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Fundamental Link between Folding States and Functional States of Proteins

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Abstract: Folding and function of proteins are two aspects of proteins which are usually considered as basically unrelated phenomena that are optimized by evolution independently. From the funnel model of folding/unfolding and the associated energy landscape, we infer the paradigm that the minimum number of folding intermediates is determined by the number of all functional states of a protein ("essential" folding intermediates). Here, we demonstrate the supposed fundamental link using the Ras protein complexed with the GTP analogue GppNHp that occurs in two structural states coexisting in solution. State 2 was shown earlier to represent the effector interacting state, and the function of state 1 was hitherto unknown. By ^{31}P NMR spectroscopy, we demonstrate that state 1 represents the conformation interacting with guanine nucleotide exchange factors (GEFs). Denaturation experiments of the protein with a chaotropic reagent show that both functional states coexist during folding and unfolding. Application of high pressure represents another perturbation of the energy landscape, leading to an increased population of the state 1 as observed by NMR spectroscopy. The specific volume difference between the two states ΔV_{12} is 17.2 $\pm$ 0.5 mL mol^{-1}, indicating that state 1 represents a more open conformation of the protein. The free energies of stabilization for state 1 and state 2 at 278 K can be determined as 8.3 and 9.8 kJ mol^{-1}, respectively.

Introduction

The theory of protein folding is a well-developed field in protein science (see Onuchic et al.[1]), where in the past, various models were proposed which differ essentially in the focus on special aspects of protein folding but not in the general theory. The funnel model[2,3] allows one to describe all of these theories in a common picture. In this picture, the reduced Gibbs free energy of stabilization G_{micro} is plotted as a function of a general conformational variable Φ, which is projected for the sake of simplicity to one or two dimensions. Functional aspects of proteins, such as conformational changes of proteins in protein−protein interaction, are usually not discussed in this framework but are the domain of structural biology. In fact, the scientific community involved in these two fields has developed largely independently in history. Outside the folding community, a similar description, the energy landscape concept based on the same basic thermodynamic principles, has been developed at the same time by Frauenfelder and Wolynes.[4] They show that the energy landscape is rugged but has also deeper energy minima they call taxonomic states. This concept has been studied experimentally in detail on myoglobin,[5,6] and a very recent paper also shows the connection with protein dynamics.[7]

A consequence of classical thermodynamics especially well-described by the energy landscape model is the fact that all possible conformational states of a protein with inclusion of all functional states are coexisting in solution. Generalization of the two-state Monod−Wyman−Changeux model for cooperative binding,[8] which assumes that structural states with different ligand binding affinities are coexisting and are only selected by ligand binding, leads to a picture where these preformed binding states are part of the energy landscape that is rapidly scanned by the protein. A well-studied early experimental example is the Ras protein, where such a model was applied for the explanation of the experimental data. Here, two conformational states, state 1 and state 2, were detected by NMR spectroscopy;[9] state 2 was immediately identified as the effector binding state, and the nature of state 1 was not clear until now.

Very recently, the energy landscape model including preformed structural states has become rather popular, and different aspects of this model have been reported in excellent reviews. There intermediates seem to be ubiquitous species on folding energy landscapes.[10] Ligand binding effects on the energy landscape of a protein were recently reviewed by Lee and Craik.[11] PDZ and SH3 domains are examples for a pretransition

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state prior to ligand binding and finally undergo a conformational change to the final complex upon binding.[12] It has been shown that protein dynamics is linked to enzyme catalysis[13,14] as well as to signaling pathways[15] and allosteric ligand binding.[16] Especially the ability of signaling proteins to respond to signals and pass them on depends on the features of the energy landscape where the functional states crucial for signaling seem to be located in the lower energy regions of the overall folding landscape, that is, they can be regarded as excited states. The ability of proteins to adapt and evolve new functions or dysfunctions[17] seems to depend on packing modes of proteins; that is, poorly packed, disordered, and conformationally diverse proteins are evolutionary wanted for selection.[18]

There are four main features to optimize simultaneously when nature has to evolve sequences of soluble proteins: (I) The protein must fulfill its biological function, which in most cases includes multiple conformational states and the interaction with other proteins and/or ligands. (II) It must fold properly under *in vivo* conditions. (III) It must be stable enough to fulfill its function in a given environment and for a given lifetime. (IV) It should not aggregate unspecifically with itself or with the other proteins which are present in solution in extremely high concentrations[19] (total protein and RNA concentration inside a cell of *Escherichia coli* is about 300−400 mg/mL; see Ellis[20]). Other properties such as localization may pose additional constraints to the evolution.

In the present paper, we want to unify important aspects of folding, function, and evolution in the picture of energy landscape and apply it to the Ras protein. Since the nature of the second conformational state of Ras could not be revealed until now, we will also present experimental data that clarify its role.

Methods

Protein Purification. Wild-type and T35S mutant of Ras(1−189) were expressed in *Escherichia coli* and purified as described before.[21] Nucleotide exchange to GppNHp was done using alkaline phosphatase treatment as described by John et al.[22] Free nucleotide and phosphates were removed by gel filtration. The final purity of the protein was >95% as judged by SDS poly acrylamide gel electrophoresis. Ras binding domain of c-Raf-1 (aa 51−131) was prepared as described before.[23] Sos mutant (W729E) (aa 564−1049) was expressed and purified as described by Sondermann et al.[24]

Protein Denaturation Experiments with Guanidine Hydrochloride. To 1.5 mM Ras in 40 mM Hepes/NaOH pH 7.4, 10 mM $MgCl_2$, 2 mM DTE, 10% D_2O, and 0.1 mM DSS, 3 or 5 M GdmCl in same buffer was titrated. Care was taken that pH of the solutions was adjusted. Before performing the measurement, the sample was incubated for 45 min.

NMR Spectroscopy. High-pressure NMR spectra were recorded with a Bruker DRX-600 NMR spectrometer operating at ^{1}H and ^{31}P resonance frequencies of 600.1 and 242.9 MHz, respectively. ^{31}P NMR measurements were performed in a 5 mm selective phosphorus probe. High-pressure NMR spectroscopy was performed with an on-line high-pressure system using a high-sensitivity sapphire cell developed in our laboratory.[25] Typically, 2−3 mM Ras protein solutions in buffer A (40 mM Hepes/NaOH pH 7.4, 10 mM $MgCl_2$, 2 mM DTE) contained 10% D_2O as a lock signal; 0.1 mM DSS was added to calibrate the spectra by indirect referencing. A Ξ value of 0.4048073561 was used for phosphorus referencing as reported by Maurer and Kalbitzer.[26] GdmCl titration measurements were performed with a Bruker Avance 500 spectrometer using a selective 10 mm ^{31}P probe and 10 mm Shigemi tubes at a spectrometer frequency of 500.1 MHz for protons and 202.4 MHz for ^{31}P, respectively. Measurements were performed using 70° pulses and a total repetition time of 7 s. Protons were decoupled during data acquisition. The temperature was 278 K.

Data Evaluation. Equilibrium constants were obtained fitting Lorentzian functions to the resonances. Under conditions used, the integrals are proportional to the concentration of the nuclei in the sample. The equilibrium constant is defined as $K_{12} = [2]/[1]$. The pressure dependence of the equilibrium constant K_{12} is defined as $K_{12} = \exp(-\Delta G_{12}/RT)$ with $\Delta G_{12} = \Delta G^0{}_{12} + \Delta V_{12}(p - p_0) + {}^1/_2\Delta\beta_{12}(p - p_0)^2$. ΔG^0 is the free energy at ambient pressure; ΔV_{12} is the partial molar volume difference with $\Delta V_{12} = V_2 - V_1$, p the pressure, and $\Delta\beta_{12}$ the corresponding compressibility difference. For the fit of the data, the term in quadratic p has been neglected. As usual, the dependence of the free energy difference ΔG_{ab} for a transition between states a and b on the concentration c of the denaturant was approximated by $\Delta G_{ab} = \Delta G_{ab}{}^0 + m_{ab}c$, with m_{ab} a constant of proportionality, the equilibrium m-value.[27,28]

Results and Discussion

1. The Hypothesis. The funnel model for folding and unfolding of proteins connects in an easily understandable way the thermodynamics of folding with the polypeptide structure. Several different representations of the folding funnel were proposed in literature, Dill and Chan[2] plot ΔG_{micro}, the free energy reduced by the conformational entropy as a function of the spatial coordinate Φ. The spatial coordinate Φ is usually projected into one or two dimensions for a graphical representation (Figure 1). In this picture, all specific folding models can, in principle, be represented. The native, lowest energy state of the protein is depicted as the global minimum of the funnel. Folding intermediates are represented as local minima which can widely differ in depth and shape (Figure 1). In thermal equilibrium, the whole surface of the n-dimensional funnel is populated with populations determined by Boltzmann's law. As long as a perturbation does not change the shape of the funnel, folding and unfolding are equivalent processes. This also means that any local minimum (intermediate state) is populated in thermal equilibrium and is accessible in some extent during unfolding and folding.

When assuming that, from the point of view of protein conformational space, all functional states have to be preformed (=sterically possible) and thus have principally to coexist in solution, as a direct inference from the funnel model of folding/

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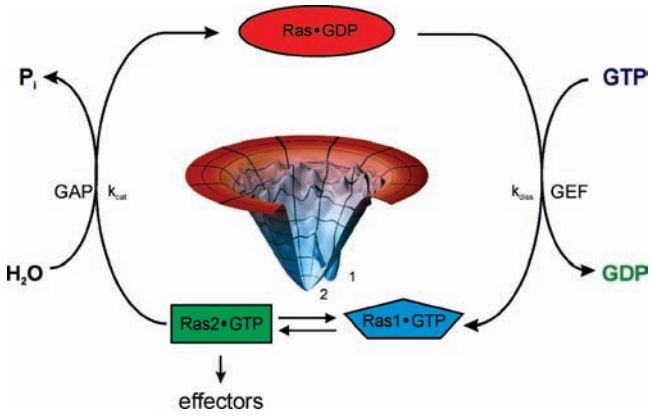

Figure 1. Functional states of Ras and its relation to the folding funnel. The Ras cycle including the functional states 1 and 2 of Ras and schematic folding funnel is shown. Here, the free energy of the microstates $G_{micro}(\Phi)$ is plotted for two components of the conformational space Φ. For Ras·Mg^{2+}·GppNHp at 278 K, the difference of the free energies ΔG_{12} is -1.48 kJ mol^{-1} and the difference of the free activation energies $\Delta G_{12}^{\ddagger}$ is 42 kJ mol^{-1}.[34,41] GAP, GTPase activating protein; GEF, guanine nucleotide exchange factor; Ras1 and Ras2, Ras in conformational state 1 and state 2.

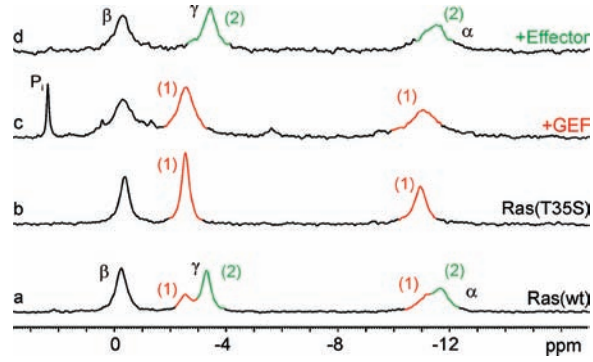

Figure 2. Main functional states of Ras·Mg^{2+}·GppNHp as detected by ^{31}P NMR spectroscopy. The samples contained 1 mM Ras·Mg^{2+}·GppNHp in buffer A (40 mM Hepes/NaOH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM DTE). Ras(wt)·Mg^{2+}·GppNHp (spectrum a), Ras(T35S)·Mg^{2+}·GppNHp (spectrum b), Ras(T35S) Mg^{2+} GppNHp in complex with Sos(W729E) (spectrum c), Ras(T35S) Mg^{2+} GppNHp in complex with Raf-RBD (spectrum d).

unfolding, one can conclude that all functional states of the protein should also be folding/unfolding intermediates. The direct conclusion is that the minimum number of folding intermediates is given by the number of functional states required. They present essential folding intermediates. However, not all folding states (optional folding intermediates) have to be simultaneously important for function. In nonequilibrium experiments, the practical importance of these states depends also on kinetic parameters as well as on details of the perturbation itself. In many cases, they may also not be detected in folding/unfolding experiments since either their relative populations are too small to be observed by standard methods or since the method used is not sufficiently sensitive for their detection. The latter may be the case if, for example, the environment of the fluorescence tag used does not change in two native states or if, in CD experiments, the relative contributions of secondary elements are unchanged in the two states.

A general connection between protein folding, conformational dynamics, and function has been observed earlier in different systems and shows that this is a more general property of proteins not restricted to the Ras protein considered here. However, this connection has been mainly described under the aspect of evolutionary competition not as a basic phenomenon. It is also sometimes called as negative design when, for example, the formation of folding intermediates may lead to aggregation and amyloid formation.[29−31] A recently published example is the folding of the colicin binding protein Im7[32] that shows a highly populated on-pathway folding intermediate. Many residues that are forming the non-native contacts are involved in the recognition of colicin toxins.[33] A similar conformer may be involved in the suggested dual recognition mechanism of toxins.

2. Experimental Test System, the Ras-Dependent Signal Transduction. For an experimental test of the above inferences, the Ras protein was used, which functions as a central molecular switch in cellular signal transduction. The Ras protein is the prototype member of the Ras superfamily with more than 100 different guanine nucleotide-binding (GNB) proteins of different subfamilies. The GNB proteins regulate a diverse array of signal transduction reactions and/or transport processes in cells. They cycle between two main structural states, stabilized by GDP and GTP, respectively. In the GDP bound state (Figure 1), the affinity to effectors is low, and in the GTP bound state, the affinity to effector proteins is high and thus Ras can transmit a signal induced by an exchange of GDP by GTP by the guanine nucleotide exchange factors (GEFs). The activation cycle is finished by GTP hydrolysis catalyzed by GTPase activating proteins (GAPs). In light of the multiple state model, as a direct consequence of that cycle, at least three conformational substates of Ras·Mg^{2+}·GTP must coexist in solution, states that correspond to the complexes with GEFs, effectors, and GAPs. Two main conformational states (state 1 and state 2) of Ras·Mg^{2+}·GppNHp can be directly observed by ^{31}P NMR spectroscopy, which are characterized by different chemical shift values for the resonances of the α- and γ-phosphate groups (Figure 2a).[9,34] In wild-type protein, the relative concentration of state 2 to state 1 is 1.9. In the partial loss-of-function mutant Ras(T35S), the equilibrium is shifted almost completely to state 1 (Figure 2b).[34,35] It has been shown by NMR spectroscopy as well as kinetic experiments that Ras can properly interact with effector proteins only in state 2.[9,34,35] Addition of effectors such as Raf kinase leads to a reduction of the NMR lines assigned to state 1 and an increase of that corresponding to state 2 (Figure 2b,d). The biological function of state 1 observable by NMR spectroscopy was for a long time a puzzling question but represents most probably a functional state. However, indepen-

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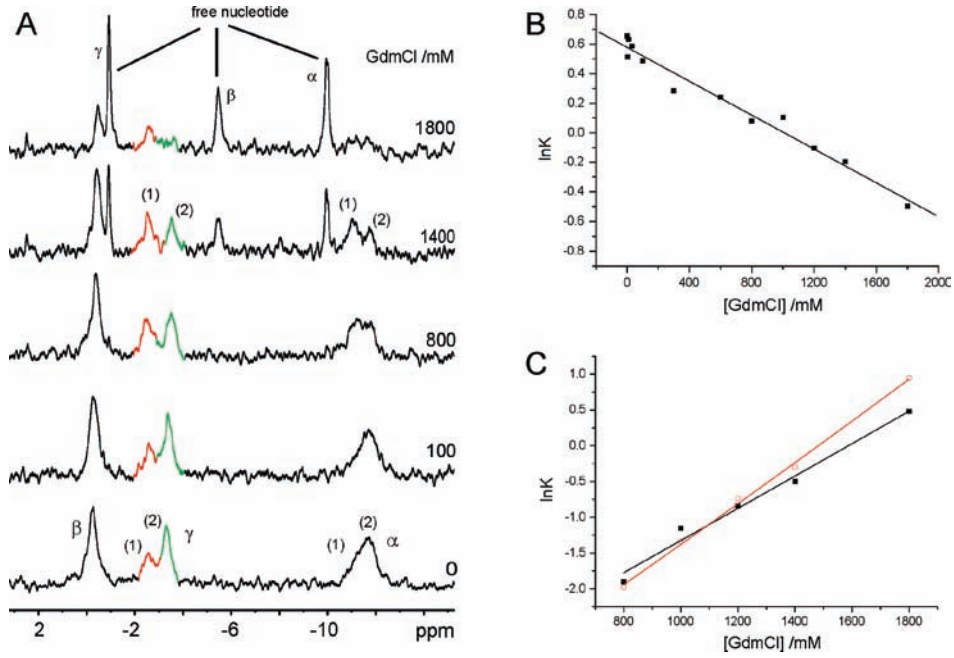

Figure 3. Conformational equilibria in the presence of GdmCl. (A) Dependence of the conformational equilibria of Ras·Mg^{2+}·GppNHp on the concentration c of GdmCl at 278 K as obtained by the ^{31}P NMR spectroscopy. The sample contained 1.4 mM Ras in 40 mM Hepes/NaOH pH 7.4, 10 mM $MgCl_2$, 2 mM DTE, 10% D_2O, and 0.1 mM DSS. The concentration of GdmCl is indicated. (***B***) Plot of ln K_{12} with K_{12} = [state 2]/[state 1] on the concentration of GdmCl. The function was fitted assuming a linear dependence of ΔG on the GdmCl concentration ($\Delta G_{12}{}^0 = -1.48$ kJ mol^{-1}; $m_{12} = 0.84 \pm 0.03$ kJ mol^{-1} M^{-1}). (C) Plot of ln K_{D1} (black squares) and ln K_{D2} (red circles) between denatured protein [D] (represented by the signal of released nucleotide) and Ras protein in state 1 or state 2, respectively, on the concentration of GdmCl. Using a linear fit of the data resulted in $\Delta G_{1D}{}^0 = 8.3$ kJ mol^{-1}, $m_{1D} = -19.1 \pm 1.3$ kJ mol^{-1} M^{-1} for ln K_{D1} and $\Delta G_{2D}{}^0 = 9.8$ kJ mol^{-1}, $m_{2D} = -28.4 \pm 1.9$ kJ mol^{-1} M^{-1} for ln K_{D2}.

dent of the specific function of state 1, its stabilization by small ligands enables a novel approach to weaken the Ras effector interaction and thus to interrupt the signal transduction via oncogenic Ras.[36]

3. Biological Function of State 1 in Ras Nucleotide Triphosphate Complexes. For using the Ras system as a test system, the function of state 1 has to be elucidated. Recently, it has been suggested that state 1 may correspond to the GEF binding state.[37] Figure 2 shows that this is indeed the case: The wild-type guanine nucleotide exchange factor Sos (son-of-sevenless) contains a catalytic and a regulatory Ras-binding site,[38] but the mutant Sos(W729E) used here has only the catalytic binding site.[24] As it is apparent from Figure 2c, Sos(W729E) binds only to state 1 because, exclusively, the correspondent ^{31}P resonance lines are influenced by the addition of the exchange factor that introduces a strong, selective line broadening caused by the increase of molecular mass by the complex formation with Sos.

4. Detection of the Two Conformational States in the Folding−Unfolding Energy Landscape. If our hypothesis is correct that functional states are part of the unfolding and folding pathway, they should be present at least in small populations also in standard unfolding experiments. Therefore, we studied the unfolding with one of the most commonly used chaotropic reagent, GdmCl, by NMR spectroscopy (Figure 3). As required from our theoretical considerations, at intermediate concentrations, the population of state 1 increases again with the concentration of GdmCl. In addition, at higher concentrations of GdmCl, the signals of free Mg^{2+}·GppNHp also become observable. This is indicative for a beginning denaturation of the protein leading to a release of the bound ligand. The unfolding experiment can also be performed in the inverse manner by diluting a Ras solution containing a high concentration of GdmCl stepwise. As to be expected, the relative population of state 1 decreases with decreasing concentration of GdmCl, and at lower concentrations of GdmCl, again state 2 dominates (data not shown).

It could be possible (but is not very likely) that the conformation induced by the addition of GdmCl would have a similar ^{31}P NMR spectrum as state 1, although it corresponds to a partly denatured state. Complete denaturation leads to a different, characteristic ^{31}P NMR spectrum (Figure 3) since here the signal of Mg^{2+}·GppNHp released from the protein can be observed (Figure 3). Such a spectrum can be observed at GdmCl concentrations above 700 mM for the wild-type protein. When performing the same experiments with the Ras mutant Ras(T35S), which exists predominantly in conformational state 1, the spectral changes are more easy to interpret. At GdmCl concentrations up to 700 mM, spectral changes are not detectable in the ^{31}P NMR spectrum (Figure 4A) as well as in the ^{1}H NMR spectrum (not shown). This means the protein stays in the structural state it has been in the absence of GdmCl, namely, state 1. As in wild-type protein, at concentrations higher than 700 mM GdmCl, free Mg^{2+}·GppNHp can be observed (Figure 4A). This makes it quite unlikely that GdmCl creates a new state in wild-type protein but not in the mutant protein.

Before denaturation, the wild-type protein behaves in the presence of GdmCl as in the absence of GdmCl: when the effector Raf−RBD is added at a GdmCl concentration of 1 M, the equilibrium between states 1 and 2 is shifted from $K_{12} = 1$ again almost completely to state 2 (Figure 4B). The nucleotide

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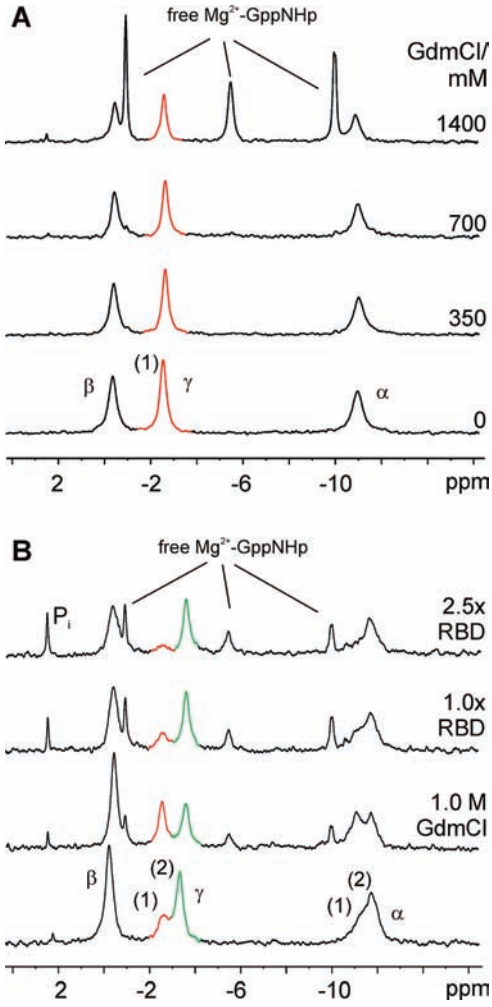

Figure 4. Effect of GdmCl on the state 1 mutant Ras(T35S) and its effector interaction. (A) To 2.2 mM Ras(T35S)·Mg^{2+}·GppNHp in buffer A (40 mM Hepes/NaOH pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 2 mM DTE, 10% D_2O, and 0.1 mM DSS) were added appropriate aliquots of GdmCl in buffer A. The final concentrations of GdmCl are indicated. (B) To initially 1.3 mM Ras(wt)·Mg^{2+}·GppNHp in buffer A (bottom spectrum) were added appropriate aliquots of GdmCl in buffer A. Afterward, a highly concentrated Raf−RBD solution in buffer A containing 1 M GdmCl was added in 1-fold and 2.5-fold molar excess. All measurements were performed at 278 K.

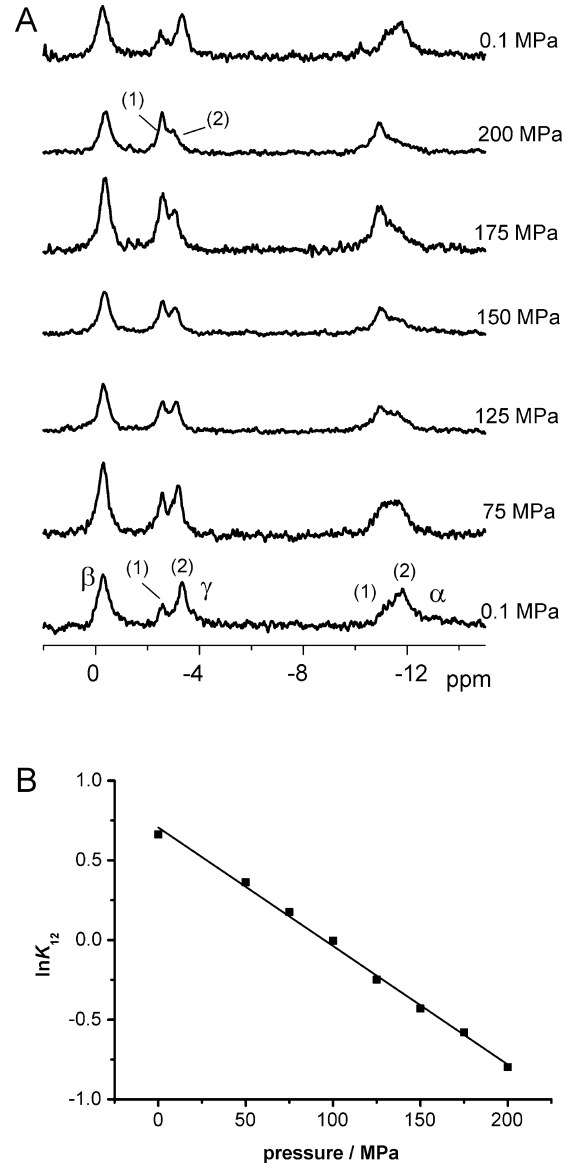

Figure 5. Conformational equilibria as a function of pressure. (A) Pressure dependence of the conformational equilibria as obtained by the ^{31}P NMR signals of Ras(wt)·Mg^{2+}·GppNHp at 278 K. The sample contained 3 mM Ras in buffer A. The respective pressure is indicated. (B) Plot of the ln K_{12} (K_{12}, equilibrium constant between state 1 and 2) as a function of pressure p. A fit of the data gives a specific molar volume change ΔV_{12} of 17.2 ± 0.5 mL mol^{-1}.

released cannot be recovered by the addition of Ras−RBD; that is, a renaturation does not occur after addition of Raf−RBD. In summary, the assumption that GdmCl only shifts the equilibrium between states 1 and 2 most probably holds.

5. Perturbation of the Folding Funnel by Application of Pressure. Application of pressure to proteins represents a new tool to study intermediate states of proteins in solution since it modifies only slightly the fundamental conformations of a protein but changes the population of conformational states by selecting structures with smaller specific volumes V^0 (for recent reviews, see refs 39, 40); that is, "excited" states (states with lower free energy of stabilization at normal pressure) can be detected. Because the direct structural changes induced by pressure are rather small (the typical volume change at 200 MPa is about 0.3%), it is rather straightforward to extrapolate the ambient-pressure structure from the observed high-pressure structures.

Because of its low magnetogyric ratio and the lack of directly bonded protons allowing indirect detection, phosphorus resonance spectroscopy is an inherently insensitive method. With the small volumes of standard glass high-pressure cell, nucleotides bound to a protein would not be detectable with sufficient sensitivity even at the concentrations of Ras of approximately 2 mM used here. However, the newly developed sapphire cell system[25] allows the recording of sufficient spectra (Figure 5A) at a ^{31}P NMR resonance frequency of 242.9 MHz. At low temperature, two sets of resonance lines corresponding to the three phosphate groups of the bound GTP analogue GppNHp can be observed, which coalesce at increasing temperatures. At 278 K, the free energy of activation for the transition $\Delta G_{12}^{\ddagger}$

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between the two states is 42 kJ mol^{-1} and the Gibbs energy ΔG_{12} is -1.48 kJ mol^{-1}, favoring state 2.[34,41] With increasing pressure, the equilibrium between the two states is shifted to state 1 (Figure 5A). At the maximum pressure of 200 MPa obtainable in the sapphire system, the equilibrium between the two states is inversed, leading to a substantial decrease of K_{12} from 1.9 to 0.44. A plot of ln K_{12} as a function of pressure p is shown in Figure 5B. A fit of the data gives a difference of specific volumes $\Delta V_{12} = V_2 - V_1$ for the two states of 17.2 ± 0.5 mL mol^{-1}.

Pressure usually favors structures with higher water-exposed surfaces since the density of bound water is smaller than that of the bulk water. This means that state 1 is most probably characterized by a structure with enlarged protein surface; a possible mechanism would be a partial opening of the nucleotide binding pocket as it is necessary for the GEF-catalyzed nucleotide released.

6. General Consequences of the Paradigm. We have shown that the two conformational states experimentally observed by ^{31}P NMR spectroscopy are both functional states of the Ras protein; state 1 is the interaction state with guanine exchange factors (GEF), and state 2 is the interaction state with effectors. According to theory and experiment, they are also essential folding/unfolding states. Thus the conformational states occurring in folding and required for function are closely related. The question arises of why the link between folding states and functional states has not been explicitly described earlier. From theory, one would conclude the following: When the essential intermediate states cannot be detected, it is only a sign that the experimental method used was not adequate (sensitive enough) for this purpose. Conversely, folding/unfolding experiments can be used to find possible new functional states; in fact, in some respect, it is already done by the use of high-pressure spectroscopy. The identification of these states may be especially important to find inactive states for drug design.[34,42] Another hypothesis can be deduced with regard to protein evolution. Since functional states and essential folding states are directly coupled, evolution has to optimize two features simultaneously, a smooth folding funnel and different functional states. When this cannot be done satisfactorily for both features, then optimization of function has the higher priority. For proper folding of specialized, well-known proteins, so-called chaperones are then required. An important example here is actin, a multipurpose protein occurring in monomeric and filamentous form and interacting with a multitude of proteins. In the absence of the specialized chaperone system of prefoldin[43] and TriC,[44] only misfolded actin can be found.

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Supporting Information Available: Complete ref 6. This material is available free of charge via the Internet at http://pubs.acs.org.

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