FLUORESCENCE STUDY OF GUANIDINE HYDROCHLORIDE DENATURATION OF THYMIDYLATE SYNTHETASE[1]

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Summary: The denaturation of thymidylate synthetase by guanidine hydrochloride has been studied using both the intrinsic fluorescence of the protein, and the polarization of the 1-dimethyl aminonaphthalene 5-sulfonyl conjugate of the protein. The polarization of the conjugate shows two transitions. The first transition, complete by 2.3 M guanidine, involves swelling or elongation of the protein; the second, complete by 5.5 M guanidine, is associated with unfolding of the protein. The Stokes' shift of the intrinsic protein fluorescence reflects a transition which is complete by 5.0 M guanidine hydrochloride.

Thymidylate synthetase (methylene tetrahydrofolate: deoxyuridine-5'-monophosphate C-methyl transferase EC 2.1.1.b) catalyzes the conversion of deoxyuridylate to thymidylate, with the concomitant reaction of 5,10-methylene tetrahydrofolate to form 7,8-dihydrofolate (1,2). This enzyme is the target for 5-fluorodeoxyuridylate, the active form of the antitumor agent 5-fluorouracil. This investigation of the chemical denaturation of thymidylate synthetase was undertaken as part of an effort to elucidate the mechanism of action of this enzyme in terms of its physico-chemical properties. The enzyme, purified to homogeneity from amethopterin-resistant Lactobacillus casei, has a molecular weight of 70,000 (3), and is composed of two similar or identical subunits (4). Dissociation into subunits occurs in sodium dodecyl sulfate (5), or 5 M guanidine hydrochloride (Gdn·HCl)[2].

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[2]Abbreviations: GdnHCl, guanidine hydrochloride; dansyl, 1-dimethyl aminonaphthalene-5-sulfonyl.

Fluorescence spectroscopy is a sensitive tool for monitoring conformational changes in proteins. The change in emission intensity and Stokes' shift can give information about structural transitions (6,7). In addition, fluorescence polarization often yields detailed information about the size and shape of proteins; in some cases, the internal motion of proteins can also be measured (8,9). For linearly polarized excitation, the polarization, P, is defined as $P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$, where $I_{\parallel}$ is the emission intensity with the analyzer oriented vertically, and $I_{\perp}$ is the emission intensity with the analyzer horizontal. The polarization is related to the size of the molecule by the following equation:

$$\frac{1}{P} - \frac{1}{3} = \left(\frac{1}{P_o} - \frac{1}{3}\right)\left(1 + \frac{RT\tau}{\eta V}\right) \qquad (1)$$

where P_o is the polarization in absence of Brownian rotation, τ is the lifetime of the fluorophor, η is the viscosity, and the other parameters have their usual significance. The rotational relaxation time is given by $\rho = 3\eta V/RT$, where V is the molecular volume. Variations in ρ with denaturant concentration reflect changes in rigidity, size, or shape of the protein (10).

Materials and Methods

Dansyl chloride (Eastman Organic), quinine sulfate (Aldrich), D,L-tryptophan (Sigma Chemical Co.), were used without further purification. Thymidylate synthetase was purified to homogeneity from amethopterin-resistant Lactobacillus casei by a modification (11,12) of the method of Dunlap et al. (3).

Fluorescence spectra were recorded on a Perkin Elmer Model 303A Spectrofluorimeter. For unconjugated protein excitation was at 280nm (2nm slit width), and the maximum emission varied from 337 to 350nm (18nm slit width). The polarizations of the dansylated protein were obtained by excitation at 333nm (5nm slit width) with emission at 510nm (40nm slit width). All spectra were recorded in the ratio mode. To

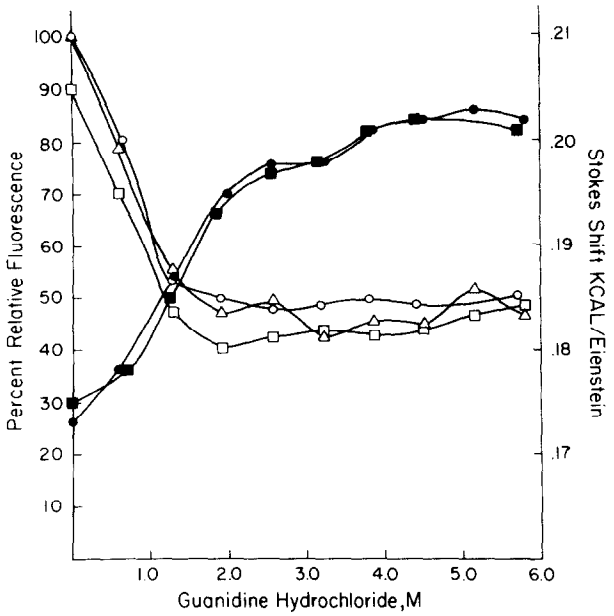

Figure 1. The intrinsic fluorescence and Stokes' shift of unlabeled thymidylate synthetase (20μg/ml) of various concentrations of GdnHCl (0.002 M potassium phosphate buffer, pH 6.8, 0.025 M KCl, 0.25 mM EDTA, 5 mM 2-mercaptoethanol). Run 1: intensity after 30 min ○ ; Run 2: intensity within 3 min △, intensity after 15 hrs □. Stokes' shift: Run 1: ● ; Run 2: ■ .

compensate for any fluctuations in lamp intensity quinine sulfate in 0.1 N H_2SO_4 or tryptophan in water was used as a reference. Emission intensities were also corrected for volume changes upon addition of GdnHCl. Since viscosity increases with increasing GdnHCl concentration, all polarizations were normalized to the viscosity of water using the data of Kawahara and Tanford (13). Concentrations of GdnHCl were determined from the refractive index (14). The solution was maintained at constant temperature by means of a flow cell.

Dansylation was accomplished in the usual manner (15): 20mg thymidylate

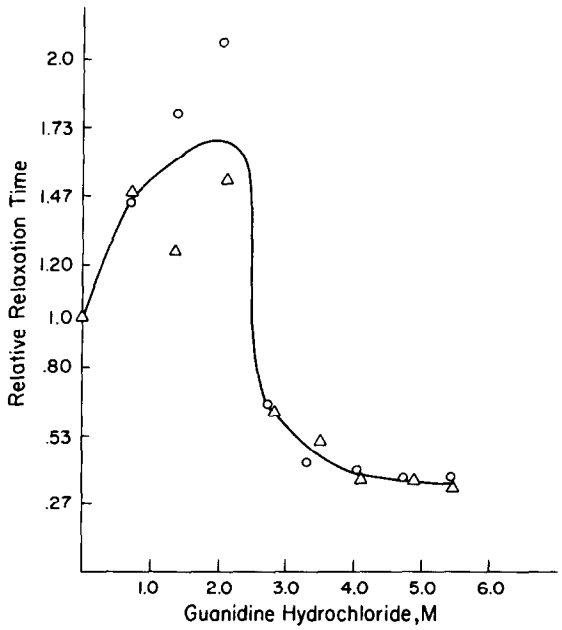

Figure 2. Relative relaxation times of dansylated thymidylate synthetase (87.5μg/ml) as a function of GdnHCl concentration (in 0.020 M potassium phosphate buffer, pH 6.8, 0.025 M KCl, 0.25 mM EDTA, and 1.0 mM 2-mercaptoethanol. Run 1: ○ ; Run 2: △ .

synthetase was first dialyzed against 1% sodium bicarbonate, at pH 8.3. After dialysis, the protein solution was made 2.89 mM in deoxyuridylic acid. A small volume of dansyl chloride in acetone was then added, and the reaction allowed to proceed 40.5 hr at 5°C. The conjugate was purified by dialyzing twice against 6 1. of 0.08 M pH 6.8 potassium phosphate buffer, containing 0.1 M potassium chloride, 0.001 M EDTA, and 0.020 M 2-mercaptoethanol. The labeling ratio, 0.78, was determined by using a molar extinction coefficient of 3400 $M^{-1}cm^{-1}$ at 340nm for the conjugate, and correcting for protein absorbance at 340nm. The specific activity was 3.52 units/mg. Full activity was retained only with substrate protection during labeling. The polarization of the conjugate decreased

over a period of one week, but the original polarization was obtained when the sample was dialyzed against buffer containing fresh 2-mercaptoethano

Results and Discussion

The effect of GdnHCl concentration on the intrinsic fluorescence of unlabeled thymidylate synthetase is shown in Figure 1. These data have been corrected for a GdnHCl concentration-dependent quenching (up to 9% by 6 M GdnHCl). The changes in intensity shown in Figure 1 reflect a change in protein quantum yield. Since the protein quantum yield is dependent on conformation, a conformational change, which occurs between 0 and 2 M GdnHCl, is indicated.

Figure 1 also shows the variation in Stokes' shift with GdnHCl concentration. The increase in Stokes' shift is associated with an increase in the polarity of the fluorophor environment as the protein swells or unfolds in the presence of denaturant. This transition is complete by 5.0 M GdnHCl.

Figure 2 shows the relative relaxation times of the protein (calculated from equation 1) as a function of GdnHCl concentration at pH 6.8. Between 0 and 2.3 M GdnHCl, the relative relaxation times increase by a factor of about 1.5. Therefore, in this range, the protein is either aggregating, elongating, or expanding (16,17). Aggregation, at least at the protein concentrations used in this study, appears unlikely in view of the absence of a change in polarization upon addition of unlabeled protein to dansylated thymidylate synthetase (in 0.72 and 1.30 M GdnHCl). The relative relaxation time of the protein decreases to about 33% of its initial value between 2.3 and 5.5 M GdnHCl. Such a decrease in relaxation time would be expected for unfolding of the protein accompanied by separation of the subunits. Concomitant NMR spectral studies confirm that by about 5 M GdnHCl, the spectrum of the protein agrees with that predicted for a random coil (18). Unfolding and separation of subunits appear to be highly cooperative processes. The absence of a break in

the denaturation curve (2.3 - 5.0 M GdnHCl) does not support the existence of an intermediate state in which separation of subunits has occurred with retention of tertiary structure. However, additional experiments now in progress at this laboratory are required to conclusively investigate this possibility.

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