Stability of a thermophilic TIM-barrel enzyme: indole-3-glycerol phosphate synthase from the thermophilic archaeon *Sulfolobus solfataricus**

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The stability and activity of indole-3-glycerol phosphate synthase from *Sulfolobus solfataricus* were studied as a function of pH and temperature. In this paper we focus on three points: (1) the long-term stability of the protein to irreversible denaturation at high temperature; (2) the short-term stability of the protein to reversible temperature-driven unfolding; and (3) the dependence of its activity on temperature. Results can be summarized as follows: (a) the same first-order kinetic constant ($0.020 \pm 0.003 \text{ min}^{-1}$) was determined at different pH values (6.5, 8.0 and 9.5) from long-term stability experiments at 80 °C; (b) short-term stability experiments revealed different behaviour in two different pH ranges (6.5–8.0, 8.5–9.5), suggesting that the melting temperature is higher at alkaline than at neutral pH; (c)

the dependence of activity on temperature was investigated at pH 7.0 and 9.0, and a discontinuity was observed in the Arrhenius plot of k_{cat} values at pH 9.0. We also investigated the stability in the presence of guanidinium chloride at 20 °C either at pH 7.0 or at pH 9.0, and we present data that indicate that the unfolding mechanism closely approaches a two-state model at pH 7.0 and a more complex mechanism at pH 9.0. Satisfactory fitting of the equilibrium unfolding transition obtained by fluorescence measurements at pH 9.0 required a model that involves a stable intermediate in addition to the native and unfolded forms. At 20 °C the folded conformation is more stable than the unfolded conformation by $(14.7 \pm 1.2) \text{ kJ/mol}$ at pH 7.0 and by $(25.5 \pm 1.8) \text{ kJ/mol}$ at pH 9.0.

INTRODUCTION

Indole-3-glycerol phosphate synthase (IGPS), an enzyme of the tryptophan biosynthetic pathway, catalyses the conversion of 1-[(2-carboxyphenyl)amino]-1-deoxyribulose 5-phosphate to indole-3-glycerol phosphate. IGPS has been found to occur in several prokaryotic and eukaryotic micro-organisms: in some cases, such as in *Bacillus subtilis* [1,2] and in *Pseudomonas putida* [3], it is a monomeric and a monofunctional enzyme; in others, such as in *Escherichia coli* [4–6] it is associated with other enzymic activities in a multidomain and multifunctional polypeptide. In addition, it can be found associated with (phosphoribosyl)anthranilate isomerase in a loose multienzyme complex, as is the case in *Brevibacterium flavum* [7].

In previous papers we reported the cloning [8] and expression in *E. coli* [9] of the gene encoding IGPS from *Sulfolobus solfataricus* (IGPSSs), a thermophilic archaeon that grows optimally at 85 °C and pH 3.5. The recombinant protein was found to be monomeric and monofunctional. Indirect analyses such as hydropathy and chain flexibility profiles, as well as helical wheel analysis and examination of the CD spectrum, allowed us to confidently predict an $(\alpha/\beta)_8$ barrel fold, namely an eight-fold parallel β -barrel with α -helices on the outside connecting the β -strands, preceded at the N-terminus by a peptide of about 40 amino acids. These analyses were based on sequence alignment of IGPSSs with the sequence of IGPS from *E. coli* (IGPSEc), which is the sequence of the first 254 amino acids of the bifunctional *E. coli* protein indole-3-glycerol phosphate syn-

thase: *N*-(5'-phosphoribosyl)anthranilate isomerase whose three-dimensional structure is known [10]. Crystallographic analysis has recently confirmed our prediction [11,12].

The ease of obtaining large amounts of this protein, its small size (248 amino acids), its monomeric structure and the absence of disulphide bonds, as well as the interest because of its archaeal origin, make IGPSSs a good model system for studying the energetics and mechanism of protein unfolding.

MATERIALS AND METHODS

Materials

Pure guanidinium chloride (ULTROL Grade) was purchased from Calbiochem. The concentration of guanidinium chloride was determined refractrometrically using tabulated values of refractive index [13]. 1-[(2-Carboxyphenyl)amino]-1-deoxyribulose 5-phosphate was chemically synthesized [14]. All other chemicals were reagent grade from Sigma. All buffers were prepared at room temperature and the pH was modified taking into account an appropriate $\Delta pK_a/\text{deg}$: -0.011 for Mes, -0.020 for Tes, -0.0085 for Pipes, -0.009 for 2-(*N*-cyclohexylamino)-ethanesulphonic acid (Ches) and -0.031 for Tris [15].

Protein purification and enzyme assay

IGPSSs was expressed in *E. coli* and purified using a modification of a protocol previously described [9]. The protein extract was

Abbreviations used: IGPS, indole-3-glycerol phosphate synthase (EC 4.1.1.48); IGPSSs, indole-3-glycerol phosphate synthase from *Sulfolobus solfataricus*; IGPSEc, indole-3-glycerol phosphate synthase from *Escherichia coli*; Ches, 2-(*N*-cyclohexylamino)-ethanesulphonic acid; GdmCl, guanidinium chloride.

* Dedicated to Professor A. Ballio on the occasion of his 75th birthday.

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heated for 10 min at 75 °C, ammonium sulphate was added to 40 % saturation and the clear supernatant was loaded onto an affinity column of anti-IGPSSs antibodies: the column and the sample were previously equilibrated in 50 mM Tris/HCl, pH 7.5, the column was washed with 20 mM Tris/HCl, pH 7.5/2 M KCl and eluted with the equilibration buffer containing 3 M guanidinium chloride (GdmCl).

Standard conditions for the enzyme assay were: 20 mM Tris/HCl, pH 7.5, 60 °C and 0.3 mM 1-[(2-carboxyphenyl)amino]-1-deoxyribulose 5-phosphate. The activity was determined by measuring the increase of absorbance intensity at 280 nm due to the conversion of substrate to indole-3-glycerol phosphate. It was considered that the complete conversion of 0.10 mM 1-[(2-carboxyphenyl)amino]-1-deoxyribulose 5-phosphate to indole-3-glycerol phosphate results in an increase in absorbance at 280 nm of 0.448 [5].

The specific activity of the purified enzyme under standard conditions was 5.7 units/mg. Protein concentration was determined using the Bio-Rad Protein Assay System [16].

Thermal properties

(a) Long-term stability experiments were performed at 80 °C using two different IGPSSs concentrations (0.1 and 0.4 mg/ml) at pH 6.5 in 20 mM Mes/NaOH ($I = 0.018$ M), at pH 8.0 in 20 mM Tes/NaOH ($I = 0.020$ M) and at pH 9.5 in 20 mM Ches/NaOH ($I = 0.017$ M). Samples were incubated for various periods of time, cooled in ice and assayed under standard conditions at 60 °C. (b) Short-term stability of IGPSSs at different pH values was followed by recording the CD spectrum in the wavelength range 200–250 nm and in the temperature range 25–95 °C, and measuring the ellipticity at 220 nm. For each pH value different samples were prepared to a final concentration of 0.2 mg/ml: each sample was equilibrated at the chosen temperature for 4 min before recording the CD spectrum. The following buffers were used at the various pH values: 20 mM Mes/NaOH, pH 6.5 ($I = 0.014$ M), 20 mM Tes/NaOH, pH 7.0 ($I = 0.005$ M), 7.5 ($I = 0.010$ M) and 8.0 ($I = 0.015$ M), 20 mM Ches/NaOH, pH 8.5 ($I = 0.003$ M) and 9.5 ($I = 0.012$ M), 20 mM Tris/HCl, pH 7.0 ($I = 0.019$ M) and 9.0 ($I = 0.003$ M). The measurements were performed in a Jasco J-500 spectropolarimeter equipped with a thermostatically controlled cuvette holder using 0.1 cm path length cuvette. The temperature of each sample was directly monitored in the cell using a thermistor sensor. (c) The temperature dependence of the fluorescence emission spectrum was examined by increasing the temperature from 25 to 75 °C at approx. 0.5 °C/min. Excitation was at 295 nm and the protein (0.05 mg/ml) was equilibrated in either 20 mM Ches/NaOH, pH 9.0 ($I = 0.007$ M) or 20 mM Pipes/NaOH, pH 7.0 ($I = 0.012$ M). The experiment was also performed using Tris/HCl buffer (20 mM), pH 7.0 ($I = 0.019$ M) or pH 9.0 ($I = 0.003$ M): to take into account the relatively high $\Delta pK_a/\text{deg}$ of Tris/HCl, different samples were prepared by diluting a concentrated sample of protein in an appropriate volume of buffer that had been previously equilibrated at the chosen temperature. A Perkin Elmer MPF66B spectrofluorimeter was used and the sample was stirred continuously to minimize the temperature gradient in the cell (0.5 × 1 cm). The temperature of the samples was directly monitored in the cell using a thermistor sensor. (d) The activity at different temperatures ranging from 25 to 75 °C, was measured by monitoring the rate of increase in absorbance at 280 nm when the enzyme was added to a reaction mixture containing 0.3 mM of 1-[(2-carboxyphenyl)amino]-1-deoxyribulose 5-phosphate in 20 mM Tris/HCl, pH 7.0

or pH 9.0, that had previously been equilibrated at the desired temperature.

Guanidinium chloride-induced denaturation

IGPS (0.05 mg/ml) was incubated at 20 °C in 20 mM Tris/HCl, pH 7.0 or 9.0 (using a thermostatically controlled water bath), in the presence of various concentrations of GdmCl until unfolding equilibrium was obtained. Preliminary experiments were carried out by fluorescence measurements to determine how long an incubation period was necessary for equilibrium to be reached. Incubation at different GdmCl concentrations was allowed to proceed for known periods of time ranging from 5 min to 24 h, after which the fluorescence emission spectrum (excitation at 295 nm) was recorded. Equilibrium was attained within the first 4 h of incubation; in fact no further changes of the emission spectrum were observed at a longer incubation time at any guanidinium concentration, and therefore a 4 h incubation was considered adequate to reach equilibrium in the subsequent experiments.

Guanidinium-induced unfolding was monitored by measuring the CD at 220 nm or the shift of the fluorescence emission maximum (excitation at 295 nm) as a function of denaturant concentration. CD measurements were performed at 20 °C on a JASCO J-500 spectropolarimeter using a 0.5 cm path length cell. Molar ellipticities were calculated from observed ellipticity values, θ_{obs} , according to:

$$[\theta]_{220} = (100 \theta_{\text{obs}} M_r) / (l c) \quad (1)$$

where θ_{obs} is the measured ellipticity in deg, l is the cell path length in cm, c is the protein concentration expressed in mg/ml, and M_r is the relative molecular mass of the protein. Fluorescence measurements were performed at 20 °C on a Perkin Elmer MPF66B spectrofluorimeter in a 0.5 × 1 cm cell.

The experimental data were fitted to equation (2) [17] using a non-linear least-squares method to obtain the m -values and $\Delta G_u^{\text{H}_2\text{O}}$:

$$y_{\text{obs}} = \frac{(y_n + m_n [\text{GdmCl}]) - (y_u + m_u [\text{GdmCl}])}{1 + K} + (y_u + m_u [\text{GdmCl}]) \quad (2)$$

where y_{obs} is the experimental value observed, y_n and y_u are the values for the native and the unfolded protein at 0 M denaturant, m_n and m_u are the slopes of the pre- and post-transitional regions respectively, K is defined by equation (3) for a two-state model ($N \rightleftharpoons U$) (N = native, U = unfolded) or equation (4) [18] for a three-state model ($N \rightleftharpoons I \rightleftharpoons U$) (I = intermediate):

$$K = \exp - \{(\Delta G_u^{\text{H}_2\text{O}} - m[\text{GdmCl}]) / RT\} \quad (3)$$

where $\Delta G_u^{\text{H}_2\text{O}}$ is the difference in Gibbs free energy between the native and unfolded protein at 0 M denaturant, R is the gas constant [8.314 J/(mol·K)], T is the absolute temperature and $m = d\Delta G_u/d[\text{GdmCl}]$;

$$K = (K_1 K_2 + Z K_1) / [1 + (1 - Z) K_1] \quad (4)$$

where K_1 is the constant for native–intermediate equilibrium while K_2 is the constant for intermediate–denatured equilibrium and both were assumed to depend on guanidinium concentration according to equation (3); Z is the fractional change in the parameters in the transition from N to I and is defined by

$Z = (y_i - y_n)/(y_u - y_n)$, where y_i is the value of a given parameter for the intermediate species, I . In this case $\Delta G_u^{\text{H}_2\text{O}}$ is the difference in Gibbs free energy between the native and intermediate ($\Delta G_{u1}^{\text{H}_2\text{O}}$) or between the intermediate and the unfolded protein ($\Delta G_{u2}^{\text{H}_2\text{O}}$) at 0 M denaturant.

The fraction of unfolded protein was derived from the experimental data by assuming two-state transition of unfolding according to equation (5):

$$f_u = (y_n - y_{\text{obs}})/(y_n - y_u) \quad (5)$$

while the fractions of the native f_n , intermediate f_i and unfolded f_u forms at various concentrations of denaturant in the case of a three-state transition model can be calculated by equations (6), (7) and (8):

$$f_i/f_n = K_1 = \exp\{-(\Delta G_{u1}^{\text{H}_2\text{O}} - m[\text{GdmCl}])/RT\} \quad (6)$$

$$f_u/f_i = K_2 = \exp\{-(\Delta G_{u2}^{\text{H}_2\text{O}} - m[\text{GdmCl}])/RT\} \quad (7)$$

$$f_n + f_i + f_u = 1 \quad (8)$$

Renaturation of denatured protein was induced by diluting the samples with buffer to obtain the chosen final concentration of denaturant. Samples containing 0.225 mg/ml of IGPSSs were equilibrated in 5.0 M GdmCl then diluted 4.5-fold by addition of buffer or buffer containing 1.13 M GdmCl. Final concentrations of denaturant were 1.1 M or 2.0 M.

For both CD and fluorescence measurements the blank spectrum was subtracted for each sample.

The fit was achieved using the non-linear Levenberg–Marquardt method with constrained range 0–1 on the Z parameter. The errors on the single fitting parameters ($\Delta G_u^{\text{H}_2\text{O}}$, m , Z) were calculated with a confidence limit of 95.4% by the Monte Carlo simulation method [19].

RESULTS

Thermal properties of recombinant IGPSSs

Thermoresistance

Stability against irreversible processes (i.e. thermoresistance or long-term stability) at 80 °C was measured in 20 mM Mes/

Figure 1 Thermal unfolding of IGPSSs as a function of pH

Melting profiles were determined following the increase in ellipticity at 220 nm using samples (0.2 mg/ml) equilibrated at different pH values. The buffers used were: Mes/NaOH, pH 6.5, Tes/NaOH, pH 7.0, 7.5 and 8.0, Ches/NaOH, pH 8.5 and 9.5. A different batch of buffer was prepared for each temperature. The sample was allowed to equilibrate for 4 min before recording the CD spectrum.

Figure 2 Shift of fluorescence emission maximum wavelength of IGPSSs as a function of temperature

The sample, 0.05 mg/ml in 20 mM Pipes/NaOH, pH 7.0 (open circles) and in 20 mM Ches/NaOH, pH 9.0 (solid circles), was progressively heated (0.5 °C/min) from 25 to 70 °C and then slowly cooled again (triangles).

NaOH, pH 6.5, 20 mM Tes/NaOH, pH 8.0 or 20 mM Ches/NaOH, pH 9.5. No change in light scattering was observed upon heating, and the stability of IGPSSs did not depend on pH (in the range examined) or on protein concentration (in the range 0.1–0.4 mg/ml). The first-order kinetic constant for thermal inactivation was determined to be $0.020 \pm 0.003 \text{ min}^{-1}$.

Thermostability

Stability of IGPS against thermally induced reversible unfolding processes (i.e. thermostability or short-term stability) was investigated by CD measurements at 220 nm, using different buffer conditions ranging from pH 6.5 to 9.5 in the temperature range 25–95 °C (Figure 1). In order to investigate thermostability over this pH range, different buffers were required: Tes/NaOH, Mes/NaOH and Ches/NaOH.

Our study confirmed that melting profiles are strongly influenced by pH, and that thermostability clearly increases at alkaline pH; in particular it was found that two ranges of pH, 6.5–8.0 and 8.5–9.5, can be distinguished: thermostability of IGPSSs is similar within each range but changes dramatically moving from one range to the other. To confirm that the observed increase in thermostability results from the increased pH and is not due to the stabilizing effect of the buffer used, the experiments were repeated using Tris/HCl buffer at pH 7.0 and 9.0; similar results were obtained (results not shown).

Fluorescence emission spectra (excitation at 295 nm) were recorded as a function of temperature either at pH 7.0 in Pipes/NaOH and in Tris/HCl buffers, or at pH 9.0 in Ches/NaOH and in Tris/HCl buffers, in the temperature range 25–75 °C. The temperature dependence of the wavelength of the emission maximum was different at the two different pH values: as shown in Figure 2, at pH 7.0 no significant change was observed over the temperature range 25–75 °C, while at pH 9.0 there is a reversible increase in the maximum emission wavelength with temperature starting from approx. 50 °C.

Thermophilicity

As is the case for other enzymes isolated from *S. solfataricus* (see [20–22]) IGPSSs is an extremely thermophilic enzyme. k_{cat} values

Figure 3 Arrhenius plots for the catalytic constant of IGPSSs

The k_{cat} values for the enzymic reaction were determined spectrophotometrically at different temperatures in 20 mM Tris/HCl at pH 7.0 (circles) and at pH 9.0 (squares) using saturating substrate concentrations.

Figure 4 Guanidinium chloride-induced unfolding transitions of IGPSSs

The transitions were measured by change of maximum fluorescence emission after excitation at 295 nm (panel A) and by CD at 220 nm (panel B) of protein samples (0.05 mg/ml) equilibrated in 20 mM Tris/HCl, pH 7.0 (open symbols) or 9.0 (solid symbols), at 20 °C. Each sample was allowed to equilibrate for 4 h before measuring the optical parameters. Refolding data obtained by dilution of unfolded samples are also shown (triangles).

at various temperatures were used to establish the maximal activity temperature (higher than 80 °C both at pH 7.0 and 9.0, results not shown) and to obtain an Arrhenius-type plot as shown in Figure 3. A unique straight line with a slope of 85 kJ/mol can fit k_{cat} values obtained in Tris/HCl, pH 7.0, while

values obtained at pH 9.0 can be best fitted by two straight lines with different slopes, 34 and 63 kJ/mol, that cross at approx. 50 °C.

Guanidinium-induced denaturation

Unfolding of IGPS at 20 °C in 20 mM Tris/HCl, pH 7.0 or 9.0, was induced by addition of GdmCl. Samples were incubated at different concentrations of the denaturant, ranging from 0 to 6.0 M. CD at 220 nm and shift of fluorescence emission maximum (excitation at 295 nm) were measured to monitor the GdmCl-induced unfolding of IGPS to probe changes in secondary and tertiary structure respectively (Figure 4). The reversibility of the unfolding transition was verified fluorimetrically following refolding after full denaturation and upon dilution with 20 mM Tris/HCl, pH 7.0 and 9.0, at 20 °C.

Figure 4 shows the guanidinium-induced unfolding curves for IGPS at pH 7.0 and 9.0 monitored by fluorescence and CD. Independent analyses of the individual curves shown in this Figure using equations (2) and (3) and assuming a two-state mechanism for unfolding were used to obtain values for $\Delta G_{\text{u}}^{\text{H}_2\text{O}}$ and the co-operativity parameter m . Values calculated using the different techniques are in good agreement at pH 7.0, but not at pH 9.0 (Table 1). Figure 5 shows the values of the fraction of protein unfolded as a function of denaturant concentration, calculated using equation (5). This calculation also shows good agreement between the fluorescence and CD data at pH 7.0, but not at pH 9.0. The coincidence of the transition curves at pH 7.0 indicates that under this condition the conformational changes that expose the tryptophan residue to the solvent also disrupt the secondary structure of the protein. At pH 9.0 fluorescence data do not fit very well to the two-state model, and a more complex model obtained by incorporating a third species, an intermediate that is partially unfolded, was used.

In order to characterize these transitions and to obtain quantitative thermodynamic parameters for the unfolding process, both fluorescence and CD data at pH 9.0 were computer fitted to a three-state model using equations (2), (3) and (4). As a comparison, the data collected at pH 7.0 were also analysed using a three-state model. The values for $\Delta G_{\text{u1}}^{\text{H}_2\text{O}}$, $\Delta G_{\text{u2}}^{\text{H}_2\text{O}}$ and Z , are shown in Table 1. At pH 7.0 both sets of data fit very well to either the two-state or the three-state model over the entire transition region as far as the χ^2 values are concerned, while the magnitude of Z , for both wavelength of fluorescence emission maximum and ellipticity at 220 nm, indicates that the intermediate in the three-state model is almost identical with the native state of the protein. Therefore we conclude that the unfolding transition of IGPS at pH 7.0 can be correctly described by a two-state model. Data sets at pH 9.0 were also fitted to a three-state model but CD data could be fitted either to a two-state or a three-state model; the three-state model provided a better fit to the fluorescence data. The magnitude of Z obtained from fluorescence measurements (0.14) indicates that about one-seventh of the total change of the fluorescence parameter observed in complete unfolding occurs in the transition from the native to the intermediate form. In addition, the Z value obtained from CD measurements (approx. 10^{-7}) is very close to zero. Thus the intermediate retains almost completely the native secondary structure and substantial tertiary structure.

The fractions of the native (f_{n}), intermediate (f_{i}) and unfolded (f_{u}) forms at various guanidinium concentrations were calculated from fluorescence data at pH 9.0 using equations (6), (7) and (8) and the values are shown in Figure 6. According to this model, the intermediate is the predominant species in solution at 1.5 M

Table 1 Thermodynamic parameters for the guanidinium-induced unfolding transitions of IGPSSs

The parameters were determined by fluorescence and CD at 20 °C at pH 7.0 and 9.0 using a two- or three-state model and were derived from data shown in Figure 4 using the non-linear Levenberg–Marquardt method. Errors were calculated by the Monte Carlo simulation method [19].

Technique	Two-state			Three-state					
	$\Delta G_{\text{H}_2\text{O}}^0$ (kJ/mol)	m (kJ · mol ⁻¹ · M ⁻¹)	χ^2	$\Delta G_{\text{H}_2\text{O}}^0$ (kJ/mol)	$\Delta G_{\text{H}_2\text{O}}^0$ (kJ/mol)	m_1 (kJ · mol ⁻¹ · M ⁻¹)	m_2 (kJ · mol ⁻¹ · M ⁻¹)	Z	χ^2
CD, pH 7.0	15.2 ± 1.1	6.2 ± 0.6	2.3	9.9 ± 1.2	4.7 ± 1.2	0.2 ± 0.1	5.8 ± 0.7	~ 10 ⁻⁶	2.4
Fluorescence, pH 7.0	14.1 ± 1.3	6.6 ± 0.7	12.5	12.1 ± 1.2	1.6 ± 1.2	1.0 ± 0.4	5.2 ± 4.1	~ 10 ⁻⁴	13.8
CD, pH 9.0	23.3 ± 1.5	9.2 ± 0.6	1.8	18.7 ± 1.3	7.3 ± 1.3	6.6 ± 1.7	4.8 ± 1.4	~ 10 ⁻⁷	1.7
Fluorescence, pH 9.0	10.1 ± 1.1	5.2 ± 0.6	9.3	18.4 ± 1.2	9.3 ± 0.8	16.5 ± 2.8	4.5 ± 0.4	0.14 ± 0.06	2.2

Figure 5 Two-state analysis of the guanidinium chloride-induced unfolding transitions

The fraction of unfolded protein, $f_u = (y_n - y_{\text{obs}})/(y_n - y_d)$, was derived from the experimental data by assuming a two-state transition mechanism of unfolding. Ellipticity at 220 nm (squares) and fluorescence maximum (circles) were used as different probes. The experiments were performed at 20 °C in 20 mM Tris/HCl, pH 7.0 (open symbols) and in 20 mM Tris/HCl, pH 9.0 (solid symbols). The protein concentration was 0.05 mg/ml.

Figure 6 Native (N), intermediate (I) and unfolded (U) states as fractions of the protein population during denaturation of IGPSSs as a function of guanidinium chloride concentration in 20 mM Tris/HCl, pH 9.0, 20 °C

The partition of states was calculated assuming a three-state model using fluorescence data:

$$f_i/f_n = \exp - \{(\Delta G_{\text{H}_2\text{O}}^0 - m[\text{GdmCl}])/RT\}, f_u/f_i = \exp - \{(\Delta G_{\text{H}_2\text{O}}^0 - m[\text{GdmCl}])/RT\},$$

$$f_n + f_i + f_u = 1.$$

GdmCl, while the unfolded species appears at 1.0 M GdmCl and becomes the predominant species at concentrations above 2.0 M.

DISCUSSION

IGPSSs is a relatively small protein (248 amino acids) for which an $(\alpha/\beta)_8$ barrel fold preceded at the N-terminus by a peptide of about 40 amino acids has been predicted [9]. Recent X-ray diffraction studies confirmed our prediction [11,12].

IGPSSs is quite stable against irreversible thermal denaturation: aggregation was not observed upon heating of IGPSSs (0.1 and 0.4 mg/ml) at 80 °C within the pH range explored (6.5–9.5). Moreover thermoinactivation is not dependent on initial enzyme concentration or pH, and all kinetics match the first-order law with correlation coefficients of 0.94. Since in IGPSSs there are no cysteines, it can be proposed that irreversible thermal inactivation is a monomolecular process due to the deamidation of asparagine and glutamine or to the formation of incorrect structures [23]. The two processes should provide different but balanced contributions at the different pHs, since it is well known that deamidation is more pronounced at alkaline rather than at neutral pH.

A peculiarity of IGPSSs, which was observed from the comparison with the analogous enzyme from *E. coli*, is a higher content of charged amino acids (31 % compared with 22 % of the mesophilic enzyme). Moreover the α -helices of the $(\alpha/\beta)_8$ barrel display a pronounced amphipaticity and possess negatively charged amino acids in the proximity of their amino termini. These findings support the hypothesis that salt bridges and polar interaction could play a role in the stabilization of IGPSSs. For this reason the influence of pH on the stability of IGPSSs was investigated.

It is a familiar assertion that globular proteins are more stable near their isoelectric points [24]. IGPSSs is an exception: in fact it is more stable to thermal unfolding at alkaline than at neutral pH despite the fact that it has a slightly acidic isoelectric point (pI = 6.0) (Figure 1). Melting profiles that were drawn from 25 to 95 °C, recording ellipticity at 220 nm, shift to higher temperatures on moving from the pH range 6.5–8.0 to the pH range 8.5–9.5.

Tryptophan fluorescence of IGPSSs was measured selectively by excitation at 295 nm in the temperature range 25–75 °C. When the wavelength of the emission maximum was plotted against temperature, the graph showed an increase in wavelength with increasing temperature at pH 9.0 starting from about 50 °C, but was insensitive to temperature changes at pH 7.0 (Figure 2). IGPSSs contains only one tryptophan residue, that is located at

position 8 in the amino acid sequence, so it is outside the TIM-barrel structure and it could be quite exposed to the solvent. This is also confirmed by the wavelength of the emission maximum, which is 338 nm at 25 °C at both pHs. The shift of emission maximum to higher wavelengths occurs only at alkaline pH and suggests that the tryptophan becomes more exposed to the solvent [25]. The change of fluorescence emission spectrum of IGPSSs at pH 9.0 as a function of temperature does not match the ellipticity data at 220 nm under the same conditions. In fact, ellipticity does not show significant changes over the temperature range 25–75 °C at pH 9.0 (Figure 1). Taking into account these results, we can assume a conformational change induced by temperature occurring only at pH 9.0, which modifies the tryptophan-8 environment and, possibly, the tertiary structure of the protein but not its secondary structure.

Evidence for the existence of an intermediate along the thermal-induced unfolding pathway of IGPSSs at alkaline pH derives also from analysis of the Arrhenius plot shown in Figure 3. The Arrhenius plot at pH 9.0 is clearly non-linear in the temperature range 25–75 °C with a discontinuity at approx. 50 °C, while at pH 7.0 in the same temperature range the plot is linear (Figure 3).

A possible hypothesis to explain the discontinuity in the Arrhenius plot involves an equilibrium between two conformational states of the protein, each with a different catalytic competence, which changes with temperature [26]. Experimental evidence has been produced in the case of some enzymes favoring this hypothesis [27,28]. As observed for most other enzymes showing discontinuity of the Arrhenius plot (see [27]), the activation energy for IGPSSs at pH 9.0 is higher at lower temperature (63 kJ/mol compared with 34 kJ/mol at high temperature). These data could be interpreted in terms of different flexibility of the catalytic site of the enzyme in the two different temperature ranges. Both the discontinuity at about 50 °C in the Arrhenius plot and the shift of the tryptophan fluorescence maximum starting at about 50 °C are suggestive of a common structural transition.

The occurrence of an intermediate along the thermal unfolding pathway at alkaline pH could be accompanied by the occurrence of an intermediate along the guanidinium-induced unfolding pathway at the same pH. For this reason fluorescence emission spectra and CD spectra were recorded as a function of GdmCl concentration at pH 7.0 and 9.0. Experimental data were processed according to a two- or a three-state model (Table 1). At pH 7.0 the estimates of both $\Delta G_u^{H_2O}$ (15.2 ± 1.1 and 14.1 ± 1.3 kJ/mol) and m [6.2 ± 0.6 and 6.6 ± 0.7 kJ/(mol·M)] are in close agreement when the CD and fluorescence data are independently fitted to a two-state model indicating that this model is probably correct. An independent analysis by a two-state model of the data at pH 9.0 gave two different values for $\Delta G_u^{H_2O}$ and for m : 23.3 ± 1.5 and 10.1 ± 1.1 kJ/mol and 9.2 ± 0.6 and 5.2 ± 0.6 kJ/(mol·M) respectively. At this pH the two-state model is adequate to describe the CD data but a three-state model is required to fit fluorescence data, indicating that a stable intermediate is probably present at this pH and that this intermediate differs from the native state only in the tertiary structure [the $\Delta G_u^{H_2O}$ value for the total process of unfolding, $(18.4 \pm 9.3) \pm (1.2 \pm 0.8)$ kJ/mol, is in good agreement with the $\Delta G_u^{H_2O}$ value determined by two-state fitting of CD data, (23.3 ± 1.5) kJ/mol]. The nature of the intermediates that occur

during temperature-driven and guanidinium-driven unfolding of IGPSSs is not clear at this stage. However, both intermediates have a far-UV CD spectrum similar to that of the native protein and display a red shift of the maximum of tryptophan emission fluorescence. It is possible that on increasing pH from 7.0 to 9.0, a group such as a lysine and/or an arginine and/or a tyrosine residue with an abnormally low pK_a or the N-terminal amino group is titrated and this results in the differential stabilization of the native and intermediate states. The N-terminal amino group plays a special role in IGPSSs; in fact it is involved in two out of five salt bridges that clamp the N-terminal peptide to the $(\alpha/\beta)_8$ core [11,12]. Its titration could result in the weakening of the interaction between the N-terminal peptide and the $(\alpha/\beta)_8$ barrel, in the loss of co-operation of the subdomains and in a three-state denaturation mechanism.

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