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Ligand binding to a high-energy partially unfolded protein

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Abstract: The conformational energy landscape of a protein determines populations of all possible conformations of the protein and also determines the kinetics of the conversion between the conformations. Interaction with ligands influences the conformational energy landscapes of proteins and shifts populations of proteins in different conformational states. To investigate the effect of ligand binding on partial unfolding of a protein, we use *Escherichia coli* dihydrofolate reductase (DHFR) and its functional ligand $NADP^+$ as a model system. We previously identified a partially unfolded form of DHFR that is populated under native conditions. In this report, we determined the free energy for partial unfolding of DHFR at varying concentrations of $NADP^+$ and found that $NADP^+$ binds to the partially unfolded form as well as the native form. DHFR unfolds partially without releasing the ligand, though the binding affinity for $NADP^+$ is diminished upon partial unfolding. Based on known crystallographic structures of $NADP^+$-bound DHFR and the model of the partially unfolded protein we previously determined, we propose that the adenosine-binding domain of DHFR remains folded in the partially unfolded form and interacts with the adenosine moiety of $NADP^+$. Our result demonstrates that ligand binding may affect the conformational free energy of not only native forms but also high-energy non-native forms.

Keywords: DHFR; $NADP^+$; partial unfolding; proteolysis; ligand binding

Introduction

Proteins assume various conformations even under native conditions.[1] The experimentally observed structures are ensemble-averaged structures and dominated by the most populated compactly folded forms (the native forms). Though much less in population than the native forms, various non-native conformations, including partially or even globally unfolded forms, also exist in equilibrium with the native forms.[2–4] The conformational energy landscape of a protein determines the relative populations of all the possible conformations and the kinetics of conversion between the conformations.[5] Protein folding can be described as a process in which a protein finds the global minimum by traversing through multiple local energy minima along one or more low-energy paths on its conformational energy landscape.[6–8]

The environment and chemical components of the system have critical influences on the conformational energy landscapes of proteins. Proteins denature at an elevated temperature or at a high concentration of a chemical denaturant because the native forms are no longer at global minima in their conformational energy landscapes.[9] Ligand binding is also coupled with shifts in the population of each conformation on the conformational energy landscape of proteins, which is strongly relevant to protein functions, such as enzyme catalysis, allostery,

Abbreviations: DHFR, C85A/C152S *E. coli* dihydrofolate reductase; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; $NADP^+$, β-nicotinamide adenine dinucleotide phosphate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

Additional Supporting Information may be found in the online version of this article.

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and signal transduction.[10–12] In most cases, we observe the consequence of ligand binding only on the structure of the native form, for example, the transition from an apo-form to a ligand-bound form. However, ligand binding may alter the relative energies of multiple non-native conformations of a protein and reshape the conformational energy landscape of the protein.[10] Interactions between ligands and non-native conformations have significant implications in protein folding. Proteins frequently form partially unfolded intermediates along their folding routes on the conformational energy landscapes.[13] Alteration of the energy of the folding intermediates by interactions with ligands would affect the rate and the efficiency of folding.[14] When multiple routes are available for folding, interactions with ligands may dictate which route is most favorable.[15] Designing a ligand that can modulate folding of a protein through interactions with folding intermediates is a potential way to improve folding efficiency of a target protein. Ligands with this property could also be used as drugs to facilitate folding of proteins with disease-causing mutations. Therefore, the complete picture of the effect of ligand binding on the conformational energy landscape requires elucidation of the effect of ligand binding on these non-native conformations.

In this study, we investigate the effect of $NADP^+$ on partial unfolding of *Escherichia coli* dihydrofolate reductase (DHFR; EC 1.5.1.3). We have recently demonstrated the presence of a high-energy partially unfolded form of DHFR that is transiently populated under native conditions.[16] Using native-state proteolysis, we determined the free energy required for this partial unfolding (4.9 kcal/mol). Also, analyzing the effect of mutations, we determined the structure of the partially unfolded form. Our results suggested that two loops near the active site of the protein (F-G loop and Met20 loop; Fig. 1) are mostly unfolded, and the hydrophobic cluster covered by these loops is disrupted and exposed to solvent. Still, the adenosine-binding domain of DHFR remains mostly folded in the partially unfolded form. Here we examine the effect of $NADP^+$ on the energetics of partial unfolding in DHFR using native-state proteolysis. For its catalysis, DHFR interacts with two substrates (NADPH and dihydrofolate) and two products ($NADP^+$ and tetrahydrofolate). It is well known that DHFR experiences a series of conformational changes in its catalytic cycle according to the bound ligands.[17,18] With an understanding of the structure of the partially unfolded form and the energetics of partial unfolding, we now probe the effect of ligand binding on a high-energy non-native conformation of DHFR. As the ligand, we chose $NADP^+$, which is one of the products from the catalytic reaction. Moreover, due to the relatively weak binding affinity of $NADP^+$, we can investigate partial unfolding of DHFR under conditions ranging from non-saturating to saturating concentrations of $NADP^+$. As well as on wild-type DHFR, we also investigate the effect of $NADP^+$ on the partial unfolding of I61A, I91A, and L110V DHFR to gain insight on the effect of $NADP^+$ on the structure of the partially unfolded DHFR.

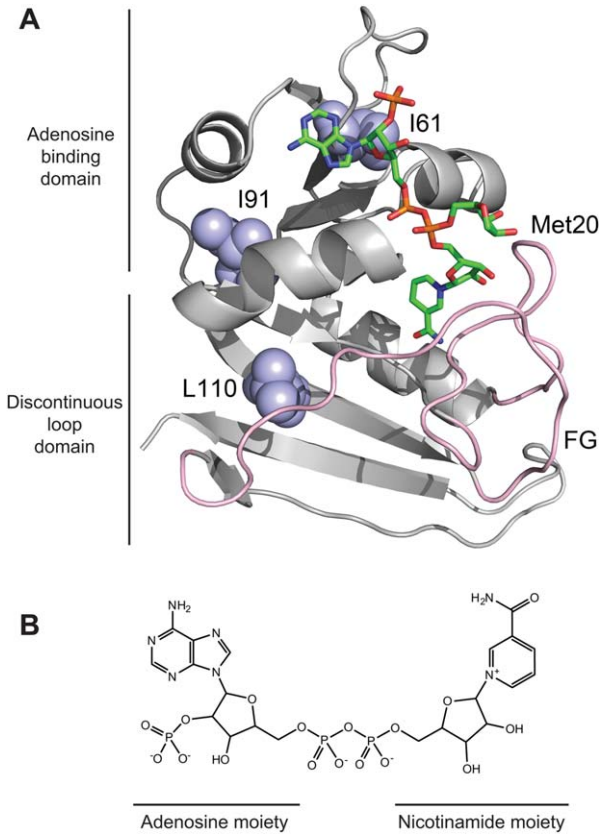


Figure 1. The structure of $NADP^+$-bound DHFR and $NADP^+$. (A) The backbone of DHFR (PDB: 1RX9) is represented as a ribbon with $NADP^+$ as sticks. The sugar group of the nicotinamide moiety is observed in two different conformations in the crystallographic structure. The nicotinamide group is not observable in the conformation pointing to bulk water. Ile61, Ile91, and Leu110 are shown in light blue spheres. The F-G and Met20 loops are colored pink. The image was created with PyMOL. (B) The chemical structure of $NADP^+$ is shown. The adenosine and nicotinamide moieties are indicated accordingly.

Results

Kinetic model for native-state proteolysis with a ligand

We have previously investigated the partially unfolded form of DHFR by native-state proteolysis.[16] From native-state proteolysis, we determine the equilibrium constant for partial unfolding by comparing the proteolysis rate of a protein by a non-specific protease with that of an unstructured

peptide substrate by the same protease. The typical kinetic model for native-state proteolysis is:

$$\mathrm{N} \overset{K_{op}}{\rightleftharpoons} \mathrm{C} \overset{k_{int}}{\longrightarrow} \text{Cleaved}$$

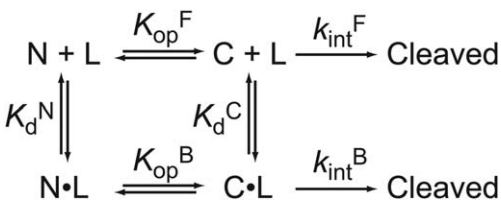
Scheme 1. •••

In Scheme 1, K_{op} is the equilibrium constant between the native form (N) and the cleavable form (C), and k_{int} is the rate constant for the proteolysis of the cleavable form. When the proteolysis step is rate-limiting (EX2-like condition), the apparent rate constant for overall proteolysis (k_p) is expressed as:

$$k_p = K_{op} k_{int}. \tag{1}$$

By determining k_p experimentally and approximating k_{int} with the rate constant for proteolysis of a peptide substrate, one can calculate K_{op} by Eq. (1) and also the free energy for unfolding to the cleavable form ($\Delta G_{op}{}^{\circ}$) from K_{op}. By applying this approach to DHFR, we found that proteolysis of DHFR by thermolysin occurs through a partially unfolded form, which is similar to a known folding intermediate (I_{HF}) of the protein.[16]

To determine the effect of ligand binding on the partial unfolding in DHFR, we modified the kinetic model. Assuming that both the native and partially unfolded form of DHFR can bind a ligand, we used the following kinetic model with four states:



Scheme 2. •••

The four states are the free native form (N), the free partially-unfolded form (C), the bound native form (N·L), and the bound partially-unfolded form (C·L). K_{op}^{F} and K_{op}^{B} are the equilibrium constants for partial unfolding in the free form and the bound form, respectively. K_d^N and K_d^C are the dissociation equilibrium constants for the native form and the partially unfolded form, respectively. k_{int}^{F} and k_{int}^{B} are the rate constant for the proteolysis of the free partially-unfolded form (C) and the bound partially-unfolded form (C·L), respectively. An analogous model has been used to assess ligand binding to a partially unfolded form by native-state hydrogen exchange.[19]

When the proteolysis step is rate-limiting in Scheme 2, the four states can be assumed to be in equilibrium. Also, we approximate both k_{int}^{F} and k_{int}^{B} with k_{int} determined with a peptide substrate. Because thermolysin is a non-specific protease and partial unfolding in DHFR is relatively large in scale, k_{int} is not likely to change significantly upon binding to a ligand. Under this condition, the apparent rate constant for overall proteolysis (k_p) is determined by the product of k_{int} and the fraction of the protein in the cleavable forms (C and C·L). Therefore, the apparent rate constant for overall proteolysis (k_p) is expressed as a function of [L]:

$$k_p = \left(\frac{K_{op}^{F} K_d{}^{N} + K_{op}^{B}[L]}{\left(1+K_{op}^{F}\right) K_d{}^{N} + \left(1+K_{op}^{B}\right)[L]} \right) k_{int} \tag{2}$$

Because $K_{op}{}^{F} << 1$ and $K_{op}{}^{B} << 1$, Eq. (2) is simplified to:

$$k_p = \left(\frac{K_{op}^{F} K_d{}^{N} + K_{op}^{B}[L]}{K_d{}^{N} + [L]} \right) k_{int} \tag{3}$$

We define the apparent free energy required for partial unfolding at a given ligand concentration (ΔG_{app}°) as

$$\Delta G_{app}^{\circ} = -RT \ln \left(k_p / k_{int} \right) \tag{4}$$

By combining Eq. (3) and Eq. (4), we obtain

$$\Delta G_{app}^{\circ} = -RT \ln \left(\frac{K_{op}^{F} K_d{}^{N} + K_{op}^{B}[L]}{K_d{}^{N} + [L]} \right) \tag{5}$$

At a very low ligand concentration, ΔG_{app}° would converge to ΔG_{op}^{Fo} (= –RT ln K_{op}^{F}), the free energy for partial unfolding in the free form. At a very high ligand concentration, ΔG_{app}° would converge at ΔG_{op}^{Bo} (= –RTlnK_{op}^{B}), the free energy for partial unfolding in the bound form. By setting K_{op}^{B} to 0, Eq. (5) is recast to explain the effect of ligand concentration on ΔG_{app}° for a model in which proteolysis occurs only through the free partially-unfolded form (C):

$$\Delta G_{app}^{\circ} = RT \ln \left(\frac{K_{op}^{F} K_d^{N}}{K_d^{N} + [L]} \right) \tag{6}$$

Different from Eq. (5), $\Delta G^{\circ}{}_{app}$ in this case increases infinitely as the ligand concentration increases.

Native-state proteolysis in the presence of NADP^{+}

As the ligand for this study, we chose NADP^{+}, one of the products of DHFR. During its catalytic cycle,

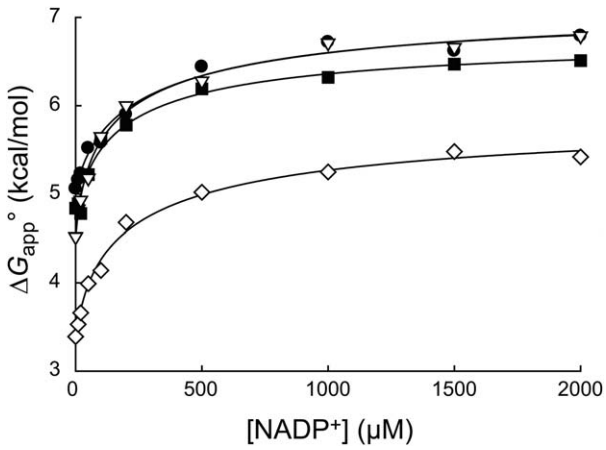


Figure 2. Effect of $NADP^+$ binding on partial unfolding. The apparent free energy for partial unfolding (ΔG°_{app}) of wild-type (•), I61A (▽), I91A (■), and L110V (◇) DHFR is plotted against the concentration of $NADP^+$. The curves were fit to Eq. (5). The standard errors of ΔG°_{app} are smaller than the size of the symbols.

DHFR does not exist in a binary complex with $NADP^+$ but with NADPH. Still, we used $NADP^+$ instead of NADPH because the binding affinity of $NADP^+$ ($K_d = 24$ μ*M*)[20] is more suitable than that of NADPH ($K_d = 0.33$ μ*M*)[20] for native-state proteolysis. To know the free ligand concentration ([L]) accurately, we need a ligand concentration significantly greater than the protein concentration. In the case of NADPH, the minimum concentration of a ligand necessary for our assay (~10 μ*M*) is much greater than its K_d. In the case of $NADP^+$, we can use ligand concentrations both below and above the K_d value, and therefore the use of Eq. (5) is valid.

In the presence of $NADP^+$, we investigated partial unfolding of I61A, I91A, and L110V DHFR as well as wild-type DHFR. We chose the three DHFR variants to probe the structure of the potential $NADP^+$-bound partially unfolded form. According to our previous study, Ile61 and Ile91 maintain a large portion of their native contacts in the partially unfolded form while Leu110 loses most of its native contacts in the partially unfolded form.[16] Also, the three residues do not contact $NADP^+$ directly in the native structure (Fig. 1). We determined the rate of proteolysis of DHFR by thermolysin at varying concentrations of $NADP^+$ (0.010–2.0 m*M*) (Supporting Information Fig. S1). We calculated ΔG°_{app} from k_p for each $NADP^+$ concentration using Eq. (4). When ΔG°_{app} is plotted against $NADP^+$ concentration, ΔG°_{app} increases as $NADP^+$ concentration increases (Fig. 2). This increase in ΔG°_{app} indicates that $NADP^+$ suppresses partial unfolding of DHFR. As our kinetic model predicts, ΔG°_{app} values approach a plateau at high concentrations of $NADP^+$.

The curve for each variant was fit to Eq. (5) to determine the values of K^F_{op}, K^B_{op}, and K^N_d (Table I). The K^B_{op} values for all four curves clearly have nonzero values ($P < 0.001$), which indicate that DHFR can access the partially unfolded form without releasing the ligand. In other words, $NADP^+$ can bind to the partially unfolded form of DHFR. As observed in the trend in Figure 2, the K^B_{op} values are much smaller than the K^F_{op} values. Binding of $NADP^+$ suppresses the partial unfolding of DHFR.

The similar K^N_d values of the DHFR variants to that of wild-type DHFR (Table I) show that the mutations do not affect binding to $NADP^+$. To confirm the validity of our kinetic model, we determined K^N_d independently by isothermal titration calorimetry (Table I). For wild-type and I61A DHFR, the K^N_d values determined by isothermal titration calorimetry agree well with those determined by proteolysis kinetics within the error. For I91A and L110V, the K^N_d values determined by isothermal titration calorimetry are about 2–3 times higher than those determined by proteolysis kinetics. Still, the K^N_d values within a similar range confirm that the kinetic model is valid.

The thermodynamic cycle in Scheme 1 also allows us to determine the dissociation constant for the $NADP^+$-bound partially unfolded form (K^C_d) from the three other equilibrium constants using the relationship of $K^B_{op}K^C_d = K^N_d K^F_{op}$. For the four DHFRs, K^C_d ranges from 0.8 to 1.7 m*M* (Table I). The similar K^C_d values for wild-type DHFR and the variants indicate that the mutations do not interfere with the binding of $NADP^+$ to the partially unfolded form. The K^C_d values much greater than K^N_d also show that partial unfolding diminishes the binding affinity to $NADP^+$.

Table I. *Equilibrium Constants for Partial Unfolding and $NADP^+$ Dissociation*

DHFR	K^F_{op} ($\times 10^{-6}$)[a]	K^B_{op} ($\times 10^{-6}$)[a]	K^N_d (μ*M*)[a]	$K^N_{d\,ITC}$ (μ*M*)[b]	K^C_d (μ*M*)[c]
Wild type	190 ± 20	5.5 ± 1.5	47 ± 11	34 ± 6	1600 ± 600
I61A	470 ± 50	5.9 ± 1.1	17 ± 3	24 ± 2	1400 ± 400
I91A	330 ± 50	11 ± 3	33 ± 9	60 ± 7	1000 ± 400
L110V	3400 ± 300	49 ± 11	26 ± 5	52 ± 4	1800 ± 600

[a] K^F_{op}, K^B_{op}, and K^N_d were determined by fitting to Eq. (5).
[b] $K^N_{d\,ITC}$ was determined by isothermal titration calorimetry.
[c] K^C_d was calculated from the relationship of $K^B_{op}K^C_d = K^N_d K^F_{op}$.
The standard errors of $K^F_{op}, K^B_{op}, K^N_d$, and $K^N_{d\,ITC}$ are from curve-fitting. The standard errors of K^C_d are from error propagation.

Table II. *Free Energies for Partial Unfolding in the Free and Bound Form of DHFR*

DHFR	ΔG_{op}^{Fo} (kcal/mol)[a]	$\Delta\Delta G_{op}^{Fo}$ (kcal/mol)[b]	ΔG_{op}^{Bo} (kcal/mol)[a]	$\Delta\Delta G_{op}^{Bo}$ (kcal/mol)[c]	$\Delta\Delta G_{op}^{\circ}$ (kcal/mol)[d]
Wild Type	5.06 ± 0.06	–	7.1 ± 0.2	–	2.0 ± 0.2
I61A	4.52 ± 0.06	-0.54 ± 0.08	7.1 ± 0.1	0.0 ± 0.2	2.6 ± 0.1
I91A	4.73 ± 0.09	-0.33 ± 0.11	6.7 ± 0.2	-0.4 ± 0.3	2.0 ± 0.2
L110V	3.35 ± 0.05	-1.71 ± 0.08	5.9 ± 0.1	-1.2 ± 0.2	2.6 ± 0.1

[a] ΔG_{op}° values were calculated with the equilibrium constants in Table I.
[b] $\Delta\Delta G_{op}^{Fo} = \Delta G_{op}^{Fo}(\text{mutant}) - \Delta G_{op}^{Fo}(\text{wild type})$.
[c] $\Delta\Delta G_{op}^{Bo} = \Delta G_{op}^{Bo}(\text{mutant}) - \Delta G_{op}^{Bo}(\text{wild type})$.
[d] $\Delta\Delta G_{op}^{\circ} = \Delta G_{op}^{Bo} - \Delta G_{op}^{Fo}$.
The standard errors are from error propagation.

We calculated ΔG_{op}^{Fo} and ΔG_{op}^{Bo} from K_{op}^{F} and K_{op}^{B}, respectively (Table II). The ΔG_{op}^{Fo} values are close to 5 kcal/mol for wild-type, I61A, and I91A DHFR and 3.4 kcal/mol for L110V DHFR. ΔG_{op}^{Fo} matches very well to the ΔG_{op}^{Fo} value that we previously determined by native-state proteolysis of the free form of each variant (Table II).[16] The ΔG_{op}^{Bo} values are close to 7 kcal/mol for wild-type, I61A, and I91A DHFR and 6.0 kcal/mol for L110V DHFR. The effects of mutations on ΔG_{op}^{Bo} ($\Delta\Delta G_{op}^{Bo}$) are quite similar to those on ΔG_{op}^{Fo} ($\Delta\Delta G_{op}^{Fo}$). At least near the mutated residues, the structure of the bound partially-unfolded form is similar to that of the free partially-unfolded form. The difference between ΔG_{op}^{Bo} and ΔG_{op}^{Fo} ($\Delta\Delta G_{op}$) shows the degree of the protection against partial unfolding by $NADP^+$. For wild-type DHFR, partial unfolding in the $NADP^+$-bound form requires 2.2 kcal/mol more of free energy than partial unfolding in the free form. $\Delta\Delta G_{op}$ is similar for the three mutant DHFRs as well.

To demonstrate visually the effect of $NADP^+$ binding on partial unfolding, we present our results in energy diagrams (Fig. 3). The diagrams show the relative free energy of the native and the partially unfolded forms in the presence of 10 m*M* $NADP^+$ as well as the relative free energies of each form in free DHFR. We only measured proteolysis of DHFR in the presence of up to 2.0 m*M* $NADP^+$. However, to show ΔG_{op}^{Bo} in the diagram, we calculated the free energies at 10 m*M* $NADP^+$ with which ΔG_{app} would converge at ΔG_{op}^{Bo} [Eq. (5) and Fig. 2]. As shown in the diagram, the partially unfolded form is stabilized by $NADP^+$, though not as significantly as the native form is. Additional increases in the ligand concentration will not increase the energy difference between the native and the partially unfolded forms, because both forms are saturated with $NADP^+$, and the energy of both forms decrease to the same extent with the increase in the concentration of $NADP^+$. For comparison, the energy diagram at 2.0 m*M* $NADP^+$, with which the partially unfolded forms are not fully saturated, is also shown in Supporting Information Figure S2.

Discussion

We have studied partial unfolding of DHFR by native-state proteolysis in the presence of $NADP^+$ to understand the effects of this ligand on the protein's conformational energy landscape, specifically interaction with a partially unfolded form. $NADP^+$ slows

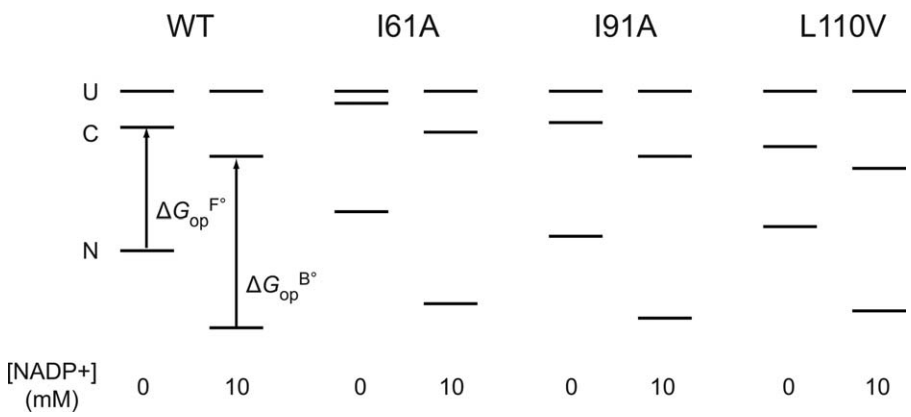


Figure 3. Consequences of $NADP^+$ binding on free energy. The diagram is scaled according to the relative free energy of the native (N), partially unfolded (C), and globally unfolded (U) forms in the presence and absence of 10 m*M* $NADP^+$. The global stabilities of wild-type DHFR and the variants are from our previous study.[16] We estimated the effect of 10 m*M* $NADP^+$ on the stability of the native form of DHFR with the $NADP^+$ concentration and K_d^N using the relationship of $\Delta\Delta G_{unf}^{\circ} = RT\ln(1+[L]/K_d)$,[41] where R is the gas constant and T is the temperature in Kelvin. We determined the relative free energy of the free and bound partially unfolded forms by adding $\Delta G_{op}^{F\circ}$ and $\Delta G_{op}^{B\circ}$ to the relative free energy of the free and bound native forms, respectively. Here we assume that proteolysis occurs mostly through bound partially-unfolded forms at 10 m*M* $NADP^+$.

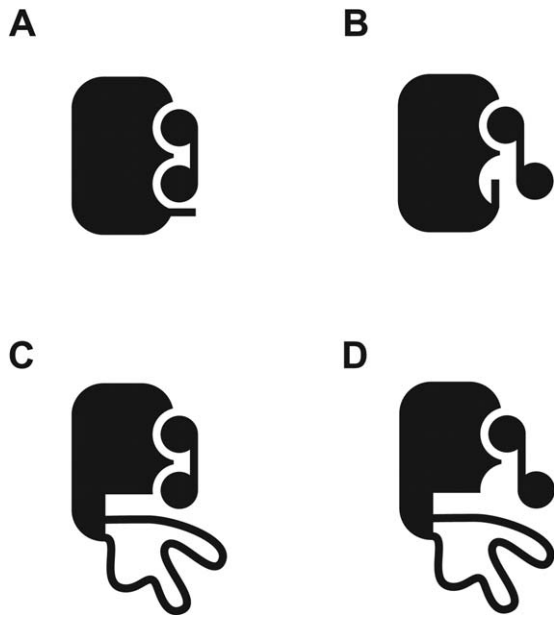


Figure 4. Binding of $NADP^+$ to the native and the partially unfolded DHFR. The modes of interactions between $NADP^+$ and DHFR in the native and the partially unfolded forms are shown in schematic presentations. (A) $NADP^+$ bound to the native DHFR in the closed conformation. (B) $NADP^+$ bound to the native DHFR in the occluded conformation. The nicotinamide moiety is not in the binding site. (C) $NADP^+$ bound to the partially unfolded DHFR with the nicotinamide moiety in the conformation analogous to that in panel A. (D) $NADP^+$ bound to the partially unfolded DHFR with the nicotinamide moiety in the conformation analogous to that in panel B.

proteolysis but does not slow it infinitely. As the kinetic model in Scheme 2 predicts, proteolysis occurs through the $NADP^+$-bound partially-unfolded form at high concentrations of the ligand. Proteolysis through the $NADP^+$-bound form is slower than through the free form because $NADP^+$ binds to the native form more tightly than to the cleavable form (Tables I and II).

Structure of $NADP^+$-bound partially-unfolded form

Based on our knowledge of the cleavable form, we make some inferences about the structural origin of its lower affinity for $NADP^+$. In the previous investigation, we found that the cluster of Leu8, Leu110, and Leu112 loses most of its native contacts in the partially unfolded form.[16] We infer that the F-G and Met20 loops that these residues contact (Fig. 1) are unfolded in the partially unfolded form. These loops close over the nicotinamide moiety of $NADP^+$ in the native structure (Fig. 1). The lower affinity of $NADP^+$ to the partially unfolded form likely results from unfolding of the binding site for the nicotinamide moiety. Still, $NADP^+$ binds to the partially unfolded form through the interaction between the adenosine moiety of $NADP^+$ and the adenosine-binding domain, which remains folded in the partially unfolded form.

Crystallographic structures of NADPH- or $NADP^+$-bound DHFR also reveal that the binding of the adenosine and nicotinamide moieties can be uncoupled.[21] The crystallographic structure of NADPH-bound DHFR showed only 75% occupancy of the nicotinamide moiety in the binding pocket.[21] In the case of $NADP^+$, occupancy of the nicotinamide moiety is even lower so that the electron density of the moiety is almost undetectable. The structure shown in Figure 1 also shows two different conformations of the nicotinamide moiety. In one conformation, the nicotinamide moiety is properly bound to the binding pocket. In the other conformation, however, the nicotinamide moiety points to the bulk water, and only the ribose group is visible. Figure 4 shows schematic representations of the two different conformations of $NADP^+$. These structural observations suggest that the adenosine moiety contributes much more greatly to the binding affinity of $NADP^+$ to DHFR than the nicotinamide moiety. Our finding that $NADP^+$ still binds to the partially unfolded form is consistent with the structural studies. The unfolding of the FG and Met20 loops in the partially unfolded form diminishes, but does not abolish, the binding of $NADP^+$. The association of the adenosine moiety of $NADP^+$ with the native-like adenosine-binding domain and the plausible interaction with the diphosphate group seem primarily responsible for the binding of $NADP^+$ to the partially unfolded form [Fig. 4(C,D)].

The uncoupling of binding of the adenosine and nicotinamide moieties of $NADP^+$ is relevant to the catalytic mechanism of DHFR.[21,22] DHFR utilizes NADPH to reduce 7,8-dihydrofolate to 5,6,7,8-tetrahydrofolate. After NADPH is oxidized, and prior to release of $NADP^+$ from DHFR, the active site is occluded by the Met20 loop and the nicotinamide moiety is no longer bound; this transient form associates with only the adenosine moiety [Fig. 4(B)].[22] After release of $NADP^+$, the adenosine moiety of NADPH binds DHFR, while the nicotinamide site still remains occluded. It is not until 5,6,7,8-tetrahydrofolate is released that the Met20 loop opens, allowing the nicotinamide of NADPH to bind the active site.

A computational study showed that the $NADP^+$ binding site is energetically independent of the region that we found to be unfolded in the partially unfolded form. Pan *et al*. investigated the connection of $NADP^+$ binding and folding of DHFR with the COREX algorithm.[23] This model assesses an ensemble of conformational states in which a unique set of residues are unfolded, and the population of each state is calculated according to the energetic penalty of unfolding. Using this model, they determined the probability of each residue to be folded if the entire $NADP^+$ binding site is folded. The $NADP^+$ binding site has few correlations with residues in the β strands F (containing L110), G, and H. The F-G loop even shows negative correlation with the $NADP^+$

binding site; the loop is less likely to be unfolded when the $NADP^+$ binding site is unfolded. The absence of positive correlation between the $NADP^+$ binding site and the region unfolded in the cleavable form is consistent with our observation that DHFR can still unfold partially with bound $NADP^+$.

Ligand binding by partially unfolded forms

Ligand binding by partially unfolded forms has also been observed in other proteins. The effects of partial unfolding on ligand binding span a broad spectrum. A protein may maintain its full binding affinity or lose its affinity completely to a ligand upon partial unfolding. Partial unfolding of *E. coli* maltose binding protein is not affected by the presence of its ligand.[24] Proteolysis of the protein by thermolysin occurs through unfolding of two C-terminal helices, and the rate of proteolysis is identical whether maltose is present or not. When hydrogen exchange of cytochrome c was monitored in the presence of antibodies, protection factors of a group of residues in cytochrome *c* are shown to increase by the binding of E3 and C3 antibodies.[25] Because the change in protection factors is not even across the structure of cytochrome c, the antibodies also likely stabilize a partially unfolded form of cytochrome c. Free energies for opening of free and bound SH3 domain in the presence of a peptide ligand were investigated using the same model that we use in this study.[19] Some residues of SH3 do exchange through a bound form, but these residues do not map to a common element of the structure. This pattern of exchange in SH3 is thought to result from the promotion of local fluctuations relative to global unfolding upon binding, as opposed to binding of a partially unfolded form. The complex of S protein with S peptide (ribonuclease S) did not show any evidence of unfolding in the bound form.[26] It was found that exchange occurs only through the dissociated complex, the extended S peptide and a partially unfolded S protein. Whether a protein can unfold partially without releasing its ligand must be dependent on the structural cooperativity between the unfolding site and the ligand binding site. In a highly cooperative system, partial unfolding would be strongly coupled with release of the ligand, and the bound partially unfolded form would be extremely unfavorable and not detectable. In a less cooperative system, a protein would unfold partially with a ligand bound, though with reduced binding affinity. The uncoupling in the binding of the adenosine moiety and the nicotinamide moiety of $NADP^+$ to DHFR results in somewhat low cooperativity between the partial unfolding and binding of $NADP^+$ in DHFR, which allows us to detect the bound partially unfolded form under native conditions.

Our findings support the growing evidence that small molecules can interact with non-native forms of proteins during folding. Cofactors and ligands have been found to interact with proteins before translation is complete,[27] and metal cofactors can interact with intermediate forms and even unfolded forms.[28] Also, some inhibitors stabilize transition states of protein folding,[29,30] and an enzyme was shown to acquire partial catalytic activity in a folding intermediate form.[31] We previously discovered that ATP binds to a folding intermediate of glyceraldehyde-3-phosphate dehydrogenase but does not bind to the native form.[14] Binding with non-native forms may facilitate folding by protecting folding intermediates against misfolding or degradation or by enhancing folding rates.

Frieden's group has conducted refolding experiments of DHFR in the presence of 100 μM $NADP^+$.[32] However, they did not observe any influence of $NADP^+$ on the amplitude of the fluorescence during the formation of I_1 or the following intermediates I_2 or I_3, and concluded that $NADP^+$ only binds the native form. We measured a K_d value of 1.4 mM for binding of $NADP^+$ to the partially unfolded form, which we believe is equivalent to I_1. Our result is consistent with what Frieden's group observed because only 7% of the partially unfolded form or I_1 would be bound to $NADP^+$ in the presence of 100 μM $NADP^+$.

The ligand binding that we observed may be relevant to DHFR *in vivo*. The cellular concentrations of $NADP^+$ and NADPH in *E. coli* are estimated to be 2 μM and 300 μM, respectively.[33] Assuming similar binding of NADPH to the partially unfolded form as $NADP^+$ ($K_d \sim 1.4$ mM), we estimate ~20% of the kinetic intermediate bound to NADPH in *E. coli*. The binding affinity of NADPH to the native DHFR is ~70-fold greater than that of $NADP^+$.[20] Though the binding site for the nicotinamide moiety is largely unfolded, it is still possible that NADPH also binds to the partially unfolded form better than $NADP^+$ and associates with the folding intermediate to a greater extent than we estimate here.

Here we show that $NADP^+$ not only binds to the native form but also a partially unfolded form of DHFR. Although binding to the partially unfolded form is weak, $NADP^+$ or NADPH may provide DHFR with a mild stabilization or protection during folding. Our result contributes to a body of evidence that demonstrates a ligand can affect the entire conformational energy landscape of a protein, not just the stability of the native form.

Materials and Methods

Materials

We expressed DHFR in *E. coli* BL21(DE3)pLysS cells grown to OD_{600} of 0.6 and induced with 500 μM isopropyl-β-D-thiogalactopyranoside (IPTG). We purified DHFR by DEAE Sepharose Fast Flow (GE

Healthcare Life Sciences; Piscataway, NJ) anion exchange chromatography and Superdex 200 (GE Healthcare Life Sciences; Piscataway, NJ) size exclusion chromatography. The production of I61A, I91A, and L110V DHFR has been reported previously.[16] All the DHFR proteins we used in this study have C85A/C152S mutations.[34] For simplicity, we refer to C85A/C152S DHFR as wild-type DHFR. We dissolved lyophilized thermolysin (Type X; Sigma-Aldrich; St. Louis, MO) in 2.5*M* NaCl, 10 m*M* $CaCl_2$.[35] Concentrations of all proteins were determined by absorbance at 280 nm using extinction coefficients determined according to their amino acid composition.[36] Less than 10% of purified DHFR was bound to the cofactor, NADPH, as determined by NADPH titration. $NADP^+$ monosodium salt (Sigma-Aldrich; St. Louis, MO) was dissolved in 20 m*M* Tris-HCl (pH 8.0) containing 50 m*M* NaCl, and the pH was adjusted to 8.0 with NaOH. The concentration of $NADP^+$ was determined by absorbance at 260 nm according to an extinction coefficient of 18,000M^{-1} cm^{-1}.[37]

Proteolysis

We initiated proteolysis of DHFR by adding thermolysin to DHFR in buffer to achieve final conditions of 0.10 mg/mL DHFR, 20 m*M* Tris-HCl buffer (pH 8.0), 100 m*M* NaCl, 10 m*M* $CaCl_2$, 80 μg/mL Thermolysin, and varying concentrations of $NADP^+$ (0.010–2.0 m*M*). At the desired time, 15-μL aliquots were quenched with 5 μL of 50 m*M* EDTA. Quenched samples were separated by SDS-PAGE. Gels were stained with SYPRO Red Protein Gel Stain (Life Technologies; Grand Island, NY), and fluorescent images were taken with a Typhoon scanner (GE Healthcare Life Sciences; Piscataway, NJ). Intact protein gel bands were quantified from images with ImageJ software. The apparent rate constants of proteolysis (k_{obs}) were determined by fitting the change in band intensity over time to a first-order rate equation in OriginPro 8.5.1 (OriginLab; Northampton, MA). We have recently reported that, due to product inhibition, proteolysis of intact proteins may deviate from first-order kinetics, and k_{obs} may be underestimated.[38] In DHFR, however, we have shown that ΔG_{op}° is still well consistent with ΔG_{unf}°,[16] which confirms that ΔG_{op}° is determined reliably by native-state proteolysis.

We calculated ΔG_{app}° from k_{obs} and k_{int} according to Eq. (4). We estimated k_{int} using the k_{cat}/K_m value for cleavage of a generic thermolysin substrate, 2-aminobenzoyl-Ala-Gly-Leu-Ala-4-nitrobenzylamide.[39] We confirmed that $NADP^+$ does not inhibit thermolysin up to 2.0 m*M* $NADP^+$. We determined K_{op}^{F}, K_{op}^{B}, and K_d^N by fitting the plot of ΔG_{app}° versus [$NADP^+$] in Figure 2 to Eq. (5).

In our analysis with Eq. (5), we used the total $NADP^+$ concentration instead of free $NADP^+$ concentration. When the $NADP^+$ concentration is as low as 10 μ*M*, the free $NADP^+$ concentration changes up to ~10% from the release of bound $NADP^+$ during proteolysis. This change in the free $NADP^+$ concentration is negligible when the $NADP^+$ concentration is greater than 100 μ*M*. For simplicity, we did not count in this change in the $NADP^+$ concentration in our analysis of proteolysis kinetics. When we used the initial free $NADP^+$ concentration instead of the total $NADP^+$ concentration in Eq. (5), the change in the fitting parameters was negligible.

Isothermal titration calorimetry

Isothermal titration calorimetry was performed using a MicroCal iTC200 (GE Healthcare Life Sciences; Piscataway, NJ) calorimeter. We prepared $NADP^+$ and DHFRs in 20 m*M* Tris–HCl buffer (pH 8.0) containing 100 m*M* NaCl, and 10 m*M* $CaCl_2$. 1.5 m*M* $NADP^+$ was titrated in 21 injections into 58 μ*M* (L110V) or 76 μ*M* (wild-type, I61A, and I91A) DHFR with 3-min intervals. Thermograms were automatically integrated with NITPIC[40] and fit with SEDPHAT, assuming a single $NADP^+$ binding site per DHFR molecule.

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