Spectrophotometric and Fluorometric Study of the Denaturation of *Euglena* Cytochrome *c*-552†

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ABSTRACT: The denaturation of *Euglena gracilis* cytochrome *c*-552 by urea and guanidine·HCl is evidenced by a remarkable enhancement of tryptophan fluorescence and by changes in the absorption spectrum. Comparison of the free energy of unfolding at pH 7.0 with corresponding values reported for horse cytochrome *c* indicates that cytochrome *c*-552 is considerably less stable. Spectra of unfolded cytochrome *c*-552 are pH dependent. p*K*s of 5.9 in 6 M guanidine and 7.1 in 8 M urea were evaluated for a spin transition, which may be assigned to deprotonation of the single histidyl residue of cytochrome *c*-552. The alkaline-denatured forms are of low spin and lack the 695-nm absorption band; the acid-denatured forms exhibit spectra characteristic of a high-spin iron. Comparison of the denaturation curves at the alkaline and acid ends of the transition indicates that the alkaline unfolding, leading to the formation of the low-spin denatured form, requires significantly lower concentrations of the denaturing agents, especially in the case of urea. Opposite results were obtained for horse cytochrome *c*. Cytochrome *c*-552 possesses a remarkable stability towards acid pH. Iodide quenching studies reveal heterogeneous accessibility of the two tryptophanyl residues toward the quencher at low concentrations of the denaturant and equal exposure at advanced stages of denaturation. These results imply that the unfolding of cytochrome *c*-552 does not represent a true two-state equilibrium.

Two main approaches marked previous denaturation studies of cytochromes *c*: one was to investigate the effect of various unfolding conditions on one typical representative of the class, horse cytochrome *c*; another was to compare cytochrome *c* of different primary structures. The second approach implied a search for correlations between the primary structures of the cytochromes and the stability of their native states, measured in terms of resistance to denaturation. Cytochrome *c* employed hitherto for these studies belonged to the homologous group of mitochondrial electron carriers characterized by a common, well-defined tertiary structure (Dickerson et al., 1976).

Taking the second approach we studied the denaturation of *Euglena* cytochrome *c*-552, an electron carrier in the photosynthetic electron-transfer chain. Concerning the primary structure (Pettigrew, 1974) and electrostatic charge, cytochrome *c*-552 bears no resemblance to mitochondrial cytochromes *c*. The net charge of the oxidized protein at neutral pH, calculated from its amino acid composition, is −8, in contrast to the respective value of +8 for horse cytochrome *c*. In both cytochromes, however, methionine and histidine coordination to the heme iron was established (Keller et al., 1977) and both share a similar reactivity (Ben Hayyim & Schejter, 1974; Aviram et al., 1976). In view of the suggestion of a common-fold and evolutionary origin for algal photosynthetic and mitochondrial respiratory cytochromes *c* (Dickerson et al., 1976), it was of interest to extend the comparison between the two groups to the process of denaturation. The denaturation studies of cytochrome *c*-552 by urea, Gdn·HCl,[1] and acid, as followed by absorption spectrophotometry and fluorometry, are reported below.

Experimental Procedure

Euglena gracilis cytochrome *c*-552 was purified as described by Pettigrew (1974). Horse heart cytochrome *c* type III was obtained from the Sigma Chemical Co. and used without purification. Aristar urea and Gdn·HCl were purchased from BDH Chemicals, Ltd.

Spectrophotometric measurements were performed on a Cary 118 spectrophotometer. Fluorescence was measured on a Hitachi-Perkin-Elmer spectrofluorometer using excitation and emission wavelengths at 295 and 350 nm, respectively. Whenever necessary, the fluorescence of the protein-free solutions was subtracted. All measurements were done at 23 °C, 15–30 min after addition of the denaturing agents. No time-dependent changes were detected during this period of time.

Iodide quenching was determined by titrating 2.5-mL aliquots of 10^{-6} M solutions of cytochrome *c*-552 in buffered urea or Gdn·HCl with a 5 M KI solution of the same concentration of the denaturant and 10^{-4} M in sodium thiosulfate. Fluorescence readings were corrected for the dilution of the protein. At concentrations employed, KI had no effect on the visible spectrum of the hemoprotein.

Results

The unfolding of the cytochrome *c*-552 molecule is accompanied by a significant enhancement of its tryptophan fluorescence, quenched in the native neutral protein by a radiationless energy transfer to the heme group (Aviram et al., 1976). Relative fluorescence values at different urea and Gdn·HCl concentrations at pH 7.0 are shown in Figure 1. The midpoints of these titrations are 4.6 M for urea and 2.5 M for Gdn·HCl. The maximal fluorescence yield, relative to that measured for an equimolar (in tryptophan) solution of *N*-acetyltryptophanamide, amounts to 83% in urea (8 M) and 71% in Gdn·HCl (6 M), pH 7.0.

The effects of urea and guanidine were also evident in the optical spectrum of *c*-552, mainly in the Soret and the 695-nm absorption regions; the 695-nm band disappeared while the

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[1] Abbreviations used: Gdn·HCl, guanidine hydrochloride; *c*-552, cytochrome *c*-552.

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TABLE I: Parameters of Denaturation of Cytochrome *c*-552.

Denaturant	$\Delta G_{D,H_2O}$ (kcal mol^{-1})[a]	m (cal mol^{-1} M^{-1})[a]	$\Delta G_{D,H_2O}$ (kcal mol^{-1})[b]	Δn[b]	$[D]_{1/2}$ (M)[c]
Gdn·HCl, pH 7.0	3.72	1460	7.51	15.2	2.5
Urea, pH 7.0	3.23	680	7.31	6.9	4.6
Urea, pH 5.0	5.72	915	11.2[d]	9.2[d]	6.3
Urea, pH 9.0					2.12

[a] From eq 3. [b] From eq 4, using $k = 1.2$. Activity of the denaturant was calculated as explained in the text. [c] The midpoint of the titration curve. [d] The correlation coefficient of the regression line is 0.944.

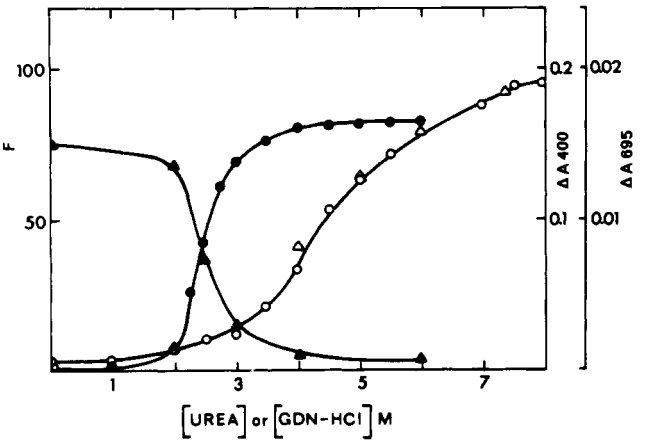

FIGURE 1: Fluorometric (○,●) and spectrophotometric (△,▲) titration of *c*-552 with urea (○,△) and Gdn·HCl (●,▲), pH 7.0, 50 mM phosphate buffer. The concentration of *c*-552 was 2×10^{-6} M for fluorometric measurements 3×10^{-6} M in the spectrophotometric titration by urea at 400 nm (△), and 10^{-5} M in the titration by Gdn·HCl at 695 nm (▲), 23 °C.

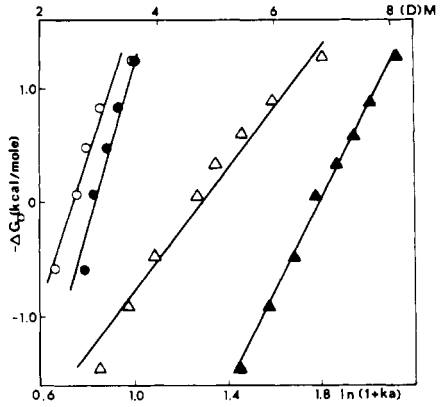

FIGURE 2: Variation of ΔG_D calculated from the data of Figure 1 with the concentration of the denaturant according to eq 3 (○,△) and with ln $(1 + ka)$ according to eq 4 (●,▲): (○,●) Gdn·HCl; (△,▲) urea. Calculations are described in the text.

Soret peak shifted to the blue and gained intensity. These changes occurred concomitantly with fluorescence enhancement (Figure 1).

The data of Figure 1 were analyzed in terms of a two-state mechanism. The apparent equilibrium constant, K_D, between the denatured and native forms was calculated using:

$$K_D = \frac{F - F_N}{F_D - F} \quad (1)$$

where F is the observed fluorescence intensity and F_N and F_D represent fluorescence intensities of the native and fully denatured states. Values of ΔG_D, the apparent free energy of denaturation, were obtained using:

$$\Delta G_D = -RT \ln K_D \quad (2)$$

Least-squares analysis was used to fit the values of ΔG_D to the equation:

$$\Delta G_D = \Delta G_{D,H_2O} - m[D] \quad (3)$$

$\Delta G_{D,H_2O}$ represents the free energy of unfolding in the absence of denaturant D and [D] is the concentration of the denaturant. By extending the linear variation of ΔG_D with D to zero concentration of the denaturant, values of $\Delta G_{D,H_2O}$ and m listed in Table I were obtained. The corresponding plots are shown in Figure 2.

The free energy of unfolding, $\Delta G_{D,H_2O}$, was also estimated by assuming that denaturation results from binding of the denaturant to the protein according to the equation:

$$\Delta G_D = \Delta G_{D,H_2O} - \Delta nRT \ln (1 + ka) \quad (4)$$

where k, a, and Δn represent, respectively, the binding constant of the denaturant to the protein, the activity of the denaturant, and the difference in the number of binding sites in the unfolded and native protein. The mean ion activity of Gdn·HCl was calculated from the equation of Aune & Tanford (1969) and urea activity was determined from the data of Ellerton & Dunlop (1966). For both denaturants a k value of 1.2 was used (Puett, 1973). The resulting estimates of $\Delta G_{D,H_2O}$ and Δn are listed in Table I, and the plots are shown in Figure 2.

Spectra of *c*-552 in 6 M Gdn·HCl and 8 M urea were pH dependent, as shown in Figures 3 and 4. These pH-dependent transitions may be defined as a low spin–high spin interconversion. In the alkaline side of the transition (above pH 8 in urea and pH 7 in guanidine) both denatured species were of low spin, devoid of the 695-nm band, and with the Soret maximum at 404 nm. Upon acidification, conversion to high-spin forms was evidenced by the appearance of bands at 620 and 490 nm, and a Soret band maximum positioned at 398 nm (Figures 3 and 4). The pKs for these heme-linked ionizations were 7.1 and 5.9 in 8 M urea and in 6 M guanidine, respectively. Using the initial and final absorbancy values corresponding to the low-spin and high-spin denatured cytochrome *c*-552, fractions of these forms at each pH were estimated; plots of the logarithms of their ratios against pH were linear for both urea and guanidine (not shown). The estimated slopes were 1.18 for urea and 1.28 for guanidine, suggesting one proton dissociation.

In general, this behavior of *c*-552 is similar to that reported for horse cytochrome *c*, with respect to the spectral and fluorescence changes upon denaturation and the pH-dependent high spin–low spin transition (Tsong, 1974; Babul & Stellwagen, 1971). In the horse protein pKs of 5.0 and 5.1 for 9 M urea and 6 M Gdn·HCl were evaluated for these transitions (Tsong, 1975) and attributed to the protonation of His-18, the residue that occupies the fifth coordination position of the iron

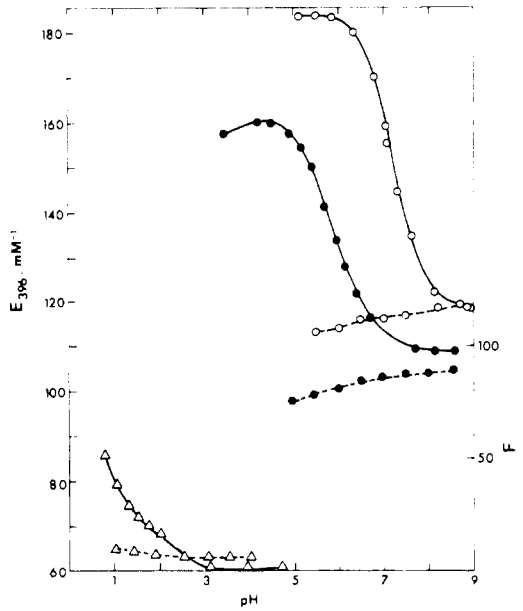

FIGURE 3: Spectrophotometric titration curves of *c*-552 (5×10^{-6} M) in aqueous solutions (△), in 6 M Gdn·HCl (●), and 8 M urea (○). Dashed lines represent the corresponding changes in tryptophan fluorescence measured in 2×10^{-6} M solutions of *c*-552, 23 °C. The pH was varied by addition of NaOH or HCl to the measuring cuvette.

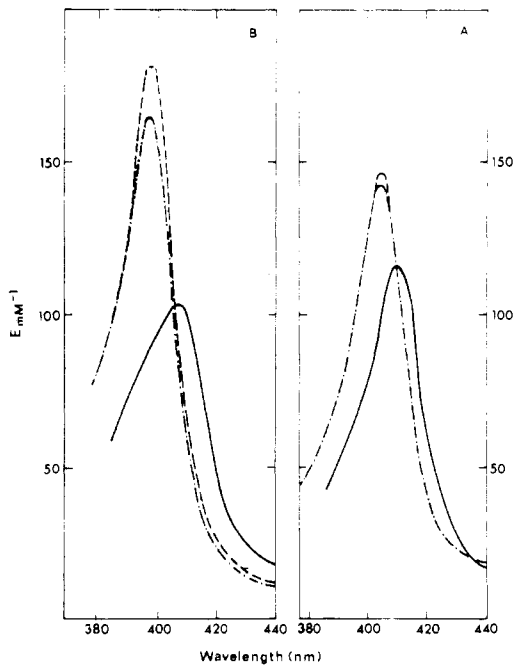

FIGURE 4: Soret absorption peak of *c*-552: (—) aqueous solution; (- - -) 8 M urea; (·····) 6 M Gdn·HCl; (A) alkaline forms, pH 7.0 native, pH 7.2 Gdn·HCl; pH 9.0 urea; (B) acid forms, pH 0.95 native; pH 5 urea; pH 4.4 Gdn·HCl. For experimental conditions see Figure 3.

(Stellwagen, 1971). Disruption of the iron–protein bonds by acidification below the above-mentioned p*K*s was accompanied in horse cytochrome *c* by an additional fluorescence enhancement (Tsong, 1975). In *c*-552, however, formation of the high-spin species in the presence of denaturant caused a small but definite decrease in the relative fluorescence yield (Figure 3).

It was of interest to investigate whether the alkaline and acid species of *c*-552 are equally susceptible to denaturation. Figure 5 summarizes changes in fluorescence measured in guanidine (pH 4.0 and 7.0) and urea (pH 5.0 and 9.0). In guanidine, the

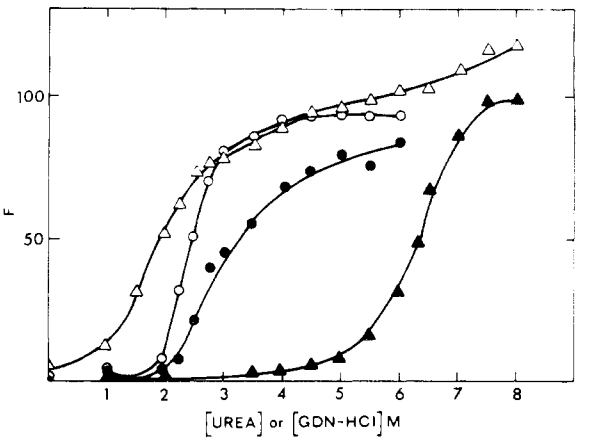

FIGURE 5: Fluorescence titrations curves of *c*-552 in 8 M urea [(△) pH 9.0; (▲) pH 5.0)] and 6 M Gdn·HCl [(○) pH 7.0; (●) pH 4.0)]; 50 mM buffers: acetate for pH 4.0 and 5.0, phosphate, pH 7.0; Tris-HCl, pH 9.0; 23 °C.

dependence on the concentration of denaturant at the alkaline end (pH 7.0) is somewhat steeper and the concentration required for 50% reaction lower. In urea the differences are much more pronounced. The curve at pH 9.0 indicates that denaturation occurs in two stages: in the first stage the fluorescence yield increases in a cooperative manner with a midpoint at 1.7 M urea. This reaction, taking place at low urea concentrations, accounts for about 60% of the total enhancement. Above 2.5 M urea, the fluorescence increases almost linearly with the concentration of the denaturant. Since it is evident that at pH 9.0 the assumption of a two-state mechanism is not justified, neither of the two models could be applied. At the other end of the pH-dependent transition (pH 5.0) the curve is steep and monophasic and requires 6.3 M urea for 50% reaction. The calculated parameters characterizing denaturation at this pH are listed in Table I. While in cytochrome *c*-552 significantly lower concentrations of denaturant were required for the formation of the low-spin denatured species, the opposite was demonstrated for horse cytochrome *c* (Figure 6). In the latter, lower concentrations of urea cause unfolding at lower pH, where the denatured form is of high spin (Tsong, 1975).

In native respiratory cytochromes *c* in aqueous solution, displacement of the protein ligands from iron coordination and their replacement by the solvent occur at low pH with an apparent p*K* of 2.5 (Knapp & Pace, 1974; Lanir & Aviram, 1975). This process is characterized spectroscopically by the appearance of a high-spin species, and, fluorimetrically, by the enhancement of tryptophan emission (Tsong, 1975). These criteria, as well as optical rotatory dispersion measurements (Knapp & Pace, 1974), indicate that the acidic ionization represents unfolding of the protein. *Euglena c*-552 is, however, much more stable in acid solution, as indicated by the very small spectroscopic and fluorescence changes at low pH (Figure 3). The optical spectrum at a pH as low as 0.9 is still not characteristic of a high-spin cytochrome *c* species. Although formation of a high-spin band at 620 nm may be noticed (Figure 7), the 695-nm band is still evident and the Soret peak, contrary to the expected intensification, loses intensity (Figure 4). These changes are almost entirely reversible.

From kinetic studies by Ikai et al. (1973) and equilibrium measurements by Myer (1968) and Stellwagen (1968), it was concluded that denaturation of horse cytochrome *c* is not a true two-state phenomenon. In the present study this question was approached through the iodide fluorescence quenching method as employed by Lehrer (1971), taking advantage of the pres-

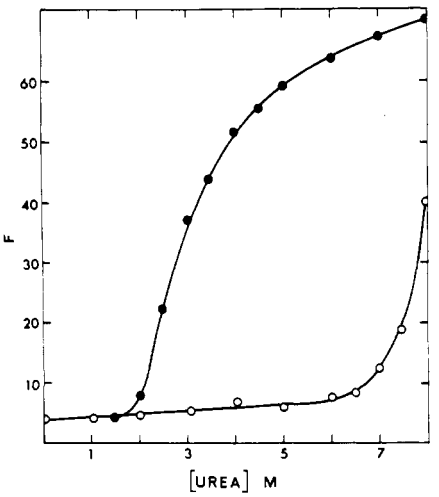

FIGURE 6: Fluorescence titration curves of horse ferricytochrome *c* (2×10^{-6} M) in 8 M urea; (○) pH 7.0; (●) pH 3.5; 23 °C.

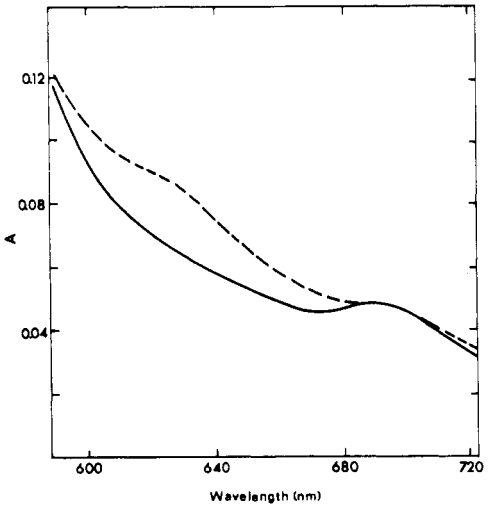

FIGURE 7: Spectrum of an aqueous solution of *c*-552: (—) pH 7.0; (---) pH 1.17 (acidified with HCl).

ence of two tryptophan residues in the molecule. Iodide quenching at different concentrations of the denaturing agents (pH 7.0) was followed to obtain information about accessibility of the two tryptophans toward the quencher at different stages of denaturation.

Iodide quenches tryptophan fluorescence, mainly by a collisional mechanism (Lehrer, 1971). In the case of a monotryptophan protein, the fluorescence quenching should obey, therefore, the Stern–Volmer equation:

$$\frac{F_0}{F} = 1 + K_Q[X] \qquad (5)$$

where F_0 and F are the fluorescence intensities at 0 concentration and the concentration of the quencher ([X]), and K_Q is the quenching constant. Similarly, a linear dependence of F_0/F on the concentration of a quencher is expected in denatured multitryptophan proteins (Lehrer, 1971; Eftink & Ghiron, 1976). In the case of multitryptophan proteins in which the fluorescing residues differ in accessibility toward a dynamic quencher, a downward curvature of the plot of F_0/F vs. [X] is to be expected.

The internal energy transfer from tryptophan to heme precludes the use of fluorescence measurements in native hemoproteins. This obstacle, as shown above, is removed upon denaturation. Upon application of the iodide quenching method to *c*-552 in the presence of either urea or guanidine, the following results were obtained. At pH 7.0, at concentrations above 3 M for guanidine and 5 M for urea, the Stern–Volmer plots were linear, indicating homogeneous accessibility of the fluorescing residues (Figures 8 and 9). At 2 M guanidine and 3 and 4 M urea, the plots bent downward, indicating heterogeneity in exposure.

Discussion

The unfolding of cytochrome *c*-552 by urea and Gdn·HCl was followed by absorption and tryptophan fluorescence measurements. In native cytochrome *c*-552 the fluorescence of the two tryptophanyl residues is quenched by energy transfer to heme (Weber & Teale, 1959; Aviram et al., 1976). Unfolding increases the fluorescence yield by changing the position (distance and/or angle) of the fluorophores with respect to the prosthetic group. As indicated by Figure 1, fluorescence changes at pH 7.0 coincide with spectral effects. The finding that two independent physical methods give similar results justified the assumption of a two-state transition (Tanford, 1968). $\Delta G_{D,H_2O}$ values obtained from Figure 3 correlating linearly ΔG_D with the concentration of denaturants were 3.23 and 3.72 kcal mol^{-1} for urea and Gdn·HCl, respectively (Table I). For horse cytochrome *c* at pH 6.5 a value of 7.27 kcal mol^{-1} was reported for Gdn·HCl (Knapp & Pace, 1974) and a value of 7.44 kcal mol^{-1} was calculated for urea from the results of Tsong (1976). Thus, stability of cytochrome *c*-552 appears to be distinctly lower. The values of m are also lower in our case: 680 and 1460 cal mol^{-1} M^{-1} in urea and Gdn·HCl, corresponding to 990 and 3010 cal mol^{-1} M^{-1} for horse cytochrome *c*. This may reflect a smaller number of residues exposed to the denaturant in the unfolding process (Tanford, 1968) and a lower molecular weight of cytochrome *c*-552. The ratios of m(Gdn·HCl)/m(urea) are 2.14 in cytochrome *c*-552 and 3.04 in horse cytochrome *c*, suggesting that the unfolding unit is more nonpolar in the algal protein (Greene & Pace, 1974).

Comparison of the results obtained using eq 4, assuming binding of denaturant to the protein, leads to similar conclusions. $\Delta G_{D,H_2O}$ of 7.51 kcal mol^{-1} for cytochrome *c*-552 in Gdn·HCl (Table I) is to be compared to 12.7 kcal mol^{-1}, reported by Knapp & Pace (1974); the corresponding estimates of Δn are 15.1 and 26.4 and indicate again a significantly lower number of residues exposed to the denaturant in cytochrome *c*-552. It must be remembered, however, that these conclusions are based on the assumption of a two-state transition, which leads to lower estimates for $\Delta G_{D,H_2O}$ and m or Δn for unfolding reactions with considerable fractions of stable intermediates (Tanford, 1968).

Iodide quenching studies (Figures 8 and 9) suggest indeed that, in the case of cytochrome *c*-552, unfolding by Gdn·HCl and urea involves formation of stable intermediates. In the native molecule at neutral pH, the fluorescence of both tryptophanyl residues is quenched by energy transfer to heme (Weber & Teale, 1959). In the presence of guanidine and urea at concentrations corresponding to 10–30% of total fluorescence enhancement (Figure 1) the Stern–Volmer plots exhibit a downward curvature diagnostic of different exposure of the tryptophans to the quencher. At higher concentrations of denaturants, the plots were linear as expected for denatured proteins. In the case of a two-state mechanism the denatured molecules, detected by fluorescence as a result of a conformational transition, would have both tryptophans equally accessible to iodide.

The optical spectrum of cytochrome *c*-552 unfolded in 8 M

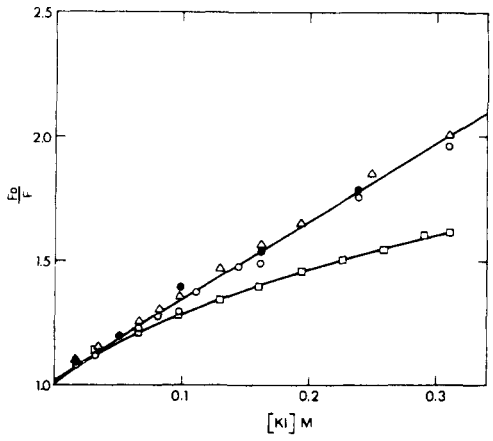

FIGURE 8: Stern–Volmer plots of iodide quenching of *c*-552 at different concentrations of Gdn·HCl: (□) 2 M; (△) 3 M; (●) 5 M; (○) 6 M; pH 7.0; 10 mM phosphate buffer. Experimental conditions are described in the text.

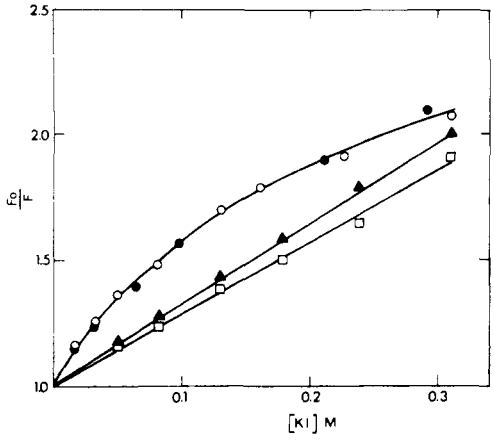

FIGURE 9: Stern–Volmer plots of iodide quenching of *c*-552 at different concentrations of urea: (○) 3 M; (●) 4 M; (▲) 6 M; (□) 8 M; pH 7.0; 10 mM phosphate buffer. Experimental conditions are described in the text.

urea and 6 M Gdn·HCl solution is pH dependent. At the alkaline side of the ionization shown in Figure 3 the spectrum is characteristic of a low-spin hemoprotein. The 695-nm absorption band attributed to the iron–methionine linkage in native ferricytochrome *c*-552 (Ben Hayyim & Schejter, 1974; Aviram et al., 1976) is absent; the Soret peak shifted and intensified (Figure 4A).

Coordination of two histidyl residues was suggested by Babul & Stellwagen (1971), to explain a similar low-spin type spectrum of urea and Gdn·HCl denatured horse heart cytochrome *c* at pH 7.0. In the horse protein, p*K*s of 5.1 in 6 M guanidine and 5.0 in 9 M urea were determined for the pH-dependent transition leading to the formation of an unfolded, high-spin species (Tsong, 1975). In the present study corresponding p*K*s of 5.9 and 7.1 were obtained, closer to the intrinsic p*K* of the histidine imidazole group in the presence of denaturant (Babul & Stellwagen, 1971). Hill coefficients calculated for the ionizations shown in Figure 3 are close to unity and are compatible with single proton dissociation. These findings support the participation of histidine in the high spin–low spin transition in *Euglena c*-552. The only histidine present in the *c*-552 molecule is the one which ligates the prosthetic group (Pettigrew, 1974); in view of kinetic (Aviram & Krauss, 1974) and modification studies of horse cytochrome (Pettigrew et al., 1976) suggesting that a low-spin form may exist with histidine as the only protein ligand, it seems to us that this may be true also for cytochrome *c*-552. Based on this assumption in the high-spin unfolding protein both methionine and histidine ligands are replaced by the solvent, while in the low-spin unfolded form solvent occupies only the sixth coordination position of the iron. In the native cytochrome *c*-552 the intrinsic pK = 6.5 of the histidyl ligand is lowered by at least 5 pH units (see below); in the denatured protein this acidifying effect is almost lost, as evidenced by p*K*s of 5.9 and 7.1 for guanidine and urea, respectively.

The profiles of unfolding at both pH ends of the ionization (Figure 5) are entirely different, especially in urea. Unfolding at alkaline pH, leading to a denatured state in which the native histidyl ligand is retained, occurs at distinctly lower concentrations of urea (Figure 5 and Table I). Formation of a denatured form in which both ligands are replaced by the solvent requires more urea, but the transition is sharper, indicating higher cooperativity.

Opposite results were obtained when horse cytochrome *c* was titrated fluorimetrically with urea at both ends of the high spin–low spin transition of pH 5.0 (Tsong, 1975). Figure 6 indicates that in the acid side of the transition (pH 3.5) the susceptibility toward urea is markedly increased as compared to pH 7.0. Assuming that both alkaline- and acid-denatured forms in the mitochondrial and photosynthetic cytochromes *c* are unfolded to a similar extent, horse cytochrome *c* appears to be most resistant to denaturation at higher pH, while cytochrome *c*-552 is most stable at acid pH. This is evidenced by the decrease of $\Delta G_{D,H_2O}$ values estimated for urea denaturation of cytochrome *c*-552 with pH (Table I). The *m* values exhibit a similar pH dependence (Table I). To account for a lower number of residues exposed upon unfolding at higher pH, different native states have to be assumed, with alkalinization per se favoring unfolding. In fact, it was shown (Aviram et al., 1976) that the pH-dependent transition of pK = 10, for the disappearance of the 695-nm band, is accompanied in cytochrome *c*-552 by an enhancement of fluorescence amounting to 60% of the intensity found in 8 M urea, pH 7.0. In addition, if denaturation at higher pH involves large proportions of stable intermediates a lower value of *m* can be expected. The biphasic curve of unfolding of cytochrome *c*-552 at pH 9.0 (Figure 5) is indeed indicative of a non-two-state mechanism.

The assumption that the alkaline-denatured state represents a high degree of unfolding, in spite of the preservation of histidine coordination to the iron, is justified by its high fluorescence intensity (Figure 3), exceeding slightly the intensity of the high-spin denatured species. In this respect also cytochrome *c*-552 differs from horse cytochrome *c*, where the transition to high spin is accompanied by additional enhancement of fluorescence (Tsong, 1975). The increased stability of native cytochrome *c*-552 at lower pHs, deduced from experiments with urea and Gdn·HCl, is further supported by titrations at low pH.

While horse, cow, *Candida*, and yeast cytochromes *c* were reported to unfold with midpoints above pH 2.5 (Knapp & Pace, 1974; Polastro et al., 1976), as indicated by spectral, optical rotatory, and fluorescence changes, cytochrome *c*-552 below pH 1.0 still possesses the 695-nm absorption band, has a very low fluorescence yield (Figure 3), and its Soret peak, contrary to the expected hyperchromic effect, loses intensity (Figure 4). Since the iron ligands are identical in both classes of cytochrome *c* (Ben Hayyim & Schejter, 1974; Pettigrew, 1974), the different stability at acid pH is most probably due to different composition of the domain of the protein chain which enfolds the prosthetic group, and to the different charges on the protein. Respiratory cytochromes *c* at low pH are highly

charged polycations, and the electrostatic repulsions between the numerous positive charges located at their surfaces may well contribute to their destabilization and consequent unfolding. In cytochrome *c*-552 the corresponding destabilization phenomenon should be much weaker since there could exist at low pH no more than 6 positive charges distributed over a similar area (Pettigrew, 1974). The persistence of the native closed-crevice structure at acid pH, as visualized by the absence of spectral changes, indicates also that the degree of ionization of the carboxyl groups does not affect the folding of the chain around the heme.

Addition of neutral salts to horse cytochrome *c* unfolded by acid results in partial refolding, dependent on the nature of the anion and its concentration (Aviram, 1973; Lanir & Aviram, 1975). The stability of cytochrome *c*-552 in solutions of HCl would reflect a relatively higher affinity for chloride. Since, however, chloride is the weakest of a series of anions effective in stabilization of horse cytochrome *c*, this hypothesis can be experimentally tested only by direct binding experiments.

References

Aune, K. C., & Tanford, C. (1969) *Biochemistry 8,* 4586–4590.

Aviram, I. (1973) *J. Biol. Chem. 248,* 1894–1896.

Aviram, I., & Krauss, Y. (1974) *J. Biol. Chem. 249,* 2575–2578.

Aviram, I., Pettigrew, G. W., & Schejter, A. (1976) *Biochemistry 15,* 635–637.

Babul, N., & Stellwagen, E. (1971) *Biopolymers 10,* 2359–2361.

Ben Hayyim, G., & Schejter, A. (1974) *Eur. J. Biochem. 40,* 569–573.

Boeri, E., Ehrenberg, A., Paul, K. G., & Theorell, H. (1959) *Biochim. Biophys. Acta 12,* 273–282.

Dickerson, R. E., Timkowich, R., & Almassy, R. J. (1976) *J. Mol. Biol. 100,* 473–491.

Eftink, M. R., & Ghiron, C. A. (1976) *Biochemistry 15,* 672–680.

Ellerton, H. D., & Dunlop, P. J. (1966) *J. Phys. Chem. 70,* 1831–1837.

Greene, R. F., Jr., & Pace, C. N. (1974) *J. Biol. Chem. 249,* 5388–5393.

Ikai, A., Fish, W. W., & Tanford, C. (1973) *J. Mol. Biol. 73,* 165–184.

Keller, R. M., Wuthrich, K., & Schejter, A. (1977) *Biochim. Biophys. Acta 491,* 409–413.

Knapp, J. A., & Pace, C. N. (1974) *Biochemistry 13,* 1289–1294.

Lanir, A., & Aviram, I. (1975) *Arch. Biochem. Biophys. 166,* 439–455.

Lehrer, S. S. (1971) *Biochemistry 10,* 3254–3263.

Myer, Y. P. (1968) *Biochemistry 7,* 765–776.

Pettigrew, G. W. (1974) *Biochem. J. 139,* 449–459.

Pettigrew, G. W., Aviram, I., & Schejter, A. (1976) *Biochem. Biophys. Res. Commun. 68,* 807–813.

Polastro, E., Looze, Y., & Leonis, J. (1976) *Biochim. Biophys. Acta 446,* 310–320.

Puett, D. (1973) *J. Biol. Chem. 248,* 4623–4634.

Stellwagen, E. (1968) *Biochemistry 7,* 2893–2898.

Tanford, C. (1968) *Adv. Protein Chem. 23,* 121–282.

Tsong, T. Y. (1974) *J. Biol. Chem. 249,* 1988–1990.

Tsong, T. Y. (1975) *Biochemistry 14,* 1542–1547.

Tsong, T. Y. (1976) *Biochemistry 15,* 5467–5473.

Weber, G., & Teale (1959) *Discuss. Faraday Soc. 27,* 134–141.

Spin-Labeled Ribonuclease A. Effects of Chemical, Enzymatic, and Physical Modifications on Enzyme Conformation†

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ABSTRACT: 3-SLHis-105-RNase A is an active derivative of ribonuclease A (RNase A) spin-labeled at the 3 position of the imidazole ring of histidine-105. The spin-labeled enzyme has been modified by urea denaturation, reduction, reduction–carboxymethylation, performic acid oxidation, and digestion with proteolytic enzymes in order to monitor changes in the geometry of the protein by changes in the electron paramagnetic resonance (EPR) spectrum of the nitroxide spin-label probe. The results of these experiments indicate that the spin-label attached to histidine-105 of RNase A is sensitive to modifications affecting the conformational integrity of the molecule and to the reconstituting effects of various active-center ligands.

Proteins are dynamic macromolecules whose conformational properties relate closely to biological activity. Consequently, many studies of proteins have involved investigation of their conformational properties. Spin-labeling, or labeling a molecule with a stable organic free radical, has been introduced (Stone et al., 1965; Ohnishi and McConnell, 1965) as a possible means of measuring subtle structural changes in biological macromolecules. Reaction of bovine pancreatic ribonuclease A (RNase A) with the nitroxide spin-label *N*-(2,2,5,5-tetramethyl-3-pyrrolidinyl-1-oxy)bromoacetamide at pH 5.5 or 6.5 results in alkylation at the 3 position of the imidazole ring of histidine-105 (Daniel et al., 1973). The 3-SLHis-105-RNase A,[1] ~85% as active as RNase A, is inactivated by iodoacetate

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[1] Abbreviations used are: EPR, electron paramagnetic resonance; RNase A, ribonuclease A; 3-SLHis-105-RNase A or SL-RNase A, ri-

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