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Phenylalanine-to-Tyrosine Singlet Energy Transfer in the Archaebacterial Histone-like Protein HTa†

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Appendix: Analysis of Energy Transfer and Fluorescence Lifetime Data

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ABSTRACT: The Archaebacterium *Thermoplasma acidophilum* has a histone-like protein (HTa) abundantly associated with its deoxyribonucleic acid. Each native tetrameric complex of HTa contains 20 phenylalanine residues, 4 tyrosine residues, and no tryptophan. When the protein was excited by radiation at 252 nm, which is a wavelength absorbed predominantly by phenylalanine, the fluorescent emission was mostly from tyrosine. According to the excitation spectrum for this tyrosine fluorescence, the cause was energy transfer from phenylalanine, which occurred with about 50% efficiency. When the tyrosine residues were removed enzymatically, the exicted-state lifetime of the phenylalanine residues nearly doubled. Because of energy transfer, the tyrosine emission had two apparent fluorescence decay lifetimes; one lifetime (3.9 ns) was that of tyrosine while the second (12.1 ns) corresponded to the excited state of phenylalanine.

The histone-like protein HTa[1] is abundantly associated with the DNA of the Archaebacterium *Thermoplasma acidophilum* (Searcy, 1975). It functions to protect DNA from thermal denaturation (Stein & Searcy, 1978). In contrast to other known bacterial histone-like proteins (Pettijohn, 1988), HTa remains stably bound to DNA in salt concentrations up to 10-fold higher than is physiological (Searcy, 1976). Thus, the nucleoprotein has been easily isolated and studied (Searcy & Stein, 1980).

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[1] Abbreviations: HTa, the histone-like protein of *Thermoplasma acidophilum*; SDS, sodium dodecyl sulfate.

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When associated with HTa, the DNA is condensed into a chain of globular particles resembling eukaryotic nucleosomes (Searcy & Stein, 1980). Either in association with DNA or free in solution the protein HTa is found as an oligomer of four identical subunits. Each tetramer contains 20 phenylalanine residues, 4 tyrosine residues, and no tryptophan (DeLange et al., 1981). This combination of aromatic residues gives the protein unusual ultraviolet absorbance and fluorescence properties. Here we describe some of these properties, including fluorescence energy transfer, and compare HTa to model oligopeptides of related compositions.

The protein HTa is highly basic, similar to eukaryotic histones, with lysine and arginine residues accounting for 22% of the total. In each tetramer at neutral pH there is a calculated net ionic charge of +52. Previously we reported fluorescence studies indicating that the tyrosine residues are both solvent-accessible and in a particularly cationic environment (Searcy et al., 1988).

HTa is a member of an homologous family of eubacterial histone-like proteins that include *Escherichia coli* HU and the *Bacillus stearothermophilus* protein HBs (Drlica & Rouvière-Yaniv, 1987). The amino acid sequences of HBs and HTa are 32% identical. The crystallographic structure of HBs has been partially solved (Tanaka et al., 1984). In HBs there are 8 phenylalanine residues buried in the core of each protein dimer. If HTa is folded in a similar pattern, then all 20 phenylalanine residues should be buried within the core region of the protein.

Since clustered aromatic residues contribute significantly to thermal stability (Burley & Petsko, 1985), the phenylalanine-rich composition of HTa is probably an adaptation to heat. Within the "HU" family of homologous proteins, those from the more thermophilic organisms tend to contain more phenylalanine. For example, HU from *E. coli* contains 3 phenylalanine residues per polypeptide chain, *B. stearothermophilus* contains 4, and *T. acidophilum* contains 5. Thus, the unusual optical properties of HTa may be a fortuitous consequence of thermal adaptation.

The COOH-terminal sequence of HTa is ...-Lys-Ile-Lys-Tyr-Gln-Gln-COOH (DeLange et al., 1981). The residues can be removed sequentially by carboxypeptidase A. Because different residues are removed at different rates by the enzyme, the terminal three residues including the tyrosine should be removed most rapidly. Then the next several residues will be digested at a slower rate (Ambler, 1967). Therefore, carboxypeptidase A digestion was predicted to produce a fairly homogeneous preparation of tyrosine-free protein, but with most of the protein still intact. Using such a preparation, it was possible to characterize the protein's phenylalanine fluorescence in the absence of tyrosine. Consequently, we have been able to obtain a fairly complete understanding of the fluorescence and energy-transfer properties of the protein HTa.

Experimental Procedures

Preparation. T. acidophilum (strain 122-1B2) was cultured and stored at −70 °C (Searcy & Stein, 1980). HTa was purified as described previously (Searcy et al., 1988).

Fluorescence Measurements. An Aminco SPF-500 spectrofluorometer was used with quartz "Suprasil" fluorometer cells (path lengths 5 mm × 5 mm). Entrance and exit slits were set to band-passes of 4 nm. The sample was thermostated to 20.0 °C.

(*1*) *Corrected Excitation Spectra.* Attenuation of the excitation beam by the sample (=inner filter effect) was corrected essentially as described by Brand and Witholt (1967). Emission intensities were recorded as ratios to excitation intensities using Rhodamine 6G (3 g/L in ethylene glycol) in the reference beam. A small additional correction to the excitation spectra was determined by using a standard in the sample beam (Rhodamine 101; 3 g/L in glycerol) measured in a 56° front face cuvette (Eisinger & Flores, 1979). After these corrections the excitation spectrum of tyrosine matched its absorption spectrum (see later).

(*2*) *Emission spectra* were corrected against a light-scattering aluminum standard. A slight spectral unevenness in the aluminum was corrected by using measurements from a dual-beam spectrophotometer modified for reflectance. Following these corrections the emission spectrum of benzene (330 μM in cyclohexane) was similar to that of a published standard (Berlman, 1965).

(*3*) *Fluorescence lifetimes* were measured by using the single-photon technique in a Model 199 spectrofluorimeter (Edinburgh Instruments, Edinburgh, U.K.). All slit widths were set to a band-pass of 10.6 nm. Calibration of the time scale was electronic. The fluorescence decay profiles were analyzed by "reconvolution fits" to either single-lifetime or double-lifetime models (Grinvald & Steinberg, 1974) by using computer software supplied by Edinburgh Instruments.

Carboxypeptidase Treatment. Carboxypeptidase A (bovine pancreas, Boehringer-Mannheim) was treated with phenylmethanesulfonyl fluoride (Ambler, 1967). HTa (100 μM in 20 mL of 0.75 M NaCl, 1 mM EDTA, and 10 mM Tris–cacodylate buffer, pH 8.0) was digested with 1.5 μM carboxypeptidase A for 2 h at 40 °C. Then an additional 1.5 μM carboxypeptidase A was added and incubation continued for 18 h. The solution was diluted with an equal volume of water and adjusted to pH 7.0, and the HTa was adsorbed by passage through DNA–cellulose (DeLange et al., 1981). When the column was washed with an increasing salt gradient, HTa eluted near 0.68 M NaCl (see Results).

Sources of Model Polypeptides. Lys-Phe-Lys, Lys-Phe-Gly-Lys, and Ac-Phe-Tyr-NH_2 were obtained from Bachem AG, Bubendorf, Switzerland). Lys-Tyr-Lys-ethylamide was synthesized by Dr. R. Mayer, Centre de Biophysique Moléculaire, CNRS, Orléans, France. L-Amino acid residues were used throughout.

Other Procedures. The concentrations of the protein HTa were measured by dissolving the protein in 0.1 M NaOH and measuring the tyrosinate absorbance at 293 nm (ϵ = 2381 M^{-1} cm^{-1}; Fasman, 1976). According to these measurements the decadic extinction coefficient of the native protein at 276 nm (pH 7.0) was 1440 ± 75 (SE) M^{-1} cm^{-1}, which is insignificantly different from that of free tyrosine (ϵ = 1405 M^{-1} cm^{-1}; Fasman, 1976). Optical density due to light scattering was routinely subtracted by exponential extrapolation from measurements at 330–370 nm (Schauenstein & Bayzer, 1955). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS) was performed in 18% acrylamide and 0.48% *N,N'*-methylenebis(acrylamide), and otherwise as described by Laemmli (1970).

Results

Ultraviolet Absorbance Spectrum of HTa. The absorbance spectrum of HTa (Figure 1) is attributable to its high content of phenylalanine and the absence of tryptophan. The multiple phenylalanine peaks are both red-shifted and hypochromic. After subtraction of the tyrosine absorbance and correction for light scattering the remaining absorbance near 260 nm was 0.84 of that expected of free phenylalanine. This red shift and hypochromicity both disappeared when the protein was boiled in 6 M guanidinium chloride (not shown). The tyrosine absorbance was red-shifted also but was not hypochromic.

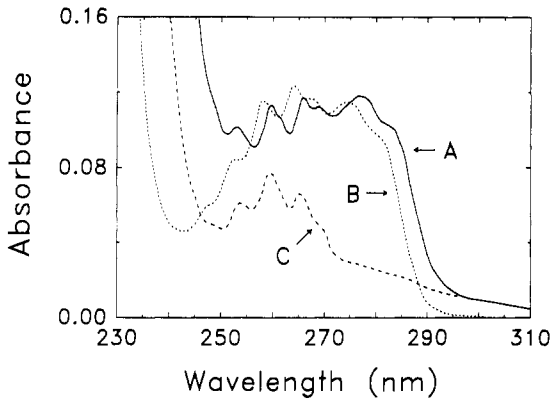

FIGURE 1: Ultraviolet absorbance spectra. (A) Native protein HTa (80 μM). (B) Mixture of aromatic amino acids equivalent to that in the protein (80 μM tyrosine plus 400 μM phenylalanine). (C) HTa (80 μM) that has been digested with carboxypeptidase in order to remove the tyrosine. Solvent: 0.75 M NaCl and 10 mM sodium cacodylate (pH 7.0).

Preparation of Carboxypeptidase-Treated HTa. Previous observations suggested that the tyrosine residues might be involved in DNA binding (Searcy et al., 1988). In order to remove the tyrosine residues, HTa was digested with carboxypeptidase A. The enzyme-treated protein was adsorbed onto DNA–cellulose and then eluted with an increasing gradient of NaCl. The protein emerged in two peaks. The first peak (fraction A) eluted at about 0.40 M NaCl and the second peak (fraction B) at about 0.68 M NaCl. There was no detectable tyrosine absorbance in either fraction. For comparison, untreated native HTa eluted from DNA–cellulose near 0.75 M NaCl.

When the digested protein was analyzed by SDS–polyacrylamide gel electrophoresis, both fractions A and B migrated as single bands. Fraction B migrated with a mobility identical with that of the undigested protein, while fraction A migrated 3% faster.

In the native protein the phenylalanine absorbance peaks were shifted +1.5 nm relative to those of free phenylalanine (Figure 1). In fraction B the shift was +1.5 nm also, but in fraction A the spectral shift was +1.0 nm. These observations suggest that fraction A may have been overdigested or partially denatured. In the work that follows, fraction B was used exclusively.

In fraction B (Figure 1) there was a broad absorbance shoulder from 275 to 310 nm, some of which was due to contamination with tryptophan (see below). Also, part of the shoulder was due to light scattering, which otherwise was subtracted routinely from the absorbance measurements (see Experimental Procedures).

When analyzed by gel-exclusion chromatography on Sephadex G-75, fraction B eluted with an apparent molecular weight of 40 000. The monomer of HTa has a calculated molecular weight (M_r) of 9934 (DeLange et al., 1981). Thus, the tetrameric structure of the protein remained unaffected by the carboxypeptidase treatment. Presumably, only the carboxy-terminal three amino acid residues were removed.

In the fluorescence emission spectrum of fraction B no tyrosine fluorescence was detected (see later). However, a trace of tryptophan-like fluorescence appeared which was not originally present in the undigested HTa. This contamination could not be separated by chromatography on DNA–cellulose or on Sephadex G-75. Presumably, it was caused by a tightly adsorbed fragment of the carboxypeptidase, which is an enzyme rich in tryptophan. From calculations based upon the intensity of the fluorescence, about 2% of the enzyme-treated

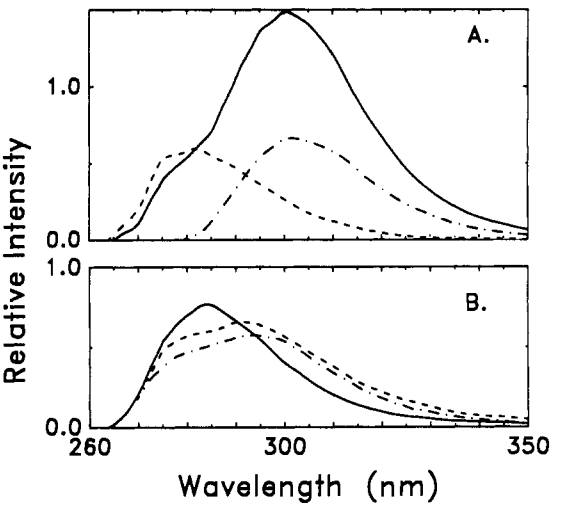

FIGURE 2: Corrected fluorescence emission spectra. (A) (—) 80 μM native HTa; (---) 400 μM phenylalanine; (-·-) 80 μM tyrosine. (B) (—) HTa digested with carboxypeptidase A so as to remove the tyrosine residue and then reisolated in 0.75 M NaCl buffer. (---) Undigested HTa in 0.5% sodium dodecyl sulfate and 10 mM sodium cacodylate (pH 7.0). (-·-) Undigested HTa in 6 M guanidinium chloride and 10 mM sodium cacodylate (pH 7.0). The data were normalized so that the tyrosine and phenylalanine emissions, if added together, equal 1.0 maximum. Solvent: 0.75 M NaCl and 10 mM sodium cacodylate (pH 7.0), except for those samples in denaturing solvents. Excitation: 252 nm.

tetrameric cores were associated with tryptophan. Thus, in the experiments that follow it was necessary to subtract the tryptophan fluorescence from some of the data (see the footnote to Table I).

Fluorescence Spectra. The fluorescence emission spectrum of the protein HTa is shown in Figure 2. The emission spectrum was similar in shape to that of tyrosine, but was exceptionally intense. When the protein was excited at a wavelength of 252 nm the total tyrosine fluorescence was greater than could be accounted for by a mixture of tyrosine plus phenylalanine as free amino acids. When the protein was dissolved in either sodium dodecyl sulfate or 6 M guanidinium chloride (Figure 2B), the tyrosine emission decreased, suggesting that the intense fluorescence depended upon the conformation of the protein.

Although tyrosine emission predominated, there was detectable phenylalanine emission. In Figure 2A the phenylalanine emission can be seen as a shoulder near 275 nm. When the protein was dissolved in a denaturing solvent, the tyrosine emission was reduced and the phenylalanine emission became more apparent (Figure 2B). When the tyrosine residues were removed by carboxypeptidase treatment, the phenylalanine fluorescence increased (Figure 2B). Together, these observations suggest that phenylalanine-to-tyrosine energy transfer is the explanation for the intense tyrosine emission in the native protein.

The efficiency of energy transfer can be measured by analysis of the excitation spectrum. If there is no energy transfer, then for tyrosine emission the excitation spectrum should be similar to its ultraviolet absorbance spectrum (Longworth, 1971). In contrast, if energy is absorbed by phenylalanine and then transferred to tyrosine, the observed excitation spectrum for tyrosine emission should resemble the sum of the phenylalanine and tyrosine absorbance spectra.

Experimentally, the excitation spectrum of HTa was midway between that of tyrosine alone and that of tyrosine plus phenylalanine in the same concentrations as found in the protein (Figure 3A). Thus, the efficiency of energy transfer

Table I: Relative Fluorescence Intensities (F_{rel}) of Tyrosine and Phenylalanine Residues and Their Apparent Excited-State Lifetimes (τ)[a]

excitation wavelength residues excited emission wavelength emission detected sample	252 nm Phe and Tyr 280 nm Phe F_{rel} (τ)	282 nm Tyr 330 nm Tyr F_{rel} (τ)	252 nm Phe and Tyr 330 nm Tyr F_{rel} (τ_1; τ_2)	χ^2
solvent: 0.75 M NaCl				
Phe	1.00 (7.0 ns)	0	0	
Tyr	0	1.00 (3.3 ns)	1.00 (–; 3.3 ns, 100%)	1.21
Phe + Tyr	0.96 (7.0 ns)	1.00 (3.3 ns)	1.02 (–; 3.3 ns, 100%)	1.28
HTa (native)	0.90 (12.0 ns)	0.93 (4.0 ns)	1.95 (12.1 ns, 44%; 3.9 ns, 56%)	1.60
HTa carboxypeptidase core	1.22 (22.5 ns)	0.01[b]	0.01[b]	
solvent: 6 M guanidinium chloride				
Phe	1.08 (8.3 ns)	0	0.10	
Tyr	0.01	0.98 (3.4 ns)	0.86 (–; 3.3 ns, 100%)	1.40
HTa	0.51 (8.1 ns)	0.44 (2.5 ns)	0.71 (8.4 ns, 19%; 2.2 ns, 81%)	1.95
solvent: 0.5% sodium dodecyl sulfate				
Phe	1.03 (7.8 ns)	0	0.03	
Tyr	0.02	0.96 (3.4 ns)	0.87 (–; 3.4 ns, 100%)	1.69
HTa	0.86 (16.1 ns)	0.36 (3.2 ns)	0.83 (14.8 ns, 24%; 2.7 ns, 76%)	3.81
solvent: 0.75 M NaCl				
Lys-Phe-Lys	0.91 (6.2 ns)	0.01	0.01	
Lys-Phe-Gly-Lys	0.90 (6.3 ns)	0	0	
Lys-Tyr-Lys-NH-EtOH	0.03	0.31 (1.3 ns)	0.33	
Ac-Phe-Tyr-NH_2	0.06	0.31 (1.3 ns)	0.47 (–; 1.3 ns, 100%)	1.11
solvent: 0.2 M NaCl, 10 mM Tris (pH 7.8)				
HTa		0.81 (3.8 ns)	1.69 (12.2 ns, 39%; 3.5 ns, 61%)	1.34
HTa + 60 mM sodium phosphate		0.29 (1.8 ns)	0.63 (11.8 ns, 35%; 1.9 ns, 65%)	1.36

[a] The concentration of HTa was 80 μmol of polypeptide chain/L. Each polypeptide chain contains 1 tyrosine residue and 5 phenylalanine residues. Other solutions were adjusted to equivalent concentrations. Solutions were buffered to pH 7.0 with 10 mM sodium cacodylate. Interference between phenylalanine and tyrosine was corrected as follows: 6% of the intensity measured at 280 nm was subtracted from that at 330 nm; 1% of the intensity at 330 nm was subtracted from that at 280 nm. The percent emission at 330 nm due to each lifetime component was calculated by using eq 22 in the Appendix. [b] The carboxypeptidase-treated material contained tryptophan-like fluorescence. The tryptophan contribution was estimated from the emission at >360 nm and subtracted from the data. Before this correction the intensity values in columns 3 and 4 were 0.08 and 0.14, respectively.

was near 50%; i.e., 50% of the energy absorbed by the phenylalanine residues was transferred to tyrosine residues. When the protein was dissolved in 6 M guanidinium chloride, the efficiency of energy transfer fell to near 25% (Figure 3B).

As a control, the excitation spectrum of free tyrosine was measured and found to match its ultraviolet absorbance spectrum, as expected (Figure 3A).

Fluorescence Intensities. Table I summarizes the fluorescence properties of HTa at certain key wavelengths. The wavelengths were chosen so as to excite only tyrosine (282 nm) or predominantly phenylalanine (252 nm). Unfortunately, with these two amino acids there is no wavelength that can excite phenylalanine only. Nonetheless, fluorescence emissions could be measured separately by using 280 nm for phenylalanine and 330 nm for tyrosine.

The tyrosine fluorescence of the native protein was unusually intense (Table I, column 3). Most comparable proteins have fluorescence intensities near 0.2 relative to free tyrosine (Longworth, 1971). The phenylalanine residues also were fluorescent, particularly after carboxypeptidase treatment. The quantum yield depended upon the conformation of the protein, decreasing when the protein was dissolved in a denaturing solvent. As a control, when the free amino acids were dissolved in the same denaturing solvents, their fluorescence properties were only slightly affected.

Energy transfer was apparent when the protein was excited at 252 nm, which is a wavelength absorbed primarily by the phenylalanine residues. The tyrosine emission at 330 nm was nearly double that of free tyrosine. In contrast, when the protein was excited at a wavelength that does not excite phenylalanine (282 nm), the emission from the protein at 330 nm dropped to below that of free tyrosine.

Mixtures of free amino acids at concentrations equivalent to those in HTa showed no indication of energy transfer. This is as expected, since molecules in dilute solution are not close

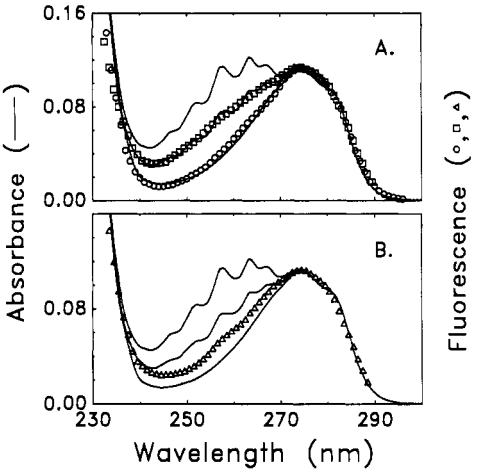

FIGURE 3: Corrected fluorescence excitation spectra (symbols) superimposed upon the ultraviolet absorbance spectra of mixtures of tyrosine plus phenylalanine. (A) (□) Native HTa or (○) tyrosine, both 80 μM in 0.75 M NaCl and 10 mM sodium cacodylate (pH 7.0). (B) HTa freshly boiled in 6 M guanidinium chloride and 10 mM sodium cacodylate (pH 7.0). The fluorescence spectra were corrected for "inner filter effect" and for other systematic errors as described under Experimental Procedures. Fluorescence emissions were measured at 330 nm. The continuous lines are absorbance spectra for 80 μM tyrosine plus 0, 200, or 400 μM phenylalanine in 0.75 M NaCl buffer.

enough for energy transfer to occur (Eisinger et al., 1969). In contrast, with the model peptide Ac-Phe-Tyr-NH_2 energy transfer was clearly evident.

Fluorescence Lifetime Measurements. Fluorescence lifetime measurements are summarized in Table I. When the native protein was excited at 282 nm, the excited-state lifetime of the tyrosine residue was 4.0 ns. This compares to 3.3 ns for free tyrosine. In HTa the phenylalanine residue lifetime also was exceptionally long. Thus, the excited-state lifetimes were

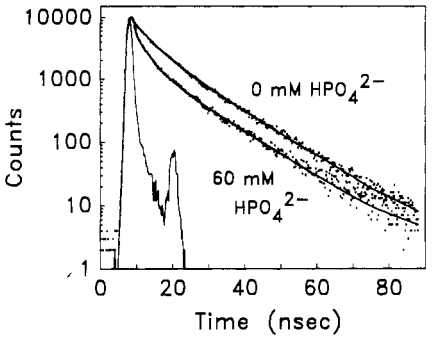

FIGURE 4: Fluorescence emission decay curves. HTa (160 μM) in 0.2 M NaCl and 10 mM Tris (pH 7.8) was excited at 252 nm. Emission was measured at 330 nm. The solutions contained either 0 or 60 mM sodium phosphate (pH 7.8). The lower left solid line is the "lamp profile" obtained by measuring light scattering from colloidal SiO_2 at 330 nm; the double peak is an "echo" that occurs in the photomultiplier tube.

Table II: Protein HTa Fluorescence Decay Lifetime Components at Different Emission Wavelengths[a]

wavelengths (nm)		lifetime (ns), % contribution		
excitation	emission	τ_1	τ_2	χ^2
252	310	12.2, 47	3.9, 53	1.66
252	320	12.0, 47	3.9, 53	1.77
252	330	12.1, 44	3.9, 56	1.60
252	340	12.1, 41	3.7, 59	1.80

[a] Solvent: 0.75 M NaCl and 10 mM cacodylate, pH 7.0. Other conditions as in Table I.

longer than those of the free amino acids, but the quantum yields were less. These data seem to contradict the rule that for a given fluorophore the lifetime should be proportional to the quantum efficiency. Nonetheless, the observations can be explained if some of the aromatic residues are not fluorescent (see Discussion).

When native HTa was excited at 252 nm and the fluorescence decay measured at 330 nm, there were two lifetime components (Table I and Figure 4). The shorter component was that of tyrosine while the longer component corresponded to the excited-state lifetime of phenylalanine. Phenylalanine residues are not expected to emit any fluorescence at 330 nm; nevertheless, the possibility was tested. If the 12.1-ns component were a direct phenylalanine emission, then the emission spectra of the tyrosine and phenylalanine residues would be overlapping at 330 nm. At another wavelength the ratio of these two emissions should be different. When actually measured, the two lifetimes remained in essentially constant ratio at wavelengths from 310 to 340 nm (see percent contributions, Table II). Thus, the data indicate that the two lifetime components emitted at 330 nm are in fact both emitted by tyrosine residues.

When the tyrosine residues were removed by carboxypeptidase treatment, the excited-state lifetime of the phenylalanine increased nearly 2-fold (Table I). This is consistent with the theory of energy transfer, since if the acceptor residues are removed, then the donor residues remain in the excited state longer. An accurate estimate of the efficiency of energy transfer can be obtained from this increase in lifetime [see Cantor and Schimmel (1980)]. Thus, efficiency (E) = $1.0 - \tau_{ET}/\tau_0 = (1.0 - 12.0\ \text{ns})/22.5\ \text{ns} = 0.47$, where τ_{ET} is the phenylalanine lifetime in the intact protein and τ_0 is that in HTa from which the tyrosine has been removed. This calculation is in good agreement with the earlier estimate of 0.5 obtained from the excitation spectrum.

When the protein was dissolved in 6 M guanidinium chloride, the phenylalanine and tyrosine lifetimes both decreased, consistent with their diminished fluorescence intensities. However, when the protein was dissolved in 0.5% sodium dodecyl sulfate, the phenylalanine lifetime increased while the intensity of its emission decreased. When the protein samples were stored after being boiled, the phenylalanine lifetimes increased up to 3 times of that in the protein. Presumably, the explanation was that some of the phenylalanine residues were incorporated into detergent micelles where they were relatively protected from nonradiative deexcitation. Since this phenomenon increased upon aging, data are shown for only samples that had been freshly boiled. Even so, it is evident that sodium dodecyl sulfate is not a good denaturant for this type of study.

When the protein was dissolved in 6 M guanidinium chloride, the 12.1-ns component of the tyrosine emission decreased from 44% to 19%. This is consistent with its cause being energy transfer, which should decrease as the protein unfolds and the residues become more widely separated.

The dipeptide Ac-Phe-Tyr-NH_2 shows efficient phenylalanine-to-tyrosine energy transfer, as noted earlier. Nevertheless, when it was excited at 252 nm, there was only a single detectable fluorescence lifetime: that of tyrosine. Because of the close proximity of the donor and acceptor residues in this dipeptide, rapid energy transfer is anticipated. Thus the excited-state lifetime of the phenylalanine was so shortened by energy transfer that only the tyrosine lifetime was detected.

Quenching by Phosphate. Previously, we reported that the tyrosine fluorescence in HTa is quenched by phosphate ions (Searcy et al., 1988). This type of quenching should reduce both the quantum efficiency and the excited-state lifetime proportionately. Such was indeed the case for the tyrosine residues; 60 mM phosphate quenched both the fluorescence intensity and the lifetime (Table I). In contrast, the lifetime of the 12.1-ns component was nearly unaffected by 60 mM phosphate, although its intensity was reduced. The fluorescence decay curves are shown in Figure 4. The lower curve, recorded in the presence of phosphate, shows the two lifetimes most conspicuously because the effect of phosphate was to shorten the shorter lifetime component, thus increasing the contrast between it and the longer component.

Discussion

In HTa the efficiency of fluorescence energy transfer can be defined as the fraction of ultraviolet energy absorbed by phenylalanine residues that is transferred to fluorescent tyrosine residues. Energy-transfer efficiency can be calculated from several types of measurement (Cantor & Schimmel, 1980), including (1) analysis of the excitation spectrum for tyrosine emission and (2) measurement of the effect of energy transfer upon the excited-state lifetime of the phenylalanine residues. In the present study each of these techniques yielded similar estimates for the efficiency of energy transfer, $E = 0.5$.

The second technique (donor lifetime analysis) can be affected by any changes that occur in the donor environment when the acceptor residue is removed. Thus, it is important that the conformation of the protein HTa not be changed by the removal of the carboxy-terminal residues. In this study, the hypochromicity and spectral red shift of the phenylalanine residues were not affected when these residues were removed, and the tetrameric structure of the protein remained intact, suggesting that indeed the conformation of the protein core was not seriously affected. Nevertheless, when the tyrosine residues were removed, the ratio between phenylalanine lifetime and fluorescence intensity did not remain constant (see below), suggesting that some of the residues may have changed. Apparently, this effect was compensated for by

another factor; one of the tyrosine residues is nonfluorescent. This is explained as follows.

For a given residue the excited-state lifetime should be proportional to the fluorescence quantum yield [see Cantor and Schimmel (1980)]. Nonetheless, when the residues in native HTa are compared to the free amino acids, the observed fluorescence lifetimes are longer than correspond to the intensities. This can be explained if there is a heterogeneous population of residues. Thus, some residues might be hyperfluorescent (accounting for the long lifetimes) while other residues are nonfluorescent (accounting for the low average quantum yield). According to this model, the intensity and lifetime data can be used to estimate the fraction of residues that are fluorescent.

For the tyrosine data a correction should be made because the fluorescence emission spectrum of the protein is shifted +2 nm relative to free tyrosine (Searcy et al., 1988). This affects the intrinsic rate of fluorescence emission (Cantor & Schimmel, 1980). However, if the rate constants for nonradiative deexcitation are unaffected by the spectral shift, then there should be only a slight effect upon the observed tyrosine lifetime. Thus, the calculated fraction of tyrosine residues that are fluorescent is 77%, or 3 of the 4 tyrosine residues present in each HTa tetramer. This confirms our previous pH titration measurements (Searcy et al., 1988). Thus, in native HTa the 4 tyrosine residues are not all in identical environments, and the tetrameric complex cannot be symmetrical.

For the phenylalanine residues also, the quantum yields do not correlate with the observed lifetimes. In native HTa the phenylalanine environment is clearly perturbed, as shown by the absorbance hypochromism and red shift. The hypochromism reflects a lower dipole transition probability, and thus a slower rate of fluorescence emission (Cantor & Schimmel, 1980). Consequently, more energy is channeled into nonradiative pathways of deexcitation, and the fluorescence quantum efficiency should be significantly reduced. More precise predictions would be conjectural. In conclusion, we surmise that all of the phenylalanine residues in native HTa may be fluorescent.

The presence of nonfluorescent tyrosine residues causes a complication in the interpretation of the energy-transfer data. Nonfluorescent residues should not affect the excitation spectrum method for measuring energy transfer since by definition only transfer to fluorescent residues is important. In contrast, the donor lifetime method will overestimate the efficiency of energy transfer since the nonfluorescent tyrosine residue probably causes some quenching of the phenylalanine residues and is removed by the carboxypeptidase treatment. Presumably, this error was compensated for by certain of the phenylalanine residues becoming nonfluorescent during the carboxypeptidase treatment.

The kinetic rate of energy transfer can be calculated from the effect upon the fluorescence decay rate of the donor residue when the acceptor residue is removed (Cantor & Schimmel, 1980):

$$1/\tau = k_T + \sum k \quad (1)$$

where τ is the excited-state lifetime of the donor residue and k_T is the rate constant for energy transfer. $\sum k$ is the sum of all other rate constants for deexcitation and is assumed to remain constant whether the acceptor residue is present or absent. Thus

$$\tau_0/\tau_{ET} = 1 + k_T\tau_0 \quad (2)$$

where τ_0 is the fluorescence decay lifetime of phenylalanine in carboxypeptidase-treated HTa and τ_{ET} is that in native HTa. From the lifetime data (Table I) we calculate that the kinetic rate constant for energy transfer from phenylalanine to tyrosine is $k_T = 3.9 \times 10^7\ s^{-1}$.

In a mixture of free phenylalanine and tyrosine equivalent to that in the protein there was no detectable energy transfer. A small fraction of phenylalanine fluorescence should be reabsorbed by the tyrosine. Such emission–reabsorption is different from nonradiative energy transfer and does not depend upon close proximity of donor and acceptor residues. The magnitude of this process was estimated by calculation and is too low to measure.

When HTa was excited by a pulse of radiation at 252 nm, the fluorescence at 330 nm had two lifetimes: a shorter lifetime characteristic of tyrosine and a longer lifetime characteristic of phenylalanine. Both lifetime components were emitted from tyrosine residues. The phenomenon can be explained as follows. When a pulse of radiation excites both types of residues, tyrosines that have been excited directly decay with a fluorescence lifetime typical for the residue (approximately 4 ns). In addition, a population of tyrosine residues is excited also via energy transfer. This population comes into a steady state between excitation via energy transfer and deexcitation via radiative and nonradiative processes. Overall, this population decays at the same rate that the population of excited phenylalanines decays. Thus, the situation may be thought of as viewing the phenylalanine residues through a tyrosine "pipe". Such a phenomenon is observable only if the lifetime of the donor is longer than that of the acceptor, since only then can the steady state develop. Thus the rate of energy transfer must be relatively slow, since energy transfer deexcites the donor residue. For example, with the dipeptide Ac-Phe-Tyr-NH_2 the phenylalanine lifetime was not detectable in the tyrosine emission; energy transfer was too rapid, making the phenylalanine lifetime too short. A mathematical treatment is shown in the Appendix.

The phosphate-quenching data confirm this explanation. The phenylalanine residues are presumed to be buried and inaccessible to external quenching agents such as phosphate ions. Thus, their excited-state lifetimes are unaffected by added phosphate. Nonetheless, via energy transfer the emission at 330 nm passes through the tyrosine residues, which are quenched by phosphate. Thus, adding phosphate quenched both the intensity and the lifetime of the tyrosine component but affected only the intensity and not the lifetime of the phenylalanine (12-ns) component.

The efficiency of energy transfer can be used to estimate the average distance between the donor and acceptor residues (Eisinger et al., 1969). In HTa, the Förster critical distance (R_0) is 1.35 nm [calculated by using eq 3 of Eisinger et al. (1969) with $\Phi_D = 0.13$ calculated from the lifetime data relative to $\Phi = 0.04$ for phenylalanine in air-equilibrated water, 20 °C, pH 7]. Then from the rate of energy transfer we estimate the phenylalanine-to-tyrosine distance to be 1.4 nm. This is consistent with the X-ray diffraction structure of the homologous protein HBs (Tanaka et al., 1984). When HTa was boiled in 6 M guanidinium chloride, the estimated energy-transfer distance increased to 1.6 nm. In the primary sequence of HTa there are 8 residues from the tyrosine to the nearest phenylalanine residue. If the polypeptide chain were fully extended, then the intervening distance would be about 2.9 nm. If it were in an α-helix, then the distance would be 1.2 nm. Thus, the distances estimated by energy transfer appear to be reasonable.

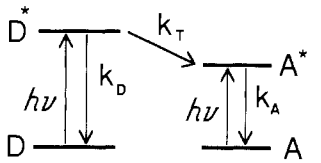

FIGURE 5: General model for energy transfer and decay. Excitation energy is transferred with kinetic rate constant k_T from excited donor group D* to acceptor group A, raising it to excited state A*. In addition, both D and A can be excited by directly absorbed radiation ($h\nu$). Excited-state decay occurs with rate constants k_D and k_A, each combining several different mechanisms of deexcitation. One component of k_A is fluorescence emission from A*, which was measured experimentally.

The results obtained in this study provide an interesting example of a protein that lacks tryptophan residues. This circumstance allowed us to measure the parameters of phenylalanine-to-tyrosine energy transfer. The combination of energy transfer plus the long excited-state lifetime of the phenylalanine residues resulted in two lifetime components in the tyrosine emission. These unusual properties are a chance consequence of the protein's amino acid composition, which is presumed to be a result of adaptation to life at high temperatures.

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APPENDIX: ANALYSIS OF ENERGY TRANSFER AND FLUORESCENCE LIFETIME DATA

The model for energy transfer is diagrammed in Figure 5. The excitation energy is absorbed primarily by donor residue D which then donates energy to acceptor residue A. Experimentally, the concentration of excited A is measured by the intensity of its fluorescent emission. In relation to HTa, D is phenylalanine and A is tyrosine.

Model and Definitions. [D*] is the concentration of excited donor and [A*] the concentration of excited acceptor. The mixture is excited by a brief pulse of radiation and then allowed to decay. Let

$$I_0 = [D^*]_0 + [A^*]_0 \tag{3}$$

where the subscript "0" signifies time $t = 0$ after a brief excitatory pulse. At this moment both A and D have been excited by direct absorption. I_0 is equivalent to the total light absorbed. Also by definition let

$$\gamma = [D^*]_0/[A^*]_0 \tag{4}$$

where γ is equivalent to the ratio of the absorbances of D and A. By rearrangement of eq 3 and 4

$$[D^*]_0 = \gamma I_0/(\gamma + 1) \tag{5}$$

and

$$[A^*]_0 = I_0/(\gamma + 1) \tag{6}$$

By inspection of Figure 5

$$d[D^*]/dt = -(k_T + k_D)[D^*] \tag{7}$$

Following integration and evaluation at $t = 0$

$$[D^*] = \frac{I_0\gamma}{\gamma + 1} e^{-(k_T + k_D)t} \tag{8}$$

For A*

$$d[A^*]/dt = k_T[D^*] - k_A[A^*] \tag{9}$$

$$d^2[A^*]/dt^2 = k_T\, d[D^*]/dt - k_A\, d[A^*]/dt \tag{10}$$

Substituting eq 7 and 9 into eq 10, one obtains

$$\frac{d^2[A^*]}{dt^2} + (k_T + k_D + k_A)\frac{d[A^*]}{dt} + k_A(k_T + k_D)[A^*] = 0 \tag{11}$$

This is a second-order linear homogeneous differential equation. It can be integrated to an expression of the form

$$[A^*] = \beta_1 e^{t/\tau_1} + \beta_2 e^{t/\tau_2} \tag{12}$$

From the algorithm for this integral

$$\tau_1 = -1/(k_T + k_D) \quad \text{and} \quad \tau_2 = -1/k_A \tag{13}$$

τ_1 and τ_2 are the inverse of the kinetic decay constants, which by definition are the excited-state lifetimes of D and A. Expressions for β_1 and β_2 can be obtained as follows. Equation 12 is differentiated and evaluated at $t = 0$ to obtain

$$d[A^*]/dt|_0 = \beta_1/\tau_1 + \beta_2/\tau_2 \tag{14}$$

Separately, when expressions 8 and 12 are substituted into eq 9 and evaluated at $t = 0$, one finds

$$d[A^*]/dt|_0 = k_T I_0\gamma/(\gamma + 1) - k_A(\beta_1 + \beta_2) \tag{15}$$