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Peptide Inhibitors of ADP-Ribosylation by Pertussis Toxin Are Substrates with Affinities Comparable to Those of the Trimeric GTP-Binding Proteins

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SUMMARY

Pertussis toxin (PTX) ADP-ribosylates α subunits of GTP-binding proteins (G proteins) when they are in association with $\beta\gamma$ dimers, and free α subunits are thought not to be substrates under standard assay conditions. We now report the rather unexpected discovery that synthetic peptides encompassing the last 10–20 amino acids of α subunits of PTX-sensitive G proteins are substrates for PTX by themselves and in the absence of $\beta\gamma$ dimers. As determined for G_{i3}, the K_m of PTX for the 20-amino acid carboxyl-terminal peptide is 10-fold higher than that for the trimeric G protein. Interestingly, PTX ADP-ribosylates the free full length α_{i3} subunit with a K_m not different from that of the trimer but with a V_{max} that is only 1% of that with which it ADP-ribosylates the trimer. It follows that the primary role of $\beta\gamma$ dimers in ADP-ribosylation of G proteins is one of increasing the V_{max} of the reaction without affecting the K_m of the substrate for the toxin. Mutant peptides lacking the ADP-ribose acceptor site act as competitive inhibitors.

Whooping cough is an infectious disease caused by *Bordetella* pertussis. It is accepted that most of the clinical symptoms are due to the activity of PTX (also termed islet-activating protein), which is the major exotoxin of this bacterium (1, 2). PTX affects cellular metabolism by ADP-ribosylating, and thereby uncoupling from their receptors, a subclass of heterotrimeric G proteins (3–5). The G protein subclass includes three types of G_i, two types of G_o, and transducin; there may be more. On the basis of studies with the G protein transducin, it has been proposed that the site of ADP-ribosylation is a cysteine at position −4 from the carboxyl terminus of the α subunits of these proteins (6).

It is becoming increasingly apparent that synthetic fragments of complex proteins may mimic functional aspects of the domain from which they derive, especially if they are part of domains that define protein-protein interactions. Examples of functional mimicry of this kind are fragments of autoinhibitory domains of protein kinases that act as inhibitors, such as calmodulin kinase-281–302, myosin light chain kinase-480–501, and protein kinase C-19–36 (reviewed in Ref. 7); fragments of inhibitory proteins that mimic the inhibitory action of the parent protein, such as the protein kinase inhibitor peptide that blocks the active state of the catalytic subunit of the cAMP-dependent protein kinase (8); and fragments of proteins that block interaction of the G proteins with their upstream or downstream regulatory elements, such as transducin-311–328, which impedes formation of *meta*-rhodopsin, the form that light-activated rhodopsin adopts upon interaction with transducin (9), and the *rab* "effector region" peptide, which blocks vesicular protein transfer (10). Examples of this kind include also peptides that act as artificial substrates and simple competitive inhibitors of enzyme substrate sites, such as CAAX box-containing peptides that block farnesyl-PP:CAAX-protein farnesyl transferase where CAAX denotes a carboxyl terminus of composition cystein-(alyphatic amino acid)$_2$-any amino acid) (11) and peptides that, rather than blocking, mimic the function of the domain of origin, such as the 15-amino acid peptide derived from the carboxyl-terminal end of the third intracellular loop of the human β_2-adrenergic receptor (12).

In the studies reported below we have investigated whether synthetic peptides encompassing the site of G protein α subunits that is thought to be ADP-ribosylated by PTX would interfere with ADP-ribosylation. Although this was indeed found to be the case, we also found that these peptides are themselves substrates. This is surprising, because in the past

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ABBREVIATIONS: PTX, pertussis toxin; α_x, α subunit of the G_x GTP-binding protein; G protein, heterotrimeric GTP-binding protein of $\alpha\beta\gamma$ subunit composition; TCA, trichloroacetic acid; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

we (13) as well as others (e.g., Ref. 14) have assumed, on the basis of original work with purified brain G protein α subunits (15), that α subunits are substrates for PTX only after they combine with $\beta\gamma$ dimers, i.e., when in their heterotrimeric state. In addition, the results extend the experimental basis for the proposal, based previously only on sequence similarity between PTX-sensitive α subunits and the PTX-sensitive transducin α subunit, that the cysteine at position −4 of the carboxyl terminus is the site ADP-ribosylated by PTX in the α subunits of G_i and G_o proteins.

Materials and Methods

[^{32}P]NAD was obtained at 200–400 Ci/mmol from the Molecular Endocrinology Core Laboratory of the Baylor College Diabetes and Endocrinology Research Center, where it was synthesized according to the method of Cassel and Pfeuffer (16) from [α-^{32}P]ATP, prepared in turn according to the method of Walseth and Johnson (17).

Synthetic peptides (Fig. 1) were prepared by the Baylor College of Medicine Protein Sequencing and Peptide Synthesis Facility, headed by Dr. Richard Cook, using an automated Applied Biosystems peptide synthesizer. The primary structure of the products was confirmed by analysis of their amino acid composition. The purity of the final products was >95%, as seen by high pressure liquid chromatography on a Waters C18-μBondapac column eluted with an acetonitrile/trifluoroacetic acid gradient.

ADP-ribosylations of G protein α subunits were carried out in 0.5-ml conical polypropylene (Eppendorf) tubes in a darkened laboratory. Unless specified otherwise, incubations were for 30 min at 32° in 15 μl containing 10 μg/ml activated PTX (obtained by pretreatment at 250 μg/ml for 30 min at 32° with 50 mM dithiothreitol, 1 mM adenylylimidodiphosphate, and 0.05% BSA, 1 μM [^{32}P]NAD (~2 × 10^6 cpm), 15 nM recombinant human α_{i3} plus 15 nM bovine brain $\beta\gamma$ dimers or 15 nM each of purified human erythrocyte G_{i2} and G_{i3}, 0.04 mM adenylylimidodiphosphate, 1 mM ATP, 100 μM guanosine 5′-(2-thio)diphosphate, 2 mM dithiothreitol, 0.2% Lubrol PX, 0.02% BSA, 10 mM thymidine, 15 mM Tris·HCl, pH 8.0, and 2.5 μl of 0.1 M NH_4OH, without or with peptides as indicated. The reactions were stopped by addition of an equal volume of 2 × concentrated Laemmli sample buffer (18) and were subjected without further treatment to urea gradient SDS-PAGE (19, 20) to separate the ADP-ribosylated α subunits from the reaction reagents. The gel slabs were stained with Coomassie blue to confirm equal loading of the samples, dried, and autoradiographed to quantify ADP-ribosylation.

Incorporation of [^{32}P]ADP-ribose from [^{32}P]NAD into α_{i3}C20 peptides was determined by a filtration assay, as follows. Peptides were incubated as described in the previous paragraph, with the following changes: the final volume was 10 μl, the [^{32}P]NAD concentration was 20 μM (5 × 10^6 cpm/10-μl reaction volume), and incubations were for 3 hr at 4°. The reactions were terminated by diluting replicate 2.5-μl aliquots in 12- × 100-mm glass test tubes with 2 ml of 10% TCA containing 0.5 μg/ml unlabeled α_{i3}C20 peptide. After 40 min on ice, the samples were filtered through 25-mm Schleicher & Schüll BA-85 nitrocellulose filters that had been presoaked in 1 mM NAD, using a 12-place Millipore filtration manifold. The tubes were rinsed twice with 2 ml of 10% TCA and the filters were washed twice with 5 ml of 10% TCA. The filters were then soaked in 3.5 ml of liquid scintillation fluid for 30 min and counted in a refrigerated (10°) Beckman LSC-1800 liquid scintillation counter. Background counts were between 0.005 and 0.010% of total counts added.

Recombinant (human) α_{i3} protein was synthesized by *Spodoptera frugiperda*-9 (Sf9) cells infected with a recombinant *Autographa californica* polyhedrosis virus (baculovirus) in which the open reading frame of the polyhedrin gene had been replaced by the open reading frame of human $G_{i3}\alpha$ (21, 22) by using the procedures of Summers and collaborators (23, 24). The recombinant protein was then purified to >90% purity (25).[2] Human erythrocyte G_{i2} and G_{i3} and bovine brain $\beta\gamma$ dimers were purified as described previously (26–28). Reconstitution

A.

α Subunit	-20	-15	-10	PTX	Last aa #
G_s	V F N D C R D I I Q R M H L R Q Y E L	L			-394
G_{i1}	V F D A V T D V I I K N N L K D C G L	F			-355
G_{i2}	V F D A V T D V I I K N N L K D C G L	F			-354
G_{i3}	V F D A V T D V I I K N N L K E C G L	Y			-354
G_{o1}	V F D A V T D I I I A N N L R G C G L	Y			-354
G_{o2}	V F D A V T D V I I A K N L R G C G L	Y			-354
G_{oX1}	V F D A V T D V I I A K N L R G C G L	Y			-354
T_{rod}	V F D A V T D I I I K E N L K D C G L	F			-350
G_z	V F D A V T D V I I Q N N L K Y I G L	C			-355
$G_{q/11}$	V F A A V K D T I L Q L N L K E Y N L	V			-359

B. Gα

Peptide	-20	-15	-10	-5	Soluble
α_{i3}C20	V F D A V T D V I I K N N L K E C G L Y				Yes
α_{i3}C20(C->S)	V F D A V T D V I I K N N L K E S G L Y				Yes
α_s C20(Y->C)	V F N D C R D I I Q R M H L R Q C E L L				Yes
α_s C20(QYE->DCG)	V F N D C R D I I Q R M H L R D C G L L				Yes
α_{oX1}C20	V F D A V T D V I I A Y N L R G C G L Y				Yes
α_{i3}C15	T D V I I K N N L K E C G L Y				Yes
α_{i1}C15/α_{i2}C15	T D V I I K N N L K D C G L F				Yes
α_{o1}C15	T D I I I A N N L R G C G L Y				Yes
α_{o2}C15	T D V I I A K N L R G C G L Y				No
α_{oX1}C15	T D V I I A Y N L R G C G L Y				No
α_{i3}C10	K N N L K E C G L Y				Yes

C.

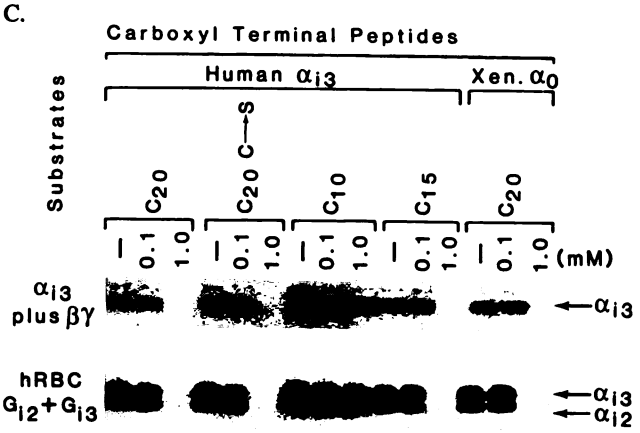

Fig. 1. Inhibition by α subunit carboxyl-terminal peptides of PTX-mediated ADP-ribosylation of trimeric G proteins. A, Carboxyl-terminal amino acid composition of G protein α subunits. Sequences were derived from published nucleotide sequences of their respective cDNAs. For definition of $G_s\alpha$, $G_{i1}\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, $G_{o1}\alpha$, $T_{rod}\alpha$ (also termed rod cell $G_t\alpha$), and $G_z\alpha$, see Lochrie and Simon (30) and Birnbaumer (25). $G_{o2}\alpha$ is from Hsu *et al.* (31), *X. laevis* G_o ($G_{oXI}\alpha$ or *Xen.* α_o) is from Olate *et al.* (32), and $G_q\alpha$ and $G_{11}\alpha$ are from Strathman and Simon (33). *Box* encloses sequences identical to that of $G_{i3}\alpha$; *bold-type C*, cysteine at position −4 that has been determined in transducin α (G_t) to be the site of ADP-ribosylation by PTX (6). B, Synthetic peptides prepared for the studies in this report. Peptides were custom synthesized by the Protein Sequencing and Peptide Synthesis Facility of Baylor College of Medicine, under the direction of Dr. Richard A. Cook. All were analyzed for amino acid composition. The structures of α_{i3}C20, α_{i3}C20 (C→S), α_{i3}C15, and α_{i2}C15 were confirmed further by sequencing. Boxes highlight location of amino acid substitutions. *Soluble*, denotes whether or not it was possible to prepare 6 mM stock solutions in 0.1 M NH_4OH. All peptides were insoluble in 10% TCA. C, Effect of peptides on PTX-mediated ADP-ribosylation of reconstituted and native trimeric G proteins. Synthetic peptides were added at 0.1 and 1 mM to reactions in which trimeric G proteins were [^{32}P]ADP-ribosylated by PTX in the presence of [^{32}P]NAD. The reaction products were analyzed by urea gradient SDS-PAGE as described in Materials and Methods. The figure shows photographs of the areas of the autoradiograms that correspond to the ADP-ribosylated α subunits. hRBC, human red blood cell.

[2] R. Graf, R. Mattera, J. Codina, M. K. Estes, and L. Birnbaumer. A truncated form of recombinant α subunit of G_{i3}: reduced affinity for $\beta\gamma$ dimer and guanine nucleotide. Submitted for publication.

of trimer with recombinant α_{i3} and bovine brain $\beta\gamma$ was obtained by coincubating 1 μM levels of each for 30 min at 4° in the presence of 10 mM Na-HEPES, pH 8.0, 1 mM EDTA, 20 mM β-mercaptoethanol, 0.1% Lubrol PX, 200 mM NaCl, 0.05% BSA, and 30% ethylene glycol.

Results and Discussion

Fig. 1A shows most of the known carboxyl-terminal amino acid sequences of G protein α subunits, including those that are known to be PTX substrates, as deduced from their respective cDNAs (29–33). For our initial experiments, we synthesized peptide α_{i3}C20, composed of the last 20 amino acids of the α subunit of the G_{i3} protein, and added it to reactions in which we ADP-ribosylated human erythrocyte G_{i3}. ADP-ribosylation of the G protein was carried out in the presence of ^{32}P-labeled NAD, the donor of the [^{32}P]ADP-ribose moiety, and we visualized the covalent modification of the protein by autoradiography after separation of the reaction products by SDS-PAGE. We found that α_{i3}C20 inhibited in a concentration-dependent manner the incorporation of ADP-ribose into the $G_{i3}\alpha$ subunit. We next synthesized the panel of carboxyl-terminal peptides listed in Fig. 1B, in order to obtain further insight into the ability of these peptides to inhibit ADP-ribosylation of native G_{i3}, of native G_{i2}, and of a reconstituted $\alpha_{i3}\beta\gamma$ trimer. A typical result is shown in Fig. 1C. The inhibitory effect of the peptides did not discriminate between native trimers (mixture of human erythrocyte G_{i2} and G_{i3}) (22) and trimers reconstituted from α_{i3} made by the baculovirus/Sf9 cell expression system (human sequence) and native $\beta\gamma$ dimers purified from bovine brain. We found that ADP-ribosylation of all three substrates was inhibited by both a human α_{i3}C20 and a *Xenopus laevis* α_oC20 peptide and that the effectiveness of the inhibition was essentially unchanged by mutating to serine the cysteine at position −4 from the carboxyl terminus, which is the position cognate to the one that is ADP-ribosylated in the α chains of transducin (Fig. 1C). Decreasing the length of the peptide to 15 amino acids did not impair the blocking effect, but decreasing it to 10 amino acids decreased dramatically its ability to interfere with ADP-ribosylation of native and reconstituted G proteins (Fig. 1C). No noticeable inhibition of ADP-ribosylation was seen with up to 1 mM α_{o1}C15, α_{i2}C15, α_sC20 (Y→C), or α_sC20 (QYE→DCG) (for composition, see Fig. 1B), even though the latter type of replacements convert full length α_s into a substrate for PTX (34). α_{o2}C15 and α_{oXl}C15 (Fig. 1B) were difficult to solubilize under solvent conditions that did not interfere with the ADP-ribosylation reaction and were not tested further for inhibitory effects.

Upon examining the autoradiograms, we noticed that the presence of the α_{i3}C20 peptide led consistently to the appearance of a diffuse band of radioactivity near the dye front (data not shown). This led us to suspect that the C20 peptide might itself be a PTX substrate. Its insolubility in TCA (see legend to Fig. 1B) allowed us to develop a filtration assay to test directly for ADP-ribosylation of α_{i3}C20 by PTX. Indeed, incubation of α_{i3}C20 with PTX and [^{32}P]NAD led to ADP-ribosylation of the peptide itself, in the absence of any other G protein component. In addition, we found that essentially all of the peptides derived from PTX-sensitive α subunits that we could test were acceptor substrates for the ADP-ribosylatransferase activity of PTX.

The following criteria lead us to conclude that α_{i3}C20 is a *bona fide* substrate for the ADP-ribosylating activity of PTX and that the site of ADP-ribosylation is the cysteine at position −4 from the carboxyl terminus. 1) The apparent K_m for NAD for incorporation of NAD-derived radioactivity into α_{i3}C20 is 14–20 μM (three experiments; Fig. 2), which is essentially indistinguishable from the K_m of 20–30 μM determined previously by us for ADP-ribosylation of native human G_i (35) and by Moss *et al.* (36) for ADP-ribosylation of native bovine transducin. 2) Pretreatment of the α_{i3}C20 peptide with iodoacetic acid, to carboxymethylate the sulfhydryl group of the cysteine at position −4 from the carboxyl terminus, inhibited both the appearance of radioactivity near the dye front in autoradiograms and incorporation of radioactivity into TCA-precipitated α_{i3}C20. 3) Substitution of serine for the cysteine at position −4, to give α_{i3}C20 (C→S), led to loss of incorporation of radioactivity into the mutated peptide analyzed either by TCA precipitation or by SDS-PAGE plus autoradiography. 4) Addition of "mutant" α_{i3}C20 (C→S) to ADP-ribosylation reactions resulted in a concentration-dependent inhibition of incorporation of radioactivity not only into complete α_{i3} chains but also into α_{i3}C20.

Using reaction conditions that were linear as a function of time and toxin concentration, we determined the apparent K_m values and enzyme reaction velocities for the various peptides shown in Fig. 3 and Table 1. The results indicated that reduction of the length of the α_{i3} peptide made it progressively a poorer substrate, much as the ability of the peptides to block ADP-ribosylation was reduced. The loss of blocking ability might have suggested that reduction of chain length leads to reduction in potency, i.e., affinity of the peptide for the toxin. However, the kinetic analysis of Fig. 3 and Table 1 indicated that, whereas there is indeed a reduction in the apparent K_m with reduction of chain length (K_m for C20/K_m for C10 = 0.37), there is a 15–20 times more marked effect of chain length on the V_{max} with which PTX ADP-ribosylates the peptides, i.e., on the sum of the catalytic rate of transfer of the ADP-ribosyl moiety onto the cysteine plus the rate at which the reaction products exit from the catalytic site so as to allow for binding

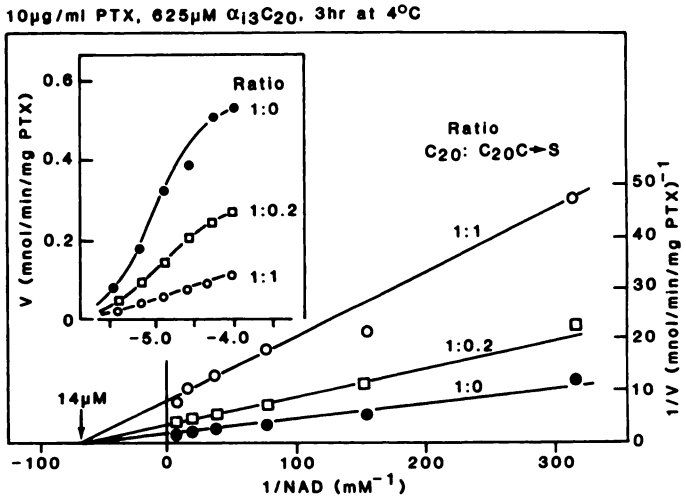

Fig. 2. Effect of varying the concentration of NAD on the rate of ADP-ribosylation of peptide α_{i3}C20 and inhibition of peptide ADP-ribosylation by α_{i3}C20 (C→S). Incubations were as described in Materials and Methods, for 3 hr at 4°, and reactions were stopped by dilution into 10% TCA. [^{32}P]ADP-ribose incorporated was quantified by collecting the insoluble reaction product on Schleicher and Schüll membranes and determining the ^{32}P by liquid scintillation counting.

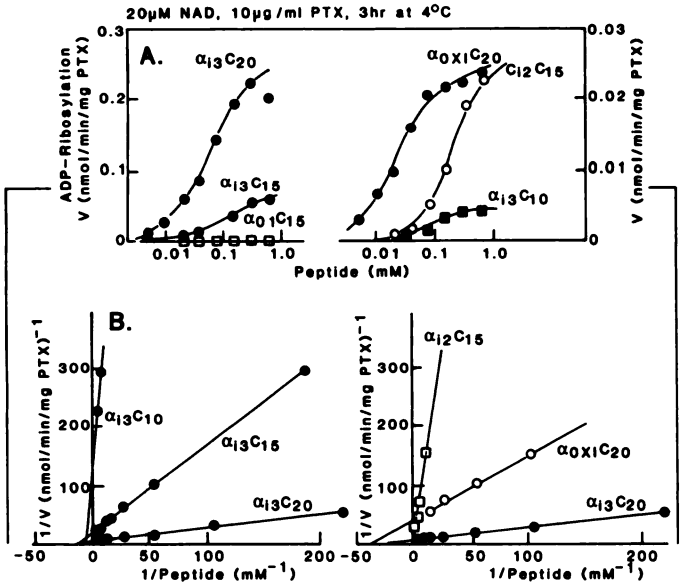

Fig. 3. ADP-ribosylation of various carboxyl-terminal peptides of G protein α subunits by PTX. A, Effect of varying the concentration of peptides on the rate of ADP-ribosylation by PTX. Note the 10-fold change in scale of *y*-axis between *left* and *right*. *B*, Lineweaver-Burk transformation of data shown in A. ADP-ribosylations were as described in Materials and Methods. The peptides are those described in Fig. 1B. Peptides α_sC20 (Y→C) and α_sC20 (DCG) showed negligible incorporation (not shown), as did peptide α_{o1}C15 (A, *left*).

TABLE 1

ADP-ribosylation by PTX of carboxyl-terminal peptides of α subunits of G proteins

ADP-ribosylation reactions were at 4° for 3 hr with 20 μM NAD and 10 μg/ml activated PTX. Peptides are those described in Fig. 1B. Apparent affinities (apparent K_m values) and V_{max} values were estimated from Lineweaver-Burk plots, such as illustrated in Fig. 3. Results are means of values obtained in three independent experiments, which agreed within 10% of the mean and of which one is represented graphically in Fig. 3.

Peptide	Apparent K_m	V_{max}
	μM	pmol/min/mg of PTX
α_{i3}C20	60	244
α_{i3}C15	120	42
α_{i3}C10	160	5.5
α_{oX1}C20	30	26
$\alpha_{i1/2}$C15	480	15

of new substrate (V_{max} for ADP-ribosylation of C20/V_{max} for ADP-ribosylation of C10 = 44).

We next compared, under the same assay conditions, the substrate ability of the α_{i3}C20 peptide with those of the full length α_{i3}, a "poor" substrate, and the reconstituted $\alpha_{i3}\beta\gamma$ trimer, a "good" substrate. Before the studies described above with peptides, we had thought intuitively that the effect of $\beta\gamma$ on ADP-ribosylation of α_{i3} by PTX was primarily to increase its binding to the toxin. This would have been seen in kinetic studies as a shift in its apparent K_m for PTX. However, we found that the primary effect of trimer formation is to change the V_{max} with which PTX ADP-ribosylates the α subunit [apparent K_m for $\alpha_{i3}\beta\gamma$/apparent K_m for α_{i3} = 0.6 (two experiments; range, 0.5 to 0.7); V_{max} for $\alpha_{i3}\beta\gamma/V_{max}$ for α_{i3} = 101 (two experiments; range, 90 to 112)] (Fig. 4; Table 2). Addition of $\beta\gamma$ to the α_{i3}C20 peptide before ADP-ribosylation was without effect (Fig. 4; Table 2). Thus, the change of V_{max} for α_{i3} seen upon trimer formation appears to be due to a change in the

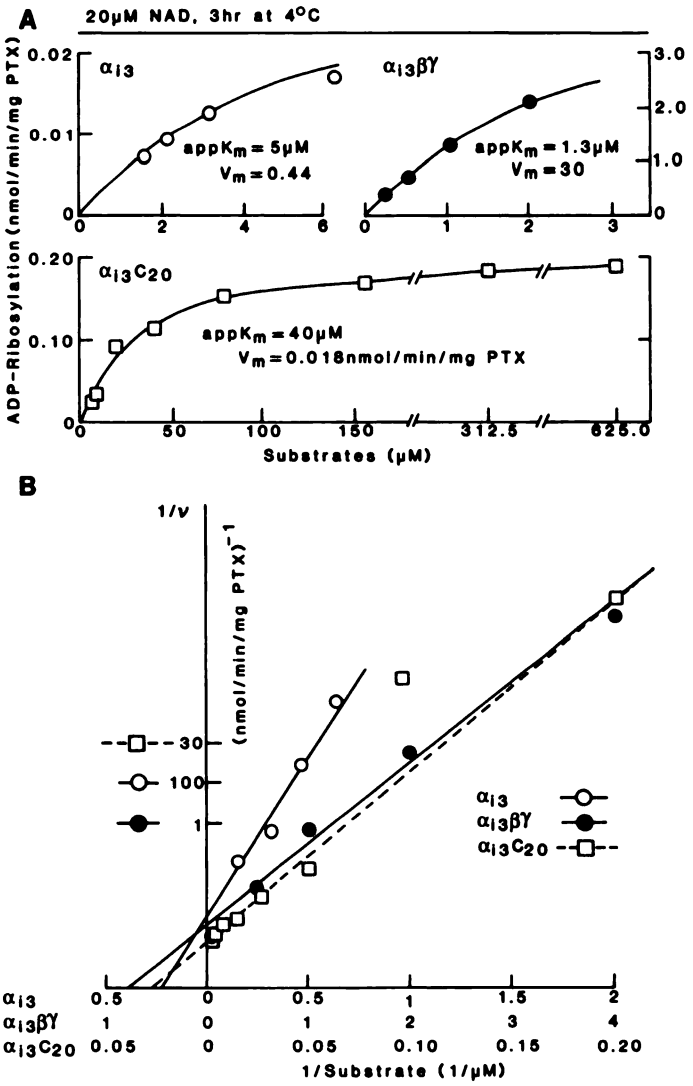

Fig. 4. Kinetic basis for the effect of $\beta\gamma$ dimers in promoting ADP-ribosylation of α subunits of G proteins. Assay conditions and quantification of ADP-ribosylation were as described in Materials and Methods. *A*, Effect of varying the concentrations of PTX substrates on their rate of ADP-ribosylation. *B*, Lineweaver-Burk transformation of the data presented in A.

TABLE 2

Comparison of ADP-ribosylation of reconstituted $\alpha_{i3}\beta\gamma$ trimer, α_{i3} monomer, and α_{i3}C20 peptide

Conditions were as for Table 1 except that NAD was 10 μM. Numbers are averages of results from two independent experiments, which agreed within 10% of the mean and of which one is presented in Fig. 4.

Substrate	Apparent K_m	V_{max}
	μM	pmol/min/mg of PTX
α_{i3} monomer	5	30.5
$\alpha_{i3}\beta\gamma$ trimer	3	3100
α_{i3}C20	50	212
α_{i3}C20 + $\beta\gamma$	45	195

conformation of α_{i3}, rather than an allosteric effect of $\beta\gamma$ on PTX that is independent of its association with α_{i3}.

In conclusion, we have shown that small peptides, of 10–20 amino acids in length, having the sequence composition of the carboxyl termini of the α subunits of PTX-sensitive G proteins are both inhibitors of the ADP-ribosylation of the G proteins

and substrates of the transferase activity of the toxin. ADP-ribosylation of the peptides by the toxin was unaffected by addition of G protein $\beta\gamma$ dimers, whereas that of a natural full length α chain, which by itself is a substrate that is ADP-ribosylated more slowly than the peptide analogs, was enhanced by interaction with $\beta\gamma$. In our studies trimer formation enhanced the rate of ADP-ribosylation by a factor of 100. A study by Cortina *et al.* (37) comparing the rates at which a recombinant S1 subunit of PTX ADP-ribosylated transducin α (α_T) and holotransducin ($\alpha_T\beta\gamma$) found a rate difference of 16-fold in favor of the heterotrimer. Interestingly, a mutant S1 subunit (C180) lacking its last 55 amino acids failed to show this difference, thus providing independent evidence for separate modes of interaction of the toxin with α and $\beta\gamma$ subunits. Comparison of the apparent affinities for PTX and the rates at which PTX ADP-ribosylated a limited number of carboxyl-terminal peptides indicates that it will be possible to determine detailed structure-activity relations and raises the possibility that such peptide analogs of PTX-sensitive α chains could be developed into inhibitors of PTX action.

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