Reversible dissociation and unfolding of the dimeric protein thymidylate synthase

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Abstract

Conditions for in vitro unfolding and refolding of dimeric thymidylate synthase from *Lactobacillus casei* were found. Ultraviolet difference and circular dichroism spectra showed that the enzyme was completely unfolded at concentrations of urea over 5.5 M. As measured by restoration of enzyme activity, refolding was accomplished when 0.5 M potassium chloride was included in the refolding mixture. Recombination of subunits from catalytically inactive mutant homodimers to form an active hybrid dimer was achieved under these unfolding–refolding conditions, demonstrating a monomer to dimer association step.

Keywords: dimerization; folding; oligomerization; thymidylate synthase

Thymidylate synthase (TS) is a dimer of two identical, 35-kDa subunits (Kinemage 1) that catalyzes the conversion of deoxyuridine monophosphate (dUMP) and 5,10-methylenetetrahydrofolate (CH₂H₄folate) to dTMP and 7,8-dihydrofolate (H₂folate). TS provides the only de novo pathway to deoxythymidine monophosphate (dTMP) production and has been studied extensively from the standpoints of structure, function, and inhibition (Santi & Danenberg, 1984). The crystal structure, which has been determined to 2.3 Å resolution for *Lactobacillus casei* TS (Hardy et al., 1987; Perry et al., 1990; Finer-Moore, unpubl.), shows that the monomers form dimer contacts primarily between two, five-stranded beta sheets that maintain a unique +28° dihedral angle between strand directions (Kinemage 4). Three of these strands form a beta kink that creates part of the active site pocket of the second monomer. Further, two active site arginine residues (178' and 179') are donated from the opposing monomer and coordinate with the phosphate moiety of the substrate (Kinemage 3). Thus, the dimeric structure of TS is essential for the function of the enzyme.

In order to study interactions at the dimer interface of TS, we sought conditions that would allow the separation, unfolding, and reassembly of subunits. To date,

there is no evidence that the dimer can be dissociated and reformed. In the present work, we show that TS can be denatured to unfolded monomers in urea and subsequently refolded to its catalytically active native state.

Results and discussion

The concentration of urea required to unfold TS was determined using UV and CD spectroscopies. The difference in environment of tryptophan residues between native and unfolded protein was monitored by the UV absorbance change at 294 nm (Fig. 1). Three zones were apparent along the equilibrium unfolding transition: (1) a range below 3.5 M urea where spectral changes were not observed, (2) a sharp transition between 3.5 M and 5.5 M denaturant, and (3) a range above 5.5 M urea where no further change in absorbance was detected. The ΔG_{H_2O} calculated for this equilibrium unfolding transition at 0 M urea was 19.1 kcal mol⁻¹ (see Materials and methods). To ascertain whether secondary structure was lost at high urea concentration, the CD spectrum of TS equilibrated in 8.0 M urea was compared with that of native protein (Fig. 2). Without urea, a strong signal at 222 nm characteristic of α -helical secondary structure ($\theta_{222} = -19,203$ deg-cm² dmol⁻¹) was observed. In 0.8 M urea, the concentration present in the refolding mixture, the molar ellipticity was $-23,600$ deg-cm² dmol⁻¹ (spectrum not shown). The spectrum of TS equilibrated in 8.0 M urea

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Fig. 1. Equilibrium unfolding of TS in urea monitored by the difference in tryptophan absorbance between unfolded and native protein at 294 nm. Measurements were made at room temperature in folding buffer containing various urea concentrations. The solid line indicates the fit to a two-state model where a native dimer and unfolded monomer are present at equilibrium (see Materials and methods).

Fig. 2. Circular dichroism spectra of native TS in folding buffer (●) and TS in folding buffer plus 8.0 M urea (■). Data below 215 nm were not recorded for the denatured sample due to the high absorbance of urea.

showed no appreciable secondary structure ($\theta_{222} = -2,306$ deg-cm² dmol⁻¹). Thus, TS appears to be completely unfolded at urea concentrations above 5.5 M.

Refolding of 8.0 M urea-denatured TS was monitored by the recovery of enzymatic activity upon removal of denaturant (Table 1). In preliminary experiments, no recovery of activity was observed after extensive dialysis of urea from the solution of inactivated protein. However, 15% of the activity was regained upon a 10-fold, rapid dilution of the denatured protein into buffer. Other workers have observed that additives such as substrates, glycerol (Lee & Timasheff, 1977), or KCl (Hermann et al., 1981) may enhance refolding of proteins into their native forms. Thus, refolding of urea-denatured TS was performed by rapid dilution in the presence of the putative

Table 1. Conditions for reversible unfolding-refolding of TS

Condition ^a	[Urea], M, initial	[Urea], M, final	% Recovery
Control	0	0	100
Dialysis ^b	8.0	0	ND ^c
No additive	8.0	0.8	15
1 mM dUMP	8.0	0.8	32
5% Glycerol	8.0	0.8	41
0.5 M KCl	8.0	0.8	69
0.5 M KCl, 1 mM dUMP	8.0	0.8	61
0.5 M KCl, 5% glycerol	8.0	0.8	66
0.5 M KCl, 5% glycerol 1 mM dUMP	8.0	0.8	64

^a Unfolding-refolding experiments were performed in 20 mM KHPO₄, pH 7.0, 0.1 mM EDTA, 1 mM DTT at pH 7.0 and 4 °C plus the indicated additive. The protein was allowed to unfold at the indicated urea concentration for 4 h and refold for at least 2 h.

^b The unfolded protein solution (100 μ L) was equilibrated in 1 L of buffer containing 20 mM KHPO₄, pH 7.0, 0.1 mM EDTA, 1.0 mM DTT at 4 °C for at least 3 h. The dialysis was repeated three times.

^c None detected.

stabilizing agents glycerol and KCl, and the substrate dUMP. Neither 1.0 mM dUMP nor 5% glycerol gave a significant improvement in recovery of activity. However, with 0.5 M KCl present in the folding buffer, 69% of the enzyme activity was recovered. A study of the effect of KCl concentration in phosphate buffer (Fig. 3) shows that optimal activity was regained when refolding was performed in 0.5 M KCl. Combinations of dUMP and glycerol with KCl in the refolding mix did not enhance the recovery of enzymatic activity.

A time course for unfolding and refolding was measured to obtain an estimate of the endpoints of the reac-

Fig. 3. Effect of KCl concentration on specific activity when present in the assay mixture (●) and in the refolding mixture (■).

tions. For the time course of unfolding, aliquots of TS in phosphate buffer and 8.0 M urea were removed at various times and initial velocities were determined upon a 100-fold dilution in the standard assay buffer (Fig. 4). A 97% decrease in activity was observed within 5 min. The 3% residual activity, which remained constant up to 1 h, was probably due to refolding in the assay buffer during initial velocity measurements. The time course of refolding was measured by unfolding TS in phosphate buffer and 8.0 M urea for 1 h followed by refolding in phosphate buffer containing 0.5 M KCl. Aliquots assayed for activity at various times showed that refolding continues over at least 35 min and is complete after approximately 1 h (Fig. 4).

Because residual KCl and urea from unfolding and refolding mixtures were present in the standard assay buffer, the direct effect of these components on enzymatic activity was determined. Concentrations of KCl up to 200 mM had little effect on TS activity when added to the assay cuvette (Fig. 3). Further increases in salt concentration showed small decreases in activity with 77% activity remaining at 750 mM KCl. When present in the assay cuvette, urea concentrations up to 100 mM had no effect on the initial velocity of the reaction (Fig. 5). Reduced activity was observed at concentrations of 1.0 M urea and higher. Under optimum refolding conditions, the assay cuvette contained 50 mM KCl and 80 mM urea; thus, the above results show that regain of enzymatic activity upon refolding was not the result of enhanced dTMP production by either urea or salt. When TS was equilibrated in either 50 mM or 100 mM urea for 1 h prior to measurement of activity, the specific activity was reduced by 30% (Fig. 5). This 30% reduction in activity is consistent with the result that, even in the presence of 0.5 M KCl, only ~70% of the enzymatic activity was recovered upon refolding. This decrease in activity may be the result of a small conformational change that is not detectable by ei-

Fig. 4. Specific activity as a function of time monitoring the refolding reaction from 8.0 M to 0.8 M urea in folding buffer (■) and the unfolding reaction in phosphate buffer plus 8.0 M urea (●).

Fig. 5. Activity of TS with varying concentrations of urea in the assay cuvette (solid bars). The shaded bars indicate activity measurements of TS after pre-equilibration of TS in the assay buffer at the specified urea concentration for 1 h at room temperature. The enzymatic reaction was initiated with the addition of dUMP.

ther CD or UV spectroscopies (see above). Alternatively, the protein may incur chemical damage upon prolonged exposure to denaturant.

Previously, Freidkin and Donovan (1972) reported that refolding of the 7.0 M urea-denatured *Escherichia coli* TS dimer was a unimolecular process and concluded that unfolding was not accompanied by dissociation into monomeric species. In order to prove that the folding reaction includes a monomer-dimer association step, a catalytically active hybrid dimer was formed from two inactive mutant homodimers. Previously, we have shown that the Cys 198/Ser and Arg 178/Phe TS mutants are individually catalytically inactive (Climie et al., 1990). However, coexpression of these two mutant proteins in the same *E. coli* host cell provides a catalytically active heterodimer in which Arg 178 of the Cys 198/Ser subunit is contributed across the dimer interface to the active site of the Arg 178/Phe subunit (Kinemage 3; unpubl.). Purified preparations of either mutant treated under conditions for reversible unfolding-refolding described above did not result in gain of activity upon refolding (Table 2).

Table 2. Activities of homodimeric and heterodimeric Cys 198/Ser and Arg 179/Phe mutants after unfolding and refolding^a

Sample	Specific activity
Wild type	2.0
Cys 198/Ser	ND ^b
Arg 179/Phe	ND
Cys 198/Ser; Arg 179/Phe	0.5

^a All protein samples were unfolded and refolded in 20 mM K₂HPO₄, pH 7.0, 0.5 M KCl, 0.1 mM EDTA, and 1 mM DTT at 4 °C.

^b None detected.

However, unfolding and refolding of an equimolar mixture of these mutants produced a hybrid protein that attained 25% activity compared to the wild type control (Table 2). The mutant hybrid represents the activity expected upon refolding where a 1:1:2 ratio of homodimer:homodimer:hybrid is present and one active site per hybrid dimer is competent. The formation of a catalytically active heterodimer containing monomers from two inactive mutant homodimers definitively shows that dissociated subunits refold and reassemble into a kinetically competent dimer.

Having found conditions for the reversible folding of structured dimers from unfolded monomers of TS, both the folding and oligomerization processes can be examined. Future experiments will also involve optimizing the refolding conditions in order to approach 100% recovery of enzymatic activity.

Materials and methods

Recombinant wild-type *L. casei* TS was prepared from pKPTS (Pinter et al., 1988). The Cys 198/Ser and Arg 178/Phe TS mutants were prepared from pSCTS9 as described (Climie & Santi, 1990) and purified by phosphocellulose (Wataya et al., 1977) and hydroxyapatite chromatography (Santi et al., 1990). All preparations were homogeneous by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Unfolding buffer contained 20 mM KHPO₄, pH 7.0, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1.0 mM dithiothreitol (DTT), and 8.0 M urea with or without additive (KCl, 5% glycerol, or 1.0 mM dUMP). TS (0.3 mg/mL in 100 μ L) was kept in unfolding buffer at 4 °C for at least 1 h. Refolding was initiated by the addition of 900 μ L of 20 mM KHPO₄, pH 7.0, 0.1 mM EDTA, 1.0 mM DTT (phosphate buffer), and either 0.5 M KCl, 5% glycerol, or 1.0 mM dUMP to 100 μ L of the unfolding solution. Unless otherwise specified, protein was allowed to refold for at least 2 h at 4 °C.

Enzymatic activity was monitored by spectrophotometric assay of the conversion of CH₂H₄folate to H₂folate under previously described conditions (Pogolotti et al., 1986). Assay solutions contained 40 μ M dUMP, 100 μ M CH₂H₄folate, and between 0.1 and 0.5 μ M enzyme. Protein concentration was determined either by Coomassie blue staining (Read & Northcote, 1981) or spectrophotometrically using an extinction coefficient of 107,000 M⁻¹ cm⁻¹ at 278 nm (Santi et al., 1974). A unit of activity is defined as the amount of enzyme necessary to produce 1 μ M dTMP per minute.

The urea-induced unfolding transition of TS was measured by the change in environment of buried tryptophan residues upon exposure to solvent. The difference in absorption between native TS and protein titrated with urea was recorded at 294 nm on a Hewlett Packard 8452A diode array spectrophotometer, using the tandem cell technique

(Herskovitz, 1967). Equilibrium measurements were made in 20 mM KHPO₄, pH 7.0, 0.5 M KCl, 0.1 mM EDTA, 1 mM DTT (folding buffer), and varying urea concentration after all optical changes were complete. The protein concentration was 2.4 μ M in each sample. A two-state model, whereby a native dimer (N) and unfolded monomer (U), was assumed. These data were converted to the apparent fraction of unfolded form by the equation

$$F_{app} = (\epsilon_{obs} - \epsilon_N) / (\epsilon_U - \epsilon_N),$$

where ϵ_{obs} refers to the extinction coefficient observed at a particular urea concentration and ϵ_N and ϵ_U are the extinction coefficients of native and unfolded protein, respectively. The Gibbs free energy was calculated from

$$F_{app} = ((K^2 + 8K[P_{tot}])^{1/2} - K) / 4[P_{tot}],$$

where K is the equilibrium constant between N and $2U$. In accordance with Schellman (1978), a linear dependence of ΔG on urea concentration was assumed.

CD spectra were measured on a Jasco J-500A Spectropolarimeter. Samples contained 0.3 mg/mL protein in 20 mM KHPO₄, pH 7.0, 0.5 M KCl, 0.1 mM EDTA, 1 mM DTT, and either 0, 0.8, or 8.0 M urea. Samples were loaded in a 1-mm quartz cuvette, and the molar ellipticity was calculated at 222 nm.

CH₂H₄folate was prepared by a reported method (Bruce & Santi, 1982) and dUMP was purchased from Sigma. Ultra pure urea was obtained from Boehringer Mannheim. All other chemicals were obtained from commercial sources and were the highest grade available.

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