Visualization of a Slow, ATP-induced Structural Transition in the Bacterial Molecular Chaperone DnaK*

(Received for publication, November 21, 1997, and in revised form, January 23, 1998)

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Recent reports have shown that the binding of ATP to a 70-kDa molecular chaperone induces a rapid global conformational transition from a “high affinity” state to a “low affinity” state, where these states are defined by tight and weak binding to (poly)peptides, respectively. To complete the activity cycle, a chaperone molecule must ultimately return to the high affinity state. In this report, this return to the high affinity state was studied using a chemical cross-linking assay in conjunction with SDS-polyacrylamide gel electrophoresis. The basis for this assay is that in the absence of nucleotide or in the presence of ADP, conditions that stabilize the high affinity state, cross-linking of the Escherichia coli molecular chaperone DnaK yielded two monomeric forms, with apparent molecular masses of 70 kDa (77%) and 90 kDa (23%), whereas cross-linking yielded only the 70-kDa monomeric form in the presence of ATP. This ATP-dependent difference in cross-linking was used to follow the kinetics of the low affinity to high affinity transition under single turnover conditions. The rate of this transition ($k_{obs} = 3.4 \pm 0.6 \times 10^{-4} \text{s}^{-1}$ at 25 °C) is almost identical to the reported rate of ATP hydrolysis ($k_{ap} = 2.7 (\pm 0.7) \times 10^{-4} \text{s}^{-1}$ at 22 °C). These results are consistent with a two-step sequential reaction where rate-limiting ATP hydrolysis precedes the conformational change. Models for the formation of two cross-linked DnaK monomers in the absence of ATP are discussed.

The highly conserved and ubiquitous 70-kDa family of molecular chaperones, which include stress induced (Hsp70)† and constitutively expressed (Hsc70) variants, promote protein–protein interactions via the of coupling ATP binding and hydrolysis to selective substrate binding and release. Molecular chaperones use this activity cycle to perform diverse biological processes such as the stabilization of partially unfolded and nascent proteins, protein translocation, and protein complex assembly and disassembly under normal growth and stress conditions (for reviews, see Refs. 1–3). The underlying molecular events that enable the coupling of ATP binding and hydrolysis to selective substrate binding and release are poorly understood.

The three-dimensional structure of the two separate 70-kDa chaperone functional domains have been determined. The NH$_2$-terminal domain of Hsc70 binds nucleotide in the base of a cleft formed by two subdomains (4). Two K$^+$ ions and a Mg$^{2+}$ ion are cofactors in the ATPase reaction by interacting with nucleotides in this cleft (5). The requirement for K$^+$ is specific, because when K$^+$ is replaced by Na$^+$, (i) the rate of Hsc70-catalyzed ATP hydrolysis is five times slower (6), and (ii) DnaK-protein complexes do not dissociate in the presence of ATP (7). The COOH-terminal domain of DnaK (residues 389–607) binds a peptide substrate in a channel formed by the loops from a β-sandwich (8). The molecular mechanism which couples the activities of the two domains is not understood.

Several lines of evidence indicate that ATP induces a global structural transition in a 70-kDa chaperone molecule from a high affinity state to a low affinity state (9–13). The high affinity state tightly binds (poly)peptides, whereas the low affinity state weakly binds (poly)peptides. Significantly, the induction of this structural transition is a consequence of ATP binding and not hydrolysis, because the rate of the transition ($0.7 \text{s}^{-1}$) (14) is much faster than chaperone-catalyzed ATP hydrolysis ($0.003–0.0005 \text{s}^{-1}$) (7, 15, 16). It appears that ATP hydrolysis is involved in the reverse transition, that is, the return from the low affinity state to the high affinity state (12, 14, 17). Small-angle x-ray scattering experiments conducted on recombinant bovine Hsc70 have indicated that the reverse reaction occurs in at least two steps, where the hydrolysis of ATP is followed by rate-limiting product release (14).

In this report, a chemical cross-linking/SDS-PAGE assay was used to visualize the slow transition from the low affinity state to the high affinity state of the Escherichia coli 70-kDa molecular chaperone DnaK under single turnover conditions. The results are consistent with a two-step sequential reaction where ATP hydrolysis is rate-limiting rather than product release, and ATP hydrolysis induces a second reaction, a structural transition in the chaperone molecule. This assay can also be used to probe the effect of other parameters such as salt, temperature, and even cochaperones, on the kinetics of the transition.

EXPERIMENTAL PROCEDURES

DnaK Purification—DnaK was isolated from the E. coli strain RLM893 (18) as described previously (19). The protein was maintained in a K$^+$/HEPES sample buffer (25 mM HEPES, 50 mM KCl, 5 mM MgCl$_2$, 5 mM mercaptoethanol at pH 7.0) and stored at 4 °C prior to use. Protein concentration was determined by the Bio-Rad assay using bovine serum albumin as a standard and verified spectrophotometrically ($ε_{280} = 15.8 \times 10^3 \text{M}^{-1} \text{cm}^{-1}$) (20). SDS-PAGE analysis demonstrated that the DnaK preparations were >95% pure. For some of the cross-linking and fluorescence experiments DnaK was dialyzed into a Na$^+$/HEPES sample buffer (25 mM HEPES, 50 mM NaCl, 5 mM MgCl$_2$, 5 mM 2-mercaptoethanol at pH = 7.0). All reagents were purchased from Sigma.

Cross-linking/SDS-PAGE—Samples of DnaK were cross-linked for 2 min at 25 °C with glutaraldehyde (final concentration: 11 mM), and the reaction was stopped by the addition of an excess of glycine (final

* This work was supported in part by grants from the American Cancer Society and the National Institutes of Health (to S. N. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: Hsp70, 70-kDa heat shock protein; AMP-PNP, adenylyl-5’-yl imidodiphosphate; Hsc70, 70-kDa heat shock cognate protein; PAGE, polyacrylamide gel electrophoresis.
RESULTS

In the course of conducting cross-linking experiments to determine the conditions that promote the oligomerization of DnaK (21), we have noticed that the treatment of DnaK (20 μM) with glutaraldehyde produces two distinct monomer bands as well as dimer and trimer bands (Fig. 1A). On a 4–12% polyacrylamide denaturing gel, one monomer band is centered at a molecular mass of 70 kDa, as expected, while the upper monomer band is centered at an apparent molecular mass of 90 kDa. The broad protein bands are probably due to variations in concentration. Cross-linked samples were loaded onto either a 4–12% linear gradient denaturing polyacrylamide gel or a 10% denaturing polyacrylamide gel. Conditions: [DnaK] = 20 μM (A), 6 μM (B); [nucleotide] = 2.0 mM; temperature = 25 °C. Sample buffer was K+/HEPES. The molecular mass standards were myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), fructose-6-phosphate kinase (84 kDa), bovine serum albumin (66 kDa), and glutamic dehydrogenase (55 kDa).

Effect of Nucleotide on Cross-linking—Fig. 1B shows the effect of nucleotides on the cross-linking of DnaK. DnaK was cross-linked in the presence of ADP (2.0 mM), GTP, CTP, and UTP, also abolished the upper monomer band (data not shown). On the other hand, cross-linking in the presence of ATP (2.0 mM) abolished the upper monomer band (lane 4). Other nucleotide triphosphates, GTP, CTP, and UTP, also abolished the upper monomer band (data not shown). On the other hand, cross-linking in the presence of AMP-PNP (2.0 mM), a nonhydrolyzable analog of ATP, had no effect on the upper band (lane 5). On the basis of these results, we conclude that the abolition of the upper monomer band is a consequence of a highly specific conformational change in DnaK, induced by the action of hydrolyzable nucleotide triphosphates. The γ-phosphate group -O-P-O_3^- of the nucleotide triphosphate is required for the induction of the conformational change since the nucleotide triphosphate AMP-PNP, where the γ-phosphate group is -NH-P-O_3^- did not induce the conformational change. Possibly the nitrogen atom linkage in AMP-PNP modifies the coordination of Mg^2+, which in turn prevents the proper docking of AMP-PNP within the ATP binding site of DnaK.

Two models are proposed to explain the above results (Fig. 2). In both models: (i) E and E** stand for the high and low affinity states of DnaK, respectively. E_a and E_b are isomers of the high affinity state (see “Discussion”). cl indicates cross-linking.

The value for the equilibrium dissociation constant for ATP binding to DnaK has an error of 20%, while the values for the kinetic constants have 5–10% error.
apparent molecular mass higher than 70 kDa; whereas, the lower band is probably due to a population of DnaK monomers with a relatively small number of intramolecular cross-links. ATP binding to the E state produces the \(E^{**}\) state, which happens to cross-link homogeneously, yielding a single monomer band. Model 2 is a three-state mechanism defined by two distinct E state isomers \(E_u \Leftrightarrow E_h\) and one \(E^{**}\) state. Since there are two E state isomers, cross-linking produces two bands. Upon the addition of ATP, either both E states convert to the \(E^{**}\) state directly, or one state converts to the \(E^{**}\) state and the equilibrium readjusts. Both paths lead to the \(E^{**}\) state, which yields one band on cross-linking. An alternative interpretation of the gel data that does not assume the validity of (iii) is discussed below.

Chromatography—If Model 2 is correct it should be possible to separate the E state isomers. In an attempt to separate the putative isomers, DnaK was electrophoresed on both a 10–15% and 15–20% linear gradient nondenaturing polyacrylamide gel, and, in each case, a single band was observed. Although unsuccessful in our attempts to separate these putative isomers, Model 2 should not be ruled out, as discussed below.

Equilibrium Experiments—The thermodynamics of ATP binding to DnaK were investigated by exploiting the different cross-linking patterns of DnaK in the absence and presence of ATP. Samples of DnaK (6.0 \(\mu M\)) with varying amounts of ATP were incubated for 1 min at 25 °C and then cross-linked. Fig. 3A shows the relative amounts of the two monomer bands before and after the addition of ATP. The upper monomer band was abolished when \([ATP]/[DnaK] \geq 1.6\), indicating that the population of the \(E^{**}\) state was also abolished.

The data in Fig. 3A were used to estimate the apparent equilibrium dissociation constant \(K_d\) for ATP binding to DnaK according to Model 1 (Fig. 2). Each lane in the gel was scanned to determine the fraction of the upper monomer band \(f_E\), which is an indicator of the amount of the E state, and then \(f_E\) was plotted as a function of the total concentration of ATP (Fig. 3B). The apparent equilibrium dissociation constant for the reaction \(E^{**}\cdot\text{ATP} \Leftrightarrow \text{ATP} + \text{E}^{**}\) is related to \(K_d\) according to Equation 1,

\[
1/f_E = 1 + ([ATP])_{free}/K_d \tag{1}
\]

where \([ATP])_{free} = [ATP]_0 - [E_u](1 - f_E). The plot of \(1/f_E\) versus \([ATP]\) is linear (Fig. 3B, inset), and the reciprocal of the slope yields a \(K_d\) value of 0.19 \((\pm 0.04) \mu M\) that was determined from pre-steady state kinetic experiments (17).

Single Turnover Experiments—Since the upper monomer band is abolished at near stochiometric concentrations of ATP, the assay is an ideal way to monitor the kinetics of structural transitions in a DnaK monomer that occur during a single turnover. Single turnover experiments at 25 °C were conducted by adding a stoichiometric amount of ATP (6.0 \(\mu M\)) to a solution of DnaK (6.0 \(\mu M\)) in a K\(^+\)/HEPES buffer (Fig. 4A). Aliquots were removed at the indicated times and cross-linked as out-

![Fig. 3. Thermodynamics of ATP binding to DnaK. A, Samples of DnaK (6.0 \(\mu M\)) were incubated with ATP for 1 min at 25 °C and then the reaction was halted by cross-linking. The first lane shows protein standards; the other lanes show increasing concentrations of ATP, as indicated. B, plot of the relative amount of the E state \(f_E\) versus the total amount of ATP. Values for \(f_E\) are given by \(f_E = (P - P_{min})/(P_{max} - P_{min})\), where \(P\) is the percentage of the apparent molecular mass 90-kDa band, determined by scanning each lane in Fig. 3A. \(P = 100 	imes I\) (apparent molecular mass 90-kDa band/\(\Sigma\), where \(I\) (apparent molecular mass 90-kDa band) is the intensity of the monomer band at 90-kDa, and \(\Sigma\) is sum of the intensities of both monomer bands. \(P_{max}\) is the percentage of the apparent molecular mass 90-kDa band in the absence of ATP; \(P_{min}\) is the percentage of the apparent molecular mass 90-kDa band at a large excess of ATP. The dotted line is to help guide the eye. Inset, plot of \(1/f_E\) versus \([ATP])_{free}. Values for \([ATP])_{free}\) were estimated using the following relation: \([ATP])_{free} = [ATP]_0 - [E_u](1 - f_E)\). Data were fit to the equation \(1/f_E = 1 + ([ATP])_{free}/K_d\) (solid line), yielding \(K_d = 0.19 \pm 0.04 \mu M\) \((R^2 = 0.995)\).](http://www.jbc.org/content/273/13/9746)
Kinetics of an ATP-induced Conformational Change in DnaK

Fig. 5. Single turnover kinetics were monitored by fluorescence spectroscopy. Changes in the intrinsic fluorescence of DnaK (K/Hepes buffer) were monitored after the addition of a stoichiometric amount of ATP. The change in fluorescence followed \( F(t) = \Delta F(1 - e^{-kt}) + \gamma \) (solid line), with \( \Delta F = 2.32 \times 10^{-6} \) (±200), \( k_{obs} = 2.65 \times (0.61) \times 10^{-4} \) s\(^{-1}\), and \( \gamma = 1.07 \times 10^{4} \) (±300) (\( R^2 = 0.999 \)). The gel kinetic data (○) from Fig. 4B are superimposed on the fluorescence data. Inset, fluorescence (line)- and gel (○)-detected single turnover experiments were also conducted in a Na\(^+/\)Hepes buffer. Conditions for fluorescence experiments: [DnaK] = [ATP] = 1.0 \( \mu \)M; temperature = 25 °C; \( \lambda_{ex} = 295 \) nm (bandwidth = 3 nm); \( \lambda_{em} = 340 \) nm (bandwidth = 5 nm).

The major finding in this study was that the gel-detected and fluorescence-detected single turnover experiments showed that ATP induces a rapid conformational change in a molecule of DnaK. For example, the addition of a stoichiometric amount of ATP to DnaK lead to the elimination of the upper monomer band (Fig. 4A) within 2 min or less. Similarly, the addition of a stoichiometric amount of ATP to DnaK resulted in a rapid reduction in fluorescence (Fig. 5). Since the rapid conformational change and the rapid spectral change occur much faster than the steady state rate of ATP hydrolysis (25), and because no rapid burst phase of ATP hydrolysis has been detected in any single turnover experiments (7), we conclude that the conformational change and the attending spectral change are due to ATP binding to DnaK. This conformational change is consistent with our claim that the abolition of the upper monomer band is an indicator of the high affinity state to low affinity state transition.

DISCUSSION

Since Na\(^+\) ions inhibit the high affinity state to low affinity state transition (7), Na\(^+\) ions should also inhibit the ATP-induced elimination of the upper monomer band. To test this idea, single turnover gel- and fluorescence-detected experiments were also conducted in a Na\(^+/\)Hepes buffer. Using the gel assay we found that Na\(^+\) ions almost completely prevented the ATP-induced elimination of the upper monomer band (Fig. 5, inset). Similarly, Na\(^+\) ions both attenuated the initial reduction in fluorescence upon the addition of ATP and retarded the rate of reappearance of the fluorescence. That Na\(^+\) ions inhibit the ATP-induced abolition of the upper monomer band is consistent with our claim that the abolition of the upper monomer band is an indicator of the high affinity state to low affinity state transition.

\[
E^{**} \rightarrow E^{\text{ADP}}
\]

However, it is unlikely that the cleavage of the \( \gamma \)-phosphate group of ATP and a global conformational change in DnaK occur at precisely the same instant. A more likely explanation is that ATP hydrolysis precedes the conformational change, according to Reaction 2.

\[
E^{**} \rightarrow E^{\text{ADP}} \rightarrow E^{\text{ADP}}
\]

We therefore attempted to fit the single turnover kinetic data to Equation 2, which describes product formation according to the above two-step mechanism, where

\[
S(t) = A \left( 1 - \frac{k_{e} e^{-kt} - k_{ad} e^{-kt}}{k_{ad} - k_{e}} \right) + B.
\]
$S(t)$ is the signal due to the product, $A$ is the amplitude, $k_{by}$ and $k_e$ are the first-order rate constants defined in Reaction 2, and $B$ is the offset. A similar function has been used to fit single turnover data from fluorescence experiments (14, 17). Unfortunately, due to the small number of data points, the gel kinetic data (Fig. 4B) could not be reasonably fit to Equation 2. On the other hand, the large number of data points in the fluorescence traces allowed those traces (Fig. 5) to be fit to Equation 2. The best fit curves were nearly superimposable on the data, and the best fit values for $k_{by}$ and $k_e$ were $3.0 \pm 0.2 \times 10^{-4}$ s$^{-1}$ and 1.9 $(\pm 0.5) \times 10^{-3}$ s$^{-1}$, respectively. It is noteworthy that when ATP hydrolysis is rate-limiting ($k_{by} < k_e$), Equation 2 simplifies to

$$S(t) = A(1 - e^{-k_{by}t}) + B,$$

which explains why the single turnover data followed single exponential kinetics and why $k_{obs}$ = $k_{by}$. In conclusion, the single turnover kinetic data from both assays are consistent with Reaction 2, where ATP hydrolysis occurs first and is rate-limiting.\(^3\)

On the basis of this work, we conclude that the mechanistic details of how the bacterial Hsp70 and the eukaryotic cytosolic Hsc70 couple ATP hydrolysis to structural transitions are different. Single turnover experiments on bovine Hsc70 have revealed the same two-step mechanism as shown in Reaction 2 (14); however, the rate-limiting step for this eukaryotic cytosolic Hsc70 is not the first step, ATP hydrolysis, it is the second step, product release. This mechanistic difference is probably related in some way to the fact that the bacterial Hsp70 chaperone DnaK depends on the cochaperones DnaJ and GrpE to accelerate ATP hydrolysis and ADP release, respectively, whereas the eukaryotic cytosolic Hsc70 chaperone is GrpE-independent (26, 27).

Although the cross-linking results in this report were interpreted in terms of Model 1, there are two reasons why Model 2 should not be discarded. First, a genetically engineered COOH-terminal fragment of DnaK, with a peptide in the binding site, crystallized in two different forms (8). In one crystal form the $\alpha$-helical lid blocks the deep peptide binding channel, while in the other crystal form the $\alpha$-helical lid is rotated away from the channel, making the channel more accessible. Possibly, these two different crystals represent the two different $E$ state isomers. Second, hexokinase, which has an ATP and glucose binding core that is identical in tertiary structure to the ATP-binding core of 70-kDa molecular chaperones (4), equilibrates between closed and open forms in the absence of glucose (28, 29). Given the similar tertiary structures of hexokinase and the 70-kDa chaperones, it is reasonable to postulate that the ATPase domain of 70-kDa chaperones also equilibrates between closed and open forms in the absence of substrates, as indicated in Model 2. Clearly, more experiments are required before the presence of the two monomers bands can be definitively assigned to a specific model.

Last, we address one other interpretation of the gel results. Suppose that the high affinity species, which migrates as the apparent molecular mass 90-kDa species after cross-linking, and the low affinity species, which migrates as the 70-kDa species after cross-linking, are in equilibrium in the absence of ATP, the equilibrium lies to the low affinity state, and ATP binding shifts the equilibrium completely to the low affinity state. Specifically, suppose that 30% of the DnaK molecules populate the high affinity state and 70% of the DnaK molecules populate the low affinity state in the absence of ATP; cross-linking such a sample would yield a doublet, exactly as shown in Fig. 2. On the other hand, because ATP binding shifts the equilibrium to the low affinity state, cross-linking would yield a singlet, exactly as shown in Fig. 2. In this interpretation of the gel data, polypeptide binding should shift the population of molecules from the low affinity to the high affinity state. But we have been unable to cause such a shift by incubating DnaK with a large excess of peptide and then cross-linking. In addition, the idea that DnaK predominantly populates the low affinity state in the absence of ATP also contradicts results from structural (12, 13) and kinetic (15) studies, which have shown that in the absence of ATP 70-kDa chaperone molecules populate the high affinity state. We believe the best explanation of the gel results is Model 1, where in the absence of ATP all DnaK molecules populate the high affinity state, which happens to cross-link heterogeneously. Whichever model turns out to be correct, the low affinity to high affinity structural transition in DnaK molecules occurs at exactly the same rate as DnaK-catalyzed ATP hydrolysis, consistent with Reaction 2.

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\[^3\text{It is theoretically possible that the slow } E^{++} \rightarrow E \text{ transition in DnaK is due to the two-step reaction } E^{++}\text{-ATP} \rightarrow E\text{-ATP} \rightarrow E\text{-ADP}, \text{ where a rate-limiting conformational change precedes ATP hydrolysis.}\]
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J. Biol. Chem. 1998, 273:9744-9748.
doi: 10.1074/jbc.273.16.9744

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