Protein Science (1996), 5:2506–2513. Cambridge University Press. Printed in the USA.

Significant hydrogen exchange protection in GroEL-bound DHFR is maintained during iterative rounds of substrate cycling

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(RECEIVED July 31, 1996; ACCEPTED September 26, 1996)

Abstract

An unresolved key issue in the mechanism of protein folding assisted by the molecular chaperone GroEL is the nature of the substrate protein bound to the chaperonin at different stages of its reaction cycle. Here we describe the conformational properties of human dihydrofolate reductase (DHFR) bound to GroEL at different stages of its ATP-driven folding reaction, determined by hydrogen exchange labeling and electrospray ionization mass spectrometry. Considerable protection involving about 20 hydrogens is observed in DHFR bound to GroEL in the absence of ATP. Analysis of the line width of peaks in the mass spectra, together with fluorescence quenching and ANS binding studies, suggest that the bound DHFR is partially folded, but contains stable structure in a small region of the polypeptide chain. DHFR rebound to GroEL 3 min after initiating its folding by the addition of MgATP was also examined by hydrogen exchange, fluorescence quenching, and ANS binding. The results indicate that the extent of protection of the substrate protein rebound to GroEL is indistinguishable from that of the initial bound state. Despite this, small differences in the quenching coefficient and ANS binding properties are observed in the rebound state. On the basis of these results, we suggest that GroEL-assisted folding of DHFR occurs by minor structural adjustments to the partially folded substrate protein during iterative cycling, rather than by complete unfolding of this protein substrate on the chaperonin surface.

Keywords: hydrogen exchange; mass spectrometry; molecular chaperones; substrate cycling

Molecular chaperones are believed to protect nascent or unfolded polypeptide chains from aggregation and to assist protein folding in the cell. Their interaction with newly synthesized proteins has been described as a sequential pathway of assisted folding (Langer et al., 1992), in which the chaperonins complete folding by guiding molecules to their ultimate native structures (Jaenicke, 1993; Ellis, 1994; Hartl et al., 1994; Hartl, 1996). One representative of this class of chaperones, the tetradecameric bacterial chaperonin GroEL, has been studied in great detail, and models for its reaction cycle with different combinations of protein substrate, nucleotides, and GroES have been proposed (Jackson et al., 1993; Martin et al., 1993; Todd et al., 1994; Burston et al., 1995; Mayhew et al., 1996; Weissman et al., 1996). Although it is now generally accepted that partially folded molecules bind and fold within the GroEL central cavity (Braig et al., 1993; Chen et al., 1994; Mayhew et al., 1996; Thiyagarajan et al., 1996; Weissman et al., 1996), there is still little information at the molecular level on the mechanism by which GroEL facilitates protein folding. Models exist involving unfoldase activity (Jackson et al., 1993; Zahn et al., 1996), prevention of aggregation by sequestration of intermediates (Buchner et al., 1991), and dissociation of early aggregates (Todd et al., 1994; Weissman et al., 1994; Ranson et al., 1995). A full understanding of the molecular mechanism of chaperonin-assisted folding, therefore, will require information at the molecular level about the nature of intermediates bound to GroEL throughout the entire pathway of GroEL-assisted folding.

In order to address these issues, we have developed a methodology to study hydrogen exchange protection in GroEL-bound proteins using electrospray ionization mass spectrometry (ESI-MS) (Fig. 1) (Robinson et al., 1994). In addition to having significant advantages over NMR methods in terms of the amount of protein sample required (typically 2–3 mg GroEL per complete ESI-MS timecourse), and that exchange is measured directly in the complex without the need to first dissociate and refold the protein (Zahn et al., 1994, 1996), ESI MS has the unique advan-

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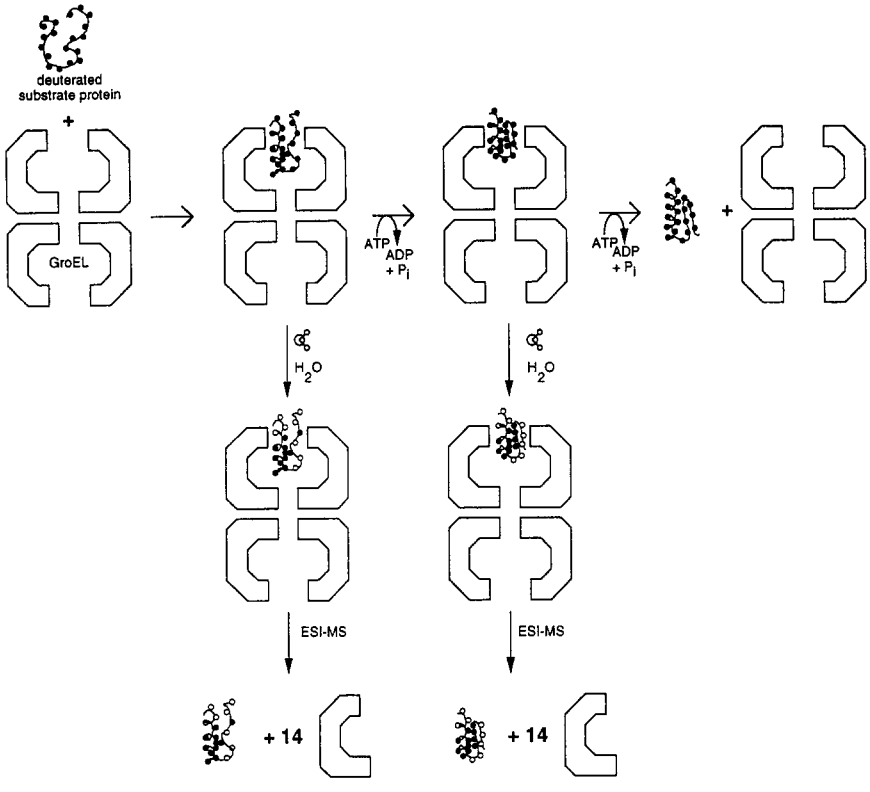

Fig. 1. Schematic diagram of the experiment designed to measure hydrogen exchange in GroEL-bound DHFR by ESI MS at different stages of its ATP-dependent folding reaction. First, all exchangeable sites in DHFR were exchanged for deuterium by incubation of the protein in 6 M guanidine deuterochloride in D_2O solution. A complex was then formed by diluting the protein into a D_2O solution containing GroEL (but lacking nucleotides and GroES) and buffer exchanged into D_2O pD 5.0. Hydrogen exchange was initiated by a further 10-fold dilution of the complex into H_2O. After different periods of time, the sample was introduced into the mass spectrometer. In the gas phase, the GroEL oligomer dissociates into monomers, releasing the substrate protein. The experiment has been designed and tested to ensure that no further hydrogen exchange occurs within the mass spectrometer (Robinson et al., 1994). The rate of hydrogen exchange was directly determined from the mass of the DHFR as a function of the exchange time. In a second experiment, folding of DHFR from its complex with GroEL was initiated in D_2O solution by the addition of MgATP. During this incubation time, DHFR molecules start to fold, but no hydrogen exchange takes place. The reaction was quenched after 3 min by the addition of EDTA (also in D_2O), allowing DHFR molecules to rebind to the chaperone. Hydrogen exchange of these molecules was then initiated by dilution into H_2O and the mass as a function of time after the dilution was measured by ESI MS as described above.

tage that information about the distribution of different populations of molecules can be inferred from analysis of the charge state distribution. In hydrogen exchange experiments, this information can also be extracted from an analysis of the linewidths of peaks in the mass spectrum (Robinson et al., 1994). Using this methodology, we previously observed that the GroEL-bound state of a three-disulfide derivative of bovine α-lactalbumin ([3SS]-BLA, Ewbank & Creighton, 1989; Hayer-Hartl et al., 1994) consists of a distinct population of molecules that are weakly protected from hydrogen exchange, resembling a molten globule state in free solution (Robinson et al., 1994).

In this paper, we describe experiments that extend our previous methodology and that have allowed us to analyze the hydrogen exchange protection of human DHFR, bound to GroEL at two different stages of the chaperonin-assisted folding reaction (Fig. 1). DHFR is an ideal protein for these studies because the native protein has been studied in great detail by NMR (Stockman et al., 1991, 1992) and X-ray (Davies et al., 1990) methods, and much is known about the spontaneous folding pathway of the protein from *Escherichia coli* (Jennings et al., 1993; Jones & Matthews, 1995). By contrast with *E. coli* DHFR (Clark et al., 1996; Rospert et al., 1996), however, several mammalian DHFR species, although they can be refolded reversibly, form a well-defined stable complex with GroEL in the absence of nucleotides (Martin et al., 1991; Viitanen et al., 1991; Mayhew et al., 1996). The protein can then be refolded from the GroEL-bound state by the addition of ATP (Martin et al., 1991). Although GroES is not essential for refolding, in its presence, DHFR folds more efficiently (Martin et al., 1991; Viitanen et al., 1991) to the native structure while it is completely encapsulated by the chaperone machinery (Mayhew et al., 1996).

Results and discussion

The ESI mass spectrum of DHFR introduced into the mass spectrometer from its native conformation at pH 5.0, 20 °C, is shown in Figure 2A. The spectrum exhibits two characteristic features, the first arising from incomplete processing of the recombinant protein, which results in doublets of peaks at each charge state. These correspond in mass to molecules with and without an N-terminal methionine residue (the mass difference is 131 ± 1 Da). The second characteristic feature arises from the bimodal distribution of charge states, the series at higher *m/z* (labeled C and D), which is the major component under these conditions, having a mass (21,990.9 ± 2.4 and 21,865.6 ± 1.9 Da) much larger than that expected from the amino acid sequence (21,452.8 and 21,321.6 Da for species with and without the N-terminal methionine, respectively). The mass of DHFR molecules obtained under denaturing conditions [50% (v/v) acetonitrile, 1% (v/v) formic acid, 50 °C], by contrast (21,452.9 ± 0.8 and 21,321.8 ± 0.7 Da), corresponds closely to the calculated mass, confirming the fidelity of its amino acid sequence. The masses observed for series C and D correspond closely to that expected for DHFR molecules to which the substrate has remained bound, even in the gas phase of the mass spectrometer, the two series corresponding to DHFR molecules (with and without the N-terminal methionine) to which dihydrofolate (used to elute DHFR from the affinity column during its preparation) and an additional glycerol molecule (used to stabilize the preparation) have remained bound (expected mass 21,998.3 and 21,857.1 Da, respectively). The larger deviation from the expected mass for this series arises as a consequence of peak broadening, a phenomenonknown to be associated with ESI MS of noncovalent complexes (Loo, 1995). The preservation of the non-

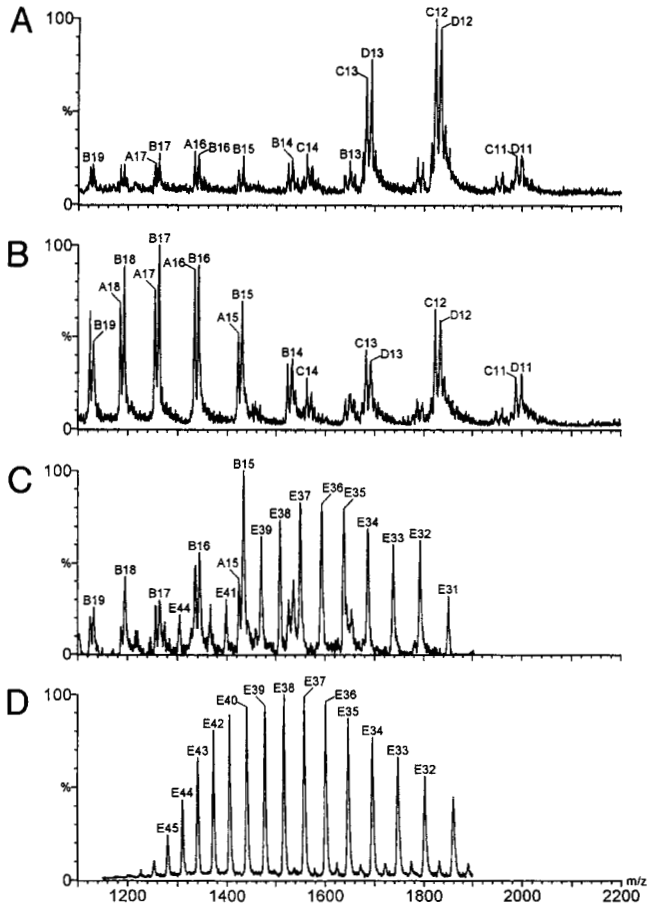

Fig. 2. ESI mass spectra of different conformations of DHFR. **A:** Native DHFR (obtained from H_2O, pH 5.0, 20 °C). **B:** DHFR partially unfolded in H_2O at pH 5.0, 50 °C. **C:** DHFR–GroEL complex (H_2O, pH 5.0, 20 °C). **D:** GroEL alone (H_2O, pH 5.0, 20 °C). In all of the spectra shown, charge state series arising from GroEL are labeled E, those labeled A, B, C, and D arise from DHFR.

covalent DHFR–ligand complex in the gas phase is consistent with the high affinity of DHFR for its substrate (Schweitzer et al., 1989) and substantiates the view from other studies of ligand binding by mass spectrometry, that ionic interactions (which are important in the DHFR active site (Oefner et al., 1988) become favorable in the gas phase (Robinson et al., 1996).

The second charge state series, labeled A and B, appears in the spectrum at a lower *m/z* than that of ligand-bound DHFR and corresponds in mass to that expected of the ligand-free protein (observed masses 21,321.4 ± 2.7 and 21,452.4 ± 3.4 Da). We interpret these series, therefore, as representing a population of about 20% of DHFR molecules that are partially unfolded and unable to bind substrate under these conditions. This view is supported by the extended nature of the charge state series, which covers a much wider range (+19 to +13) than that observed for the series corresponding to the ligand-bound protein. This is further substantiated by the ESI mass spectrum of DHFR obtained from aqueous solution at pH 5.0, 50 °C, in which the more highly charged series (A and B), which corresponds to the more unfolded species, is prominent (Fig. 2B).

The ESI mass spectrum of the GroEL–DHFR complex formed by diluting DHFR denatured in 6 M guanidine hydrochloride into a solution of the chaperonin (see methods) is shown in Figure 2C. Two distinct series of peaks can be seen clearly in the spectrum. The first, arising from DHFR, can be identified readily by its characteristic peak doublets (labeled A and B) and is confirmed as being the ligand-free species by its mass. The species with and without the N-terminal methionine are present in approximately equal proportions, indicating that the presence of the additional methionine does not affect the binding of DHFR to the chaperonin. The second series (labeled E) corresponds in mass to the GroEL monomer (57,197.8 Da), the mass of which is entirely consistent with the revised gene sequence (Horovitz et al., 1993). Given that the conditions of the mass spectrometry were identical to those used to obtain the spectrum of native DHFR (Fig. 2A), information about the conformation of the bound ligand can be immediately drawn from the appearance of the charge state series. Thus, the maximum at +15 and the extended nature of the charge state series are indicative of binding of a relatively unfolded protein. An ESI mass spectrum of GroEL without its protein ligand, also obtained under identical conditions, is shown in Figure 2D. The spectrum is not significantly different to that of GroEL complexed with DHFR, demonstrating, in accord with data from electron microscopy (Braig et al., 1993; Chen et al., 1994), that extensive conformational changes do not occur in the chaperonin upon ligand binding. Interestingly, however, a small amount of GroEL dimers is visible in the spectrum of GroEL in Figure 2D. These peaks (which lie between charge states +31 and +37) are not apparent in the spectrum of the GroEL–DHFR complex, even though the spectra were obtained under identical conditions. This might reflect differences in the nature of the cooperative interactions around and between the GroEL subunits when the substrate binds, in accord with previous observations (Mendoza & Horowitz, 1994; Yifrach & Horovitz, 1996).

To examine the conformation of DHFR folding intermediates bound to GroEL in more detail, the hydrogen exchange properties of the DHFR molecules bound within the GroEL central cavity were analyzed by ESI MS, using the method described (Robinson et al., 1994) and shown in Figure 1. The hydrogen exchange properties of the DHFR bound to GroEL are shown and compared with those of native DHFR (in the presence of bound dihydrofolate) and with those expected for a fully unstructured protein with the sequence of human DHFR, in Figure 3A. As demonstrated previously for [3SS]-BLA (Robinson et al., 1994), it was found that DHFR can only be detected in the mass spectrum if it was bound by GroEL (equimolar mixtures of the two proteins revealed only the presence of GroEL). This, along with gel filtration analysis, confirmed that the hydrogen exchange protection was not attributable to small amounts of DHFR that might have dissociated from the complex prior to its analysis by ESI MS. As judged by ESI MS, about 70 hydrogens are highly protected from exchange in native DHFR after an exchange time of 3 h at pH 5.0, 20 °C. These data are in accord with site-specific measurements of amide exchange by NMR, which have demonstrated that the majority of these slowly exchanging amides are located in secondary structural elements in the native structure (Stockman et al., 1992). By contrast with native DHFR, however, GroEL-bound DHFR is only weakly protected from exchange, only about 15 deuterons remaining after 3 h under these conditions. After an exchange time of 20 h, all of the deuterons have exchanged with the solvent, demonstrating the significance of their protection at the earlier times.

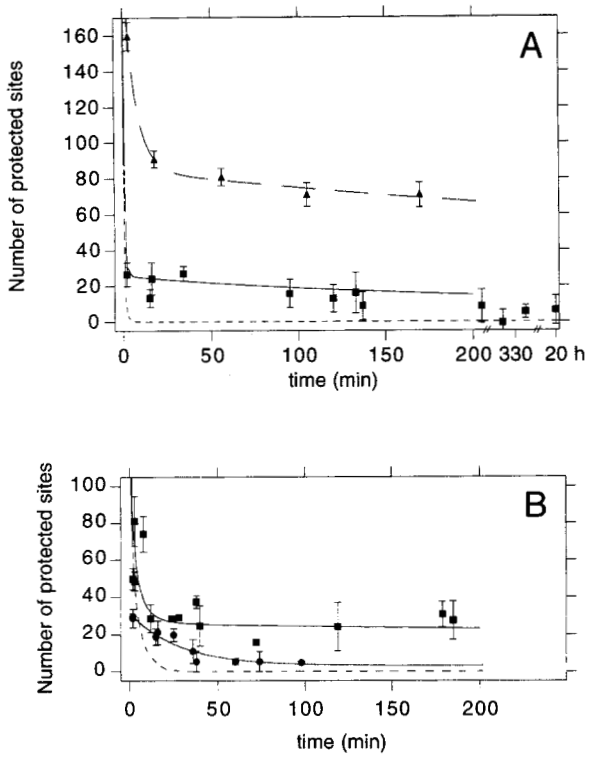

Fig. 3. Hydrogen exchange kinetics of GroEL-bound folding intermediates of DHFR at pH 5.0. **A:** Exchange at 20 °C of (▲) native DHFR (DHF-bound); (■) GroEL-bound DHFR and (– – –) and the curve expected for DHFR in a completely unstructured conformation under these conditions. **B:** Exchange of (■) GroEL-bound DHFR at 4 °C; (●) GroEL-bound [3SS]-BLA at 4 °C (Robinson et al., 1994); and (– – –) the curve expected for DHFR in a completely unstructured conformation at pH 5.0, 4 °C. In (**A**) and (**B**), the curve fitted to the exchange data for GroEL-bound DHFR is simulated based on the binding of a partially folded state of DHFR with a highly protected core involving 26 amides (see text). The protection factors of amides in the core were assumed to be 1,000; all other exchangeable sites were given a protection factor of 1.0. The fits to the other curves are for illustrative purposes only.

To compare the protection observed in GroEL-bound DHFR with that observed previously for GroEL-bound [3SS]-BLA (Robinson et al., 1994), the hydrogen exchange experiment was repeated at 4 °C. The results (Fig. 3B) show a dramatic difference in the degree of protection of the two substrate proteins. Whereas the exchange in GroEL-bound [3SS]-BLA is complete within about 2 h, some 20 deuterons remain in GroEL-bound DHFR after this time. The latter sites do not exchange over the entire time course of the experiment (3 h), demonstrating that they represent sites that are highly protected from hydrogen exchange. Assuming that these sites are amides and that the central cavity of GroEL is solvent filled, the protection factor of these sites must be at least 1,000. If side-chain hydrogen bonds contribute to the observed highly protected sites, their protection factors will exceed 10^4 (Bai et al., 1993; Connelly et al., 1993).

An alternative explanation that is consistent with the data would be the existence of two distinct populations of DHFR bound to GroEL, one population (representing about one third of molecules) being highly protected from exchange, and the second (corresponding to two-thirds of molecules) having structure that is not protective against hydrogen exchange. Although these two models cannot be distinguished easily by NMR methods, they can be distinguished by ESI MS, because the peak widths in the mass spectrum reflect the distribution of species populated (Miranker et al., 1993, 1996; Robinson et al., 1994). Such an analysis was therefore performed on the charge state series labeled A in Figure 2C arising from GroEL-bound DHFR (such an analysis was not possible on series B because substantial overlap occurs between this charge state series and that arising from the GroEL monomer). The mass transformed spectrum of GroEL-bound DHFR, 92 min after the initiation of hydrogen exchange at 4 °C, is shown in Figure 4. The peak is relatively narrow, revealing a distinct population of molecules, more protected than the unfolded state under these conditions, and considerably less protected than the native state of DHFR. More importantly, however, this analysis shows that there are virtually no molecules (<5%) with native-like protection in GroEL-bound DHFR, ruling out the possibility that the highly protected sites arise from a minor population of native-like molecules. The data suggest instead that GroEL-bound DHFR is a distinct partially folded species, containing stable structure in a small region of the polypeptide chain.

It is interesting in this regard that an intermediate formed within 141 ms of the initiation of folding of *E. coli* DHFR (unassisted by GroEL) protects a similar number of amides from hydrogen exchange, their protection factors exceeding 100 after this refolding time (Jennings et al., 1993). These sites are located throughout the eight-stranded β-sheet in DHFR, demonstrating the formation of a native-like topology early in folding (Jennings et al., 1993). The close similarity of the hydrogen exchange properties of the kinetic folding intermediate with those of GroEL-bound DHFR suggests the intriguing possibility that a similar intermediate could be the substrate for GroEL. Indeed, a simulation of the hydrogen exchange kinetics, assuming that amides corresponding to those protected in the early folding intermediate of *E. coli* DHFR are protected in the GroEL-bound state of human DHFR (Fig. 5), and that the protection factor of these amides is 1,000, describes the experi-

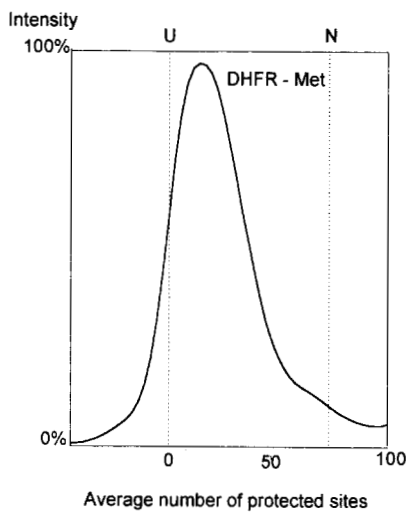

Fig. 4. Analysis of the linewidth of the molecular ion arising from GroEL-bound DHFR, 92 min after the initiation of hydrogen exchange at 20 °C. The charge series A was transformed onto a scale of the average number of protected sites. The ESI mass spectrum was analyzed by maximum entropy methods (Ferridge et al., 1992). Positions of unprotected species (U) and species with native-like protection (N) under the same conditions are indicated.

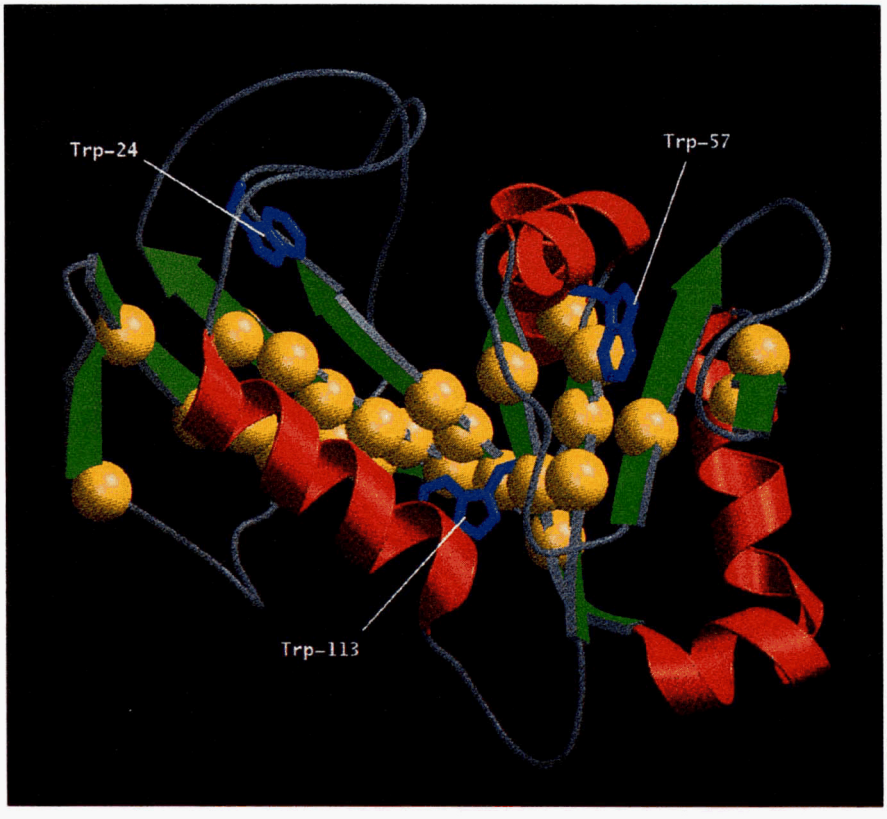

Fig. 5. Molecular graphics representation of the structure of human DHFR (PDB file 2DHF). The 26 amides that are protected from hydrogen exchange within 141 ms of the initiation of folding of the *E. coli* protein (Jones & Matthews, 1995) were identified in the human structure by sequence alignment. The positions of these amides are shown as yellow spheres. The three tryptophan residues are also shown. In native DHFR, the three tryptophan residues are deeply buried in the hydrophobic core of the molecule (Davies et al., 1990), two tryptophan residues (Trp 57 and Trp 113) are completely buried from solvent, and Trp 24 exposes 15 Å^2 to the solvent (the surface area of a fully exposed tryptophan residue is about 120 Å^2 [Kabsch & Sander, 1983]). The figure was produced using the program Raster3D (Bacon & Anderson, 1988; Merritt & Murphy, 1994).

mental data well at both 4 °C and 20 °C (Fig. 3A,B). Furthermore, fluorescence quenching experiments, in which the accessibility of tryptophan residues to iodide ions was measured (Eftink & Ghiron, 1981), indicate that the tryptophan residues in GroEL-bound DHFR (Fig. 5) are relatively inaccessible to the iodide ions, their Stern–Volmer constant (4.2 M^{-1}) approaching that of native DHFR (2.6 M^{-1}) and being markedly different from that (13.1 M^{-1}) of a fully exposed tryptophan residue (determined for the amino acid N-acetyl-Trp-amide) under the same conditions. Thus, despite the fact that GroEL-bound DHFR contains a small stable core that sequesters the three tryptophan residues from solvent, the molecule nevertheless retains many of the characteristics of a molten globule state (the ability to bind ANS, a λ_{max} of tryptophan fluorescence (347 nm) intermediate between that of the native (342 nm) and denatured (356 nm) states, and proteinase sensitivity), consistent with a partially folded state with affinity for GroEL.

Although a contribution to protection by binding of DHFR to GroEL cannot be ruled out, the similarity in both the nature and extent of protection of GroEL-bound DHFR with that of the transient intermediate suggests that protected sites in the former may lie within a native-like core. Although evidence for such a model cannot be obtained directly from the ESI MS experiments described here, independent investigations using NMR methods to detect amide protection site-specifically in GroEL-bound DHFR (M. Goldberg & A. Horwich, pers. comm.) are fully consistent with this view. In the experiments by Goldberg and Horwich, however, the degree of protection observed in GroEL-bound DHFR is lower than that determined by ESI MS. Although the higher stability of GroEL-bound DHFR measured by ESI MS could arise from differences in the experimental conditions, the major difference in the two approaches is that, whereas exchange is monitored indirectly by NMR methods after release and refolding of the substrate protein, exchange by ESI MS is measured directly in the complex. Thus, although it cannot be ruled out that the origin of weakly protected sites in GroEL-bound DHFR observed by NMR might be unrelated to those observed by ESI MS, the body of data described here would suggest that this is not the case.

Complete chaperonin-assisted folding of DHFR to its native state requires several cycles of ATP hydrolysis, which is accompanied by iterative rounds of binding and release of the substrate protein to/from the surface of the GroEL central cavity (Martin et al., 1993). Although ATP hydrolysis is required for GroEL-assisted folding of many proteins, there is little detailed knowledge about the role that substrate cycling plays in the mechanism of GroEL-assisted folding. We have now extended our hydrogen exchange protocol to enable us to examine this issue directly by measuring the hydrogen exchange properties of DHFR rebound to GroEL several minutes after initiating its folding from the GroEL-bound state by the addition of MgATP (Fig. 1). The results (Fig. 6A,B) show the remarkable finding that, at both 4 °C and

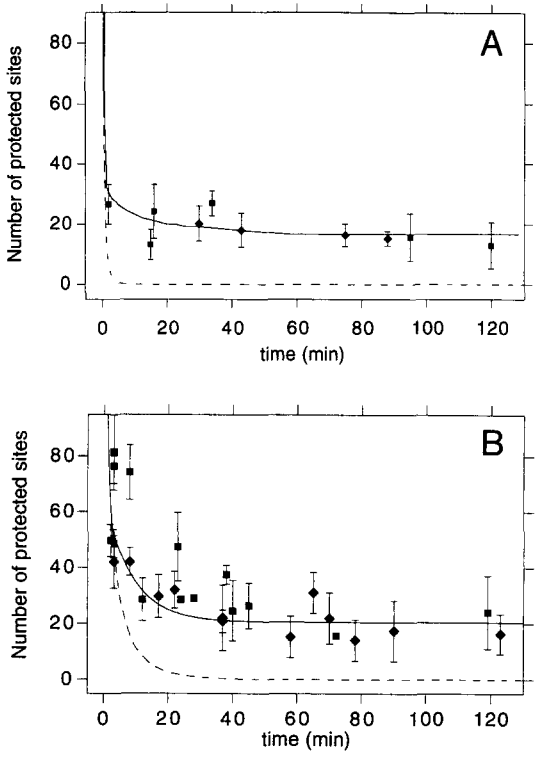

Fig. 6. Hydrogen exchange kinetics of DHFR rebound to GroEL after several rounds of substrate cycling. **A:** Exchange profile of DHFR rebound to GroEL, after initiating the folding of the GroEL-bound protein for 3 min by the addition of ATP (◆). The data are compared with those of DHFR bound to GroEL in the absence of ATP hydrolysis (the initial bound state (■) [taken from Fig. 3A]) and (– – –) the profile expected for exchange in a completely unstructured polypeptide with the sequence of DHFR. The data were acquired at pH 5.0, 20 °C. **B:** Same as (A), but at 4 °C.

20 °C, the hydrogen exchange properties of DHFR rebound to GroEL are indistinguishable from those of the initial bound state. In addition, both the charge-state distribution and the linewidth of the peaks in the spectrum of the rebound protein are indistinguishable from those of the protein bound initially. Despite this, a decrease in the quenching coefficient occurs when DHFR rebinds to GroEL (the Stern–Volmer constant is reduced to a value [2.7 M^{-1}] close to that of the native protein [2.6 M^{-1}]) and the molecule shows a slightly reduced ability to bind ANS (the fluorescence intensity is decreased by 35% relative to that of the initial bound state, but no change in λ_{max} is observed), suggesting that minor structural rearrangements occur upon rebinding to the chaperonin, but that these do not involve substantial changes in hydrogen exchange protection.

Several mechanisms of GroEL-assisted folding have been proposed, ranging from binding of progressively more native-like states during repeated rounds of substrate cycling (Martin et al., 1991; Lilie & Buchner, 1995), to complete unfolding of the substrate protein on the chaperonin surface (Zahn et al., 1996). Our data suggest that GroEL-mediated folding of DHFR does not involve the release of progressively more native-like states from the chaperonin surface as folding proceeds. They also suggest that complete unfolding of DHFR molecules does not occur upon rebinding to the chaperonin. The data suggest instead that molecules that rebind to GroEL either have not folded further, or that they undergo limited unfolding on the GroEL surface, reforming molecules that resemble closely the initial bound state. Given that the half-life for folding of DHFR is about 3 min (Martin et al., 1991) and that the turnover time of GroEL is about 12 s (Todd et al., 1994), the former is unlikely.

A mechanism of GroEL-assisted folding of DHFR involving maintenance of substantial protection on the GroEL surface contrasts with the results obtained when the small protein, barnase, binds to GroEL, in which global unfolding on the chaperonin surface was found to occur (Zahn et al., 1996). Our results suggest, therefore, that the degree of unfolding of a protein substrate on the GroEL surface can vary, presumably depending upon a delicate balance of the rates and pathways of folding, the stability of partially folded states, the topology and size of the native protein, and the affinities of different intermediates for GroEL. At least in the case of chaperonin-assisted folding of DHFR (in the presence of ATP), GroEL appears to play the role of a proofreader, facilitating the completion of folding by the reorganization or limited unfolding of partially folded states during substrate cycling, rather than by reinitiating the entire folding reaction from a fully unfolded (unprotected) state.

Materials and methods

Materials

Ultrapure guanidine hydrochloride was obtained from ICN, other chemicals were from Sigma. Guanidine hydrochloride was deuterated by 10 rounds of recrystallization from D_2O. Human DHFR was expressed and purified from *E. coli* BL21 cells transformed with pKT7HDR (Schweitzer et al., 1989). Expression was induced with 0.5 mM IPTG. Following lysis of the cells, and ammonium sulfate fractionation, DHFR was purified on methotrexate (MTX)-agarose essentially as described (Schweitzer et al., 1989), except that, in our experiments, the DHFR-containing extract was loaded onto the MTX-agarose column in 10 mM potassium phosphate, pH 5.6. The column (2.5 × 10 cm) was equilibrated with 200 mL of the same buffer containing 0.5 M NaCl and then with the same volume of buffer alone. DHFR was eluted from the column with 0.1 M K_2HPO_4 containing 200 μM dihydrofolate. The fractions containing DHFR were pooled, concentrated, and buffer exchanged into 50 mM MOPS, pH 7.4, containing 100 mM NaCl and 5 mM $MgCl_2$ using a Sephacryl S100 column. The resulting protein was concentrated and stored at −20 °C in 5% (v/v) glycerol. GroEL was overexpressed and purified as described (Martin et al., 1991) and stored as a concentrated stock solution (17 mg/mL in MOPS, pH 7.2) at −80 °C. The protein was diluted before its use to a concentration of ca. 3 mg/mL.

Sample preparation

To form a complex with GroEL, DHFR that had been denatured in 6 M guanidine hydrochloride (10 min, 20 °C) was diluted 25-fold into 0.1 mM DTT in H_2O, pH 7.0 containing GroEL (a 1.2-fold molar excess of DHFR over $GroEL_{14}$ was used). After 10 min at 20 °C, samples were concentrated and buffer-exchanged into H_2O/formic acid, pH 5.0, by ultrafiltration (Centricon 100, Amicon). The final sample volume was typically 50–80 μL containing 2.5–5.0 nmol of complex. Samples were kept on ice and used within 24 h.

To produce samples of DHFR rebound to GroEL after several rounds of iterative cycling, a GroEL–DHFR complex was formed

as described above. Folding of DHFR was then initiated by the addition of 1 mM ATP/2 mM magnesium acetate/50 mM KCl and was allowed to proceed for 3 min at pH 7.0, 20 °C. Under these conditions, complete refolding of GroEL-bound DHFR occurs with a half-time of approximately 3 min (Martin et al., 1991). The reaction was then quenched by the addition of 3 mM EDTA. DHFR molecules that remained complexed to GroEL after this time were then repurified and buffer exchanged into the H_2O/formic acid buffer, pH 5.0, as described above. The sample was kept on ice and used within 1 h. The integrity and stability of all complexes used for ESI-MS was verified by size-exclusion chromatography, using a miniature S-300 column. Column fractions were analyzed by SDS-PAGE following standard procedures (Groß et al., 1994).

For the preparation of GroEL–DHFR complexes in which all exchangeable sites in the substrate protein had been exchanged with deuterium, the above procedures were repeated, except that all solvents were replaced with D_2O and guanidine deuterochloride (see above) was used.

Mass spectrometry

All mass spectra were recorded on Platform Electrospray Mass Spectrometer (Micromass) operating in the positive ion mode with a capillary voltage typically at 2.8 kV and a counter electrode voltage of 0.4 kV. The instrument was operated without source heating and with cooling of the nebulizer gas. Samples (10 μL) were introduced via a Rheodyne injector and pumped with a solvent delivery module (Michrom. Bioresources) at a flow rate of 10 μL/min.

For exchange measurements into H_2O solution, the electrospray interface was equilibrated in 10% D_2O/90% H_2O to match the isotopic composition of the exchange buffer and to prevent mixing of solvent during introduction of samples into the mass spectrometer. All mass spectra were calibrated against hen egg white lysozyme and represent the average of 10 scans with minimal smoothing. The mass differences measured between the fully proteated protein and partially deuterated GroEL-bound DHFR were corrected for the 10% residual D_2O.

The hydrogen exchange properties of the native state of DHFR were measured by 10-fold dilution into D_2O pD 5.0. This avoided the need to unfold the protein to enable deuteration of all exchangeable sites and to refold the protein prior to its analysis. In these experiments, the electrospray interface was equilibrated overnight in 90% D_2O/10% H_2O to match the exchange conditions in the protein solution and the mass difference was again corrected for the isotopic content of the solvent.

Measurements of hydrogen exchange by mass spectrometry

In the majority of experiments, hydrogen exchange was monitored by diluting initially deuterated samples of DHFR 10-fold into H_2O solution at pH 5.0. For native DHFR, however, exchange was monitored by diluting the proteated sample directly into D_2O solution, pD 5.0. Mass spectra were acquired at different times after the dilution; these were obtained as described above. The number of deuterons (or hydrogens) remaining in the sample as a function of the exchange time was monitored and the data corrected for the 10% residual D_2O (or H_2O) in the exchange buffer. The exchange profile expected for a completely unstructured protein with the sequence of DHFR was calculated from the amino acid sequence of the protein, taking into consideration the 157 exchangeable side-chain hydrogens, the temperature and pH of the solution, and whether exchange is measured into D_2O or H_2O solution (Bai et al., 1993; Connelly et al., 1993).

Fluorescence spectroscopy

The quenching constant (K_{sv}) of native DHFR, of GroEL-bound DHFR complexes at different stages of chaperonin-facilitated folding (see above), and of the amino acid derivative N-acetyl-Trp-amide was determined using a Perkin Elmer LS50B spectrofluorimeter. Tryptophan fluorescence intensity (at the λ_{max}) as a function of the concentration of KI added to the buffer solution was determined using an excitation wavelength of 295 nm in 1 cm cells. All experiments were performed in 10 mM sodium acetate buffer, pH 5.0, 20 °C. The ionic strength of the samples was kept constant throughout the experiment by the addition of KCl. The final protein concentration of DHFR in each experiment was 512 nM and the concentration of KI was varied, in 50 or 100 mM increments, from 0 to 0.6 M. The data were plotted in the form I_0/I versus the concentration of iodide added (where I_0 is the fluorescence intensity in the absence of iodide, and I the fluorescence intensity in the presence of iodide). The data were fitted to a straight line plot, the gradient of which revealed the Stern–Volmer constant (Eftink & Ghiron, 1981).

Binding of the hydrophobic dye ANS was monitored using excitation at 390 nm and monitoring fluorescence from 420 to 520 nm. The dye was added in 40-fold molar excess over DHFR (0.6 μM to 24 μM) from a 100-fold concentrated stock solution.

Acknowledgments

This is a contribution from the Oxford Centre for Molecular Sciences, which is supported by the BBSRC, EPSRC, and MRC. The project is also supported by a grant from the NIH (F.U.H.). M.G. received long-term fellowships from EMBO (1993–1994) and FEBS (1994–1996). M.M. was supported in part by a HFSPO postdoctoral fellowship. C.V.R. and S.E.R. thank the Royal Society for support. Help and advice from Drs. Chris Dobson, Robin Aplin, and Robin Procter are gratefully acknowledged. We thank Matt Goldberg and Art Horwich for sharing their data on exchange protection in GroEL-bound DHFR with us prior to their publication.

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