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Promotion of the in Vitro Renaturation of Dodecameric Glutamine Synthetase from *Escherichia coli* in the Presence of GroEL (Chaperonin-60) and ATP†

Mark T. Fisher*
Laboratory of Biochemistry, National Institutes of Health, National Heart Lung and Blood Institute, Bethesda, Maryland 20892

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ABSTRACT: The folding and assembly of dodecameric glutamine synthetase (GS) from *Escherichia coli* was examined in the absence and presence of the *E. coli* heat shock protein, GroEL (chaperonin-60). At nonphysiological temperatures (15–20 °C), unfolded GS spontaneously renatured to 80–90% of its original activity in the absence of GroEL. At near-physiological temperatures (37 °C), only 20–40% of the original activity returns. Under the latter solution conditions, GroEL and ATP enhance the extent of GS renaturation to 70–80% of the original activity at 37 °C. In the absence of ATP, GroEL arrests the renaturation of unfolded GS by forming a stable binary complex. The addition of ATP to this complex resulted in the release of GS subunits and formation of active dodecameric GS. The order of addition of ATP or unfolded GS to GroEL results in differences in the $t_{1/2}$ values where half-maximal GS activity is attained. At a constant GS concentration, the formation of the GroEL·GS complex followed by ATP addition resulted in approximately a 2-fold increase in the observed $t_{1/2}$ value compared to that observed when GroEL was preincubated with ATP before the GS renaturation reaction was initiated. These differences in renaturation rates may be related to binding affinity differences between the ATP-free and -bound GroEL conformer for unfolded or partially folded protein substrates [Badcoe, I. G., Smith, C. J., Wood, S., Halsall, D. J., Holbrook, J. J., Lund, P., & Clarke, A. R. (1991) *Biochemistry 30*, 9195–9200]. Although the smaller chaperonin protein, GroES (chaperonin-10), was not required for the in vitro renaturation of GS, the renaturation rates were accelerated when it was included in the reaction mixture. The addition of ATP analogues, adenosine 5′-*O*-thiomonophosphate (ATP-γ-S) and 5′-adenylylimidodiphosphate (AMP-PNP), to the arrested complex also results in the release of GS from GroEL but with slower renaturation rates and/or lower extents of GS renaturation. This suggests that ATP hydrolysis by GroEL is not required to initiate GS renaturation.

The elucidation of the physical processes which dictate protein folding has been the focus of intense research for the past three decades. Results from numerous in vitro protein folding experiments have demonstrated that extended or unfolded polypeptide chains spontaneously fold to a more compact structure. All the necessary information that is required to attain the correct folded protein structure is ultimately contained in the primary amino acid sequence. Elegant studies by King and colleagues have revealed that the primary sequence also dictates both the kinetics of formation and the folded structure of partially folded intermediates (Mitraki et al., 1991). The proper formation of these intermediates appears to be a crucial process in determining the correct folding pathway. In vivo, proteins fold in a highly complex heterogeneous environment which can potentially provide numerous interactive surfaces that can severely inhibit productive folding. The formation of folding intermediates which possess a strong tendency to form inactive aggregates usually results in the formation of inclusion bodies inside the cell. The accumulation of insoluble and/or misfolded proteins is often encountered during heat shock and overproduction of recombinant proteins (Nguyen et al., 1989; Hart et al., 1990). Although a number of oligomeric and monomeric proteins have been successfully refolded following denaturation, these reactions are typically initiated under nonphysiological solution conditions characterized by low temperatures, low protein concentrations, the

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* Address correspondence to the author at NHLBI/NIH, Building 3, Room 207, Bethesda, MD 20892.

presence of denaturant or detergent, and varying salt concentrations (Hermann et al., 1983; Martel & Garel, 1984; Vaucheret et al., 1987; Mendoza et al., 1991a).

Anfinsen and co-workers postulated that the proper folding of some proteins in vivo may require specific factors which would evidently guide them toward a properly folded protein structure (Epstein et al., 1963). It now appears that some proteins, called molecular chaperones or chaperonins, are needed to prevent a few nascent proteins from following nonproductive aggregation pathways as these proteins fold and/or assemble [for review, see Ellis and van der Vies (1991)]. Current experimental evidence suggests that some newly synthesized proteins absolutely require the interaction with chaperonin proteins in order to achieve properly folded protein structures (Goloubinoff et al., 1989a; Van Dyk et al., 1989). Elucidating the mechanism(s) by which these chaperonins prevent nonspecific protein aggregation events and promote the correct protein structure formation now appears to be an extremely important consideration for defining how proteins fold in vivo.

The GroE chaperonins were first demonstrated to be required for proper oligomer formation while investigators were examining various mutants in proper λ coat protein assembly in *Escherichia coli* (Sternberg 1973; Georgopoulos et al., 1973). It was observed that host mutations which result in lower levels of GroEL led to the improper assembly of a λ phage dodecameric head coat protein. The *E. coli* chaperonin system has been shown to promote or enhance the folding and assembly of a number of proteins in vivo and in vitro (Goloubinoff et al., 1989a,b). Thus far, ribulose-bisphosphate carboxylase (Goloubinoff et al., 1989b; Viitanen et al., 1990), pre-β-lactamase (Laminet et al., 1990), citrate synthase (Buchner et al., 1990), and rhodanese (Mendoza et al., 1991b,c; Martin et al., 1991) have been demonstrated to require some or all of the GroE components in order to fold properly in vitro.

E. coli glutamine synthetase (GS)[1] is an oligomeric protein which might require the assistance of chaperonin proteins in order to ensure proper assembly under physiological solution conditions. Recent studies have indicated that the chloroplast chaperonins are transiently associated with glutamine synthetase subunits after these subunits are transported into the chloroplast (Lubben et al., 1989). *E. coli* GS is a dodecamer (M_r 622 000) which is assembled in two face-to-face hexameric rings. The 12 active sites of GS are located at heterologous subunit interfaces in a side to side configuration within the hexameric ring. The up–down dimer contacts between the two stacked hexamers are somewhat unusual in that each subunit carboxyl-terminal α-helix, called a "helical thong", is anchored into the opposing subunit (Almassey et al., 1986; Yamashita et al., 1989). Therefore, the monomer can only attain its proper tertiary structure when it is associated with a number of other subunits in the quaternary structure. In view of the fact that the individual GS monomer can exist as a partially folded intermediate (Hunt & Ginsburg, 1972) that must associate with other subunits in such a unique manner, the question remains as to whether the chaperonin protein GroEL would promote the renaturation of GS from unfolded monomers to form the correct dodecameric structure. The results presented here demonstrate that, at near-physiological temperatures, the renaturation of unfolded GS was significantly enhanced in the presence of GroEL and ATP.

[1] Abbreviations: GS, glutamine synthetase, GnHCl, guanidine hydrochloride; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; AMP-PNP, 5′-adenylylimidodiphosphate; ATP-γ-S, adenosine 5′-*O*-thiomonophosphate; rubisco, ribulose-1,5-bisphosphate carboxylase; SDS, sodium dodecyl sulfate.

Materials and Methods

Chemicals. Glutamate, ATP, ATP-γ-S, and AMP-PNP were purchased from Sigma Chemical Co. Chelex 100 resin was purchased from Sigma and was prepared as previously described (Hunt & Ginsburg, 1980). Ultrapure urea, GnHCl, and Tris-HCl were purchased from Schwarz/Mann Biotech. Ultrapure ammonium sulfate was purchased from Bethesda Research Laboratories.

Purification of GS. Glutamine synthetase (GS) was isolated from *E. coli* (YMC10/pgln6) which overproduces the enzyme (Backman et al., 1981). GS was purified using the zinc-induced aggregation method (Miller et al., 1974) followed by ammonium sulfate and acetone treatment (Woolfolk et al., 1966). Protein concentrations were determined by absorbance measurements at 290 nm using A_{290} of a 0.1% solution (1 cm) = 0.387 (Shapiro & Stadtman, 1970). GS activity was monitored using the γ-glutamyl transferase assay method (Stadtman et al., 1979). The GS preparations contained approximately 0.7–1.5 adenylylated subunits per dodecamer and possessed transferase activities of 120–130 units/mg.

Growth of E. coli for GroEL Preparation. The growth of the cells and the purification protocol used here was a modification of published procedures (Chandrasekhar et al., 1986; Burton & Eisenberg, 1980). *E. coli* K-12 was grown in a 10-L fermentor using a tryptone/yeast extract containing 15 g of tryptone, 10 g of yeast extract, and 5 g of NaCl per liter. The cells were grown to an OD of 0.6 at 37 °C and were heat shocked to 43 °C until the cells went into a stationary growth phase (2–3 h). The cells were harvested by centrifugation and frozen in liquid N_2.

Purification of GroEL. Cells (50 g) were suspended in 160 mL of lysis buffer A containing 50 mM Tris-HCl, 1 mM EDTA, 200 μg/mL lysozyme, 0.1 M ammonium sulfate, 1 mM DTT, 10% (w/v) sucrose, 10% glycerol, and 0.18 M spermidine HCl at pH 8.0. The suspension was stirred at 4 °C for 45 min, and the temperature was increased to 37 °C for 15 min. DNase and RNase were added at 10 μg/mL, and the solution was stirred for an additional 5 min. The cell debris was removed by centrifugation at 4 °C, and the pellet was washed with half the original lysis buffer volume. The wash was centrifuged, and the supernatants were combined. GroEL was precipitated from the solution between 35 and 50% ammonium sulfate saturation at 4 °C. The ammonium sulfate pellet was resuspended in an equal volume of gel filtration buffer B containing 50 mM Tris-HCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, and 1 M KCl at pH 8.0 at 4 °C. The redissolved pellet was loaded onto a Sephycryl HR S-400 column (5 × 100 cm column) and eluted at a flow rate of 15 cm h^{-1}. The protein peak containing GroEL eluted just after the void volume. GroEL purification was followed by monitoring the prominent protein migrating at approximately 66 kDa on SDS–PAGE. The GroEL-containing fractions were pooled and dialyzed against buffer C containing 50 mM Tris-HCl, 50 mM KCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol at pH 7.5 at 4 °C. This sample was loaded onto a Whatman DE52 cellulose column equilibrated in buffer C. The column was washed with four column volumes of low-salt buffer, and GroEL was eluted from the column using a salt gradient of 50–400 mM KCl; GroEL eluted between 200 and 300 mM KCl. GroEL-containing fractions with a absorbance ratio of A_{280}/A_{260} no less than 1.1 were combined. The pooled fractions were dialyzed against buffer D containing 20 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5 at 4 °C and brought to a final

concentration of 2 mg/mL protein as assessed by a Bradford assay using GS as a standard. The sample was then applied to an Affigel-Blue column (Bio-Rad) previously washed with 6 M GnHCl and reequilibrated in buffer D. The protein sample was loaded onto the column and was allowed to equilibrate for 15 min at 4 °C. The column was washed with buffer D, and the eluant contained GroEL. This procedure removed contaminating glutamine synthetase as assessed by the γ-glutamyl transferase assay (Burton & Eisenberg, 1980). At this point, the purified GroEL fraction ran as a single band on SDS–PAGE. The absorbance maximum for the purified sample used in the current studies was 275 nm. Gel filtration analysis indicated that the native molecular mass was 810 kDa. The protein possessed ATPase activity as measured by the methods outlined by Hendrix (1979). GroEL was dialyzed against 50 mM Tris-HCl, pH 7.5 (at 25 °C) and 0.6 mM EDTA and was concentrated using a Amincon centricon filtration device (30 000 M_r cutoff). The GroEL solution (approximately 4–5 mg/mL) was sterile filtered and kept at 4 °C until further use. GroEL was purified to apparent homogeneity as assessed by gel electrophoresis in the presence of 1% SDS. In addition, the absence of any contaminants migrating in the same position as lysozyme indicated that the small chaperonin protein, GroES, did not copurify with GroEL.

The concentration of GroEL has been determined spectrophotometrically by a number of investigators. Unfortunately, there is no agreement among these investigators as to which extinction coefficient is valid. For example, extinction coefficients of 2.38, 1.2, and 0.9 × 10^4 M^{-1} cm^{-1} at 280 nm were estimated by Viitanen et al. (1990), Lissen et al. (1990), and Badcoe et al. (1991), respectively. Using a second-derivative multicomponent fitting analysis procedure previously described (Federici & Levine, 1982), the purified samples used in this particular study were estimated to contain between 0.2 and 0.3 tryptophan residues per GroEL monomer. Examination of the second-derivative spectrum indicated that there was a broad featureless decline in absorbance from 290 to 296 nm without the characteristic trough at 291 and peak at 295 nm indicative of a substantial tryptophan contribution. From the multicomponent fitting analysis, the molar amounts of tyrosine and phenylalanine for GnHCl unfolded GroEL were found to be equivalent. This result agrees with the expected ratio derived from the amino acid sequence of seven tyrosine and seven phenylalanine residues (Hemmingsen et al., 1989). The concentration of the GroEL protomer was determined by the molar amounts of tyrosine and phenylalanine indicated by the multicomponent fit analysis. Since GroEL was isolated in the presence of a tryptophan contaminant, the observed extinction coefficient is, therefore, dependent on the preparation. The calculated extinction coefficient for four individual GroEL preparations was determined to be 1.22 ± 0.4 M^{-1} cm^{-1} per subunit at 280 nm. This value agrees with the latter two molar extinction coefficients rather than the former estimate. Purified GroES (>95%) was a generous gift from Dr. E. Eisenstein (Center for Advanced Research in Biotechnology, Rockville, MD).

Denaturation of Glutamine Synthetase. Concentrated samples of glutamine synthetase (1–2 mg/mL) were denatured in either 6 M GnHCl or 8 M urea, 50 mM Tris-HCl, pH 8.5 (at 0 °C), 1 mM EDTA, and 1 mM DTT at 0 °C for a minimum of 4 h. After this incubation period, the GS sample was inactive, the dodecamer was dissociated into monomers, and no further spectral changes were observed as monitored by circular dichroism (CD), tryptophan fluorescence, and second-derivative UV absorption spectroscopies. Spectral analysis of the denatured protein indicated that the tryptophan and tyrosine residues were completely exposed to solvent and no residual secondary structure was evident as assessed by circular dichroism spectroscopy. These results support those of earlier denaturation studies (Woolfolk et al., 1966; Hunt & Ginsburg, 1972). At any given protein concentration, the time-dependent renaturation profiles remained the same regardless of the amount of time GS was subjected to denaturing conditions from 4 to 24 h. This suggests that there were no further slow reversible or irreversible reactions occurring once the denatured GS monomers had formed.

Renaturation of GS. Renaturation of GS was initiated by rapidly diluting away the denaturant with renaturation buffers, resulting in a 100-fold dilution of GS. Under nonphysiological temperatures (15–20 °C), the buffer conditions which resulted in optimal renaturation consisted of 50 mM Tris-HCl, pH 7.5, at 20 °C, 30 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 400 mM KCl, and 0.8 M urea (buffer I). At near-physiological temperatures (37 °C), the renaturation buffer consisted of 50 mM Tris-HCl, pH 7.7, at 37 °C, 10 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 1 μM bovine serum albumin (BSA), and 1 mM EDTA (buffer II). GroEL ± 5 mM ATP was diluted into the renaturation buffer at indicated concentrations (Figures 4 and 5) prior to initiating the refolding of GS.

GS assays were linear in time with all GS concentrations examined. Upon initiating the GS renaturation, initial aliquots were taken to determine the activity. The initial time points (time = 30 s) showed no GS activity. This indicates that GS cannot renature in the presence of the γ-glutamyl transferase assay mixture. This result agrees with earlier studies of Ciardi et al. (1973).

Native Electrophoretic Separation of GS Renaturation Reaction Products. Native gradient-pore gel electrophoresis was used to examine the time-dependent changes of the protein species present during GroEL-dependent GS renaturation. At specified times during renaturation, the protein species present were separated using a precast, continuous 4–15% polyacrylamide gradient, Phastgel. The renaturation reactions were initiated at staggered times, and the samples were applied onto the gradient gel simultaneously using a Pharmacia Phast electrophoretic separation system. The separated protein species were visualized by Coomassie staining.

RESULTS

The Renaturation of Unfolded GS without GroEL. The assembly and renaturation of GS was initiated by dilution of the denatured monomers into buffer I at 20 °C (see Materials and Methods). These solution conditions were originally used by Ciardi et al. (1973) during assembly studies of hybrid GS oligomers. Profiles of time-dependent activity regain at four different GS concentrations are illustrated in Figure 1. The typical GS activity regain profile shows an initial lag followed by a faster phase. The initial time course of the GS renaturation reaction appears to reach a maximal rate of change around 100 μg/mL GS concentration. The initial lag phase is still present at higher GS concentrations. This may indicate that there are some slow unimolecular refolding steps that must occur prior to the assembly reactions (Figure 1, inset). No changes in activity were observed after 24 h. The slow rise in activity between 1 and 24 h, as well as the final extent of renaturation, is highly dependent on the initial concentration of GS present (Figure 1). At low protein concentrations, the slow rise in activity probably reflects slow association reactions. At nonphysiological temperature (20 °C), the optimal concentration for renaturation of GS was found to be 25 μg/mL or 480 nM subunit. At this concentration, the extent of re-

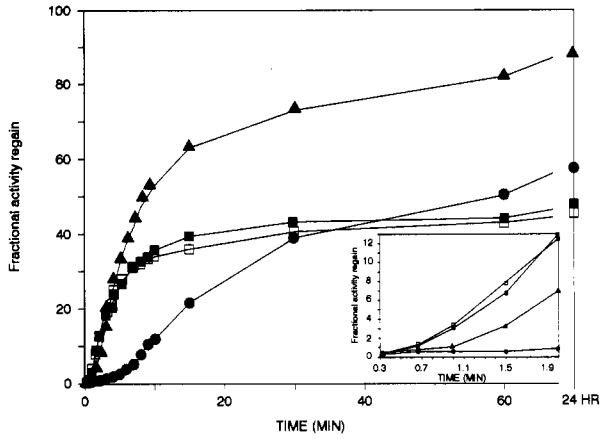

FIGURE 1: Renaturation of GS at nonphysiological temperatures (20 °C, no GroEL) in buffer I (see Materials and Methods). The initial GS concentrations were 10 (●), 25 (▲), 100 (■), and 200 μg/mL (□). GS was denatured and renatured in buffer I as indicated under Materials and Methods. The optimal regain of activity was achieved when the final concentration of refolding GS was 25 μg/mL (480 nM monomers). (Inset) Normalized rates of renaturation illustrate that the observed lag in the renaturation rate profile decreases with the concomitant increase in the reactivation rates as the initial concentration of unfolded GS is increased. At higher GS concentrations, there was no further acceleration in the initial rates of change for the renaturation profile.

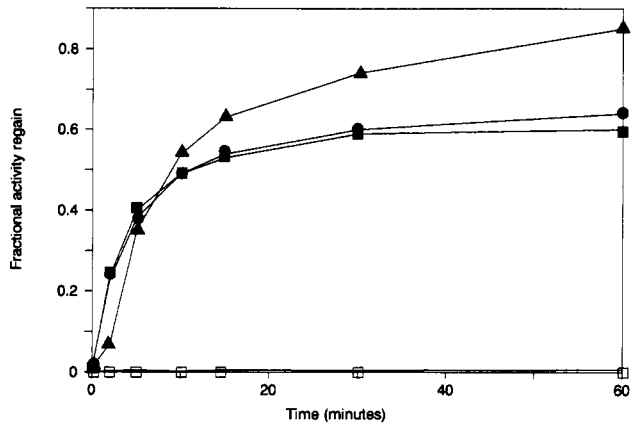

FIGURE 2: Optimal renaturation of GS (25 μg/mL) depends on defined buffer conditions. The necessity of some of the solution components used in the renaturation buffer I (see Materials and Methods) was examined by deleting each component independently. When the GS renaturation was examined at low ionic strength (no KCl, ■) and low urea concentrations (0.08 mM urea, ●), the initial activity rate profiles were more rapid than those observed under the optimal conditions (▲). The final extent of renaturation under low ionic strength and urea concentrations was substantially lower than that observed under the optimal conditions. The lower trace shows that no active GS oligomers formed when Mn^{2+} or Mg^{2+} ions were absent from the renaturation mixture (□).

naturation is 87 ± 5% of the original activity after 24 h.

The extent and activity rate profiles during GS renaturation are not only dependent on the initial protein concentration, changes in the buffer conditions such as decreases in the ionic strength and/or urea concentrations (0.8–0.08 M) also resulted in significantly faster reactivation rates in the renaturation time courses (Figure 2). However, the final extent of GS renaturation was decreased at low ionic strength and low urea concentrations. Higher ionic strength conditions and moderate urea concentrations may substantially inhibit the formation of misfolded GS aggregates by increasing reshuffling and/or refolding reactions. Curiously, when MgADP or MgATP (5 mM) was initially added to the renaturation buffer (buffer I, 10 mM Mg^{2+}) prior to initiating the GS renaturation, the

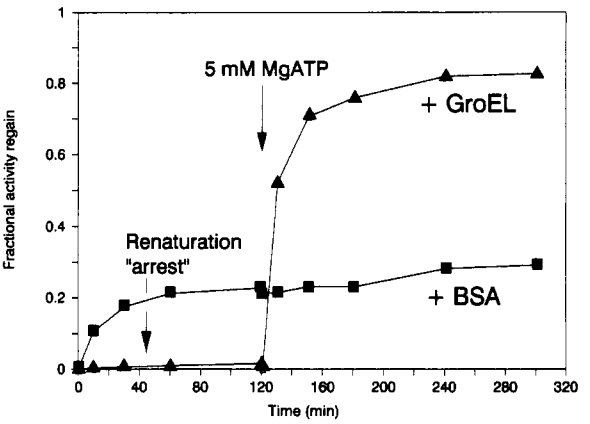

FIGURE 3: Initial GS renaturation profiles comparing the reconstitution buffer II containing bovine serum albumin (BSA) (■) and GroEL (▲). As indicated in the text, the extent of GS renaturation at near-physiological temperatures (37 °C) is significantly decreased compared the extent of renaturation observed under optimal conditions illustrated in Figure 1. In the presence of GroEL, GS monomers were still structurally competent to undergo refolding and renaturation but remained in an arrested state until MgATP is added. In the presence of ATP, the activity of the once arrested form of GS returns to 78% of the original GS activity. The concentrations of GS monomers and GroEL oligomers were 0.38 and 0.78 μM, respectively.

rates of GS renaturation were actually slower than those rates observed with the metal ions alone (data not shown).

Effect of Metal on the Renaturation Profiles. The renaturation of unfolded GS absolutely requires Mn^{2+} or Mg^{2+} metal ions. Optimal activity regain was observed at metal ion concentrations either ≥1 mM Mn^{2+} or 10 mM Mg^{2+}. In the absence of these metal ions, no activity regain was observed (Figure 2). The inclusion of metal ions in the renaturation buffer was found to be required to induce the oligomerization of GS as observed by light scattering analysis (data not shown). If metal ions were added to the renaturation mixture after 1 h at 15–20 °C, the activity followed the same activity renaturation profiles as was observed if the metal ions were initially present. This suggests that there is a stable intermediate of GS that forms in the absence of metal ions. The extent of the activity regain under these conditions is temperature dependent. When Mg^{2+} ions were added to a refolded GS sample that was incubated at 37 °C for 1 h in the absence of metal ions, the final extent of renaturation was only 10–15% of the original activity.

Renaturation of GS in the Presence of GroEL. At near-physiological solution temperatures (37 °C) (buffer II), spontaneous GS renaturation was observed but the maximal extent of GS activity regain was dramatically decreased (Figure 3). The spontaneous GS renaturation experiments were performed in parallel with the renaturation experiments in the presence of GroEL. At the GS concentrations used (0.38 μM subunit), both the extent and kinetics of the spontaneous GS renaturation did not exhibit any significant dependence on the BSA concentration (≥0.8 μM).

GroEL Alone Arrests the Renaturation of Unfolded GS Monomers. When GS renaturation was attempted in the presence of a 2-fold molar excess of GroEL oligomer to GS monomer (0.8 and 0.38 μM, respectively), no regain in GS activity could be observed prior to adding ATP (Figure 3). At 37 °C, the GS subunits in this "arrested complex" remained in forms that were competent to assemble into active GS oligomers for as long as 20 h until ATP was added to initiate renaturation. GroEL also arrests the renaturation of GS under optimal solution conditions (buffer I) at 20 °C. The addition of unfolded GS monomers (0.38 μM) in buffer II at 37 °C

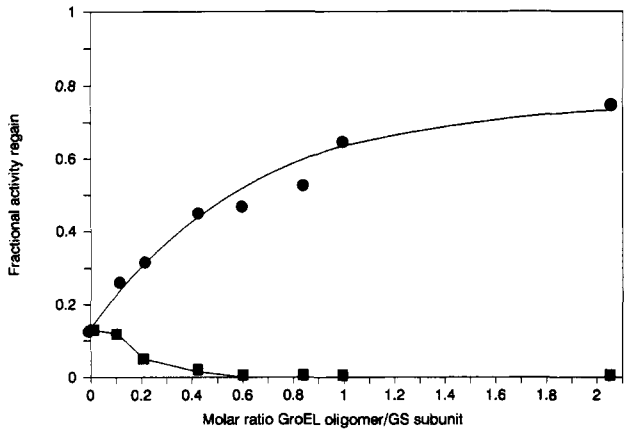

FIGURE 4: Renaturation of active GS (0.38 μM subunit) in buffer II (no BSA present) as a function of increasing GroEL concentration. The lower curve (■) represents the GS activity regain after 1 h in the presence of increasing concentrations of GroEL. The upper curve (●) represents the extent of GS activity regain 2 h after 5 mM ATP was added these same samples represented as closed squares.

containing GroEL in molar ratios exceeding 0.5 (GroEL oligomer:GS monomer) resulted in an arrest of the renaturation reaction in the absence of ATP (Figure 4).

Addition of ATP to the "GroEL·GS Complex" Initiates Renaturation. The addition of ATP to the GroEL·GS complex initiates the renaturation of GS (Figure 3). The extent of the renaturation depends on increasing amounts of GroEL relative to GS monomer (Figure 4). At an initial molar ratio of at least 2 GroEL oligomers to 1 GS monomer, the activity was observed to return to 76 ± 5% of the original activity after 2 h. The final extent of GS renaturation does not increase significantly above the molar ratio of 2. Using the same renaturation solution conditions, control experiments indicated that native GS activity was unaffected by the presence of similar concentrations of GroEL. This suggests that GroEL does not interact with the native form of GS. When the initial Mg^{2+} concentration was increased from 10 to 20 mM, the additional metal ion concentration did not relieve the observed renaturation arrest by GroEL.

If unfolded GS was allowed to renature in the BSA control (no GroEL) for 1 h and GroEL and ATP were then added at this time, there was no effect on rate or extent of the observed renaturation (data not shown). This indicates that GroEL cannot rescue misfolded GS aggregates once they have formed. The presence of BSA in the GS renaturation solution containing GroEL did not affect the extent or the rate of GS activity regain following ATP addition. From these results, it appears that native BSA does not complete with the unfolded or partially folded GS subunits for binding sites on GroEL.

The Renaturation Rate Profile Depends on the Order of Addition of Unfolded GS and MgATP to GroEL. The GroEL-dependent renaturation time course illustrated in Figure 5 shows that changes in the order of addition of unfolded GS and MgATP to GroEL result in significant differences in the renaturation profiles. The rates of the spontaneous and GroEL-assisted GS renaturation were estimated by examining the time, $t_{1/2}$, where the activity regain was half of the maximal extent (observed at 24 h) of reaction at a constant GS monomer concentration of 0.38 μM. The observed $t_{1/2}$ values were found to be the same ($t_{1/2} \approx 6$ min) for the spontaneous (no GroEL) and GroEL-dependent GS renaturations when GroEL and ATP were mixed prior to initiating the GS renaturation reaction. In addition, the normalized renaturation profiles for the above conditions were coincident (Figure 5, inset). This indicates that GroEL alone

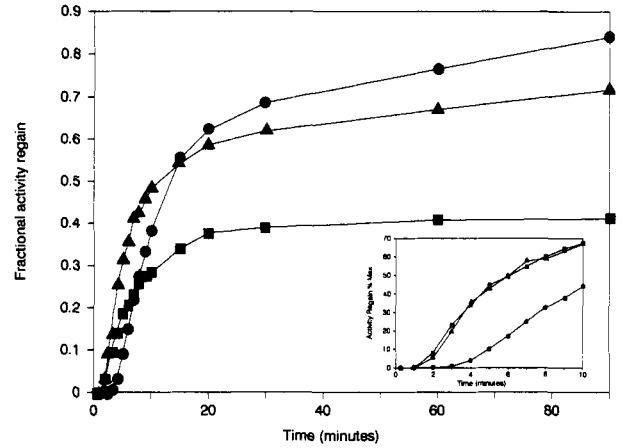

FIGURE 5: Renaturation of GS with GroEL alone and the effects of the order of addition of ATP and unfolded GS to GroEL. The final GS concentration used in this experiment was 0.38 μM protomer. The representative reaction profiles (in buffer II, 10 mM Mg^{2+}) are of the (1) spontaneous GS renaturation with 5 mM ATP alone (■), (2) preincubated GroEL and 5 mM ATP for 1 min (▲), and (3) preformed GroEL·GS complex (●). The fractional increase in activity was significantly higher when the GS renaturation was initiated in the presence of ATP and GroEL as opposed to the spontaneous renaturation with ATP alone. When GroEL (0.8 μM) and unfolded GS were premixed prior to addition of ATP (5 mM), the final extent of the total renaturation increased compared with that observed when GroEL and ATP were premixed before starting the GS renaturation. (Inset) When GroEL was incubated with ATP prior to initiating the GS renaturation reaction, a comparison of the normalized rates of GS renaturation in the absence and presence of GroEL suggests that GroEL itself does not accelerate the GS activity regain rate. The observed lag time and $t_{1/2}$ values were the same with and without GroEL (1 and 6 min, respectively). In contrast, the observed lag was extended and the $t_{1/2}$ increased to 3 and 10 min, respectively, when the GroEL·GS complex was allowed to form before initiating the renaturation reaction with ATP. The observed lag times and $t_{1/2}$ values were found to be the same in four separate trials.

does not accelerate the observed renaturation of GS at this particular GS concentration. The identical lag times may indicate that there is also no rate enhancement of the initial slow reactions (refolding monomer and/or correct dimer formation) which must occur prior to the assembly of GS into active oligomers.

When the GroEL·GS complex was formed prior to the addition of ATP, the initial lag in the formation of active GS was extended to 3 min (Figure 5, inset) and the observed $t_{1/2}$ value increased to 10 min (Figure 5). Interestingly, the final extent of the renaturation reaction was always consistently higher in experiments where the GroEL·GS complex was allowed to form prior to adding ATP. In four separate trials, the extent of GS renaturation assayed at 24 h for the preformed complex was 11 ± 2% higher than the final activity observed if GroEL was incubated with ATP (~1 min) prior to initiating the GS renaturation reaction (Figure 5). When GroEL was preincubated with ATP prior to GS renaturation, both the rate and the extent of GS activity regain was found to be independent of the time of GroEL·ATP preincubation up to at least 5 min This suggests that the depletion of ATP in the renaturation buffer due to the intrinsic ATPase activity of GroEL alone during the preincubation times indicated above were not enough to affect the renaturation of GS.

GroES Accelerates the Renaturation Rate of the GroEL-Dependent GS renaturation. For comparative purposes, experiments examining the effect of GroES on the GroEL-assisted GS renaturation were performed in parallel with those where only GroEL was used. If GroES, GroEL, and ATP were present in the initial renaturation solution, the $t_{1/2}$ for

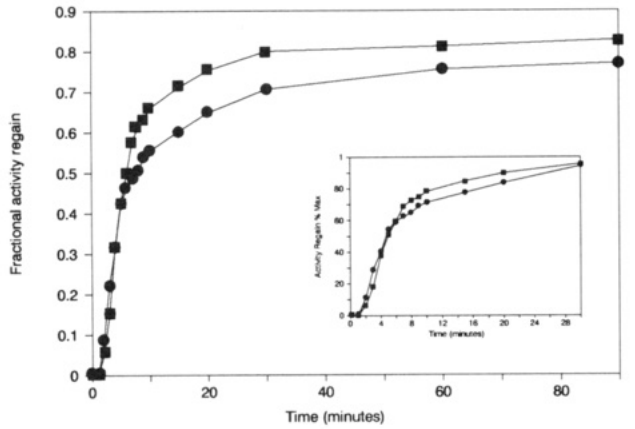

FIGURE 6: Effect of GroES on the GroEL-dependent renaturation of GS (buffer II). The addition of GroES to GroEL-dependent renaturation of GS accelerated the GS renaturation when the initial species was either the preformed GroEL·GS (■) or the premixed GroEL·ATP complexes (●). The extent of the lag time and the observed $t_{1/2}$ were the same for both renaturation conditions (1 and 5 min, respectively) in four separate trials. The concentrations of GroEL, GroES, and ATP were 1.6 μM oligomer, 0.8 μM oligomer, and 5 mM, respectively. (Inset) The normalized time-dependent renaturation profiles show that the inclusion of GroES in the renaturation mixture eliminates the observed order of addition differences with GroEL alone (Figure 5).

Table I

nucleotide added	% activity regain[a] 2 h	24 h
ATP	75	78
ADP	0	0
ATP-γ-S	30	72
AMP-PNP	16	34

[a] The nucleotide was added 2 h after denatured GS was diluted (0.38 μM subunits) into reconstitution buffer II in the presence of 780 nM of GroEL. Initial aliquots of the renaturation mixtures taken 30 s after nucleotide addition showed no GS activity. GS activity after 2 and 24 h is indicated. The final concentration of each nucleotide in the reconstitution buffer was 5 mM in the presence of 10 mM $MgCl_2$.

GS renaturation was consistently lower than that observed with GroEL and ATP alone (Figure 6). The decrease in the $t_{1/2}$ value was approximately 1 min. When GroES was initially present with the preformed complex, the ATP-induced GS renaturation rates were characterized by substantial decreases in the initial lag times and $t_{1/2}$ values (2 and 4 min, respectively) compared with those values observed with GroEL·GS alone. Regardless of the defined order of addition, the time-dependent renaturation changes observed in the normalized profiles were essentially the same when GroES is included (Figure 6, inset). Thus, it appears that GroES eliminates differences due to the order of addition observed when GroEL alone was present during the GS renaturation (Figure 5). For all the GS renaturation experiments illustrated in this work, the time-dependent renaturation profiles were found to be sigmoidal.

GroEL-Dependent Renaturation of GS in the Presence of ATP Analogues. When the ATP analogues adenosine 5′-*O*-thiomonophosphate (ATP-γ-S) and 5′-adenylylimidodiphosphate (AMP-PNP) were added to the arrested GroEL·GS complex at 37 °C, the GS activity slowly returned as a function of time (Table I). With ATP, the final extent of renaturation remained constant over 22 h, indicating that a stable active oligomer of GS had formed. Although ATP is a substrate for GS, the inclusion of MgATP in the near-physiological renaturation buffer does not enhance the extent or kinetics of the spontaneous GS renaturation (no GroEL) relative to the rate

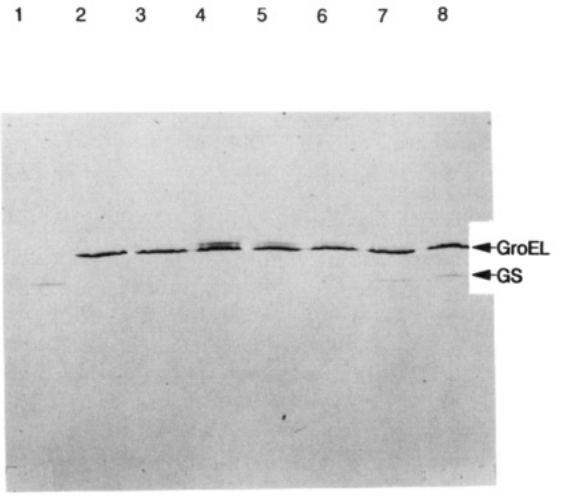

FIGURE 7: Native gel electrophoresis of the GroEL before and after unfolded GS addition. After ATP addition, the oligomerization of GS in buffer II was observed after 1.5 h. Lanes 1–8 are samples containing (1) native GS (622 kDa) 20 μg/mL, (2) GroEL (802 kDa) 150 μg/mL, (3) GroEL (802 kDa) 150 μg/mL + 5 mM ATP + 0.05 M GnHCl, (4) GroEL + unfolded GS (no ATP) (0.05 M GnHCl), (lanes 5–8) GroEL + unfolded GS + 5 mM ATP at (5) 4 min, (6) 10 min, (7) 30 min, and (8) 2 h after ATP addition. The appearance of the GS dodecamer is evident at the later times.

profile observed with Mg^{2+} alone (Figures 3 and 5). In addition, AMP-PNP and ATP-γ-S did not affect the extent and kinetics of the spontaneous GS renaturation reaction (data not shown).

When ATP-γ-S was added to the GroEL·GS complex, the GS activity eventually returned to the renaturation levels observed with ATP although the renaturation rate was significantly slower than that observed with ATP (Table I). For AMP-PNP, the final extent of renaturation at 24 h was substantially lower than those observed in the presence of ATP or ATP-γ-S. After 24 h, the extent of renaturation was comparable to that observed for the controls where GroEL was absent (Figure 3). No GS renaturation was observed when ADP was added to the GroEL·GS complex (Table I).

Native Gel Electrophoresis Indicates the Formation of Dodecameric GS. Native gel electrophoresis of the renaturation mixture was performed to determine if the renaturation of GS resulted in dodecamer formation. Interestingly, the native gel shows a band migrating slower than the major GroEL protein band only when unfolded GS was added to GroEL (Figure 7). When ATP was added, the progression of the GS renaturation was examined by separating the protein species on the native gel at various times. The slower migrating protein band gradually disappeared (Figure 7, lanes 4–6), and formation of a protein band consistent with the migration of the native GS dodecamer is observed a later times (20 min). The intensity of the protein staining of the slower band migrating above the native GroEL was found to be dependent on the concentrations of both the unfolded GS species and the GroEL oligomer (data not shown) and suggests that the identity of this upper band is a complex between GroEL and GS.[2]

[2] When the upper band was excised from the gel and the proteins were rerun on an SDS gel and silver stained, protein bands migrating with the molecular masses consistent with those of GS and GroEL were found. In addition, preliminary western analysis using antibodies to *E. coli* GS indicated that only the upper band contained GS and that this band slowly disappeared when ATP was added and dodecameric GS appeared at later time points. These results will be presented in their entirety in an ensuing paper.

Discussion

The in vitro renaturation of dodecameric glutamine synthetase (GS) from unfolded monomers at near-physiological temperatures was significantly enhanced when GroEL and ATP were present. GroES is not absolutely required under these conditions although the renaturation rates are increased in the presence of GroES. In the presence of K^+ and MgATP, GroEL must alter the kinetic pathway of refolding or reassociation of GS to favor the correct assembly of dodecameric GS at near-physiological temperatures since significant GS renaturation can be observed at nonphysiological temperatures.

The in vitro folding reactions of a number of proteins has been demonstrated to be assisted by the *E. coli* chaperonin system. However, some proteins do not require the assistance of all the chaperonin components. Numerous experiments using different unfolded proteins as substrates have indicated that GroES is not absolutely required for release and proper folding of some GroEL-bound proteins. For example, the renaturation of β-lactamase, dihydrofolate reductase (DHFR), and lactate dehydrogenase can be initiated with GroEL, K^+, and MgATP alone (Laminet et al., 1990: Martin et al., 1991; Viitanen et al., 1991; Badcoe et al., 1991). In another case, a precursor protein bound to GroEL, proOmpA, was released from GroEL in the absence of both ATP and GroES and binds to SecB to form a stable complex (Lecker et al., 1989). On the other hand, the reconstitution of active ribulose bisphosphate carboxylase (rubisco), rhodanese, and citrate synthase are accomplished only in the presence of GroEL, GroES, K^+ and MgATP (Goloubinoff et al., 1989b; Viitanen et al., 1990; Buchner et al., 1990; Mendoza et al., 1991b). A small amount of rubisco and rhodanese were released from GroEL after the addition of MgATP alone, but the dissociated proteins were not active and tended to form inactive aggregates (Goloubinoff et al., 1989b; Martin et al., 1991). Clearly, not all renaturation reactions require both chaperonin proteins to promote correct protein folding. Since the binding affinities between these various unfolded proteins and GroEL are almost certainly different, it is tempting to speculate that those proteins which require both chaperonin components along with ATP for renaturation may bind to GroEL with larger affinities than do those proteins which require only GroEL and ATP for successful renaturation. However, this hypothesis remains to be tested.

The reassociation and renaturation of GS was inhibited in the presence of GroEL. This result strongly suggests that GroEL forms a complex with unfolded or partially folded GS subunits to prevent the reassociation. Native gel electrophoresis showed the appearance of an upper band migrating slower than native GroEL. It is conceivable that this upper band represents a complex between GroEL and unfolded or partially folded GS subunits. After ATP was added, this protein band slowly disappeared with a concomitant increase in a band consistent with the formation of dodecameric GS. No activity was observed in renaturation arrested samples for as long as 20 h at 37 °C until ATP was added. Aliquots of these long-term arrested samples subjected to native gel electrophoresis clearly showed the presence of this slower migrating band. Evidently, GroEL can maintain GS subunits in conformations that are competent to undergo proper oligomerization for a long period of time and can inhibit nonproductive interactions.

The GS renaturation rate profile was sigmoidal under all conditions examined here. The renaturation rate profile of GS always exhibited an initial lag with a clear dependence on the initial protein concentration (Figure 1, inset). This could be due to slow refolding reactions of the monomer prior to the correct formation of dimers and may be the rate-limiting step in the renaturation process. Alternatively, since the formation of the correct dimer requires the insertion of the carboxyl terminus into another monomer, the rate-limiting reaction could also be due to the correct refolding of the dimer.

In the presence of GroEL alone, the duration in the lag for the GS renaturation reaction was increased if the arrested complex was formed prior to adding ATP (Figure 6). Conversely, if GroEL was incubated with ATP before the GS renaturation was initiated, the lag in the renaturation profile was the same as that observed in the spontaneous GS renaturation experiments. Whether GroEL undergoes a significant conformational change after ATP addition or hydrolysis is a key question which remains to be addressed. Since the observed lag in activity directly reflects the renaturation of GS, this extended lag could be due to slower rates of release of GS from the GroEL surface when the complex is formed prior to ATP addition. Curiously, the extent of GS renaturation reaction was always enhanced if the GS renaturation was arrested by GroEL prior to the addition of ATP. This observation may indicate that the ATP-free form of GroEL will bind to unfolded or partially folded GS monomers more effectively than the ATP-bound form. If this is true, then the ATP-bound form of GroEL may not compete with the nonproductive reactions as effectively as does the ATP-free form. A similar hypothesis has been proposed to explain the observed differences between ATP (or AMP-PNP)-free and -bound forms of GroEL during the refolding of *Bacillus stearothermophilus* lactate dehydrogenous (Badcoe et al., 1991).

Although GroES is not absolutely required to ensure the proper release and association of GS in vitro, the presence of GroES effects the observed renaturation rates. GroES appears to eliminate the observed differences in the renaturation profiles for the GroEL-dependent GS renaturation conditions which are dependent on the order of addition of ATP and unfolded GS to GroEL (Figures 5 and 6). Since the GS renaturation rate was found to be concentration dependent, the GroES-induced attenuation of the observed lag and the acceleration in the observed GroEL-dependent GS renaturation rate could be due to an increased release of GS monomers which are competent to form active GS oligomers. Experimental results of Martin et al. (1991) have suggested that GroES does not appear to function by simply displacing a bound folding intermediate from the binding sites of GroEL. Clearly, GroES must somehow influence the conformation of GroEL to accelerate the refolding and reassociation reaction since GroES alone does not affect the extent and rates of GS renaturation.

The mechanism governing the release of unfolded or partially folded subuntis of GS from GroEL is further complicated by the fact that ATP hydrolysis does not appear to be required for the renaturation of GS to begin. The ATP analogues ATP-γ-S and AMP-PNP also led to some activity regain although the rates and the extent of renaturation were lower for these respective analogues. This might indicate that, in some cases, simply binding ATP or an equivalent structure is sufficient to disrupt the complex between GroEL and GS. In the presence of ATP-γ-S, the extent of renaturation slowly approaches that observed for ATP after 24 h. In light of these data, it is interesting to note that ATP-γ-S is hydrolyzable although the rates are typically 100-fold less than those observed for ATP. Whether the large extent of renaturation observed with ATP-γ-S is due to this slow hydrolysis is not clear at this time. Recently, Gatenby and co-workers have

also noted that there is partial discharge of bound dihydrofolate reductase from GroEL in the presence of the ATP analogues used in this current work (Viitanen et al., 1991). Similarly, lactate dehydrogenase appears to be released from GroEL when AMP-PNP is added to the complex (Badcoe et al., 1991). Clearly, the role of ATP hydrolysis remains unclear.

The stoichiometry of the GroEL·GS complex is unknown at present. A preliminary estimate of this ratio based on measuring the remaining activity in the presence of increasing concentrations of GroEL (Figure 4) was not attempted because the GS monomer is inactive. In addition, the individual GS subunits have a tendency to form active as well as inactive aggregates. The minimum number of subunits which could theoretically result in the construction of the GS active site is a tetramer (Maurizi & Ginsburg, 1982; Almassey et al., 1986). Activity measurements with isolated GS tetramers with some sulfhydryl groups modified with 5-thio-2-nitrobenzoic acid (TNB) were found to yield substantially lower activities than what was expected (Maurizi & Ginsburg, 1982). However, experimental estimates of the binding stoichiometry between other protein systems and GroEL appear to be in the range of one monomeric unit bound to a single GroEL tetradecamer (Laminet et al., 1989; Mendoza et al., 1991c; Martin et al., 1991; Badcoe et al., 1991).

The extent of the activity regain of GS was enhanced as the concentration of GroEL oligomer was increased. At a high molar ratio of GroEL oligomer to GS subunit, the additional activity regain might be due to simple mass action effects. Increases in the GroEL concentration would increase the collisional frequency to favor GroEL–GS interactions as opposed to forming native and nonnative GS aggregates. GroEL does not appear to be able to rescue misfolded GS complexes. It is evident from the present work and earlier studies (Goloubinoff et al., 1989b; Buchner et al., 1990) that there exists a limited time window where GroEL can interact with a particular form of refolding GS monomers. A ratio of 3 GroEL oligomers to 1 rubisco monomer was observed to give the optimal regain in activity for in vitro reconstitution experiments (Goloubinoff et al., 1989). The demonstration that an excess amount of GroEL is effective for the in vitro renaturation of various proteins might be relevant considering that the natural in vivo concentration of GroEL is 2% of the total protein in *E. coli* at 37 °C (Neidhardt et al., 1981).

In summary, the renaturation of unfolded *E. coli* glutamine synthetase subunits to form the native dodecamer is enhanced in the presence of ATP, K^+, and GroEL. The inclusion of GroES in this renaturation system accelerates the rate of GS renaturation. The observation that the order of addition of ATP and unfolded GS to GroEL resulted in substantial differences in the GS renaturation rate profile coupled with the observation that GroES eliminated these differences illustrates the importance in understanding how ATP and GroES affect the conformation of GroEL. Elucidation of these changes at the molecular level are paramount in deciphering the mechanisms of chaperonin-assisted protein folding. In addition, determining the stoichiometry of the GS subunit interaction with GroEL and the mechanism of the GroEL-assisted GS assembly will provide important information for resolving the question of how the GroE system promotes correct protein assembly for large oligomers.

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Registry No. GS, 9023-70-5; ATP, 56-65-5; ATPγS, 35094-46-3; AMP-PNP, 25612-73-1.

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Receptor-Induced Switch in Site–Site Cooperativity during Iron Release by Transferrin†

Pawan K. Bali‡ and Philip Aisen*,‡,§
Department of Physiology and Biophysics and Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York 10461

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ABSTRACT: Iron removal by PP_i from the N- and C-terminal binding sites of both free and receptor-complexed transferrin, when the partner site remains occupied with kinetically inert Co(III), has been studied at pH 7.4 and 5.6, at 25 °C. At extracellular pH, 7.4, the C-terminal site of free mixed-metal proteins is slightly more labile than its N-terminal counterpart in releasing iron to 0.05 M PP_i. The rate and extent of iron removal are retarded from both sites when transferrins are receptor-bound. At endosomal pH, 5.6, the two sites exhibit greater kinetic heterogeneity in iron release to 0.005 M PP_i. The N-terminal site is 6 times more facile in relinquishing iron than the C-terminal site when mixed-metal transferrins are free. However, the two sites are affected oppositely upon binding to the receptor. Iron release from the C-terminal site of receptor-complexed Co_N–transferrin–Fe_C is 4 times faster than that from receptor-free protein. In contrast, iron removal from the N-terminal site of receptor-complexed Fe_N–transferrin–Co_C is slowed by a factor of 2 compared to that from free protein. These results help explain our previous observation of a receptor-induced switch in site lability during iron removal from diferric transferrin at pH 5.6 (Bali & Aisen, 1991). Site–site cooperative interactions between the two sites of doubly-occupied transferrin during iron release are altered upon binding to receptor at pH 5.6. Iron in the otherwise weaker binding site of the N-terminal lobe is stabilized, while iron in the relatively stable binding site of the C-terminal lobe is labilized.

Transferrin, in providing iron for the needs of iron-dependent cells, is first seized by a specific receptor on the cell surface, then internalized by the cell into an acidified endosome where iron release takes place, and finally returned to the cell surface where it is freed, depleted of iron but otherwise intact, for another cycle of iron transport (Dautry-Varsat et al., 1983; Klausner et al., 1983). Throughout its journey in the cell transferrin remains attached to its receptor. We have recently reported that, in addition to its long-recognized function of capturing diferric transferrin from the circulation for internalization into an endocytotic vesicle, the transferrin receptor also modulates the kinetics of iron release from transferrin (Bali et al., 1991b). At extracellular pH, 7.4, the receptor impedes the release of iron from diferric transferrin, while at endosomal pH, 5.6, it facilitates release to a suitable acceptor of Fe(III).

The effect of receptor is particularly striking at the iron-binding site in the C-terminal lobe of transferrin, since at endosomal pH iron is released with greater facility from this site than from the much weaker binding site in the N-terminal lobe when diferric transferrin is complexed to its receptor (Bali & Aisen, 1991). However, release of iron from monoferric transferrins at pH 5.6 is faster from the N-terminal site, whether the proteins are bound to receptor or free. This seeming inconsistency in the behavior of monoferric and diferric transferrins prompted us to examine whether site–site interactions, when both sites of transferrin are occupied by metal ions, further modulate the kinetics of iron release. Because simultaneous release of iron from each site of diferric transferrin complicates the independent determination of release rates from the two sites of the protein, we have turned for our studies to mixed-metal transferrins, with Fe(III) at one site of the protein and Co(III) at the other. Cobalt(III) is a substitutionally inert species that remains bound to transferrin during the course of iron release, thereby making it possible to examine the effect of occupancy of one site on iron release from the other whether the proteins are free or complexed to receptor.

Materials and Methods

Human serum transferrin was purchased from Boehringer-Mannheim and purified as previously reported (Aisen et al., 1978). Amersham Corporation supplied ^{59}Fe as $^{59}FeCl_3$. Radiolabeled Fe_N–Tf–Co_C[1] and Co_N–Tf–Fe_C were prepared

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* To whom correspondence should be addressed.
‡ Department of Physiology and Biophysics.
§ Department of Medicine.

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