to unusual mitochondrial evolution.

The deep split in coral mitochondrial lineages traces evolutionary events that predate skeleton formation and are thus invisible in the fossil record. Combined molecular and traditional analyses suggest that there was repeated evolution of the scleractinian skeleton in early seas. Understanding the selective scenario that led to such major convergent events may help to illuminate the evolutionary and ecological basis for the diversity of scleractinians and the complex ecosystems they support.

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11. Initial work with *Tubastraea coccinea* used mtDNA primers 16Sar and 16Sbr (*12*) and a hydroid primer [primer 1 (*13*)].
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14. Genomic DNA extractions were from living, frozen, or ethanol-preserved colonies (*8*, *12*). Highly enriched mtDNA was from the ProMega Wizard Minipreps system (*8*) [K. B. Beckman, M. F. Smith, C. Orrego, *Promega Notes* **43**, 10 (1993)]. Coral-specific primers (16Sd-L, 5′-GGTGAGACCTGCCCAATGGTT-3′, and 16Sc-H, 5′-AACAGCGCAATAACGTTTGAGAG-3′) amplified a 406- to 565-base pair (bp) segment (*8*), which was cloned and sequenced (*8*) with primers 16Sd-L, 16Sc-H, 16Se-H (5′-CGCCTTTAAAAAAGTAAC-3′), and 16Sf-L (5′-CTACATCCAAATTGTTAGAC-3′). Sequences have been deposited in GenBank (accession numbers L75992–L76024 and L76132). Replicate PCR sequences revealed most corals to be homoplasmic. However, three were heteroplasmic with infrequent amplifications of sequences from the opposite major clade. These results were repeatable from different DNA extractions and different colonies and were not found to result from contamination (*8*). The source of heteroplasmy of corals is unknown; hypotheses for the source include hybridization and unusual modes of mtDNA transmission (*8*).
15. Scleractinian sequences were aligned to each other and to sequences from a hydrozoan (*13*) and *Renilla* [D. Bridge, C. W. Cunningham, R. DeSalle, L. W. Buss, *Mol. Biol. Evol.* **12**, 679 (1995)], an anthozoan in the order Octocorallia. This alignment (577 bp, of which 315 bp are variable and 210 bp are informative) includes 16 insertion-deletion events, which are not used in phylogenetic analyses, that map onto clades observed on the phylogenetic tree.
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# Catalysis of Amide Proton Exchange by the Molecular Chaperones GroEL and SecB


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Hydrogen-deuterium exchange of 39 amide protons of *Bacillus amyloliquefaciens* ribonuclease (barnase) was analyzed by two-dimensional nuclear magnetic resonance in the presence of micromolar concentrations of the molecular chaperones GroEL and SecB. Both chaperones bound to native barnase under physiological conditions and catalyzed exchange of deeply buried amide protons with solvent. Such exchange required complete unfolding of barnase, which occurred in the complex with the chaperones. Subsequent collapse of unfolded barnase to the exchange-protected folding intermediate was markedly slowed in the presence of GroEL or SecB. Thus, both chaperones have the potential to correct misfolding in proteins by annealing.


Molecular chaperones contribute to the folding, assembly, and transport of proteins (*1*). Two homo-oligomeric molecular chaperones have been identified in *Escherichia coli*: the tetradecameric chaperonin GroEL (*2*) and the tetrameric SecB (*3*). GroEL is composed of 57-kD subunits, each of which has three functional domains, arranged as a hollow cylinder of two stacked rings with sevenfold symmetry (*4*). The SecB protein is composed of 17-kD subunits (*3*, *5*). It has been proposed that GroEL acts as a "folding cage" (*6*), in which aggregation of incompletely folded proteins is prevented (*7*). It has also been proposed that chaperonins act as "unfoldases" (*8*), using protein-protein binding energy to reverse incorrect interactions in proteins (*9*). A correction mechanism implies that GroEL would be able to bind a fully unfolded protein. Amide proton exchange has been used to analyze the GroEL-bound state of cyclophilin A (*10*) and α-lactalbumin (*11*), and, in both instances, has shown that the secondary structure of the GroEL-bound substrate is markedly destabilized. However, these and other studies (*12*) could not clearly define to what extent a protein is unfolded in the complex with a chaperone.

Here, we used barnase as a model substrate to investigate the GroEL- and SecB-bound conformations by nuclear magnetic resonance (NMR). This technique is a powerful tool for investigating both the structure and dynamics of proteins with high resolution (*13*). However, although small peptides have been studied with magnetization transfer (*14*), the large molecular mass of most of the chaperones does not allow direct observation of complexed polypeptide chains by NMR because of line broadening when bound to the slowly tumbling chaperone. Amide proton exchange detected by NMR may be applied to provide detailed information on the secondary and tertiary structure of a protein complexed with a chaperone. Chaperone-mediated unfolding of a protein should result in an increase in rate constants of amide proton exchange ($k_{ex}^{obs}$). Barnase is a particularly suitable substrate for such studies for two reasons. First, the amide protons that exchange in response to local fluctuations in structure and those that require complete unfolding for exchange to occur have been identified (*15*, *16*). Exchange of 15 of the 39 protected amide protons of barnase (Fig. 1A) occurs only from the fully unfolded state; these are termed globally exchanging protons. Most of these protons are located in the central β sheet of barnase. Sixteen amide protons exchange predominantly in response to local fluctuations or "breathing" of the native structure; these are referred to as locally exchanging protons and are mostly situated in the α helices and loop regions. Eight protons exchange by a mixture of the two mechanisms. The second reason that barnase is an appropriate sub-


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strate is that the binding of the enzyme to both GroEL and SecB is reversible at physiological pH and temperature (*17*), unlike the binding of cyclophilin A to GroEL (*10*), thus allowing turnover of substrate molecules during the exchange experiment and, consequently, the use of catalytic amounts of chaperone.

Exchange kinetics of locally and globally exchanging amide protons of $^{15}N$-labeled barnase in $D_2O$ result in an exponential decay in NMR signal intensity (Fig. 2C) (*15, 16*). In general, local exchange is faster than global exchange. Three important characteristics were apparent when amide proton exchange of barnase was determined in the presence of 0.002 to 0.009 molar equivalents of unlabeled GroEL (Fig. 2A) or SecB (Fig. 2B) (*18*):

1) There was an apparent decrease in the initial signal intensity of all the 39 measurable amide protons as a result of line broadening (Fig. 2), which increased with increasing chaperone concentration, a phenomenon noticed also for cyclophilin A (*10*). The line broadening was a specific effect as verified by the addition of 10 mM of the magnesium salt of adenosine diphosphate (MgADP), which decreases the affinity of GroEL for proteins (9) and, in this instance, restored much of the sharpness of the signal. The line broadening resulted from rapid binding of native barnase to and dissociation from the slowly tumbling chaperone on the NMR time scale (*13, 19*). A native barnase–chaperone complex with a dissociation constant in the millimolar range must exist to account for the observed line broadening.

2) The exchange rate constants of some of the amide protons that are known to exchange by global unfolding of the native protein increased. Exchange of the locally exchanging amide protons was less affected by GroEL and SecB. The relative observed exchange rate constants of the 39 measurable amide protons of barnase in the presence and absence of chaperone were assessed (Fig. 3). The presence of GroEL (Fig. 3A) or SecB (Fig. 3C) increased the exchange rate constants of the locally exchanging protons by at most a factor of 2 or 3. In contrast, exchange of the globally exchanging protons was increased by a factor of 4 to 25 by GroEL and by a factor of 3 to 15 by SecB. When the experiment with GroEL was performed under similar conditions but in the presence of 10 mM MgADP, the pattern of catalysis of amide proton exchange was similar but was char-

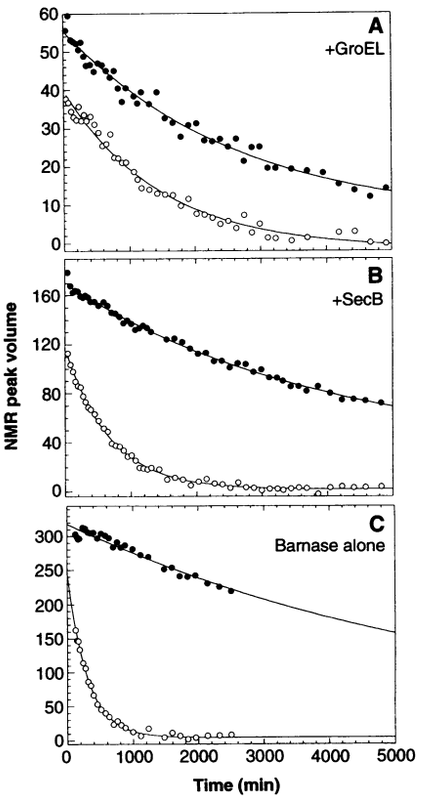


**Fig. 2.** Hydrogen-deuterium exchange of 2.4 mM barnase at 33°C in the presence of 5 μM GroEL (**A**) or SecB (**B**) or in the absence of chaperone (**C**). The time-dependent decreases in NMR peak volume of the backbone amide protons of residues Leu[14] (●), which exchanges by global unfolding, and Ala[11] (○), which exchanges by local unfolding, are shown. The pD values of the samples were 6.9 (A), 7.2 (B), and 7.6 (C). The rate of decay of Ala[11] was the same in all three experiments when corrected for pD. In contrast, the presence of chaperone increased the rate of exchange of the globally exchanging residue Leu[14]. For the exchange experiments in the absence of chaperone, 22 mg of $^{15}N$-labeled, lyophilized barnase was dissolved in 550 μl of NMR buffer [20 mM deuterated imidazole (pD 6.7) and 0.05% $NaN_3$ (*15, 16*), or 20 mM deuterated imidazole (pD 7.1), 0.05% $NaN_3$, and 10 mM MgADP], and the solution was centrifuged to remove insoluble protein, immediately transferred to an NMR tube, and placed in the magnetic field. For the exchange experiments in the presence of chaperones, unlabeled GroEL (±10 mM MgADP) or SecB and $^{15}N$-labeled barnase solutions, each in NMR buffer, were mixed and centrifuged before placing the sample in the magnet. $^1H$-$^{15}N$ heteronuclear single quantum coherence spectra were acquired on a Bruker AMX500 spectrometer as a series of 40-min experiments over 4 days. The time base for the decay of the signals was taken to be the middle of the acquisition time for each spectrum. The volume integrals of the cross peaks were calculated for each spectrum with the Bruker program UXNMR, and the decays were fitted to a single exponential decay with Kaleidagraph (Abelbeck Software). The final pD of the sample was measured at the temperature of the exchange experiment, taking into account the isotope effect: $pD = pH_{read} + 0.4$ (*31*). GroEL and barnase were expressed and purified as described (*32*). The cloning and purification of SecB will be described in (*29*).

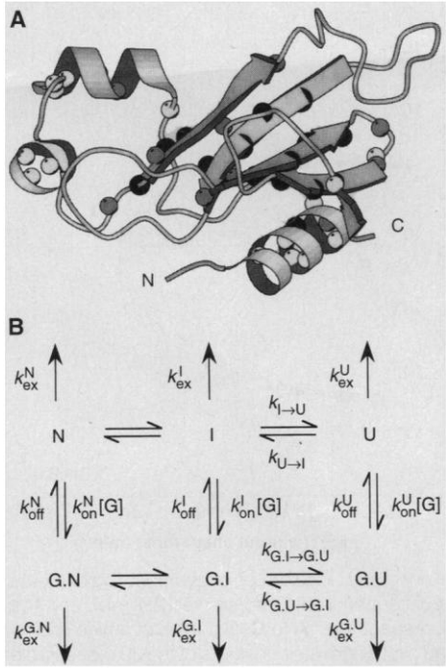


**Fig. 1.** (**A**) Structure of the polypeptide backbone of barnase with identification of amide protons that exchange by global unfolding (black) (residues 14, 25, 50, 52, 72 to 75, 88 to 91, and 97 to 99), local "breathing" (white) (residues 10 to 13, 16, 17, 26, 31, 33, 35, 45, 87, 94, 95, and 107), or a mixture of these mechanisms (gray) (residues 15, 30, 46, 49, 51, 53, 56, and 76) (*15, 16*). The locally exchanging indole NH proton of Trp[71] is not indicated. The figure was prepared with the program MOLSCRIPT (*30*). N and C, $NH_2$- and COOH-termini, respectively. (**B**) Minimal scheme for exchange of amide protons and deuterons at equilibrium. [G], [N], [I], and [U] are the concentrations of GroEL or SecB and of the unligated native, intermediate, and unfolded states of barnase, respectively. Because the total concentration of N, $[N]_{total}$, is much higher than those of the other reagents, $[N] \approx [N]_{total}$. The concentration of U is given by $[U] = K_{U\text{-}N}[N]$, where $K_{U\text{-}N}$ is the equilibrium constant for unfolding. Also, $[G.U] = [U][G]/K_d^{G.U}$, where $K_d^{G.U}$ is the dissociation constant. Combining the last three equations gives $[G.U] = [N]_{total}[G]K_{U\text{-}N}/K_d^{G.U}$. If the rate constants for interconversion of the folding states are higher than those for exchange (under EX2 conditions), then the observed rate constant for exchange is given simply by $k_{ex}^{obs} = (k_{ex}^{N}[N] + k_{ex}^{I}[I] + k_{ex}^{U}[U] + k_{ex}^{G.N}[G.N] + k_{ex}^{G.I}[G.I] + k_{ex}^{G.U}[G.U])/[N]$. If, however, the exchange rate constants are comparable to those for interconversion, then more complex equations must be used, and the rate law for exchange may depend on which is the fastest route from N to the exchange-competent folding state. For example, if G.U is generated fastest from the route G + U → G.U, then the rate of exchange via G.U is given by $k_{ex}^{obs}{}_{(G.U)} = k_{on}^{U}[G][U]k_{ex}^{G.U}/[N](k_{ex}^{G.U} + k_{off}^{U})$. Under EX1 conditions, $k_{ex}^{G.U} \gg k_{off}^{U}$, and so $k_{ex}^{obs}{}_{(G.U)} = k_{on}^{U}[G][U]/[N]$; that is, the rate-determining step for EX1 is the association of G and U, and therefore depends on [G]. The rate increases as the concentration of unligated G increases and that of ligated G decreases. If, on the other hand, G.U is generated primarily from the route G.I → G.U, then the rate of exchange via G.U is given by $k_{ex}^{obs}{}_{(G.U)} = k_{G.I\rightarrow G.U}[G.I]k_{ex}^{G.U}/[N](k_{ex}^{G.U} + k_{G.U\rightarrow G.I})$. Under EX1 conditions, $k_{ex}^{G.U} \gg k_{G.U\rightarrow G.I}$, and so $k_{ex}^{obs}{}_{(G.U)} = k_{G.I\rightarrow G.U}[G.I]/[N]$; that is, the rate increases as the concentration of [G.I] increases.

**Table 1.** Rate constants for exchange of globally exchanging amide protons of barnase in the presence of chaperone. Amide proton exchange experiments were performed as described in Fig. 2. The concentrations of barnase and MgADP were 2.4 and 10 mM, respectively. [Chaperone] is the total concentration of GroEL or SecB, and $k_{ex}^{obs}$ is the average of the observed EX1 exchange rate constants for the global residues. Values for $k_{ex}^{obs}$/[chaperone] increased with increasing pD and temperature ($T$), but to a lesser extent than the observed rate constant in the absence of chaperone; this finding indicated that exchange in the presence of chaperone does not depend on the intrinsic rate constant for exchange (*22*), in agreement with the proposed mechanism.

| Chaperone | $T$ (°C) | pD | [Chaperone] (μM) | $k_{ex}^{obs}$ ($10^{-4}$ $min^{-1}$) | $k_{ex}^{obs}$/[chaperone] ($M^{-1}$ $min^{-1}$) |
|---|---|---|---|---|---|
| GroEL | 33 | 6.9 | 5 | 3 | 60 |
| GroEL | 37 | 6.4 | 5 | 29 | 560 |
| GroEL | 37 | 6.8 | 5 | 46 | 870 |
| GroEL-ADP | 33 | 6.5 | 21 | 1 | 4 |
| GroEL-ADP | 33 | 6.6 | 11 | 1 | 8 |
| GroEL-ADP | 33 | 7.1 | 5 | 2 | 29 |
| SecB | 33 | 6.5 | 16 | 1 | 9 |
| SecB | 33 | 7.2 | 16 | 4 | 56 |
| SecB | 33 | 7.9 | 16 | 16 | 96 |

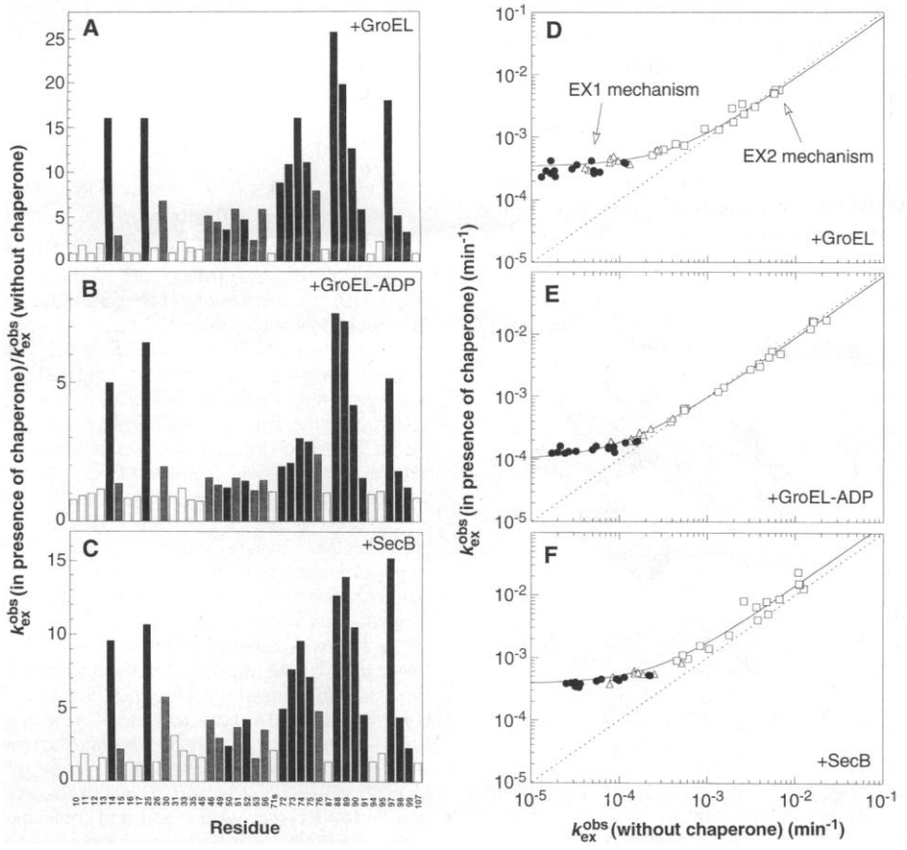


**Fig. 3.** Amide proton exchange of barnase in the presence and absence of chaperone. Experiments were performed at 33°C as described in Fig. 2. The concentration of barnase was 2.4 mM, and the concentrations of GroEL and SecB were 5 and 16 μM, respectively. (**A** to **C**) Catalysis of amide proton exchange by GroEL and SecB. The ratios of the observed exchange rate constants of the 39 measurable amide protons of barnase (Fig. 1A) in the presence of GroEL (A), GroEL-ADP (B), or SecB (C) to those in the absence of chaperone are shown. Locally and globally exchanging amide protons, and amide protons exchanging by a mixture of both mechanisms, are indicated by white, black, and gray bars, respectively. The values of the observed exchange rate constant in the absence of chaperone were corrected for pD to the pD of the experiment in the presence of chaperone with the use of the intrinsic rate (*22*). The indole NH proton of $Trp^{71}$ is indicated as 71s. (**D** to **F**) Amide proton exchange by an EX1 mechanism in the barnase-chaperone complex. The observed exchange rate constants of the 39 measurable amide protons of barnase in the presence of GroEL (D), GroEL-ADP (E), or SecB (F) are plotted against those in the absence of chaperone. Global (●), mixed (△), and local (□) amide protons are indicated. The dashed lines indicate what would be observed without any catalysis by the chaperones. Amide protons exchanging by an EX1 or an EX2 mechanism are indicated.

acterized by lower absolute values than those in the absence of nucleotide (Fig. 3B). Measurement of exchange at equilibrium in the absence of chaperone (*16*) revealed no detectable exchange from the folding intermediate of barnase (*20*). The observation that exchange of all the globally exchanging amide protons was catalyzed by GroEL, GroEL-ADP, and SecB indicates that the chaperones bind a fully unfolded state of barnase.

3) When the observed exchange rate constants in the presence of GroEL, GroEL-ADP, or SecB were plotted against those in the absence of chaperones (Fig. 3, D to F), the data points for the locally exchanging protons lay on a straight line with a slope of 1 and an intercept of 0, indicating negligible catalysis of their exchange. The observed rate constants for the 15 globally exchanging amide protons, however, tended to a constant, limiting value, which implied a changeover from an EX2 to an EX1 mechanism (*21*). In an EX2 mechanism, the observed rate constant for exchange is given by the product of the equilibrium constant for the formation of an unprotected state from the protected state, and the intrinsic rate constant for exchange ($k_{int}$). The value of $k_{int}$ varies for each site (*22*). The criterion for an EX2 mechanism is that the rate constant for reprotection is much greater than $k_{int}$. At the other extreme, when $k_{int}$ is much greater than the rate constant for reprotection, the mechanism becomes EX1, and the observed rate constant for exchange becomes independent of $k_{int}$ and is simply equal to the rate constant for the formation of the unprotected state. Exchange of all the measurable amide protons of barnase in the absence of chaperone occurs by an EX2 mechanism under these experimental conditions (*16*). Thus, the exchange mechanism in the presence of chaperone ranges from an EX2 mechanism for the faster, locally exchanging sites to an EX1 mechanism for the slower, globally exchanging sites (*23*), and this was observed under all the conditions tested (Table 1).

The complex (G.U) between fully unfolded barnase (U) and chaperone (G) can be generated by two routes in the scheme shown in Fig. 1B: G + U → G.U and G.I → G.U (*24*). We could distinguish between the two mechanisms from the effects of ADP, which increases the rate of folding of GroEL-bound barnase and increases the equilibrium constant for dissociation of bound protein without affecting the on-rate (*25*). The addition of 10 mM MgADP decreased the limiting value of the rate constant for the EX1 mechanism (Table 1). Thus, exchange took place after unfolding of the folding intermediate in the complex with the chaperone. The subsequent col-

lapse of the unfolded state to the folding intermediate was slowed by a factor of $>10^3$ by the chaperones (*26*).

The amide proton exchange data do not require that the major chaperone-bound state of barnase is the unfolded conformation; the data merely require that barnase becomes transiently fully unfolded in order for exchange to occur. Indeed, the kinetics of refolding of barnase in the presence of GroEL or SecB are consistent with the intermediate state being the major bound species (*27–29*). A small amount of transient unfolding, detectable by amide proton exchange, would not be detected in attempts to measure the gross unfolding of the protein by direct spectroscopic techniques (*7*, *9*, *12*), and transient unfolding is all that is required to correct misfolding. Such a transient unfolding is an annealing process. Our data are consistent with both chaperones having a dual role with regard to protein folding: (i) They recognize and bind to partially folded proteins. By this mechanism, GroEL prevents formation of irreversible aggregates, which would lead to off-pathway reactions (*7*), and SecB holds the substrate protein in a state competent for membrane transport (*3*). (ii) GroEL and SecB use protein-protein binding energy to denature the bound polypeptide chain to its fully unfolded state, allowing misfolded protein states to be corrected and guided back to the productive folding or transport pathway.

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17. S. Perrett, R. Zahn, G. Stenberg, A. R. Fersht, in preparation.
18. The concentrations of GroEL and SecB always refer to the oligomeric proteins.
19. It may seem strange that a small amount of GroEL or SecB can appreciably broaden the line widths in barnase. However, if the line width of a signal in free barnase is $W_F$ and that when the enzyme is bound to chaperone is $W_B$, and the fractions of free and complexed barnase are $f_F$ and $f_B$, respectively, then the line width that is observed if barnase associates and dissociates rapidly ($W_{obs}$) is given by $W_F f_F + W_B f_B$. Because $W_B$ is very large, it makes a substantial contribution to $W_{obs}$ even when $f_B$ is low.
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23. In the presence of chaperone, the observed exchange rate constant is given by the sum of the overall exchange rate constants for each of the various species present—namely, free native barnase $k^{obs}_{ex\,(N)}$, free folding intermediate $k^{obs}_{ex\,(I)}$, free unfolded barnase $k^{obs}_{ex\,(U)}$, and the respective chaperone-bound states $k^{obs}_{ex\,(G.N)}$, $k^{obs}_{ex\,(G.I)}$, and $k^{obs}_{ex\,(G.U)}$. The observed exchange rate constants of the locally exchanging amide protons are approximately equal to $k^{obs}_{ex\,(N)}$ in both the presence and absence of chaperones because it is by far the largest term at the low concentrations of chaperone used in the exchange experiments. In the presence of chaperone, the slowly exchanging amide protons tend to exchange with the single limiting rate constant $k^{obs}_{ex\,(G.U)}$.
24. The rate laws in Fig. 1B fit all the known data. If, however, the scheme is less complicated and G.N is converted to G.U directly, or if there is a more complicated scheme in which there is another state (G.X) from which exchange occurs, the same types of rate laws still apply.
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26. If, in order for an EX1 mechanism to occur, the lowest intrinsic rate constant for exchange (*22*) must be greater than five times the rate constant for the reversion of the unfolded state to the folding intermediate, then the rate constant of G.U → G.I must be $<20\ min^{-1}$. At the other extreme, and by a similar argument, the rate constant for the reversion of free barnase, exchanging under EX2 conditions, must be $>2 \times 10^4\ min^{-1}$.
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# Protein Kinase N (PKN) and PKN-Related Protein Rhophilin as Targets of Small GTPase Rho


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The Rho guanosine 5′-triphosphatase (GTPase) cycles between the active guanosine triphosphate (GTP)–bound form and the inactive guanosine diphosphate–bound form and regulates cell adhesion and cytokinesis, but how it exerts these actions is unknown. The yeast two-hybrid system was used to clone a complementary DNA for a protein (designated Rhophilin) that specifically bound to GTP-Rho. The Rho-binding domain of this protein has 40 percent identity with a putative regulatory domain of a protein kinase, PKN. PKN itself bound to GTP-Rho and was activated by this binding both in vitro and in vivo. This study indicates that a serine-threonine protein kinase is a Rho effector and presents an amino acid sequence motif for binding to GTP-Rho that may be shared by a family of Rho target proteins.


The Ras-related small GTPase Rho regulates certain types of actin-based cytoskeletal structures, including focal adhesions, stress fibers, and the contractile ring, and works as a switch in stimulus-evoked cell adhesion (*1*) and cytokinesis (*2*). Rho also functions to regulate smooth muscle contraction (*3*), transcription (*4*), and cell proliferation (*5*). However, the biochemical mechanism of these actions is unknown. Although protein kinases (*6*), lipid kinases, and a phospholipase (*7*) have been implicated in the Rho signaling pathway, the direct binding and activation of these molecules by activated GTP-bound Rho has not been demonstrated.

To isolate complementary DNAs (cDNAs) encoding proteins that bound to GTP-Rho, human RhoA truncated at


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