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Protein fragments as probes in the study of protein folding mechanisms: Differential effects of dihydrofolate reductase fragments on the refolding of the intact protein

JOHN G. HALL AND CARL FRIEDEN

Department of Biochemistry and Molecular Biophysics, Box 8094, Division of Biology and Biomedical Sciences, Washington University School of Medicine, Saint Louis, MO 63110

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ABSTRACT We describe an approach for investigating the protein folding process, using protein fragments as inhibitory probes of the refolding protein. The refolding of *Escherichia coli* dihydrofolate reductase (EC 1.5.1.3), reversibly unfolded in 7 M urea, was monitored by the reappearance of enzyme activity after diluting the unfolded enzyme into low urea concentrations (≤2 M) in the presence of substrates. Of eight protein fragments produced by limited proteolysis of the 159-residue enzyme, three isolated peptides—Ser-49/Glu-90, Ile-91/Glu-154, and Gln-102/Glu-154—were evaluated for their effects on the recovery of the refolding protein's enzymatic activity. By this criterion, 13 μM peptide Gln-102/Glu-154 inhibits the refolding of 0.015 μM enzyme by ≈80%, while the related peptide, Ile-91/Glu-154, and peptide Ser-49/Glu-90 at the same concentration inhibit the recoverable activity of the refolding enzyme by ≤20%. None of these three peptides has any significant effect on the activity of the folded enzyme. Our results indicate that peptides may inhibit refolding differentially and that these effects may be extremely sensitive to fragment sequence and composition. We suggest that peptide specificity in the inhibition of protein folding may be exploited as a structural probe of protein folding mechanisms.

A major barrier in the determination of protein folding mechanisms has been the difficulty in characterizing the structural nature of intermediate states in the folding pathway. The reason for this is the great cooperativity of the folding process and, as a consequence, the low equilibrium concentrations of any intermediates. Various experimental approaches to characterizing intermediates in folding pathways have included spectroscopic techniques such as circular dichroism (1–4) and NMR (5, 6), amide proton labeling techniques coupled with two-dimensional NMR methods (7, 8), and the trapping of disulfide intermediates accumulating during the reoxidation of a disulfide-containing protein, such as bovine pancreatic trypsin inhibitor (9).

A different experimental approach is to use fragments of proteins, produced by limited proteolytic cleavage or by chemical synthesis, as probes of the possible nature of intermediates in the folding process. For example, mapping the differential accessibility of susceptible peptide bonds to proteases at different stages in folding has been used to try to reconstruct general folding patterns (10). In other work, protein fragments have been combined to try to understand how the different moieties of a protein complement to produce the final secondary and tertiary structure of the folded protein. The kinetic and structural investigations of the complementation of S-peptide and S-protein of ribonuclease (2, 11–14) and the complementation of various combinations of overlapping fragments of staphylococcal nuclease (15, 16) are models of this approach.

Although protein fragments have been employed to investigate the nature of the interactions between elements of secondary structure, protein fragments or peptides apparently have not been used specifically to probe the folding of the intact protein. We thought that this might be a potentially useful complementary approach to the study of protein folding mechanisms. Any secondary structure present in peptides or fragments of a protein might serve as initiation sites around which the protein may fold to an intermediate state and become trapped, thereby retarding folding to the native conformation. Alternatively, such initiation sites might accelerate the folding process. These effects could be varied and quite specific. That such a technique is feasible is suggested by observations that under favorable conditions even small peptides (<15 residues) may exhibit some secondary structure in aqueous solution. For example, the C- and S-peptides of ribonuclease A, encompassing the NH_2-terminal α-helix, exhibit significant helical structure in solution at low temperatures (17–19). Even in cases where peptides exhibit no or very limited secondary structure in aqueous solution, the latent structure of a protein fragment may be realized upon interaction with other protein fragments or with another protein. This is the case in the interaction of S-protein and S-peptide of ribonuclease (2) as well as for the binding of calmodulin-binding peptide to calmodulin, which induces α-helix formation in the peptide as it binds (20). In some cases, peptide secondary structure can be induced via concentration-dependent effects of the peptide itself (21). Experiments with bovine growth hormone have shown that a peptide containing about 50% helical structure in solution can interfere with the self-association of the refolding intact protein (21, 22).

Here we describe the differential effects of several peptide fragments, derived from the limited proteolytic cleavage of dihydrofolate reductase (DHFR; 5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase, EC 1.5.1.3), on the attainment of the folded, enzymatically active, state. Our results suggest that this experimental approach may be useful in the characterization of intermediates in the folding process.

MATERIALS AND METHODS

Materials. Urea solutions used in refolding experiments were made with 8 M stock solutions, prepared by dissolving ultrapure urea (Schwarz/Mann) in deionized, distilled water. Urea solutions were deionized by adding 1 g of mixed-bed ion-exchange resin (Bio-Rad AG 501-X8) per 150 g of urea and stirring at room temperature for 1 hr. This solution was then filtered through a 0.2-μm Millipore filter and stored at −70°C until used. [Bis(2-hydroxyethyl)amino]tris(hydroxy-

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Abbreviation: DHFR, dihydrofolate reductase.

methyl)methane (Bistris), reduced nicotinamide adenine dinucleotide phosphate (NADPH), and methotrexate were obtained from Sigma. Staphylococcal V8 protease was obtained from Boehringer Mannheim. Dihydrofolate was prepared from folic acid (23) and stored at −70°C in 5 mM HCl containing 50 mM 2-mercaptoethanol. All other chemicals were reagent grade.

Protein Purification. DHFR was isolated from *Escherichia coli* strain HB101 containing the plasmid pTY1, a derivative of pBR322 containing a 1-kilobase insert coding for *E. coli* DHFR. Protein purification followed standard procedures (24, 25). To rid enzyme preparations of residual folate left from the methotrexate affinity-chromatography step, the purified enzyme was brought to 90% saturation with solid ammonium sulfate. The precipitate was pelleted and redissolved in 50 mM potassium phosphate/2.5 mM dithioerythritol/1 mM disodium ethylenediaminetetraacetate (Na_2-EDTA)/3 M guanidine hydrochloride, pH 7.0 at 4°C, and dialyzed exhaustively against the same buffer. The enzyme was renatured by dialysis against the same buffer without guanidine hydrochloride. The enzyme was then centrifuged at 10,000 × *g* for 30 min to remove any denatured enzyme. The soluble, renatured enzyme was decanted, brought to 90% saturation with solid ammonium sulfate, and stored at −20°C. The specific enzyme activity (26) of these preparations was identical to that of the enzyme freshly eluted from the final chromatographic step. Observations of single bands on $NaDodSO_4$/polyacrylamide gels established the purity of enzyme preparations. Protein concentration was determined both by active-site titration with methotrexate (27) and by absorbance at 280 nm, based on an extinction coefficient of $3.11 \times 10^4\ M^{-1} \cdot cm^{-1}$ (27).

Preparation of DHFR Fragments. Purified wild-type DHFR (50–100 mg) was dialyzed against 0.1 M ammonium bicarbonate/2.5 mM dithioerythritol/1 mM Na_2EDTA/4 M urea, pH 7.8 at 0°C. The dialyzed enzyme solution was brought to 3.2 mg/ml in the same buffer and digested with 0.5% (wt/wt) staphylococcal V8 protease for 2 hr at 37°C. V8 protease digestion is only partial under these conditions, with hydrolysis specifically occurring on the COOH-terminal side of glutamic residues (28). Digestion was stopped by cooling the digest in an ice bath and subsequently adding 10 mM diisopropyl fluorophosphate. After incubation at room temperature for 1 hr, the DHFR digest was dialyzed three times against 60 volumes of 0.1 M ammonium formate/0.02% thiodiglycol, pH 3.0 at 4°C with formic acid. The digest was then lyophilized and stored at −70°C. Dialysis and lyophilization had no significant effects on the $NaDodSO_4$ electrophoretic profile of the DHFR digest.

FPLC Isolation of DHFR Fragments. The dialyzed and lyophilized DHFR digest was redissolved in 25 mM sodium formate/0.02% thiodiglycol/8 M urea, adjusted to pH 4.0 with formic acid. A volume containing 25 mg was loaded onto a Pharmacia Mono S HR16/10 FPLC (fast protein liquid chromatography) column equilibrated with the above buffer. DHFR fragments were eluted with 640 ml of a 0–0.22 M sodium chloride gradient at a flow rate of 8 ml/min. Monitoring the eluates at 280 nm indicated eight major peptide peaks. Fractions from each of these peaks were pooled, dialyzed exhaustively against 0.1 M ammonium formate/0.04% thiodiglycol at pH 3.0, lyophilized, and stored at −70°C. Aliquots of peak fraction pools were lyophilized and stored separately for later analysis.

DHFR Refolding Assays. The time course of the reappearance of enzymatic activity upon dilution of the unfolded enzyme in 7 M urea to refolding conditions (≤2 M urea) was used to monitor the refolding of DHFR. In standard assays, progress curves were recorded over 9 min at 10°C with a Hewlett–Packard 8452A diode-array spectrophotometer at several analytical (338–342 nm) and reference (406–410 nm) wavelengths. Assays were conducted in a thermally equilibrated microcuvette containing 0.35 ml of 0.05 M Bistris buffer/250 μM Na_2EDTA/1 mM dithioerythritol/0–3 M urea/50 μM NADPH/40 μM dihydrofolate, pH 7.2. Equilibrium unfolding and refolding experiments, conducted under these assay conditions with a SPEX spectrofluorometer at 290-nm excitation and 376-nm emission wavelengths, indicated complete reversibility of the folding transition.

The effects of individual DHFR peptides on the refolding enzyme were tested by equilibrating an aliquot of DHFR fragment in the cuvette, containing buffer, urea (usually 2 M), and substrates. An aliquot of unfolded enzyme then was added to start the assay. Control assays lacked added peptide. Final peptide and refolding enzyme concentrations were approximately 13 μM and 0.015 μM, respectively, giving a ratio of peptide to enzyme of about 900:1. Data were stored on a Hewlett–Packard microcomputer interfaced with the spectrophotometer and were transferred later to a VAX mainframe computer for subsequent analyses.

RESULTS AND DISCUSSION

Sequence Identities of Isolated DHFR Peptides. Three V8 protease fragments of DHFR were isolated in sufficient quantities to allow characterization of their effects on the refolding of the intact protein. The identities of these peptides were inferred from determination of both the sequence of the six NH_2-terminal residues by Edman degradation and the amino acid composition of the peptide. Peptide Ile-91/Glu-154 consists of 64 residues, Ile-91 through Glu-154 in the intact protein, and has a molecular mass of 7426 Da. Peptide Gln-102/Glu-154 is 53 residues long and has a molecular mass of 6251 Da. It has the same sequence as Ile-91/Glu-154 except for the truncation of the NH_2 terminus by 11 residues. The third isolated peptide, Ser-49/Glu-90, is 42 residues long, Ser-49 through Glu-90 in the intact protein, and at 4449 Da is somewhat smaller than the other two peptides. On the basis of $NaDodSO_4$ and native polyacrylamide gel electrophoresis, NH_2-terminal sequence analysis, and amino acid compositions, peptides Ile-91/Glu-154 and Ser-49/Glu-90 were determined to be essentially pure, whereas the peptide Gln-102/Glu-154 preparation was contaminated with peptide Ile-91/Glu-154 by ≈8%. A V8 protease digest of 100 mg of enzyme typically yielded 3–7 mg of these three purified fragments. Other peptide peaks obtained by FPLC ion-exchange chromatography either were contaminated with other peptides or gave yields too low to allow further characterization.

Differential Effects of Peptides upon DHFR Refolding. The progress of DHFR refolding, as monitored by the reappearance of enzymatic activity over the course of the assay, characteristically shows a long lag phase (>2 min) as the enzyme refolds in 2 M urea at 10°C. The three peptides have strikingly different effects on the final extent of enzymatic activity recovered under refolding conditions. At peptide/enzyme ratios of ≈900:1 (e.g., 13 μM:0.015 μM), peptide Gln-102/Glu-154 lowers the final activity by about 80%, whereas peptides Ile-91/Glu-154 and Ser-49/Glu-90 inhibit the refolding enzyme by only about 20% (Table 1). Typical progress curves for refolding assays with and without added peptide Gln-102/Glu-154 are shown in Fig. 1. The progress curves are qualitatively similar: both reach a steady state after ≈300 sec and the progress curves superimpose closely for all three peptides when the progress curves of refolding assays with added peptide are rescaled to that of the refolding assays without peptide (data not shown). These observations indicate that the effects of peptide Gln-102/Glu-154 and of the other peptides on the refolding protein are complete within the 5- to 10-sec dead time of manual mixing. The differential effects of these three peptides are observed only under refolding conditions; the peptides have no appreciable effect

Table 1. Differential inhibition of DHFR refolding by DHFR peptides, as monitored by the recovery of enzymatic activity

Assay conditions*	Peptide	% of control†	95% C.L.‡
7 M → 2 M urea	Ile-91/Glu-154	80.9 (6)	54.0–97.5
	Gln-102/Glu-154	22.8 (6)	17.0–29.2
	Ser-49/Glu-90	83.7 (6)	67.8–94.9
2 M → 2 M urea	Ile-91/Glu-154	101.8 (6)	99.9–108.6
	Gln-102/Glu-154	100.1 (6)	96.8–105.2
	Ser-49/Glu-90	95.9 (3)	81.4–100.1

*Unfolded (at 7 M urea) or folded (at 2 M urea) DHFR was diluted to 0.015 μM into the assay solution, previously equilibrated in the presence or absence of added peptide (13 μM) at 10°C for 10 min at a final urea concentration of 2 M.

†Peptide effects on enzyme activity are expressed as percentages of control assays without added peptide. Sample sizes are given in parentheses.

‡Means and confidence limits (C.L.) were calculated for arcsin transformations of percentage data (29).

on the activity of the folded enzyme with our conditions (Table 1).

The differential effects of these three DHFR fragments on the refolding enzyme do not depend on the initial presence or absence of substrates. In assays where the enzyme is refolded with peptide before substrates are added to start the assay, the results are the same as in the usual assay procedure where the substrates and peptide are equilibrated in the spectrophotometer cell before the unfolded enzyme is added.

DHFR refolds faster when refolded at lower urea concentrations (30). Under these conditions peptide Gln-102/Glu-154 inhibits refolding of DHFR less well. For example, refolding DHFR from 6 M to 0.6 M urea in the presence of 32 μM peptide Gln-102/Glu-154 inhibits the recovery of enzyme activity by about 65%, while refolding to 2 M and 3 M (at the beginning and the middle of the unfolding transition) inhibits the refolding by greater than 90% and 95%, respectively.

The inhibition of the recovery of enzymatic activity by the refolding enzyme is dependent upon the concentration of peptide Gln-102/Glu-154 but not upon the concentrations of peptide Ile-91/Glu-154 or Ser-49/Glu-90 (Fig. 2). Inhibition by Gln-102/Glu-154 shows a saturation effect with 50%

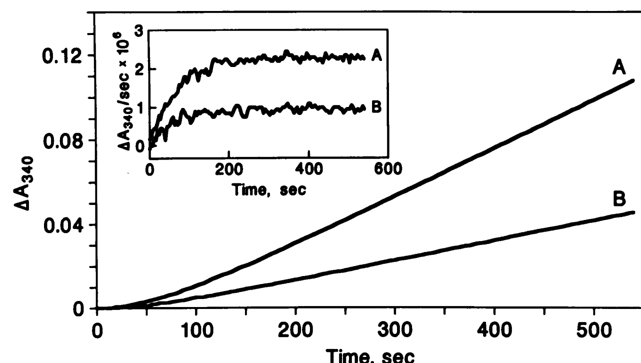

FIG. 1. Inhibitory effect of 6 μM peptide Gln-102/Glu-154 upon the recovery of enzymatic activity of DHFR (0.015 μM), refolded from 7 M to 2 M urea. Curve A, refolding assay without added peptide; curve B, refolding assay with 6 μM peptide Gln-102/Glu-154 equilibrated at 2 M urea in the presence of the substrates NADPH (50 μM) and dihydrofolate (40 μM). (*Inset*) Data were replotted to depict the recovery of reaction velocity of refolding DHFR in the absence (profile A) or presence (profile B) of 6 μM peptide Gln-102/Glu-154. Reaction velocities were calculated for successive pairs of absorbance data values, which were collected at 4-sec intervals. Replot data points represent the running averages of five such calculations. ΔA_{340} indicates absorbance change calculated for the mean of three signal wavelengths (338, 340, and 342 nm) minus the mean of three reference wavelengths (406, 408, and 410 nm).

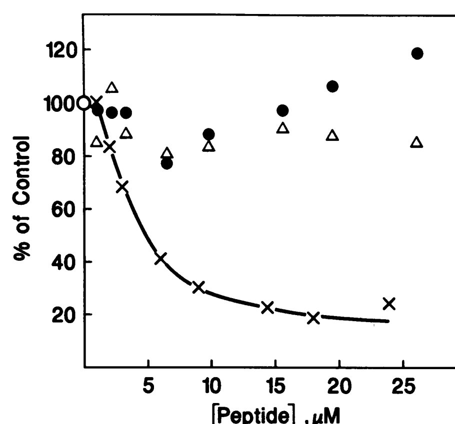

FIG. 2. Concentration dependence of peptide inhibition of the recovery of DHFR enzymatic activity for peptides Ile-91/Glu-154 (●), Gln-102/Glu-154 (×), and Ser-49/Glu-90 (△).

inhibition of the refolding enzyme occurring at a peptide concentration of about 5 μM.

Several considerations tend to rule out the possibility that our observations are a consequence of general aggregation phenomena. (*i*) The inhibitory effect is highly specific: both a peptide related in sequence to Gln-102/Glu-154 (i.e., Ile-91/Glu-154) and an unrelated one (Ser-49/Glu-90) show relatively little inhibitory effect. (*ii*) The phenomenon is only apparent with the refolding enzyme; the folded enzyme is generally little affected by the presence of any of the peptides. (*iii*) Although peptide Ile-91/Glu-154 has a greater tendency to aggregate than Gln-102/Glu-154, it exhibits a relatively small inhibitory effect on the refolding enzyme.

Potential Modes for Peptide Inhibition of Refolding DHFR. Observations with Gln-102/Glu-154 on refolding DHFR imply the possibility that a stable peptide–enzyme complex forms under these refolding conditions. Examination of progress curves provides no evidence that substrates can reverse the inhibition by peptide Gln-102/Glu-154. Enzymatic activity increases to a steady-state velocity after 4–5 min with or without added peptide. If substrates were capable of pulling enzyme out of an initial misfolded enzyme–peptide complex, one would expect to see a longer lag phase in the activity curve. These observations are supported by an experiment in which 50 μM NADPH was added to a mixture of 4.6 μM peptide Gln-102/Glu-154 and 0.015 μM enzyme, which had been refolded in the presence of the peptide at 2 M urea and equilibrated for 30 min. This mixture of refolded enzyme and peptide was incubated with NADPH for an additional 60 min in an attempt to "pull" active enzyme out of this hypothetical enzyme–peptide complex. The second substrate, dihydrofolate (40 μM), was then added to start the assay. This experiment, however, provided no evidence for a "rescue" of active enzyme from the peptide-inhibited state by NADPH. This would suggest that the binding of peptide Gln-102/Glu-154 to refolding enzyme is not significantly reversible by substrate.

The specific mechanism for the inhibitory effect on the refolding enzyme that we observe with peptide Gln-102/Glu-154 is not known at this time. Gln-102/Glu-154 may possess significant secondary structure in solution that could serve as initiation sites around which the protein might fold incorrectly. However, the presence of 2 M urea in most of our refolding assays would tend to destabilize any residual structure that would be present in our peptides. Alternatively, the refolding protein might induce formation of structure in the interacting peptide that in turn may lead to protein misfolding around this structure. High ratios of peptide Gln-102/Glu-154 (i.e., 200- to 1000-fold molar excess) to refolding DHFR are required to achieve an inhibitory effect.

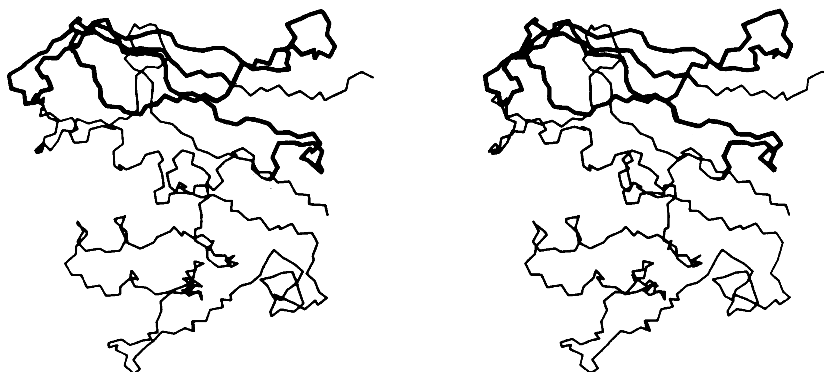

FIG. 3. Stereo diagram of the polypeptide backbone of *E. coli* DHFR, based on the crystal structure of the binary complex with methotrexate. To indicate the relationship between the sequence corresponding to peptide Gln-102/Glu-154 and the rest of the protein, sequence Gln-102 through Glu-154 is represented by bold line. Note the looping of residues 8–13 into contacts with sequence Gln-102/Glu-154.

If peptide inhibition is due to competition between peptide structure (either intrinsic or induced) and corresponding structural elements in the intact protein during refolding, then this result would be expected because of the high relative concentrations of peptide necessary to overcome its entropic disadvantage.

Proposing a structural explanation for the differential effects of these three DHFR fragments on the refolding enzyme is clearly speculative. However, an examination of the crystal structure of the binary complex of methotrexate and the enzyme, isolated from *E. coli* strain MB1428 (31), suggests an interesting way in which peptide Gln-102/Glu-154 might interact with stretches of the protein during folding (Fig. 3). The sequence of our enzyme differs from MB1428 DHFR at four positions: Asn-37, Asp-87, Asp-142, and Glu-154, instead of Asp-37, Asn-87, Asn-142, and Lys-154, respectively. Assuming that these substitutions exert minimal effects on the overall structure of our enzyme, we see that peptide Gln-102/Glu-154 includes most of the three COOH-terminal β-strands present in the intact enzyme. Residues 113–125—comprising part of one of these β-strands (βF) and part of an extended loop structure—form a long loop that wraps around the hydrophobic base of a finger-like loop (residues 8–13) that follows the NH_2-terminal β-strand in the sequence, βA (31). This loop-within-a-loop structure is an unusual feature of this protein. The NH_2-terminal residues 1–18 and the sequence corresponding to peptide Gln-102/Glu-154 form extensive contacts in the native protein via β-sheet formation and this loop–loop interaction. Together, they form an almost autonomous structural unit that is physically separated from the rest of the protein by the major structural feature of DHFR, a pronounced central cleft that cuts across an entire face of the enzyme. By competing with the corresponding sequence of the intact enzyme during refolding, peptide Gln-102/Glu-154 might interact with the NH_2-terminal moiety of this "domain"—particularly the finger-like loop—and prevent formation of the final, folded conformation.

The loss of the effect of Gln-102/Glu-154 by the addition of 11 residues to the NH_2 terminus is also of interest. One possible explanation is that intrapeptide interactions (e.g., hydrophobic interactions) between this 11-residue sequence and the rest of the peptide might be responsible for blocking the ability of the rest of the peptide to interact specifically with the refolding protein.

In summary, we have demonstrated marked differential inhibitory effects of DHFR fragments on the refolding protein, as detected by the recovery of the enzymatic activity of the refolding protein. Our observations with three DHFR fragments imply that fairly specific interactions between peptide and enzyme and/or between peptide moieties are required for the inhibition of the refolding pathway. A small change in sequence appears sufficient to alter these effects significantly. This experimental approach involving the use of protein fragments does not appear to have been used before for specifically probing the folding mechanism of an intact protein. Our results demonstrate that this approach is a potentially powerful one, since different synthesized peptides or protein fragments of widely varying sequence could be used to pinpoint what areas of a protein are important in the folding process. In some instances, the peptide–protein complexes that are formed might be stable and subsequently isolated. This would permit the eventual physical characterization of the misfolded enzyme.

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