The Function of Interdomain Interactions in Controlling Nucleotide Exchange Rates in Transducin*

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The intramolecular contacts in heterotrimeric G proteins that determine the rates of basal and receptor-stimulated nucleotide exchange are not fully understood. The α subunit of heterotrimeric G proteins consists of two domains: a Ras-like domain with structural homology to the monomeric G protein Ras and a helical domain comprised of six α-helices. The bound nucleotide lies in a deep cleft between the two domains. Exchange of the bound nucleotide may involve opening of this cleft. Thus interactions between the domains may affect the rate of nucleotide exchange in G proteins. We have tested this hypothesis in the α subunit of the rod cell G protein transducin (G\textsubscript{αi}). Site-directed mutations were prepared in a series of residues located at the interdomain interface. The proteins were expressed in vitro in a reticulocyte lysate system. The rates of basal and rhodopsin-catalyzed nucleotide exchange were determined using a trypsin digestion assay specifically adapted for kinetic measurements. Charge-altering substitutions of two residues at the interdomain interface, Lys\textsubscript{276} and Lys\textsubscript{277}, increased basal nucleotide exchange rates modestly (5–10-fold). However, we found no evidence that interactions spanning the two domains in G\textsubscript{αi} significantly affected either basal or rhodopsin-catalyzed nucleotide exchange rates. These results suggest that opening of the interdomain cleft is not an energetic barrier to nucleotide exchange in G\textsubscript{αi}. Experiments with G\textsubscript{αi} suggest by comparison that the organization and function of the interdomain region differ among various G protein subtypes.

Transducin (G\textsubscript{αi}) is the heterotrimeric guanine-nucleotide binding regulatory protein (G protein) of the rod cell. In the GDP-bound state, the α subunit of transducin (G\textsubscript{αi}) does not signal. Following exchange of GDP for GTP, which is catalyzed by photoactivated rhodopsin (R\textsuperscript{*}), G\textsubscript{αi} signals to its downstream effector. Physiologically, the detection of dim light requires that the basal nucleotide exchange rates of G\textsubscript{αi} be very low to prevent background noise and that R\textsuperscript{*}-catalyzed exchange be very efficient, to ensure consistent detection and amplification of light signals.

G\textsubscript{αi} consists of two domains: a Ras-like domain, which is structurally similar to the monomeric G protein p21\textsubscript{ras}, and a helical domain, which is unique to the heterotrimeric G proteins (1). The bound nucleotide lies in a deep cleft between the two domains (Fig. 1A). Although the discovery of this arrangement initially prompted speculation that nucleotide exchange would involve opening of the interdomain cleft (1), and that interactions between the domains might affect the rate of nucleotide exchange, the intramolecular contacts in G\textsubscript{αi} that determine the rates of nucleotide exchange remain to be elucidated.

There is evidence that structures that do not directly interact with the nucleotide can modulate both the basal and the receptor-catalyzed rates of nucleotide exchange. For example, although the direct contacts between the protein and the nucleotide are virtually the same in closely related subtypes of G protein, the rates of basal nucleotide exchange vary widely. Furthermore, R\textsuperscript{*} tremendously accelerates nucleotide exchange, yet available evidence indicates that it does not directly contact the nucleotide binding site (2).

One of the regions of the G protein hypothesized to control nucleotide release rates without directly contacting the nucleotide is the interdomain interface. The interface is composed of contacts adjacent to the nucleotide and also interactions that are distant from the nucleotide (Fig. 1). These latter interactions involve residues located on the αD-αE loop (amino acid residues 139–147 of G\textsubscript{αi}) of the helical domain, the Switch III region (residues 227–238), and the αG region (residues 269–277) of the Ras-like domain (Fig. 1B). These interactions have been implicated in mediating the lower rate of dissociation of GTP\textsubscript{S} relative to GDP in G\textsubscript{αi} (3) and in affecting the basal nucleotide exchange rates in G\textsubscript{αi} (4) and G\textsubscript{αi} (5, 6). Additionally, studies in G\textsubscript{αi} have suggested that interdomain interactions are involved in mediating rapid nucleotide exchange catalyzed by the β\textsubscript{3}-adrenergic receptor (7, 8).

We have studied the function of several residues of G\textsubscript{αi} that are located at the interdomain interface but do not contact the nucleotide. A number of site-directed mutations of these residues were constructed. The well documented difficulties in expressing and purifying recombinant G\textsubscript{αi} were overcome by expressing the mutant proteins in vitro in a rabbit reticulocyte lysate system. The rates of basal and R\textsuperscript{*}-catalyzed nucleotide exchange.
exchange were measured using a trypsin digestion assay specifically adapted for kinetic measurements. Alteration of two conserved lysine residues, Lys<sup>273</sup> and Lys<sup>276</sup>, increased the rate of spontaneous nucleotide exchange 5–10-fold. However, in contrast to what would be predicted based on published structural and biochemical studies, we found no evidence that interactions that span the domain interface were important in either maintaining the low rate of basal nucleotide exchange or in supporting the high rate of R*-catalyzed exchange in Go<sub>i</sub>. Experiments with Go<sub>i</sub>, demonstrated that conserved lysine residues serve different roles in Go<sub>j</sub> than in Go<sub>i</sub>. In general, the function of the interdomain region appears to differ among various G protein subtypes.

**Experimental Procedures**

**Reagents—** Buffers, nucleotides, protease inhibitors, and salts were from Sigma or Roche Molecular Biochemicals. [35S]Methionine was purchased from PerkinElmer Life Sciences. TPCK-treated trypsin was from Worthington Biochemicals. Synthetic oligonucleotides were purchased from PerkinElmer Life Sciences. Trypsin was from Sigma or Roche Molecular Biochemicals. [35S]Methionine was added from a 1 Ci/mmol stock solution in 0.17 mM AlCl<sub>3</sub> and 10 mM NaF added from separate stock solutions, or for the basal exchange time course in a R*-catalyzed activation assay, GDP/AlF<sub>4</sub>, was replaced. Activation kinetics were analyzed by plotting the fraction of Go<sub>i</sub> activated as a function of time. In the basal exchange assay, the data were fit to a single exponential rise to a maximum equation of the form: percent activated = c + 100(1 − exp(−kt)). The apparent rate constants derived from the fits are presented in Table I.

For experiments conducted with Go<sub>j</sub>, the intensity of the GDP-dependent band could not be determined reliably due to nonspecific background intensities in the region of the gel where the GDP band migrated. This background was present even in undigested samples of Go<sub>i</sub>. Since the ratio of the GTP<sub>j</sub>-dependent band to the sum of the GTP<sub>j</sub>S and GDP bands could not be determined, the intensity of the GTP<sub>j</sub>S band was expressed as a fraction of the total intensity in each lane. Activation time courses were plotted as the change in this intensity over time. For fully activated Go<sub>j</sub>, the GTP<sub>j</sub>S band accounted for roughly 25–30% of the intensity in the lane.

**Control Reactions for Trypsin Proteolysis of Go<sub>i</sub>—** Control reactions were performed on each sample to check the quantity and apparent molecular mass of the expressed protein, as well as the digest patterns following incubation with GDP and GDP/AlF<sub>4</sub>. From each 100-μl sample of translated G<sub>a</sub>, 30 μl was removed and combined with 100 μM GDP and 30 μl, one 8-μl aliquot was mock-digested with digest control buffer (“undigested”). The remaining 14 μl was mixed with 8-μl aliquots were removed and digested (“+GDP/AlF<sub>4</sub>”).

**Basal Nucleotide Exchange Time Course of Go<sub>i</sub>, G<sub>i</sub>—** Samples (70 μl each) of translated Go<sub>i</sub> or mutant Go<sub>i</sub> in Buffer A were quickly warmed to room temperature in a water bath, and GTP<sub>i</sub>S was added to a final concentration of 100 μM. Aliquots (8 μl) were withdrawn at 1, 2, 3, 4, and 6 h following GTP<sub>i</sub>S addition and digested. The activity of the protein following 6-h incubation at room temperature was investigated by digestion of R<sup>6</sup> and G<sub>i</sub>β<sub>3</sub> (30 μl each, see below) and incubation under room temperature for 20 min. A final 8-μl aliquot was then removed and digested. For Go<sub>i</sub>α samples, which activated very quickly, aliquots were taken for a 180-min time course, and the R<sup>*</sup>G<sub>i</sub>β<sub>3</sub> mix was not added.

**R<sup>*</sup>/G<sub>i</sub>β<sub>3</sub>-catalyzed Activation Time Course of Go<sub>i</sub>, G<sub>i</sub>β<sub>3</sub>—** Samples (70 μl each) of translated Go<sub>i</sub> or mutant Go<sub>i</sub> in Buffer A were quickly warmed to room temperature in a water bath. A mixture of R<sup>*</sup> and GTP<sub>i</sub>S (4 μl) was added to the sample yielding a final concentration of 30 nM R<sup>*</sup> and 14 μM GTP<sub>i</sub>S. Immediately after the addition to the reaction, the rhodopsin was photolyzed by illumination for 15 s with a fiber optic cable connected to a Dolan Jenner lamp equipped with a >495-nm long-pass filter. The samples were incubated at room temperature under illumination. Aliquots (8 μl) were withdrawn and digested at 1, 2, 3, 5, 10, and 20 min following addition of the R<sup>*</sup>GTP<sub>i</sub>S mix.

**Preparation of Go<sub>i</sub>, G<sub>i</sub>α, G<sub>i</sub>β<sub>3</sub>, and Rhodopsin from Bovine Reti-
**TABLE I**

| Mutant         | \( k_{\text{app}} \) | Fold increase | Mutant         | \( k_{\text{app}} \) | Fold increase |
|----------------|-----------------|--------------|----------------|-----------------|--------------|
| Wild-type      | 8.6 ± 0.7       | 1.0 ± 0.1    | D227N          | 3.4 ± 0.6       | 0.4 ± 0.1    |
| S140A          | 14.7 ± 4.6      | 1.7 ± 0.6    | M228L          | 4.0 ± 0.4       | 0.5 ± 0.1    |
| S140N          | 7.8 ± 2.1       | 0.9 ± 0.3    | V231A          | 4.6 ± 0.6       | 0.5 ± 0.1    |
| S140R          | 12.1 ± 2.6      | 1.4 ± 0.3    | D227A          | 5.0 ± 1.1       | 0.6 ± 1.1    |
| Q143A          | 6.1 ± 1.4       | 0.7 ± 0.2    | Q143A          | 6.1 ± 1.4       | 0.7 ± 0.2    |
| D227N          | 5.0 ± 1.1       | 0.6 ± 1.1    | M228Q          | 6.2 ± 0.6       | 0.7 ± 0.1    |
| D227N          | 3.4 ± 0.6       | 0.4 ± 0.1    | V231W          | 6.4 ± 1.1       | 0.7 ± 0.1    |
| M228A          | 14.6 ± 0.7      | 1.7 ± 0.2    | S140A/D227N    | 7.4 ± 0.7       | 0.9 ± 0.1    |
| M228L          | 4.0 ± 0.4       | 0.5 ± 0.1    | S140N          | 7.8 ± 2.1       | 0.9 ± 0.3    |
| M228Q          | 6.2 ± 0.6       | 0.5 ± 0.1    | Wild-type      | 8.6 ± 0.7       | 1.0 ± 0.3    |
| V231A          | 4.6 ± 0.6       | 0.5 ± 0.1    | K276R          | 8.8 ± 2.5       | 1.0 ± 0.3    |
| V231W          | 6.4 ± 1.1       | 0.7 ± 0.1    | S140A/Q143A    | 10.4 ± 4.7      | 1.2 ± 0.3    |
| K273A          | 39.0 ± 22.6     | 4.6 ± 2.7    | K275A          | 12.0 ± 1.4      | 1.4 ± 0.6    |
| K275A          | 12.0 ± 1.4      | 1.4 ± 0.2    | S140R          | 12.1 ± 2.6      | 1.4 ± 0.2    |
| K276A          | 45.1 ± 7.5      | 5.3 ± 1.0    | D227N/K273A    | 14.5 ± 2.7      | 1.7 ± 0.3    |
| K276E          | 98.8 ± 29.1     | 11.5 ± 3.5   | M228A          | 14.6 ± 0.7      | 1.7 ± 0.3    |
| K276F          | 8.8 ± 2.5       | 1.0 ± 0.3    | S140A          | 14.7 ± 4.6      | 1.7 ± 0.2    |
| S140A/Q143A    | 10.4 ± 4.7      | 1.2 ± 0.6    | D227A/K276A    | 14.8 ± 7.3      | 1.7 ± 0.9    |
| S140A/K276A    | 67.3 ± 14.0     | 7.9 ± 1.8    | D227N/K276A    | 20.3 ± 4.7      | 2.4 ± 0.6    |
| S140A/D227N    | 7.4 ± 0.7       | 0.9 ± 0.1    | K273A          | 39.0 ± 22.6     | 4.6 ± 2.7    |
| S140R/K276A    | 48.0 ± 14.6     | 5.6 ± 1.8    | K276A          | 45.1 ± 7.5      | 5.3 ± 1.0    |
| D227A/K276A    | 14.8 ± 7.3      | 1.7 ± 0.9    | S140R/K276A    | 45.8 ± 14.6     | 5.6 ± 1.8    |
| D227N/K273A    | 14.5 ± 2.7      | 1.7 ± 0.3    | S140A/K276A    | 67.3 ± 14.0     | 7.9 ± 1.8    |
| D227N/K276A    | 20.3 ± 4.7      | 2.4 ± 0.6    | K276E          | 98.8 ± 29.1     | 11.5 ± 3.5    |

*The apparent rate constants were derived from fits of each data set to the exponential rise equation, \( y = c + 100(1 - \exp(-kt)) \). Each mutant was assayed at least three times (WT Goα was assayed 26 times), and an independent fit was made to each data set. The values reported are the mean \( k_{\text{app}} \times 10^4 \text{min}^{-1} \pm 2 \times \text{S.E.} \). The catalyzed activation rate of wild-type Goα was determined to be 5390 ± 802 in the presence of 30 nM photoactivated rhodopsin, 30 nM Gβγ, and 14 μM GTPγS. This is a 629 ± 107-fold increase over the basal (uncatalyzed) rate.

The fold increase in the rate of the mutant relative to that of wild-type is calculated as \( k_{\text{app}(\text{mutant})}/k_{\text{app}(\text{wild-type})} \).

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Interdomain Interactions in Transducin

A series of site-directed mutants of Goα with replacements of residues located at the interdomain interface was prepared. Sites were selected for mutation based on their position in the crystal structure of GDP-bound Goα (18) (Fig. 1), as well as their importance in Goα1 and Goα suggested by published studies (see below). Selected residues were replaced with alanine, or with the amino acid present in the homologous position of either Goα1 or Goα, and/or with amino acids reported to cause altered phenotypes in Goα1 or Goα. For each mutant Goα, the rates of both basal (i.e. uncatalyzed) and Rα-catalyzed nucleotide exchange were measured.

Expression of Goα in Vitro and Trypsin Digestion Assay of Nucleotide Binding and Exchange—All Goα constructs were expressed in vitro in a coupled transcription/translation rabbit reticulocyte lysate system. Time course experiments confirmed that in vitro expression was maximal in 90 min (not shown).

Typical reactions with plasmids encoding Goα or Goα mutant genes yielded one major protein band at the expected molecular mass of ~40-kDa (Fig. 2). Generally 80–90% of the total intensity in the lane was in the one band. Expressed Goα was digested with trypsin following various treatments. Inactive, GDP-bound Goα was prepared by incubating in vitro translated Goα with 100 μM GDP. Trypsin proteolysis of this sample resulted in the formation of a ~23-kDa fragment (Fig. 2).

The active conformation was prepared by incubating Goα with GDP and AIFγ. AIFγ is known to bind to Goα and simulate the presence of the γ-phosphate of GTP. Therefore, a conformation nearly identical to the activated GTP-bound conformation is induced (19). Digestion of the AIFγ-activated Goα yielded a ~34-kDa band and no ~23-kDa band. Similarly, activation of Goα with GTPγS yielded an identical ~34-kDa band following trypsin digestion (Fig. 2).

In all cases, trypsin proteolysis produced a variety of lower molecular weight fragments. Some of these were the smaller polypeptides that were cleaved to produce the ~23- and ~34-kDa fragments. Others likely resulted from extensive proteolysis of protein that was not properly folded. This is consistent with the large number of potential trypsin sites present in the primary structure of Goα and the relatively small number of accessible sites in the properly folded tertiary structure. The fraction of the total pool of translated Goα that was properly folded and functional was estimated from the ratio of the intensities of the ~34-kDa band following GDP/AIFγ treatment (i.e. the properly folded, activable pool) to that of the ~40-kDa...
The rate of trypsin proteolysis of GDP- and GDP/AlF$_4$-treated Go$_i$ and Go$_i$ was measured in vitro. The rate of nucleotide exchange of each sample was determined by monitoring the fraction of Go$_i$ in the active conformation in a partially activated lane. The fraction of Go$_i$ in the active conformation in a partially activated sample was calculated as the intensity of the ~34-kDa band (i.e. the activated Go$_i$) divided by the sum of the intensities of the ~23- and ~34-kDa bands. The sum of the intensities of the ~23- and ~34-kDa bands is indicative of the total pool of functional Go$_i$ in the sample. This calculation is therefore internally normalized to the total amount of functional Go$_i$ in each aliquot and does not require comparison with the ~34-kDa band of a separate sample (such as one in a completely activated lane).

The rate of nucleotide exchange of each sample was determined by monitoring the fraction of Go$_i$ in the active conformation at specific times following addition of GTP-$\gamma$S. In the basal exchange rate assay, Go$_i$ was 31% activated at 6 h following GTP-$\gamma$S addition (Fig. 2A). The activity of Go$_i$ following the 6-h incubation was confirmed by demonstrating that addition of rhodopsin and G$\beta$$\gamma$S could fully activate the remaining Go$_i$ (Fig. 2B). In comparison, Go$_i$ A322S (a mutant known to display high nucleotide exchange rates in Go$_i$ (21) and Go$_i$
In R*-catalyzed assays, Gαt was nearly 100% activated in 20 min (Fig. 2B). Under the conditions of the assay, the rate of Gαt activation was found to be dependent on the concentration of rhodopsin from 0–100 nM and sensitive to the presence of added Gβγ, (not shown). Some residual activation was observed in the absence of added Gβγ, probably as a result of small quantities of Gβγ present in the reticulocyte lysate or the rhodopsin preparations. No rhodopsin-catalyzed activation was observed in the dark (data not shown). Additionally, mutant G348P, which was previously reported to be unable to bind rhodopsin (23), was not activated by R* in this assay (not shown).

To our knowledge, this is the first report in which trypsin proteolysis of in vitro translated Gαt has been used to measure the kinetics of R*-catalyzed nucleotide exchange.

Analysis of Single Amino Acid Replacements in the Interdomain Interface of Gαt—Two residues in the helical domain, Ser140 and Gln143, extend toward and interact with residues from the Ras-like domain. Ser140 was replaced with alanine, arginine (the homologous residue in Gαi1), and asparagine (the homologous residue in Gσi). Gln143 was mutated to alanine and was also combined with S140A in a S140A/Q143A double mutant. None of these mutations substantially altered the rate of basal (Fig. 3A, Table I) or R*-catalyzed nucleotide exchange (not shown).

Several residues in the Switch III region of Gαt were altered by site-directed mutagenesis. The crystal structure suggests that Asp227 participates in hydrogen bonds with both Ser140 and Glu276 (Fig. 1). This residue was replaced with both asparagine (the homologous residue in Gαi1) and with alanine. The adjacent Met228 was replaced with alanine, leucine (the equivalent in Gαi1), and glutamine (the equivalent of a mutation in Gαt, reported to increase the GDP release rate (4)). Val231 was replaced with alanine and with tryptophan. The V231I mutant was prepared to simulate a naturally occurring mutation in the homologous residue of Gαo, Arg236, which was found in a patient with Albright’s hereditary osteodystrophy (5). None of these mutations was found to substantially alter either the basal (Fig. 3B and C, and Table I) or the R*-catalyzed activation rates (not shown). However, several mutations caused slight (~2-fold), but reproducible reductions in the basal nucleotide exchange rate. These mutations include D227A, D227N, V231A, and M228L (Table I).

Three lysine residues in the αG region of Gαt were studied. Lys273 and Lys276 are oriented toward Asp227 of Switch III (Fig. 1). In addition, the crystal structure of Gαt-GDP indicates that Lys276 forms hydrogen bonds with Ser140 of the helical domain (Fig. 1). Lys273 extends out toward the solvent (Fig. 1). Lys273, Lys275, and Lys276 were each replaced with alanine. In addition, Lys276 was replaced with glutamic acid and with arginine. The K273A, K276A, and K276E mutations all resulted in significantly increased rates of basal nucleotide exchange, from 5–10-fold above wild-type (Fig. 3D). The K276R mutation did not affect nucleotide exchange rates, nor did mutation of Lys275. None of the mutations substantially altered the rate of R*-catalyzed nucleotide exchange (not shown).

Analysis of Double Amino Acid Replacements—To probe for functional interactions between pairs of residues, a series of double amino acid replacements was prepared by site-directed mutagenesis (Table I). The K276A/D227N, K276A/D227A, and K273A/D227A/D227N double replacements all displayed slower basal rates of nucleotide exchange than the corresponding single replacements of Lys276 or Lys273 (Fig. 4). The double mutants displayed faster exchange kinetics than wild-type Gαt, however. Combining amino acid replacements at positions Lys276 and Ser140 revealed that the effects on basal exchange rates of each individual mutation were roughly additive (Fig. 4). A combination of amino acid replacements at positions 140 and 227, S140A/D227N, had similar exchange kinetics to that of wild-type Gαt (Table I).

Analysis of Gαt Mutants—Gαt is 66% identical to Gαo at the primary structure level and very similar at the tertiary structure level (Fig. 1C). However, the basal nucleotide exchange rate of Gαt has been reported to be significantly higher than that of Gαo (24). We confirmed this observation in studies with recombinant Gαt and retinal Gαt in a fluorescence activation assay (Fig. 5A). The rate of basal nucleotide exchange as monitored by increases in fluorescence was much greater in Gαt than in Gαo at 25 °C. However, the rate and the magnitude of fluorescence change were comparable when each protein was fully activated with excess AlF6–.

Gαt was expressed in vitro and studied by trypsin digestion.

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2 E. P. Marin, unpublished observation.
SDS-PAGE analysis indicated that a ~38-kDa band was produced following full activation with either GTP$\gamma$S or GDP/AlF$_4$ and that a smaller fragment resulting from digestion in the presence of GDP. The intensity of the smaller GDP-dependent band of Go$_t$ could not be accurately quantitated due to reproducible nonspecific background in that portion of the gel. Therefore, the method for determining the fraction of in vitro translated Go$_t$ activated in an aliquot was modified from the “ratio” method used for Go$_i$. In each aliquot, the intensity of the ~38-kDa band was determined as a fraction of the total intensity in the lane, and the time course of activation was plotted as a change in this fraction over time (Fig. 5B). This analysis suggests that basal nucleotide exchange of GDP for GTP$\gamma$S by Go$_t$ is complete in 1 h, significantly faster than that by Go$_i$, which was only 32% complete in 6 h. The $t_{1/2}$ for activation of Go$_t$ (~20 min) was comparable in both the fluorescence and the trypsin protection assays (Fig. 5).

A series of site-directed mutants was prepared in Go$_t$, to probe for similarities between the functions of interdomain residues in Go$_i$ and Go$_t$. The equivalent of Lys$^{273}$ and Lys$^{276}$ of Go$_i$ were conserved in Go$_t$ as Lys$^{277}$ and Lys$^{280}$, respectively (Fig. 1). Both were replaced with alanine, expressed in vitro, and the basal nucleotide exchange rates of the resulting mutants were determined. Neither of these mutations (K277A and K280A) increased the basal activation rate of Go$_t$, appreciably (Fig. 5B). Additionally, Arg$^{144}$ of Go$_i$, the homolog of Ser$^{140}$ in Go$_t$, was replaced with serine. Since the Ser$^{140}$ of Go$_t$ forms hydrogen bonds with Lys$^{276}$, it was hypothesized that in Go$_t$, the replacement of Arg$^{144}$ with serine might alter the position of Lys$^{280}$ (Lys$^{276}$ in Go$_i$) to resemble the Go$_i$ conformation and lower the basal rate of nucleotide exchange. However, the opposite was observed. The Go$_t$ R144S mutant exhibited accelerated nucleotide exchange rates (Fig. 5B), consistent with previous reports of mutagenesis at the Arg$^{144}$ position (4).

**DISCUSSION**

**Analysis of Trypsin Digest Products of In Vitro Translated Go$_t$ to Evaluate Nucleotide Exchange Kinetics**—Since Go$_t$ is refractory to expression in bacteria (24) and is cumbersome to express in insect cells (16), we chose to express Go$_t$ in vitro and analyze nucleotide exchange rates using trypsin digestion. The pattern of proteolytic fragments resulting from trypsin digestion directly reflects the conformation of Go$_i$ and, therefore, the identity of the bound nucleotide (25, 26). Trypsin proteolysis of expressed Go$_t$ yielded ~23-kDa fragments following incubation with GDP and ~34-kDa fragments following activation with either AlF$_4$ or GTP$\gamma$S (Fig. 2). These observations are consistent with previously published results (23, 27).

Trypsin proteolysis of in vitro translated Go$_t$ allowed for the precise quantitation of basal and R$^*$-catalyzed nucleotide exchange rates. By using both the ~23- and the ~34-kDa bands, the calculation of nucleotide exchange rates was internally normalized and took into consideration both the GDP- and the GTP$\gamma$S-bound fractions. As a result, the data were very reproducible, and samples in which only a small fraction of the total expressed protein was functional could be analyzed. Additionally, the methodology was rapid enough to allow for the characterization of relatively large numbers of mutants in parallel.

The kinetic parameters for Go$_t$ activation derived from the trypsin digestion assay are consistent with data reported using other traditional methodologies (24, 28) as well as with analysis of retinal Go$_t$ studied with the fluorescence activation assay (Fig. 5). Additionally, analysis of the mutant A322S by trypsin proteolysis indicated that the basal rate of activation was >60-fold greater than that of wild-type Go$_i$ (not shown). This result is also in agreement with published data using different methodologies on the analogous mutation in Go$_a$ and in Go$_{i1}$ (21, 22).
Several control reactions demonstrated the fidelity of the R*\textsuperscript{a}-catalyzed assay. The rate of rhodopsin-dependent activation was sensitive to light (not shown), to the concentration of rhodopsin from 0–100 nM, and to the presence of added Gβγ. A mutation near the carboxyl terminus of Gα\textsubscript{t} (G348P) that was previously reported to disrupt rhodopsin-transducin interactions (23) was not activated (not shown).

This expression and assay system offers additional flexibility not explored in the present work. Expressed recombinant rhodopsin could be used in place of retinal rhodopsin to test the combined effects of mutations of rhodopsin and transducin.\textsuperscript{3} Other proteins (e.g. Gβγ mutants, regulator of G protein signaling proteins, etc.), could be co-translated with Gα\textsubscript{t} in the in vitro system (25). Furthermore, this system will likely prove useful in the kinetic characterization of other G protein subtypes, such as cone transducin, which are difficult to express heterologously.

\textbf{Site-directed Mutation of Lys\textsubscript{273} or Lys\textsubscript{276} Increases Basal Nucleotide Exchange Rates—Replacement of Lys\textsubscript{276} or Lys\textsubscript{273} with alanine increased the basal rate of nucleotide exchange \textasciitilde5-fold in Gα\textsubscript{t} (Fig. 3D, Table I). Replacement of the adjacent Lys\textsubscript{275}, which in the crystal structure is oriented toward the solvent (Fig. 1), had no effect. Replacement of Lys\textsubscript{276} with a negatively charged glutamic acid increased basal nucleotide exchange rates more dramatically (\textasciitilde10-fold) than the neutral alanine replacement mutant. However, mutation to a positively charged arginine did not alter the activation rate. Together, these results suggest that the function of Lys\textsubscript{276} and Lys\textsubscript{273} is dependent on positive charge and orientation toward the interior of the protein. Interestingly, in the activated, GTP-bound structure of Gα\textsubscript{t}, Lys\textsubscript{276} is rotated outward toward the solvent relative to the GDP-bound conformation (Fig. 1B). The K276A mutant may anticipate this active conformation.

Lys\textsubscript{276} and Lys\textsubscript{273} both lie in the third of four regions of Gα\textsubscript{t} for which there is no homologous sequence in the monomeric G protein Ras. The four regions are known as Insert 1 through Insert 4 (1). Specific functions have been attributed to Insert 1 (the helical domain), Insert 2 (the Switch III region), and Insert 4 (which may interact with heptahelical receptors). The present work is the first to identify a functional role for residues in Insert 3 of Gα\textsubscript{t}.

The structure of GDP-bound Gα\textsubscript{t} reveals that Lys\textsubscript{276} and Lys\textsubscript{273} lie near to and possibly form ionic interactions with Asp\textsuperscript{227}. This observation suggests that the reason for accelerated nucleotide exchange rates caused by mutations of Lys\textsubscript{276} and Lys\textsubscript{273} might involve disruption of interactions with Asp\textsuperscript{227}. To test this hypothesis, the K276A and K273A mutations were combined with mutation of D227N to produce the K276A/D227N and K273A/D227N double mutants. If the effects of K276A were due solely to breaking of an interaction with Asp\textsuperscript{227}, then the Lys\textsubscript{276} mutation should not increase the rate of nucleotide exchange in the context of D227N or D227A mutants. Indeed, the increase in the basal rate of activation caused by the K276A and K273A mutations was reduced (from \textasciitilde5-fold to \textasciitilde2-fold relative to wild-type) when combined with D227N (Fig. 4). However, since the rate of the D227N mutant alone is 2-fold slower than that of wild-type, the effect of the K276A mutation is roughly the same (i.e. a \textasciitilde5-fold increase in rate) whether introduced into a wild-type or a D227N background. Similar results were obtained with a D227A mutation. Thus, the origin of the increase in basal nucleotide exchange rates by the K276A and K273A mutation is not merely due to disruption of interactions with Asp\textsuperscript{227}.

Lys\textsubscript{276} also appears to interact across the interdomain interface with S140. Replacement of Ser\textsuperscript{140} with alanine, which would have disrupted hydrogen bonding to K276A, did not affect the rate of activation. Thus, breaking of the putative Ser\textsuperscript{140}-Lys\textsubscript{276} interaction does not fully explain the effects of the K276A mutation. There appear to be other unidentified requirements for positively charged side chains in the aG region to maintain low basal rates of nucleotide exchange.

Mutation of Asp\textsuperscript{227} slows the rate of basal nucleotide exchange, both in the context of the wild-type protein as well as in the K276A mutant (Fig. 4). This is surprising since Gα\textsubscript{t} has an extremely low rate of basal nucleotide exchange, as is demanded by the low background noise required for sensitive light detection by photoreceptors. Other mutations in the Switch III regions also appear to slightly reduce the basal rate of nucleotide exchange, including M228L and V231A (Fig. 3; Table I). The origins of these effects, which are relatively small, are unclear. Previously, it has been demonstrated that the entire Switch III region can be deleted from Gα\textsubscript{t} without disrupting the ability to bind nucleotides (29). The rates of nucleotide exchange in these Switch III-deleted constructs were not characterized, but such studies might illuminate the role of Switch III in facilitating or impeding nucleotide exchange.

\textbf{Interdomain Interactions in Gα\textsubscript{t} Do Not Affect Basal or Rhodopsin-catalyzed Nucleotide Exchange Rates—When the structure of Gα\textsubscript{t} was determined, the nucleotide was found to reside in a deep cleft between the Ras-like domain and the helical domain (1). It was proposed that rhodopsin might accelerate the nucleotide exchange rate by opening the cleft (1, 30). Similarly, interactions between these domains could control the rate of basal nucleotide exchange. Gα\textsubscript{t} has a very low rate of basal nucleotide exchange as compared with related G proteins and a very high rate of R*\textsuperscript{a}-catalyzed exchange. If interdomain interactions were important mediators of either of these processes, one might expect nucleotide exchange rates in Gα\textsubscript{t} to be particularly sensitive to mutation of residues involved in those interactions. However, none of the Gα\textsubscript{t} mutants characterized in this report significantly affected either basal or R*\textsuperscript{a}-catalyzed rates.

Close analyses of G protein structures indicate that opening of the interdomain cleft is not necessarily an energetic barrier to nucleotide release. The helical domain does not contribute many contacts to the nucleotide binding pocket, and certain monomeric G proteins, which do not have a helical domain at all, release GDP more slowly than some heterotrimeric G proteins subtypes (31). A crystal structure of the Gα\textsubscript{t} mutant A326S, which releases GDP \textasciitilde250-fold faster than wild-type Gα\textsubscript{t}, does not reveal any alteration in the interdomain interactions, suggesting that an open cleft is not a prerequisite of fast nucleotide exchange (21). In addition, the reported increases in nucleotide release rates resulting from mutations at the interdomain interface of Gα34 and Gα12 are relatively modest (\textasciitilde5–10-fold) as compared with mutations in other regions of G proteins hypothesized to be involved in regulating nucleotide exchange rates. For example, we observed \textasciitilde150-fold increases in nucleotide exchange rates in mutations of certain residues of the o5-helix of Gα12, a structure implicated in the mechanism of rhodopsin-catalyzed activation (34). In summary, the opening of the interdomain cleft may not necessarily be a rate-determining step in nucleotide exchange in G proteins.

The \textbf{Function of Residues at the Interdomain Interface Differ} among Gα\textsubscript{a}, Gα\textsubscript{i1}, and Gα\textsubscript{g}.—Many of the residues mutated in this study, such as Ser\textsuperscript{140}, Glu\textsuperscript{143}, Met\textsuperscript{228}, and Val\textsuperscript{231}, have been previously found to alter basal or receptor-catalyzed nucleotide exchange rates in the related G proteins, Gα\textsubscript{a} and Gα\textsubscript{i1}. In Gα\textsubscript{a}, a mutation in the Switch III region, R258W, has been previously found to alter basal or receptor-catalyzed nucleotide exchange rates in the related G proteins, Gα\textsubscript{a} and Gα\textsubscript{i1}. In Gα\textsubscript{i1}, a mutation in the Switch III region, R258W (corre-
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...sponding to Val231 in Gαt), was found in a patient with Albright’s hereditary osteodystrophy (5). Biochemical studies indicated that replacement of Arg258 to tryptophan and to alanine, as well as alteration of a proposed interacting residue, Gln170 of the helical domain (corresponding to Gln143 in Gαt), led to increases in the basal nucleotide exchange rate (5, 6). These mutations were hypothesized to widen the interdomain cleft. Other studies in Gαt found that mutation of Arg258 or Asn167 (corresponding to Ser140 in Gαt) disrupted receptor-catalyzed activation (8), suggesting that the receptor induces structural changes that are communicated across the interdomain interface in Gαt. Mutation of either Leu232 or Arg144 (corresponding to Met228 and Ser140, respectively, in Gαt) increased the basal nucleotide exchange rate by disrupting a proposed interdomain hydrophobic interaction (4). The effects of mutating Arg144 were corroborated by the results of the R144S mutant in the current work (Fig. 5B).

The residues analogous to those proposed to interact with each other across the interdomain interface in Gαt and Gαi1 are also potentially interacting in Gαt (Fig. 1B). In many cases, however, the amino acids are not conserved. For example, Val231 and Gln143 of Gαt, which correspond to the proposed interaction between Arg258 and Gln170 in Gαt, are adjacent. Ser140 and Met228 (corresponding to Arg144 and Leu232 of Gαi1) and Ser140 and Asp227 (corresponding to the proposed interaction of Asn167 and Asn254 of Gαt (8)) are similarly adjacent. However, in contrast to the results with Gαt and Gαi1, replacement of these residues in Gαt did not affect nucleotide exchange rates. These results suggest that the interdomain interface residues are functionally different in Gαt than in Gαi1 and Gαt. Counterintuitively, Gαt and Gαi1, which exchange nucleotides faster than Gαt, appear to have tighter and more sensitive interactions across the interdomain interface than those of Gαt.

Both Lys273 and Lys276 of Gαt are conserved in Gαt. However, the structure of GDP-bound Gαt reveals that Lys280 (cognate to Lys276 of Gαt) is oriented towards the solvent instead of toward the Switch III region as in Gαt (Fig. 1C). Functionally, mutation of Lys280 and Lys277 to alanine did not lead to increases in nucleotide exchange rates in Gαt, as was observed in Gαt. Thus, conserved residues, Lys276 and Lys277 of Gαt, are found to serve different roles and to assume different structures in closely related G proteins.

In conclusion, the data in this report ascribe a role to Lys273 and Lys276 in the αG region of Gαt in maintaining low basal rates of nucleotide exchange. However, unlike in Gαi1 and Gαt, interactions that span the interdomain interface do not appear to be important in regulating either basal or rhodopsin-catalyzed nucleotide exchange rates. Differences exist in the organization of the interdomain interface among Gαt, Gαi1, and Gαs, and even between conserved residues in Gαi1 and Gαt.

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