Effect of gangliosides and substrate analogues on the hydrolysis of nicotinamide adenine dinucleotide by choleragen

(adenylate cyclase/diphtheria toxin/ADP-ribosylation)

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ABSTRACT Choleragen and its A protomer catalyzed the hydrolysis of NAD to ADP-ribose and nicotinamide. NADase activity was inhibited by gangliosides GM1 (galactosyl-N-acetylgalactosaminyl-[N-acetylneuraminyl]-galactosylglucosylceramide), GM2 (N-acetylgalactosaminyl-[N-acetylneuraminyl]-galactosylglucosylceramide), GM1 (N-acetylneuraminylgalactosylglucosylceramide), and GDIa (N-acetylgalactosaminyl-N-acetylneuraminyl-galactosylglucosylceramide). These gangliosides also increased the intensity of the tryptophan fluorescence of the isolated A protomer (λmax = 358 nm). GM1 but not GM2, GM2, and GDIa caused a "blue shift" in the fluorescence spectrum of the B protomer. These results are consistent with other evidence that the specificity of GM1 as the choleragen receptor resides in its carbohydrate moiety.

The NADase activity of choleragen was similar to that of diphtheria toxin previously described (J. Kandel, R. J. Collister & D. W. Chung (1974) J. Biol. Chem. 249, 2068–2077). As with diphtheria toxin, analogues of NAD were inhibitory, adenine being the most effective. Significant inhibition was also noted with adenosine, AMP, ADP-ribose, nicotinamide, nicotinamide mononucleotide, and NADP. NADP was hydrolyzed only slowly by choleragen. In the NADase reaction catalyzed by diphtheria toxin, water serves as an acceptor for the ADP-ribose moiety of NAD in lieu of the natural acceptor molecule, which is elongation factor II (Kandel et al., 1974). It seems probable that the natural protein acceptor for ADP-ribose in the reaction catalyzed by choleragen is adenylate cyclase or a protein component of a cyclase complex that regulates enzymatic activity.

The effects of choleragen on vertebrate cells are believed to be mediated through the activation of adenylate cyclase (1). The initial event appears to be the binding of the B protomer of choleragen to a cell surface receptor, the monosialoganglioside GM1 (galactosyl-N-acetylgalactosaminyl-[N-acetylneuraminyl]-galactosylglucosylceramide) (8–7). The A protomer is then thought to activate the cyclase in a reaction that may be dependent on NAD (8–14). The activation process has not been defined; some workers feel that an enzymatic reaction is involved (9), while others believe that activation involves the direct binding of choleragen to the cyclase (10, 15, 16).

We recently reported that choleragen and its A protomer catalyze the hydrolysis of NAD to ADP-ribose and nicotinamide (17). This abortive reaction is similar to that catalyzed by the active fragment A of diphtheria toxin (18), which inhibits protein synthesis as a result of the NAD-dependent ADP-ribosylation of elongation factor II (19). We proposed, therefore, that choleragen activates adenylate cyclase through an NAD-dependent enzymatic reaction, which probably involves the

ADP-ribosylation of a protein (17). The present studies further characterize the NADase activity of choleragen, and demonstrate that gangliosides can inhibit the NADase activity of the isolated A protomer.

EXPERIMENTAL PROCEDURE

NADase Assay. NADase activity was determined as described (17). Assay mixtures contained potassium phosphate (pH 7.0), dithiothreitol, [carboxyl-14C]NAD, and other additions at the concentrations indicated. After incubation at 37° as indicated, duplicate 0.1-ml samples were transferred to columns of Dowex-1, and [14C]nicotinamide was eluted for radioassay as described (17). The same procedure was used for assay of hydrolysis of [carboxyl-14C]NADP (17). The reaction was initiated by the addition of choleragen or A protomer in 50 mM Tris-Cl, 200 mM NaCl, 1 mM EDTA, 3 mM NaN3, pH 7.5. The protomers of choleragen were prepared by the method of Finklestein et al. (20), and exhibited a single band on polyacrylamide gel electrophoresis (Fig. 1). Protein was determined as described by Lowry et al. (21).

Choleragen was purchased from Schwarz/Mann; [carboxyl-14C]nicotinamide adenine dinucleotide (50 mCi/mmol) and [carboxyl-14C]nicotinamide adenine dinucleotide phosphate (55 mCi/mmol) from Amersham/Searle; dithiothreitol from Calbiochem or Schwarz/Mann; NAD, NADP, AMP, GMP, IMP, CMP, adenine, adenosine, ADP-ribose, ADP, nicotinamide, and nicotinamide mononucleotide from Sigma; and Bio-Gel P-60 from Bio-Rad.

A Perkin-Elmer model MPF-4 fluorescence spectrophotometer equipped with a temperature-controlled cell holder was used for the fluorescence studies. The excitation wavelength was 292 nm, and excitation and emission slits were 3 nm. Samples were maintained at 25° with a Lauda K-2/R cooling bath. Intensity measurements are relative to the control protein solution in each case, and appropriate blanks were subtracted from each sample. Gangliosides were obtained as described and were at least 99% pure, as assessed by thin-layer chromatography (22, 23).

RESULTS

Effect of gangliosides on NADase activity of choleragen and A protomer

Incubation of choleragen with GM1 (up to 50 μM) prior to assay resulted in inhibition of NAD hydrolysis (Fig. 2). The NADase activity was also inhibited by GMI (N-acetylgalactosaminyl-galactosylglucosylceramide), GM2 (N-acetylgalactosaminyl-[N-acetylneuraminyl]-galactosylglucosylceramide), GM3 (N-acetylgalactosaminyl-N-acetylneuraminyl-galactosylglucosylceramide), and GM1 (N-acetylgalactosaminyl-N-acetylneuraminyl-galactosylglucosylceramide), although GM1

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74
Biochemistry: Moss et al.

Fig. 1. Polyacrylamide gel electrophoresis of the A and B protomers of choleragen. The A and B protomers were separated by chromatography on Bio-Gel P-60 in 0.5 M urea, 0.1 M glycine, pH 3.2 (20) and dialyzed against 50 mM Tris-Cl, 200 mM NaCl, 1 mM EDTA, 3 mM NaN₃, pH 7.5. The A (21 µg) and B (32 µg) protomers were mixed with 0.1% sodium dodecyl sulfate, 24% sucrose, and 15 µl of reservoir buffer without sodium dodecyl sulfate in a total volume of 0.1 ml and applied to polyacrylamide gels containing 0.1% sodium dodecyl sulfate. Reservoir buffer contained 36 mM Tris, 30 mM sodium phosphate, 1 mM EDTA, 0.1% sodium dodecyl sulfate, pH 7.55. The gels were run as described (17). The gels were then stained with Coomassie brilliant blue. (Left) A protomer; (right) B protomer.

Fig. 2. Effect of GM₁ on NAD hydrolysis by choleragen. Choleragen (50 µg in 10 µl) was incubated with the indicated concentrations of GM₁ in 0.15 ml for 15 min at 23°C. Assay reaction mix (0.15 ml) was added and incubation continued for 60 min at 37°C. The final concentrations of reactants are given in Table 1. Each point represents the mean of duplicate assays.

was the most effective ganglioside (Table 1). The B protomer did not appear to be required for ganglioside inhibition, since, as demonstrated in Table 2, the NADase activity of the isolated A protomer was inhibited by GM₁.

Effect of gangliosides on tryptophanyl fluorescence of A and B protomers

As shown in Fig. 3, the fluorescence spectrum of the A protomer had an λ_max = 328 nm. Under assay conditions, additions of GM₁, GM₂, GM₃, or GD₁α to the A protomer resulted in small increases in fluorescence intensity, but no shift in λ_max (data not shown). The fluorescence spectrum of the B protomer was "blue shifted" by 15 nm in the presence of 50 µM GM₁ (Fig. 4). Increasing the GM₁ concentration did not further shift the peak. The spectrum in the presence of GM₁ exhibited an asymmetric peak, in contrast to that obtained in the absence of ganglioside. GM₃, GM₂, and GD₁α did not significantly change the fluorescence spectrum of the B protomer.

Inhibition of NAD hydrolysis by substrate analogues

NAD hydrolysis by choleragen was competitively inhibited by substrate analogues. Of the compounds tested, adenine was...

Table 1. Effect of gangliosides on NAD hydrolysis by choleragen

<table>
<thead>
<tr>
<th>Ganglioside added</th>
<th>NADase activity (nmol/min·mg)</th>
<th>% Control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>11.9</td>
<td>100</td>
</tr>
<tr>
<td>GM₃, 37 µM</td>
<td>10.3</td>
<td>87</td>
</tr>
<tr>
<td>GM₂, 32 µM</td>
<td>8.5</td>
<td>71</td>
</tr>
<tr>
<td>GM₁, 47 µM</td>
<td>6.4</td>
<td>54</td>
</tr>
<tr>
<td>GD₁α, 43 µM</td>
<td>8.2</td>
<td>69</td>
</tr>
</tbody>
</table>

Choleragen (50 µg in 10 µl) was incubated with or without gangliosides, as indicated, in a final volume of 0.15 ml for 15 min at 23°C. Assay reaction mix (0.15 ml) was then added and incubation continued for 60 min at 37°C. The final concentrations of reactants were 200 mM potassium phosphate (pH 7.0), 20 mM dithiothreitol, and 2 mM [carboxyl-¹⁴C]NAD (48,000 cpm). Means of values from duplicate determinations are reported.
Table 2. Inhibition by GM₁ of NAD hydrolysis by choleragen and A protomer

<table>
<thead>
<tr>
<th>Ganglioside added</th>
<th>NADase activity (nmol/min-mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Choleragen</td>
</tr>
<tr>
<td>None</td>
<td>12.2 (100)*</td>
</tr>
<tr>
<td>GM₁, 48 μM</td>
<td>6.3 (52)</td>
</tr>
</tbody>
</table>

Choleragen (50 μg in 10 μl) or A protomer (12.9 μg in 30 μl) was incubated with or without GM₁ for 20 min at 23° in a volume of 0.14 ml. Assay reaction mix (0.16 ml) was added, bringing the final concentrations of reactants to 2 mM [carbonyl-14C]NAD (40,000 cpm), 20 mM dithiothreitol, and 200 mM potassium phosphate, pH 7.0. Assay mixtures were incubated for 60 min at 37°. Means of values from duplicate assays are reported.

* NADase activity as percentage of control in the absence of ganglioside.

Clearly the most potent (Table 3). The Kᵢ for adenine (3 mM) was similar to the Kᵢ for NAD determined previously (17). Substitutions at the N-7 position of the purine ring, while increasing the structural similarity to NAD, clearly reduced the effectiveness of the compound as an inhibitor. The Kᵢ for adenosine was 4.5 times greater than that for adenine; the Kᵢ values for AMP, ADP, and ADP-ribose were an order of magnitude greater than that for adenine. Nicotinamide, although lacking the charge on the ring nitrogen, also inhibited the NADase activity of choleragen. Nicotinamide mononucleotide, which, like NAD, has a positively charged nitrogen linked to ribosylphosphate, was clearly less effective than nicotinamide as an inhibitor.

As might be expected from the potency of adenine as an inhibitor, substitutions on the purine ring were critical in determining the ability of derivatives to inhibit the NADase activity (Table 4). When compared at concentrations of 20 mM, AMP was a more effective inhibitor than GMP. IMP and CMP did not significantly inhibit the NADase activity.

NADP, at a concentration of 20 mM, inhibited the hydrolysis of NAD (Table 4). When 2 mM [carbonyl-14C]NADP was substituted for NAD in the standard assay, the amount of [carbonyl-14C]nicotinamide formed was less than 1% that observed with [carbonyl-14C]NAD.

**DISCUSSION**

We previously found that choleragen and its A protomer can catalyze the hydrolysis of NAD to ADP-ribose and nicotinamide (17). By analogy with the reactions catalyzed by diphtheria toxin (18), we proposed that choleragen activation of adenylate

Table 3. Competitive inhibition of NAD hydrolysis by adenine and nicotinamide derivatives

<table>
<thead>
<tr>
<th>Addition</th>
<th>Kᵢ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenine</td>
<td>3</td>
</tr>
<tr>
<td>Adenosine</td>
<td>14</td>
</tr>
<tr>
<td>AMP</td>
<td>38</td>
</tr>
<tr>
<td>ADP</td>
<td>34</td>
</tr>
<tr>
<td>ADP-ribose</td>
<td>60</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>42</td>
</tr>
<tr>
<td>Nicotinamide mononucleotide</td>
<td>160</td>
</tr>
</tbody>
</table>

Reaction mixtures contained 400 mM potassium phosphate, pH 7.0, 20 mM dithiothreitol, and [carbonyl-14C]NAD (48,000 cpm), ranging in concentration from 0.6 to 6.0 mM (seven concentrations). A minimum of four concentrations of inhibitor were added for each concentration of NAD. Reactions were initiated with 0.05 mg of choleragen (final volume 0.3 ml), and assay mixtures were incubated for 90 min at 37°. Lineweaver-Burk plots (1/activity against 1/substrate at constant inhibitor concentration) were used to calculate the Kᵢ and determine the type of inhibition.
cyclase probably proceeded through an ADP-ribosylated intermediate (17). In the studies reported here, NAD hydrolysis by choleragen was competitively inhibited by analogues of NAD. The relative potencies of these inhibitors are similar to those noted previously with diphtheria toxin (18). Adenine was the most effective inhibitor, followed by adenosine; AMP, ADP, and ADP-ribose were clearly less effective. Similarly, nicotinamide was a more potent inhibitor than nicotinamide mononucleotide. Choleragen differs from diphtheria toxin, however, in that the $K_m$ for NAD hydrolysis by the A protomer was more than two orders of magnitude higher than the $K_m$ for NAD obtained with the A fragment of diphtheria toxin. In addition, the $K_i$ values for the substrate analogues were 1–2 orders of magnitude higher with choleragen than with diphtheria toxin. These quantitative differences could be related to the higher salt concentrations used in our assays. NADP, which cannot serve as a substrate for diphtheria toxin (18), was hydrolyzed by choleragen at less than 1% the rate of NAD. NADP in high concentrations did inhibit the hydrolysis of NAD by choleragen, suggesting that it may gain access to the catalytic site, but not be properly positioned for the disruption of the ribosyl nicotinamide bond. It appears that there are marked similarities between substrate and inhibitor specificities of the catalytic sites of choleragen and those of diphtheria toxin.

Gangliosides, particularly $G_{M_1}$, have been reported to alter the fluorescence of choleragen, presumably through interaction with the B protomer (24). In our studies, $G_{M_1}$, but not $G_{M_2}, G_{M_3}$ or $G_{D_1A_4}$, caused a change in the fluorescence spectrum of the isolated B protomer, which is interpreted as indicative of a shift of the tryptophanyl residue(s) to a less polar environment. The interaction of the B protomer with $G_{M_1}$, which is accompanied by a "blue shift" in fluorescence, may, when choleragen binds to $G_{M_1}$ on a cell surface, promote dissociation of the A protomer or facilitate its interaction with other lipids in the plasma membrane or both. The results of the fluorescence studies are consistent with other evidence (3, 26–28) that the determinants of the specificity of $G_{M_1}$ as the choleragen receptor reside in the carbohydrate moiety of the ganglioside and that this portion of the molecule is involved in its interaction with the B protomer of choleragen.

It can be inferred from the fluorescence spectrum of the isolated A protomer ($\lambda_{max} = 328$ nm) that the tryptophanyl residues were located in a nonpolar environment. Therefore, additional hydrophobic interactions involving the tryptophanyl residues of the A protomer would not be expected to cause a "blue shift" in the fluorescence spectrum. The fluorescence intensity of the A protomer, however, determined under the conditions used for NADase assay, was modified by gangliosides $G_{M_1}, G_{M_2}, G_{M_3}$, and $G_{D_1A_4}$. The NADase activities of choleragen and of the A protomer were inhibited by $G_{M_1}, G_{M_2}, G_{M_3}$, and $G_{D_1A_4}$ were somewhat less inhibitory. Under the conditions in the NADase assay, choleragen is believed to be dissociated into A and B protomers and the A protomer further dissociated (due to the presence of dithiothreitol) into $A_1$ and $A_2$ subunits. The evidence that NADase activity which comigrates with the

### Table 4. Inhibition of NAD hydrolysis by substrate analogues

<table>
<thead>
<tr>
<th>Addition</th>
<th>NADase activity (nmol/min-mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>31.8</td>
</tr>
<tr>
<td>Adenine (5 mM)</td>
<td>14.5</td>
</tr>
<tr>
<td>AMP (20 mM)</td>
<td>21.3</td>
</tr>
<tr>
<td>GMP (20 mM)</td>
<td>27.4</td>
</tr>
<tr>
<td>IMP (20 mM)</td>
<td>31.0</td>
</tr>
<tr>
<td>CMP (20 mM)</td>
<td>30.9</td>
</tr>
<tr>
<td>NADP (10 mM)</td>
<td>33.8</td>
</tr>
<tr>
<td>(20 mM)</td>
<td>21.5</td>
</tr>
<tr>
<td>(50 mM)</td>
<td>12.7</td>
</tr>
</tbody>
</table>

Assay mixtures contained 400 mM potassium phosphate, pH 7.0, 20 mM dithiothreitol, 2 mM [carboxyl-14C]NAD (42,000 cpm), and the indicated additions in a final volume of 0.3 ml. Reactions were initiated with 0.05 mg of choleragen, and mixtures were incubated at 37° for 90 min.

A protomer and choleragen (17) was present in a preparation of A protomer, which exhibited one band on polyacrylamide gel electrophoresis, and was inhibited by gangliosides at concentrations that caused changes in the fluorescence intensity, suggests that the A protomer, not a contaminant, is indeed responsible for the observed NAD hydrolysis.

The effects on fluorescence and catalytic activity of the A protomer are apparently somewhat less ganglioside-specific than the $G_{M_1}$-B protomer association and could be the result of nonspecific hydrophobic lipid-protein interactions. It is difficult, however, from these studies to draw any firm conclusions concerning molecular mechanisms since the behavior of gangliosides in aqueous solution is complex. At high ganglioside concentrations (about 100 μM) micelles are formed, which dissociate only slowly on dilution (29). In contrast to an ideal system where monomers exist in a rapidly reversible equilibrium with micelles, aqueous solutions of gangliosides also contain small ganglioside complexes, e.g., dimers and trimers (29), and gangliosides in any or all of these forms could interact with proteins in differing ways.

Cuatrecasas and coworkers (10, 15, 16) have proposed that activation of adenylate cyclase is a direct result of the interaction of choleragen (or a portion of it) with the enzyme protein. We believe it more probable, however, that cyclase activation by choleragen is the consequence of an enzymatic reaction, as suggested earlier by Gill and King (8). The NADase activity (Eq. 1) exhibited by the A protomer of choleragen, like that of fragment A of diphtheria toxin (18), represents transfer of the ADP-ribose moiety of NAD to water. In this reaction water substitutes for

\[
\text{NAD + water} \xrightarrow{\text{(choleragen) A protomer}} \text{ADP-ribose + nicotinamide}
\]

[1]

NAD + protein acceptor

\[
\xrightarrow{\text{A protomer}} \text{ADP-ribose-protein acceptor + nicotinamide}
\]

[2]

the natural acceptor molecule, which, in the case of diphtheria toxin, is elongation factor II (19). We have not identified a natural protein acceptor for ADP-ribose in the reaction cata-

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5. Choleragen can bind to liposomes containing $G_{M_1}$ and cause the release of trapped glucose from the liposomes (ref. 25; J. Moss, R. L. Richards, C. R. Alving, and P. H. Fishman, J. Biol. Chem., in press). The B protomer is as effective as choleragen in releasing trapped glucose, whereas the A protomer is ineffective; the A protomer but not the B protomer, however, can bind to ganglioside-free liposomes (J. Moss et al., J. Biol. Chem., in press). These results are consistent with the hydrophobic nature of the A protomer (16) and its ability to interact with biological membranes.

lyzed by cholera, but expect that it is adenylate cyclase or a protein component of a cyclase complex that regulates enzymatic activity.

We thank Miss Sally Stanley for her expert assistance.