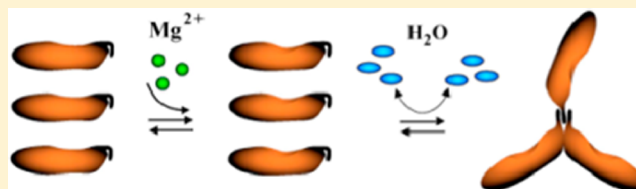


Energetics of the *Escherichia coli* DnaT Protein Trimerization ReactionMichal R. Szymanski,[†] Maria J. Jezewska,[†] and Włodzimierz Bujalowski^{*,‡}

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ABSTRACT: Thermodynamic and structural characteristics of the *Escherichia coli* DnaT protein trimerization reaction have been quantitatively examined using fluorescence anisotropy and analytical ultracentrifugation methods. Binding of magnesium to the DnaT monomers regulates the intrinsic affinity of the DnaT trimerization reaction. Comparison between the DnaT trimer and the isolated N-terminal core domain suggests that magnesium binds to the N-terminal domain but does not associate with the C-terminal region of the protein. The magnesium binding process is complex and involves approximately three Mg^{2+} cations per protein monomer. The observed effect seems to be specific for Mg^{2+} . In the examined salt concentration range, monovalent cations and anions do not affect the trimer assembly process. However, magnesium affects neither the cooperativity of the trimerization reaction nor the GnHCl-induced trimer dissociation, strongly indicating that Mg^{2+} indirectly stabilizes the trimer through the induced changes in the monomer structures. Nevertheless, formation of the trimer also involves specific conformational changes of the monomers, which are independent of the presence of magnesium. Binding of Mg^{2+} cations dramatically changes the thermodynamic functions of the DnaT trimerization, transforming the reaction from a temperature-dependent to temperature-independent process. Highly cooperative dissociation of the trimer by GnHCl indicates that both interacting sites of the monomer, located on the N-terminal core domain and formed by the small C-terminal region, are intimately integrated with the entire protein structure. In the intact protein, the C-terminal region most probably interacts with the corresponding binding site on the N-terminal domain of the monomer. Functional implications of these findings are discussed.



The DnaT protein is an essential replication protein in *Escherichia coli* that plays a fundamental role in the assembly of the primosome, a multiple-protein–nucleic acid complex, which catalyzes priming of the DNA strand during the DNA replication process.^{1–7} The protein was originally discovered to be a critical factor during synthesis of the complementary DNA strand of phage ϕ X174 DNA.^{1,4} Current data show that the assembly of the primosome is a fundamental step in the restart of the stalled replication fork at the damaged DNA sites.^{6,7} Subsequent studies indicated that the role of the DnaT protein is to provide a specific recognition marker within the primosome structure for the primary replicative helicase, the DnaB protein, which allows the helicase to enter and become the integral part of the assembly.^{1,4,5,8,9} The DnaT protein monomer contains 179 amino acids with a molecular weight of ~ 19455 .^{10,11} Although early biochemical data indicated that the native protein forms a homotrimer, the presence of monomer, dimer, tetramer, and pentamer forms was also proposed, with the molecular weight of the native protein ranging from ~ 61000 to ~ 110000 , depending on the presence of magnesium.¹⁰

In the preceding paper (DOI: 10.1021/bi301568w), we addressed the fundamental characteristics of the DnaT protein trimerization reaction. In solution, the protein exists as a monomer–trimer equilibrium system, without the presence of any specific oligomeric states larger than a trimer. The

trimerization reaction is highly cooperative, without the detectable presence of the intervening dimer. Nevertheless, because of the modest affinity, the DnaT protein forms a mixture of the monomer and trimer states with the 3:1 molar ratio, at physiological concentrations in the *E. coli* cell. The protein monomer consists of a large, N-terminal core domain and a small C-terminal region. It also possesses two structurally different, interacting sites located on the N-terminal core domain and the small C-terminal region. While the interaction site located on the N-terminal core domain stabilizes the initial dimer, the final attachment of the third monomer in the trimer is achieved through the C-terminal region (DOI: 10.1021/bi301568w). The data indicate that each monomer in the trimer is in contact with the two remaining monomers forming a starlike global structure.

Although the DnaT protein has been recognized as a key factor in the activities of the primosome, little is known about its functional behavior.^{5,10} Prior to this work, the very functional form of the DnaT in the primosome structure was still under the debate, with both the trimer and the monomer proposed as possible ingredients of the primosome.⁵ Nevertheless, these early data suggest that oligomerization and

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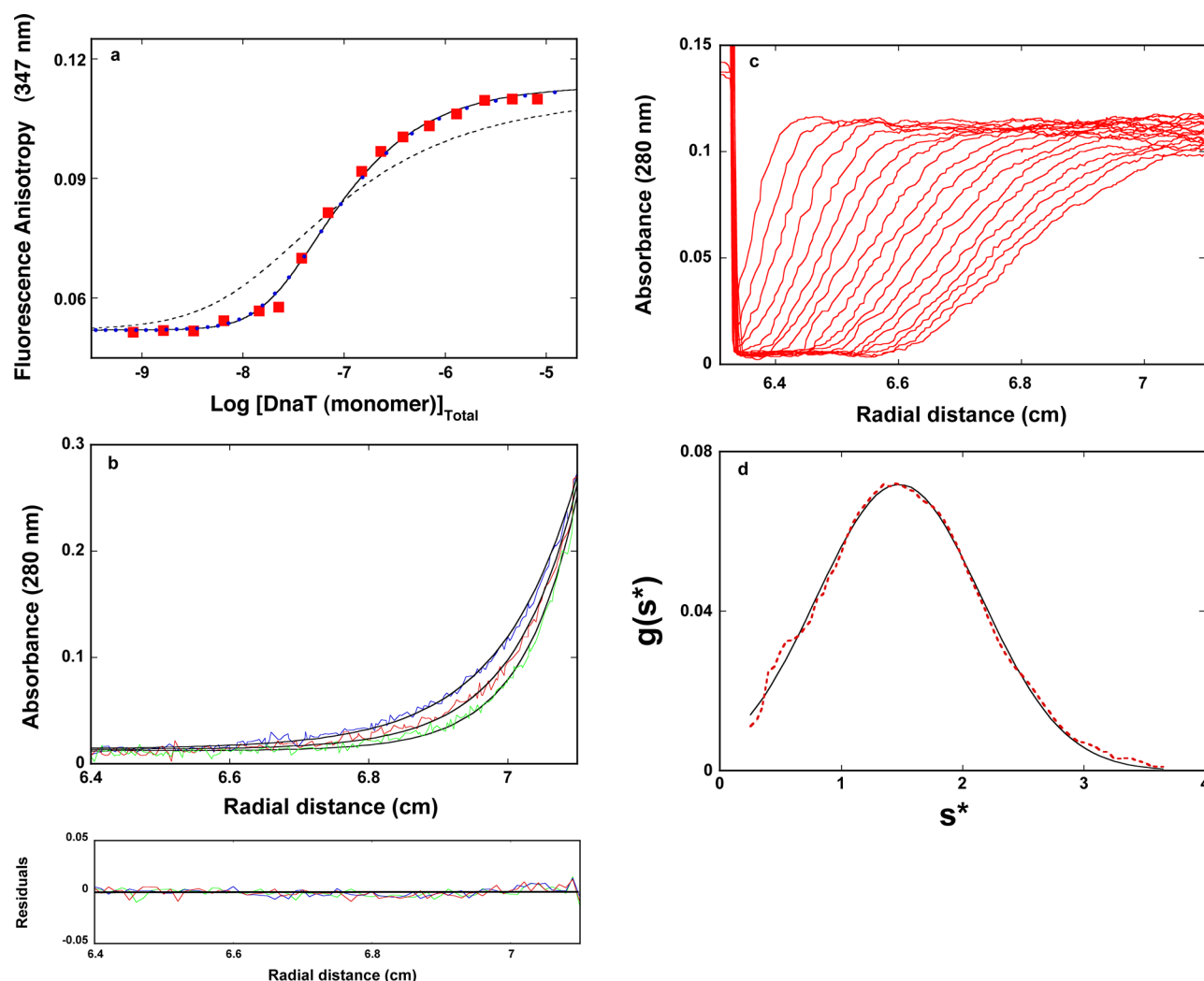


Figure 1. (a) Dependence of the DnaT fluorescence anisotropy upon the total DnaT monomer concentration ($\lambda_{\text{ex}} = 296 \text{ nm}$, $\lambda_{\text{em}} = 347 \text{ nm}$) in buffer C (pH 7.0, 20 °C), containing 0.1 mM EDTA and no magnesium (Materials and Methods). The solid line is the nonlinear least-squares fit of the titration curve, using the trimer model described by eqs 3–7 (DOI: 10.1021/bi301568w), with the following values: $K_T = 1.8 \times 10^{14} \text{ M}^{-2}$, $r_M = 0.0520$, and $r_T = 0.1133$. The dashed line is the best fit of the titration curve using the monomer \leftrightarrow dimer \leftrightarrow trimer model described by eqs 8–13 (DOI: 10.1021/bi301568w), with the following values: $K_M = 1.6 \times 10^7 \text{ M}^{-1}$, $r_M = 0.0520$, $r_D = 0.080$, and $r_T = 0.1133$. The blue circles represent the nonlinear least-squares fit of the titration curve using the cooperative monomer \leftrightarrow dimer \leftrightarrow trimer model (eq 21a, DOI: 10.1021/bi301568w), with the following values: $K_M = 5 \times 10^5 \text{ M}^{-1}$, $\sigma = 750 \pm 100$, $r_M = 0.0520$, $r_D = 0.080$, and $r_T = 0.1133$. (b) Sedimentation equilibrium concentration profiles of the DnaT protein under the same buffer conditions without magnesium. The concentration of the protein is $2.86 \times 10^{-6} \text{ M}$ (monomer). The profiles were recorded at 280 nm and at 14000 (blue), 16000 (red), and 18000 rpm (green). The solid lines are nonlinear least-squares fits to a single-exponential function (DOI: 10.1021/bi301568w, eq 2), with a single species having a molecular weight of 62000 ± 4000 (Materials and Methods). The bottom panel shows the residuals of the fits. (c) Sedimentation velocity absorption profiles at 280 nm of the DnaT protein in buffer C (pH 7.0, 25 °C), containing 0.1 mM EDTA and no magnesium. The DnaT concentration is $4.29 \times 10^{-6} \text{ M}$ (monomer), at 50000 rpm. (d) Apparent sedimentation coefficient distribution, $g(s^*)$, as a function of the radial sedimentation coefficient coordinate, s^* , obtained from the time derivatives of the DnaT protein sedimentation profiles in panel c. The solid line is the nonlinear least-squares fit of the distribution using the software provided by the manufacturer (Materials and Methods).

disassembly of the protein oligomers may be a specific part of the primosome assembly process. On the other hand, how these processes are controlled by temperature, solution conditions, salt, and magnesium is unknown. Nothing is known about the role of the protein structure in the stability of the trimer. The discovery of two different interacting sites on the DnaT monomer, which differently stabilize the trimer, adds additional complexity (DOI: 10.1021/bi301568w). Nothing is known about the control exerted by the solution variables on these two very different interactions.

In this work, we report quantitative analyses of the energetics of the DnaT trimerization process. Specific binding of Mg^{2+}

cations to the DnaT monomers regulates the intrinsic affinity of the DnaT trimerization reaction. Magnesium binds to the N-terminal domain of the protein in the process that involves approximately three Mg^{2+} cations per DnaT monomer. Mg^{2+} cations indirectly stabilize the DnaT trimer through the induced changes in the structures of the monomers. Both interacting sites of the DnaT protein are intimately integrated with the entire protein structure. In the intact protein, the C-terminal region constitutively interacts with the binding site on the N-terminal domain.

MATERIALS AND METHODS

Reagents and Buffers. All solutions were made with distilled and deionized >18 MΩ (Milli-Q Plus) water. All chemicals were reagent grade. Buffer C consists of 10 mM sodium cacodylate adjusted to pH 7.0 with HCl, 1 mM DTT, 100 mM NaCl, 5 mM MgCl₂, and 25% (w/v) glycerol.^{12–15} Buffer C, without magnesium, contains 0.1 mM EDTA. The temperature and salt concentrations are indicated in the text.

DnaT Protein. The *E. coli* DnaT protein has been isolated as described in the preceding paper (DOI: 10.1021/bi301568w). The protein concentration was spectrophotometrically determined, with an extinction coefficient ϵ_{280} of $2.7960 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$ (monomer) obtained using an approach based on Edelhoch's method.^{12–17}

Fluorescence Measurements. All steady-state fluorescence titrations were performed using the ISS (Urbana, IL) PC-1 spectrofluorometer as previously described by us.^{18–25} The state of the DnaT protein oligomerization has been analyzed, using the steady-state fluorescence anisotropy, r , of the protein tryptophan residues.^{26–32} The dynamic quenching data were analyzed using the Stern–Volmer equation

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \quad (1)$$

where F_0 is the fluorescence intensity of the sample prior to the addition of the quencher, F is the intensity in the presence of the quencher, K_{SV} is the Stern–Volmer quenching constant, and $[Q]$ is the molar concentration of the quencher.^{33–35}

Analytical Ultracentrifugation Measurements. Analytical, equilibrium, and velocity ultracentrifugation experiments were performed with an Optima XL-A analytical ultracentrifuge (Beckman Inc., Palo Alto, CA), using double-sector charcoal-filled 12 mm centerpieces, as previously described by us.^{36–43} The values of sedimentation coefficients, $s_{20,w}$, were corrected to $s_{20,w}^\circ$ for solvent viscosity and temperature to standard conditions.⁴⁴

RESULTS

Effect of Magnesium on the DnaT Protein Trimerization. Early studies of the DnaT protein suggested that the presence of magnesium in solution might significantly affect the oligomeric state of the protein (see above).¹⁰ Therefore, we address the effect of Mg²⁺ on the protein trimerization reaction. The fluorescence anisotropy dependence upon the total DnaT concentration (monomer) obtained in buffer C (pH 7.0, 20 °C), containing 0.1 mM EDTA and no magnesium, is shown in Figure 1a. Similar to the data obtained in the presence of Mg²⁺ (DOI: 10.1021/bi301568w), the titration curve spans less than ~2 orders of magnitude on the protein total monomer concentration scale between 10 and 90% of the observed signal, indicating that the monomer–trimer reaction is predominantly observed.²⁶ The solid line in Figure 1a is the nonlinear least-squares fit of the experimental curve using the monomer ↔ trimer model (DOI: 10.1021/bi301568w, eqs 3–7). The fit provides an excellent description of the experimental curve, indicating that both the exclusive formation of the trimer and the cooperative character of the trimerization process are not affected by the absence of magnesium, and provides the following values: $K_T = (1.8 \pm 0.3) \times 10^{14} \text{ M}^{-2}$, $r_M = 0.052 \pm 0.001$, and $r_T = 0.011 \pm 0.001$. The values of the fluorescence anisotropies of the monomer and the trimer are also very similar to the same parameters obtained in the presence of

Mg²⁺. Nevertheless, K_T has a lower value than in the presence of magnesium [$K_T = (3.5 \pm 0.6) \times 10^{14} \text{ M}^{-2}$ (DOI: 10.1021/bi301568w; see below)]. The dashed line in Figure 1a is the best fit of the experimental titration curve, using the monomer ↔ dimer ↔ trimer model (DOI: 10.1021/bi301568w, eqs 8–13). Clearly, the thermodynamic model, which assumes the presence of a significant population of the dimer, does not adequately describe the experimental data.

As we discuss in the preceding paper (DOI: 10.1021/bi301568w), a more complex model includes two binding parameters, the intrinsic binding constant, K_M , and the cooperativity parameter, σ (DOI: 10.1021/bi301568w, eq 21a). However, the high cooperativity of the system precludes any unique and independent determination of K_M and σ , because of their highly correlated values, and the analysis, using the model, renders only the maximal possible value of the intrinsic association constant, K_M , and the minimal value of σ . The nonlinear least-squares fit of the titration curve using the model defined by eq 21a (DOI: 10.1021/bi301568w) is included in Figure 1a. The plot has been obtained by setting a given value of K_M and fitting σ and r_D . The fit provides the maximal possible value of K_M [$(5 \pm 1) \times 10^5 \text{ M}^{-1}$] and the minimal value of σ (750 ± 100).

The sedimentation equilibrium profiles of the DnaT protein in buffer C (pH 7.0, 20 °C), containing 0.1 mM EDTA and no magnesium, recorded at the protein absorption band (280 nm), at different rotational speeds, are shown in Figure 1b.^{36–43} The selected protein concentrations [$2.86 \times 10^{-6} \text{ M}$ (monomer)] correspond to the final plateaus observed in the fluorescence anisotropy titrations (Figure 1a). The smooth solid lines in Figure 1b are the nonlinear least-squares fits, using the single-exponential function (DOI: 10.1021/bi301568w, eq 2). The fits indicate the presence of a single molecular species with a molecular weight of 62000 ± 4000 . Adding additional exponents does not improve the statistics of the fits (data not shown). With a molecular weight of the DnaT monomer of ~19455, these results establish that, at high protein concentrations and in the absence of magnesium, the DnaT protein exists in solution as a stable trimer, which is in full agreement with the fluorescence anisotropy data (Figure 1a).

Global Conformation of the DnaT Protein Trimer in the Absence of Magnesium. The sedimentation velocity profiles (monitored at 280 nm) of the DnaT protein in buffer C (pH 7.0, 25 °C), containing 0.1 mM EDTA and no magnesium, are shown in Figure 1c. The concentration of the protein is $4.29 \times 10^{-6} \text{ M}$ (monomer). The profiles clearly show that there is a single moving boundary, indicating the presence of a single molecular entity.^{41–44} The sedimentation coefficient of the protein, $s_{20,w}$, has been obtained using the time-derivative approach as shown in Figure 1b [refs 45 and 46 and the preceding paper (DOI: 10.1021/bi301568w)]. Like the buffer conditions containing magnesium, the value of $s_{20,w}$ shows little, if any, dependence upon the protein concentration, in the protein concentrations range where only the stable trimer is present (data not shown). The obtained value of the sedimentation coefficient of the DnaT protein in the absence of magnesium ($s_{20,w}^\circ$) is $3.5 \pm 0.1 \text{ S}$, i.e., within experimental accuracy, the same as that obtained in the presence of magnesium (DOI: 10.1021/bi301568w). In other words, Mg²⁺ cations have no effect on the global conformation of the DnaT trimer (DOI: 10.1021/bi301568w) (see Discussion).

Dynamic Fluorescence Quenching of the DnaT Monomer and Trimer in the Absence and Presence of

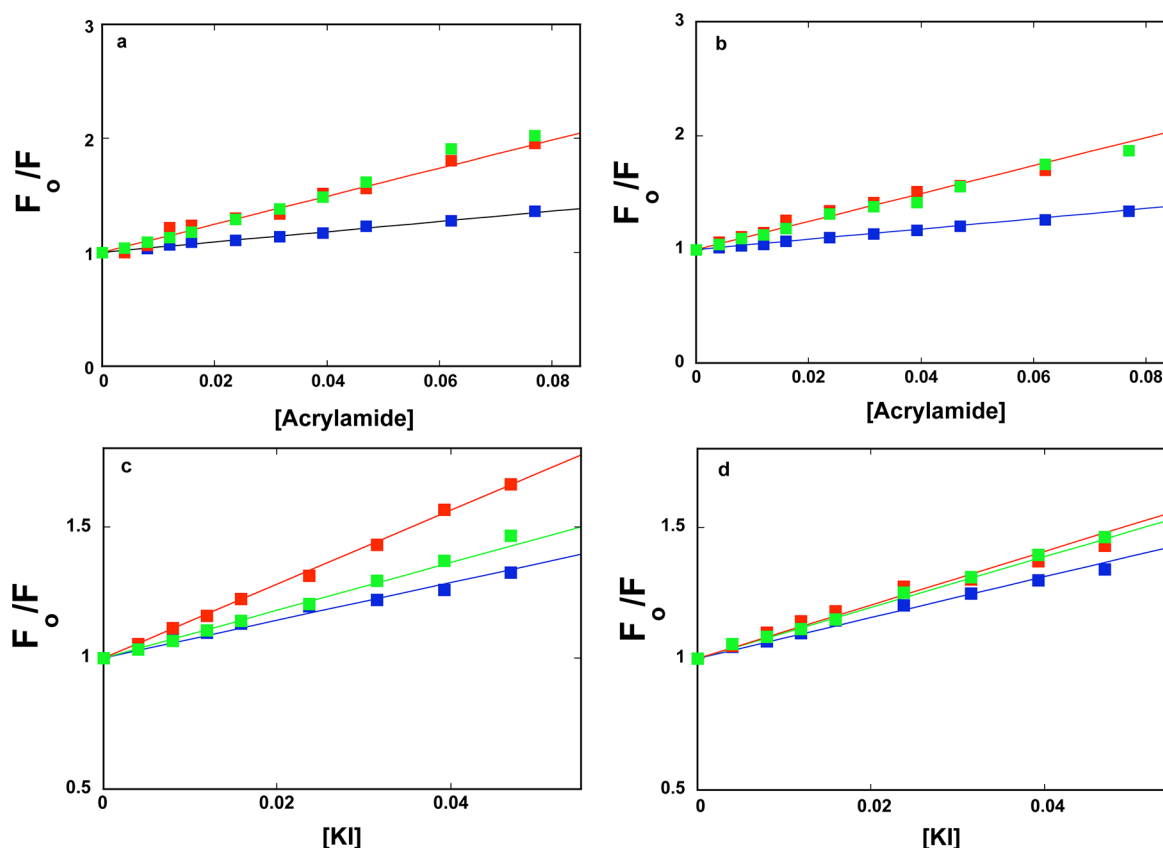


Figure 2. (a) Stern–Volmer plots of the DnaT fluorescence quenching in buffer C (pH 7.0, 20 °C), containing 0.1 mM EDTA and no magnesium, with acrylamide as the dynamic fluorescence quencher. The protein concentrations are (red squares) 3.0×10^{-8} M (monomer) and (blue squares) 3.18×10^{-6} M (monomer). (b) Stern–Volmer plots (Materials and Methods) of the DnaT fluorescence quenching in buffer C (pH 7.0, 20 °C) with acrylamide as the dynamic fluorescence quencher ($\lambda_{\text{ex}} = 296$ nm; $\lambda_{\text{em}} = 347$ nm). The protein concentrations are (red squares) 3.0×10^{-8} M (monomer) and (blue squares) 3.25×10^{-6} M (monomer). In both panels, Stern–Volmer plots of NATA (3.18×10^{-6} M) fluorescence quenching under the corresponding buffer conditions are shown (green squares). (c) Stern–Volmer plots of the DnaT fluorescence quenching in buffer C (pH 7.0, 20 °C), containing 0.1 mM EDTA and no magnesium, with I^- as the dynamic fluorescence quencher. The protein concentrations are (red squares) 3.0×10^{-8} M (monomer) and (blue squares) 3.18×10^{-6} M (monomer). (d) Stern–Volmer plots of the DnaT fluorescence quenching in buffer C (pH 7.0, 20 °C) with I^- as the dynamic fluorescence quencher. The protein concentrations are (red squares) 3.0×10^{-8} M (monomer) and (blue squares) 3.25×10^{-6} M (monomer). In both panels, Stern–Volmer plots of NATA (3.18×10^{-6} M) fluorescence quenching under the corresponding buffer conditions are shown (green squares).

Magnesium. Both acrylamide and iodide anions, I^- , are efficient dynamic quenchers of the protein tryptophan fluorescence.^{33–35} While neutral acrylamide provides information about the steric access of the tryptophan residues in the protein, the negatively charged iodide accesses tryptophans on the protein surface and senses the local environment of the fluorophore.^{33–35} The quenching studies have been performed with DnaT solutions of 3×10^{-8} M (monomer), where the protein is predominantly in the monomer state, and 3.25×10^{-6} M (monomer), where the DnaT forms a stable trimer (Figure 1a,b).

Stern–Volmer plots of the DnaT protein monomer and the trimer with acrylamide as the dynamic quencher, in the absence and presence of Mg^{2+} , are shown in panels a and b of Figure 2, respectively. For comparison, the Stern–Volmer plots for NATA, under the corresponding solution conditions, are also included in the figures. Within experimental accuracy, the plots are linear. The solid lines in panels a and b of Figure 2 are the linear least-squares fits to eq 1, which provide the Stern–Volmer quenching constants for the DnaT monomer ($K_{\text{SV}} = 12.3 \pm 1 \text{ M}^{-1}$) and the trimer ($K_{\text{SV}} = 4.5 \pm 0.5 \text{ M}^{-1}$), respectively, in a manner independent of the presence or

absence of magnesium. Thus, magnesium does not change the acrylamide accessibility of the tryptophan residues in the monomer and trimer (see Discussion). Nevertheless, the data show that the tryptophan residues are significantly more accessible to acrylamide in the monomer than in the trimer. In fact, the accessibility of the tryptophans in the DnaT monomer is practically the same as the accessibility of the free NATA in solution ($K_{\text{SV}} = 12.3 \pm 1 \text{ M}^{-1}$) (Figure 2a,b).

The situation is different in the case of the quenching by iodide anions, with corresponding Stern–Volmer plots shown in panels c and d of Figure 2. In the absence of Mg^{2+} , the Stern–Volmer quenching constants are $14.1 \pm 1.2 \text{ M}^{-1}$ for the monomer and $7.2 \pm 0.7 \text{ M}^{-1}$ for the trimer. In the presence of Mg^{2+} , the corresponding Stern–Volmer constants are 10.2 ± 1.1 and $7.8 \pm 0.7 \text{ M}^{-1}$, respectively. First, the tryptophan residues are still considerably more accessible to I^- in the monomer than in the trimer. Second, magnesium significantly changes the iodide accessibility of the tryptophan residues of the monomer, while the accessibility of the residues in the trimer remains, within experimental accuracy, unaffected by Mg^{2+} . The fluorescence quenching data provide the first indication that magnesium cations bind to the DnaT

monomers prior to their entry into the trimer structure (see below). Interestingly, the quenching of the tryptophan residues in the monomer by Γ^- , in the absence of Mg^{2+} , is even more efficient than that observed for free NATA ($K_{SV} = 9.1 \pm 0.9 \text{ M}^{-1}$), under the same buffer conditions (Figure 2c). The presence of magnesium (Figure 2d) reduces the quenching of the tryptophan residues in the monomer to that of NATA ($K_{SV} = 9.8 \pm 0.9 \text{ M}^{-1}$) (see Discussion).

Effect of Magnesium on the Free Energy of DnaT Trimerization. Analysis of the effect of temperature on the DnaT trimerization reaction showed that the presence of Mg^{2+} cations mostly affects the free energy of trimerization at the highest examined temperature (see below). Therefore, to increase the resolution, we address the effect of magnesium on the trimerization constant, K_T , at 35 °C. Figure 3 shows the

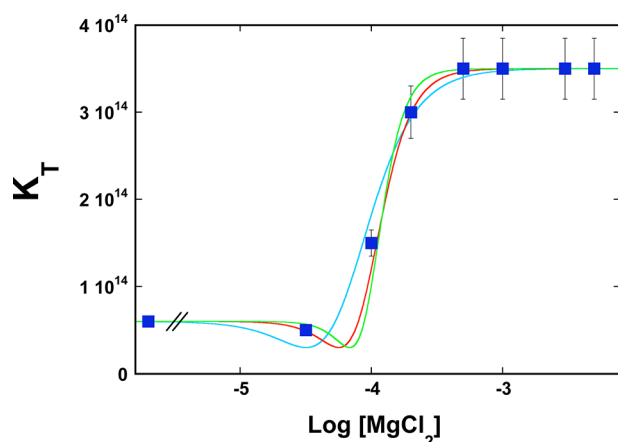


Figure 3. Dependence of the DnaT trimerization constant, K_T , upon the logarithm of MgCl_2 concentration in solution, in buffer C (pH 7.0, 35 °C). The solid red line is the nonlinear least-squares fit of the plot to eq 6 with an n of 3 and a K_n of $2.3 \times 10^{12} \text{ M}^{-3}$. For comparison, the best fits using $n = 2$ (turquoise) and $n = 4$ (green) are also included.

dependence of K_T upon the logarithm of the magnesium concentration. The plot clearly indicates a complex behavior of the examined system. The value of K_T remains unchanged from 0 to $\sim 3 \times 10^{-5} \text{ M}$ MgCl_2 . However, above $\sim 1 \times 10^{-4} \text{ M}$ MgCl_2 , K_T steeply increases and reaches a plateau at $\sim 5 \times 10^{-4} \text{ M}$ MgCl_2 , indicating that the binding process saturates at higher MgCl_2 concentrations. Because NaCl does not affect the DnaT trimerization at the same or higher salt concentration range (data not shown), neither monovalent cations nor anions participate in the reaction, and the observed effect must be due to the specific binding of magnesium cations.

Notice that magnesium does not affect the global structure of the DnaT trimer (Figure 1c,d). Also, the fluorescence quenching data indicate that the presence of Mg^{2+} cations affects the monomer structure only prior to the trimerization reaction (Figure 2). Therefore, the simplest thermodynamic model, which describes the effect of magnesium on K_T , includes the binding of Mg^{2+} cations to the monomer, which indirectly affects the monomer–monomer interactions in the trimer (see below).^{47–52} The system is described by the following equilibria:



The trimerization constant, K_T , in the absence of Mg^{2+} is described by eq 4 in the preceding paper (DOI: 10.1021/bi301568w). The magnesium binding constant for the monomer, K_n , and the trimerization constant, K_{T_n} , in the presence of Mg^{2+} , are defined as

$$K_n = \frac{[\text{M}_n]_F}{[\text{M}]_F [\text{Mg}^{2+}]^n} \quad (5a)$$

and

$$K_{T_n} = \frac{[\text{T}_n]_F}{[\text{M}_n]_F^3} \quad (5b)$$

The observed, overall trimerization constant, $K_{T_{\text{obs}}}$, at any MgCl_2 concentration is then^{47–52}

$$K_{T_{\text{obs}}} = \frac{K_T (1 + K_n K_n^3 [\text{Mg}^{2+}]^n)^{3n}}{(1 + K_n [\text{Mg}^{2+}]^n)^3} \quad (6)$$

At a high magnesium concentration ($[\text{MgCl}_2] \rightarrow \infty$), $K_{T_{\text{obs}}} = K_T = 3.5 \times 10^{14} \text{ M}^{-2}$ (DOI: 10.1021/bi301568w). The value of K_T in the absence of Mg^{2+} at 35 °C is also known ($K_T = 6 \times 10^{13} \text{ M}^{-2}$) (Figure 3). Therefore, there are only two independent parameters, K_n and n , in eq 6, which have to be determined. The solid red line in Figure 3 is the nonlinear least-squares fit of eq 6 to the observed dependence of the trimerization constant upon MgCl_2 concentration, which provides the following values: $n = 3 \pm 1$, and $K_n = (2.3 \pm 0.7) \times 10^{12} \text{ M}^{-3}$. The apparent, intrinsic binding constant of Mg^{2+} , $K_{n_{\text{int}}} = (K_n)^{1/3} = (1.3 \pm 0.3) \times 10^4 \text{ M}^{-1}$. For comparison, the best fits using $n = 2$ (turquoise) and $n = 4$ (green) are also shown in Figure 3 (see Discussion).

Effect of Temperature on the DnaT Protein Trimerization in the Presence and Absence of Magnesium. We address the nature of the DnaT trimerization process by examining the effect of temperature on the reaction. The dependence of the DnaT protein fluorescence anisotropy upon the total monomer concentration, in buffer C (pH 7.0), obtained at different temperatures, is shown in Figure 4a. The steady-state anisotropy of the monomer becomes lower at higher temperatures, while the steady-state anisotropy of the trimer, as well as the midpoint of the titration curves, is only slightly affected by temperature. The solid lines in Figure 4a are the nonlinear least-squares fits of the experimental titration curves, using the monomer \leftrightarrow trimer model (DOI: 10.1021/bi301568w, eqs 3–7). Analogous anisotropy titrations, obtained in the absence of magnesium, are shown in Figure 4b. Similarly, the increase in temperature predominantly affects the steady-state anisotropy of the monomer. Although the changing plateaus of the plots obscure the visual evaluation of the behavior of the system, the midpoints of the titration curves are more pronouncedly shifted toward higher protein concentrations than in the presence of Mg^{2+} , indicating a lower affinity at the higher temperature.

The dependencies of the trimerization constant, K_T , upon the reciprocal of the temperature (kelvin) (van't Hoff plot), in the presence and absence of magnesium, are shown in Figure 4c.^{19,20,53} The plots are linear in the examined temperature range. The solid lines are linear least-squares fits to the van't Hoff equation: $\partial \ln K_T / \partial (1/T) = -\Delta H/R$. Thus, in the presence

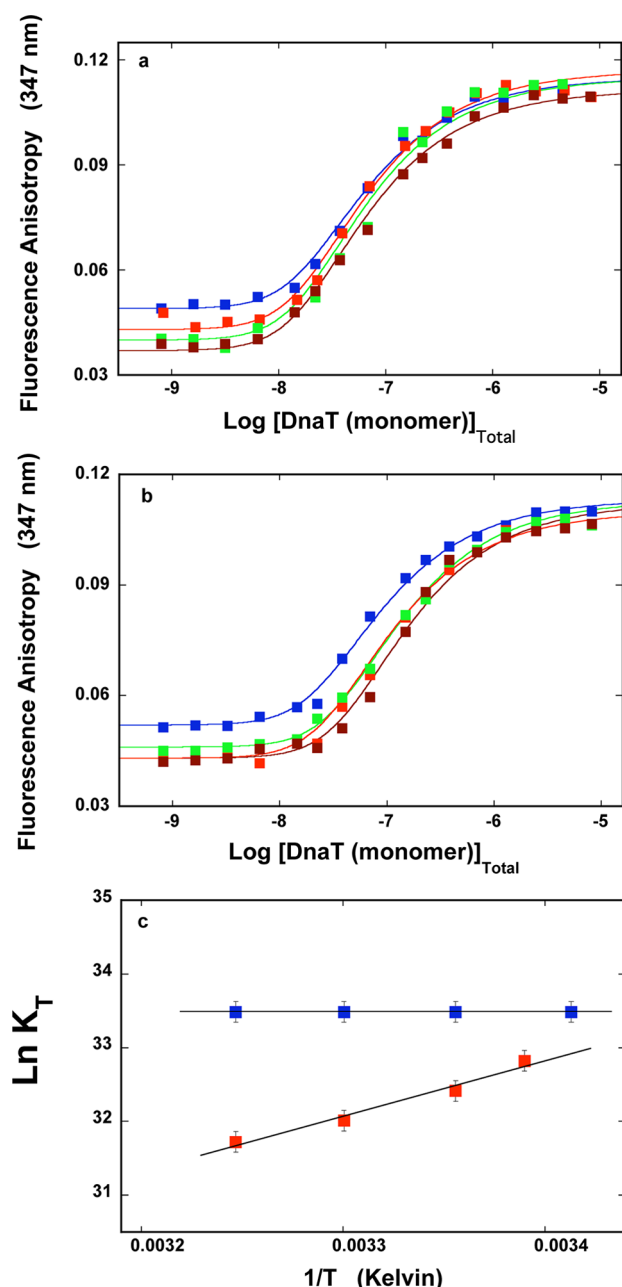


Figure 4. (a) Dependence of the DnaT fluorescence anisotropy upon the total DnaT monomer concentration ($\lambda_{\text{ex}} = 296 \text{ nm}$; $\lambda_{\text{em}} = 347 \text{ nm}$) in buffer C (pH 7.0), recorded at different temperatures: (blue squares) 20, (red squares) 25, (green squares) 30, and (maroon squares) 35 °C. The solid lines are the nonlinear least-squares fits of the titration curves, using the trimer model described by eqs 3–7 (DOI: 10.1021/bi301568w). (b) Dependence of DnaT fluorescence anisotropy on the total DnaT monomer concentration ($\lambda_{\text{ex}} = 296 \text{ nm}$; $\lambda_{\text{em}} = 347 \text{ nm}$) in buffer C (pH 7.0), containing 0.1 mM EDTA and no magnesium, recorded at different temperatures: (blue squares) 20, (red squares) 25, (green squares) 30, and (maroon squares) 35 °C. The solid lines are the nonlinear least-squares fits of the titration curves, using the trimer model described by eqs 3–7 (DOI: 10.1021/bi301568w). (c) Dependence of the trimerization constant, K_T , upon the reciprocal of temperature (kelvin) (van't Hoff plot), in the presence (blue squares) and absence (red squares) of magnesium. The solid lines are linear least-squares fits to the van't Hoff equation (details in the text).

of magnesium, trimer formation is characterized by the apparent enthalpy change ($\Delta H^\circ = 0 \pm 2 \text{ kcal/mol}$). In other words, the interactions are completely driven by the apparent entropy change, ΔS° . The situation is very different in the absence of magnesium, where the trimerization process is characterized by the negative apparent enthalpy change ($\Delta H^\circ = -15 \pm 2 \text{ kcal/mol}$), indicating that the trimerization reaction is driven by the apparent enthalpy. Using the standard thermodynamic formulas [$\Delta G^\circ = -RT \ln K_T$, at 20 °C, and $\Delta S^\circ = (-\Delta G^\circ + \Delta H^\circ)/T$], one obtains the apparent entropy changes (ΔS°), characterizing the trimerization process in the presence and absence of magnesium: ≈ 67 and $\approx -116 \text{ cal mol}^{-1} \text{ deg}^{-1}$, respectively (see Discussion).

Effect of Solvent on the DnaT Protein Trimerization in the Presence and Absence of Magnesium. To obtain further insight into the nature of the DnaT oligomerization reaction, we examined the effect of solvent on protein trimer formation. The dependence of fluorescence anisotropy upon the total DnaT concentration (monomer) obtained in buffer C (pH 7.0, 25 °C), containing different concentrations of the neutral solute, glycerol (w/v %), is shown in Figure 5a. Corresponding fluorescence anisotropy titrations obtained in the absence of Mg^{2+} are shown in Figure 5b. In the presence of magnesium, increasing the glycerol concentration, from 10 to 25% (w/v), has an only slight effect on the midpoint of the titration curves, indicating similar affinities in the examined solute concentration range. In the absence of Mg^{2+} , the titration curves shift toward higher total protein concentrations at higher glycerol concentrations, indicating a significant decrease in the monomer–monomer affinity. Analogous titrations have been performed in the presence of another neutral solute, sucrose, which showed very similar effects on the fluorescence anisotropy titration curves, in the presence and absence of Mg^{2+} (data not shown). The solid lines in panels a and b of Figure 5 are nonlinear least-squares fits of the experimental titration curves to the monomer \leftrightarrow trimer model (DOI: 10.1021/bi301568w, eqs 3–7), with the trimerization binding constant, K_T , as a fitting parameter.

The effect of the “neutral” solute on the macromolecular interactions can be complicated by the presence of preferential interactions.^{54,55} However, the very similar effect of two different solutes on the same interactions strongly suggests that the selected solutes do not affect the DnaT oligomerization through preferential interactions with the protein (see below).^{54,55} The simplest thermodynamic model of the observed solute effect is that the trimerization is affected by the changes of the water concentration in the sample and the reaction can be described by a general linkage equation $3\text{M} \leftrightarrow \text{T} \pm n\text{H}_2\text{O}$.^{47–52} It should be stressed that, even at the maximal concentration of the applied solutes [25% (w/v) glycerol or sucrose], the water activity coefficient is practically ~ 1 and the water concentration, instead of its activity, can be used in the analysis.^{56,57} Moreover, very small effects of the selected solutes on the water activity are completely absorbed by the error in the determination of the trimerization constant, which is 10–15% and is included in errors of the determined slopes of the log–log plots (see below).^{47,50,51}

The dependencies of the logarithm of the trimerization constant, K_T , upon the logarithm of H_2O concentration (log–log plots), determined in the presence and absence of magnesium and with glycerol as the neutral solute, are shown in Figure 5c.^{58,59} The plots are linear in the examined solute concentration ranges. The slopes of the plots are $\partial \log K_T / \partial$

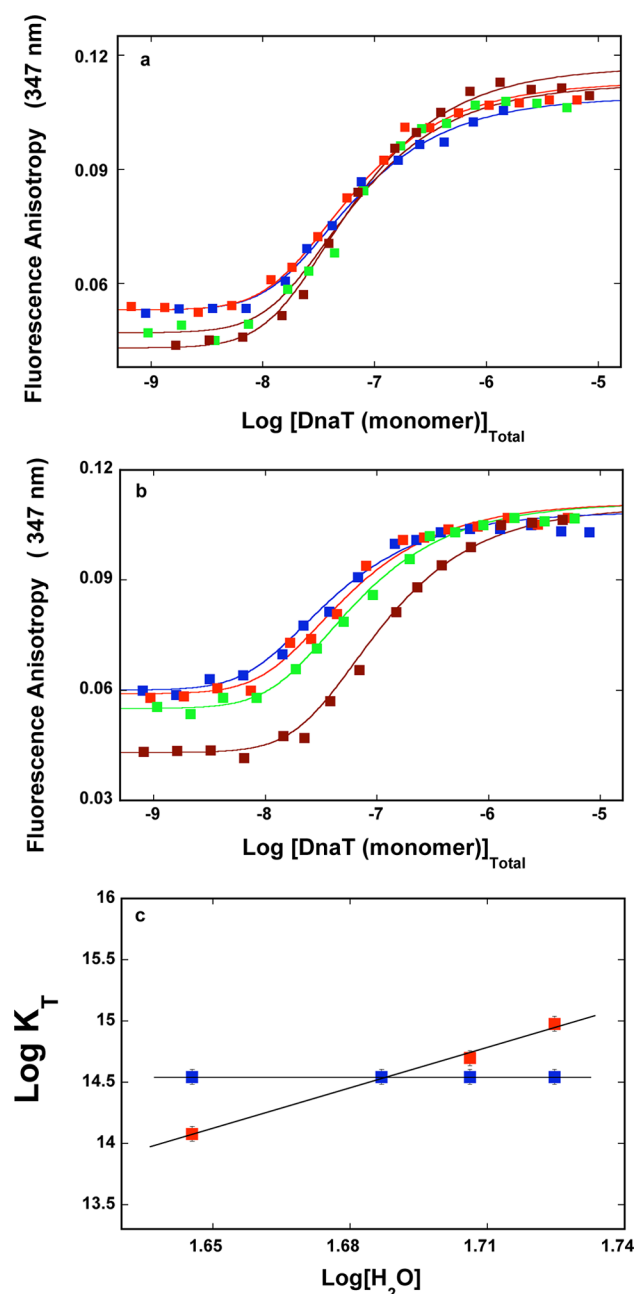


Figure 5. (a) Dependence of the DnaT fluorescence anisotropy upon the total DnaT monomer concentration ($\lambda_{\text{ex}} = 296$ nm; $\lambda_{\text{em}} = 347$ nm) in buffer C (pH 7.0, 25 °C) containing different concentrations of glycerol (w/v): (blue squares) 10%, (red squares) 15%, (green squares) 20%, and (maroon squares) 25%. The solid lines are the nonlinear least-squares fits of the titration curves, using the trimer model described by eqs 3–7 (DOI: 10.1021/bi301568w). (b) Dependence of DnaT fluorescence anisotropy on the total DnaT monomer concentration ($\lambda_{\text{ex}} = 296$ nm; $\lambda_{\text{em}} = 347$ nm) in buffer C (pH 7.0, 25 °C) containing 0.1 mM EDTA and no magnesium, and different concentrations of glycerol (w/v): (blue squares) 10%, (red squares) 15%, (green squares) 20%, and (maroon squares) 25%. The solid lines are the nonlinear least-squares fits of the titration curves, using the trimer model described by eqs 3–7 (DOI: 10.1021/bi301568w). (c) Dependence of the trimerization constant, K_T , upon the water concentration in the sample in the presence (blue squares) and absence (red squares) of magnesium. The solid lines are linear least-squares fits with the slopes $\partial \log K_T / \partial \log [\text{H}_2\text{O}] = 0$ in the presence of Mg^{2+} and $\partial \log K_T / \partial \log [\text{H}_2\text{O}] = 11.1$ in the absence of Mg^{2+} (details in the text).

$\log[\text{H}_2\text{O}] = 0 \pm 1.6$ in the presence of Mg^{2+} and $\partial \log K_T / \partial \log [\text{H}_2\text{O}] = 11.1 \pm 3.5$ in the absence of Mg^{2+} . The values of the analogous slopes of the log–log plots, obtained in the presence of sucrose, are $\partial \log K_T / \partial \log [\text{H}_2\text{O}] = 1 \pm 1.6$ and $\partial \log K_T / \partial \log [\text{H}_2\text{O}] = 10.3 \pm 2.8$ (data not shown). The very similar values of the slopes of the log–log plots, obtained for different solutes, reinforce the conclusion that the water concentration is the dominant factor in the observed solute effects.^{54,55} The results indicate that the DnaT trimerization, in the presence of magnesium, is not accompanied by any significant, apparent water uptake and/or release, while in the absence of magnesium, the formation of the trimer is accompanied by the apparent, net uptake of ~ 11 water molecules^{58,59} (see Discussion).

Effect of Guanidine Hydrochloride on the DnaT Trimer Stability in the Presence and Absence of Magnesium. The analysis has so far focused on DnaT trimer formation. In the next set of experiments, we address the role of the DnaT structure in trimer stability, by examining the dissociation reaction of the trimer in the presence and absence of magnesium. The DnaT trimer dissociation has been induced using guanidine hydrochloride (GnHCl), which is a potent denaturant of the protein secondary and tertiary structures.^{60–62} The dependence of the fluorescence anisotropy of the DnaT protein [2.91×10^{-6} M (monomer)] upon the concentration of GnHCl in buffer C (pH 7.0, 25 °C) is shown in Figure 6a. At this protein concentration, in the absence of GnHCl, the DnaT protein primarily exists as a trimer (DOI: 10.1021/bi301568w). The value of the fluorescence anisotropy, corresponding to the trimer ($r \sim 0.111$), remains unchanged up to ~ 4 M GnHCl. As the GnHCl concentration increases, the anisotropy steeply decreases, reaching a plateau ($r \sim 0.088$) at ~ 4.8 M GnHCl, indicating a highly cooperative transition.^{47–52,60–62} The corresponding plot, obtained in the absence of Mg^{2+} , is shown in Figure 6b. The dependence of DnaT protein anisotropy upon GnHCl concentration is similar to that obtained in the presence of Mg^{2+} . However, the observed transition is much less cooperative, as compared to the transition observed in the buffer containing Mg^{2+} (see below).^{47–52,60–62}

It should be noted that the anisotropies of the system at plateaus, obtained at the high GnHCl concentration, are significantly higher than the anisotropies (~ 0.05) of the native DnaT monomer (Figures 1a and 4a,b). This is true both in the presence and in the absence of magnesium (Figure 6a,b). Therefore, the oligomeric state of the protein at high GnHCl concentrations has been addressed using the sedimentation equilibrium method (DOI: 10.1021/bi301568w). Figure 6c shows the sedimentation equilibrium profile of the DnaT protein in buffer C (pH 7.0, 25 °C), containing 5.4 M GnHCl recorded at the protein absorption band (280 nm). An analogous profile recorded in the absence of magnesium is shown in Figure 6d. The protein concentrations are 3.25×10^{-6} M (monomer). The smooth solid lines in panels a and b of Figure 6 are nonlinear least-squares fits, using the single-exponential function (DOI: 10.1021/bi301568w, eq 2). The fits provide excellent descriptions of the experimental sedimentation equilibrium profiles, indicating the presence of a single species with molecular weights of 18950 ± 2000 and 19500 ± 2000 in the presence and absence of Mg^{2+} , respectively. Thus, it is clear that the molecular entity observed at high GnHCl concentrations (Figure 6c,d) is the denatured DnaT monomer,

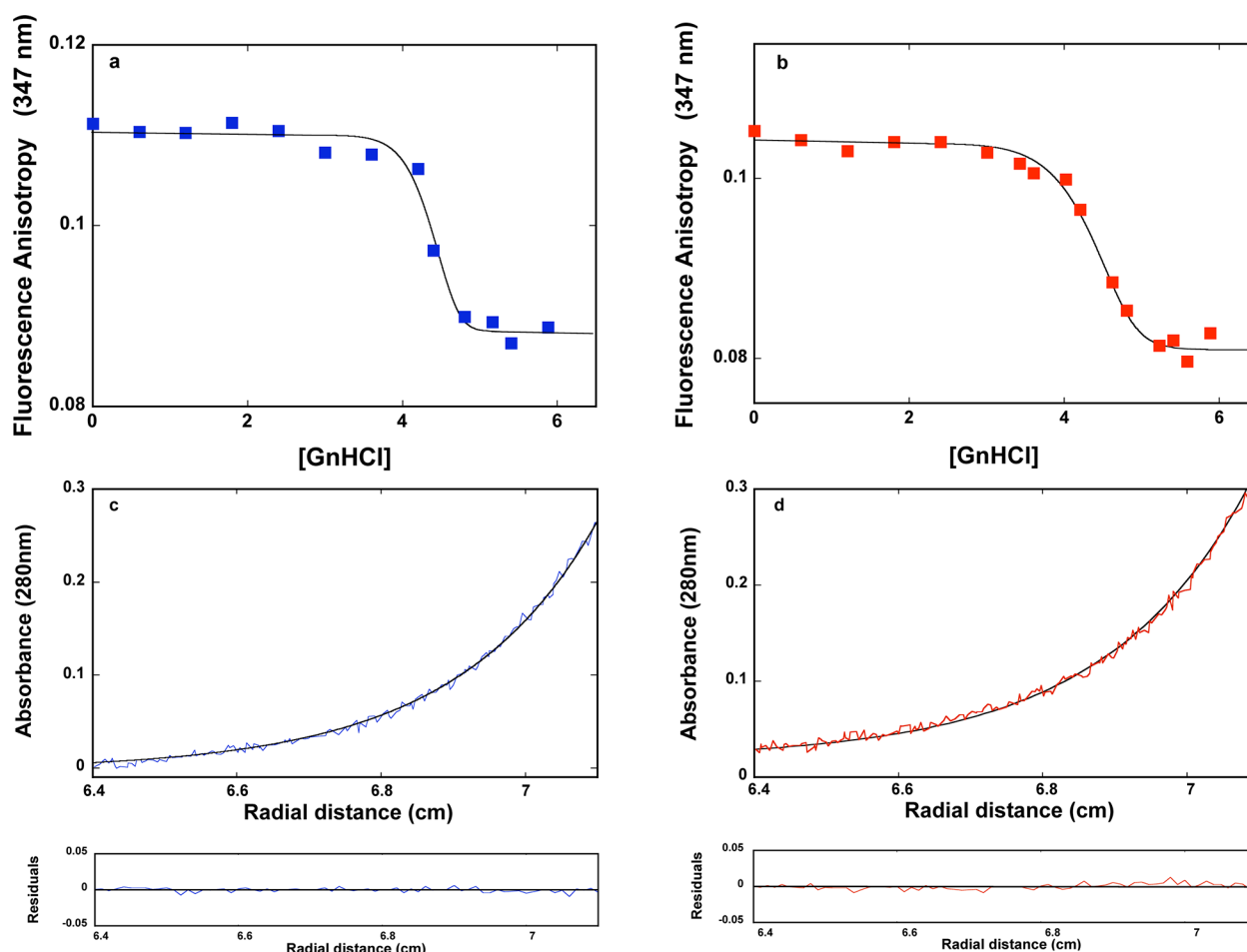


Figure 6. (a) Dependence of the fluorescence anisotropy of the DnaT protein [2.91×10^{-6} M (monomer)] upon the concentration of GnHCl in buffer C (pH 7.0, 25 °C). The solid line is the nonlinear least-squares fit of the experimental titration curve to eqs 7–14, where $p = 18$ and $K_G = 4.0 \times 10^{-11}$ M $^{-18}$. (b) Dependence of the fluorescence anisotropy of the DnaT protein [2.91×10^{-6} M (monomer)] upon the concentration of GnHCl in buffer C (pH 7.0, 25 °C), containing 0.1 mM EDTA and no magnesium. The solid line is the nonlinear least-squares fit of the experimental titration curve to eqs 7–14, where $p = 10$ and $K_G = 3.4 \times 10^{-6}$ M $^{-10}$ (details in the text). (c) Sedimentation equilibrium concentration profiles of the DnaT protein in buffer C (pH 7.0, 25 °C). The concentration of the protein is 3.25×10^{-6} M (monomer). The profile was recorded at 280 nm and 27000 rpm. The smooth solid line is the nonlinear least-squares fit to a single-exponential function (DOI: 10.1021/bi301568w, eq 2), with a single species having a molecular weight of 18950 (Materials and Methods). The bottom panel shows the residuals of the fit. (d) Sedimentation equilibrium concentration profiles of the DnaT protein in buffer C (pH 7.0, 25 °C). The concentration of the protein was 3.25×10^{-6} M (monomer). The profile was recorded at 280 nm and 27000 rpm. The smooth solid line is the nonlinear least-squares fit to a single-exponential function (DOI: 10.1021/bi301568w, eq 2), with a single species having a molecular weight of 19500 (Materials and Methods). The bottom panel shows the residuals of the fit.

characterized by the fluorescence anisotropy being higher than that of the native monomer (see Discussion).

There are several aspects of the observed transitions, which are peculiar to the examined system. The locations of the midpoints of the titration curves at high GnHCl concentrations are where the protein secondary structure is usually denatured (Figure 6a,b).^{60–62} High cooperativities of the observed GnHCl-induced transitions are striking and indicate the lack of any intermediate states of the trimer. The cooperativity of the transition is particularly pronounced in the presence of magnesium cations, which are bound to the monomers; i.e., they do not directly stabilize the trimer (Figure 2) (see Discussion). Finally, the protein is exclusively in the denatured monomer state, at the saturating GnHCl concentration. All these features strongly suggest that the dissociation occurs practically as a single step, without any trimer or dimer interventions, and results from the denaturation of the native (secondary and tertiary) structures of the DnaT monomers.

Thus, the simplest thermodynamic model of the observed effect of GnHCl on the DnaT trimer dissociation includes the following equilibria:^{47–52}



and



The trimer dissociation constant in the absence of GnHCl is $1/K_T$, and the constant for the binding of GnHCl to the monomer, K_G , is defined as

$$K_G = \frac{[M]_G}{[M]_F [\text{GnHCl}]^p} \quad (8)$$

The total DnaT monomer concentration in the sample is defined by the mass conservation expression, as

$$[M]_T = [M]_F + [M]_G + 3[T]_F \quad (9a)$$

and

$$[M]_T = [M]_F + [M]_F K_G [GnHCl]^p + 3[M]_F^3 K_T \quad (9b)$$

However, the analysis is complicated by the fact that there are two independent variables, $[M]_F$ and $[GnHCl]$, two simultaneous equilibria, and the analytical expression for the concentration of the free DnaT monomer cannot be obtained. Below, we present an algorithm, which allows the analysis of these simultaneous equilibria. Moreover, the presented algorithm can be applied to more complex situations.

The concentration of the monomer state at any GnHCl concentration is defined as a function of the sum of concentrations, $[M]_F + [M]_G$. The sum, $[M]_F + [M]_G$, is then treated as the independent variable. Because the concentration of the free trimer, $[T]_F = ([M]_T - ([M]_F + [M]_G))/3$, the observed, overall trimer dissociation constant, K_{TD} , in the presence of GnHCl is

$$K_{TD} = \frac{3([M]_F + [M]_G)^3}{[M]_T - ([M]_F + [M]_G)} \quad (10)$$

The same overall trimer dissociation constant, K_{TD} , can exclusively be expressed using $[GnHCl]$, as

$$K_{TD} = \frac{(1 + K_G [GnHCl]^p)^3}{K_T} \quad (11)$$

From eq 11, one obtains $[GnHCl]$ as a function of K_{TD} as

$$[GnHCl] = \left[\frac{(K_{TD} K_T)^{1/3} - 1}{K_G} \right]^{1/p} \quad (12)$$

The anisotropy of the sample is defined as

$$r = r_{M_F} f_{M_F} + r_{M_G} f_{M_G} + r_T f_T \quad (13)$$

where r_{M_F} , r_{M_G} , and r_T are fluorescence anisotropies of the free native monomer, denatured monomer associated with GnHCl, and the trimer, respectively. The quantities f_{M_F} , f_{M_G} , and f_T are the corresponding fractional contributions of the three species to the total emission, F_T , of the sample defined as

$$F_T = F_{M_F} [M]_F + F_{M_G} [M]_G + F_{Tr} [T]_F \quad (14a)$$

Then

$$f_M = \frac{F_{M_F} [M]_F}{F_T} \quad (14b)$$

$$f_D = \frac{F_{M_G} [M]_G}{F_T} \quad (14c)$$

and

$$f_T = \frac{F_{Tr} [T]_F}{F_T} \quad (14d)$$

where F_{M_F} , F_{M_G} , and F_{Tr} are molar fluorescence intensities of the monomer states and the trimer, respectively.

The values of r_{M_F} , r_{M_G} , r_T , F_{M_F} , F_{M_G} , F_{Tr} , and K_T are known. Also, $[M]_T$ is known. Thus, there are only two unknown parameters in eqs 8–14, K_G and p . The algorithm for the simulation and fitting is as follows. (1) K_{TD} is calculated from eq 10, using the sum $[M]_F + [M]_G$ as the independent variable. (2) $[GnHCl]$ is calculated, using the obtained K_{TD} , from eq 12.

(3) $[T]_F$ is calculated as $[M]_T - ([M]_F + [M]_G)/3$. (4) $[M]_F$ is calculated using the obtained $[T]_F$ and the known trimerization constant, K_T (DOI: 10.1021/bi301568w). (5) $[M]_G$ is calculated from eq 9a. (6) F_T , f_{M_F} , f_{M_G} , and f_T are calculated from eqs 14a–14d. (7) Finally, the fluorescence anisotropy, r , is calculated from eq 13.

The solid lines in panels a and b of Figure 6 are nonlinear least-squares fits of the experimental DnaT dissociation curves to eqs 7–14, which provide the following values: $p = 18 \pm 2$ and $K_G = (4.0 \pm 1.5) \times 10^{-11} \text{ M}^{-18}$ in the presence of Mg^{2+} , and $p = 10 \pm 2$ and $K_G = (3.4 \pm 1.1) \times 10^{-6} \text{ M}^{-10}$ in the absence of magnesium. Thus, the apparent intrinsic affinities of GnHCl for the DnaT monomer $[(4 \times 10^{-11} \text{ M}^{-18})^{1/18} \approx 0.26 \text{ M}^{-1}]$ and $(3.4 \times 10^{-6} \text{ M}^{-10})^{1/10} = 0.28 \text{ M}^{-1}]$ are virtually the same in the presence and absence of Mg^{2+} , respectively. Nevertheless, in the presence of Mg^{2+} , the system is significantly more cooperative than in the absence of magnesium, as reflected by the considerably larger value of p (see Discussion).

Effect of Temperature on the DnaT N-Terminal Core Domain Dimerization in the Presence and Absence of Magnesium. The dependence of the fluorescence anisotropy of the DnaT N-terminal core domain upon the total, domain monomer concentration in buffer C (pH 7.0), obtained at different temperatures, is shown in Figure 7a. Similar to the behavior of the intact DnaT trimer, the steady-state anisotropy of the domain monomer becomes lower at higher temperatures, while the steady-state anisotropy of the domain dimer is only slightly affected by the temperature. Analogous anisotropy titrations, in the absence of magnesium, are shown in Figure 7b. Correspondingly, the increase in temperature predominantly affects the steady-state anisotropy of the domain monomer, although to a lesser extent than in the presence of magnesium. As a result of changing plateaus, visual inspection of the titration curves does not provide an easy visual evaluation of the behavior of the system (see above). The solid lines in Figure 7a are the nonlinear least-squares fits of the experimental titration curves, using the monomer \leftrightarrow dimer model, with the dimerization constant, K_D , as the fitting parameter (DOI: 10.1021/bi301568w, eqs 16–20).

The van't Hoff plots of the dimerization constant, K_D , as a function of the reciprocal temperature (kelvin) in the presence and absence of magnesium, are shown in Figure 7c. The solid lines are linear least-squares fits to the van't Hoff equation: $\partial \ln K_D / d(1/T) = -\Delta H^\circ / R$. In the presence of magnesium, the formation of the domain dimer is characterized by the small, positive apparent enthalpy change ($\Delta H^\circ = 2.2 \pm 2 \text{ kcal/mol}$). Thus, the interactions are driven by the apparent entropy change, ΔS° , as observed for trimer formation (Figure 4c). In the absence of magnesium, the N-terminal core domain dimerization is characterized by the negative apparent enthalpy change ($\Delta H^\circ = -4.6 \pm 2 \text{ kcal/mol}$). This value is significantly lower than the ΔH° of $-15 \pm 2 \text{ kcal/mol}$, obtained for the trimerization reaction (Figure 4c). The corresponding apparent entropy changes (ΔS°), characterizing the dimerization process of the isolated, N-terminal core domain in the presence and absence of magnesium, are ≈ 26 and $\approx 17 \text{ cal mol}^{-1} \text{ deg}^{-1}$, respectively (see Discussion).

Effect of Guanidine Hydrochloride on the Stability of the DnaT N-Terminal Core Domain Dimer. The dependence of the fluorescence anisotropy of the DnaT N-terminal core domain $[1.18 \times 10^{-6} \text{ M (monomer)}]$ upon the

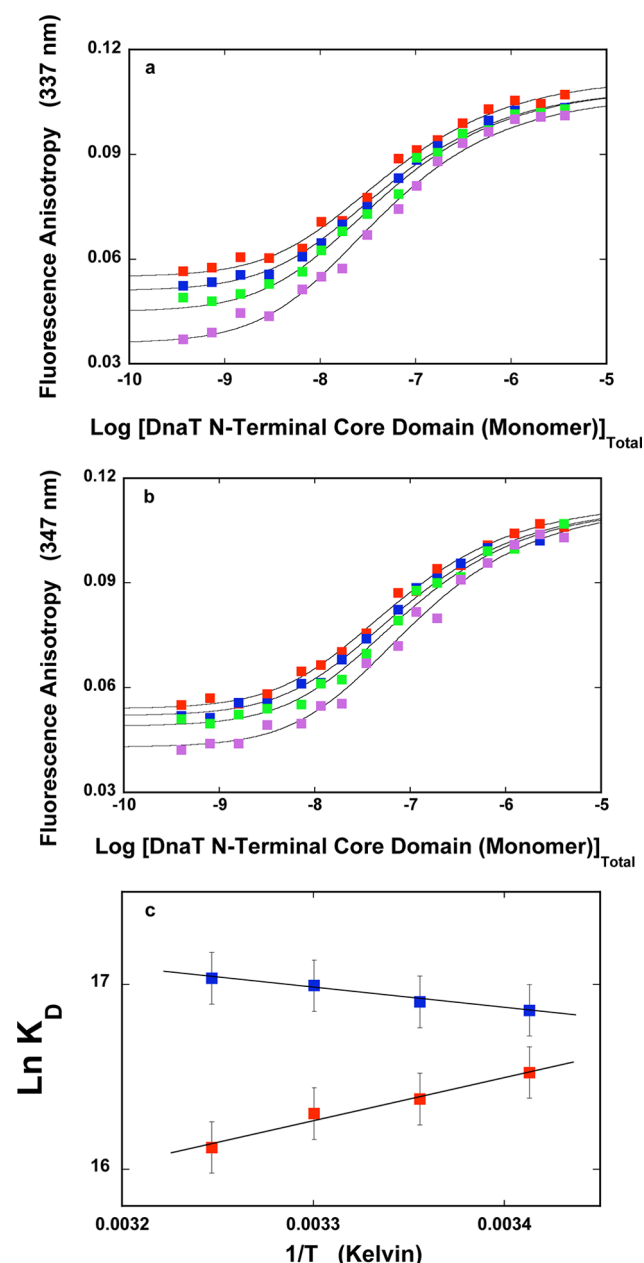


Figure 7. (a) Dependence of the fluorescence anisotropy of the DnaT N-terminal core domain upon the total domain monomer concentration ($\lambda_{\text{ex}} = 296 \text{ nm}$; $\lambda_{\text{em}} = 347 \text{ nm}$) in buffer C (pH 7.0), recorded at different temperatures: (red squares) 20, (blue squares) 25, (green squares) 30, and (magenta squares) 35 °C. The solid lines are the nonlinear least-squares fits of the titration curves, using the trimer model described by eqs 3–7 (DOI: 10.1021/bi301568w). (b) Dependence of the DnaT fluorescence anisotropy upon the total DnaT monomer concentration ($\lambda_{\text{ex}} = 295 \text{ nm}$; $\lambda_{\text{em}} = 347 \text{ nm}$) in buffer C (pH 7.0), containing 0.1 mM EDTA and no magnesium, recorded at different temperatures: (red squares) 20, (blue squares) 25, (green squares) 30, and (magenta squares) 35 °C. The solid lines are the nonlinear least-squares fits of the titration curves, using the trimer model (DOI: 10.1021/bi301568w, eqs 3–7). (c) Dependence of the trimerization constant, K_T , upon the reciprocal of the temperature (kelvin) (van't Hoff plot), in the presence (blue squares) and absence (red squares) of magnesium. The solid lines are linear least-squares fits to the van't Hoff equation (details in the text).

concentration of GnHCl in buffer C (pH 7.0, 25 °C) is shown in Figure 8a. In the presence of magnesium, the observed effect

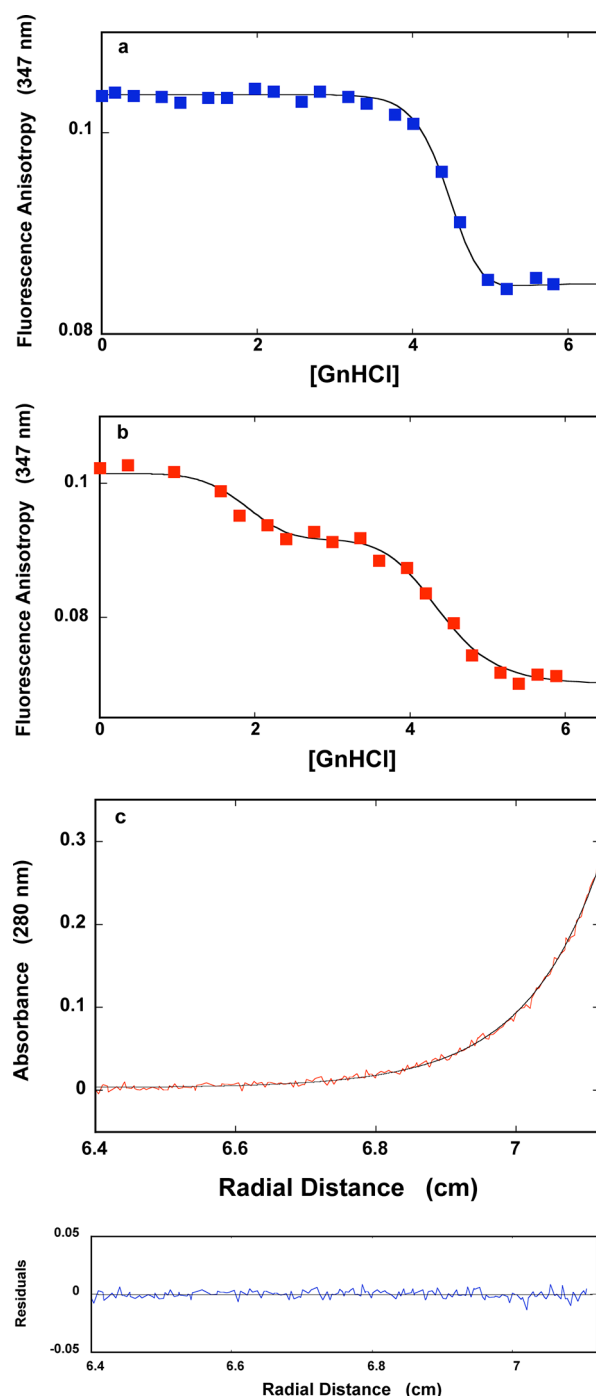


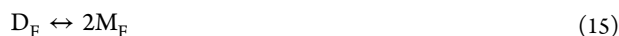
Figure 8. (a) Dependence of the fluorescence anisotropy of the DnaT N-terminal core domain [$1.18 \times 10^{-6} \text{ M}$ (monomer)] upon the concentration of GnHCl in buffer C (pH 7.0, 25 °C). The solid line is the nonlinear least-squares fit of the experimental titration curve to eqs 7–14, where $p = 16$, $K_G = 1.7 \times 10^{-10} \text{ M}^{-16}$, $r_{\text{M}_F} = 0.055$, $r_{\text{M}_G} = 0.085$, and $r_D = 0.113$. (b) Dependence of the fluorescence anisotropy of the DnaT protein [$1.18 \times 10^{-6} \text{ M}$ (monomer)] upon the concentration of GnHCl in buffer C (pH 7.0, 25 °C), containing 0.1 mM EDTA and no magnesium. The smooth solid line is the nonlinear least-squares fit of the experimental titration curve to eqs 21–25, where $m = 5$ and $q = 12$: $K_M = 1.1 \times 10^{-1} \text{ M}^{-1}$, $K_Q = 2.1 \times 10^{-8} \text{ M}^{-12}$, $r_{\text{M}_F} = 0.054$, $r_{\text{M}_M} = 0.0925$, $r_{\text{M}_Q} = 0.070$, and $r_D = 0.1133$ (details in the text). (c) Sedimentation equilibrium concentration profiles of the DnaT N-terminal core domain in buffer C (pH 7.0, 25 °C), containing 0.1 mM EDTA and no magnesium, and 3.36 M GnHCl. The concentration of

Figure 8. continued

the protein was 3.25×10^{-6} M (monomer). The profile was recorded at 280 nm and 27000 rpm. The smooth solid line is the nonlinear least-squares fit to a single-exponential function (DOI: 10.1021/bi301568w, eq 2), with a single species having a molecular weight of 16467 (Materials and Methods). The bottom panel shows the residuals of the fit.

of GnHCl on the fluorescence anisotropy of the isolated domain dimer is very similar to the effect observed in the case of the protein trimer (Figure 6a). The value of the anisotropy, corresponding to the dimer ($r \sim 0.105$), remains unchanged up to ~ 4 M GnHCl. Further, an increase in the GnHCl concentration causes the anisotropy to steeply decrease and reach a plateau ($r \sim 0.085$) at ≈ 5 M GnHCl. Sedimentation equilibrium data recorded at 5.4 M GnHCl indicate the presence of a single species with a molecular weight of 18950 ± 2000 (data not shown). Thus, the molecular entity observed at high GnHCl concentrations is the monomer of the denatured, N-terminal core domain (see Discussion).

In the presence of magnesium, like the effect of GnHCl on the trimerization reaction (see above), the simplest thermodynamic model of the observed GnHCl effect on the dimer dissociation of the N-terminal core domain includes the following equilibria:^{47–52}



and



The dimer dissociation constant in the absence of GnHCl is $1/K_D$ (DOI: 10.1021/bi301568w, eq 17), and the constant for binding of GnHCl to the domain monomer, K_G , is defined by eq 8. The total domain monomer concentration in the sample is then described by the mass conservation expression, as

$$[M]_T = [M]_F + [M]_G + 2[D]_F \quad (17a)$$

and

$$[M]_T = [M]_F + [M]_F K_G [\text{GnHCl}]^p + 2[M]_F^2 K_D \quad (17b)$$

Unlike in the case of the trimer, the expression described above can be analytically solved, providing the concentration of the free domain monomer as a function of GnHCl concentration, for example

$$[M]_F = \{[(1 + K_G [\text{GnHCl}]^p)^2 + 8K_D [M]_T]^{0.5} - (1 + K_G [\text{GnHCl}]^p)\} / (4K_D) \quad (18)$$

The anisotropy of the sample is then defined as

$$r = r_{M_F} f_{M_F} + r_{M_G} f_{M_G} + r_{D_F} f_{D_F} \quad (19)$$

where r_{M_F} , r_{M_G} , and r_{D_F} are fluorescence anisotropies of the free native monomer, denatured monomer associated with GnHCl, and dimer, respectively. The quantities f_{M_F} , f_{M_G} , and f_{D_F} are the corresponding fractional contributions of corresponding species to the total emission, F_T , of the sample defined as

$$F_T = F_{M_F} [M]_F + F_{M_G} [M]_G + F_{D_F} [D]_F \quad (20a)$$

with

$$f_{M_F} = \frac{F_{M_F} [M]_F}{F_T} \quad (20b)$$

$$f_{M_G} = \frac{F_{M_G} [M]_G}{F_T} \quad (20c)$$

and

$$f_{D_F} = \frac{F_{D_F} [D]_F}{F_T} \quad (20d)$$

where F_{M_F} , F_{M_G} , and F_{D_F} are molar fluorescence intensities of the two monomer states and the dimer, respectively. In the expressions given above, r_{M_F} , r_{M_G} , r_{D_F} , F_{M_F} , F_{M_G} , F_{D_F} , K_D , and $[M]_T$ are known and the value of $(K_G)^{1/p}$ can be estimated from the midpoint of the titration curve, leaving only one unknown parameter, p . Nevertheless, we treated K_G as a fitting parameter. The solid line in Figure 8a is the nonlinear least-squares fit of the experimental dissociation curve of the N-terminal domain dimer by GnHCl in the presence of magnesium to eqs 15–20, which provides the following values: $p = 16 \pm 2$, and $K_G = (1.7 \pm 0.5) \times 10^{-10} \text{ M}^{-16}$. Thus, the value of p and the apparent intrinsic affinity of GnHCl for the N-terminal domain monomer, $(1.7 \times 10^{-10})^{1/16} \approx 0.25 \text{ M}^{-1}$, are very similar to the corresponding parameters obtained in the case of the intact DnaT protein trimer (Figure 6a) (see Discussion).

Very different behavior is observed in the absence of magnesium. The dependence of the fluorescence anisotropy of the DnaT N-terminal core domain upon the concentration of GnHCl in the absence of magnesium is shown in Figure 8b. There are clearly two transitions induced by GnHCl with midpoints at ~ 1.9 and ~ 4.5 M GnHCl. Figure 8c shows the sedimentation equilibrium profile of the DnaT N-terminal domain in the presence of 3.36 M GnHCl, recorded at the protein absorption band (280 nm). The selected concentration of GnHCl corresponds to the first plateau separating the two transitions in Figure 8b. The smooth solid line in Figure 8c is the nonlinear least-squares fit, using the single-exponential function (DOI: 10.1021/bi301568w, eq 2). The fit provides an excellent description of the experimental sedimentation profile, indicating the presence of a single species with a molecular weight of 16500 ± 1500 . Thus, in spite of the high fluorescence anisotropy, the molecular entity, induced in the first transition at low GnHCl concentrations in the absence of Mg^{2+} , is the monomer of the N-terminal domain (Figure 8c) (see Discussion). Analogous sedimentation equilibrium experiments in the presence of 5.5 M GnHCl and in the absence of magnesium showed that the plateau at high GnHCl concentrations also corresponds to the isolated, N-terminal domain monomer (data not shown).

In the absence of magnesium, we must consider two transitions both leading to the dissociation of the domain dimer. The simplest thermodynamic model of the observed GnHCl effect, which can account for the observed behavior, includes the following equilibria:^{47–52}



and



The GnHCl binding constants for M_F and M_M , K_M and K_Q , respectively, are defined by expressions analogous to eq 8. The total concentration of the domain monomer in the sample is defined by the mass conservation expression, as

$$[M]_T = [M]_F + [M]_M + [M]_Q + 2[D]_F \quad (22a)$$

and

$$[M]_T = [M]_F + [M]_F K_G [GnHCl]^p + 2[M]_F^2 K_D \quad (22b)$$

The analytical solution for the concentration of the free domain monomer as a function of GnHCl concentration is

$$[M]_F = \frac{(R^2 + 8K_D[M]_T)^{0.5} - R}{4K_D} \quad (23a)$$

where

$$R = 1 + K_M[GnHCl]^M + K_M K_Q [GnHCl]^{M+Q} \quad (23b)$$

The anisotropy of the sample is defined as

$$r = r_{M_F} f_{M_F} + r_{M_M} f_{M_M} + r_{M_Q} f_{M_Q} + r_D f_D \quad (24)$$

where r_{M_F} , r_{M_M} , r_{M_Q} , and r_D are fluorescence anisotropies of the corresponding monomer states and the dimer. The quantities f_{M_F} , f_{M_M} , f_{M_Q} , and f_D are the corresponding fractional contributions of all species to the total emission, F_T , for example

$$F_T = F_{M_F}[M]_F + F_{M_M}[M]_M + F_{M_Q}[M]_Q + F_D[D]_F \quad (25a)$$

with

$$f_{M_F} = \frac{F_{M_F}[M]_F}{F_T} \quad (25b)$$

$$f_{M_M} = \frac{F_{M_M}[M]_M}{F_T} \quad (25c)$$

$$f_{M_Q} = \frac{F_{M_Q}[M]_Q}{F_T} \quad (25d)$$

and

$$f_D = \frac{F_D[D]_F}{F_T} \quad (25e)$$

where F_{M_F} , F_{M_M} , F_{M_Q} , and F_D are molar fluorescence intensities of the monomer states and the dimer, respectively. In the expressions given above, r_{M_F} , r_{M_M} , r_{M_Q} , r_D , K_D , and $[M]_T$ are known. F_{M_F} , F_{M_M} , F_{M_Q} , and F_D are also known from fluorescence spectra of the system at corresponding GnHCl concentrations (see above). The values of $(K_M)^{1/m}$ and $(K_Q)^{1/q}$ can be estimated from the midpoints of both transitions, leaving m and q as independent, unknown parameters. The solid line in Figure 8b is the nonlinear least-squares fit of the experimental dissociation curve of the N-terminal domain dimer in the absence of magnesium with m and q as fitting parameters to eqs 21–25. The fit provides a remarkably good description of the experimental titration curve with the following values: $m = 5 \pm 1$, $q = 12 \pm 2$, $K_M = (1.1 \pm 0.3) \times 10^{-1} \text{ M}^{-1}$, and $K_Q = (2.1 \pm 0.6) \times 10^{-8} \text{ M}^{-12}$. The apparent intrinsic affinity of GnHCl for the N-terminal domain monomer in the first transition is

$(K_M)^{1/m} \approx 0.64 \text{ M}^{-1}$, while in the second transition, $(K_Q)^{1/q} \approx 0.23 \text{ M}^{-1}$ (see Discussion). Thus, although the apparent affinity of the GnHCl is higher for the first transition, the analogous quantity for the second transition is very similar to the value obtained for trimerization of the intact protein in the absence of magnesium (see Discussion).

DISCUSSION

Binding of Magnesium Cations to the DnaT Monomers Regulates the Intrinsic Affinity of the DnaT Trimerization Reaction without Affecting the Cooperativity of the Assembly Reaction. A characteristic aspect of the effect of magnesium on the DnaT trimerization reaction is its specificity. NaCl does not affect the trimerization to any extent, in the same salt concentration range (data not shown), strongly suggesting that monovalent cations and anions do not participate in the trimer assembly process. Moreover, the specificity is also reflected in the high apparent intrinsic affinity, which is on the order of $\sim 1.3 \times 10^4 \text{ M}^{-1}$, indicating that the DnaT monomer possesses specific Mg^{2+} cation binding sites (Figure 3). A peculiar feature of the observed effect is that the presence of magnesium does not affect the cooperativity of the trimerization reaction. Consequently, the dimer population is undetectable, in the absence and presence of Mg^{2+} (Figure 1a; DOI: 10.1021/bi301568w). The independence of the cooperativity of the trimerization reaction upon magnesium cations provides the first indication that Mg^{2+} cations do not directly stabilize the DnaT trimer. Otherwise, the cooperativity of the trimerization would be significantly diminished in the absence of magnesium, which is not experimentally observed (Figure 1a) (see below).

Magnesium Cations Affect the Structure of the DnaT Monomers Prior to Trimer Assembly. Neither the global structure of the DnaT trimer (Figure 1c,d) nor the local accessibility of protein tryptophan residues in the trimer is affected by the presence of magnesium, as tested using two different dynamic quenchers (Figure 2). On the other hand, Mg^{2+} cations induce significant changes in the monomer conformation. Assessment of the acrylamide and I^- quenching data of the monomer indicates that Mg^{2+} binding does not change the solvent accessibility of the tryptophan residues in the monomer, reflected in the unchanged acrylamide accessibility, but changes the structure of the protein matrix surrounding them (Figure 2). In the absence of magnesium, the tryptophan residues in the monomer are characterized by an iodide quenching constant even higher than that of the free NATA, indicating the presence of an excess of positive charges around the tryptophan residues, which attract negatively charged iodide anions.^{33–35}

Binding of Mg^{2+} reduces the extent of quenching of the monomer tryptophan fluorescence by I^- to that of NATA. The effect is not due to the simple presence of bound Mg^{2+} cations, which would increase the positive charge and lead to the amplification, or the reduction of the iodide quenching efficiency. Thus, magnesium binding to the monomer induces changes in the structure of the protein matrix, which, in turn, favor the formation of the trimer. These data together with the fact that the cooperativity of the trimerization reaction is not affected by magnesium, i.e., Mg^{2+} cations do not directly stabilize the trimer (see above), and the lack of any effect of Mg^{2+} on the trimer global structure indicate that Mg^{2+} cations bind to the DnaT monomers prior to the assembly of the trimer.

Formation of the DnaT Trimer Involves Specific Conformational Changes of the Monomers That Are Independent of the Presence of Magnesium.

While the acrylamide accessibility of the tryptophans in the DnaT monomer is as high as that of the free NATA in solution, the accessibility of the same residues in the trimer is strongly diminished. Similar behavior is observed for the fluorescence quenching in the presence of iodide anions. Thus, the fluorescence quenching results show very different structures of the protein matrix surrounding the tryptophan residues in the monomer, as compared to the trimer (Figure 2). This is true in the presence and absence of magnesium, indicating that the decreased accessibility of the tryptophan residues in the trimer is induced by trimer formation, not by binding of magnesium to the monomers. This conclusion is also strongly supported by the results for the N-terminal core domain dimerization. First, analogous fluorescence quenching data of the truncated domain monomer and the dimer are very similar to that obtained for the intact monomer and the trimer (data not shown). Second, changes in conformation leading to trimer formation include the C-terminal region of the protein, which does not bind magnesium cations (DOI: 10.1021/bi301568w; see below).

Magnesium Dramatically Changes the Thermodynamic Functions of DnaT Trimerization. A striking result is the effect of magnesium on the thermodynamic functions characterizing the trimerization reaction (Figure 4c). In the presence of Mg^{2+} , the trimerization reaction is driven by the large apparent entropy change, while in the absence of magnesium, the same reaction is driven by the large apparent enthalpy change. The situation is even more complicated by the fact that in the presence of magnesium, there is no detectable participation of the water molecules in the trimerization process (Figure 5a,c). A large net release of water molecules would rather be expected, which would contribute to the observed, large, and positive change in entropy.^{58,59} For instance, in the absence of Mg^{2+} , the trimerization reaction is accompanied by an uptake of ~ 11 water molecules, which accounts for a part of the observed, large, and negative entropy change (Figure 5b,c).

The observed behavior is very complex and must include compensatory effects between protein binding processes at two different interacting sites, magnesium binding to monomers, and conformational changes of the monomers accompanying trimer formation (see above). A plausible explanation of this peculiar behavior is based on the fact that binding of magnesium is characterized by a high affinity, the trimerization process is characterized by a negative enthalpy in the absence of Mg^{2+} , and there is a net uptake of water molecules. Because magnesium cations are not present, the negative enthalpy change includes intrinsic protein association and magnesium-independent conformational changes of the monomers accompanying trimerization (Figure 4c). The intrinsic protein–protein associations would also be characterized by a positive change in entropy, masked by a large net uptake of water molecules (Figure 5c).

On the other hand, the high intrinsic affinity of magnesium cations indicates that some cations can form inner-sphere complexes; i.e., they release their hydration layers, a process characterized by a large positive enthalpy change.⁶³ Therefore, in the presence of magnesium, conformational changes of the monomers induced by trimer formation may also induce formation of an inner-sphere complex by some of the bound magnesium cations. The positive enthalpy of the cation

dehydration would compensate for the intrinsic negative enthalpy of the protein–protein interactions in the trimerization reaction, observed in the absence of magnesium. In such a case, the water molecules released from the dehydrated cations would compensate for the intrinsic water uptake accompanying the intrinsic association process. As a result, in the presence of magnesium, the apparent $\Delta H^\circ \approx 0$, ΔS° is positive, and $d \log K_T/d \log [H_2O] \approx 0$, as experimentally observed (Figures 4c and 5c).

Highly Cooperative Dissociation of the DnaT Trimer by GnHCl Indicates a Significant Integration of the DnaT Monomer Interacting Sites with the Entire Structure of the Protein Stabilized by Magnesium.

Like the high cooperativity of the trimerization process, the dissociation of the DnaT trimer by the protein denaturant, GnHCl, is also characterized by a very high cooperativity (Figure 6a,b). Moreover, the dissociation process of the DnaT trimer as well as the N-terminal core domain dimer occurs in the GnHCl concentration range where the secondary structure of the protein is destabilized.^{60–62} It should be stressed that although we use binding models to describe the effect of GnHCl on the dissociation of the DnaT trimer and the N-terminal domain dimer, the values of the obtained exponents are measures of the structural cooperativity of the protein, which are analogous to the well-known Hill coefficient in the binding studies and do not necessarily mirror the average numbers of bound GnHCl molecules.⁵²

First, the very high cooperativity of the dissociation process indicates that a large cooperative unit of the protein structure, i.e., a large structural part of the protein responding to GnHCl, is subjected to destabilization by GnHCl, without any detectable intervening intermediates.^{52,60–62} The only and final product is the denatured monomer, characterized by the fluorescence anisotropy being higher than the fluorescence anisotropy of the native monomer. These data indicate that the DnaT dissociation by GnHCl occurs as a result of denaturation of most of the secondary structures of the monomers constituting the trimer. Second, both interacting sites of the DnaT monomer, located on the N-terminal core domain and formed by the small C-terminal region, must be intimately integrated with the entire protein structure. If the sites were to any extent structurally autonomous, then a trimer intermediate with partially denatured monomers would be a part of the transition process, which is not experimentally observed.

Third, the apparent affinity of GnHCl for the protein is not affected by the presence of magnesium, although the cooperativity of trimer dissociation is (Figure 6a,b). Note that the midpoints of the titration curves in panels a and b of Figure 6 are, within experimental accuracy, at the same locations on the GnHCl concentration scale. These results provide additional strong support for the conclusion that Mg^{2+} cations do not directly stabilize the trimer; i.e., they do not bind at the monomer–monomer interfaces in the trimer, because otherwise the titration curve would be shifted toward a high GnHCl concentration in the presence of magnesium, which is not observed (Figure 6a,b). Rather, binding of magnesium results in an increase in the size of the protein cooperative unit within each monomer subjected to denaturation. Such a process can occur without affecting the apparent GnHCl affinity.

The results of the effect of GnHCl on the N-terminal core domain dimer provide further strong support for the conclusion that the binding site on the N-terminal domain is an integral part of the protein structure without any significant structural

autonomy from the protein. Thus, in the presence of magnesium, the dimer disintegrates directly to denatured monomers, without the appearance of any detectable intermediate(s). The dissociation occurs at the same GnHCl concentration as trimer dissociation, where the secondary structure of the protein is destabilized by the denaturant.^{60–62} The monomer cooperative unit is characterized by a p of ≈ 16 , i.e., very similar to the value of ≈ 18 , obtained for the monomers in the trimer. A collateral result of the very similar behavior of the monomers in the trimer and the isolated domain dimer in the presence of magnesium is that Mg^{2+} cations do not seem to bind to the C-terminal region of the protein but only to the large N-terminal core domain.

In the absence of magnesium, the lack of the C-terminal region leads to the disintegration of the domain dimer into monomers already at a significantly lower GnHCl concentration (Figure 8b). Thus, even limited changes in the protein structure, induced at the lower GnHCl concentration, affect the interacting site of the N-terminal domain. The first transition is much less cooperative ($m \approx 5$), and the monomers possess a fluorescence anisotropy ($r \approx 0.09$) higher than that of the native monomers ($r \approx 0.05$) (Figure 1a), most probably preserving their secondary structure. As the GnHCl concentration increases, the secondary structure of the monomers is further destabilized, reaching the fully denatured state at high GnHCl concentrations (Figure 8b). The cooperative unit of the second transition is characterized by a q of ≈ 12 , i.e., very similar to the p of ≈ 10 obtained for the analogous transition of the intact monomer in the trimer (Figures 6b and 8b).

The Small C-Terminal Region of the DnaT Protein May Engage in Interactions with the N-Terminal Domain Prior to the Formation of the Trimer. Formation of the isolated, N-terminal core domain dimer is characterized by apparent enthalpy changes (ΔH°) of ≈ 2.2 and ≈ -4.6 kcal/mol, in the presence and absence of magnesium, respectively (Figure 7c). Thus, under both solution conditions, the values of enthalpy changes are more positive than those observed in the case of the trimerization reaction of the intact protein, although the difference is more pronounced in the absence of magnesium (Figure 4c). Nevertheless, these data strongly suggest that the lack of the C-terminal region does not affect the major characteristics of the monomer–monomer interactions in the domain dimer, as compared to the trimer; i.e., the enthalpy change in the presence of magnesium is much more positive than in the absence of Mg^{2+} cations.

Recall that the affinity of the monomers in the domain dimer is significantly higher than the affinity among the monomers in the trimer; i.e., the flexible, small C-terminal region must hinder interactions between monomers through the sites located on the corresponding N-terminal domains, prior to trimer formation (DOI: 10.1021/bi301568w). The data suggest the simplest explanation of the same direction in the observed enthalpy changes in the presence and absence of magnesium, in domain dimer formation. In the intact protein, the C-terminal region interacts with the corresponding binding site on the N-terminal domain of the monomer. A simple steric hindrance would not contribute to the enthalpy change. Separation of the C-terminal regions from the N-terminal domain, formation of the additional interacting sites by the regions, and engagement of the sites in interactions with the third monomer in the trimer contribute an unfavorable enthalpy change to the overall enthalpy of trimer formation and to the accompanying entropy changes.

Functional Implications. In spite of the key physiological role of the DnaT protein in primosome assembly and primosome activities, the mechanistic details of exactly how the protein fulfills its role are unknown.^{3,5,7,10} As we mentioned before, both the monomer and the trimer have been suggested as the oligomeric state of the DnaT protein in the functional primosome.⁵ Results described here and in the preceding paper (DOI: 10.1021/bi301568w) show that the protein exists as a 3:1 molar mixture of the monomer and trimer forms in the cell at all physiological temperatures. Thus, both oligomeric states are physiological states of the DnaT protein.

Our current thinking, based on these results, is that these two physiological oligomeric states have different and specific functions at different stages of the primosome activities. The discovered highly cooperative character of the DnaT trimerization reaction strongly suggests that in the primosome assembly processes, the trimer is the major state in which the DnaT protein enters the assembling process of the primosome scaffold. Moreover, the trimer would also provide the protein with an increased number of interacting sites necessary to efficiently recognize the formed PriA–PriB–DNA complex.¹² In this context, the engagement of both interacting sites, one located on the N-terminal core domain and another in the C-terminal region, in the stabilization of the trimer structure indicates that the DnaT molecule possesses another binding site(s) that serves to recognize the ingredients of the primosome scaffold.

However, once the trimer is in the primosome, the strong dependence of DnaT trimer integrity on the conformational states of the constituting monomers controlled by magnesium binding suggests that strong interactions with the primosome ingredients affect the oligomeric state of the bound protein. The high cooperativity of trimer dissociation and the rather modest intrinsic affinity among the monomers in the trimer ensure an efficient transformation into the monomer state modulated by magnesium binding. Thus, the trimer would be a transient form of the DnaT protein, in terms of the primosome functions, which would be operational in the recognition stages of the primosome assembly reaction. The remaining monomeric protein within the primosome scaffold now has two interacting areas, on the N-terminal core domain and the C-terminal region, free to engage the DnaB–DnaC complex. Consequently, the monomer form would be the DnaT state in the postassembly activities of the primosome.⁵ Our laboratory is currently examining these possibilities.

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■ ABBREVIATIONS

NATA, N-acetyl-L-tryptophanamide; EDTA, ethylenediamine-tetraacetic acid; GnHCl, guanidine hydrochloride.

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