## Dimeric Tyrosyl-tRNA Synthetase from *Bacillus stearothermophilus* Unfolds through a Monomeric Intermediate

A QUANTITATIVE ANALYSIS UNDER EQUILIBRIUM CONDITIONS\*

(Received for publication, February 2, 1998, and in revised form, April 15, 1998)

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Tyrosyl-tRNA synthetase from Bacillus stearothermophilus comprises an N-terminal domain (residues 1-319), which is dimeric and forms tyrosyladenylate, and a C-terminal domain (residues 320-419), which binds the anticodon arm of tRNA<sup>Tyr</sup>. The N-terminal domain has the characteristic fold of the class I aminoacyl-tRNA synthetases. The unfolding of the N-terminal domain by urea at 25 °C under equilibrium conditions was monitored by its intensities of light emission at 330 and 350 nm, the ratio of these intensities, its ellipticity at 229 nm, and its partition coefficient, in spectrofluorometry, circular dichroism, and size-exclusion chromatography experiments, respectively. These experiments showed the existence of an equilibrium between the native dimeric state of the N-terminal domain, a monomeric intermediate state, and the unfolded state. The intermediate was compact and had secondary structure, and its tryptophan residues were partially buried. These properties of the intermediate and its inability to bind 1-anilino-8-naphthalenesulfonate showed that it was not in a molten globular state. The variation of free energy  $\Delta G(H_2O)$  and its coefficient *m* of dependence on the concentration of urea were, respectively,  $13.8 \pm 0.2$ kcal·mol<sup>-1</sup> and 0.9  $\pm$  0.1 kcal·mol<sup>-1</sup>·M<sup>-1</sup> for the dissociation of the native dimer and 13.9  $\pm$  0.6 kcal·mol<sup>-1</sup> and 2.5  $\pm$  0.1 kcal·mol<sup>-1</sup>·M<sup>-1</sup> for the unfolding of the monomeric intermediate.

The unfolding of proteins by denaturing agents under thermodynamic equilibrium conditions is useful to characterize their unfolding mechanism and to quantify their conformational stability. Most quantitative studies of unfolding have been performed on soluble monomeric proteins or on dimeric proteins that unfold according to a two-state mechanism, *i.e.* without an intermediate state between the native protein and the unfolded polypeptide (1). Very few quantitative studies have dealt with dimeric proteins that unfold through an intermediate, whether monomeric (2, 3) or dimeric (4, 5), yet most cellular proteins of therapeutic interest are dimeric, and it is important to be able to compare their stability with those of engineered mutants.

The aminoacyl-tRNA synthetases are divided into two classes. The 10 enzymes of class I are characterized by the common fold of their catalytic domain and the existence of two conserved sequence motifs, involved in the binding of ATP. All of the aminoacyl-tRNA synthetases of class I are monomeric, except for the tyrosyl- and tryptophanyl-tRNA synthetases, which are dimeric and structurally very homologous (6–8). Tyrosyl-tRNA synthetase (TyrRS)<sup>1</sup> catalyzes the aminoacylation of tRNA<sup>Tyr</sup> in a two-step reaction. Tyrosine is first activated with ATP to form tyrosyladenylate, and then this intermediate is attacked by tRNA<sup>Tyr</sup> to form tyrosyl-tRNA<sup>Tyr</sup> and AMP. TyrRS shows half-of-the-sites reactivity since it binds only one molecule of tyrosine and one molecule of tRNA<sup>Tyr</sup> per molecule of dimer (9, 10).

The structure of TyrRS from *Bacillus stearothermophilus* has been determined at high resolution (11). Each subunit of TyrRS comprises two structural domains in the crystal structure, an N-terminal domain (residues 1–319) and a C-terminal domain (residues 320–419). The N-terminal domain contains the interface of dimerization and the binding sites for tyrosine, tyrosyladenylate, and the acceptor arm of tRNA<sup>Tyr</sup>. Its isolated form (*i.e.* unlinked to the other domain) is dimeric, forms tyrosyladenylate normally, but does not bind tRNA<sup>Tyr</sup> (12). The integrated form and the isolated form of the N-terminal domain have the same crystal structure (11, 13). The C-terminal domain is disordered in the crystals of full-length TyrRS. It is essential for the binding of tRNA<sup>Tyr</sup> to TyrRS (12, 14).

We have chosen to study the N- and C-terminal domains of TyrRS separately to characterize its unfolding mechanism and its stability. In a previous study, we have shown that the isolated form of the C-terminal domain,  $TyrRS(\Delta 3)$ , has secondary structure, is compact, and unfolds through a cooperative transition (15). In the present work, we studied the unfolding of the isolated N-terminal domain,  $TyrRS(\Delta 1)$ , by urea under equilibrium conditions. We detected a monomeric intermediate between the native dimeric state of the N-terminal domain and its unfolded state and quantified the variation of free energy and its dependence on the concentration of urea for each of the corresponding conformational transitions. This work opens new prospects for the study of TyrRS by a mutational approach. For example, it will be possible to analyze the recognition between the subunits, the transmission of information between the active sites across the subunit interface, or the molecular bases for the hyperstability of TyrRS from B. stearothermophilus. Moreover, the quantitative thermodynamic analysis developed in this work should be applicable to other dimeric or oligomeric proteins.

## MATERIALS AND METHODS

Proteins and General Conditions—TyrRS( $\Delta 1)$  was overexpressed from phage M13-BY( $\Delta 1)$  and purified as described (15, 16). The purified

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 $<sup>^1</sup>$  The abbreviations used are: TyrRS, tyrosyl-tRNA synthetase; ANS, 1-anilino-8-naphthalenesulfonate; fast-SEC, fast size-exclusion chromatography;  $\Delta ASA$ , variation of accessible surface area.

 $TyrRS(\Delta 1)$  was >95% pure as judged by gel electrophoresis. It was stored at -70 °C in 20 mM Tris-HCl (pH 7.78) and 5 mM 2-mercaptoethanol. The concentration of protein in the purified samples of TyrRS( $\Delta 1$ ) was measured with the Bio-Rad protein assay kit using bovine serum albumin as a standard. The molecular mass of  $TyrRS(\Delta 1)$ was taken as 36,324 Da/subunit (12). Ultrapure urea was purchased from ICN and used directly as provided. All the experiments were performed at 25 °C. The reactions of unfolding and refolding of TyrRS( $\Delta 1$ ) in the presence of urea were performed and brought to equilibrium as described (15). In particular, all the measurements were done after a prolonged incubation of the reaction mixtures at 25 °C, between 12 and 16 h, a time after which the equilibrium between the different conformational states of  $TyrRS(\Delta 1)$  is reached (15). The concentration of urea in the reaction mixtures was measured with a refractometer and a precision of 0.01 M after the completion of each experiment.

Intrinsic Fluorescence Experiments—The samples were excited at 278 nm; the slit width was equal to 2.5 nm for the excitation light and 5 nm for the emission. The spectra of fluorescence intensity were recorded between 310 and 380 nm with a Quanta Master spectrofluorometer (Photon Technology International). The signal was acquired for 1 s at each wavelength, and the increment of wavelength was equal to 1 nm. The intensities of fluorescence at 330 nm were measured with a Perkin-Elmer LS-5B spectrofluorometer. The signal was averaged during 17 s. The fluorescence signal for the protein was corrected by substraction of the signal for the solvent alone.

Circular Dichroism Experiments—Far-UV CD experiments were performed with a Jobin-Yvon CD6 apparatus. The spectra were recorded between 210 and 250 nm with a 0.2-cm path length cell when the concentration of TyrRS( $\Delta$ 1) was equal to 100 µg/ml and between 225 and 250 nm with a 1.0-cm path length cell when its concentration was 20 µg/ml. The signal was acquired for 1 s at each wavelength, and the increment of wavelength was equal to 1 nm. The signal for the protein was corrected by substraction of the signal for the solvent alone.

Size-exclusion Chromatography-The hydrodynamic properties of  $TyrRS(\Delta 1)$  and their variations with the concentration of urea were measured by size-exclusion chromatography through a Superdex 200 HR 10/30 column connected to a fast protein liquid chromatography system (Amersham Pharmacia Biotech). The effluent was continuously monitored at  $A_{280 \text{ nm}}$ . Before each chromatographic run, the column was equilibrated with more than 4 column volumes of elution buffer. An aliquot (100  $\mu$ l) of either an unfolding reaction or a refolding reaction, containing 100  $\mu$ g/ml TyrRS( $\Delta$ 1), was injected at the top of the column. The column was eluted with the same buffer that was used in the unfolding or refolding reaction. The measures were expressed using the partition coefficient  $K_{av} = (V_e - V_O)/(V_t - V_0)$ , where  $V_e$  is the elution volume corresponding to the maximum of the protein elution peak and  $V_t$  and  $V_0$  are the total volume and the void volume of the column, respectively. The relation between the Stokes radius  $(R_S)$  and the rate of elution  $(1000/V_e)$  was established with the proteins in the calibration kits from Amersham Pharmacia Biotech. Blue dextran 200 and acetone were used to measure  $V_0$  and  $V_t$ , respectively. All the chromatographic runs were performed at a flow rate of 0.3 ml/min and at room temperature (22-26 °C).

Pyrophosphate Exchange—The active-site titration and the pyrophosphate exchange assay were performed essentially as described (16, 17) with the following modifications. TyrRS(A1) (0.40  $\mu$ M active sites) was first unfolded by different concentrations of urea as described above. The pyrophosphate exchange reaction was then started by diluting four times the unfolded enzyme in a reaction mixture containing the various substrates ([<sup>32</sup>P]pyrophosphate, ATP, and tyrosine) and the same concentration of urea as in the unfolding reaction.

ANS Binding—A stock solution of ANS (either 28 or 280 mM in methanol) was prepared, and its concentration was determined using a molar extinction coefficient equal to  $6.8 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$  at 370 nm in methanol (18). ANS (0.5  $\mu$ l, either 14 or 140  $\mu$ M final concentration) was added to a pre-equilibrated unfolding reaction of TyrRS( $\Delta$ 1) (1 ml, 100  $\mu$ g/ml = 0.1  $\mu$ M protein) or to a control reaction without protein. The mixture was incubated for 1 h, and then the fluorescence spectrum of ANS was recorded between 400 and 600 nm, with excitation at 380 nm.

Analysis of the Unfolding Profiles—The profiles of unfolding of TyrRS( $\Delta$ 1) by urea, monitored by its fluorescence intensity or its CD ellipticity, were analyzed with the thermodynamic models described in Table I. Equation 13 was fitted to the unfolding profiles with the software pro Fit 5.0 for Macintosh (Cherwell Scientific Publishing Ltd., Oxford, United Kingdom) and 10<sup>6</sup> iterations of the Monte Carlo algorithm, followed by the Levenberg-Marquardt algorithm. The error on the concentration of urea (x) was set up to zero, and the error on the



FIG. 1. Unfolding of TyrRS( $\Delta 1$ ) by urea, as monitored by its fluorescence intensity (excitation at 278 nm and emission at 330 nm). The concentration of TyrRS( $\Delta 1$ ) was 10 µg/ml. The corrected signal (Equation 6 in Table I) is given along the *y* axis. The solid line was obtained by fitting Equation 13 (mechanism with a monomeric intermediate) in Table I to the experimental data.

signal (Y) was set up to unknown for both algorithms. The fitting range for the Monte Carlo algorithm was set up to the default one, *i.e.* ±10% of the starting values of the parameters. However, the autosearch option was activated so that the limits of the parameters were adapted during the fit. Care was taken to reset these limits to ±10% of the starting values before each new fit. The values of the molar fractions  $f_n$ ,  $f_i$ , and  $f_u$  (Equations 10–12) and of their roots were calculated with the same software.

## RESULTS

Unfolding Monitored by Fluorescence Intensity-The sequence of  $TyrRS(\Delta 1)$  contains six tryptophan residues (at positions 9, 97, 126, 196, 240, and 255) and 11 tyrosine residues. Several tryptophan residues are buried inside the protein, according to its crystal structure (13). We recorded the fluorescence emission spectra of  $TyrRS(\Delta 1)$  in 0 and 8 M urea at an excitation wavelength of 278 nm. The spectrum of unfolded  $\mathrm{TyrRS}(\Delta 1)$  had a  $\lambda_{\mathrm{max}}$  at 349 nm, a value similar to that of tryptophan as a free amino acid. The  $\lambda_{max}$  of native TyrRS( $\Delta 1)$ was blue-shifted to 341 nm, and this shift was accompanied by an increase in intensity. The blue shift and the increase in intensity confirmed that some of the tryptophan side chains were buried in the hydrophobic interior of the native protein. The difference in fluorescence intensity between the native and unfolded states of  $TyrRS(\Delta 1)$  was maximal at an emission wavelength of 330 nm. This difference was larger when the excitation wavelength was equal to 278 nm than when it was 295 nm, as expected since both tyrosine and tryptophan are excited at 278 nm, whereas only tryptophan is excited at 295 nm. We therefore used the intensity of fluorescence emission at 330 nm, upon excitation at 278 nm, to monitor the unfolding of TyrRS( $\Delta 1$ ) by urea under equilibrium conditions. Fig. 1 shows the unfolding profile of  $TyrRS(\Delta 1)$  at a concentration of 10  $\mu$ g/ml. The fluorescence intensity remained roughly constant at low concentrations of urea, decreased non-linearly between 2 and 6 M urea, and then increased linearly at high concentrations of urea.

The molar fractions of the different conformational states depend on the total concentration of protein for a dimer (Table I). In contrast, the equilibrium constants K,  $K_1$ , and  $K_2$  between these states and the associated free energies  $\Delta G$ ,  $\Delta G_1$ , and  $\Delta G_2$  do not depend on this concentration and should remain constant when it varies. We used this invariability of the thermodynamic parameters as a criterion to characterize the mechanism through which TyrRS( $\Delta 1$ ) unfolds. We recorded its profile of unfolding by urea, monitored by its intensity of fluorescence, at different concentrations of protein, 10 µg/ml (138 nM), 5 µg/ml (69 nM), and 2.5 µg/ml (34 nM). Each profile was recorded three times in independent experiments. We fitted Equation

## Unfolding of the Tyrosyl-tRNA Synthetase Dimer

#### TABLE I

### Equations for the equilibrium unfolding of dimeric proteins, as monitored by spectrometry

The abbreviations used are:  $N_2$ , native dimeric state;  $J_2$ , dimeric intermediate state; I, monomeric intermediate state; U, unfolded state; C, total concentration of protein (M), expressed as dimer equivalent; x, concentration of denaturant (M); Y', measured global signal; Y, corrected signal;  $Y_n$ ,  $Y_j$ ,  $Y_j$ , and  $Y_u$ , molar signals of the various protein states;  $Y_d$ , molar signal of the denaturant; T, temperature (K); R, gas constant. The other parameters are defined below. The final fitting equation was obtained by replacing  $Y_n$ ,  $Y_j$ ,  $Y_j$ ,  $Y_u$ ,  $f_n$ ,  $f_i$ ,  $f_j$ , and  $f_u$  with their expressions as a function of x in Equation 13. Equations 7 and 8 have already been discussed (31, 34).

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13, which links the global intensity of fluorescence and the concentration of urea, to the unfolding profiles for each of the three mechanisms in Table I, *i.e.* without an intermediate state between the native and unfolded states, with a monomeric intermediate, or with a dimeric intermediate. The thermodynamic parameters corresponding to these fittings varied with the total concentration of protein for the unfolding mechanism without an intermediate state or with a dimeric intermediate. In contrast, they remained constant, within experimental error, for the mechanism with a monomeric intermediate (Table II). Thus, the values of the thermodynamic parameters and their comparison at different protein concentrations showed that  $TyrRS(\Delta 1)$  unfolded through a monomeric intermediate.

We recorded the statistical parameter  $\chi^2$ , which measures the agreement between the function and the experimental values, for each experimental unfolding profile and each mechanism in Table I. We found that the relative values of  $\chi^2$  were equal to 2.4  $\pm$  0.3, 1.3  $\pm$  0.1, and 1.0 (mean  $\pm$  S.E. in nine independent experiments) for the mechanisms without an intermediate, with a monomeric intermediate, and with a dimeric intermediate, respectively. Thus, the equation corresponding to the mechanism with a dimeric intermediate gave the best formal fit to the experimental data, even though it did not satisfy the thermodynamic criteria described above. To reconcile the conclusions obtained with the thermodynamic and fitting criteria, we established the equation describing the mechanism with both a dimeric intermediate and a monomeric intermediate. This equation (not shown) comprised a total of 11 fitting parameters. The S.D. values that were associated with these parameters during the fitting process were very large, which indicated that the parameters were too numerous to be accurately determined by the fitting process.

Characteristic Parameters of Unfolding Monitored by Fluorescence Intensity—We calculated the molar fractions  $f_n$ ,  $f_i$ , and  $f_u$  of the different conformational states of TyrRS( $\Delta 1$ ) from the thermodynamic parameters for an unfolding mechanism with a monomeric intermediate. We deduced several characteristic

## TABLE II

Comparison of different mechanisms for the unfolding of TyrRS( $\Delta 1$ ) The notations are the same as described for Table I. The unfolding of TyrRS( $\Delta 1$ ) was monitored by its fluorescence intensity, as described in the legend to Fig. 1. The thermodynamic parameters listed in the first column were obtained by fitting equation 13 in Table I to the experimental data. Each entry corresponds to the mean  $\pm$  S.E. of three independent experiments.

	$TyrRS(\Delta 1)$ concentration			
	10.0 µg/ml	$5.0 \ \mu \text{g/ml}$	$2.5 \ \mu \mathrm{g/ml}$	
Without intermediate				
$m \; (\text{kcal} \cdot \text{mol}^{-1} \cdot \text{M}^{-1})$	$2.5\pm0.4$	$2.7 \pm 1.1$	$1.4\pm0.2$	
$\Delta G(\mathrm{H}_{2}\mathrm{O}) \; (\mathrm{kcal} \cdot \mathrm{mol}^{-1})$	$22.4\pm2.2$	$23.5\pm6.0$	$15.9 \pm 1.3$	
Dimeric intermediate				
$m_1 (\text{kcal} \cdot \text{mol}^{-1} \cdot \text{M}^{-1})$	$2.5\pm1.0$	$1.0\pm0.1$	$1.0\pm0.3$	
$m_2$ (kcal $\cdot$ mol <sup>-1</sup> $\cdot$ M <sup>-1</sup> )	$3.4\pm0.6$	$5.8\pm0.2$	$9.8\pm1.9$	
$\Delta \tilde{G}_{1}(\mathrm{H}_{2}\mathrm{O}) \; (\mathrm{kcal} \cdot \mathrm{mol}^{-1})$	$7.5\pm3.4$	$3.5\pm0.2$	$4.1\pm0.9$	
$\Delta G_2(H_2O)$ (kcal $\cdot$ mol <sup>-1</sup> )	$27.7 \pm 3.1$	$40.6 \pm 1.1$	$62.9\pm9.8$	
Monomeric intermediate				
$m_1$ (kcal · mol <sup>-1</sup> · M <sup>-1</sup> )	$0.88\pm0.06$	$0.76\pm0.03$	$1.07\pm0.12$	
$m_2$ (kcal $\cdot$ mol <sup>-1</sup> $\cdot$ M <sup>-1</sup> )	$2.57 \pm 0.16$	$2.61\pm0.03$	$2.25\pm0.15$	
$\Delta \tilde{G}_{1}(\mathrm{H}_{2}\mathrm{O}) \; (\mathrm{kcal} \cdot \mathrm{mol}^{-1})$	$13.8\pm0.4$	$13.6\pm0.4$	$13.87\pm0.04$	
$\Delta G_2(H_2O) (\text{kcal} \cdot \text{mol}^{-1})$	$14.9\pm1.0$	$14.5\pm0.4$	$12.3\pm1.0$	

concentrations of urea from these molar fractions:  $f_n^{-1}(0.5)$ , at which half of the TyrRS( $\Delta 1$ ) molecules were dissociated;  $f_i^{-1}(\max f_i)$ , at which the concentration of monomeric intermediate was maximal; and  $f_u^{-1}(0.5)$ , at which half of the molecules were unfolded. Table III gives the average values of these characteristic concentrations for the experiments performed at 10, 5, and 2.5  $\mu$ g/ml. These values showed an effect of the total concentration of protein on the molar fractions of the different conformational states, as expected for the unfolding of a dimeric protein.

Table IV gives the global average values of the thermodynamic parameters, calculated from the results of nine experiments performed at three different protein concentrations. The unfolding reaction is characterized by four thermodynamic parameters: the free energies for the dissociation of the native

#### TABLE III

# Characteristic concentrations of urea for the unfolding of $TyrRS(\Delta I)$ , as monitored by its fluorescence intensity

The notations are the same as described for Table I. The abbreviations used are:  $\max f_i$ , maximal value of  $f_i$ ;  $f_i^{-1}(\max f_i)$ , concentration of urea at which  $f_i$  is maximal;  $f_n^{-1}(0.5)$  and  $f_u^{-1}(0.5)$ , concentrations of urea (x) at which  $f_n(x) = 0.5$  and  $f_u(x) = 0.5$ , respectively. Thermodynamic parameters were obtained by fitting Equation 13 (mechanism with a monomeric intermediate) in Table I to the experimental data. The values of the molar fractions  $f_n$ ,  $f_i$ , and  $f_u$  and of their roots were calculated from these thermodynamic parameters using Equations 10-12. Each entry corresponds to the mean  $\pm$  S.E. of three independent experiments.

	Ty	$TyrRs(\Delta 1)$ concentration			
	10.0 $\mu$ g/ml	5.0 $\mu$ g/ml	$2.5 \ \mu g/ml$		
$\frac{\max f_i}{f_i^{-1}(\max f_i) (\mathbf{M})}$	$\begin{array}{c} 0.47 \pm 0.03 \\ 5.52 \pm 0.15 \end{array}$	$\begin{array}{c} 0.43 \pm 0.12 \\ 5.27 \pm 0.02 \end{array}$	$\begin{array}{c} 0.72 \pm 0.08 \\ 4.86 \pm 0.20 \end{array}$		
$f_n^{-1}(0.5)$ (M) $f_u^{-1}(0.5)$ (M)	$5.3 \pm 0.1 \\ 5.88 \pm 0.16$	$5.0 \pm 0.2 \\ 5.65 \pm 0.05$	$\begin{array}{c} 3.9 \pm 0.4 \\ 5.47 \pm 0.11 \end{array}$		

#### TABLE IV Mean parameters for the unfolding of $TyrRS(\Delta 1)$ , as monitored by its fluorescence intensity

The notations are the same as described for Table I. The parameters were obtained by fitting Equation 13 (mechanism with a monomeric intermediate) in Table I to the experimental data. The molar signals of the different conformational states,  $Y_n = y_n + m_n x$  and  $Y_i$  and  $Y_u = y_u + m_u x$ , where x = [urea], were expressed as fractions of the molar signal of the native protein in the absence of urea,  $y_n$ . Each entry corresponds to the mean  $\pm$  S.E. of nine independent experiments at three different concentrations of TyrRS( $\Delta$ 1): 2.5, 5.0, and 10.0  $\mu$ g/ml.

Parameter	Mean $\pm$ S.E.
$m_1 (\text{kcal} \cdot \text{mol}^{-1} \cdot \text{M}^{-1})$	$0.90\pm0.06$
$\Delta \dot{G}_1(\mathrm{H}_2\mathrm{O}) \; (\mathrm{kcal} \cdot \mathrm{mol}^{-1})$	$13.75\pm0.16$
$m_2$ (kcal · mol <sup>-1</sup> · M <sup>-1</sup> )	$2.48\pm0.09$
$\Delta \bar{G}_2(\mathrm{H}_2\mathrm{O}) \; (\mathrm{kcal} \cdot \mathrm{mol}^{-1})$	$13.87\pm0.59$
$m_n (M^{-1})$	$0.025\pm0.006$
$y_n$	1
$\overline{Y_i}$	$0.098 \pm 0.008$
$m_{\mu} (M^{-1})$	$0.0061 \pm 0.0006$
$y_u$	$0.076\pm0.010$

dimer and for the unfolding of the monomeric intermediate in the absence of urea ( $\Delta G_1(\mathrm{H}_2\mathrm{O})$  and  $\Delta G_2(\mathrm{H}_2\mathrm{O})$ , respectively) and their coefficients of dependence on the concentration of urea ( $m_1$  and  $m_2$ , respectively). The values of the equilibrium constants  $K_1(\mathrm{H}_2\mathrm{O})$  and  $K_2(\mathrm{H}_2\mathrm{O})$ , calculated from them by Equation 8 in Table I, were 84 pM and 68  $\times$  10<sup>-12</sup>, respectively. The values of the total parameters  $\Delta G(\mathrm{H}_2\mathrm{O})$  and m, calculated by Equation 14 in Table I, were 41  $\pm$  1 kcal·mol<sup>-1</sup> and 5.9  $\pm$  0.2 kcal·mol<sup>-1</sup>·M<sup>-1</sup>, respectively.

Whereas the thermodynamic parameters  $\Delta G_1$ ,  $\Delta G_2$ ,  $m_1$ , and  $m_2$  are characteristic of the studied molecule and can be directly compared between experiments, the molar signals of the different conformational states  $(Y_n, Y_i, \text{ and } Y_u)$  depend on the experimental setting. However, they can be compared between experiments if they are expressed as fractions of the molar signal of the native protein in the absence of urea  $(y_n)$  provided that the native protein is the only conformational state under these conditions. Table IV gives the average values of these molar signals, calculated from the results of the same nine independent experiments. The molar fluorescence of the monomeric intermediate in 0 M urea was equal to 9.8% of its value for the dimeric protein and therefore to 19.6% of its value for one subunit of the native protein. The molar fluorescence of the unfolded monomer in 0 M urea was equal to 15.2% of its value for one subunit of the native protein and to 78% of its value for the monomeric intermediate.

Fig. 2 gives the molar fractions  $f_n$ ,  $f_i$ , and  $f_u$  of the different



FIG. 2. Fraction of each TyrRS( $\Delta 1$ ) state as a function of urea concentration in the unfolding process. The fractions  $f_n$ ,  $f_i$ , and  $f_u$  of TyrRS( $\Delta 1$ ) in the native, monomeric, and unfolded states were calculated using Equations 10–12 in Table I, the mean thermodynamic parameters in Table IV, and concentrations of TyrRS( $\Delta 1$ ) equal to 2.5, 5.0, and 10  $\mu$ g/ml. See also Table III.



FIG. 3. Unfolding of TyrRS( $\Delta 1$ ) by urea, as monitored by its CD ellipticity at 229 nm. The concentration of TyrRS( $\Delta 1$ ) was equal to 100 µg/ml. The corrected signal (Equation 6 in Table I) is given along the *y* axis. The solid line was obtained by fitting Equation 13 (mechanism with a monomeric intermediate) in Table I to the experimental data. *mdeg*, millidegrees.

conformational states of TyrRS( $\Delta 1$ ) as functions of the concentration of urea, at the three protein concentrations used experimentally. These fractions were calculated from the global average values of the thermodynamic parameters given in Table IV.

Unfolding Monitored by Circular Dichroism-We used the circular dichroism of  $TyrRS(\Delta 1)$  in the absorption band of the peptide bond (from 200 to 230 nm) to monitor the variation of its content in secondary structure during its unfolding by urea. We first recorded the CD spectra of  $TyrRS(\Delta 1)$  in 0 and 8 M urea. Unfolded TyrRS( $\Delta 1$ ) showed no ellipticity for wavelengths >212 nm. In contrast, native TyrRS( $\Delta 1$ ) showed a broad peak of negative ellipticity, around 222 nm, consistent with its high helical content in the crystal structure (13). We used higher total concentrations of  $TyrRS(\Delta 1)$  in the CD experiments (20  $\mu$ g/ml (0.28  $\mu$ M) and 100  $\mu$ g/ml (1.4  $\mu$ M)) than in the fluorescence experiments for sensitivity reasons. We chose a wavelength equal to 229 nm to monitor quantitatively the unfolding of  $TyrRS(\Delta 1)$  for the same reason. Fig. 3 shows the unfolding profile of TyrRS( $\Delta 1$ ) at 100  $\mu$ g/ml, monitored by CD at 229 nm under equilibrium conditions. The ellipticity of  $TyrRS(\Delta 1)$  decreased slowly and linearly between 0 and 4.5 m urea, increased sharply and non-linearly between 4.5 and 6 M urea, and then increased slowly and linearly between 6 and 8.5 м urea.

Because the ellipticity of molecules can be considered as an additive property, we could fit the equations in Table I to the unfolding profiles of  $TyrRS(\Delta 1)$  monitored by CD. Table V gives

## TABLE V

Comparison of the characteristic parameters for the unfolding of  $TyrRS(\Delta 1)$  by urea, as monitored by its CD ellipticity and its fluorescence intensity The notations are as described for Tables I and III. The values of the parameters in the second and third columns correspond to the fitting of Equation 13 (mechanism without intermediate) to the CD data. Those in the fourth and fifth columns correspond to the fitting of Equation 13 (mechanism with a monomeric intermediate). The values of the four thermodynamic parameters in the sixth and seventh columns were taken from Table IV. The values of the molar fractions  $f_n$ ,  $f_i$ , and  $f_u$  and of their roots were calculated from the thermodynamic parameters in the first four rows using Equations 10–12 and TyrRS( $\Delta 1$ ) concentrations of 20 and 100  $\mu$ g/ml.

C (µg/ml) Intermediate Method	20 None CD	100 None CD	20 Monomer CD	100 Monomer CD	20 Monomer Fluorescence (simulated)	100 Monomer Fluorescence (simulated)
$ \begin{array}{c} \hline m_1 \; (\mathrm{kcal} \cdot \mathrm{mol}^{-1} \cdot \mathrm{M}^{-1}) \\ m_2 \; (\mathrm{kcal} \cdot \mathrm{mol}^{-1} \cdot \mathrm{M}^{-1}) \end{array} $	$4.43 \pm 0.03 \ \mathrm{NA}^a$	5.6 + 0.5 NA	$\begin{array}{c} 0.80 \pm 0.06 \\ 2.94 \pm 0.04 \end{array}$	$\begin{array}{c} 1.4 \pm 1.1 \\ 2.90 \pm 0.03 \end{array}$	$\begin{array}{c} 0.90 \pm 0.06 \\ 2.48 \pm 0.09 \end{array}$	$\begin{array}{c} 0.90 \pm 0.06 \\ 2.48 \pm 0.09 \end{array}$
$\Delta G_1(\mathrm{H}_2\mathrm{O}) \; (\mathrm{kcal} \cdot \mathrm{mol}^{-1}) \\ \Delta G_2(\mathrm{H}_2\mathrm{O}) \; (\mathrm{kcal} \cdot \mathrm{mol}^{-1})$	$33.34 \pm 0.05$ NA	$39 \pm 3$ NA	$\begin{array}{c} 13.95 \pm 0.00 \\ 16.1 \pm 0.3 \end{array}$	$17 \pm 6 \\ 15.5 \pm 0.3$	$\begin{array}{c} 13.7 \pm 0.2 \\ 13.9 \pm 0.6 \end{array}$	$13.7 \pm 0.2 \\ 13.9 \pm 0.6$
$\max f_i$ $f^{-1}(0,5)$ (M)	NA 5.60	NA 5.60	0.23 5.54	0.12 5.59	0.37 5.36	0.21 5.68
$f_i^{-1}(\max f_i) (M) f_u^{-1}(0.5) (M)$	NA 5.60	NA 5.60	5.43 5.69	$5.53 \\ 5.65$	5.42 5.72	5.60 5.84

<sup>*a*</sup> NA, not applicable.

the corresponding thermodynamic parameters and characteristic concentrations of urea and compares them with the results of the fluorescence experiments. We first tried the mechanism with a monomeric intermediate. When the concentration of TyrRS( $\Delta 1$ ) was equal to 20  $\mu$ g/ml, the thermodynamic parameters deduced from the CD experiments were close to those predicted from the fluorescence experiments. When  $TyrRS(\Delta 1)$ was at 100  $\mu$ g/ml, the S.D. values that were associated with the values of  $m_1$  and  $\Delta G_1(H_2O)$  during the fitting process were high. Moreover, the characteristic concentrations of urea deduced from the CD experiments, in particular  $f_n^{-1}(0.5)$ , varied less with the concentration of  $TyrRS(\Delta 1)$  than predicted from the fluorescence experiments. We also tried the mechanism without an intermediate. The values of m and  $\Delta G(H_2O)$  deduced from the CD experiments and the mechanism without an intermediate were close to the values of the total m and  $\Delta G(H_2O)$  parameters deduced from the fluorescence experiments and the mechanism with a monomeric intermediate,  $5.9 \pm 0.2 \text{ kcal·mol}^{-1} \text{·M}^{-1}$  and  $41 \pm 1 \text{ kcal·mol}^{-1}$ , respectively, especially at higher concentrations of  $TyrRS(\Delta 1)$ . However, the urea concentration of half-transition,  $f_n^{-1}(0.5) = 5.60$  M, deduced from the CD experiments did not vary with the concentration of  $TyrRS(\Delta 1)$ . These results showed that the CD signal was less sensitive to the dissociation of the native dimer than the fluorescence signal, but was as sensitive to the unfolding of the monomeric intermediate. They indicated that the dissociation of the subunits did not lead to a large change of the content in secondary structure.

Unfolding Monitored by Fast Size-exclusion Chromatography—Thorough studies with monomeric proteins have shown that fast size-exclusion chromatography (fast-SEC) can be used to monitor the unfolding of proteins by denaturants and to measure the molecular dimensions of their different conformational states. Fast-SEC is an inert technique, and it does not perturb the thermodynamic equilibrium between the different states of a protein (19, 20). In particular, the  $V_0$  and  $V_t$  of a Superose 12 column (Amersham Pharmacia Biotech) are practically independent of the concentration of denaturant, and a unique linear relation, valid for all the concentrations of denaturant, links the  $R_S$  of a polypeptide and its migration rate (1000/ $V_e$ ) through this type of column (20).

We used fast-SEC on a Superdex 200 HR 10/30 column to monitor the unfolding of TyrRS( $\Delta$ 1). We found that the values of  $V_0$  (6.88 ml) and  $V_t$  (19.86 ml), measured with blue dextran 200 and acetone, did not vary with the concentration of urea in the elution buffer and that a linear relation linked  $R_S$  and 1000/ $V_e$  in 0 M urea with a correlation coefficient of 0.99:  $R_S = (1.03 \pm 0.8) \cdot (1000/V_e) - (49.46 \pm 7.24)$ . We injected 100  $\mu$ l of either an unfolding reaction or a refolding reaction, containing

TyrRS( $\Delta 1$ ) at a concentration of 100 µg/ml, at the top of the column and observed that the protein eluted from the bottom of the column as a single chromatography peak, whatever the concentration of urea. This peak was narrow at low (0–3 M) and high (6.25–8 M) concentrations of urea. It was less narrow and slightly trailed toward the high values of  $V_e$  at intermediate concentrations of urea (4–6 M).

We used the partition coefficient  $K_{av} = (V_e - V_0)/(V_t - V_0)$  of  $TyrRS(\Delta 1)$  to monitor its unfolding by urea (Fig. 4). The linear decrease in  $K_{\rm av}$  between 0 and 4 M urea and then between 6.5 and 8 M urea suggested that the dimeric and unfolded forms of TyrRS( $\Delta 1$ ) swelled when the concentration of urea was increased. These swellings corresponded to increases in the value of  $R_{\rm S}$  for the dimeric protein from 32.5 Å in 0 M urea to 36.7 Å in 4 M urea and for the unfolded polypeptide from 43.7 Å in 6.5 M urea to 46.1 Å in 8 M urea. Such swellings have already been observed. One assumes that they are due to a massive penetration of the folded protein by molecules of urea, compatible with crystallographic data, and to the destruction of residual structures in the unfolded polypeptides (19, 21–23). The  $R_{\rm S}$ value for the native dimer (32.5 Å in 0 M urea) was compatible with the value that can be predicted from its molecular mass (34.7 Å) and with the dimensions of the crystal structure (34 imes $40 \times 117 \text{ Å}^3$ ;  $(3V/4\pi)^{1/3} = 33.6 \text{ Å}$ , where V is the x-ray volume) (13, 20). The  $R_S$  value for the unfolded polypeptide (46.1 Å in 8 M urea) was significantly lower than the predicted value (54 Å) (20).

The sharp increase in  $K_{\rm av}$  between 4.5 and 5.5 M urea, up to a value equal to that of the native dimer in 0 M urea, showed that the dimer of TyrRS( $\Delta 1$ ) dissociated cooperatively into a compact monomeric intermediate. Its sharp decrease between 5.5 and 6.5 M urea showed that this monomeric intermediate unfolded cooperatively.

Unfolding Monitored by the Average Exposure of the Tryptophan Residues-The tryptophan residues have a maximal emission of fluorescence around 330 nm in an apolar solvent and 350 nm in water. Therefore, the ratio  $Y_{330}/Y_{350}$ , between the fluorescence intensities of a protein at 330 nm and 350 nm, measures the average environment of its tryptophans, which can be buried in its apolar interior or exposed to the solvent at its surface. We used the ratio  $Y_{330}/Y_{350}$  to monitor the unfolding of TyrRS( $\Delta 1$ ) (at a total concentration of 10  $\mu$ g/ml) by urea under equilibrium conditions. This ratio increased slowly and linearly between 0 and 4.5 M urea, decreased sharply between 5 and 6 M urea, and then increased slowly and linearly between 7 and 9 M urea (Fig. 5). Because the ratio  $Y_{330}/Y_{350}$  is not an additive property of the molecules, we could not apply the equations in Table I. We therefore fitted a sigmoidal function to the unfolding profile. Remarkably, the urea concentration of



FIG. 4. Unfolding of TyrRS( $\Delta 1$ ) by urea, as monitored by its partition coefficient  $K_{av}$  in fast size-exclusion chromatography experiments. The injected sample (100  $\mu$ l) contained TyrRS( $\Delta 1$ ) at a concentration of 100  $\mu$ g/ml. The solid line was obtained by fitting Equation 13' (see below; corresponding to a mechanism of unfolding through a monomeric intermediate) to the experimental data. The measured signal  $Y = K_{av}$  was equal to the weighted average of the specific signals for the different conformational states of TyrRS( $\Delta 1$ ) (35) so that Equations 5 and 6 were replaced by Equation 13',  $Y = (Y_n + (2Y_i - Y_n + K_2(2Y_u - Y_n))f_i)/(1 + (1 + K_2)f_i)$ . The closed circles correspond to unfolding experiments and were the only ones to be taken into account for the fitting. The open circles overlap with the closed circles and correspond to refolding experiments.



FIG. 5. Unfolding of TyrRS( $\Delta 1$ ) by urea, as monitored by the ratio  $Y_{330}/Y_{350}$  of its fluorescence intensities at 330 and 350 nm.  $Y_{330}$  and  $Y_{350}$  are the corrected signals of fluorescence intensity (Equation 6 in Table I) upon excitation at 278 nm. The concentration of TyrRS( $\Delta 1$ ) was equal to 10  $\mu$ g/ml. The signal  $Y = Y_{330}/Y_{350}$  was assumed to vary linearly with the concentration of urea in the pre- and post-transition regions (Equation 7 in Table I). The solid line corresponds to a fit of a simple sigmoidal function to the experimental data:  $Y = Y_u + (Y_n - Y_u) \cdot (1 - 1/(1 + \exp(a(b - x))))$ .

half-transition  $b = 5.67 \pm 0.02$  M and the cooperativity coefficient  $a = (3.0 \pm 0.2)/RT$  for this sigmoid (where R is the gas constant in kcal·mol<sup>-1</sup>·K<sup>-1</sup> and T = 298.16 K) (Fig. 5) were close to the parameters  $f_u^{-1}(0.5)$  and  $m_2$  found in the fluorescence experiments above, equal to  $5.9 \pm 0.2$  M and  $2.6 \pm 0.2$  kcal·mol<sup>-1</sup>·M<sup>-1</sup>, respectively (Tables II and III). These results showed that the tryptophan residues of TyrRS( $\Delta$ 1) became exposed to the solvent mainly during the unfolding of its monomeric intermediate. We found that  $Y_{330}/Y_{350}$  measured more precisely the average environment of the tryptophan residues during the unfolding of TyrRS( $\Delta$ 1) than its  $\lambda_{\rm max}$  of emission (data not shown).

Unfolding Monitored by Activity—The truncated form of tyrosyl-tRNA synthetase, TyrRS( $\Delta 1$ ), is fully active for the reactions of tyrosyladenylate formation and pyrophosphate exchange (12). We used the latter reaction to monitor the inactivation of TyrRS( $\Delta 1$ ) by urea. The total concentration of TyrRS( $\Delta 1$ ) in the reaction was 0.1  $\mu$ M. The initial rate of pyrophosphate exchange decreased sharply when the concentration



FIG. 6. Rate of pyrophosphate exchange by TyrRS( $\Delta 1$ ) (0.1  $\mu$ M) as a function of the urea concentration. The *solid line* corresponds to a fit of an exponential function to the data:  $V_{max} = a + b \exp(-c x)$ .

of urea increased, with half-inactivation around 0.5 M urea (Fig. 6). We calculated from the results of the fluorescence experiments that, at this concentration of TyrRS( $\Delta 1$ ), 98% of the TyrRS( $\Delta 1$ ) molecules were dimeric in 0.5 M urea and that the urea concentration of half-dissociation should be equal to 5.0 M. Therefore, the results showed that the dimer of TyrRS( $\Delta 1$ ) was inactivated much before it dissociated into monomers when the concentration of urea increased. This inactivation could be due to a local conformational change of TyrRS( $\Delta 1$ ), to a weakening of the noncovalent interactions between the enzyme and the substrates for binding to the enzyme.

ANS Binding-Some proteins unfold through an intermediate conformational state that binds ANS more than the native state (24). ANS is a hydrophobic dye that fluoresces little in water, where its quantum yield is equal to 0.0091 and its maximum of emission occurs at 516 nm, much more in apolar solvents like dioxane, where its quantum yield is 0.68 and its maximum of emission occurs at 432 nm (25). We measured the binding of ANS by  $TyrRS(\Delta 1)$  in the presence of urea under equilibrium conditions. We calculated that the concentration of monomeric intermediate should be equal to 0.32  $\mu$ M in 4.5 M urea, 0.59  $\mu$ M in 5.5 M urea, and negligible in 0 and 8 M urea at the total concentration of TyrRS( $\Delta 1$ ) dimer used (1.4  $\mu$ M) according to the results of the fluorescence experiments. ANS was in either 10- or 100-fold molar excess over  $TyrRS(\Delta 1)$  and excited at 380 nm. Similar conditions have been used to show the binding of ANS to the molten globular state of other proteins (24). We compared the emission spectrum of the mixture of ANS and TyrRS( $\Delta 1$ ) with the spectrum of ANS alone by calculating the ratio of or the difference in the fluorescence intensities under the two conditions. For ANS at 14  $\mu$ M, the ratio of the intensities was at most equal to 1.32 in 0 M urea, 1.08 in 4.5 M urea, 1.10 in 5.5 M urea, and 0.98 in 8 M urea. The difference in the intensities was maximal at 502 nm in 0 M urea, 501 nm in 4.5 M urea, and 484 nm in 5.5 M urea. For ANS at 140  $\mu$ M, the difference in the fluorescence intensities was within the background signal. We concluded that  $TyrRS(\Delta 1)$ did not bind ANS, whatever its conformational state, native dimer, monomeric intermediate, or unfolded protein.

#### DISCUSSION

Experimental Conditions of the Unfolding Reaction—We used various experimental signals to monitor the unfolding of the TyrRS( $\Delta$ 1) dimer by urea. We allowed the unfolding reactions to reach equilibrium before performing any measurements (see "Materials and Methods"). In the spectrofluorometry, circular dichroism, and ANS binding experiments, this equilibrium was not perturbed by the measurement. In the fast

size-exclusion chromatography experiments, it was perturbed by a non-uniform and progressive dilution of the protein during the run. However, the elution of the protein from the column as a single chromatography peak showed that the exchange between the different conformational states of  $TyrRS(\Delta 1)$  was fast when compared with the length of the run. Therefore, the different states of  $TyrRS(\Delta 1)$  were in quasi-equilibrium.

The unfolding of TyrRS( $\Delta 1$ ) by urea is reversible, according to several criteria. The unfolding and refolding profiles of TyrRS( $\Delta 1$ ) in spectrofluorometry, urea gradient gel electrophoresis (15), and fast-SEC (Fig. 4) experiments were identical. We did not observe soluble aggregates when we analyzed the content of the refolding reactions by fast-SEC (see "Results"). The unfolding of TyrRS( $\Delta 1$ ) in 8 M urea and then its refolding do not cause a significant change in the kinetic parameters  $K_m$ for tyrosine,  $K_m$  for ATP, and  $k_{cat}$  in the pyrophosphate exchange reaction. This lack of change indicates that the reversible unfolding of TyrRS( $\Delta 1$ ) by urea has no apparent effect on its functioning (26).

Stability and Energetics of Unfolding—TyrRS( $\Delta 1$ ) unfolded according to a three-state mechanism at low concentrations in protein and to a two-state mechanism at high concentrations. We found close values for the total free energy of unfolding under the different experimental conditions, as expected (Table V). The high value of  $\Delta G(H_2O)$  (41 ± 1 kcal·mol<sup>-1</sup>) is compatible with the thermophilic origin of  $TyrRS(\Delta 1)$ . It makes  $TyrRS(\Delta 1)$  one of the two most stable dimeric enzymes, with organophosphorus hydrolase as the other one (5). Neet and Timm have shown the existence of a rough linear correlation between the number of amino acid residues (N) in the monomer and the value of  $\Delta G(H_2O)$  for a collection of dimeric proteins that unfold according to a two-state mechanism (1). The measured value of  $\Delta G(H_2O)$  for TyrRS( $\Delta 1$ ) (41  $\pm$  1 kcal·mol<sup>-1</sup>) was in reasonable agreement with the predicted value (34.4 kcal·mol<sup>-1</sup>). The measured value of  $\Delta G_2(H_2O)$  for the unfolding of the monomeric intermediate  $(13.9 \pm 0.6 \text{ kcal} \cdot \text{mol}^{-1})$  was compatible with the free energy of unfolding for soluble globular monomeric proteins (27). The values of  $\Delta G_1(H_2O)$  and  $\Delta G_2(H_2O)$ , and thus those of  $K_1(H_2O)$  and  $K_2(H_2O)$ , were close (Table IV). Thus, about one-third of the global energy of stabilization for TyrRS( $\Delta 1$ ) came from the association between the two subunits, and one-third came from the secondary and tertiary interactions stabilizing each of the two molecules of the monomeric intermediate. The closeness of the  $\Delta G_1(H_2O)$  and  $\Delta G_{2}(H_{2}O)$  values made possible the observation of a monomeric intermediate of  $TyrRS(\Delta 1)$  in the presence of urea, contrary to other dimeric proteins for which  $\Delta G_2 \ll \Delta G_1$  (i.e.  $K_2 \gg K_1$ ). Up to 21% of the subunits were in this intermediate state for a total concentration of dimer equal to 100  $\mu$ g/ml and up to 72% for a total concentration equal to 2.5  $\mu$ g/ml (Tables III and V).

The amount of tyrosyl-tRNA synthetase in an *E. coli* cell is  $\sim 1400-2000$  molecules (28, 29). One molecule/*E. coli* cell, which has a mean volume of 1.15 fl, corresponds to a molarity equal to 1.4 nm. Therefore, the calculation of  $f_n(0)$  with the thermodynamic parameters in Table IV shows that 99.7% of the TyrRS molecules should be dimeric in the cell at 25 °C. The pyrophosphate exchange and tRNA<sup>Tyr</sup> charging reactions are usually performed *in vitro* at 25 °C at concentrations of enzyme above 100 and 0.5 nm, respectively (16, 17). Our results show that most of the enzyme molecules should be dimeric at these total concentrations (99% of the molecules at 100 nm and 82% at 0.5 nm). Moreover, high concentrations of tyrosine and ATP favor dimerization (30).

Cooperativity of Unfolding—Myers et al. (31) have studied the relationship between the denaturant m value, defined by Equation 8 in Table I, and the variation of accessible surface

area ( $\Delta$ ASA) between the folded and unfolded states for a series of 45 monomeric and dimeric proteins with known crystal structures. The value of  $\Delta$ ASA is strongly correlated with the number of residues in a protein and with the value of m. The application of these relationships to the dimer of  $TyrRS(\Delta 1)$ (which comprises  $2 \times 320$  residues) and to its monomeric intermediate (320 residues) gave predicted values of m and  $m_2$ . The predicted and measured values of m (6.8 and 5.9  $\pm$  0.2 kcal·mol<sup>-1</sup>·M<sup>-1</sup>, respectively) were in reasonable agreement if one notes that the above correlations were established with proteins that were shorter than  $TyrRS(\Delta 1)$  ( $\leq 415$  residues), mainly monomeric, and unfolded according to a two-state mechanism. Comparison of the predicted and measured values of  $m_2$  (3.5 and 2.5  $\pm$  0.1 kcal·mol<sup>-1</sup>·M<sup>-1</sup>, respectively) (Table IV) showed that the unfolding of the monomeric intermediate increased the exposure of the polypeptide to the solvent slightly less than expected for a native monomeric protein of the same length. The 30% difference between the predicted and measured values could have several causes if it was significant. For example, the accessible surface area of the monomeric intermediate could be slightly larger than the areas for native monomeric proteins of the same length. Some loops at the surface of the protein, whose crystallographic B factors are high (11), could be unfolded in the intermediate. However, the hydrophobic core of the TyrRS( $\Delta 1$ ) subunits was not exposed to the solvent in the monomeric intermediate because we found that it did not bind ANS and had its tryptophan residues partially buried. We tried to apply the correlation between mand  $\Delta ASA$  to the dissociation of the TyrRS( $\Delta 1$ ) dimer. This dissociation exposes 1520 Å<sup>2</sup> of accessible surface area on each subunit and thus a total of 3040  $Å^2$  (13). The corresponding value of  $m_1$  would be 0.70 kcal·mol<sup>-1</sup>·M<sup>-1</sup>. This predicted value was in good agreement with the measured value,  $0.90 \pm 0.06$ kcal·mol<sup>-1</sup>·M<sup>-1</sup> (Table IV). We found that the value of  $m_1$  was 2.8 times lower than the value of  $m_2$ . This finding indicated that the dissociation of the  $TyrRS(\Delta 1)$  dimer increased the protein surface area exposed to the solvent much less than the unfolding of the monomeric intermediate. It was compatible with the existence of the monomeric intermediate in a folded compact state.

Comparison with the C-terminal Domain-In a previous work (15), we studied the unfolding of the disordered C-terminal domain (TyrRS( $\Delta 3$ ), residues 320-419) of tyrosyl-tRNA synthetase from B. stearothermophilus by urea under equilibrium conditions. We analyzed its unfolding profile with a twostate model and found the following values for its thermodynamic parameters:  $\Delta G(H_2O) = 4.3 \pm 0.4 \text{ kcal·mol}^{-1}, m =$  $0.65 \pm 0.08 \text{ kcal} \text{mol}^{-1} \text{M}^{-1}$ , and  $f_u^{-1}(0.5) = 6.65 \text{ M}$  urea (where the errors correspond to S.D. values in the fitting of a global equation to the fluorescence data). These data showed that the concentration of half-unfolding for  $TyrRS(\Delta 3)$  was higher than the value for the monomeric intermediate of  $TyrRS(\Delta 1)$ . Thus, TyrRS( $\Delta 3$ ) was at least as resistant to unfolding by urea as TyrRS( $\Delta 1$ ). However, the  $\Delta G(H_2O)$  stability of TyrRS( $\Delta 3$ ) was much lower than the stability of the monomeric intermediate of TyrRS( $\Delta 1$ ), because of its lower *m* value. The *m* value for TyrRS( $\Delta 3$ ) was only half of the value that could be predicted from its number of residues, 1.29  $\text{kcal·mol}^{-1} \cdot M^{-1}$  (31). This comparison suggested that the unfolding of  $TyrRS(\Delta 3)$  by urea was less cooperative than expected for a monomeric protein of the same length (15).

Unfolding Mechanism—We monitored the unfolding of TyrRS( $\Delta 1$ ) by urea using several signals. The intensity of fluorescence at 330 nm allowed us to show the existence of an equilibrium in the presence of urea among the native dimeric state of TyrRS( $\Delta 1$ ), a monomeric intermediate state, and the

unfolded state. The monomeric intermediate had a 5-fold lower molar fluorescence intensity in the absence of urea than one subunit of native dimeric TyrRS( $\Delta 1$ ). This variation of intensity could be due to global or local structural changes around some tryptophan residues or to the loss of fluorescence transfer between the tyrosines and tryptophans of one subunit and those of the other subunit upon dissociation. The ratio of the fluorescence intensities at 330 and 350 nm showed that the mean environment of the tryptophan residues became cooperatively more polar during the unfolding of the monomeric intermediate. Therefore, the monomeric intermediate had its tryptophan residues partially buried. It possessed a large part of the secondary structure of the native subunit, according to the CD ellipticity. It was compact, according to the partition coefficient in fast-SEC. It was not in a molten globular state, according to the lack of ANS binding. Finally, the activity of pyrophosphate exchange showed that the inactivation of  $TyrRS(\Delta 1)$  occurred at a much lower concentration of urea than the dissociation of its subunits. This inactivation occurred before any of the structural signals tested, and therefore, our results did not allow us to determine its structural cause. Thus, our results showed that urea led to the dissociation of the  $TyrRS(\Delta 1)$  dimer into a monomeric intermediate that had its tryptophan residues partially buried, had secondary structure, was compact, and was not a molten globule. The extrapolation of these results suggested that these different conformational states could also exist in equilibrium in the absence of urea.

Comparison with the Properties of an Interface Mutation-In TyrRS, the two symmetrical copies of Phe-164 interact with each other across the subunit interface. The side chain of Phe-164 has been changed into Asp. The mutant enzyme TyrRS (F164D) is monomeric and compact in fast-SEC experiments at high pH, which favors the ionization of the aspartate residues at position 164 and disfavors dimerization. The free monomer, unlike the dimer, does not bind tyrosine and is enzymatically inactive (30). Our work showed that wild-type  $TyrRS(\Delta 1)$  could adopt a folded compact monomeric conformation. The results were thus compatible with the data on TyrRS (F164D). A comparison between wild-type  $TyrRS(\Delta 1)$  and a F164D derivative in experiments of unfolding by urea could determine whether the mutation F164D stabilizes the monomeric intermediate by introducing a charged residue in the hydrophobic interface, which becomes exposed to the solvent upon dissociation of the subunits.

Up to now, it has never been possible to detect and thus measure the dissociation of the TyrRS and  $TyrRS(\Delta 1)$  dimers under native conditions (26, 32). Therefore, our experiments of unfolding by urea, monitored by spectrofluorometry, resulted in the first estimation of the dissociation constant  $K_1(H_2O)$  for the wild-type dimer (84 pM). The dissociation constant  $K_d$  for TyrRS (F164D) and other mutants of Phe-164 has been measured by enzyme kinetics methods (32). At low pH, which disfavors the ionization of the aspartate in position 164 and favors

dimerization, the  $K_d$  for the TyrRS (F164D) dimer is equal to 30 mM and thus  $3 \times 10^5$  times higher than the  $K_1(H_2O)$  for wildtype TyrRS( $\Delta 1$ ). At high pH, which disfavors dimerization,  $K_d$ is equal to 100 mM and thus  $\sim 10^6$  times higher than  $K_1(H_2O)$ . The corresponding variations of the free energy of dissociation  $(\Delta\Delta G)$ , on going from the wild type to mutant F164D, would be equal to 7.5 kcal·mol<sup>-1</sup> at low pH and 8.2 kcal·mol<sup>-1</sup> at high pH. It seems that the mutation F164D does not completely abolish the dimerization of TyrRS because the association between the subunits of the wild-type dimer is particularly strong. Similar experiments with the dimer of the gene V protein of bacteriophage M13, whose  $K_d$  is 1.25  $\mu$ M, led to the monomerization of the mutant protein F68D under all the pH conditions tested (33).

Acknowledgments-We thank Valérie Guez for valuable advice, Alain Chaffotte for help with the circular dichroism experiments, and Michel E. Goldberg for constant interest.

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