPS	none	106 ± 7	100
	iodoacetate		108 ± 5
DTT, CHAPS	none	117 ± 5	100
	iodoacetate		84 ± 7
ATP, CHAPS	none	90 ± 5	100
	iodoacetate		80 ± 8
ATP, DTT, CHAPS	none	71 ± 3	100
	iodoacetate		14 ± 3

[a] Pertussis toxin (1 μg) was incubated first at 37 °C for 15 min in 40 μL of buffer A: 100 mM Tris-HCl, pH 8.0 (at 30 °C), with 1 mM Na-EDTA. When present during the first incubation, DTT and ATP were 1 mM each, and CHAPS was 1% w/w. The second incubation was begun by adding 10 μL of buffer A with or without 12.5 mM iodoacetate, mixing the contents of the tube, and shifting it to 30 °C. After 15 min, the tubes were shifted to an ice bath for 10 min. The third incubation (1 h on ice) was then begun by adding 10 μL of buffer A containing 200 mM DTT to those tubes containing iodoacetate, to scavenge the iodoacetate. For those tubes *not* containing iodoacetate during the second incubation, the third incubation was begun by adding a mixture, previously incubated for 1 h on ice, of 200 mM DTT and 12.5 mM iodoacetate in buffer A. The contents of each tube were then assayed for NAD glycohydrolase activity at 37 °C for 1 h in a final volume of 100 μL, by adding 40 μL of an assay mix consisting of buffer A containing [^{3}H]NAD (final concentration in the 100 μL = 25 μM). Different assay mixes with various additions were used, so that all tubes contained 1 mM ATP and 1% w/w CHAPS in the 100 μL. For the "iced control", pertussis toxin (1 μg) was incubated on ice in 10 mM Na_xPO_4, pH 7, and 50 mM NaCl during the first three incubations. This toxin was then assayed for NAD glycohydrolase activity as described above, yielding a specific activity of 127 pmol h^{-1} (μg of toxin)$^{-1}$. The first three incubations were done under a nitrogen atmosphere in degassed solutions. The second and third incubations were done under subdued light. Each value shown is the mean of triplicate tubes ± its standard error.

These results suggest that CHAPS stabilized the structure of the toxin, thereby retarding the reduction and alkylation of the B oligomer.

Burns and Manclark (1986) recently demonstrated that the combination of ATP and CHAPS can release approximately 50% of the S1 peptide from the B oligomer. The released S1 peptide had a much higher specific NAD glycohydrolase activity than the S1 peptide that remained bound to the B oligomer, suggesting either (1) bound S1 peptides resisted reduction, (2) allosteric interactions with the B oligomer decreased the NAD glycohydrolase activity of reduced S1 peptides, or (3) the toxin preparation was heterogeneous. Considering these possibilities along with the data in Tables I and II and Figure 2 prompts consideration of several models: For example, although overnight incubation with iodoacetate nearly obliterated activity, it is possible that some S1 peptides initially resisted reduction (by 1 mM DTT) and alkylation and still expressed no NAD glycohydrolase activity upon subsequent assay (even in the presence of 20 mM DTT) because they resisted reduction. Alternatively, all S1 peptides might be reduced, but interactions with the B oligomer might not only decrease activity, but retard alkylation. Finally, of course, all S1 subunits might be reduced and alkylated. We thus sought to determine, under the conditions of activation and inacti-

Table II: Inactivation of Pertussis Toxin[a]

additions		NAD glycohydrolase act. (pmol μg^{-1} h^{-1}) [% act. with no addn to second incubn]	
first incubation	second incubation (15 min at 30 °C)	15 min at 30 °C + 6 min at 0–4 °C	15 min at 30 °C + 18 h at 0–4 °C
none	none	ND	51 [100 ± 8]
	iodoacetate	ND	59 [116 ± 18]
CHAPS	none	ND	186 [100 ● 1]
	iodoacetate	ND	171 [92 ● 3]
DTT, CHAPS	none	ND	191 [100 ● 2]
	iodoacetate	ND	151 [79 ± 1]
ATP, CHAPS	none	ND	130 [100 ± 3]
	iodoacetate	ND	110 [85 ± <1]
ATP, DTT, CHAPS	none	98 [100 ● 1]	103 [100 ● 5]
	iodoacetate	17 [17 ± 2]	3 [3 ● <1]
	none	109 [100 ± 1]	99 [100 ± 1]
	N-ethylmaleimide	16 [15 ● 1]	10 [10 ± 1]
	none	96 [100 ± 5]	98 [100 ± 2]
	chloroacetamide	76 [79 ● <1]	35 [36 ± <1]
	none	94 [100 ● 4]	98 [100 ± 4]
	chloroacetate	86 [91 ● 4]	85 [87 ± <1]

[a] The same procedure as described for Table I was used with the following differences: Each value is the mean of duplicate tubes. In some tubes, *N*-ethylmaleimide, chloroacetamide, or chloroacetate was substituted for iodoacetate. After the 30 °C incubation, the tubes were incubated for either 6 min or 18 h at 0–4 °C before the 10 μL of 200 mM DTT (with or without scavenged reagent) was added. Those tubes receiving the DTT after 6 min were stored for 19 h at 0–4 °C prior to the NAD glycohydrolase assay. Other experiments (not shown) demonstrated that the NAD glycohydrolase activity of the toxin is stabile for this period under these conditions. Those tubes receiving DTT after 18 h were further incubated for 1 h, as in Table I. All tubes were then assayed for NAD glycohydrolase activity at the same time. ND = not determined; ± = half the range of the duplicate measurements.

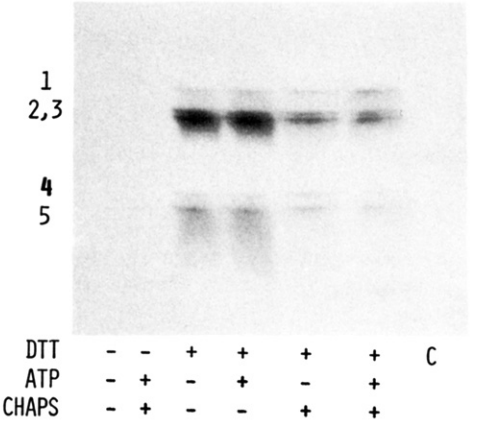

FIGURE 2: ^{14}C-Carboxymethylated pertussis toxin. Pertussis toxin was incubated either in the presence (+) or in the absence (−) of 1 mM ATP, 1 mM DTT, and 1% w/w CHAPS at 37 °C for 15 min as described in Table I. Iodo[1-^{14}C]acetate was then added (10 μL, 23 mM in buffer A), and the tubes incubated at 30 °C for 15 min and on ice for 18 h. Nonradioactive iodoacetate (10 μL, 250 mM) was then added to reduce the ^{14}C specific activity of the iodoacetate. A control tube (C) was prepared by first performing both additions of iodoacetate and then adding pertussis toxin activated with ATP, DTT, and CHAPS. An electrophoresis sample buffer containing sodium dodecyl sulfate and β-mercaptoethanol was then added, the tubes were heated (95 °C, 4 min), and the samples were electrophoresed on a one-dimensional 15% polyacrylamide gel. After electrophoresis, the gel was fixed, treated with Fluoro-Hance, dried, and used to expose X-ray film according to the instructions accompanying the Fluoro-Hance reagent. The numbers indicate the position of the various peptides of the toxin, with 1 denoting S1, 2 denoting S2, etc.

vation described herein, what fraction of the S1 *molecules* was reduced, and what fraction was alkylated.

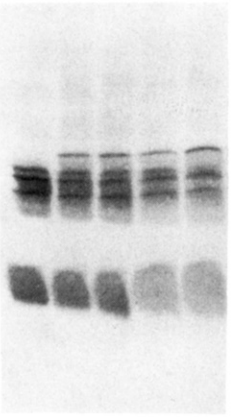

FIGURE 3: Electrophoretic mobility of pertussis toxin peptides. Pertussis toxin (2.5 μg) was incubated in the presence (+) or absence (−) of 0.5 mM DTT, 1 mM ATP, and 1% w/w CHAPS, in 50 mM Tris-HCl, pH 8, for 30 min at 37 °C in a volume of 50 μL; 10 μL of 10 mM iodoacetate was then added to prevent reoxidation of cysteine residues, and a second incubation was performed at 30 °C for 30 min in the dark. Both incubations were performed under a nitrogen atmosphere. The samples were then treated with an SDS sample buffer *not* containing β-mercaptoethanol and electrophoresed on a one-dimensional 15% polyacrylamide gel. The gel was fixed, stained with acetic acid and Serva Blue R, and dried. A photograph of the gel is shown. In the left-most and right-most lanes the three dark resolved bands are, from the top down, the S1, S2, and S3 peptides of the toxin.

To answer the first question, we took advantage of the observation that reduction of the disulfide bond in the S1 peptide retards its mobility during SDS gel electrophoresis (Sekura et al., 1983). Figure 3 shows that exposing the toxin to the combination of ATP, DTT, and CHAPS led to an essentially complete conversion of the S1 peptide to a form with reduced electrophoretic mobility. If any one of these substances was omitted, the conversion was incomplete. Thus, ATP and CHAPS allow for complete reduction of the S1 peptide.

To answer the second question, we took advantage of the expectation that carboxymethylation of −SH groups should shift the isoelectric point of the S1 peptide to a more acidic pH. Figure 4 shows that adding iodoacetate to pertussis toxin treated with ATP, DTT, and CHAPS shifted nearly all the S1 peptides. Omitting any one or more of these substances prevented iodoacetate from shifting the isoelectric point.[3] Thus, essentially all the reduced S1 peptides were carboxymethylated. Figure 4 also shows that the S1 peptide is well focused. Thus, the carboxymethylation apparently survives the overnight isoelectric focusing procedure, indicating that the modification is stabile.

Recent work (Locht & Kieth, 1986; Nicosia et al., 1986) indicates that the S1 peptide of pertussis toxin contains two cysteines, the probable sites of alkylation. It is likely that, after reduction under the conditions described above, both cysteines are alkylated. We conducted two types of experiments that indicate that NAD interacts with one of these cysteines. First, we incubated purified, reduced S1 peptide with iodo[^{14}C]-acetate in the absence or presence of NAD. After 2, 6, or 12 min, nonradioactive iodoacetate was added to essentially stop

[3] The electrophoretic mobility of the S1 peptide was examined after incubations with the combinations of ATP, DTT, and CHAPS listed in Table II, according to the procedures described under Figure 4. All combinations of two or fewer of these substances gave results (not shown) equivalent to that shown for DTT and CHAPS in Figure 4.

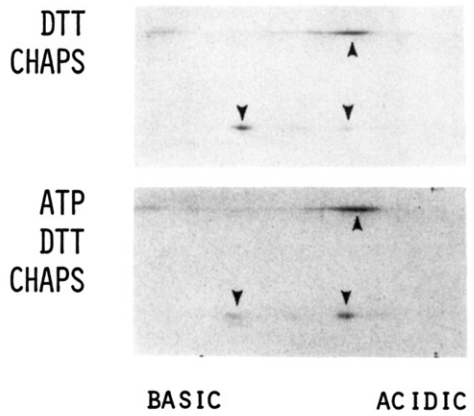

FIGURE 4: Carboxymethylation of pertussis toxin detected by isoelectric focusing. Pertussis toxin (2 μg) was incubated in 200 mM Tris-HCl, pH 8, 1 mM EDTA, 1 mM DTT, and 1% w/w CHAPS with or without 1 mM ATP for 15 min at 37 °C in a final volume of 8 μL. Compared to procedures described in Tables I and II, the volume of the reaction was reduced because of technical considerations concerning the electrophoresis procedure; the amount of toxin was increased to aid visualization of the protein after electrophoresis. These changes increased the concentration of sodium phosphate, pH 7 (present in commercial preparations of the toxin), from 4 to 20 mM in the reaction. We thus increased the concentration of Tris to maintain the pH at 8. After this first incubation, 2 μL of 12.5 mM iodoacetate in the Tris/EDTA buffer was added, and the tubes were incubated for 15 min at 30 °C and then overnight as described in Tables I and II. The samples were analyzed by two-dimensional electrophoresis. Photographs of a portion of the dried Serva Blue R stained gels are shown. An arrow pointing up indicates the position of bovine serum albumin, which was included to mark a reference isoelectric point. Arrows pointing down indicate the S1 peptide of pertussis toxin. A shift from left to right indicates a shift to a more acidic isoelectric point.

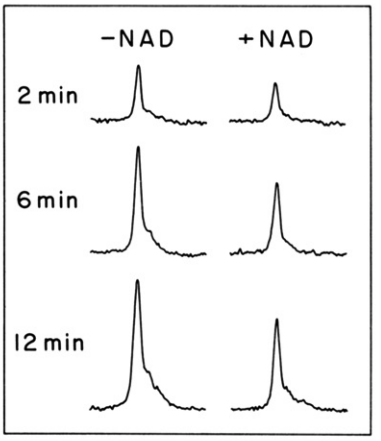

FIGURE 5: NAD inhibits the ^{14}C-carboxymethylation of the S1 peptide of pertussis toxin. A total of 40 μL of purified S1 peptide (derived from approximately 2.6 μg of toxin; see Experimental Procedures) was reduced with 1 mM DTT in the presence or absence of 1 mM NAD for 15 min at 37 °C, in borosilicate glass tubes. Iodo[^{14}C]acetic acid was added (10 μL of 12.5 mM in buffer B: 100 mM Tris-HCl, pH 8 at 30 °C, 1 mM EDTA, and 1% CHAPS), and the tubes were transferred to a 30 °C bath. After either 2, 6, or 12 min, nonradioactive iodoacetate (10 μL of 125 mM in buffer B) was added to reduce the specific radioactivity of the iodoacetate, and the tube was transferred to an ice bath. The samples were then treated and electrophoresed on 17.5% gels, and the gels were used to generate autoradiograms as described under Figure 2. Densitometric scans of the bands of exposure caused by the labeled S1 protein are shown; all six scans have the same vertical and horizontal scales. Control experiments verified that the fetuin–Sepharose yielded no ^{14}C-labeled proteins and that the addition of nonradioactive iodoacetate markedly reduced the incorporation of label.

the ^{14}C labeling of the protein. The data in Figure 5 indicate that at all time points NAD inhibited ^{14}C incorporation into the protein by about half. Second, we incubated activated pertussis toxin with bromoacetic acid (which also carboxymethylates cysteines) in the absence or presence of NAD. After 5 min a large amount of iodoacetamide (which carboxamidomethylates cysteines) was added. We previously determined that carboxamidomethylation inhibits the toxin but, in contrast to carboxymethylation, does not cause a significant shift in isoelectric point (data not shown). Thus, the addition of iodoacetamide should essentially terminate carboxymethylation and the acidic shift in isoelectric point. The results in Figure 6 show that the addition of NAD promoted the accumulation of a form of the S1 peptide with an isoelectric point intermediate between that of native (or carboxamidomethylated) and that of putative fully carboxymethylated S1 peptide. This intermediate form is probably monocarboxymethylated S1. The data support the notion that NAD retards alkylation of only one of the S1 cysteines.

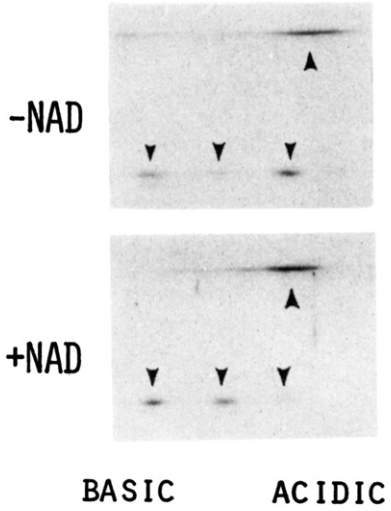

FIGURE 6: NAD alters the carboxymethylation of the S1 peptide of pertussis toxin. Pertussis toxin was activated with ATP, DTT, and CHAPS, as described under Figure 4, in either the presence or absence of 1.25 mM NAD. The samples were then processed as described under Figure 4 but with the following changes: (1) Bromoacetic acid was used instead of iodoacetic acid. (2) The 30 °C incubation with bromoacetic acid was conducted for only 5 min. (3) Following this 5-min incubation, 1 μL of 250 mM iodoacetamide was added, and the tubes were transferred to 0–4 °C for 20 h. Photographs of the stained gels are shown. An arrow pointing up indicates the position of bovine serum albumin; arrows pointing down indicate the S1 peptide of pertussis toxin. A shift from left to right indicates a shift to a more acidic isolectric point.

Discussion

Our results indicate that a free sulfhydryl group (or groups) of the S1 peptide of pertussis toxin is required for expression of its NAD glycohydrolase, and presumably its ADP-ribosyltransferase,[4] activity. Our results also indicate that NAD blocks alkylation of one of the two cysteines found in this peptide. It is worth noting that the pH optimum of the NAD glycohydrolase activity of the toxin was 8, very close to the generally accepted pK_a (pH 8.5) of cysteines in proteins (Lundblad & Noyes, 1984). This pH optimum may reflect the participation of a cysteine moiety in the NAD glycohydrolase mechanism employed by the toxin. West et al. (1985) recently determined that pertussis toxin ADP-

[4] Although detergents appear to be required for the full expression of the NAD glycohydrolase activity of pertussis toxin in vitro, they can interfere with the ADP-ribosyltransferase reaction (Moss et al., 1986; Kaslow et al., 1987). This interference may involve an interaction of the detergent with the protein substrates of the toxin. Until this issue is resolved, data obtained with an ADP-ribosyltransferase assay may be difficult to interpret.

ribosylates a cysteine residue in transducin, a retinal guanine nucleotide binding protein. It is thus tempting to speculate that the other cysteine residue of the S1 peptide that may not interact directly with NAD may instead interact with the target cysteine in the protein substrate.

Like pertussis toxin, cholera and diphtheria toxins are ADP-ribosyltransferases activated by DTT. However, the NAD glycohydrolase activity of diphtheria toxin and the toxic properties of cholera toxin are not inhibited by sulfhydryl-alkylating reagents (Carroll et al., 1985; Gill, 1976; Markel et al., 1979). In addition, neither cholera nor diphtheria toxin ADP-ribosylates a cysteine in its substrates (Moss & Vaughan, 1977; Van Ness et al., 1980; Van Dop et al., 1984). These differences suggest that the molecular mechanism by which pertussis toxin modifies its substrates is quite distinct.

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