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Asymmetric Kinetics of Protein Structural Changes

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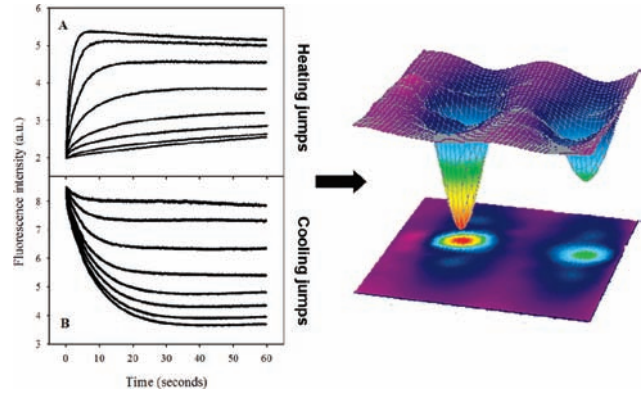

Thermodynamic and kinetic understanding of structural transformations in proteins is critical to new developments in medicine and biotechnology. These fields often require the design of mechanism-based modulators of protein function. Researchers increasingly consider these structural changes—such as folding/unfolding or shuttling between active and inactive states—within the energy landscape concept that supposes a high-dimensional, rugged conformational surface. The unevenness, or asperity, of this conformational surface results from energetic barriers and kinetic traps. However, for a large number of protein reactions, such as reversible folding/unfolding, the literature only reports simple two-state transitions, which calls into question the use of a more complex energy landscape model. The question is: are these reactions really that simple, or are we misled by a biased experimental approach? In this Account, we argue in favor of the latter possibility. Indeed, the frequently employed temperature-jump method only allows recording protein structure changes in the heating direction. Under those conditions, it might not be possible to detect other kinetic pathways that could have been taken in the cooling direction.

Recently, however, we have developed bidirectional pressure- and temperature-jump methods, which can offer new insights. Here, we show the potential of these methods both for studying protein folding/unfolding reactions, taking ribonuclease A as model, and for studying functionally relevant protein conformational changes, using the open/closed allosteric transition of tryptophan synthase. For example, the heating and cooling temperature-jump induced kinetics involved in the folding/unfolding conformational surface of ribonuclease A is illustrated above.

In both of our model systems, the kinetic transition states of several reaction steps were path-dependent, i.e. the rates and thermodynamic activation parameters depend on the direction of the applied pressure and temperature perturbation. This asymmetry suggests that proteins cope with external stress by adapting their structure to form different ensembles of conformational substates. These states are distinguished by their activation enthalpy and entropy barriers, which can be strongly negative in the folding direction. Based on our analysis of activation compressibility and heat capacity, hydration and packing defects of the kinetic transition states are also very important for determining the reaction path. We expect that a more generalized use of this experimental approach should allow researchers to obtain greater insight into the mechanisms of physiologically relevant protein structural changes.

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Introduction

Protein structural changes are macromolecular reactions which can comprise up to hundreds of individual noncovalent bond-breaking and bond-forming reactions. Examples are protein folding/unfolding reactions and enzyme structural changes shuttling between active and inactive states. The mechanism of such reactions may therefore be expected to be considerably more complex than that of chemical reactions of small molecules. Surprisingly, this is not observed. Indeed, the majority of protein kinetics obeys seemingly simple Arrhenius behavior, without reflecting the complexity of the occurring structural changes. Several reasons may explain this apparent simplicity: (i) protein reactions are mostly cooperative; (ii) usually only rate limiting reaction steps can be observed; (iii) deviations from simplicity, due to stepwise reacting single protein molecules,[1] are averaged out, because experimental constraints generally require working with micro- or millimolar protein concentrations.

In contrast, theoretical progress achieved within the last 2 decades predicts that protein structural changes must be considered in the frame of energy landscapes.[2] This concept supposes a two- or more-dimensional rugged energy surface, the asperity of which is due to energetic barriers and kinetic traps. The validity of this theory has been proven in a large number of investigations of ultra fast kinetics of protein structural changes.[3−6] Furthermore, investigation of binding of small ligands to protein, e.g. in flash-photolysis experiments, has led to a wealth of information about multiple hierarchical conformational substates.[7] Moreover, the generally irreversible protein aggregation reactions may also be seen as consistent with the concept of complex multidimensional reactions, since their kinetics are strongly dependent on a large number of external conditions.[8] There is however a difficulty for applying the energy landscape concept to the vast majority of physiologically relevant protein structural changes, such as some protein folding/unfolding reactions and protein conformational changes shuttling between active and inactive enzymes. These reactions are often reversible and they are occurring within a moderate time scale—usually within milliseconds or seconds.

The kinetics of these reactions can be studied by using different perturbation techniques, such as stopped-flow or temperature-jump (*T*-jump) induced by an infrared laser pulse. However, the resulting relaxation kinetics is usually deceivingly simple. We hypothesized that the inability to detect "asperities" of an energy landscape in such experiments is inherent to the chosen perturbation method. Due to technical constraints, with the exception of double-jump dilution stopped-flow experiments,[9] the reaction kinetics are always followed in the same direction, i.e., in the sense of protein unfolding. It is therefore not surprising that generally only rate limiting steps in the unfolding reaction are observed.

However, in complex energy landscapes it is likely that the speed of a reaction depends on which path has been taken. In order to verify experimentally this hypothesis, and to investigate the structural and energetic features of these surfaces, it would be interesting to record protein transformation kinetics in different directions, under otherwise identical final conditions. For this purpose, two experimental methods have been developed: bidirectional pressure-jump (*p*-jump) and, very recently, bidirectional temperature-jump.

In this Account we are reporting the use of bidirectional *p*- and *T*-jump devices to study two protein reactions: (i) folding and unfolding of ribonuclease A (RNase A), and (ii) the transition between the open and closed conformations of tryptophan (Trp) synthase. The use of both techniques allowed detection of path-dependent protein relaxation kinetics. The two physical perturbation parameters, pressure and temperature, appeared to affect specifically the energy landscape of both protein systems and helped in the selective analysis of different reaction paths within this landscape. The results confirm the potential of this new approach to study experimentally multidimensional protein reaction mechanisms. Before coming to its applications, we will briefly explain the use of these two kinetic methods.

Kinetic Methods

The Bidirectional *p*-Jump Method. The physical chemical basis of pressure-induced protein structural changes is explained by the relation of pressure to the chemical potential, μ.

$$(\partial\mu/\partial p)_T = V \tag{1}$$

Applied to chemical reactions under equilibrium conditions, eq 1 transforms to

$$(\partial \ln K/\partial p)_T = -\Delta V/RT \tag{2}$$

Accordingly, applying pressure to a chemical reaction will shift the equilibrium constant K to the state which has the smaller volume, V (Lange et al., 1996).[42] It is important to note that the reaction volume ΔV is not restricted to the reacting protein, but to the entire reaction system, comprising notably volumetric changes in the protein hydration

shell.[10,11] In analogy to the pressure effects on chemical equilibria, kinetic rates are also pressure dependent, according to eq 3, where

$$(\partial \ln k_i / \partial p)_T = -\Delta V^{\#} / RT \quad (3)$$

In relaxation kinetics, k_i is the individual rate constant (k_1 or k_{-1}) of a reaction described by eqs 4 and 5

$$1/\tau = k_{obs} = k_1 + k_{-1} \quad (4)$$

$$K = k_1 / k_{-1} \quad (5)$$

and $\Delta V^{\#}$ is the activation volume, i.e., the difference in volume between the reaction transition state and the starting ground state. Consequently, pressure effects on kinetics may be used both as a tool to study a reaction transition state (via its activation volume) and as a means to modulate the rate of an individual step. Indeed, in contrast to effects of temperature, elevated pressure may either accelerate or decelerate a reaction, depending on the sign of $\Delta V^{\#}$. As we will show, this property can be important for characterizing the kinetic transition state.

Which chemical interactions—in the ground state or in the transition state—are favored by high pressure? As outlined above, these interactions must result in decreased system volume. In the pressure range from atmospheric pressure to about 1.5 GPa, useful for biochemical studies, covalent bonds remain intact. That means only secondary, tertiary and quaternary protein structures are affected. In fact, pressure favors specifically the hydration of hydrophobic residues and the dissociation of electrostatic bonds, a process called "electrostriction". Hydrogen bonds appear to be affected only to a lesser degree, whereas the dissociation of stacked aromatic rings is disfavored.[12]

p-Jump experiments have been used for a long time to induce protein structure relaxations. Typically, a membrane breaking system was used to induce *p*-jumps from atmospheric to higher pressure between 5 and 20 MPa.[13] For several years bidirectional *p*-jump experiments have been possible. Their principle, as illustrated in Figure 1, is based on rapid equilibration between two independently pressurized cells. This versatile method allows upward and downward *p*-jumps of up to 100 MPa in amplitude, in the range between 0.1 and 700 MPa, with a dead-time of less than 5 ms.[14] An alternative apparatus for bidirectional *p*-jumps uses a piezoelectric stack.[15,16] UV absorbance, fluorescence, and FTIR and NMR have been used as detection methods in combination with *p*-jump devices.[17,18]

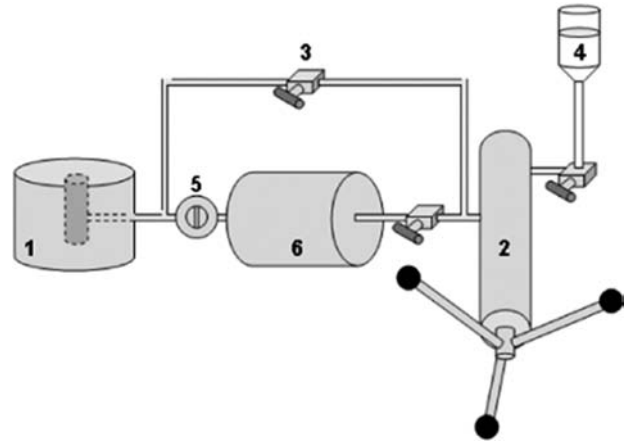

FIGURE 1. Schematic representation of a bidirectional *p*-jump device. The thermostatted high pressure optical cell (1) allows the sample cell to be pressurized up to 700 MPa. It holds a quartz sample cell, which is sealed with a polyethylene stretch. The pressure cell is interfaced to conventional spectrophotometers and fluorimeters. Pressure is generated by a manual pump (2) and controlled by high pressure valves (3). The pressure transmitting medium, water (4), is pumped through stainless steel capillaries. *p*-Jumps are carried out by opening an electrically driven pneumatic valve (5) localized between the high pressure optical cell and the ballast tank (6). The *p*-jumps consist of sudden changes of pressure (up to 100 MPa) within a range of final pressures of 0.1−600 MPa.

The Bidirectional *T*-Jump Method. The effects of temperature on protein structural changes are enthalpic and entropic in nature. The corresponding equation is

$$\ln k_i = \ln(\nu_{(T1/2)}) + \ln(\eta_{(T1/2)} / \eta_{(T)}) + (\Delta S^{\#} / R) - (\Delta H^{\#} / R)(1/T) \quad (6)$$

where the temperature dependence of the rate constant is described in the Kramer's formalism,[19] where ν and η are the pre-exponential factor and the viscosity of water at half-transition and experimental temperature, $T_{1/2}$ and T, and the superscript # refers to the kinetic transition state. Up to now, *T*-jumps have always consisted in a sudden heating of the sample, typically by a laser pulse or an electric discharge.

Very recently, a stopped-flow based *T*-jump method has been developed.[19] The principle, as illustrated in Figure 2, is simple: two solutions of different temperature are mixed in a stopped-flow apparatus. This method, termed m*T*-jump, permits temperature jumps of amplitudes between 2 and 40 °C in both heating and cooling directions, within a temperature range between 10 and 80 °C.

(1) Application to RNase A. (1a) *p*-Jumps. Pressure-induced protein structural changes occurring during protein unfolding are conveniently studied with RNase A, a model widely used to study protein unfolding.[20,21] We used Y115W RNase A, a tryptophan labeled variant, which provides an increased fluorescent signal, but exhibits a stability similar to

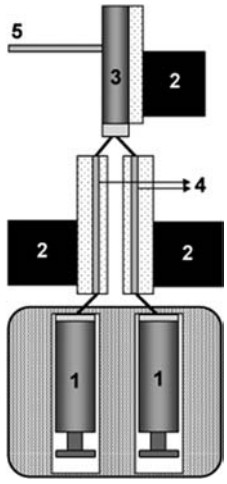

FIGURE 2. Schematic representation of a bidirectional *T*-jump device. The device is installed on a Bio-Logic stopped-flow basis. It achieves temperature changes by mixing two solutions of initially different temperatures, contained in two syringes (1). Three independent thermoelectric elements (2) are used to control the temperatures of the two storage lines (4) and of the observation cell (3). The temperature of the observation cell is monitored by a temperature probe (5) attached to its quartz surface.

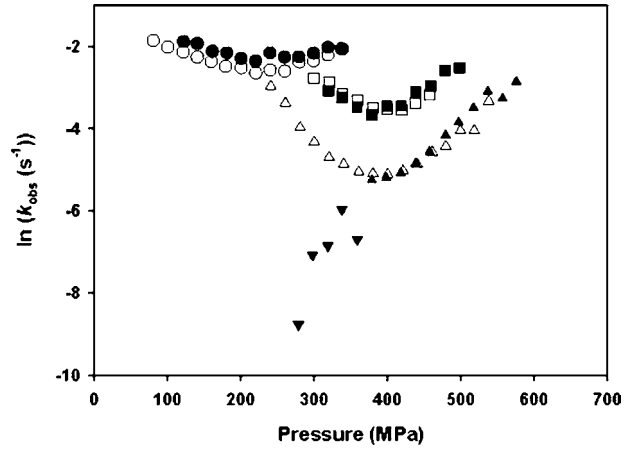

FIGURE 3. Pressure dependence of k_{obs}, determined from upward (filled symbols) and downward (open symbols) *p*-jumps at 35 °C (▲△), 50 °C (●○) and 50 °C in presence of 30% glycerol (■□). The slow phase observed after upward *p*-jumps at 35° is shown by inverted triangles. At each condition, the data from two independent experimental series were superimposed, in a way to obtain data points every 20 MPa. Reprinted with permission from ref 22. Copyright 2006 Biophysical Society.

the wild-type.[11] The pressure-unfolding process was found completely reversible up to 600 MPa, between 30 and 50 °C. A sigmoidal transition curve of its fluorescence upon pressure increase indicated a simple 2-state transition.

The kinetics of the pressure-induced folding and unfolding were studied by applying small *p*-jumps with amplitudes of 40 MPa in the whole range of the pressure-induced structural transition. Such small *p*-jump amplitudes barely affect the temperature of the solution. Yet, the *p*-jump induced folding and unfolding kinetics revealed a greater complexity than one might have expected from the simple pressure-dependent equilibrium. Whereas downward *p*-jump (folding direction) induced kinetics were always monoexponential, upward *p*-jump (unfolding direction) induced kinetics were biphasic in the lower pressure range (below the midpoint transition pressure of the unfolding reaction) and monophasic in the higher pressure range, as shown in Figure 3. The relative amplitude of the slow phase decreased as a function of both pressure and temperature. At 50 °C, only the fast phase remained. This asymmetric behavior of the observed rate constant $k_{obs(up)}$ and $k_{obs(down)}$ was unexpected. Kinetic and thermodynamic considerations predict that k_{obs} of simple relaxation kinetics should depend only on the final conditions.[22]

Different scenarios were sought to explain the path-dependency of k_{obs}. These scenarios had to account not only for the kinetic data but also for the vast structural knowledge of RNase A. The unfolding and refolding processes of RNase A have been extensively studied mainly by means of stopped-flow techniques using chemical denaturants.[9] Complex unfolding/refolding processes have been described. They involve not only conformational changes that give rise to fast-phases but *cis*−*trans* isomerization of at least three X-Pro peptide bonds,[23] present in the structure of RNase A,[24] responsible for the observed slow phases. As a possible explanation for the *p*-jump results, a two-dimensional energy surface was proposed, containing a pressure- and temperature-dependent barrier between two unfolded states differing in the isomeric state of the Asn113−Pro114 bond. To this isomerization the observed slow phase could be attributed, while the fast phase was attributed to a protein conformational change.

The thermodynamic activation parameters which were deduced from the *p*-jump experiments with the Y115W variant were used to characterize the kinetic transition state between folded and unfolded RNase A. As shown in Figure 4, the increase in volume of the unfolding transition state with respect to the unfolded state is about 50% larger at 50 °C than at 30 °C. This can be understood as a tighter packing and stronger hydration of the transition state at 30 °C with respect to that at 50 °C. Interestingly, in the presence of glycerol, the volume of the transition state at 50 °C resembles that of the protein without glycerol at 30 °C. This finding leads to a possible, though yet tentative, explanation of the protein structure stabilizing effect of glycerol. Glycerol is known for acting as an osmolyte, i.e., it binds water. Hence, it appears plausible that glycerol solvates exposed hydrophobic residues of the transition state and contributes via its bound water molecules to a stronger hydration of the transition state. And, as

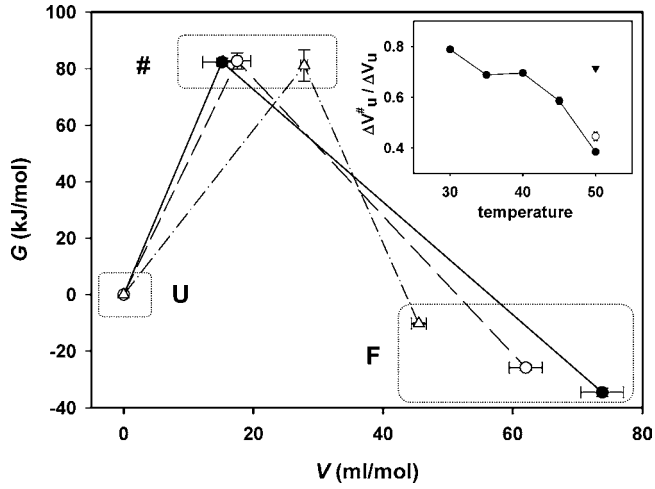

FIGURE 4. Free energy (G)−volume (V) diagram of the p-jump induced unfolding reaction (fast kinetic phase) at 30 °C (●), 50 °C (△), and 50 °C in the presence of 30% glycerol (○). The reaction goes from the folded state (F) to the unfolded state (U) via the transition state (#). The free energy and the volume of the unfolded state were set to zero. The inset shows the ratio between the activation volume, $\Delta V^{\#}$, and the reaction volume, ΔV, as a function of temperature in water (●), and in the presence of 30% glycerol (▼). Reprinted with permission from ref 22. Copyright 2006 Biophysical Society.

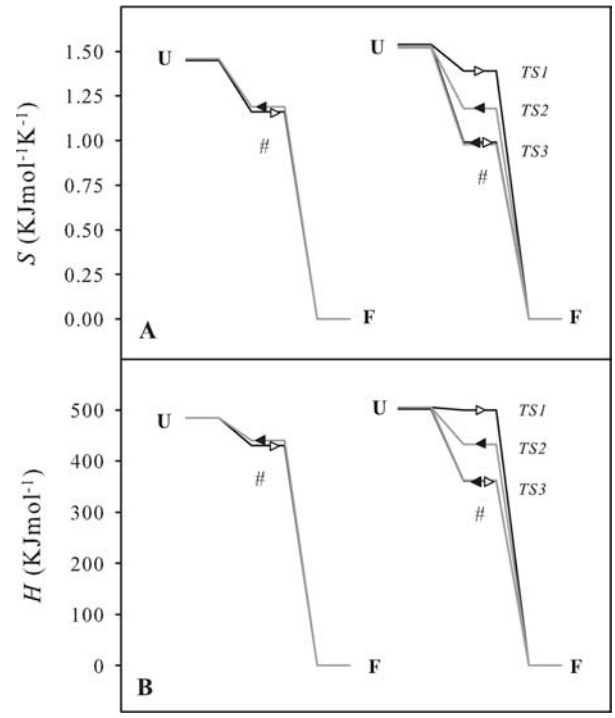

FIGURE 5. Changes of entropy (A) and enthalpy (B), determined from the temperature dependence of k_1 and k_{-1} of both heating and cooling T-jumps of RNase A wild-type (left) and Y115W variant (right). The direction of the T-jumps is indicated by arrows. Thermodynamic parameters of the folded state were set to zero. Reprinted with permission from ref 19. Copyright 2008 Biophysical Society.

explained in the Kinetic Methods section, stronger hydration results in a smaller volume. Thus, glycerol appears to exert two stabilizing effects: (i) it binds to the folded protein and provokes a diminution of its free energy, and (ii) it solvates the kinetic transition state and protects it by contributing to its hydration shell.[22]

The pressure-folding transition state of RNase A at 40 °C was also characterized by the protein engineering method or Φ-value analysis, using a set of nondisruptive variants from the main hydrophobic core of the enzyme.[25,26] The results suggested a transition state that is about 40% native-like.

(1b) *T*-Jumps. Similar to the effect of pressure, heat-induced RNase A and Y115W variant unfolding were reversible and both took place in an apparent 2-state reaction, reflected by sigmoidal fluorescence enhancements upon temperature increase. From the linear Arrhenius plots of the microscopic rate constants k_1 and k_{-1}, the thermodynamic activation parameters $\Delta H^{\#}$ and $\Delta S^{\#}$ were determined for reactions triggered by heating and cooling T-jumps.[19] As shown in Figure 5, the folding and unfolding reaction of wild-type RNase A passes via a single kinetic transition state, characterized by unique activation parameters. For the Y115W variant, the transition state (TS) is split into three. TS1 and TS2 characterize a slow kinetic phase, whereas TS3 characterizes a fast phase. Interestingly, heating and cooling T-jumps appeared to induce different folding/unfolding pathways. Heating T-jumps induced protein unfolding via TS2 and TS3, while cooling T-jumps induced folding via TS1 and TS3.

Remarkably, TS3 was characterized by a strongly negative activation enthalpy, $\Delta H^{\#}$, of the folding rate. As a consequence, the microscopic folding rate, and even k_{obs}, increase when temperature is lowered. This counterintuitive kinetic behavior results from compensating strongly negative activation entropy. The results were consistent with a path-dependent protein folding/unfolding mechanism, as depicted in Figure 6. In this scheme, the folding/unfolding kinetic model of RNase A obtained by stopped-flow[27] was taken into account to correlate the different phases to possible conformers. TS1 and TS2 are likely to reflect X-Pro114 isomerization in the folded and unfolded protein, respectively, and TS3 the local conformational change of the β-hairpin comprising Trp115. Both processes arise after the fast unfolding/folding phase, which is not observed under our experimental conditions.

(2) Application to Trp Synthase. Trp synthase is an $\alpha_2\beta_2$ heterotetrameric enzyme which catalyzes the synthesis of L-Trp from indole-3-glycerol phosphate (IGP) and L-ser with strict allosteric control of the reaction.[28] The α-subunit cata-

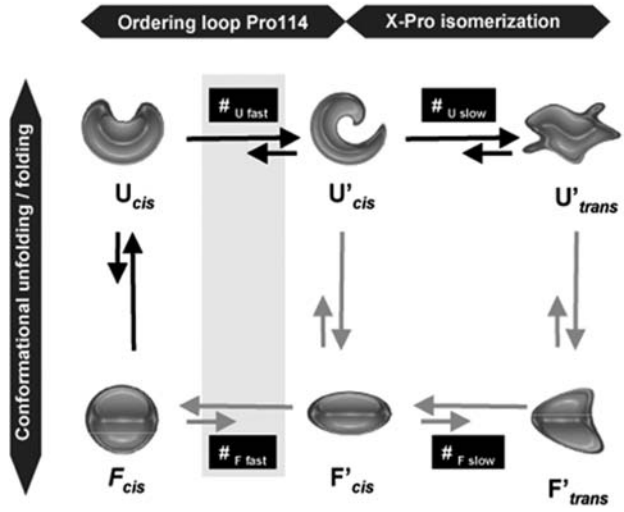

FIGURE 6. Schematic representation of the temperature-triggered folding/unfolding of RNase A. The gray square indicates the fast kinetic phases only observed for the Y115W variant. Black and gray lines denote heating and cooling *T*-jumps, respectively. Undetectable kinetic phases corresponding to conformational unfolding/folding are denoted as vertical lines. Reprinted with permission from ref 19. Copyright 2008 Biophysical Society.

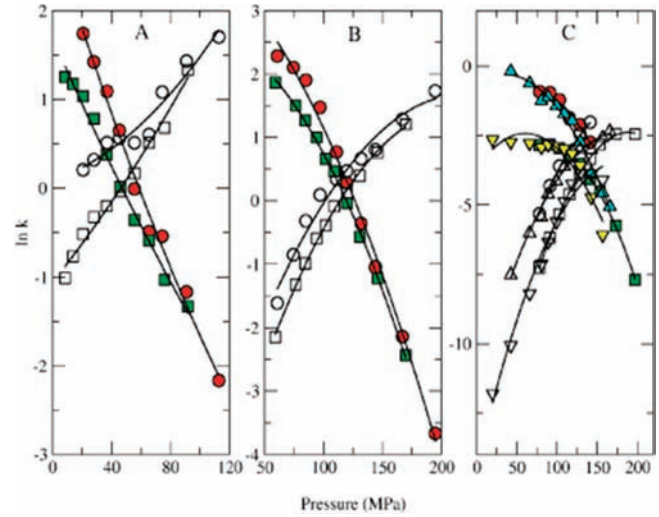

FIGURE 7. Bidirectional *p*-jump experiments modulating the open−closed allosteric transition of Trp synthase. Filled symbols correspond to the forward rate constant, k_o, and open symbols to the reverse rate constant, k_c. (A) Relaxations in the presence of Na^+. Circles indicate results from upward *p*-jumps, and squares are from downward *p*-jumps. (B) Relaxations in the presence of NH_4^+. Circles indicate results from upward *p*-jumps and squares are from downward *p*-jumps. (C) Relaxations in the presence of Na^+ and benzimidazole. Circles and squares indicate results from upward jumps, while triangles are from downward *p*-jumps.

lyzes the cleavage of indole-3-glycerol phosphate to indole and D-glyceraldehye-3-phosphate, and the β-subunit catalyzes the subsequent condensation of indole with L-Ser to give L-Trp. The intermediate indole is transferred intramolecularly between the α- and β-active sites, over a distance of 25−30 Å. For the past two decades, this protein has served as a paradigm for studying the mechanism of functional relevant protein conformational changes in a multienzyme complex. Indeed, its allosteric transition can be modulated by a wide range of physicochemical parameters, including ligands,[29−32] monovalent cations,[24,29,33−35] pH,[32] temperature,[29,32] and solvents.[36,37] We have used pressure as a perturbation tool to influence the conformational equilibrium of Trp synthase, shifting it from a high activity "closed" conformation to a low activity "open" conformation.[16,38,39] This transition is monitored easily by the absorbance (λ_{max} = 423 nm) and fluorescence (λ_{em} = 495 nm) increase arising from the pyridoxal-5′-phosphate−L-Ser Schiff's base in the open conformation.[40]

Pressure-dependent absorbance and fluorescence measurements under equilibrium conditions result in totally reversible 2-state transitions which allowed the determination of the volume change, ΔV_0, of the internal equilibrium constant, K_{eq}. Interestingly, the values of K_{eq} and ΔV_0 vary, depending on the nature of present cations and ligands, suggesting different ligand dependent structures of the conformations. This structural and mechanistic malleability was the decisive argument for choosing Trp synthase for our kinetic investigation.

The protein was subjected to upward and downward *p*-jumps of about 10 MPa within a range from atmospheric pressure up to 200 MPa.[39] From the monoexponential kinetics the individual rate constants k_o, for the closed to open direction, and k_c, for the open to closed direction were determined as a function of final pressure. As shown in Figure 7, the logarithmic plots are clearly nonlinear. In the presence of Na^+, the plots are curved upward, and in the presence of NH_4^+ and in the presence of Na^+ plus benzimidazole (BZI, an inhibitor), they are curved downward. Fitting of these rates to eq 3 required including an additional fitting parameter, the activation compressibility, $\beta_0^{\#}$, that reflects the pressure dependence of the activation volume, $\Delta V^{\#}$. The negative values of $\beta_0^{\#}$ for the NH_4^+ and Na^+ with BZI conditions indicate that the kinetic transition state is less compressible than the reactant and product states. This suggests that these transition states are more highly solvated or tightly packed than the ground states. In contrast, the positive $\beta_0^{\#}$ for the Na^+ bound enzyme suggests that the transition state is less solvated or more loosely packed than the ground states. From Figure 7 it is also obvious that the rate constants depend on whether they were measured in upward or in downward *p*-jump experiments. This means that, similarly to our observations with RNase A, the kinetics are path-dependent. This path-dependency is further illustrated in Figure 8, showing the volume profile of the

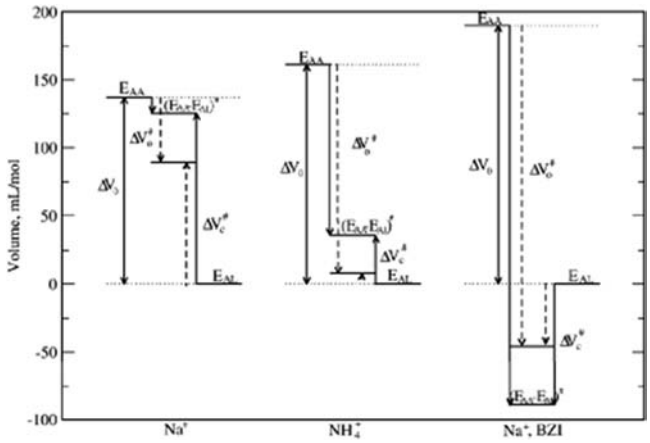

FIGURE 8. Volume changes during the allosteric conformational transition of Trp synthase. The double-headed arrows show the equilibrium reaction volumes. The solid arrows show the activation volumes for the upward *p*-jumps, and the dashed arrows show the activation volumes for the downward *p*-jumps. Reprinted with permission from ref 39. Copyright 2008 American Chemical Society.

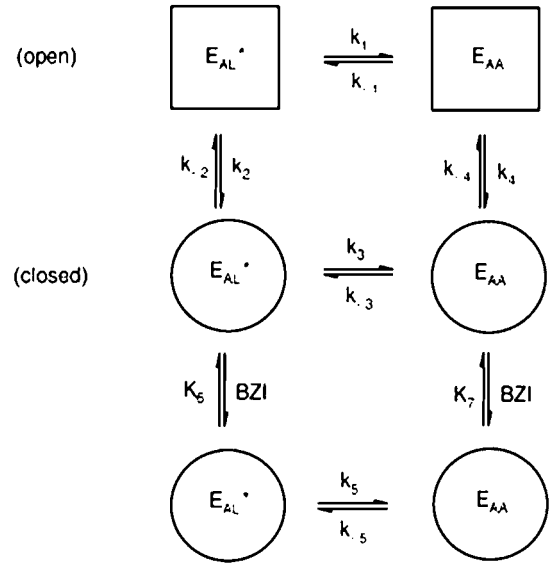

FIGURE 9. Reaction model of Trp synthase. The open conformation is represented by squares, and the closed conformation is represented by circles. E_{AL} is the external aldimine complex of Trp synthase with L-Ser, while E_{AA} is the aminoacrylate complex obtained by elimination of water from E_{AL}. Reprinted with permission from ref 39 Copyright 2008 American Chemical Society.

open (E_{AA}) to closed (E_{AL}) transition for the 3 ligand conditions in upward and downward *p*-jump experiments. As a matter of evidence, the volume of the kinetic transition state depends not only on the nature of the bound ligand but also on the direction of the experimentally induced transition.

The Na^+ form of the Trp synthase−L-Ser complex was also subjected to *T*-jump experiments.[39] The results show significant curvature of the Arrhenius plots (data not shown) and thus require a $\Delta C_p^{\#}$ term to obtain reasonable fits. The large value of $\Delta C_p^{\#}$ is consistent with significant solvation occurring in the transition state of the conformational change.

The results described above are consistent with the minimal reaction mechanism of the allosteric transition schematized in Figure 9. For the Na^+ enzyme, the relaxations involve mainly $k_3 + k_{-3}$, and the conformational transition ($k_2 + k_{-2}$) is fast. The relaxation between the open and closed conformations of E_{AA} is unlikely to contribute ($k_4 + k_{-4}$), and the aldimine (E_{AL})−aminoacrylate (E_{AA}) interconversion is a minor contribution. For the NH_4^+ form of Trp synthase, $k_3 + k_{-3}$ is fast and the conformational change is slow. This interpretation is consistent with steady state kinetic isotope effects of ^{2}H-Ser, which are near 6 for the Na^+ enzyme but almost 1 for the NH_4^+ enzyme.[41] In the case of the BZI−L-Ser complex, the kinetics is biphasic and much slower, so the major path for relaxation must involve rates $k_5 + k_{-5}$. However, the slow relaxations and large volume change for the relaxation for the BZI−L-Ser complex suggest that the binding of BZI may be associated with a conformational change.

Discussion

For RNase A folding/unfolding, as well as for the open/closed conformational change of Trp synthase, the relaxation kinetics was different when induced by upward or downward *p*-jumps. It was also different when induced by heating or cooling *T*-jumps. In both cases, the observed rate constant, k_{obs}, was significantly different when measured after *p*/*T*-jumps in opposite directions. Hence, the relaxation time, $\tau = 1/k_{obs}$, appears to be path-dependent. A path dependency in protein structural changes is understandable in reactions containing irreversible steps. However, here we are studying totally reversible monomolecular reactions. Their path-dependency can be explained by taking into account additional structural or energetic dimensions of the reaction mechanism. As a consequence, the reaction must be considered as taking place within a higher dimensional energy surface. Various energetic minima of this surface can be populated by ensembles of protein conformational substates. If the energy surface is sufficiently complex, these minima can be reached via different paths—and hence the kinetics becomes path-dependent. Thus, the above-shown bidirectional *p*- and *T*-jump experiments are in fact providing experimental evidence for the energy surface concept.

Conformational substates are well-known entities from kinetic flash techniques. However, the time scales of those kinetics are much shorter than these of protein conformational changes described here. Yet, the present results suggest

strongly that conformational substates exist also in the millisecond to second time scale.

Increased Insight into Reaction Mechanisms

The experiments performed at different temperatures and pressures permitted us to gain structural and energetic information about the kinetic transition states of distinct reaction paths on the energy surface. These parameters were notably the activation volume, the activation enthalpy, and the activation entropy. In turn, comparison of these parameters with literature data concerning simple chemical reactions, as well as with known protein structural data, permitted us to propose plausible reaction schemes for both the folding/unfolding mechanism of RNase A, and the open/closed allosteric transition of Trp synthase.

Furthermore, the activation parameters were useful for characterizing the kinetic transition states of temperature and pressure-induced unfolding reactions. As discussed in section 1b, *T*-Jumps, hydration of the transition state appears to play a major stabilizing role, preventing the protein (RNase A) unfolding. Also, in the case of Y115W RNase A variant, the bidirectional *T*-jump method revealed a manifold of transition states which was unexpected from the seemingly 2-state folding/unfolding mechanism as deduced from equilibrium measurements. The observation of path-dependent transition states was essential for designing the reaction model of Figure 6. In this model, the strongly negative $\Delta H^{\#}$ and $\Delta S^{\#}$ values observed in the fast kinetic phase could be assigned to a protein folding step involving ordering of the 112−115 β-hairpin loop of RNase.

For the open−closed conformational change of Trp synthase, application of the *p*- and *T*-jump method revealed significantly larger thermodynamic activation parameters than those observed for the folding/unfolding of RNase A. In addition, the logarithmic representations of the rate constants as a function of pressure and the inverse of temperature (Arrhenius plot) were strongly curved, indicating large activation compressibility, $\Delta\beta^{\#}$, and activation heat capacities, $\Delta C_p^{\#}$. The large values of these parameters indicate energetic barriers involving strong hydration changes. However, other effects must play also a role, since the activation parameters deduced from the big structural changes occurring in the unfolding of RNase A were much smaller. Therefore, in the case of the conformational change of Trp synthase, the kinetic transition states may reflect also significant changes in the packing of the protein.

Biological Relevance of Conformational Substates on the Energy Surface

Possibly, the path-dependency of protein structural changes via different ensembles of conformational substates is essential for both protein stability and function. As for protein stability, some degree of structural flexibility should allow protein structures to adapt smoothly to external stress, avoiding complete unfolding and aggregation, since native structures are able to transform to a variety of different conformational substates. Depending on the nature of the stress, exerted e.g. by heat, pressure or chemical compounds, the one or other conformational route may be taken. As to protein function, it is well documented that enzymes need some structural flexibility for being catalytically active. This flexibility confers to the enzyme the possibility of fine-tuning its interaction with substrates and hydration shell via a multiplay of conformational substates.

Conclusion

The results obtained with two model proteins, RNase A and Trp synthase, show the usefulness of the bidirectional *p*- and *T*-jump method for detecting and characterizing different paths of protein conformational changes via ensembles of conformational substates. The bidirectional *p*-jump method allows determination of hydration changes of kinetic transition states and exploration of volume dependent dimensions of protein structural energy landscapes. The novel bidirectional *T*-jump method informs in addition about the energetic features of the transition states. Structural and energetic characterization of these species is important for a better understanding of complex protein folding/unfolding reactions, as well as protein structural changes between states of different activities. Our hypothesis is that the possibility of a protein to adopt different routes of conformational changes is connected to protein stability and function. For a more complete understanding of these two central protein features, it would be interesting to develop further the bidirectional approach by accommodating it to other spectroscopic detection techniques. Furthermore, since both methods are technically relatively simple, they may be used without problem for the study of other proteins.

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