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Refolding of *Escherichia coli* dihydrofolate reductase: Sequential formation of substrate binding sites

(protein folding/ligand binding)

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ABSTRACT **The kinetics of refolding of *Escherichia coli* dihydrofolate reductase (EC 1.5.1.3) have been examined upon dilution of unfolded enzyme in 4.5 M urea to 1.29 M urea in 0.02 M phosphate buffer (pH 7.2) at 10°C. Changes in the intrinsic protein fluorescence on refolding are characterized by four phases. Based on changes in the amplitudes of these phases, as a consequence of quenching of the intrinsic fluorescence by ligands, it is possible to determine the step at which a ligand binds during the refolding process. The results show that either NADP or NADPH binds to the last species formed in a sequence involving three intermediates between the unfolded and native states. Dihydrofolate, on the other hand, binds during the formation of the second observed intermediate. When refolding is performed in the presence of methotrexate, an analogue of dihydrofolate, and NADPH, NADPH binds, as determined from changes in NADPH fluorescence, to the third observed intermediate rather than the last (fourth) species formed. Measurements of the recovery of enzymatic activity during refolding suggest that dihydrofolate also induces NADPH binding prior to the final observed folding phase. These results define more closely the formation of structural domains during the folding of dihydrofolate reductase.**

Dihydrofolate reductase (DHF reductase; EC 1.5.1.3) has been the subject of intense study with respect to its three-dimensional structure (1–3), its kinetic mechanism (4–7), and its folding properties (8–12). As pointed out by Touchette *et al.* (8), the *Escherichia coli* enzyme is a particularly good model for folding studies and Matthews and coworkers have characterized the wild-type enzyme as well as a number of site-directed mutants using urea as the denaturant (9–12). In addition to the wealth of available information about the *E. coli* enzyme, another distinction of this small (M_r = 17,680) globular protein is that it requires two ligands for enzymatic activity. The purpose of the present report shows that the binding sites for DHF and NADP(H) form sequentially and that the formation of these binding sites is associated with fluorescence changes observed during the folding process.

MATERIALS AND METHODS

Materials. Urea solutions used in refolding experiments were made from 10 M stock solutions, prepared by dissolving fresh ultrapure urea (Schwarz/Mann) in 0.02 M potassium phosphate buffer/0.1 mM disodium ethylenediaminetetraacetate (Na_2EDTA)/1 mM dithioerythritol, pH 7.2, at 10°C. This solution was filtered through a 0.2-μm Millipore filter and its concentration was determined with an Abbe refractometer (Bausch and Lomb). Urea solutions were stored at −20°C until used. Reduced nicotinamide adenine dinucleotide phosphate (NADPH), nicotinamide adenine dinucleotide phosphate (NADP), and methotrexate were obtained from Sigma. DHF was prepared from folic acid (13) and stored at −70°C in 5 mM HCl containing 50 mM 2-mercaptoethanol. All other chemicals were reagent grade.

Protein Purification. DHF reductase was isolated from *E. coli* strain HB101 containing the plasmid pTY1, a derivative of pBR322 containing a 2-kilobase insert coding for *E. coli* enzyme. Protein purification followed standard procedures (14, 15). 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 mM dithioerythritol/2 mM Na_2EDTA/3 M guanidine hydrochloride, pH 7.0, at 4°C and then 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, and protein concentration was determined by active-site titration with methotrexate (16). These preparations were used within 10 days.

Folding Studies. The time course of fluorescence changes was measured using an Applied Photophysics (Surrey, U.K.) stopped-flow spectrophotometer in the fluorescence mode with a path length of either 0.2 or 1.0 cm. Results were similar for either. All experiments were performed in 0.02 M phosphate buffer at 10°C, pH 7.2, containing 1 mM dithioerythritol and 0.1 mM EDTA. The drive syringes were of different volumes such that there was a 3.5-fold dilution of the urea solution containing the enzyme, the smaller syringe containing the enzyme and urea and the larger syringe containing buffer or buffer plus ligand(s). Experiments in which different ligands were compared were performed on the same day using exactly the same conditions of photomultiplier voltage and light intensity, but results from experiments performed on different days were quite reproducible. The initial concentration of urea was 4.5 M and that of the enzyme was 0.51 mg/ml. After mixing, the urea concentration was 1.29 M and the enzyme concentration was 0.15 mg/ml (8.5 μM). For measurements using the intrinsic fluorescence of the protein, the excitation wavelength was 285 nm, slit widths were set to 2 mm, and fluorescence was observed using a 305-nm cutoff filter. In the case of NADPH a 305-nm cutoff filter in conjunction with a UG1 band pass filter was used. Fluorescence data were usually collected using the dual time base mode of the instrument with 500 points being collected over the first few seconds and another 500 points collected over the next 250 sec. When changes in fluorescence of NADPH were measured, the excitation wavelength was 340 nm using the 1-cm cell and 385-nm cutoff filter. When activity measurements were made, the 1-cm absorbance cell was used at a wavelength of 340 nm.

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Abbreviation: DHF, dihydrofolate.

Data Fitting. Most of the data to be analyzed were the average of two or three individual experiments. These data could be analyzed either by using the software programs of the stopped-flow instrument or by sending the data to a microVAX computer. Exponential fits were obtained using programs provided by Applied Photophysics. In fitting, the first 30–50 msec after mixing were ignored because of mixing artifacts. Essentially all fluorescence kinetic data were best fit by a four-exponential equation. This analysis provides results in terms of four steps, each associated with an amplitude change for the given step. Attempts to fit the data with a three-exponential equation led to large systematic deviations in the residuals and thus gave poor fits. Data transferred to the microVAX could be further analyzed using kinetic simulation (17) and fitting (18) programs.

RESULTS

Changes in the Intrinsic Protein Fluorescence on Refolding. As observed by Touchette *et al.* (8), fluorescence changes on refolding DHF reductase from high to low urea concentrations first show an increase and then a decrease in fluorescence. As indicated above, the data are best fit with a sum of four exponentials representing at least four phases in refolding. Such data in the absence or presence of DHF or NADP are shown in Fig. 1. The initial concentration of urea, 4.5 M, is reasonably far above the midpoint of the urea inactivation curve (≈3.5 M under these conditions) and the final concentration (1.29 M) is well below that midpoint. Essentially similar data were obtained when the initial urea concentration was 6 M and the final concentration was 1.71 M (data not shown). Experiments that measure the total change in fluorescence as a function of urea concentration indicate that the changes observed in the kinetic measurements are approximately the same as expected for the total change for unfolded to folded forms of the enzyme under these conditions. The same observation was made by Touchette *et al.* (8) using absorbance changes. The rate constants and corresponding amplitudes for the enzyme in the absence of ligand are shown in the first line of Table 1.

It has been previously observed that when ligands such as NADP or DHF are added to native enzyme, there is a decrease in the fluorescence (4). This decrease is over and above that observed on refolding and occurs in at least two phases, a rapid phase (over in <5 msec) as a consequence of binding and then a second slower change reflecting a conformational change (19). The fluorescence changes observed in the absence of urea are also observed in the presence of 1.29 M urea under the conditions of these experiments indicating that enzyme at this urea concentration is quite similar to native enzyme (data not shown). As a consequence, substrate binding during refolding can be monitored by determining whether there are larger amplitude changes in any of the phases detected by fluorescence. Such a change in amplitude should reflect the ability of the substrate to bind to a given species since much of the ligand binding process is fast relative to the folding process. In these experiments, the ligand is added with the buffer used for diluting the urea containing the enzyme and thus is present during the refolding process.

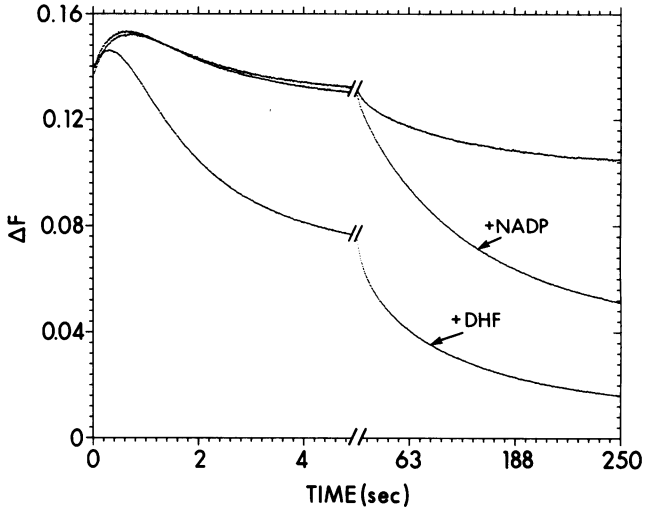

FIG. 1. Fluorescence changes observed on the refolding of enzyme from 4.5 M urea to 1.29 M urea. The data are shown as obtained from a dual time base on the stopped-flow apparatus. The curves labeled NADP and DHF were performed with each ligand added at the start of refolding process. The enzyme concentration was 8.5 μM and the experiments were performed in 0.02 M phosphate buffer (pH 7.2) at 10°C containing 1 mM dithioerythritol and 0.1 mM EDTA.

Fig. 1 shows the results using NADP or DHF and the results are tabulated in Table 1. With NADP, the results are what might be expected if the ligand binds in the final stage of refolding—i.e., the rate constants and amplitudes are quite similar to the control for the first three phases, but the amplitude of the final phase of fluorescence decrease is over 3-fold larger than for the control. As noted above, this larger decrease is a consequence of NADP binding. Similar results are observed using NADPH provided that a 355-nm band pass filter (UG1) is included before the fluorescence photomultiplier tube.

Analysis of similar data measuring the extent of the total change in the final amplitude as a function of NADP concentration yields a dissociation constant of about 15 μM, a value almost identical to that obtained by observing the quenching of enzyme fluorescence as a function of NADP concentration at 1.29 M urea (data not shown).

Fig. 1 and Table 1 also show the effect of DHF as the added ligand. In this case, the major change is observed in the amplitude of the second phase for which the decrease is 2.2-fold larger than the control. These results indicate that DHF is binding at an early step in the refolding process. Experiments as a function of DHF concentration show that the larger observed amplitude of this phase does not change at several different DHF concentrations, indicating that the dissociation constant for DHF binding is less than a few micromolar. It will be noted from Table 1 that the amplitudes of the two slower phases are also larger. This is probably a consequence of the slow DHF-induced conformational change that has been previously observed (19). It should be noted that Table 1 shows that the rate constants for the

Table 1. Rate constants and fluorescence amplitudes on refolding from 4.5 M urea to 1.29 M urea

Addition	A_1, V	k_1, sec^{-1}	A_2, V	k_2, sec^{-1}	A_3, V	k_3, sec^{-1}	A_4, V	k_4, sec^{-1}
Control	+0.49	2.1	−0.49	0.82	−0.086	0.12	−0.25	0.01
100 μM NADP	+0.44	2.3	−0.46	0.76	−0.09	0.09	−0.84	0.009
30 μM DHF	+0.56	2.6	−1.1	0.74	−0.24	0.09	−0.48	0.0095
100 μM NADP + 30 μM DHF	+0.53	2.7	−1.07	0.74	−0.24	0.078	−0.69	0.013

Experimental conditions were 10°C in 0.02 M phosphate buffer, pH 7.2/1 mM dithioerythritol/0.1 mM EDTA. Amplitude changes are in volts (−5 to +5 V full scale) with positive values reflecting an increase in fluorescence.

refolding process are essentially unchanged under the different conditions. When DHF and NADP are added at the same time, the amplitudes for the second and third phases change as expected for DHF alone, whereas that of the last phase changes somewhat less than expected for NADP alone, probably because fluorescence quenching of the native enzyme by DHF and by NADP is not additive. Methotrexate, an analogue of DHF, affected the fluorescence amplitude of an early phase and thus also binds to the early species.

Changes in the NADPH Fluorescence on Refolding. When experiments using protein fluorescence are performed using NADPH, the results are similar to those using NADP in that NADPH affects only the last phase of refolding. As will be discussed later, activity measurements imply that in the presence of DHF, NADPH binds to an earlier form of the refolding enzyme. Since the enzyme would catalyze the reaction, it is not possible to examine the binding of NADPH in the presence of DHF. Experiments have been performed using methotrexate as an analogue of DHF, but in the presence of NADPH and methotrexate, results are difficult to interpret because the quenching by methotrexate is so large it tends to obscure possible earlier changes due to NADPH binding. However, another way to measure NADPH binding is to monitor fluorescence changes in the NADPH itself rather than the protein. When NADPH is added to enzyme at the time refolding starts, a single phase of fluorescence enhancement with a rate constant of 0.007 sec^{-1} is observed. This is consistent with the rate constant obtained from the change in amplitude of the final phase of the refolding process under these conditions (Table 1). When this experiment is performed in the presence of methotrexate, a very different result is seen. In this case, there is a fluorescence decrease occurring in two phases with rate constants of 0.16 and 0.021 sec^{-1}, respectively. No fluorescence changes are observed under these conditions when methotrexate is used in the absence of NADPH and, as indicated above, methotrexate binds rapidly to a species formed early in the refolding. These results suggest that in the presence of methotrexate (and presumably DHF) NADPH can bind to the penultimate species in the refolding process. These results are consistent with activity recovery results described below. It should be emphasized that in the presence of methotrexate, the fluorescence of the bound NADPH decreases, whereas in its absence the fluorescence increases. This result may be related to the conformation of the bound NADPH in the presence or absence of methotrexate.

Activity Recovery on Refolding. Fig. 2 (curve B) shows the recovery of enzymatic activity during refolding from 4.5 M to 1.29 M urea. In this experiment both substrates, DHF and NADPH, are added at the beginning of the refolding process and the absorbance change at 340 nm, due to substrate depletion, is monitored. As expected, for the refolding experiment there is a lag of several seconds before substrate depletion begins. Also shown in Fig. 2 is the substrate depletion curve of the native enzyme at 1.29 M urea under the same conditions of substrate concentration. Not only is the enzyme active at 1.29 M urea but also it is slightly more active than enzyme in the absence of urea (data not shown). Curve C of Fig. 2 will be discussed below.

DISCUSSION

The simplest mechanism to explain the phases observed in the refolding process, as measured by changes in the intrinsic protein fluorescence, is the sequential mechanism shown in Scheme I.

$$U \xrightarrow{k_1} I1 \xrightarrow{k_2} I2 \xrightarrow{k_3} I3 \xrightarrow{k_4} N$$

Scheme I

This mechanism is somewhat simpler than that proposed by Touchette *et al.* (8) and also does not include their slowest phase as observed by absorbance changes at 293 nm. This phase [designated τ_1 by Touchette *et al.* (8)] does not appear to be necessary to explain recovery of active enzyme (see below). Using the mechanism of Scheme I, the changes in amplitudes observed upon addition of NADP or NADPH to the refolding enzyme show that these ligands bind to only the final species, designated N. DHF, on the other hand, binds early in the refolding process, presumably during the conversion of the I1 species to the I2 since the amplitude represented by I1 → I2 is increased when DHF is added at the start of refolding. Table 1 shows, in fact, that the amplitudes of the remaining two phases are also increased. As indicated earlier, this is probably a consequence of slow conformational changes induced by DHF binding.

Scheme I can, therefore, be expanded to include the binding steps as shown in Scheme II.

$$\begin{array}{l} U \rightarrow I1 \rightarrow I2 \rightarrow I3 \rightarrow N \\ \qquad\quad + \text{DHF} \\ \qquad\quad I2\text{-DHF} \rightarrow I3\text{-DHF} \rightarrow N\text{-DHF} \end{array}$$

Scheme II

The dissociation constant for DHF binding to the native enzyme in 1.29 M urea under the same experimental conditions as the refolding experiments (i.e., phosphate buffer, 10°C, pH 7.2) is 0.7 μM (data not shown). The experimental conditions use 6–8 μM enzyme and 30 μM DHF. With these concentrations, the enzyme would be at least 80% saturated with DHF even if the dissociation constant were several micromolar. As discussed earlier, different DHF concentrations do not affect amplitudes or rate constants. Hence, it is likely that the dissociation constant to the early formed

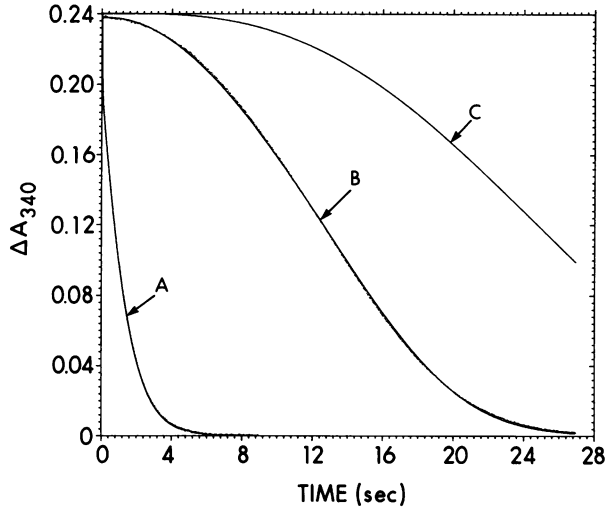

FIG. 2. Activity of native DHF reductase observed on refolding. Stopped-flow experiments were performed by measuring absorbance changes at 340 nm using 30 μM DHF and 20 μM NADPH. Curve A shows substrate disappearance in 1.29 M urea. Curve B is the activity recovery on dilution from 4.5 M to 1.29 M urea. Superimposed on the experimental data for curve A is the computer-simulated curve for a kinetic mechanism describing the progress curves for the enzyme. Using the same kinetic parameters found for curve A but starting with the unfolded enzyme and the rate constants for folding given in line 1 of Table 1 and assuming that activity is recovered with the final phase of folding gives curve C, which does not describe the real data (curve B). The simulated curve fitting the real data for activity recovery during refolding (superimposed on curve B) assumes that NADPH binds to an earlier intermediate as described in the text. Other experimental conditions are described in the legend to Fig. 1.

species is less than micromolar. Thus under the conditions of these experiments the predominant pathway of folding in the presence of 30 μM DHF is with DHF bound to all of the intermediate species.

Although NADP appears to bind to the final (N) species in the presence or absence of DHF or methotrexate, the results with NADPH differ. In the absence of methotrexate, NADPH binds to the N form as does NADP, but in the presence of methotrexate, NADPH appears to bind to species occurring during the formation of the last species rather than to the last species itself. (It should be noted, however, that the lack of a change in amplitude does not prove that a ligand is not binding but rather that if it does bind there is no effect on the protein fluorescence.) Fig. 2 shows the recovery of enzymatic activity on refolding in the presence of substrates (curve B). The kinetic mechanism for describing enzymatic activity is complex (4, 5) with the rate-limiting step for the reaction being the dissociation of tetrahydrofolate (THF) from an abortive E-THF-NADPH complex. The kinetic data can be simulated using a somewhat simpler mechanism, and results of the simulation can be superimposed on the real data with kinetic parameters used to describe substrate depletion in 1.29 M urea (curve A) also used in the simulation of the activity recovery.

Simulation of the activity in conjunction with the rate constants for the refolding shows that activity recovers much more rapidly then expected if associated only with the final phase (k = 0.01 sec^{-1}). Thus, Fig. 2 (curve C) shows the recovery expected if the activity were to recover with the last phase of the folding process. The lag is clearly too long. Rather, the activity appears to recover with a rate-limiting step of about 0.06 sec^{-1}. The binding of NADPH to an earlier step in the refolding process has already been shown by the experiment in the presence of methotrexate, and the faster recovery of activity here is consistent with the idea that DHF binding at an early phase leads to earlier NADPH binding. One can conclude from these experiments that in the absence of DHF or methotrexate, NADP(H) binds only to the N species but that in the presence of these ligands during refolding NADPH binds during the formation of the N species and this species has enzymatic activity.

It is, of course, of interest to examine the structure of the native protein relative to the sequential binding of the substrates during refolding. The refined crystal structure of the binary complex of methotrexate with *E. coli* DHF reductase has been determined by Bolin *et al.* (1). In general, methotrexate is bound in a cavity with van der Waals contacts primarily with amino acid residues in the first third of the polypeptide chain. Indeed, the fact that the residues forming the methotrexate (or DHF) site are primarily in the first third of the protein may be important in the *in vivo* folding of the protein. The pteridine ring and the *p*-aminobenzoyl group are in a hydrophobic pocket, whereas the glutamyl carboxyl groups interact strongly with the guanidinium group of Arg-57. The importance of hydrophobic residues for methotrexate binding has been examined by Singh and Benkovic (20) using site-directed mutants. Although the structure of the ternary complex of the *E. coli* enzyme with methotrexate and NADPH has not been determined, that for the *Lactobacillus casei* enzyme is available (1) and is probably similar to the *E. coli* structure. In contrast to the pocket into which methotrexate fits, NADPH binds in an extended configuration with the adenine ring binding on the surface in a shallow hydrophobic cleft while the nicotinamide portion projects into a hydrophobic pocket, a part of which is formed by the pyrazine ring of the bound methotrexate.

In terms of the model proposed in Scheme **II**, the formation of the framework for binding DHF into its pocket probably occurs during the second fluorescence phase (represented by k_2 in Scheme **I**). Garvey *et al.* (11) using site-directed mutants imply that the earliest phase represented by the fluorescence increase is the formation of a hydrophobic cluster protecting Trp-74 from the solvent. The region of the protein involved in this cluster, however, appears more related to the necessary rearrangements for binding NADP(H) on the surface in the last phase of the refolding process rather than the earlier methotrexate binding. The question of what the latter phases of folding represent will need more examination. There have been a number of folding studies on site-directed mutants carried out by Matthews and coworkers (9–12) and their results should be reexamined in view of the sequential formation of binding sites demonstrated here.

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