Identification of sites for alkylation by *N*-ethylmaleimide and pertussis toxin-catalyzed ADP-ribosylation on GTP-binding proteins

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An $\alpha\beta\gamma$-trimeric GTP-binding protein (G_o) serving as the substrate of pertussis toxin- (IAP) catalyzed ADP-ribosylation was purified from rat brain membranes. The constituent α-subunit (α_o) was alkylated with *N*-ethylmaleimide (NEM), and the functionally important sulfhydryl groups were investigated. There were at least two cysteine residues highly reactive to NEM on the GDP-bound form of α_o. These alkylations resulted in loss of its ability to be ADP-ribosylated by IAP and to associate with $\beta\gamma$, but leaving the GTP-binding site of α_o intact. The reacted cysteine residues were identified by the sequencing of tryptic fragments of α_o. One of the alkylation sites was Cys-351, which was four amino acid residues away from the carboxyl-terminus of the molecule. The Cys-351 was proven to be also a site for IAP-catalyzed ADP-ribosylation. Possible roles of cysteine residues on the α-subunit of G_o are discussed in the functions of the signal transducing protein.

Alkylation; ADP-ribosylation; GTP-binding protein; Islet-activating protein

1. INTRODUCTION

GTP-binding proteins (G proteins) are family of signal-coupling proteins that play key roles in many hormonal and sensory transduction processes in eukaryotes [1]. G proteins which have a common heterotrimeric structure consisting of an α, a β-, and a γ-subunit, carry signals from activated receptors to effectors such as enzymes or ion channels. These heterotrimeric G proteins differ from one another in their nucleotide-binding α-subunits sharing the common $\beta\gamma$-component that may act as a modulator of the accompanying α-subunits. These G proteins are also characterized by their common capabilities of being ADP-ribosylated by bacterial toxins, such as cholera and pertussis toxins (islet-activating protein; IAP). Amino acid residues modified by the two toxins have been identified only in the α-subunit (α_t) of a G protein, termed G_t or transducin. An arginine residue in the central part of α_t [2] and a cysteine four residues away from its carboxyl-terminus [3] were the ADP-ribosylation sites for cholera toxin and IAP, respectively.

The effect of IAP-induced ADP-ribosylation is unique and differs strikingly from that of the cholera toxin-induced modification; G proteins are uncoupled totally from receptors leading to the inhibition of a high-affinity agonist-binding to the receptors, upon being ADP-ribosylated by IAP, though their other functions are not affected at all [4–7]. In addition, low concentrations of *N*-ethylmaleimide (NEM), a sulfhydryl alkylating reagent, have been found to mimic the action of IAP. Treatment of cell membranes with NEM also abolished the high-affinity agonist-bindings to membrane receptors [8–10] and the receptor-mediated inhibition of adenylate cyclase [11]. Thus, sulfhydryl groups of G proteins appeared to be important for the functions of the signal-coupling proteins. In this paper, the functional consequences of alkylation by NEM of sulfhydryl groups on G_o are described with relation to the site of IAP-catalyzed ADP-ribosylation.

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Abbreviations: G_o, a GTP-binding protein of unknown function purified from brain tissues; IAP, islet-activating protein or pertussis toxin; GTPγS, guanosine 5'-(3-*O*-thio)triphosphate; NEM, *N*-ethylmaleimide; DTT, dithiothreitol

2. MATERIALS AND METHODS

2.1. *Purification of G proteins and their constituent α- and $\beta\gamma$-subunits*

The α_o- and $\beta\gamma$-subunits of G_o were purified from rat brain membranes as described in [6,12]. α_o that had been ADP-ribosylated by IAP was also prepared as described in [6]. Prior to their uses, all the purified proteins were filtered through a 10-ml column of Sephadex G-25 fine (Pharmacia-LKB Biotechnology) in 50 mM Tris-HCl (pH 7.0), 0.1 mM Na-EDTA and 0.1% Lubrol-PX, which are henceforth referred to as TEL. The molecular masses of the purified subunits were assumed to be 39 000, 36 000 and 7000 Da for α_o, β and γ, respectively.

2.2. *Alkylation by [^{3}H]NEM of sulfhydryl groups on α_o-subunit*

The number of accessible sulfhydryl groups to NEM on the purified

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α_o-subunit was determined as follows. The protein at the indicated final concentration was incubated on ice with 25 μM [^{3}H]NEM (spec. act. of 0.4 μCi/nmol) in TEL. In the control experiment, NEM was quenched with DTT at the final concentration of 1 mM before the incubation of the protein with NEM. At the indicated times, 200-μl aliquots of the reaction mixture were withdrawn, and the reaction was immediately terminated by the addition of 20 μl of 10 mM DTT. Aliquots (100 μl) of the samples were mixed with 2 ml of 20 mM Tris-HCl (pH 8.0), 25 mM $MgCl_2$ and 100 mM NaCl (TMN) and applied onto a nitrocellulose membrane filter (0.45 μM). After being washed 7 times with 2 ml of TMN, the filter was dried and counted for the amount of [^{3}H]NEM covalently incorporated into the proteins. The remaining samples were also subjected to assays of the following activities.

2.3. *Assays of activities*

Methods utilized for IAP-substrate and GTPγS-binding activities were essentially the same as those described in [6]. For assay of IAP-substrate activity, 20-μl aliquots of the above samples were mixed with 10 μl of an excess amount of intact $\beta\gamma$ and then ADP-ribosylated by preactivated IAP and [^{32}P]NAD as described in [13]. For assay of the maximum amount of GTPγS bound to α_o (i.e. intact GTP-binding site on α_o), additional 20-μl aliquots of the samples were incubated at 30°C for 30 min with 1 μM [^{35}S]GTPγS in 100 μl of TEL containing 25 mM $MgCl_2$ and $(NH_4)_2SO_4$ [14].

Competitive inhibition by GDP of [^{35}S]GTPγS binding to α_o [15] was assayed as follows. Approximately 2 nM α_o that had been alkylated by NEM or not were incubated at 20°C for 10 min with 2.5 nM [^{35}S]GTPγS (0.38 μCi/nmol) and various concentrations of GDP in 50 μl of a Mg^{2+}-free solution consisting of TEL (5 mM EDTA) and 1 mM DTT. Where indicated, $\beta\gamma$-subunits were also added to the incubation mixture. The reaction was terminated by the addition of 2 ml of ice-cold TMN and applied onto the nitrocellulose membrane filter.

2.4. *Analysis of radiolabeled peptides*

Approximately 250 μg of α_o that had been radiolabeled by [^{3}H]NEM or IAP plus [^{32}P]NAD were filtered through a 8-ml column of G-25 in TEL and then heated at 90°C for 5 min. The radiolabeled α_o-subunits were digested with tosylphenylalanyl chloromethyl ketone (TPCK)-treated trypsin at an α_o/trypsin ratio of 40:1 (w/w) in 1.8 ml of TEL at 37°C for 2 days. After centrifugation, the clear supernatant containing the cleaved peptides was applied to a column of PepPRC HR5/5 (Pharmacia-LKB) that had been equilibrated with 0.1% trifluoroacetic acid and 5% acetonitrile and then eluted with a linear gradient (55 min) of 5–40% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.7 ml/min using a Pharmacia-LKB FPLC system. The eluate absorbance at 214 nm was monitored as well as the radioactivity of ^{3}H or ^{32}P. Purified peptides containing the radioactivity were sequenced by automated Edman degradation using a gas-phase sequencer (model 470A, Applied Biosystems). Phenylthiohydantoin (PTH) amino acids were analyzed by high-performance liquid chromatography (HPLC) using a Hitachi HPLC system [16].

2.5. *Miscellaneous*

Protein was quantitated by staining with Amido black with bovine serum albumin as a standard protein [17]. The sources of other materials used are those described in [6,13,18,19].

3. RESULTS

Fig. 1 shows effects of alkylation by NEM on the various activities of α_o. After incubation of α_o with [^{3}H]NEM for the indicated periods at 0°C, the reaction was terminated and subjected to the determination of the radioactivity of ^{3}H incorporated into the α-subunit. NEM modified only two sulfhydryl groups on α_o under

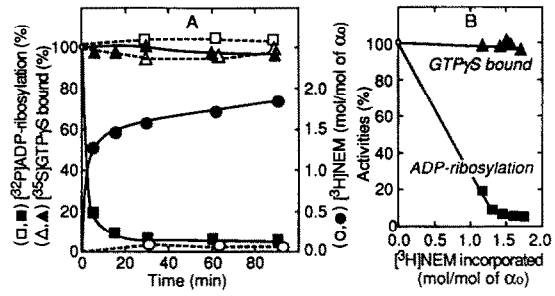

Fig. 1. Effect of alkylation by NEM on the activities of α_o. (A) Two hundred nM α_o were incubated at 0°C with 25 μM [^{3}H]NEM in 1.3 ml of TEL (●,▲,■). In the control experiments (○,△,□), NEM was quenched with 1 mM DTT before mixing of α_o with NEM. At the indicated times, 200-μl aliquots of the incubation mixture were withdrawn and mixed with 20 μl of 10 mM DTT. The sample was subjected to assays for the following activities as described in section 2.3. The amount of [^{3}H]NEM incorporated into α_o is expressed as mol/mol of α_o (○,●). GTPγS-binding (△,▲) and IAP-substrate (□,■) activities are expressed as percentages of the control values obtained at 0-time, which were 17 nmol of GTPγS bound and 15 nmol of ADP-ribosylation per mg of the purified α_o, respectively. (B) The activities of ADP-ribosylation (■) and GTPγS bound (▲) were replotted against the amount of [^{3}H]NEM incorporated into α_o.

the native (GDP-bound) state (Fig. 1A). The NEM-treated α_o was also subjected to assays for IAP-substrate and GTPγS-binding activities. The α_o very rapidly lost its ability to be ADP-ribosylated by IAP, as sulfhydryl groups on the α_o were alkylated by NEM. In contrast, GTPγS-binding activity remained intact at this level of the modification. When [^{3}H]NEM was incorporated over one mol per mol of α_o, more than 95% of the IAP-substrate activity were abolished (Fig. 1B). Thus, the modification of one sulfhydryl group, which was highly accessible to NEM, rendered the α-subunit inactive in terms of IAP-substrate activity, although the GTP-binding site of α_o remained intact.

A site for IAP-catalyzed ADP-ribosylation of transducin has been identified as a cysteine residue which is located at the fourth position from the carboxyl-terminus of the α-subunit [3]. Likewise, α_o contained a cysteine residue at the same position, based on the finding of the nucleotide sequences of cDNAs coding for the α-subunit [20]. Therefore, cysteine residues near the carboxyl-terminus might be the site for the ADP-ribosylation in all the α-subunits of IAP-substrate G proteins. Since the alkylation of α_o by NEM inhibited its ability to be the substrate for IAP (Fig. 1), it is also likely that either of the two highly reactive sulfhydryl groups on α_o is the same amino acid residue as the site for IAP-catalyzed ADP-ribosylation.

In order to locate the reactive cysteine residues within its primary structure, purified α_o-subunit that had been alkylated by [^{3}H]NEM (approximately 1.8 mol/mol of α_o) or [^{32}P]ADP-ribosylated by IAP (0.8 mol/mol of α_o) was completely digested with TPCK-treated trypsin. Fig. 2 shows the reverse-phase HPLC separation of the

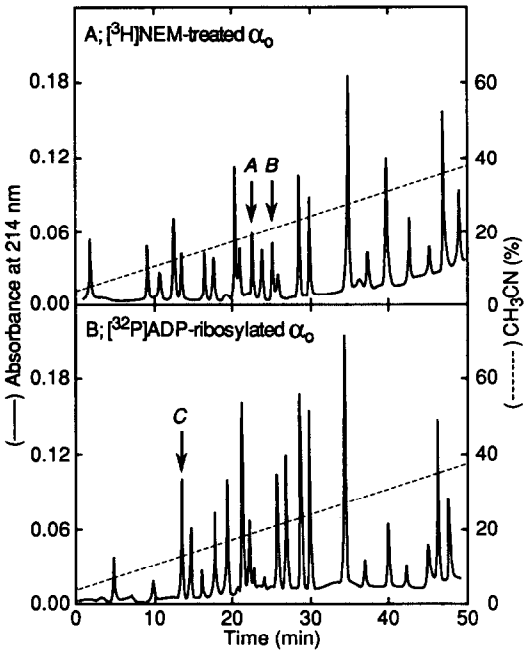

Fig. 2. HPLC separation of tryptic peptides of α_o radiolabeled by [^{3}H]NEM or [^{32}P]NAD *plus* IAP. The reaction and chromatography conditions are described in detail in section 2.4. Absorbance at 214 nm of the eluted peptides was monitored (——). (A) Alkylation of α_o by [^{3}H]NEM. (B) [^{32}P]ADP-ribosylated α_o. *Letters A–C* denote the radiolabeled peptides.

tryptic peptides of two radiolabeled α_o. A comparison of the absorbance at 214 nm of the two tryptic peptide maps shows that the patterns were virtually identical to each other with the exception of a few peaks. When α_o had been reacted with [^{3}H]NEM, two peaks contained the major radioactivity (marked *A* and *B* in Fig. 2A). However, there was only one major peak of ^{32}P-labeled peptides in the eluted fragments, if the tryptic fragments of [^{32}P]ADP-ribosylated α_o were analyzed by HPLC separation (marked *C* in Fig. 2B).

These three peaks of the tryptic peptides were sequenced by an automated Edman degradation method, and the results are shown in Fig. 3. The peaks *A* and *B* labeled by ^{3}H contained a single peptide of MVX-DVVSR (peptide A) and GXGLY (peptide B), respectively. Although the two Xs designated here were unidentified PTH-derivatives, there were significant amounts of ^{3}H-radioactivity in the PTH-derivatives eluted from the sequencer. Thus, the two Xs appeared to be cysteine residues reacted with [^{3}H]NEM. The peak *C* labeled by ^{32}P was a mixture of two peptides, but the sequences were determined as GXGLY (peptide C) and EYQLNDSAK based on the predicted amino acid sequences of the known α_o-subunit genes and cDNAs [20]. Unlike the case in ^{3}H-labeled peptides, the radioactivity of ^{32}P was not recovered in any of the eluted PTH-derivatives from the sequencer. Since the radioactivity was retained on a glass filter of the sequencer by unknown reasons, the radioactive material

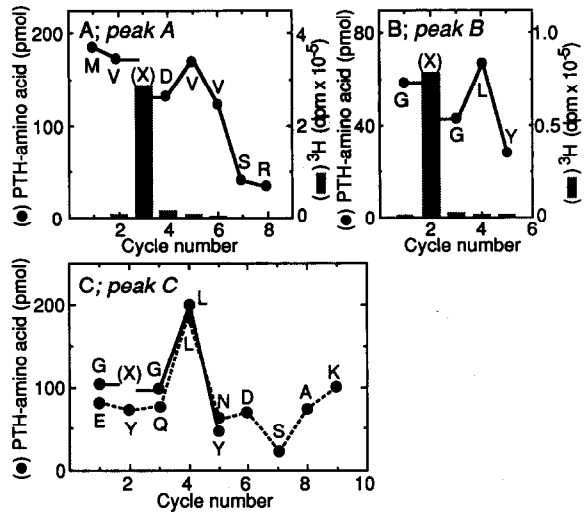

Fig. 3. Amino acid sequence analysis of the tryptic peptides containing the radioactivity of ^{3}H or ^{32}P. Peaks *A–C* in Fig. 2 were subjected to sequence analysis with a gas-phase sequencer, and the yields of PTH-amino acids at each cycle of Edman degradation are shown in the panels A, B and C, respectively. Peak *C* was a mixture of two peptides. In the panels A and B, the radioactivity of ^{3}H was also measured in each of the recovered PTH-delivatives.

was eluted from the filter by acetic acid-treatment. When the material was analyzed by a chromatography on PEI-cellulose [21], it comigrated with the same R_f value as ADP-ribose, suggesting that the amino acid X of peptide C was [^{32}P]ADP-ribosylated cysteine. The amino acid sequences for the NEM-labeled and ADP-ribosylated peptides are compared to the deduced sequences of the α-subunit of rat G_o in Fig. 4. Thus, the two reactive cysteines were identified as positions 108 and 351. The cysteine at position 351 was also proven as the site for IAP-catalyzed ADP-ribosylation.

The binding of [^{35}S]GTPγS to α_o was competitively inhibited by GDP as the concentration increased under Mg^{2+}-free conditions (Fig. 5A). There was a decrease in the concentration of GDP required for the half-maximum inhibition (IC_{50}) of the GTPγS binding, when $\beta\gamma$-subunits were added to the reaction mixture, confirming the previous findings that the affinity of α-subunit for GDP was higher in the oligomeric form than in the α-monomer [22]. The action of $\beta\gamma$ was

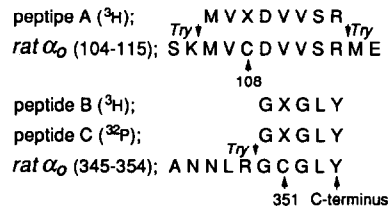

Fig. 4. Amino acid sequences of alkylated or ADP-ribosylated cysteine residues. Peptides A–C are the ones indicated in Fig. 3. Rat α_o, published by Itoh et al. [16], was deduced from cDNA cloned from a rat C6 glioma cell library.

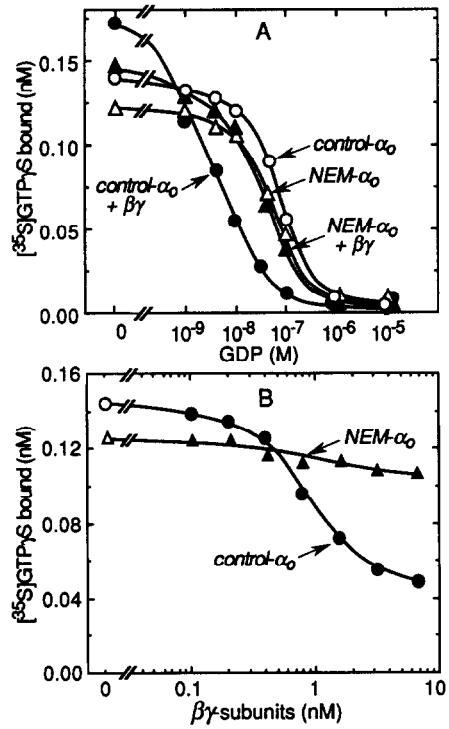

Fig. 5. Effects of alkylation by NEM on the affinity of α_o for GDP. Two hundred nM α_o were alkylated with 25 μM NEM by incubation at 0°C for 60 min in 50 μl of TEL. In the control experiment, α_o was incubated in the presence of 1 mM DTT as described above. The reaction was terminated by dilution with 450 μl of TEL (5 mM EDTA) containing 1 mM DTT. (A) Two nM α_o that had been alkylated by NEM (△,▲) or not (○,●) were incubated at 20°C for 10 min with (●,▲) or without (○,△) 4 nM $\beta\gamma$ in the presence of the indicated concentrations of GDP in 50 μl of TEL (5 mM EDTA) containing 1 mM DTT and 2.5 nM [^{35}S]GTPγS (0.38 μCi/nmol). (B) Two nM NEM-treated (△,▲) or the control (○,●) α_o were incubated with the indicated concentrations of intact $\beta\gamma$ in the presence of 25 nM GDP as in (A). [^{35}S]GTPγS binding to α_o was determined as described in section 2.3.

dependent on its concentration added to the reaction mixture, and the half-maximum effect of $\beta\gamma$ was obtained with the approximately stoichiometric amount of α_o used (Fig. 5B). Thus, the decrease in the IC_{50} of GDP was employed as an index for the association of the α-subunit with $\beta\gamma$-subunits.

When NEM-treated α_o (approximately 2 mol/mol of α_o) was incubated with [^{35}S]GTPγS and the various concentrations of GDP, the competitive inhibition by GDP was essentially the same as that observed with the control (unalkylated) α_o in accordance with the previous results in Fig. 1. However, a significant difference was noted; the $\beta\gamma$-induced decrease in the IC_{50} of GDP observed with control α_o was almost completely abolished in the NEM-treated α_o (Fig. 5). As shown in Fig. 6B, $\beta\gamma$-induced decrease in the IC_{50} of GDP was similarly observed with α_o that had been ADP-ribosylated by IAP at the position of Cys-351. Alkylation of the ADP-ribosylated α_o by NEM also resulted in

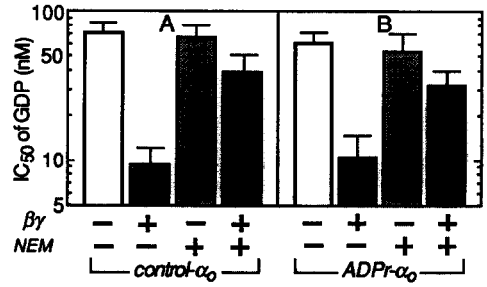

Fig. 6. Effects of alkylation by NEM on interaction between α and $\beta\gamma$. α_o that had been ADP-ribosylated by IAP (B) or not (A) was alkylated by NEM (columns 3 and 4) or not (columns 1 and 2) as described in Fig. 5. Two nM of these α-subunits were incubated at 20°C for 10 min with (columns 2 and 4) or without (columns 1 and 3) 4 nM $\beta\gamma$ in the presence of 2.5 nM [^{35}S]GTPγS and various concentrations of GDP in 50 μl of TEL (5 mM EDTA) containing 1 mM DTT. The [^{35}S]GTPγS binding curves of the α-subunits were constructed as the function of GDP added as shown in Fig. 5, and the IC_{50} value of GDP was determined in each case. The data shown are the means ± SE (error bars) from triplicate determinations.

an apparent inhibition of the $\beta\gamma$-subunits action as had been observed with the control α_o (Fig. 6A). Thus, the functionally important cysteine residue on α_o appeared to be different from Cys-351, since α_o whose Cys-351 had been marked by IAP-catalyzed ADP-ribosylation was still susceptible to the NEM-induced modification.

4. DISCUSSION

In this communication, we have studied the roles of sulfhydryl groups on the α-subunit of G_o by means of its alkylation by NEM. The alkylation of the highly susceptible sulfhydryl groups on α_o under intact conditions allowed us to determine the functionally important groups in terms of the various activities of the signal-coupling protein. The major findings obtained here are summarized as follows.

(i) There were at least two sulfhydryl groups highly reactive to NEM on the GDP-bound form of α_o resolved from $\beta\gamma$ (Fig. 1). The alkylation resulted in a complete loss of its ability being served as the substrate for IAP-catalyzed ADP-ribosylation. However, the GTP-binding site on α_o remained intact even after alkylation of the sulfhydryl groups. (ii) Amino acid residues alkylated by NEM were identified as Cys-108 and Cys-351 on the α-subunit (Figs 2–4). The Cys-351, which was the fourth residue from the carboxyl-terminus, was also proven to be the site for IAP-catalyzed ADP-ribosylation. (iii) There was another NEM-sensitive sulfhydryl group on α_o responsible for its interaction with $\beta\gamma$-subunits, since its alkylation resulted in a loss of ability to be associated with $\beta\gamma$ (Fig. 5). The alkylation of ADP-ribosylated α_o, whose Cys-351 had been masked by ADP-ribose, similarly inhibited its interaction with $\beta\gamma$ (Fig. 6). Therefore, the responsible site must be different from Cys-351 and

thus appeared to be Cys-108, though the amino acid residue on α_o remains to be determined more clearly.

The loss of IAP-substrate activity of α_o after its alkylation by NEM should be due to the prior modification of the same Cys-351 with the alkylating reagent. Indeed, the alkylation initially occurred at Cys-351 of the ADP-ribosylation site rather than at Cys-108 under our present conditions where the GDP-bound form of α_o was incubated with NEM at 0°C (data not shown). Alternatively, it might be the resultant from the inability of the modified α_o to interact with $\beta\gamma$, since α_o dissociated from $\beta\gamma$ was not the real substrate for IAP-catalyzed ADP-ribosylation [6]. However, this possibility is very unlikely, since α_o modified by ADP-ribosylation at the same Cys-351 still interacts with the $\beta\gamma$-subunits [6].

One of the cysteine residues reacted to NEM, Cys-351, is found in a region where all of the IAP-substrate G proteins are highly homologous. The cysteine was also the site for IAP-catalyzed ADP-ribosylation as had been previously reported in the α-subunit of G_t [3]. Either modification of Cys-351 by alkylation or ADP-ribosylation similarly resulted in an uncoupling of the G proteins from activated receptors; high-affinity agonist-bindings to receptors were selectively inhibited in various types of membranes that had been treated with NEM [8–10] or IAP plus NAD [4,5,23]. However, neither of the two covalent modifications Cys-351 affected the other functional parameters, such as the GTPase activity α_o or the ability of α_o to interact with $\beta\gamma$. Thus, the carboxyl-terminal regions of G protein α-subunits must be responsible for interactions with receptor molecules.

An unexpected finding in the present study was the observation that modification of Cys-108, a cysteine not present in any other α-subunit of IAP substrates at the same position, appeared to inhibit the interaction of α_o with $\beta\gamma$. Such and alkylation of Cys-108 by NEM has also been reported previously by Winslow et al. [24]. However, they proposed that the alkylation of Cys-108 completely blocked ADP-ribosylation of α_o catalyzed by IAP probably due to a large conformational change in the molecule which rendered the carboxyl-terminus inaccessible to IAP. They also suggested that the alkylation of Cys-108 did not alter the association of α_o with $\beta\gamma$ from the analysis of a sucrose density-gradient method. Thus, their findings are somewhat different from these obtained in the present studies. Such a difference might be due to the different conditions employed in the alkylation of the purified protein with NEM.

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