The ATPase activity of HscA, a specialized hsp70 molecular chaperone from Escherichia coli, is regulated by the iron-sulfur cluster assembly protein IscU and the J-type co-chaperone HscB. IscU behaves as a substrate for HscA, and HscB enhances the binding of IscU to HscA. To better understand the mechanism by which HscB and IscU regulate HscA, we examined binding of HscB to the different conformational states of HscA and the effects of HscB and IscU on the kinetics of the individual steps of the HscA ATPase reaction cycle. Affinity sensor studies revealed that whereas IscU binds both ADP (R-state) and ATP (T-state) HscA complexes, HscB interacts only with an ATP-bound state. Studies of ATPase activity under single-turnover and rapid mixing conditions showed that both IscU and HscB interact with the low peptide affinity T-state of HscA (HscA\(^{\text{T-ATP}}\)) and that both modestly accelerate (3-10-fold) the rate-determining steps in the HscA reaction cycle, \(k_{\text{hyd}}\) and \(k_{\text{T-R}}\). When present together, IscU and HscB synergistically stimulate both \(k_{\text{hyd}}\) (500-fold) and \(k_{\text{T-R}}\) (60-fold), leading to enhanced formation of the HscA-ADP-IscU complex (substrate capture). Following ADP/ATP exchange, IscU also stimulates \(k_{\text{R-T}}\) (50-fold) and thereby accelerates the rate at which the low peptide affinity HscA\(^{\text{T-ATP}}\) T-state is regenerated. Because HscA nucleotide exchange is fast, the overall rate of the chaperone cycle in vivo will be determined by the availability of the IscU-HscB substrate-co-chaperone complex.

HscA (heat shock cognate 66-kDa; Hsc66) is a specialized member of the hsp70 family of molecular chaperones that functions in the biosynthesis of iron-sulfur proteins. The biochemical properties of HscA, including slow intrinsic ATPase activity and nucleotide-dependent peptide binding (1-3), are similar to those of other hsp70s, but HscA displays distinct substrate and co-chaperone specificity. HscA interacts selectively with IscU, a 14-kDa protein proposed to serve as a scaffold for \textit{de novo} assembly of Fe-S clusters (4, 5), and may function to regulate Fe-S cluster formation and/or transfer to acceptor proteins. HscA recognizes a specific LPPVK sequence motif at positions 99–103 of IscU and is able to bind synthetic peptides containing this sequence (6-8). The crystal structure of the HscA substrate binding domain complexed with the peptide ELPPVKIHHC was recently determined (9) and revealed that the peptide binds with the opposite orientation of that observed for DnaK peptide complexes (10). The interaction of IscU with HscA is regulated by the specialized J-type co-chaperone HscB (heat shock cognate 20-kDa; Hsc20), which escorts IscU substrate to the chaperone and enhances its binding affinity (4). The molecular mechanism whereby HscB enhances HscA and IscU binding is not known but appears to involve coupling of ATP binding and hydrolysis with conformational changes that regulate substrate affinity. ATP binding to HscA leads to formation of a tense state (T) with reduced substrate affinity, and subsequent hydrolysis to ADP and phosphate results in a relaxed state (R) with increased substrate affinity (2, 3).

In an earlier study we characterized the kinetics of the individual steps of the HscA ATPase reaction cycle in the absence of auxiliary proteins to determine the intrinsic rates of conversion of HscA between its different substrate affinity states (Scheme 1) (11). HscA was found to bind ATP in a two-step process preceding ATP hydrolysis. The first step was binding of HscA and ATP, and the second was interpreted as a conformational change involving conversion of HscA from the R- to the T-state (HscA\(^{\text{R-ATP}}\) \rightarrow HscA\(^{\text{T-ATP}}\)). ATP hydrolysis occurs in the T-state, and this is followed by a conformational change that returns HscA to the R-state. ATP hydrolysis and the subsequent conformational relaxation are rate-limiting in the overall cycle and are \(>10^5\)-fold slower than release of the products ADP and phosphate. Thus, in contrast to other hsp70 isoforms that are regulated at both ATP hydrolysis and ADP/ATP exchange (12), regulation of the HscA reaction cycle is expected to occur solely at the rate-determining hydrolysis step.

The interconversion of HscA between its different conformational states is regulated by HscB and IscU, but the effects HscB and IscU have on different kinetic steps of the HscA ATPase cycle have not been investigated. Individually, HscB and IscU weakly stimulate HscA steady-state ATPase activity (maximal stimulation <10-fold), but together they synergistically stimulate HscA activity >400-fold (1, 4). In addition, HscB reduces the concentration of IscU required for half-maximal stimulation of HscA steady-state activity ~20-fold (4). Regulation of HscA ATPase and IscU binding activity may occur in a manner similar to that suggested for DnaK. The ATPase activity of DnaK is regulated by the hsp40-type co-chaperone DnaJ, and DnaJ is proposed to modulate DnaK substrate specificity by targeting peptides to the low affinity T-state of DnaK and enhancing the rate of ATP hydrolysis (13-15). Individually, DnaJ and peptide substrates stimulate DnaK \(k_{\text{hyd}}\) weakly at physiological levels (14-18), but when present together DnaJ and peptide substrates can stimulate \(k_{\text{hyd}}\) 1000-fold. This work was supported by National Institutes of Health Grant GM54264, National Institutes of Health Training Grant GM07311 (to J. J. S. and K. G. H.), National Institutes of Health F32 Fellowship GM64949-01 (to J. J. S.), and National Institutes of Health Cancerogenesis Training Program Grant 5T32CA09054 (to T. L. T). 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 1744 solely to indicate this fact.

† Both authors contributed equally to this work.
‡ Present address: Dept. of Biophysics and Biophysical Chemistry, The Johns Hopkins School of Medicine, Baltimore, MD 21205.
¶ Present address: Dept. of Physiology and Biophysics, University of California, Irvine, Irvine, CA 92697. Tel.: 949-824-6580; Fax: 949-824-8540; E-mail: lvickery@uci.edu.

Jonathan J. Silberg‡§, Tim L. Tapley‡, Kevin G. Hoff¶, and Larry E. Vickery†
Effects of HscB and IscU on $k_{hyd}$—To investigate whether $k_{hyd}$ contributes to the overall reaction rate in the presence of substrate and co-chaperone, we measured IscU and HscB effects on HscA activity under single-turnover hydrolysis conditions by monitoring the production of [$\alpha$-32P]ADP from [$\alpha$-32P]ATP. Experiments were performed using limiting [$\alpha$-32P]ATP and high concentrations of HscA to ensure that most of the nucleotide would be bound rapidly compared with the rate of hydrolysis. For these experiments, we used levels of HscB (150 $\mu$M) and IscU (150 $\mu$M) several fold higher than their apparent affinities as determined previously in steady-state ATPase assays ($K_m^{HscB} = 5–12$ $\mu$M and $K_m^{IscU} = 2/34$ $\mu$M in the presence of the ATP is hydrolyzed). The percentage of bound ligand was calculated using the Equation 1,

$$\% \text{ bound} = 100(\text{RU}_{\text{analyte}}M_r/\text{RU}_{\text{bound ligand}}M_r)$$

where $\text{RU}_{\text{analyte}}$ is the maximum response upon injection of the analyte; $\text{RU}_{\text{bound ligand}}$ is the amount of immobilized protein; and $M_r$ analyte and $M_r$ bound ligand are the molecular masses of the respective proteins used in the experiment.

Error Analysis—Kinetic values are reported as ±1 S.D. resulting from the fit to the data, with propagation of error through any subsequent calculations. Rate constants requiring a fit of $k_{on}$ (i.e. $k_{ao}$ and $k_{oa}$ for ATP) are reported as ±1 S.D. from the secondary fit, using all available values of $k_{on}$. Error bars are only shown in figures if 1 S.D. is greater than the symbol used.

RESULTS

Effects of HscB and IscU on $k_{hyd}$—To investigate whether $k_{hyd}$ contributes to the overall reaction rate in the presence of substrate and co-chaperone, we measured IscU and HscB effects on HscA activity under single-turnover hydrolysis conditions by monitoring the production of [$\alpha$-32P]ADP from [$\alpha$-32P]ATP. Experiments were performed using limiting [$\alpha$-32P]ATP and high concentrations of HscA to ensure that most of the nucleotide would be bound rapidly compared with the rate of hydrolysis. For these experiments, we used levels of HscB (150 $\mu$M) and IscU (150 $\mu$M) several fold higher than their apparent affinities as determined previously in steady-state ATPase assays ($K_m^{HscB} = 5–12$ $\mu$M and $K_m^{IscU} = 2/34$ $\mu$M in the presence of the ATP is hydrolyzed). The percentage of bound ligand was calculated using the Equation 1,

$$\% \text{ bound} = 100(\text{RU}_{\text{analyte}}M_r/\text{RU}_{\text{bound ligand}}M_r)$$

where $\text{RU}_{\text{analyte}}$ is the maximum response upon injection of the analyte; $\text{RU}_{\text{bound ligand}}$ is the amount of immobilized protein; and $M_r$ analyte and $M_r$ bound ligand are the molecular masses of the respective proteins used in the experiment.

Error Analysis—Kinetic values are reported as ±1 S.D. resulting from the fit to the data, with propagation of error through any subsequent calculations. Rate constants requiring a fit of $k_{on}$ (i.e. $k_{oa}$ and $k_{oa}$ for ATP) are reported as ±1 S.D. from the secondary fit, using all available values of $k_{on}$. Error bars are only shown in figures if 1 S.D. is greater than the symbol used.

RESULTS

Effects of HscB and IscU on $k_{hyd}$—To investigate whether $k_{hyd}$ contributes to the overall reaction rate in the presence of substrate and co-chaperone, we measured IscU and HscB effects on HscA activity under single-turnover hydrolysis conditions by monitoring the production of [$\alpha$-32P]ADP from [$\alpha$-32P]ATP. Experiments were performed using limiting [$\alpha$-32P]ATP and high concentrations of HscA to ensure that most of the nucleotide would be bound rapidly compared with the rate of hydrolysis. For these experiments, we used levels of HscB (150 $\mu$M) and IscU (150 $\mu$M) several fold higher than their apparent affinities as determined previously in steady-state ATPase assays ($K_m^{HscB} = 5–12$ $\mu$M and $K_m^{IscU} = 2/34$ $\mu$M in the presence of the ATP is hydrolyzed). The percentage of bound ligand was calculated using the Equation 1,

$$\% \text{ bound} = 100(\text{RU}_{\text{analyte}}M_r/\text{RU}_{\text{bound ligand}}M_r)$$

where $\text{RU}_{\text{analyte}}$ is the maximum response upon injection of the analyte; $\text{RU}_{\text{bound ligand}}$ is the amount of immobilized protein; and $M_r$ analyte and $M_r$ bound ligand are the molecular masses of the respective proteins used in the experiment.

Error Analysis—Kinetic values are reported as ±1 S.D. resulting from the fit to the data, with propagation of error through any subsequent calculations. Rate constants requiring a fit of $k_{on}$ (i.e. $k_{oa}$ and $k_{oa}$ for ATP) are reported as ±1 S.D. from the secondary fit, using all available values of $k_{on}$. Error bars are only shown in figures if 1 S.D. is greater than the symbol used.

RESULTS

Effects of HscB and IscU on $k_{hyd}$—To investigate whether $k_{hyd}$ contributes to the overall reaction rate in the presence of substrate and co-chaperone, we measured IscU and HscB effects on HscA activity under single-turnover hydrolysis conditions by monitoring the production of [$\alpha$-32P]ADP from [$\alpha$-32P]ATP. Experiments were performed using limiting [$\alpha$-32P]ATP and high concentrations of HscA to ensure that most of the nucleotide would be bound rapidly compared with the rate of hydrolysis. For these experiments, we used levels of HscB (150 $\mu$M) and IscU (150 $\mu$M) several fold higher than their apparent affinities as determined previously in steady-state ATPase assays ($K_m^{HscB} = 5–12$ $\mu$M and $K_m^{IscU} = 2/34$ $\mu$M in the presence of the ATP is hydrolyzed). The percentage of bound ligand was calculated using the Equation 1,

$$\% \text{ bound} = 100(\text{RU}_{\text{analyte}}M_r/\text{RU}_{\text{bound ligand}}M_r)$$

where $\text{RU}_{\text{analyte}}$ is the maximum response upon injection of the analyte; $\text{RU}_{\text{bound ligand}}$ is the amount of immobilized protein; and $M_r$ analyte and $M_r$ bound ligand are the molecular masses of the respective proteins used in the experiment.

Error Analysis—Kinetic values are reported as ±1 S.D. resulting from the fit to the data, with propagation of error through any subsequent calculations. Rate constants requiring a fit of $k_{on}$ (i.e. $k_{oa}$ and $k_{oa}$ for ATP) are reported as ±1 S.D. from the secondary fit, using all available values of $k_{on}$. Error bars are only shown in figures if 1 S.D. is greater than the symbol used.

RESULTS

Effects of HscB and IscU on $k_{hyd}$—To investigate whether $k_{hyd}$ contributes to the overall reaction rate in the presence of substrate and co-chaperone, we measured IscU and HscB effects on HscA activity under single-turnover hydrolysis conditions by monitoring the production of [$\alpha$-32P]ADP from [$\alpha$-32P]ATP. Experiments were performed using limiting [$\alpha$-32P]ATP and high concentrations of HscA to ensure that most of the nucleotide would be bound rapidly compared with the rate of hydrolysis. For these experiments, we used levels of HscB (150 $\mu$M) and IscU (150 $\mu$M) several fold higher than their apparent affinities as determined previously in steady-state ATPase assays ($K_m^{HscB} = 5–12$ $\mu$M and $K_m^{IscU} = 2/34$ $\mu$M in the presence of the ATP is hydrolyzed). The percentage of bound ligand was calculated using the Equation 1,

$$\% \text{ bound} = 100(\text{RU}_{\text{analyte}}M_r/\text{RU}_{\text{bound ligand}}M_r)$$

where $\text{RU}_{\text{analyte}}$ is the maximum response upon injection of the analyte; $\text{RU}_{\text{bound ligand}}$ is the amount of immobilized protein; and $M_r$ analyte and $M_r$ bound ligand are the molecular masses of the respective proteins used in the experiment.

Error Analysis—Kinetic values are reported as ±1 S.D. resulting from the fit to the data, with propagation of error through any subsequent calculations. Rate constants requiring a fit of $k_{on}$ (i.e. $k_{oa}$ and $k_{oa}$ for ATP) are reported as ±1 S.D. from the secondary fit, using all available values of $k_{on}$. Error bars are only shown in figures if 1 S.D. is greater than the symbol used.
HscB and IscU Regulation of HscA

**Fig. 1. Effects of IscU and HscB on ATP hydrolysis kinetics.** A–C, single-turnover ATPase activity. Reactions were performed at 23 °C and contained 150 μM HscA, 150 μM ATP, HKM buffer, and 150 μM HscB or IscU. A, HscA (k_{hyd} = 0.002 s^{-1}). B, HscA + IscU (k_{hyd} = 0.006–0.007 s^{-1}). C, HscA + HscB (k_{hyd} = 0.02 s^{-1}). In experiments containing HscB or IscU, rates were determined in reactions initiated by the final addition of ATP to a preincubated mixture of HscA and auxiliary proteins (●) or by addition of HscB or IscU to a preincubated mixture of HscA and ATP (○). The curves represent a least squares fit of Equation 2 to the data. The insets show time courses of similar reactions under steady-state conditions (1 mM ATP). A, HscA (k_{cat} = 0.002 s^{-1}), B, HscA + IscU (k_{cat} = 0.007 s^{-1}), C, HscA + HscB (k_{cat} = 0.019 s^{-1}). D, steady-state ATPase activity. Conditions were as in A–C except that reaction mixtures contained 0.5 μM HscA, 150 μM HscB, 150 μM IscU, and the ATP concentration was varied. Initial rates were determined from least squares linear regression of the data and are plotted as the rate observed (V) versus ATP concentration. A least squares fit of the data to the Michaelis-Menten equation yields a K_{m} = 49 ± 4 μM; k_{cat} = 1.14 ± 0.02 s^{-1}.

[ADP] = [ADP] \cdot (1 - e^{-k_{cat}t}) \tag{Eq. 2}

The rates of hydrolysis (k_{hyd}) observed in the presence of HscB (0.020 s^{-1}) and IscU (0.006 s^{-1}) were 10- and 3-fold greater, respectively, than observed for HscA alone (k_{hyd} = 0.002 s^{-1}). The insets in Fig. 1, A–C, show that steady-state assays performed under identical conditions, i.e. using high levels of co-chaperone and substrate, yielded similar rates suggesting ATP hydrolysis remains rate-limiting in the presence of either HscB or IscU.

Studies by others have shown that the order of addition of ATP and co-chaperones can complicate measurement of k_{hyd}. Russell et al. (14) found that a kinetic step prior to ATP hydrolysis (e.g. a first order ATP-induced conformational change) can be rate-limiting when single-turnover experiments are performed by adding ATP to a mixture of DnaK and DnaJ. It is thus possible that the rate we observed for single-turnover experiments in which ATP was added to a mixture of HscA and HscB or HscA and IscU may not represent the actual rate of ATP hydrolysis. For this reason additional experiments were carried out in which HscA was preincubated for 1 min with limiting ATP prior to addition of HscB or IscU in order to allow for formation of low peptide affinity T-state (HscA‡-ATP). The open symbols in Fig. 1, B and C, show that HscB and IscU stimulate HscA single-turnover hydrolysis activity to a similar extent regardless of the order of addition of ATP. These findings suggest that the rates observed in the presence of HscB or IscU represent rates of ATP hydrolysis.

We also investigated the effect of the combination of HscB and IscU on ATPase kinetics. When saturating levels of both auxiliary proteins were used in single-turnover experiments similar to those shown in Fig. 1, A–C, we found that >95% of the ATP was hydrolyzed at the first time point that could be acquired after initiating the reaction (~5 s; data not shown). For this reason we instead carried out steady-state ATPase measurements to assess the effect of addition of both substrate and co-chaperone. Fig. 1D shows that addition of a combination of IscU and HscB causes a dramatic stimulation of HscA ATPase activity. The actual rate of ATP hydrolysis, k_{hyd}, must be equal to or greater than the steady-state rate observed, k_{cat} = 1.14 s^{-1}. Thus HscB and IscU together stimulate k_{hyd} to a much greater level (>500-fold) than the sum of their individual stimulations (13-fold), indicating that HscB and IscU have a synergistic effect on k_{hyd}. Conversion of HscA from the R- to the T-state (k_{R→T}) must also be stimulated by HscB and/or IscU because this kinetic step is only 70-fold faster than the rate of ATP hydrolysis in the absence of auxiliary proteins (11). It is not known, however, whether k_{hyd} remains rate-limiting in the presence of HscB and IscU because other kinetic steps may be subject to regulation.

HscB Binds HscA‡-ATP and Stimulates k_{T→R}—Previous studies have shown that J-type auxiliary co-chaperones typically interact only with the ATP-bound T-state of their cognate hsp70 (27, 32, 33) and enhance formation of the ADP-bound R-state by stimulating the rate of ATP hydrolysis (13–15, 18). To investigate whether HscB binds to a unique nucleotide state of HscA, we examined ATP and ADP effects on the interactions of HscA and HscB by using SPR. In initial experiments, HscA was cross-linked to the surface of a sensor chip and exposed to HscB in the presence of either ATP or ADP. Fig. 2A shows that HscB binds immobilized HscA in the presence of ATP, and based on the strength of the signal observed, we estimated that ~22% of the immobilized HscA binds HscB by assuming for-
and yield a slope of 3.2 ± 0.6 × 10⁻⁴ M⁻¹ s⁻¹ and an \( \alpha \) intercept of 1.1 ± 0.6 s⁻¹ in the presence of HscB, and yield a slope of 2.8 ± 0.1 × 10⁻⁴ M⁻¹ s⁻¹ and an \( \alpha \) intercept of 1.3 ± 0.2 s⁻¹ in the absence of HscB. C, plot of \( k_2 \) versus ATP concentration for experiments using HscA alone (○) or a mixture of HscA and HscB (○). The dashed line represents the average value obtained for \( k_2 \), 0.10 s⁻¹, using HscA in the presence and in the absence of HscB.

A.

\[ A_t = A_0 e^{-kt} + C \]  

(B) plot of \( k_1 \) versus ATP concentration for experiments using HscA alone (○) or a mixture of HscA and HscB (○). The lines represent a least squares linear fit to the data and yield a slope of 3.2 ± 0.6 × 10⁻⁴ M⁻¹ s⁻¹ and an \( \alpha \) intercept of 1.1 ± 0.6 s⁻¹ in the presence of HscB, and yield a slope of 2.8 ± 0.1 × 10⁻⁴ M⁻¹ s⁻¹ and an \( \alpha \) intercept of 1.3 ± 0.2 s⁻¹ in the absence of HscB. C, plot of \( k_2 \) versus ATP concentration for experiments using HscA alone (○) or a mixture of HscA and HscB (○). The dashed line represents the average value obtained for \( k_2 \), 0.10 s⁻¹, using HscA in the presence and in the absence of HscB.

B.

\[ A_t = A_0 e^{-kt} + C \]  

\[ A_t = A_0 e^{-kt} + C \]

C.

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]

\[ A_t = A_0 e^{-kt} + C \]
shown in Fig. 4B, the rate of disappearance of the ATP-induced spectral change under these conditions can be described by a single exponential with $k_{obs} = 0.0164 \pm 0.0003 \text{ s}^{-1}$. This rate is 7.8-fold faster than that observed in the absence of HscB (0.0021 ± 0.0002 s$^{-1}$; data not shown), indicating that HscB stimulates $k_{R\rightarrow T}$.

IscU Enhances R→T and T→R Conversion—We also made use of ATP-induced spectral changes to characterize the effect of IscU on the kinetics of HscA and ATP binding and the R→T interconversion. As found for HscB, mixing ATP and HscA in the presence of IscU also results in a time-dependent difference absorbance spectrum immediately following ATP addition (Fig. 4C). The data can be described by a single exponential, and the rate of decay (0.0063 ± 0.0001 s$^{-1}$) is 3-fold faster than that observed in the absence of IscU (0.0021 ± 0.0002 s$^{-1}$). Thus, IscU, like HscB, stimulates the rate of conversion of HscA-ATP to the high affinity HscA-ADP R-state.

To investigate whether IscU affects the kinetics of ATP binding and/or the R→T conversion, we examined the rate of formation of the ATP-induced difference absorbance spectrum using stopped-flow methods. Fig. 5A shows a representative plot of the rate of $\Delta A_{288}$ formation upon mixing ATP with a solution containing HscA and IscU. The data fit a single exponential with $k_{obs} = 5.0 \text{ s}^{-1}$, and the residuals for this model (cf. inset, Fig. 5A) indicate the spectral change is monophasic under these conditions. This finding can be contrasted to the biphasic change observed for HscA alone (11) or in the presence of HscB (Fig. 3). To determine whether this spectral change arises from ATP binding or a subsequent unimolecular conformational change, we examined the rate of formation over a range of ATP concentrations. Fig. 5B shows that $k_{obs}$ is not affected by ATP concentration, indicating that it represents a unimolecular conformational change leading to formation of HscA T-state ($k_{R\rightarrow T}$). The rate of this transition (5.0 s$^{-1}$) is 50-fold faster than that observed for HscA alone or HscA in the presence of IscU (≈0.1 s$^{-1}$; Fig. 4). This indicates that binding of IscU strongly favors conversion of the R-state complex (HscA-ATP) to the T-state complex (HscA$^\Delta$-ATP).

Combined Effects of HscB and IscU on R→T Conversion—To characterize HscB and IscU effects on HscA conformational changes, we examined their combined effects on the ATP-induced spectral changes. In initial experiments, ATP was added to a mixture containing HscA, HscB, and IscU, and spectral changes were monitored at 280 nm for ~10 s after mixing as described in Fig. 4. No difference absorption spectrum was observed, suggesting that if an HscA:ATP intermediate was formed in the presence of HscB and IscU it was short lived as expected for $k_{cat} > 1 \text{ s}^{-1}$. To investigate this possibility, we monitored spectral changes at 280 nm in a stopped-flow spectrophotometer. Fig. 6A shows the results of rapid mixing of ATP with a mixture of HscA, HscB, and IscU. The data fit to a double exponential model, and the residuals for this model (Fig. 6A, inset) indicate that the changes observed are biphasic. A transient increase in absorbance ($k_1 = 2.1 \pm 0.2 \text{ s}^{-1}$) occurs following ATP addition as was observed in the presence of either HscB or IscU alone. However, the spectral transition rapidly decays with a rate ($k_2 = 0.13 \pm 0.01 \text{ s}^{-1}$) that is ~60-fold faster than that observed with HscA alone (0.0021 s$^{-1}$). Steady-state ATPase assays performed using identical protein concentrations yielded a rate ($k_{cat} = 0.110 \pm 0.002$) similar to $k_2$, indicating that T→R conversion and ATP hydrolysis occur concurrently in the presence of co-chaperone and substrate. The 60-fold stimulation of T→R conversion by HscB and IscU together is greater than the individual effects of HscB (~8-fold) or IscU (~3-fold; cf. Fig. 4, B and C).
IscU do not significantly alter the rates of ATP association and thus, HscB and IscU together do not significantly affect the ATP binding. Fig. 6A shows the results from ATP binding kinetics experiments performed in the presence and absence of HscB and IscU. The line represents a least squares linear fit to the data and yields a slope of $3.8 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ and a y intercept of $1.5 \text{ s}^{-1}$.

To investigate whether the initial absorbance increase results from ATP binding, we examined the dependence of $k_1$ on ATP concentration. Fig. 6B shows that the values obtained increase with ATP concentration in a linear fashion, indicating that the initial absorbance increase is associated with the bimolecular binding of HscA and ATP. The slope ($k_{a,\text{ATP}} = 3.8 \pm 0.6 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$) and y intercept ($k_{a,\text{ATP}} = 1.5 \pm 0.6 \text{ s}^{-1}$) obtained in the presence of HscB and IscU are similar to those observed in experiments performed with HscA alone ($k_{a,\text{ATP}} = 2.8 \pm 0.1 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$ and $k_{a,\text{ATP}} = 1.3 \pm 0.2 \text{ s}^{-1}$, cf. Table I). Thus, HscB and IscU together do not significantly affect the rates of ATP binding or release. The finding that HscB and IscU do not significantly alter the rates of ATP association and dissociation indicate that the decay rate ($k_2$) observed in Fig. 6A represents a lower bound on the rate of $T\to R$ conversion. The stopped-flow experiments performed in the presence of HscB and IscU were limited to using low concentrations of ATP, where ATP binding is expected to limit the rate observed for $k_2$.

**HscB Is Released Following ATP Hydrolysis**—In the absence of substrate, HscB binds to the T-state HscA$^\text{ATP}$ complex but binds weakly if at all to the R-state HscA$^\text{ADP}$ complex (see Fig. 2). It is possible, however, that IscU stabilizes HscB binding to the HscA$^\text{ADP}$ complex. To determine whether HscB is released from the HscA$^\text{ATP}$-HscB-IscU complex following ATP hydrolysis and T$\to$R conversion, we carried out SPR experiments using immobilized HscA in the presence of either ATP or ADP. Fig. 7 shows that even when IscU is present HscB binds only to the HscA$^\text{ATP}$ complex. In the presence of ADP (Fig. 7B), in contrast, addition of HscB and IscU together does not yield a signal significantly stronger than that of IscU alone. As observed previously, the rates of IscU binding and release are significantly faster in the T-state HscA$^\text{ATP}$ complex than in the R-state HscA$^\text{ADP}$ complex (3). The finding that HscB interacts weakly if at all with the HscA$^\text{ADP}$-IscU complex suggests that HscB will be rapidly released following ATP hydrolysis and T$\to$R conversion even in the presence of substrate and that HscB will not exert effects at subsequent steps in the reaction cycle.

**ADP Binding Kinetics**—In the absence of auxiliary proteins, the rate of ADP/ATP exchange is more than 6,000-fold faster than ATP hydrolysis (11). HscB does not bind to the HscA$^\text{ADP}$ complex (11), however, IscU does bind to HscA$^\text{ADP}$ complex and may regulate the kinetics of HscA and ADP binding. We were unable to measure ADP binding kinetics directly, and we therefore used isothermal titration calorimetry to investigate the effect of IscU on ADP binding. The affinity observed in the presence of IscU ($K_D = 158 \mu M$) was reduced 2.3-fold compared with that observed with HscA alone ($K_D = 68 \mu M$). This decrease in affinity could result from a slightly faster rate of ADP release or the combined effects of a slower release rate with a larger decrease in binding rate. The very fast rate of release of ADP observed in the presence of IscU ($60 \text{ s}^{-1}$) (11), however, suggests that even in the presence of IscU the overall APase reaction rate is unlikely to be limited by the rate of ADP release.

**DISCUSSION**

In an earlier report we described the kinetics of the individual steps of the HscA APase reaction cycle in the absence of auxiliary proteins (11). In the studies described here, we have investigated the mechanisms whereby the co-chaperone HscB and the substrate protein IscU regulate the ATPase and substrate-binding activities of HscA. We have used several approaches including surface plasmon resonance and steady-state and pre-steady-state measurements. The kinetic constants determined for different steps shown in Scheme 1 in the presence and absence of HscB and IscU are summarized in

**Table I**

Summary of experimental results for kinetic measurements of HscA catalyzed ATP hydrolysis in the presence and absence of HscB and IscU at 23 °C.

|       | HscA | +HscB | +IscU | +HscB +IscU |
|-------|------|-------|-------|-------------|
| $k_{a,\text{ATP}}$ (s$^{-1}$) | 0.002 | 0.019 | 0.007 | 1.14        |
| $k_{b,\text{ATP}}$ (s$^{-1}$) | 0.002 | 0.020 | 0.006 | 1.14        |
| $k_{\text{ADP}}$ (M$^{-1}$ s$^{-1}$) | 28,000 | 32,000 | ND   | 38,000      |
| $k_{\text{ATP}}$ (s$^{-1}$) | 1.3 | 1.1 | ND | 1.5         |
| $k_{\text{cat}}$ (s$^{-1}$) | 0.1 | 0.1 | 5.0 | ND          |
| $k_{\text{cat}}$ (s$^{-1}$) | 0.0021 | 0.0164 | 0.0063 | 0.13        |

$^a$ The steady-state APase rate is a lower bound on $k_{\text{cat}}$, since ATP hydrolysis is rate-limiting in the absence of auxiliary proteins.

$^b$ ND, not determined.

$^c$ The rate of $T\to R$ conversion is a lower bound, since this kinetic step could only be measured using subsaturating levels of co-chaperone and substrate.

**Fig. 7.** SPR analysis of HscB binding to HscA-IscU complexes. IscU (100 μM) or IscU + HscB (100 μM each) was injected into HKM buffer containing 5 mM dithiothreitol passing over a sensor chip containing immobilized HscA (~1800 RU) at 25 °C. A, in the presence of 1 mM ATP. B, in the presence of 1 mM ADP.

---

**TABLE I**

|       | HscA | +HscB | +IscU | +HscB +IscU |
|-------|------|-------|-------|-------------|
| $k_{a,\text{ATP}}$ (s$^{-1}$) | 0.002 | 0.019 | 0.007 | 1.14        |
| $k_{b,\text{ATP}}$ (s$^{-1}$) | 0.002 | 0.020 | 0.006 | 1.14        |
| $k_{\text{ADP}}$ (M$^{-1}$ s$^{-1}$) | 28,000 | 32,000 | ND   | 38,000      |
| $k_{\text{ATP}}$ (s$^{-1}$) | 1.3 | 1.1 | ND | 1.5         |
| $k_{\text{cat}}$ (s$^{-1}$) | 0.1 | 0.1 | 5.0 | ND          |
| $k_{\text{cat}}$ (s$^{-1}$) | 0.0021 | 0.0164 | 0.0063 | 0.13        |

$^a$ The steady-state APase rate is a lower bound on $k_{\text{cat}}$, since ATP hydrolysis is rate-limiting in the absence of auxiliary proteins.

$^b$ ND, not determined.

$^c$ The rate of $T\to R$ conversion is a lower bound, since this kinetic step could only be measured using subsaturating levels of co-chaperone and substrate.
Substrate Capture—ATP hydrolysis measurements performed under single-turnover conditions revealed that HscB and IscU individually stimulate the rates of ATP hydrolysis and T→R conversion only modestly, ~10- and 3-fold, respectively. When both co-chaperone and substrate are present, however, $k_{\text{hyd}}$ and $k_{\text{T→R}}$ were synergistically accelerated to a level that is much faster than the additive effects of the individual proteins. In the presence of saturating levels of co-chaperone and substrate, ATP hydrolysis occurred with a rate, ~1 s$^{-1}$, faster than IscU dissociation from immobilized HscA-ATP ($k_{\text{det}} \sim 0.15–0.35$ s$^{-1}$, Fig. 7) (3). T→R conversion is expected to occur concurrently with ATP hydrolysis, because $k_{\text{hyd}}$ and $k_{\text{T→R}}$ were observed to have essentially identical rates under all conditions assayed. These findings suggest that IscU will remain bound to HscA and be effectively trapped in the high affinity R-state HscA-ADP complex. HscB is expected to dissociate from this complex, because HscB does not interact with HscA-ADP even in the presence of IscU.

Substrate Release—IscU binds with high affinity to the R-state HscA-ADP complex. This complex must dissociate ADP + P$_i$ bind ATP, and undergo R→T conversion to regenerate the low affinity T-state HscA-ATP. In the absence of IscU, the rate of this process is determined by R→T conversion, because $k_{\text{R→T}}$ exhibits a rate that is $10^3$-fold slower than the intrinsic nucleotide exchange rate (~10 s$^{-1}$; cf. Ref. 11). The findings presented here show that IscU enhances R→T conversion ~50-fold. This effect reduces the lifetime of the HscA-IscU complex and serves as an auto-regulatory mechanism to allow additional cycles of substrate binding and release.

Comparison with the DnaK/DnaJ Chaperone System—The effects of IscU and HscB on HscA ATPase activity are in general similar to the effects of peptide substrates and DnaJ on DnaK. Peptide substrates stimulate DnaK ATP hydrolysis weakly (15–18), and DnaJ, when used at concentrations approximating in vivo levels, exerts a weak stimulatory effect on DnaK ATP hydrolysis (14, 15, 17, 18). Substrate-bound DnaJ dramatically accelerates the rate of DnaK ATP hydrolysis (15, 18) as we find for IscU bound to HscB. These similarities suggest that DnaJ and HscB both tightly couple regulation of ATP hydrolysis to substrate capture by the high peptide affinity conformation of their respective chaperone partners. For DnaK, the large acceleration of ATP hydrolysis has been attributed to simultaneous interactions of the DnaJ D-domain with the DnaK ATPase domain and of the peptide substrate with the DnaK substrate binding domain (15, 18, 21, 22). The similarity of the structure of the D-domain of HscB (24) to that of DnaJ (34, 35) suggests that both co-chaperones regulate the ATPase activity of HscA by a similar mechanism. Despite differences in the orientation of the peptide region of IscU bound to substrate binding domain of HscA (9) compared with the peptide complex of DnaK (10, 36–38), IscU appears to regulate the ATPase activity of HscA by a mechanism similar to that of DnaK substrates.

Although the mechanism of regulation of the ATPase activity and substrate capture by HscA and DnaK are similar, the kinetics and regulation of substrate release are different. Both HscA-ADP and DnaK-ADP substrate complexes require ADP/ATP exchange and subsequent R→T conversion to generate the low peptide affinity forms. For DnaK, nucleotide exchange is believed to determine the rate at which the low peptide affinity T-state (DnaK$^-$-ATP) is regenerated, because ADP dissociation occurs with a rate that is significantly slower than R→T conversion (16, 39–41). In the case of HscA, nucleotide exchange and R→T conversion are both fast (>1 s$^{-1}$) and are orders of magnitude faster than DnaK ADP/ATP exchange. Thus, in the absence of auxiliary proteins, the half-life of a
DnaK-substrate complex is expected to be much longer than that of the HscA-IscU complex. However, nucleotide exchange in DnaK is subject to regulation by GrpE, which accelerates ADP/ATP exchange and decreases the lifetime of bound substrates (42–44). In contrast, nucleotide exchange by HscA is not rate-limiting even in the presence of IscU and HscB and does not appear to be subject to regulation.

Comparison with Jac1 and Isu1 Regulation of Ssq1—Homologs of HscA (Ssq1), HscB (Jac1), and IscU (IscU/Isc2) are present in yeast mitochondria (45–49) and like their bacterial counterparts (50–53) have been implicated in the biogenesis of iron-sulfur proteins. Recent studies (50, 51) have shown that Ssq1, Jac1, and Isu1 interact in a manner similar to that found for HscA, HscB, and IscU, suggesting that the major function(s) of this chaperone system have been conserved through evolution. Isu1 and Jac1 cooperatively stimulate Ssq1 ATPase activity, and Jac1 enhances the binding of Isu1 to ATP-bound Ssq1 (54). In addition, the evolutionarily conserved LPPVK motif in IscU that is recognized by HscA (6, 7, 9) is critical for Isu binding to Ssq1 (55). Phylogenetic analyses, however, suggest that Ssq1 is more closely related to DnaK than to HscA (56).

Consistent with this, Ssq1 exhibits some ATPase reaction cycle kinetics that are more similar to those of DnaK than to HscA (56). Ssq1 forms nucleotide complexes that have greater stability than those of HscA, and Ssq1 nucleotide exchange is enhanced by the mitochondrial GrpE homolog Mge1. It is thus possible that eukaryotic mitochondrial chaperone systems involved in iron-sulfur protein biogenesis may employ somewhat different mechanisms of regulation.

Acknowledgment—We thank Dennis Ta for expert technical assistance.

REFERENCES

1. Vickery, L. E., Silberg, J. J., and Ta, D. T. (1997) Protein Sci. 6, 1047–1056
2. Silberg, J. J., Hoff, K. G., and Vickery, L. E. (1998) J. Bacteriol. 180, 6617–6624
3. Silberg, J. J., Hoff, K. G., Tapley, T. L., and Vickery, L. E. (2001) J. Biol. Chem. 276, 1696–1700
4. Hoff, K. G., Silberg, J. J., and Vickery, L. E. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7790–7795
5. Agar, J. N., Krebs, C., Frazzon, J., Huynh, B. H., Dean, D. R., and Johnson, M. K. (2000) Biochemistry 39, 7856–7862
6. Hoff, K. G., Ta, D. T., Tapley, T. L., Silberg, J. J., and Vickery, L. E. (2002) J. Biol. Chem. 277, 27353–27359
7. Hoff, K. G., Cupp-Vickery, J. R., and Vickery, L. E. (2003) J. Biol. Chem. 278, 37582–37589
8. Tapley, T. L., and Vickery, L. E. (2004) J. Biol. Chem. 279, 28435–28442
9. Cupp-Vickery, J., Peterson, J. C., Ta, D. T., and Vickery, L. E. (2003) J. Biol. Chem. 278, 236–244
10. Strain, J., Lorenz, C. R., Bode, J., Garland, S., Smolen, G. A., Ta, D. T., Vickery, L. E., and Culotta, V. C. (1998) J. Biol. Chem. 273, 31138–31144
11. Yusibov, V., Schuster, H. P., Pachschies, L., Bukau, B., and Reinstein, J. (1996) J. Mol. Biol. 263, 657–670
12. Mayer, M. P., Brehmer, D., Gassler, C. S., and Bukau, B. (2001) Adv. Protein Chem. 59, 1–44
13. Misselwitz, B., Staerk, O., and Rapoport, T. A. (1998) Mol. Cell 2, 593–603
14. Russell, R., Wahl Karzai, A., Meh1, A. F., and McMacken, R. (1999) Biochemistry 38, 4165–4176
15. Han, W., and Christen, P. (2003) J. Biol. Chem. 278, 19038–19043
16. Slepenskov, S. V., and Witt, S. N. (1998) Biochemistry 37, 16749–16756
17. Gassler, C. S., Buchberger, A., Laufen, T., Mayer, M. P., Schroder, H., Valen-
