Stereochemistry of the ADP-Ribosylation Catalyzed by Pertussis Toxin

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Received August 23, 1995

Bacterial toxins including cholera toxin, pertussis toxin, and diphtheria toxin catalyze the transfer of the ADP-ribose moiety from NAD^+ to a specific side chain in an acceptor protein.[1] The substrate of diphtheria toxin is the diphthamide residue of the eukaryotic elongation factor EF2,[2] and the α-subunits of several G-proteins are acceptors for the other toxins. Cholera toxin ADP-ribosylates an arginine residue of $G_{s\alpha}$,[3] and pertussis toxin ADP-ribosylates a cysteine residue, four amino acids from the C-terminus of $G_{i\alpha}$, $G_{o\alpha}$, and $G_{t\alpha}$.[4,5] Because all of these toxins are involved in the pathochemistry of infectious diseases, there is an interest to develop specific inhibitors against them. Inhibitor design requires knowledge of the enzymatic mechanism of the toxins and the stereochemistry of the products. The ADP-ribosylation catalyzed by cholera[6] and diphtheria toxins[7] occurs with inversion of the configuration at the 1′-carbon of the ribosyl ring. The resulting products from the reaction with the nucleophiles arginine and diphthamide, respectively, have all α-configuration. The stereochemistry of the reaction catalyzed by pertussis toxin is unknown.

Pertussis toxin has been reported to catalyze the transfer reaction between NAD^+ and free cysteine,[8] but McDonald et al.[9] demonstrated that the product of this reaction is a ribosylthiazolidine compound and not the expected thioglycoside. The thiazolidine forms as the nonenzymatic reaction product of cysteine and ADP-ribose, when ADP-ribose accumulates as the product of NAD^+ glycohydrolase activity.[10] There is no evidence that other low molecular weight thio compounds like *N*-acetyl cysteine, glutathione, or dithiothreitol can act as acceptors for the toxin. Recently, however, a synthetic peptide has been found to be a substrate for pertussis toxin.[11] This peptide corresponds to the last 20 amino acids of the $G_{i3\alpha}$ protein (Figure 1). High reactivity and ready synthetic access make the α_{i3}C20 peptide a suitable model compound to establish the stereochemistry of the pertussis toxin reaction. The experiments were performed using the A protomer of pertussis toxin, which is the catalytically active subunit.

Val-Phe-Asp-Arg-Val-Thr-Asp-Val-Ile-Ile-
5 10
Lys-Asn-Asn-Leu-Lys-Glu-**Cys**-Gly-Leu-Tyr
15 20

Figure 1. Amino acid sequence of the synthetic peptide α_{i3}C20. The peptide corresponds to the 20 C-terminal amino acids of the α-subunit of the G_{i3} protein. The boldface Cys 17, corresponding to Cys 391 in the protein sequence, is the target for ADP-ribosylation catalyzed by pertussis toxin.

The peptide α_{i3}C20 was synthesized by solid phase methods, purified by HPLC, and analyzed by mass spectroscopy.[12] Samples of pertussis toxin A protomer (6 μg/mL), NAD^+ (500 μM), α_{i3}C20 peptide (1 mM), dithiothreitol (20 mM), bovine serum albumin, and CHAPS (both 1%) were incubated at 4 °C for 72 h in 100 mM potassium phosphate, pH 7.5. The products were purified on DEAE Sephadex, eluting with a step gradient of ammonium bicarbonate, pH 8.0. Unreacted peptide eluted at a concentration of 150 mM, and the reaction product eluted at 1 M salt. The product was desalted on a Sephadex G-10 column and lyophilized. The peptide was ADP-ribosylated by pertussis toxin to a maximum extent of approximately 30% under these conditions. Longer incubation and higher NAD^+ concentrations did not increase the yield. The reaction was performed at 4 °C. Incubation at 37 °C resulted in the precipitation of the peptide and inactivation of the toxin. Similar results were recently reported by Barbieri and co-worker.[13] Incubation at room temperature resulted in substantial NAD^+-glycohydrolase activity, whereas at 4 °C no significant amount of ADP-ribose was detectable. Successful ADP-ribosylation was demonstrated by mass spectroscopy. The mass of the reaction product was 2796 Da (calcd: 2795 Da), whereas the peptide's mass is 2253 Da. The difference of 542 Da corresponds to the mass of the ADP-ribosyl residue. Treatment of the product with 2 mM $HgCl_2$ resulted in the release of ADP-ribose within 30 min as shown by HPLC, whereas the product was stable in the presence of hydroxylamine for several hours. This behavior is characteristic for thioglycosides[14] and confirmed that the ADP-ribose is linked to the cysteine thiol.

The configuration at the 1′-carbon of the ribosyl was established by proton NMR spectroscopy. To simplify the spectra, the ADP-ribosylated peptide was degraded by enzymatic hydrolysis using protease XVII from *Staphylococcus aureus* and carboxypeptidase A as summarized in Figure 2.[15] The nonspecific proteases pronase or proteinase K resulted in a mixture of ADP-ribosylated peptide fragments. The procedure with protease XVII and carboxypeptidase A resulted in only one product at each reaction step. The pure final product could be isolated by HPLC using DEAE anion exchange.[15] A molecular mass of 720 Da was determined by mass spectroscopy. This corresponds to an ADP-ribosylated dipeptide Cys-Gly.

The ADP-ribosyl peptide was lyophilized two times in D_2O, dried over P_2O_5, and dissolved in D_2O. Proton NMR spectra were measured at 500 MHz with solvent suppression. All

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(12) The mass of the peptide was determined to be 2250 Da (calcd: 2253). HPLC analysis showed about 80% purity.

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(15) ADP-ribosylated peptide was prepared as described. Protease XVII from *S. aureus* (10 units/mg of peptide) was added, and the mixture was incubated at 37 °C for 2 h and purified on a RP-18 Nucleosil HPLC column (7.8 × 300 mm), using a linear gradient of 50 mM ammonium acetate and methanol. Detection was at 260 nm. The ADP-ribosylated peptide fragment eluted at 25% methanol. The product was lyophilized, dissolved in 50 mM potassium phosphate, pH 7.5, and incubated with carboxypeptidase A (1 unit) at 37 °C for 1 h. The mixture was purified on a NUCLEON DEAE 60-7 HPLC column (Macherey Nagel) using a linear gradient of 100−500 mM ammonium acetate, pH 7.0. The ADP-ribosylated product eluted at 200 mM ammonium acetate.

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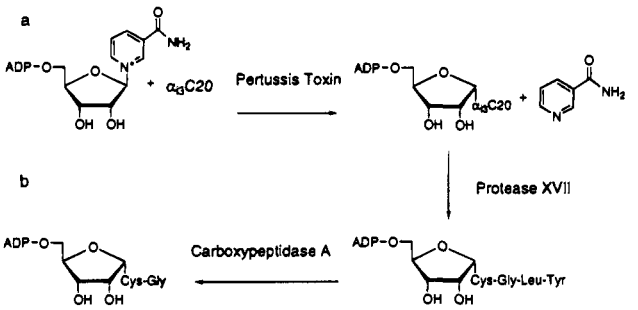

Figure 2. (a) Reaction catalyzed by pertussis toxin A subunit. The product is shown as ADP-D-ribofuranosyl-α-(α_{i3}C20). (b) Enzymatic degradation by protease XVII and carboxypeptidase A yielding ADP-D-ribofuranosyl-α-Cys-Gly.

Table 1. ^{1}H NMR Data of Cys(ADPR)-Gly[a]

proton	signal (ppm)[b]	NOE to proton
A 8	8.516	
A 2	8.269	
A 1′	6.149	(A 2′), A 4′
A 2′	4.794	(A 1′), (A 3′)
A 3′	4.556	(A 2′), A 4′, A 5′, A 5″
A 4′	4.443	A 3′, A 5′, A 5″
A 5′	4.254	A 3′, A 4′
A 5″	4.254	A 3′, A 4′
R 1′	5.429	R 2′, R 3′, R 5′, Cys β
R 2′	4.310	R 1′, R 3′
R 3′	4.226	R 1′, R 2′, R 4′, R 5′, R 5″
R 4′	4.168	R 3′, R 5″
R 5′	4.168	R 1′, R 3′, R 5″
R 5″	4.052	R 4′, R 5′
Cys α	4.084	Cys β, Cys β′
Cys β	3.147	Cys α, Cys β′, R 1′
Cys β′	3.070	Cys α, Cys β
Gly α	3.835	Gly α′
Gly α′	3.735	Gly α

[a] A: ADP moiety. R: ribosyl moiety. [b] Spectrum measured in D_2O; calibrated on the HDO signal (4.8 ppm).

signals could be assigned by the COSY spectrum (Table 1). The resonance signal of the 1′-proton of the ribosyl moiety is a doublet at 5.429 ppm, and the coupling constant is 3.9 Hz. The shift of the 1′-H of the ADP moiety is 6.149 ppm, with coupling constant 5.8 Hz. This value and also the shifts from the other signals of this moiety are in agreement with data reported earlier by Oppenheimer[6] for the cholera toxin transfer products. Comparison of the coupling constants of the 1′-protons provides an indication for the α-configuration at the ribosyl ring. The coupling constants of the α-diastereomers of related compounds had values around 4 Hz, whereas the values of the β-diastereomers were between 5 and 6 Hz.

Additional evidence for the configuration was provided by 2D NOE spectroscopy (NOESY).[16] The spectrum (Figure 3) shows a strong NOE between the 1′-H and the 2′-H (4.310 ppm) of the ribosyl. Weaker NOEs could be observed between the

(16) NOESY spectra were performed at 500 MHz in the phase-sensitive mode. Parameters: increments, 215; number of scans, 128; relaxation delay, 1.5 s; mixing time, 1.5 s; 90° pulse, 11.2 μs.

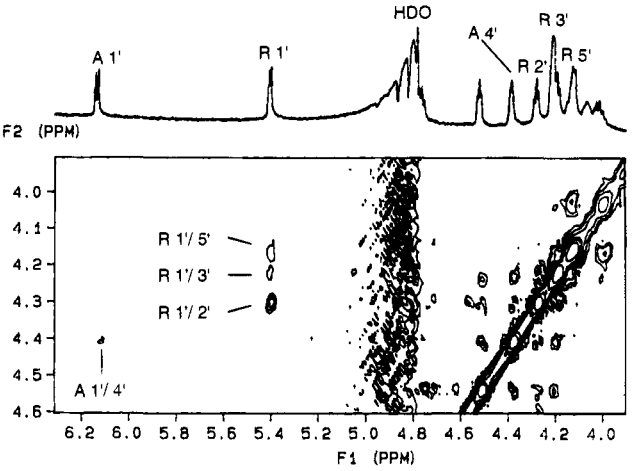

Figure 3. ^{1}H,^{1}H NOESY spectrum[16] of ADP-ribosylated Cys-Gly in D_2O. The spectrum shows the section with the cross peaks that were used to assign the configuration of the compound. A: ADP moiety. R: ribosyl moiety.

1′-H and the 3′-H (4.226 ppm) and the signal at 4.168 ppm. Through-space coupling between the 1′- and 3′-protons is possible if they are in a *cis* configuration. This is the case when the configuration at the 1′-carbon is α. The signal at 4.168 ppm represents the 4′- and 5′-protons of the ribosyl moiety. An NOE between the 1′- and 4′-protons would be consistent with a β-configuration. However, this would rule out the NOE between the 1′- and 3′-protons. We can conclude that this NOE is the result of dipolar coupling between the 1′- and one of the 5′-protons, and therefore the product must have an α-configuration. In the adenine moiety the ribose ring has a β-configuration, and only the NOE between the 1′-H and the 4′-H is detectable.

The results show that ADP-ribosylation catalyzed by pertussis toxin occurs with inversion of configuration at the 1′-carbon of the ribosyl moiety of the ADP-ribose. The stereochemistry is therefore the same as found for the bacterial ADP-ribosylating agent from cholera toxin,[6] diphtheria toxin,[7] and *Escherichia coli* enterotoxin.[17] The ADP-ribosylating toxins differ in the stereochemistry of their mechanism from NAD^+ glycohydrolase from calf-spleen[18] and pig brain,[19] which has been shown to catalyze retention of configuration. In this sense, the NAD^+ *N*-ribohydrolase/transferase family is similar to the glucosidases which include both retaining and inverting *O*-glucoside transfers.[20]

Acknowledgment. This work was supported by Research Grant AI34342 from the National Institutes of Health. The authors wish to thank Dr. Terry Dowd and Dr. Mark Girvin for their help in questions of NMR spectroscopy and Dr. Xuejun Tang for his help with mass spectrometric measurements.

JA952898B

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