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Characteristics of Tyrosinate Fluorescence Emission in α- and β-Purothionins†

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ABSTRACT: The CD, absorption, and fluorescence spectra and fluorescence lifetimes of three highly homologous, basic cytotoxic proteins isolated from wheat (α_1-, α_2-, and β-purothionins) and a moderately homologous protein isolated from *Crambe abyssinica* (crambin) have been measured. The purothionins each contain a single tyrosine, while crambin has two tyrosine residues. At neutral pH in buffered solution or in water, β-purothionin showed a single fluorescence emission peak maximal at 345 nm; α_1- and α_2-purothionins gave a double-humped emission (λ_{max} 308 and 345 nm), while crambin emitted only at 303 nm. Under acid pH conditions (<pH 3) or when denatured in 6 M guanidine hydrochloride, the spectra of the α- and β-purothionins showed predominantly the 303-nm emission (τ = 3.1 ns) while at pH >10.0 only the 345-nm emission was evinced by all three proteins. Crambin showed typical tyrosine emission in the pH range 3-9 and weak tyrosinate fluorescence at pH >10.5. From these features, and from the absorption and CD spectra, we infer that the 345-nm fluorescence emission of either α_1- or β-purothionin is from tyrosinate moieties. The purothionin emission spectra appear to be generated by excited-state proton transfer rather than from tyrosinate species in the ground state.

Protein fluorescence maximal at wavelengths greater than 310 nm is usually ascribed to the presence of tryptophan residues. In general, tyrosine fluorescence is considerably more blue shifted (λ_{max} occurs at approximately 303 nm) (Teale & Weber, 1957; Longworth, 1971; Cowgill, 1976). Recently, however, a component of tyrosine fluorescence that apparently derives from the excited state of the ionized *p*-hydroxyphenyl moiety (Cornog & Adams, 1963) has been described in several proteins containing tyrosine but no tryptophan residues (Kimura & Ting, 1971; Kimura et al., 1972; Graziani et al., 1974; Rayner et al., 1976; Szabo et al., 1978; Lim & Kimura, 1980; Jordano et al., 1983; Pundak & Roche, 1983). The characteristic feature of this fluorescence emission is that it is maximal at λ >310 nm, usually at 345 nm. Not surprisingly, such emission is likely to be masked in proteins containing both tryptophan and tyrosine residues, either by the predominance of tryptophan emission or by the occurrence of energy transfer (at rates faster than the rate of proton transfer) from tyrosine to tryptophan moieties. Despite the problems inherent in identifying tyrosinate fluorescence in the presence of tryptophan emission, its presence has been demonstrated in at least one protein, albumin (Longworth, 1981).

For tyrosinate fluorescence to occur, there is an obvious requirement for proton transfer from the phenolic moieties of tyrosine (either in the ground state or in the excited state) to an appropriate acceptor. Proton transfer from the *p*-hydroxyphenyl moiety in the excited state is far more likely because of the increased acidity of the excited state relative to the ground state. Szabo et al. (1979) quotes values of 5.4 and 10.4 for pK_a's of the phenolic hydroxyl of the excited and ground states, respectively. The proton acceptor can, in principle, be buffer species in the solvent providing they are sufficiently basic (e.g., phosphate or acetate; Szabo et al., 1979). In proteins in which tyrosinate fluorescence is evinced,

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however, it is more likely that an amino acid side chain is the proton acceptor. The tyrosyl group and the proton acceptor should, of course, be close to each other for optimal proton transfer. Obviously, study of the factors that contribute to tyrosinate fluorescence would be simplified by the availability of appropriate model proteins.

In this work, we describe the fluorescence of three proteins which appear to be excellent models for the study of tyrosinate emission. The proteins are α_1-, α_2-, and β-purothionins, toxic cationic proteins isolated from wheat (Balls & Hale, 1940; Balls et al., 1942a,b). They have the following distinct features: (i) each is of low molecular weight (~5000); (ii) the sequences of all three proteins are known, and all show strong homology (Mak, 1975; Mak & Jones, 1976; Jones & Mak, 1977); (iii) each has a single tyrosine residue and no tryptophan residues; and (iv) they are compact "globular" proteins. These three proteins are moderately homologous in amino acid sequence and probably in secondary and tertiary structure to another basic protein, crambin, which has been isolated from the seed of *Crambe abyssinica* (LeComte et al., 1981). From the high-resolution X-ray crystal structure of crambin (Teeter & Hendrickson, 1979; Hendrickson & Teeter, 1981), Llinas et al. (1980) have recently assigned several of the ^{1}H resonances in the 600-MHz spectrum of crambin, and Lecomte et al. (1981) have reported a number of the ^{1}H resonances of the purothionins. It was especially easy to assign resonances of tyrosine-13 residues of the proteins and to deduce from the data that the motions of these residues were somewhat constrained in much the same way that the homologous, conservatively substituted phenylalanine-13 of crambin appeared to be restricted (Lecomte et al., 1982; Hendrickson & Teeter, 1981).

We found that β-purothionin exhibits a fluorescence emission spectrum maximum at 345 nm while the homologous α_1- and α_2-purothionins show double-"humped" fluorescence emission spectra with peaks at 308 and 345 nm. From the effects of pH, denaturation, and chemical modification of amino acid residues on the fluorescence, and from an examination of UV absorption and CD spectra, we have concluded that the fluorescence emission at 345 nm in all three purothionins is from ionized tyrosine (i.e., tyrosinate) species. Moreover, the tyrosinate species are apparently formed only from the excited state, and their formation is probably facilitated by the presence of an adjacent proton acceptor.

Materials and Methods

All chemicals and solvents used in this study were at least of analytical reagent grade. Camphorsulfonic acid, DL-tryptophan, and L-tyrosine (99% pure) and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride, a water-soluble carbodiimide, were obtained from Aldrich Chemical Co. (Milwaukee, WI). Ultrapure guanidine hydrochloride was purchased from Heico Chemicals. Citraconic anhydride and glycine ethyl ester were purchased from Sigma Chemical Co. (St. Louis, MO). "Iodobeads" were obtained from Pierce Chemical Co.

Proteins. α_1-, α_2- and β-purothionins were extracted from the durum wheat cultivar "Vic" and from the *Aegilops squarrosa* accession G405 by the methods described by Lecomte et al. (1982). Crambin was extracted and purified from the seeds of the crucifer *Crambe abyssinica*; ground and partially defatted seeds were the kind gift of Dr. L. H. Princen of the USDA, Peoria, IL. Crambin was isolated and purified by the method of Van Etten et al. (1965). No attempt was made to separate the individual forms of crambin (Hendrickson & Teeter, 1981).

Fluorescence Measurements. Fluorescence emission spectra were measured in a thermostated SLM 4800 spectrofluorometer. All emission spectra were corrected for instrumental artifacts by use of correction factors generated on the SLM 4800 with an NBS standard lamp. Correction factors for our instrument exist only for wavelengths >300 nm. Many of the spectra reported in this paper span the range 290–400 nm and are therefore uncorrected ("technical") spectra. For measurement of spectra at temperatures <0 °C, the proteins were dissolved in glycerol/water mixtures (75:25 v/v) and taken to and maintained at −65 °C with a Neslab ULT 80 bath circulator.

Fluorescence lifetimes were measured by use of the phase-modulation method of Spencer & Weber (1969). In general, modulation frequencies of 18 and 30 MHz were employed. For these measurements, unpolarized excitation was employed (λ_{ex} 275–285 nm), but a polarizer was placed in the emission path and oriented to 35.3° ("magic angle") to eliminate the effects of Brownian rotation on τ (Spencer & Weber, 1970). Emission wavelengths were selected by use either of appropriate interference filters *or* of sharp "cut-on" (mainly Schott WG 345) filters. *p*-Terphenyl (τ = 0.93 ns) was used as a reference solution for all lifetime measurements (Lakowicz et al., 1981).

Fluorescence emission quantum yields were calculated from the simple relation

$$\frac{\theta_p}{\theta_{Tyr}} = \frac{F(\lambda_{em})_p}{F(\lambda_{em})_{Tyr}} \frac{A_{Tyr}}{A_p}$$

where θ_p and θ_{Tyr} refer to the quantum yields of the protein in question and L-tyrosine, respectively, $F(\lambda_{em})_p$ and $F(\lambda_{em})_{Tyr}$ are the areas under their corrected emission spectra (with all the instrumental conditions held the same for measurements of both spectra), and A_p and A_{Tyr} refer to the absorbances of the protein and L-tyrosine, respectively, for the excitation wavelengths used. Generally, absorbances were sufficiently low that inner filter effects could be ignored. A value of 0.15 was assumed for the quantum yield of tyrosine (Tatischeff & Klein, 1976; Chen, 1967) rather than the more frequently quoted figure of 0.21 (Teale & Weber, 1957). Inasmuch as the proteins being compared contained tyrosine but not tryptophan, the use of simple absorbance values rather than integrated (corrected) excitation spectra should not cause any marked error in the calculated yields as phenylalanine contributions would be negligible.

Absorption and CD Measurements. Absorption spectra were measured on a Carey 219 spectrophotometer. CD spectra were taken on a Jasco J-500A recording spectropolarimeter interfaced to a Jasco DP-500N data processor. The temperature of the sample compartment was controlled with a Lauda RM35 bath/circulator. Spectra were recorded at 25 °C unless otherwise stated.

The CD instrument was calibrated with a 1 mg/mL (4.3 mM) aqueous solution of 10-camphorsulfonic acid: we used a value of ϵ = 35.6 M^{-1} cm^{-1} for the molar extinction of this compound and a $\Delta\epsilon$ value of 2.37 M^{-1} cm^{-1} ($[\theta]$ = 7821 deg cm^2 $dmol^{-1}$) at 290 nm to calculate a $\Delta A/A$ of 0.0658 (θ/A = 2.17°) for 10-camphorsulfonic acid (Vallee & Holmquist, 1980). The 10-camphorsulfonic acid was recrystallized and dried prior to use. As suggested by Strickland (1974), a solution of a racemic amino acid (in this case DL-tryptophan, 99%, from Aldrich Chemicals) was used to check for instrument artifacts.

Each CD spectrum reported was the average of 16 scans with subtraction of the solvent spectrum. In general, the

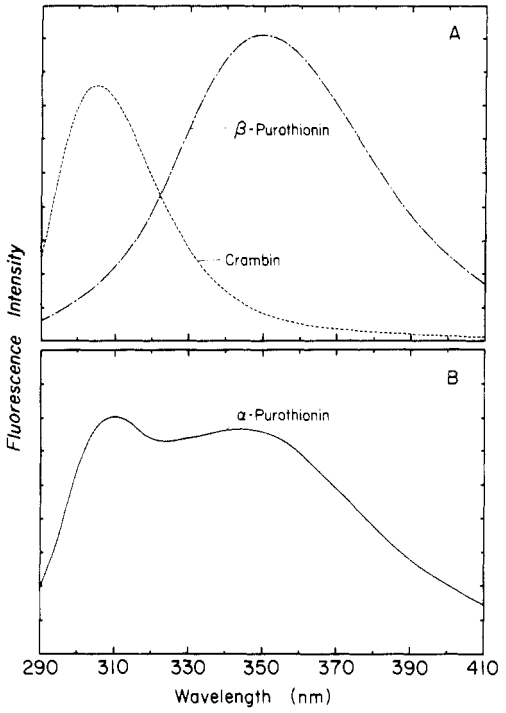

FIGURE 1: Fluorescence emission spectra of crambin, β-purothionin, and α_1-purothionin. The spectra of crambin dissolved in an ethanol/water mixture (70:30 v/v) and of β-purothionin [in 150 mM KCl and 25 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.0] are given in (A) and are depicted by (- - -) and (– - –), respectively. The spectrum of α_1-purothionin dissolved in 150 mM KCl/25 mM MOPS, pH 7.0, is given in (B). The fluorescence emission of α_2-purothionin was virtually identical with that of α_1-purothionin.

instrument was operated at scan speeds of 200 nm/min with sampling times of 10 ms and with a bandwidth of 1 nm. Protein concentrations were determined on the basis of dry weights of the lyophilized proteins assuming that the preparations (as acetate salts) contained 87% protein determined by Kjeldahl analysis. Purothionins were dissolved in a 0.15 M KCl solution buffered at pH 7.0 with 0.025 M phosphate. Crambin is insoluble in water, so the CD measurements were conducted with the protein dissolved in 67% (v/v) ethanol/water cosolvent. The apparent "pH" of this crambin solution was 6.7, and no pH buffer was subsequently added.

The units of CD are expressed as degrees centimeter squared per decimole calculated from

$$[\theta] = 100\theta_{obsd}/(lc)$$

where $[\theta]$ is the molar ellipticity, θ_{obsd} is the measured "degrees ellipticity" corrected as described above, l is the path length in centimeters, and c is the protein concentration in moles per liter.

Chemical Modification of Purothionin Amino Acid Side Chains. Citraconic anhydride was used to modify the α-amino group and ε-amino moieties of lysine residues of β-purothionin. Tyrosine was modified by iodination with Iodobeads as described by Markwell (1982); "cold" NaI was employed. An attempt was made to modify the carboxyl groups at the carboxyl terminus by incubation with 1 M glycine ethyl ester at pH 4.5 and subsequent treatment with 0.1 M 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride as described by Carraway & Koshland (1972). The reaction was allowed to proceed for 2 h at room temperature and then was quenched by the addition of 1 M sodium acetate. This reaction scheme modifies the phenolic hydroxyl, but the products of that reaction are destroyed by hydroxylamine (Carraway & Koshland, 1972). Thus, after the initial spectra were taken,

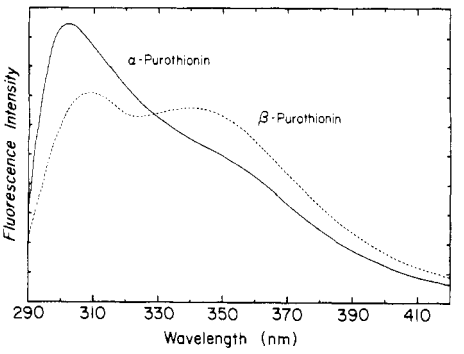

FIGURE 2: Effects of acidic conditions on the fluorescence of α_1-purothionin (—) and β-purothionin (- - -). Both purothionins were dissolved in water and taken to pH <1 by addition of HCl. The fluorescence emission spectrum of α_2-purothionin returned to that shown in Figure 1 when the pH was returned to 7.0.

the chemically modified protein was treated with 5 mM hydroxylamine at room temperature for 2 h. All reagents were finally removed by ultrafiltration of the mixture in an Amicon system fitted with a UM 05 membrane (nominal molecular weight cutoff of 500).

Results

Fluorescence emission spectra of simple tyrosine derivatives and of the proteins studied are given in Figure 1. The fluorescence spectra of *N*-acetyltyrosine ethyl ester (NATEE) and crambin exhibited a single emission λ_{max} at 303 nm (Figure 1A) and were thus typical of tyrosine in aqueous buffered solution at neutral pH. β-Purothionin also showed a single emission peak, but this was maximal at 345 nm (Figure 1A). In contrast, the fluorescence emission spectra of both α_1- and α_2-purothionin were alike and showed a double-humped form with *apparent* maxima at 308 and 348 nm, and the spectra of both proteins were independent of the buffer species used for any given pH.

The effect of pH on the fluorescence emission spectra of the purothionins is shown in Figure 2. At a pH of <2.0, the α-purothionins showed a single emission peak maximal at 303 nm. β-Purothionin, on the other hand, exhibited two emission maxima, one at 308 nm and the other at 345 nm, for up to 2 h after the protein was put into an acidic environment at pH <1. At a pH >10.0, α-purothionins had only the 345-nm emission. β-Purothionin, on the other hand, showed a single fluorescence band maximal at 345 nm at any pH >4.0. The fluorescence of both NATEE and crambin exhibited progressive decreases in yield, and their fluorescence emission spectra were red shifted as the pH was increased beyond 9.0, and finally a single weak emission band maximal at 345 nm was observed at pH >11.0 (F. G. Prendergast, unpublished results). The fluorescence emission of α_1-, α_2-, and β-purothionins returned to those shown in Figure 1 when the pH was adjusted back to 7.0. However, the fluorescence of α_1-purothionin showed a gradual change from a single peak maximal at 303 nm to the more characteristic double-humped emission over the pH range 2–5. The data were not good enough for a titration curve to be plotted, but the titratable group (or groups) that determine(s) the appearance of the 345-nm emission appeared to have a pK in the range 2–4. As noted above, the fluorescence of β-purothionin was far more resistant to pH changes, and a single emission maximum at 345 nm was obtained even at a pH as low as 2.0.

Elevation of the pH to >11.5 caused a diminution in the fluorescence yield of β-purothionin but no alteration in the position of the peak maximum. In α_1-purothionin, however, at pH 11.5 the spectrum still showed two peaks, but the

Table I: Fluorescence Quantum Yields and Lifetimes for Tyrosine, α_1- and β-Purothionins, and Crambin

sample	ϕ_p[a]	fluorescence lifetime (ns)			
		$\tau_{\phi 18}$[b]	τ_{m18}	$\tau_{\phi 30}$	τ_{m30}
D-tyrosine	0.15	3.19 (±0.08)[c]	3.04 (±0.11)	3.04 (±0.01)	3.12 (±0.02)
α-purothionin	0.011	2.95 (±0.25)	4.05 (±0.26)	3.00 (±0.2)	4.8 (±0.3)
β-purothionin	0.026	2.8 (±0.1)	3.5 (±0.2)	2.9 (±0.2)	3.5 (±0.03)
crambin[d]		0.36 (±0.08)			
α-purothionin, denatured	0.10	NM[e]	NM	2.95 (±0.1)	3.3 (±0.2)
β-purothionin, denatured	0.12	NM	NM	2.10 (±0.4)	3.45 (±0.2)
crambin, denatured	0.12	NM	NM	3.00 (±0.2)	3.2 (±0.4)

[a] Quantum yields; these were measured as described in the text. [b] τ_ϕ is the lifetime measured from the phase delay and τ_m the lifetime measured by demodulation; the subscripted number represents the modulation frequency. [c] The numbers in parentheses are the standard deviations on the fluorescence lifetimes. [d] The fluorescence lifetime of crambin was measured by use of time-resolved photon counting. The excitation source was the pulsed output of a Nd:YAG pumped dye laser frequency doubled to 290 nm and of a 15-ps pulse width (full width at half-maximum). Measurements were made at the center for Fast Kinetics Research, Austin, TX (J. Lynch and F. G. Prendergast, unpublished results). [e] NM, not measured.

long-wavelength emission had become dominant. Interestingly, neutralization of the base-treated α_1-purothionin resulted in a complete loss of the 345-nm emission, and only the 306-nm emission remained (P. D. Hampton and F. G. Prendergast, unpublished results). The quantum yields of the fluorescence emissions of the α- and β-purothionins also differed by a factor of more than 2 (Table I) and were both markedly less than that of tyrosine. Crambin, despite showing only "typical" tyrosine fluorescence, also had a very low quantum yield, less than one-tenth that of tyrosine. The quantum yields of all three proteins were enhanced when the proteins were completely denatured (Table I).

The fluorescence emission spectra of the purothionins were quite sensitive to denaturation but only minimally sensitive to temperature changes in the range 2–45 °C. Both α_1- and α_2-purothionins denatured readily in 6 M guanidine hydrochloride (Gdn·HCl) containing 0.01 M mercaptoethanol, and the denatured forms exhibited a single emission band (λ_{max} 303 nm) (Figure 3). The proteins apparently renatured when guanidine hydrochloride was dialyzed away, but the fluorescence emission did not return to "normal"; rather, the fluorescence of "renatured" α_1-purothionin showed a single peak maximal at 332 nm. β-Purothionin was markedly more resistant than either α_1- or α_2-purothionin to denaturation; even after 24 h in 6 M Gdn·HCl (0.01 M 2-mercaptoethanol), some emission of λ_{max} 345 nm remained, although most of the fluorescence evinced was now at 306-nm wavelength, i.e., was essentially identical with that of tyrosine, crambin, or α-purothionins in guanidinium chloride (Figure 3A). Upon dialysis to remove the denaturing solvent, the fluorescence of β-purothionin returned to that of the original native protein, exhibiting a single fluorescence peak of λ_{max} 345 nm (see CD Data).

In the range 2–45 °C, temperature had little effect on the fluorescence of any of the purothionins save for a reduction in the quantum yield as temperature was increased (F. Prendergast, unpublished results). At −65 °C in a glycerol glass, however, the long-wavelength (λ_{max} 345 nm) emission of all of the purothionins was markedly shifted to the blue in β-purothionin and exhibited a maximum at 323 nm (Figure 4). The fluorescence spectra of NATEE and crambin under similar conditions and the 303-nm emission of α_1-purothionin (F. G. Prendergast, unpublished results) were only minimally shifted by freezing the sample (Figure 4).

The fluorescence lifetimes of the three purothionins were difficult to measure because of their low quantum yields. The fluorescence lifetimes of L-tyrosine in aqueous buffer at pH 7.0 were similar to those reported by others (Longworth, 1971). The data show considerable heterogeneity in τ (reflected by $\tau_\phi < \tau_m$ and $\tau_{30MHz} < \tau_{18MHz}$) for β-purothionins. Heterogeneity in τ was expected for the α-purothionins since the interference filter used in the measurement of τ perforce included both the blue- and red-shifted emission bands. The fluores-

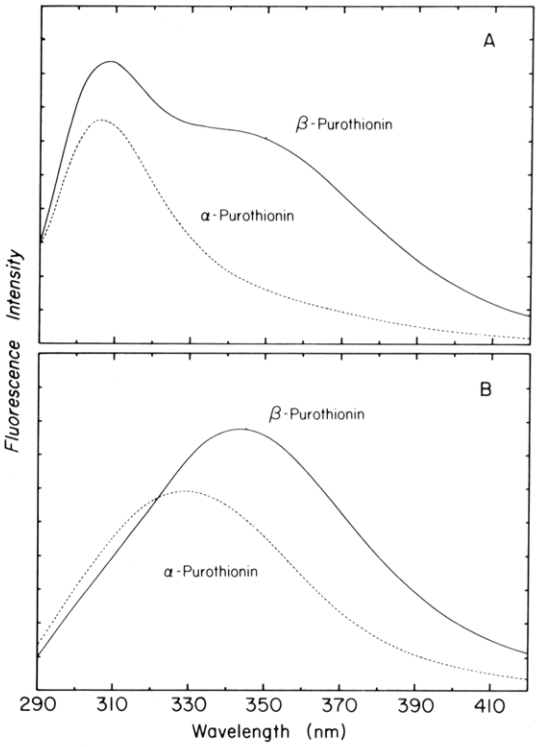

FIGURE 3: Fluorescence emission spectra of α_1- and β-purothionins denatured with 2-mercaptoethanol in 6 M guanidine hydrochloride (A) and renatured α_1- and β-purothionins (B). The spectra of α_1-purothionins are given by the dashed lines and those of β-purothionin by the solid lines. The fluorescence emission spectra of denatured crambin and α_2-purothionins are essentially the same as that of denatured α_1-purothionin. The emission λ_{max} for renatured α_1-purothionin was 332 nm and was 345 nm for renatured β-purothionin. Renaturation was effected by dialyzing away the mercaptoethanol/guanidine hydrochloride solution.

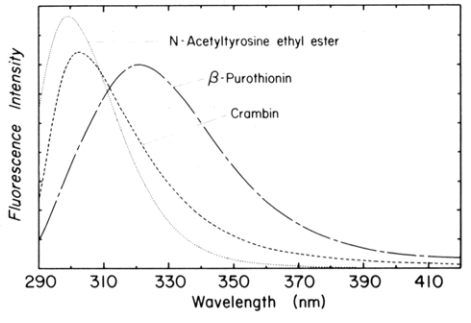

FIGURE 4: Fluorescence emission spectra of *N*-acetyltyrosine ethyl ester (···), crambin (- - -), and β-purothionins (— — —) in a vitrified glass of a glycerol/water mixture (70:30 v/v) at −65 °C.

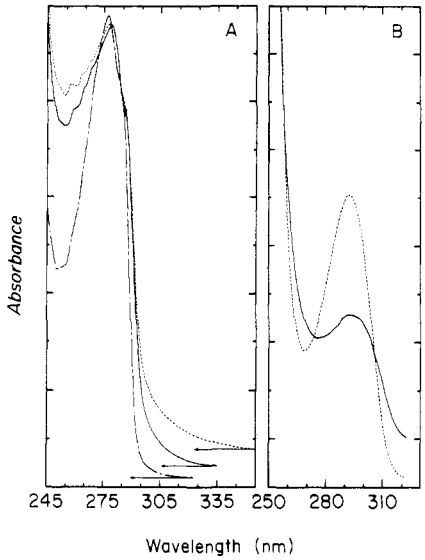

FIGURE 5: Absorption spectra of crambin (---), α_1-purothionin (—), and β-purothionin (- - -). The y axis has no values for the ordinates because the base lines for the spectra were deliberately displaced off the x axis to afford a better *qualitative* display of the long-wavelength absorption of the purothionins. The arrows indicate the base lines for the respective spectra. (B) Long-wavelength absorption spectra of NATEE (---) and β-purothionin (—) dissolved in unbuffered aqueous solution at pH 11.5. The y axis is as described for (A). The concentration of NATEE was 2×10^{-4} M, and that of β-purothionin was 10^{-4} M.

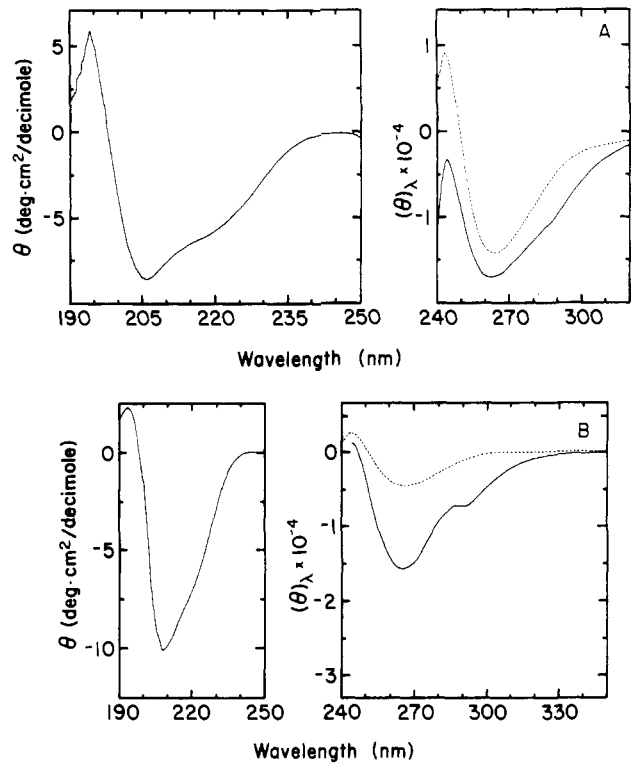

FIGURE 6: Near- and far-UV CD spectra of crambin (A) dissolved in an ethanol/water mixture (60:40 v/v) (—) or in an ethanol/water mixture (60:40) at pH 11.5 (···). (B) α_1-Purothionin dissolved in neutral solution (0.025 M sodium phosphate, pH 7.0) (—) and in basic solution (pH 11.5) (···).

cence lifetimes of the tyrosines in crambin are extremely short ($\tau < 0.5$ ns). This, taken with the quantum yield data, suggests that quenching of the emission of both tyrosines (residues 29 and 44) is due to dynamic intramolecular processes. In α- and β-purothionins denatured in guanidinium chloride, the fluorescence lifetimes of the tyrosine were very similar to those of free tyrosine, while those of denatured crambin were still short (Table I).

Absorption Spectra. The absorption spectrum for crambin was substantially the same as that of NATEE in the near-UV region. The absorption spectra of the α_1- and β-purothionins were similar and showed the general shape typical of tyrosyl absorption (Figure 5A). However, there was an obvious (though minimal) shift in the absorption λ_{max} (λ_{max} was 277.5 nm for both α_1- and β-purothionins) and a clear increase in absorbance in the 290–305-nm region in the spectra of the purothionins relative to those of NATEE or crambin. NATEE had an absorption maximum at 276 nm. (It is worth noting that the absorption maximum for crambin was also shifted, relative to that of tyrosine, to a value of 277 nm.) This band of enhanced absorption is in contrast to the distinct absorption maximum at 295 nm evinced by NATEE and β-purothionin (also by crambin and the α-purothionins) in basic (pH 11.5) solution (Figure 5B).

Fluorescence of Chemically Modified Purothionins. Iodinated purothionins were essentially nonfluorescent, while the citraconylated proteins exhibited the same fluorescence as the untreated proteins. The results of our attempts to modify the carboxyl groups of β-purothionin with glycine ethyl ester were equivocal. While there was a 50% reduction in the total fluorescence yield, there was only a slight increase in the 303-nm emission. The reduced fluorescence yield was partially regained by treatment with hydroxylamine followed by titration to pH 11.0 with sodium hydroxide.

CD Data. Near- and far-UV circular dichroic spectra of α_1-purothionin and crambin are given in Figure 6. (CD spectra of α_2- and β-purothionins were measured and were qualitatively and quantitatively similar to that of α_1-purothionin; only the spectra of α_1-purothionin are presented.) The near-UV CD spectra of α_1- and β-purothionins were clearly similar and showed some fine structure with maxima at 290 and 263–264 nm, typical of tyrosine CD bands (Edelhoch et al., 1968; Strickland, 1974). The proteins each contain a single phenylalanine residue which must contribute somewhat, but not markedly, to the CD spectrum. The spectra of the purothionins are very similar to those reported by Wada et al. (1982), although our spectra show less fine structure in the region 260–280 nm. The near-UV CD spectra of crambin and α_1- and β-purothionins dissolved in solutions at pH >11 were qualitatively similar, showing a negative peak at 263 nm and a positive peak at 243 nm (Figure 6). These peaks appeared concomitantly with an absorption band maximal at 295 nm and typical of ground-state ionized tyrosine residues. At acidic pH (pH <2), there was only a slight loss of fine structure when compared to the near-UV spectrum (taken at pH 7.0) of all three proteins.

In contrast to the distinct changes in the near-UV CD spectra of these proteins at pH >11, the far-UV CD spectra were little altered by either high (>11) or low (<2) pH. The far-UV spectra of all three purothionins were alike, and unusual. They exhibited an anomalous shape with a deep negative band at 208 nm, a very slight shoulder at 222 nm, and no positive signal in the 195–200-nm region. Such spectra imply the presence of both helical and β-sheet segments, but some other factor or factors (possibly the several disulfide bonds) clearly contribute to the far-UV CD signal. In contrast, the spectrum of crambin evinced a prominent shoulder at 222 nm, a peak at 206 nm, and a positive signal at <200 nm. An α-helical content of 60% was calculated for crambin, and this estimate of helix content is in reasonable agreement with the X-ray crystal data (Hendrickson & Teeter, 1981) and recent

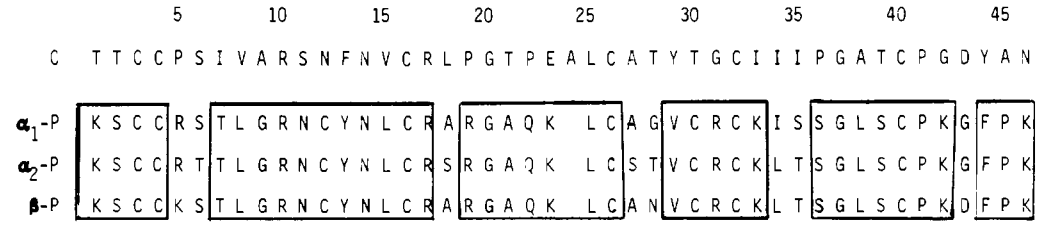

FIGURE 7: Amino acid sequences of crambin and α_1-, α_2-, and β-purothionins. The sequence data are those of Mak (1975).

CD data (Wallace et al., 1984).

Finally, although we were unable to record the entire far-UV CD spectrum of the proteins in the presence of guanidine hydrochloride and mercaptoethanol, we found a complete loss of the near-UV CD signal and of the CD spectrum in the range 220–240 nm in α_1-purothionin. From this, we infer that the protein was denatured. In β-purothionin, some secondary structure was obviously maintained even after 12 h in the denaturing solvent. The proteins were "renatured" by dialysis, initially against 1 M guanidine hydrochloride/1 mM mercaptoethanol solution, then against solutions containing progressively less guanidine hydrochloride and mercaptoethanol (five changes), and finally once against 10 nM ammonium acetate. The renatured proteins were lyophilized after dialysis against 10 mM ammonium acetate. The near- and far-UV CD spectra of "renatured" α_1- and β-purothionins returned completely to normal (P. D. Hampton and F. G. Prendergast, unpublished results).

Discussion

There are two questions that we need to answer from these data: (a) Is the fluorescence emission due to tyrosinate moieties? (b) If so, what are the structural features in the proteins that promote such fluorescence emission?

Evidence for Emission from Tyrosinate Moieties. The fluorescence of tyrosine in alkaline solution (pH 13.0) showed an emission spectrum with λ_{max} at 345 nm (Szabo et al., 1978) and a shape akin to that displayed by β-purothionin. Further, the dependence of the 303-nm fluorescence of the α_1-, α_2-, and β-purothionins on pH and on the integrity of the conformation of all the proteins argues strongly for the essential role of tyrosine ionization in their fluorescence. Finally, iodination of the sole tyrosine residue in any of the three purothionins caused irreversible loss of all fluorescence, proving that the fluorescence derives from the tyrosine moiety. These facts, taken with the absence of any other fluorophore (it is reasonable to ignore any contribution from phenylalanine), provide sufficient evidence to infer that the 345-nm fluorescence is tyrosinate emission. We have already alluded to the likely mechanisms mediating this protolysis process, as described by Szabo et al. (1978) and Rayner et al. (1978). Similar inferences have been drawn by others who have observed apparent tyrosinate emission (Kimura & Ting, 1971; Kimura et al., 1972; Graziani et al., 1974; Lim & Kimura, 1980; Jordano et al., 1983; Pundak & Roche, 1983).

The next issue of substance is whether the tyrosinate moieties are generated in the ground or excited states.

The pK_a of the phenolic moiety of tyrosine in the unexcited molecule is, at 10.4, too high to rationally postulate its deprotonation in the ground state in a typical protein except under conditions of high pH. The data presented here do not support the presence of ground-state tyrosinate moieties. Thus, while there *was* a slight red shift in the absorption maximum and an enhancement of the red-edge absorption bands in both α- and β-purothionins, there was no absorption maximum typical of tyrosinate. The perturbation of the absorption spectrum probably results from hydrogen bonding between an intramolecular acceptor and the phenolic moiety causing perturbation of ground-state vibronic transitions. Further, the near-UV CD data show apparent tyrosinate CD bands only at pH >10.0. In contrast to the data presented here, Jordano et al. (1983) describe a near-UV CD spectrum in a histone devoid of tryptophan and containing only tyrosine residues that resembled that of tyrosinate moieties. The fluorescence of that protein is maximal at 345 nm for the protein in solution at neutral pH.

Structural Basis for α- and β-Purothionin Tyrosinate Fluorescence. Excited-state proton transfer is a well-described phenomenon extensively documented by Weller (1961) and studied in some detail by Shulman and co-workers [Shulman, 1976; for a review, see Badea & Brand (1979)], and more recently by Lakowicz & Balter (1982a–c). The process is markedly promoted by the presence of proton acceptors. As Szabo and co-workers (Szabo et al., 1978; Rayner et al., 1978) have shown, conjugate bases such as acetate and phosphate promote formation of tyrosinate moieties by acting as proton acceptors. The data we have presented show that the buffer species apparently plays no role in α- and β-purothionin fluorescences, and thus the proton acceptor must be an amino acid side chain. On the basis of amino acid sequences of the toxins they studied, Szabo et al. (1978) suggested that glutamyl or aspartyl carboxylate moieties might either be hydrogen bonded to or be in sufficiently close proximity to the phenolic moiety of the susceptible tyrosine to facilitate proton transfer. Tyrosinate-like fluorescence emission has also been observed in adrenodoxin (Kimura et al., 1972; Lim & Kimura, 1980), and in this protein, there is good evidence from Raman spectroscopic data (Bicknell-Brown et al., 1981) for the existence of a distinct hydrogen bond between the tyrosine at residue 82 and a carboxylate moiety. Because the fluorescence of the tyrosyl moiety was maximal at 331 nm (cf. 345 nm for tyrosinate), Lim & Kimura (1980) considered, but did not conclude, that the fluorescence derived from tyrosinate. They were not able, however, to propose another source for this fluorescence in the absence of any other intrinsic fluorophore. Jordano et al. (1983) recently suggested that there are other examples in proteins where the phenolic moiety of the tyrosyl side chain appears to be hydrogen bonded (Szebenyi et al., 1981) but tyrosinate fluorescence is not evinced. However, in the protein to which they refer, the evidence for such a hydrogen bond is, at best, tenuous. While the conjugate bases of carboxyl groups are the most likely proton acceptors in these cases, as Longworth (1981) has pointed out, there *are* other possible proton acceptors. Histidyl and lysyl side chains are two possibilities, and both are often found hydrogen bonded to tyrosyl phenolic moieties.

What, then, is the most likely proton acceptor in the purothionins? All of the purothionins have homologous amino acid sequences (Figure 7), and there is reasonable sequence homology between the purothionins and crambin. On this basis, a similarity in the secondary and tertiary structure of all these proteins has been proposed (Lecomte et al., 1982)

from which we might infer a similar environment for tyrosine-13 of the purothionins and phenylalanine-13 of crambin. The X-ray crystallographic data on crambin show that phenylalanine-13 is ensconced in a reasonably tight slot in the protein (Hendrickson & Teeter, 1981), and this interpretation is supported by NMR data (Llinas et al., 1980; Lecomte et al., 1982; Lecomte & Llinas, 1984). Such a constraint could facilitate hydrogen bonding by reducing fluorophore mobility if the tyrosine moiety in the purothionins were indeed in a similar environment. Moreover, the fluorescence yield of a tyrosinate moiety might be enhanced by both the restricted motion and the reduced accessibility of solvent to the fluorophore, since Longworth & Rahn (1967) have shown that the quantum yield of tyrosinate emission is markedly dependent on the nature of the fluorophore's environment.

However, Lecomte et al. (1982) also drew inferences from NMR data regarding the proximity of other amino acid side chains to tyrosine-13 on the basis of the NOE spectra of the proteins. If we consider the amino acid side chains identified as close neighbors to Phe-13 in crambin (and the likely homologous groups close to tyrosine-13 in the purothionins), no candidates appear as likely proton acceptors. The analogy with crambin appears, therefore, to break down at this point. In our view, this is not really surprising given the marked disparity between the far-UV CD spectra of the purothionins and crambin. A carboxylate moiety therefore seems the most reasonable choice to be the proton acceptor, our failure to prove this by chemical modification notwithstanding. The only realistic candidate in the purothionin sequence is the carboxyl terminus since it is the only such residue common to all the purothionins. The differences in the fluorescence emission spectra of the α- and β-purothionins taken with the similarity of their far-UV CD spectra point to subtle but significant conformational differences between these proteins. But for all the purothionins and especially for β-purothionin, the insensitivity of their fluorescence emission to acid pH and the denaturing effects of guanidine hydrochloride suggests unique tertiary structural protection for the phenolic hydroxyl–proton acceptor couple.

Two other aspects of the fluorescence of these proteins merit discussion. First, although the mobility of the tyrosine moieties may be restricted, fluorescence quenching data show clearly that they *are* accessible to various quenching agents (P. D. Hampton and F. G. Prendergast, unpublished results). Second, the blue shift in the fluorescence emission when the proteins are cooled indicates sensitivity of the fluorescence to solvent conditions (Longworth & Rahn, 1967). This latter finding might explain adrenodoxin fluorescence (Kimura et al., 1972). If we assume that the 320-nm emission of β-purothionin observed at −65 °C in 75% glycerol/water glass is the most blue shifted possible for tyrosinate moieties, then the fluorescence at 331 nm observed in adrenodoxin could be due to a tyrosinate moiety in a unique environment in the protein.

Finally, we cannot determine from the available data whether the mixed fluorescence emission of the α-purothionins is a consequence of partial proton transfer during the excited state or of the existence of two distinct conformers of these proteins—the latter seems more likely in view of the fact that α_1-purothionin fails to return to its initial conformation after either treatment with guanidine hydrochloride/mercaptoethanol or treatment with alkaline solutions (see above). The data suggest that this protein might be able to adopt several conformations or at least may have several readily accessible conformational substates; i.e., the proteins are pleiomorphic. On the other hand, β-purothionin appears to exist as a single conformer, possibly stabilized by salt bridging through the "extra" carboxylate moiety at residue 42.

It seems likely to us that many more examples of tyrosinate emission will be found, often in proteins that also contain tryptophan. In such instances, the tyrosinate emission will inevitably complicate interpretation of tryptophan fluorescence, because the tyrosine absorption band may be red shifted into regions usually considered to have only indole absorption, and in part because tyrosinate emission so precisely overlaps that of tryptophan. The red-shifted tyrosinate emission is also less likely to be transferred to tryptophan because of the decrease in spectral overlap. Even though the quantum yields may be low relative to that of most tryptophan fluorescence studies, the presence of tyrosinate emission would severely complicate fluorescence decay measurements.

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Registry No. L-Tyrosine, 60-18-4; *N*-acetyltyrosine ethyl ester, 840-97-1.

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Cross-Linking of Streptomycin to the 30S Subunit of *Escherichia coli* with Phenyldiglyoxal†

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ABSTRACT: [^{3}H]Dihydrostreptomycin was covalently linked to the 30S subunit of *Escherichia coli* K12A19 with the bifunctional cross-linking reagent phenyldiglyoxal. The cross-linking was abolished under conditions that prevent the binding of streptomycin, which indicates that the cross-linking occurs at the specific binding site of streptomycin. The cross-linking involved 16S RNA and the ribosomal proteins S1, S5, S11, and S13. This suggests that the streptomycin binding site is located in the upper part of the 30S subunit, facing the 50S subunit. Unexpectedly, the same extent and pattern of cross-linking were observed with the 30S subunits from a streptomycin-resistant mutant. We have shown previously that streptomycin induces conformational changes in the ribosomes from sensitive bacteria but not from streptomycin-resistant mutants. From this and from the results in the present study, it is suggested that the binding of streptomycin to streptomycin-sensitive ribosomes is a two-step reaction wherein an initial loose interaction at the antibiotic binding site is followed by a conformational rearrangement of the ribosomal particle. The second step would tighten the association with streptomycin and cause interference with protein synthesis. That step would be lacking in streptomycin-resistant mutants.

The aminoglycoside antibiotic streptomycin interacts with the ribosome and interferes with several steps of protein synthesis in bacteria [reviewed in Vazquez (1979) and Wallace et al. (1979)]. In spite of extensive studies, the molecular mechanisms involved in the interaction of streptomycin with the ribosome are still poorly understood. The identification of the ribosomal components involved in the binding of streptomycin should provide new information on these mechanisms.

Streptomycin binds to the 30S subunit of *Escherichia coli* at a single binding site (Chang & Flaks, 1972; Schreiner & Nierhaus, 1973; Grisé-Miron & Brakier-Gingras, 1982). Affinity analogues of streptomycin have been used to probe the streptomycin binding site (Cooperman, 1980; Girshovich et al., 1976; Pongs & Erdmann, 1973; Pongs et al., 1974). In this study, we used a bifunctional cross-linking agent as a novel way to characterize the binding site of streptomycin. In this procedure, the specific complex between streptomycin and the 30S subunit can be formed before initiation of the cross-linking reaction. This is an advantage over the affinity labeling procedures which may generate unspecific reactions depending upon the reactivity of the modified streptomycin. We chose the cross-linking reagent phenyldiglyoxal, which specifically reacts with guanosine in single-stranded RNA and the guanidine groups of arginine residues in proteins and has been successfully used to make RNA–RNA and protein–RNA cross-links in the bacterial ribosome (Wagner & Garrett, 1978;

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