Quenching of Intrinsic Fluorescence of Yeast Cytochrome *c* Peroxidase by Covalently- and Noncovalently-Bound Quenchers†

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ABSTRACT: The intrinsic steady-state fluorescence of the heme enzyme cytochrome *c* peroxidase (CCP) has been characterized as a probe of its structure in solution. The fluorescence is dominated by tryptophan emission, which has a quantum yield of 7% relative to the tryptophan standard *N*-acetyltryptophanamide, and an emission maximum at 324 nm indicative of a relatively hydrophobic environment for the fluorescent residues. These fluorescence properties are consistent with the known structure of CCP; six of the seven tryptophan residues are well within quenching distance for efficient Förster energy transfer to the heme, so that the intrinsic fluorescence arises largely from Trp101 which is ~26 Å from the heme and partially buried. Quenching studies using Cs^+, I^-, and acrylamide are also consistent with this picture, since the charged species are poor quenchers, but acrylamide, which can penetrate the protein matrix, is a more effective quencher. The intrinsic fluorescence of two CCP derivatives with the quencher pentaammineruthenium(III) covalently attached to His6 and His60 has also been characterized. The His60 derivative, shown by X-ray analysis to be essentially structurally identical to native CCP, is 17% less fluorescent than native CCP, consistent with the quenching expected from distance calculations and the assignment of Trp101 as the major fluorescent center. The observed quenching of 38% in the second derivative is close to that predicted for ruthenation of His6 assuming that Trp101 is the major fluorophore. The fluorescence of compound I of CCP is also reported. This species, which has a ferryl (Fe^{IV}=O) heme and a protein radical purportedly on Trp191, exhibits 9% higher fluorescence than native CCP. This increase can be accounted for by the 12-nm red-shift in the Soret band in compound I, which reduces the efficiency of Förster energy transfer. A similar fluorescence increase is seen for the cyanide adduct of ferric CCP which also has a red-shifted Soret relative to native CCP. Thus, there is no evidence from the intrinsic fluorescence of a global protein conformational change in CCP upon compound I formation, cyanide ligation, or ruthenation, in agreement with X-ray studies.

Cytochrome *c* peroxidase (CCP)[1] catalyzes the oxidation of ferrocytochrome *c* by hydrogen peroxide. The enzyme consists of a single polypeptide of 294 residues with a single noncovalently-bound protoporphyrin IX heme (Bosshard et al., 1991). The polypeptide contains seven tryptophans, and since tryptophan fluorescence is highly sensitive to environment, these residues are useful structural probes (Burstein et al., 1973). In addition to tryptophan, CCP contains 14 tyrosine and 18 phenylalanine residues which also fluoresce on UV-excitation. However, fluorescence from tyrosine and phenylalanine is generally quenched by energy transfer to tryptophan (Lakowicz, 1983a), so that steady-state protein fluorescence is dominated by tryptophan emission. It is also well documented that heme is an efficient quencher of intrinsic protein fluorescence (Willis et al., 1990), and since the crystal structure of CCP reveals that six of the seven tryptophans are within 17 Å of the heme, substantial fluorescence quenching is expected.

The relative accessibility of tryptophan residues in a protein can be determined by the sensitivity of the intrinsic fluorescence to a variety of freely-diffusing quenchers. Quenching studies with the hydrophilic quenchers Cs^+ and I^- were carried out to determine the amount of fluorescence arising from water-accessible tryptophans. Quenching by the uncharged, polar quencher acrylamide was also examined to probe the efficiency of a quencher which can penetrate, to some extent, the hydrophobic interior of proteins (Eftink & Ghiron, 1981).

It has been observed that covalent attachment of pentaammineruthenium(III) (a_5Ru) to histidine residues efficiently quenches protein tryptophan fluorescence (Recchia et al., 1982). Two derivatives of CCP with a_5Ru attached to His6 and His60 on the surface of CCP have been isolated and characterized (Fox et al., 1990; Fox et al., in preparation). The results of studies on the covalently-bound quenchers show that this approach is complementary to the use of freely-diffusing quenchers in probing tryptophan fluorescence.

Compound I is a stable intermediate formed on the reaction of CCP with a stoichiometric amount of H_2O_2 (Bosshard et al., 1991). This intermediate contains a ferryl (Fe^{IV}=O) heme and an amino acid radical which, as recent evidence indicates, is located on Trp191 (Mauro et al., 1988; Scholes et al., 1989; Sivaraja et al., 1989; Erman et al., 1989), making CCP the first enzyme that purportedly uses a tryptophan radical during turnover. The steady-state fluorescence of compound I and that of the cyanide adduct of CCP (CCP-CN) are compared here since both species exhibit a Soret maximum that is red-shifted from that of native CCP. The Förster theory of resonance energy transfer and the crystal structure of CCP (Poulos & Finzel, 1984) are used below to

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[1] Abbreviations: CCP, cytochrome *c* peroxidase (EC 1.11.1.5); CCP-CN, cyanide adduct of CCP; compound I, two-electron-oxidized intermediate of CCP; a_5Ru, pentaammineruthenium(III) where a = NH_3; NATA, *N*-acetyltryptophanamide.

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interpret the steady-state fluorescence of compound I, of CCP-CN, and of native CCP in the presence and absence of quenchers.

EXPERIMENTAL PROCEDURES

Analytical-grade *N*-acetyltryptophanamide (NATA) and KCN were purchased from Sigma. Sodium thiosulfate was obtained from BDH. Electrophoresis-grade urea and acrylamide were obtained from Bio-Rad. Analytical-grade KI, CsCl, NaCl, and 30% (w/v, 6.8 M) hydrogen peroxide were supplied by ACP.

Preparation of Ruthenium Complexes. $[a_5RuHis](Cl)_3$ was prepared by the published procedure (Sundberg & Gupta, 1973), and the $a_5Ru(His)CCP$ derivatives were synthesized and purified as described previously (Fox et al., 1990; Fox et al., in preparation).

Sample Preparation. Stock solutions were prepared in 0.1 M sodium phosphate buffer, pH 7.0. Concentrations of stock ferric CCP and $a_5Ru(His)CCP$ were determined spectrophotometrically using $\epsilon_{408} = 98\ mM^{-1}\ cm^{-1}$ (Yonetani & Anni, 1987), and of a_5RuHis using $\epsilon_{303} = 2.1\ mM^{-1}\ cm^{-1}$ (Sundberg & Gupta, 1973). The pH of 5 N KI, CsCl, NaCl, and acrylamide was readjusted to 7.0 prior to use, and iodine precipitation was prevented by including 1 mM sodium thiosulfate in the KI stock. The salt concentration of all samples containing the ionic quenchers was adjusted to 0.73 M by adding NaCl. To form compound I, stoichiometric H_2O_2 was added to 0.85 μM CCP in the cuvette. Prior to use, the peroxide was diluted to 0.2 mM and standardized by titration vs 5.0 μM CCP. CCP-CN (0.85 μM) was prepared by adding 1 mM stock CCP to phosphate buffer containing 0.1 mM KCN.

Fluorescence Measurements. Steady-state fluorescence measurements were carried out using a Shimadzu spectrofluorophotometer (Model RF-5000) with the sample compartment maintained at 22 ± 1 °C. All emission spectra were recorded using the "very slow" (114 nm/min) scan speed of the fluorometer. The Raman peak of water was subtracted from the emission spectra, and all emission data were corrected for inner-filter effects using the formula (Lakowicz, 1983a):

$$F_c = F\ \text{antilog}[(A_{ex} + A_{em})/2] \quad (1)$$

where F_c is the corrected fluorescence intensity, F is the measured intensity, A_{ex} and A_{em} are the absorbance at the excitation and emission wavelengths, respectively. The fluorescence quantum yields for CCP in buffer and urea relative to the tryptophan standard, NATA, were determined from the ratios of the areas of the emission bands normalized for the relevant absorption at the excitation wavelength (280 nm) as discussed below.

RESULTS

Native CCP Fluorescence. Figure 1 shows the corrected steady-state emission between 300 and 450 nm of native CCP following excitation at 260, 280, and 295 nm. The emission maximum is at 324 ± 2 nm, indicating that the average tryptophan environment is hydrophobic (Lakowicz, 1983a). The excitation spectrum (not shown) is characteristic of tryptophan absorption with a maximum at 280 nm; also, the emission spectra did not show any wavelength dependence, consistent with the steady-state fluorescence being dominated by emission from tryptophan residues. To estimate the fluorescence quantum yield of native CCP relative to NATA, it is necessary to estimate the fractional absorbance of

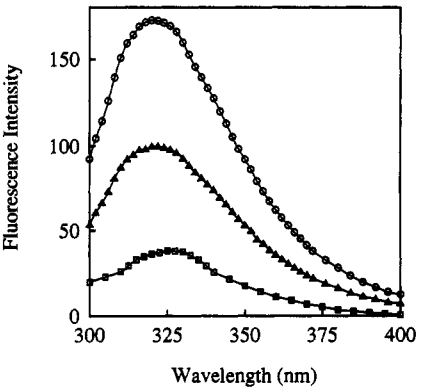

FIGURE 1: Fluorescence emission of 0.85 μM native CCP in 0.1 M phosphate buffer, pH 7.0, 22 ± 1 °C, when excited at 260 (triangles, 280 (circles), and 295 nm (squares). The data points are the fluorescence intensities corrected for inner-filter effects (F_c, eq 1). Excitation and emission slit widths were 10 and 5 nm, respectively, and the scan speed was 114 nm/min.

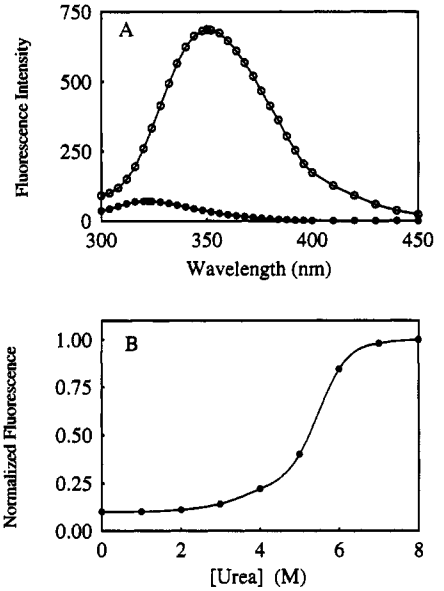

FIGURE 2: (A) Fluorescence emission of 0.85 μM CCP in 0.1 M phosphate buffer, pH 7.0, 22 ± 1 °C (closed circles), and in 8 M urea (open circles). The data points are the fluorescence intensities corrected for inner-filter effects (F_c, eq 1). (B) Normalized fluorescence intensities at 350 nm of 0.85 μM CCP vs urea concentration. The scan speed was 114 nm/min, the excitation wavelength was 280 nm, and slit widths were 5 nm.

tryptophan at the excitation wavelength. Holo- and apoCCP have molar absorptivities at 280 nm of 74 and 55 $mM^{-1}\ cm^{-1}$, respectively (Yonetani, 1967). Assuming 100% energy-transfer efficiency from tyrosine to tryptophan, all light absorbed by the apoprotein moiety will appear as tryptophan fluorescence (Lakowicz, 1983a); hence, the 280-nm absorbance of CCP was reduced by 55/74 before integrated emission intensities were normalized for the relative absorbance at 280 nm. This resulted in a fluorescence quantum yield for native CCP relative to NATA of 7%, indicating that quenching by the heme is a highly efficient process.

In 8 M urea, the emission maximum of CCP shifts to 350 ± 2 nm (Figure 2A), consistent with exposure of the tryptophans to the aqueous solvent (Lakowicz, 1983a). The fluorescence quantum yield, estimated as for the native protein, is 67% relative to NATA. However, since the efficiency of tyrosine to tryptophan energy transfer is significantly less efficient in unfolded proteins (Lakowicz, 1983a), this value is probably a lower estimate of the quantum yield. The

Table I: Relative Fluorescence Intensities (% F_c) of Native CCP in the Presence of Freely-Diffusing Quenchers in 0.1 M Phosphate Buffer, pH 7.0, 22 ± 1 °C

0.73 M quencher	% F_c[a]
none	100 ± 2
Cs^+	95 ± 2
I^-	93 ± 2
acrylamide	68 ± 3

[a] Fluorescence intensities, corrected for inner-filter effects (F_c, eq 1), were measured at the emission maximum which was centered at 324 nm for all species. Excitation was at 280 nm for Cs^+ and at 295 nm for I^- and acrylamide quenching, respectively. Excitation and emission slits were 10 and 5 nm, respectively; scan speed: 114 nm/min.

Table II: Relative Fluorescence Quantum Yields for Native CCP and a_5RuHis–CCP Derivatives

protein	quantum yield[a]
CCP	100
CCP + a_5RuHis[b]	100
a_5RuHis60–CCP	83
a_5RuHis6–CCP	62

[a] Relative fluorescence quantum yields were calculated from a ratio of the areas of the emission bands of native CCP and the a_5RuHis–CCP derivatives. See Table I for experimental conditions. [b] Noncovalent 1:1 mixture of CCP and the free a_5RuHis complex.

unfolding transition occurs between 4 and 6 M urea (Figure 2B), with half-maximum fluorescence intensity at 5.3 M urea.

Quenching of Native CCP Fluorescence by Cs^+, I^-, and Acrylamide. Table I summarizes the relative fluorescence intensity of CCP at the emission maximum (324 nm) in the presence of the freely-diffusing quenchers. At a concentration of 0.73 M, Cs^+ and I^- exhibit similar low quenching of CCP (5 and 7%, respectively; Table I), although I^- is a 6-fold more efficient quencher of free indole (Eftink & Ghiron, 1981); thus, the negative charge on CCP at pH 7.0 (Bosshard et al., 1991) may enhance the quenching efficiency of Cs^+ over I^-. However, 32% quenching is observed in the presence of 0.73 M acrylamide, which quenches free indole with the same efficiency as I^-. Hence, the relative quenching efficiencies of the polar and nonpolar species indicate that the fluorescent tryptophans are in a hydrophobic environment in CCP, consistent with the blue-shifted emission maximum (324 nm). Because of the low fluorescence quantum yield for CCP, plots of fluorescence intensity vs quencher concentration showed large scatter which precluded the estimation of Stern–Volmer quenching constants.

Fluorescence of the a_5RuHis–CCP Derivatives. The fluorescence spectra of native CCP and the a_5Ru-modified derivatives are identical, except as the data in Table II indicate, the fluorescence quantum yields relative to native CCP of the a_5RuHis6 and a_5RuHis60 derivatives are 62 and 83%, respectively. Since a 1:1 noncovalent mixture of native CCP and the free a_5RuHis complex exhibited the fluorescence intensity of CCP alone, the quenching observed for a_5RuHis-modified CCP must be due to covalently-bound a_5RuHis, and the extent of quenching clearly depends on the site of attachment of the metal complex.

Table III summarizes the fluorescence quenching of the a_5RuHis–CCP derivatives by the freely-diffusing quenchers. Cs^+ and I^- cause little or no detectable quenching of either derivative. In contrast, acrylamide quenches the a_5RuHis60 derivative by 33% (i.e., as effectively as it quenches native CCP, Table I), but the a_5RuHis6 derivative by only 8%. Thus, a_5Ru bound to His6, but *not* to His60, competes effectively with acrylamide as a quencher.

Table III: Relative Fluorescence Intensities (% F_c) of a_5RuHis–CCP Derivatives in the Presence of Freely-Diffusing Quenchers in 0.1 M Phosphate Buffer, pH 7.0, 22 ± 1 °C

	% F_c[a]	
0.73 M quencher	a_5RuHis60–CCP	a_5RuHis6–CCP
none	100 ± 2	100 ● 5
Cs^+	96 ± 3	100 ± 5
I^-	100 ● 3	100 ± 4
acrylamide	67 ± 4	92 ± 3

[a] See footnote to Table I for experimental conditions.

Table IV: Overlap Integrals (J) and R_0 for Tryptophan Fluorescence Energy Transfer in Different CCP Species[a]

protein	$J \times 10^{14}$ (cm^3 M^{-1})	R_0 (Å)
CCP[b]	5.77	35
CCP-CN[b]	2.91	31
compound I[b]	3.07	31
a_5RuHis–CCP[c]	0.102	18

[a] R_0 and J were calculated using eq 3 and 4. [b] Trp → heme energy transfer. [c] Trp → a_5RuHis energy transfer.

Quantum Yields for the Individual Tryptophan Residues in CCP and the a_5RuHis–CCP Derivatives. From the Förster theory of resonance energy transfer (Stryer, 1978), the efficiency of energy transfer (E) between the donor (Trp) and acceptor (heme or a_5RuHis) is related to the distance, r, between the acceptor and donor groups:

$$E = r^{-6}/(r^{-6} + R_0^{-6}) \quad (2)$$

where R_0 is the donor–acceptor separation at which the donor fluorescence is quenched by 50%. R_0 is defined by

$$R_0 = (9.79 \times 10^3)(\kappa^2 n^{-4} Q_a J)^{-6} \quad (3)$$

κ^2 was assumed to be 0.67 for random orientation between the donor and acceptor transition dipoles (Haas et al., 1978) since tryptophan residues possess two perpendicular emission dipoles and exhibit rotational mobility in proteins (Lakowicz et al., 1983b); the heme and a_5RuHis groups also contain electronic transitions along two and three perpendicular axes, respectively. Under these circumstances, any error in R_0 introduced by the orientation factor is expected to be negligible (Stryer, 1978). The refractive index of the medium between the donor and acceptor, n, is generally given a value of 1.4 for proteins (Stryer, 1978), and Q_a, the quantum yield for tryptophan in the absence of acceptors, is estimated to be 0.2 (Teale & Weber, 1957). The spectral overlap integral, J, between the donor emission and the acceptor absorption is calculated from

$$J = \frac{\int F_\lambda \epsilon_\lambda \lambda^4 \, d\lambda}{\int F_\lambda \, d\lambda} \quad (4)$$

where F_λ is the emission intensity of the donor at wavelength λ (in nanometers) and ϵ_λ is the extinction coefficient of the acceptor at λ (in nanometers). Overlap integrals between 300 and 400 nm were determined using eq 4 as previously described (Campbell & Dwek, 1984), and Table IV lists the calculated overlap integrals (J) and the R_0 values for Trp → heme energy transfer in CCP, CCP-CN, and compound I.

The efficiency of energy transfer (E) between donor and acceptor is also given by

$$E = 1 - Q_p/Q_a \quad (5)$$

Thus, the relative quantum yield for tryptophan in the presence

Table V: Percent Quantum Yields for Tryptophan Residues in Native CCP (% Q) and a_5RuHis–CCP Derivatives (% Q')

	native CCP		a_5RuHis60–CCP		a_5RuHis6–CCP	
	r (Å)[a]	% Q[b]	r (Å)[c]	% Q'[d]	r (Å)[c]	% Q'[d]
Trp57	15	3.37	10	0.10	6	0.03
Trp126	17	7.30	26	6.58	15	1.83
Trp223	15	3.37	29	3.17	37	3.33
Trp211	16	5.06	33	4.93	38	5.00
Trp101	26	80.9	32	78.4	21	57.9
sum[e]		100		93.2		68.4
obs[f]		100		83		62

[a] Closest distance from Fe to the Trp indole ring. [b] The % Q for each Trp in native CCP was calculated using eq 6; % Q = 0 for Trp51 and Trp191 which are within 5 Å of the heme. [c] Closest distance from the $N_{\epsilon 2}$ atom of His to the indole ring. [d] % Q' values for the a_5RuHis derivatives were calculated by multiplying the corresponding % Q for native CCP by $(1 - E')$, where E' is the Trp → a_5RuHis energy-transfer efficiency (see text). [e] Sum of % Q' for each Trp gives an estimate of the fluroescence quantum yields for the a_5RuHis derivatives relative to native CCP. [f] Observed fluorescence quantum yields for the a_5RuHis60 and a_5RuHis6 derivatives relative to native CCP (Table II).

of an acceptor, Q_p/Q_a, is $1 - E$. Assuming R_0 = 35 Å (Table IV) and the Trp-to-heme distances (r) given in Table V, values for Q_p/Q_a were calculated for the individual tryptophans in CCP, and $100/7\Sigma(Q_p/Q_a)$ = 2.6% for the seven tryptophans in CCP is in good agreement with the observed quantum yield of 7% for CCP relative to NATA. The percent contribution (% Q) of each tryptophan to the total fluorescence of CCP is

$$\% Q = \frac{100(Q_p/Q_a)}{\sum(Q_p/Q_a)} \quad (6)$$

The % Q values listed in Table V clearly show that Trp101 is expected to dominate the steady-state fluorescence of CCP, contributing >80% of the intensity.

The efficiency of Trp → a_5RuHis energy transfer (E') in CCP ruthenated at histidines can also be determined using eq 2 with R_0 = 18 Å (Table IV), and the relevant r values in Table V. Since the quantum yield of each tryptophan in the derivatives is further reduced by a factor of $1 - E'$ due to energy transfer to a_5RuHis, % Q' [= % $Q \times (1 - E')$] gives the percent contribution of each tryptophan in the derivatives relative to native CCP. From the sum of the % Q' values (Table V), fluorescence quantum yields of 68 and 93% are predicted for the a_5RuHis6 and a_5RuHis60 derivatives, respectively, relative to CCP. The observed relative yields are 62 and 83%, respectively, which are 90 ± 1% of the estimated values.

Fluorescence of Compound I and CCP-CN. Titration of CCP with 1 equiv of H_2O_2 gives 100% conversion to compound I as monitored by the Soret shift from 408 to 420 nm (Erman & Yonetani, 1975). This species is formed within the manual mixing time (3 s), and under the present experimental conditions, it has a decay half-life of ~5 h (Erman & Yonetani, 1975). The emission maximum is at 324 ± 2 nm in the spectra of both native CCP and compound I; however, the fluorescence intensity of compound I at 324 nm is 9% greater than that of native CCP. This increase may be attributed to the red-shift in the Soret band maximum since the cyanide adduct of CCP, which has a Soret maximum at 422 nm (Erman, 1974), is ~7% more fluorescent than native CCP. The smaller overlap integrals for CCP-CN and compound I compared to that for native CCP (Table IV) account for their higher fluorescence intensities.

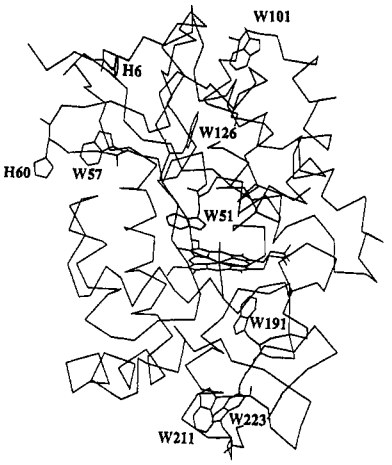

FIGURE 3: Computer graphics display of the C_α backbone of CCP showing the location of the heme, the tryptophan residues (W), and the histidine residues (H) that were ruthenated. This molecular graphics image was produced using the MidasPlus software system from the Computer Graphics Laboratory, University of California, San Francisco (Ferrin et al., 1988).

DISCUSSION

Figure 3 shows the location of the heme, His6, His60, and the tryptophan residues in CCP mapped on the C_α backbone. Trp51 and Trp191 are within 5 Å of the heme; thus, these residues are not expected to contribute to the steady-state fluorescence of CCP. Four of the remaining tryptophans are 15–17 Å from the heme, and their fluorescence should also be substantially quenched; hence, Trp101, which is 26 Å from the heme, is expected to be the main fluorophore in CCP. As can be predicted from Figure 3, Trp → heme energy transfer is an efficient process in CCP, leading to a quantum yield of 7% relative to NATA. The calculated R_0 of 35 Å for CCP is essentially the same as that calculated for cytochrome c oxidase (Hill et al., 1986), and the predicted quantum yield $[100/7\Sigma(Q_p/Q_a)]$ of 2.6% estimated using Förster theory is in reasonably good agreement with the observed value of 7%.

Consistent with extensive energy transfer to the heme, unfolding of CCP in urea results in a large increase in fluorescence as shown in Figure 2A. Also, the emission maximum red-shifts by ~25 nm to the value expected for water-solvated tryptophan (Lakowicz, 1983a), and the transition from the folded to the unfolded form of the enzyme, as monitored by fluorescence (Figure 2B), has a midpoint at 5.3 M urea. The quantum yield in 8 M urea of the unfolded enzyme is 67% relative to NATA, assuming that tyrosine to tryptophan energy transfer is 100% efficient, or 94% if no energy transfer between the aromatic residues takes place. The lower quantum yield would indicate that quenching by the heme was still occurring in the unfolded protein, which exhibits a Soret maximum at 400 nm.

Examination of the crystal structure of CCP also allows an estimation of the solvent accessibility of the tryptophan residues. Trp51 and Trp191 are buried in the protein interior; the peptide backbones of Trp126 and Trp211, and the indole rings of Trp57 and Trp101, appear to be partially solvent-exposed. The indole ring of Trp223 is fully exposed to solvent, so from its accessibility alone, we would expect Trp223 to be the most quenched by freely-diffusing quenchers. The small degree of quenching by high concentrations of both Cs^+ and I^- (Table I) indicates that the fluorescent tryptophans in CCP are not accessible to hydrophilic quenchers. This is consistent with the picture from the X-ray structure, since Trp223 is expected to account for only ~3% of the fluorescence (Table

V) due to efficient energy transfer to the heme. In contrast to the ionic quenchers, the same concentration of acrylamide quenches the fluorescence of native CCP by 32% (Table I), presumably because this apolar quencher can penetrate the protein matrix (Eftink & Ghiron, 1981). The blue-shifted emission maximum of CCP also indicates that the fluorescent tryptophans, especially Trp101, are in a hydrophobic environment (Burstein et al., 1973).

The R_0 of 18 Å calculated for Trp → a_5RuHis fluorescence energy transfer (Table IV) is similar to that calculated for α-lytic protease (16 Å), but larger than that for lysozyme (12 Å) (Recchia, 1982). However, for lysozyme, the predicted Trp → a_5RuHis energy-transfer efficiency is significantly smaller than the observed efficiency, suggesting that R_0 may be underestimated. Figure 3 also shows the position of the surface-exposed His6 and His60 residues of CCP. Covalent binding to His60 places an a_5RuHis complex within 10 Å of Trp57 (Table V), which is well within the R_0 of 18 Å estimated for CCP. Since Trp57 only contributes ~3% to the fluorescence of CCP, the 17% decrease in the fluorescence yield on attachment of the a_5Ru complex to His60 (Table II) suggests some quenching of Trp101 fluorescence. However, His60 and Trp101 are separated by 32 Å, so only ~2% quenching of Trp101 by the Ru center is expected unless other quenching mechanisms are also operative. Nonetheless, the observed fluorescence quantum yield of 83% for the a_5RuHis60 derivative, compared to native CCP, is ~89% of the estimated yield assuming Förster energy transfer to the heme and Ru centers only (Table V).

The observed quantum yield for the a_5RuHis6 derivative is 62% compared to CCP, which is 91% of the estimated relative yield (68%) (Table V). The His6–Trp101 separation is ~21 Å, so attachment of a_5Ru to His6 should significantly quench the fluorescence of Trp101, the major fluorophore in CCP, and also Trp126 and Trp57 (Table V). It is of interest to note that in addition to a_5Ru binding to His6, mass spectral analysis revealed that a second a_5Ru is coordinated to CCP in this derivative. Difference spectroscopy indicated that the second a_5Ru center is spectroscopically silent in regions where CCP does not absorb strongly. This is consistent with the fluorescence results since quenching by a_5RuHis6 alone accounts for most (90%) of the reduction in quantum yield for this derivative relative to native CCP. The fluorescence data also reveal that double-derivatization of CCP with a_5Ru does not cause any global conformational changes in the protein, consistent with the nativelike behavior of the a_5RuHis6 derivative (Fox et al., in preparation). Independent confirmation that ruthenation of His60 does not cause structural changes in CCP has been obtained from the X-ray structure of the a_5RuHis65 derivative (Fox et al., 1990).

The freely-diffusing quenchers, I^- and Cs^+, cause little or no quenching of the fluorescence of the ruthenated derivatives (Table III). Thus, the fluorescent tryptophans must be less accessible to ionic quenchers in the a_5RuHis derivatives, or the a_5RuHis centers efficiently quench the residues accessible to the ionic quenchers. In either case, the results indicate that tryptophans on the top half of CCP (Trp126, Trp57, and particularly Trp101) must be quenched by the ionic quenchers in native CCP. Acrylamide quenches the fluorescence of the a_5RuHis60 derivative by 33% but that of the a_5RuHis6 derivative by only 8%. Given the relative separations between Trp101 and the modified histidines (21 and 32 Å for His6 and His60, respectively), the acrylamide results strongly support the quantum yield calculations (Table V) which indicate that the blue-shifted emission of CCP is dominated by Trp101 fluorescence.

The fluorescence intensity of compound I is ~9% higher than that of native CCP. This may be attributed to the smaller spectral overlap integral, J, for compound I (Table IV) which arises because the Soret band of CCP red-shifts by 12 nm upon compound I formation. The similar fluorescence increase observed on CCP-CN formation supports this conclusion, since the Soret band also red-shifts upon formation of the cyanide adduct (Erman & Yonetani, 1975). The crystal structures of compound I and CCP-CN show only minor changes compared to native CCP (Edwards et al., 1987; Edwards & Poulos, 1990), consistent with the fluorescence data since tryptophan fluorescence is sensitive to protein conformation. It has been proposed that one of the oxidizing equivalents of compound I is located on Trp191 (Sivaraja et al., 1989), but since we attribute zero fluorescence quantum yield to this particular residue, no decrease in the observed fluorescence intensity of compound I relative to native CCP was predicted.

The results of this study show that the quenching of tryptophan residues in CCP and the a_5RuHis derivatives is satisfactorily described by the Förster dipole–dipole mechanism. Considering the approximations made, the experimentally observed and predicted fluorescence quantum yields are in good agreement. In particular, a meaningful comparison of the quantum yields predicted for the individual tryptophan residues using available structural data, and the observed fluorescence yields for CCP, compound I, and the a_5RuHis derivatives, is presented here. The demonstration that Trp → a_5RuHis energy transfer strongly depends on the site of ruthenation indicates that ruthenation of histidine residues in proteins should provide a useful method for probing fluorescence from selected tryptophan residues. In fact, time-resolved fluorescence measurements on ruthenated proteins should assist in the assignment of the observed lifetimes to specific residues. Thus, in addition to its utility as a paramagnetic probe in the assignment of histidine residues in NMR, the a_5RuHis group should also serve as a useful probe in fluorescence studies.

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