Exposure and Electronic Interaction of Tyrosine and Tryptophan Residues in Human Apolipoprotein A-IV†

Richard B. Weinberg*

Division of Gastroenterology, Department of Medicine, University of Texas Health Science Center, Houston, Texas 77225

Received July 7, 1987; Revised Manuscript Received October 30, 1987

ABSTRACT: We have investigated the exposure and electronic interaction of tyrosine and tryptophan residues in human apolipoprotein A-IV (apo A-IV). Differential absorption spectroscopy and chemical titration demonstrated that human apo A-IV contains six tyrosine residues, four of which are buried in the hydrophobic interior of the protein and two of which are exposed on the protein surface. Denaturation of the protein by guanidinium chloride caused progressive exposure of the buried tyrosines. The fluorescence emission spectra of apo A-IV were characterized by a blue-shifted tryptophan emission with a low relative quantum yield of 0.37 and a tyrosine emission with a relative quantum yield of 0.62. Fluorescence quenching studies demonstrated a low fractional exposure of tryptophan in the native state. Denaturation of apo A-IV was accompanied by an increase in the relative quantum yield which peaked at the denaturation midpoint. Fluorescence excitation techniques demonstrated energy transfer from tyrosine residues with a transfer efficiency of 0.40 in the native state; the efficiency was conformation dependent and decreased with protein unfolding. Fluorescence studies of tetranitromethane-modified apo A-IV suggested that a significant fraction of energy transfer proceeds from the exposed tyrosine residues. These data demonstrate the existence of intramolecular fluorescence energy transfer and tryptophan quenching in human apolipoprotein A-IV and suggest that the amino terminus of this protein is situated in a hydrophobic domain within energy-transfer range of nonvicinal tyrosine residues.

Human apolipoprotein A-IV (apo A-IV)[1] is a plasma glycoprotein of intestinal origin with a molecular weight of 46 000 (Weisgraber et al., 1978; Beisiegel & Utermann, 1979; Greene et al., 1979; Weinberg & Scanu, 1983). Recent investigations have established that apo A-IV shares many structural and biophysical properties with other apolipoproteins. Specifically, apo A-IV contains a high percentage of α-helical structure (Weinberg & Spector, 1985a) which is conferred by the presence of multiple amino acid repeats (Elshourbagy et al., 1986); as expected, these repeated helical sequences are amphipathic (Weinberg, 1987). Indeed, the lipid binding properties of apo A-IV are specifically related to the presence and stability of its secondary structure (Weinberg & Spector, 1985a). Nonetheless, certain aspects of the biochemistry of human apo A-IV are distinctive. In vitro, the association of apo A-IV with lipid is relatively labile, and it is easily displaced from the surface of model lipoproteins by other apolipoproteins (Weinberg & Spector, 1984; Rifici et al., 1985). Similarly, the in vivo affinity of apo A-IV for plasma lipoproteins is considerably weaker than for other apoproteins (Beisiegel & Utermann, 1979; Utermann & Beisiegel, 1979; Greene et al., 1980), and as much as 80% of human apo A-IV circulates as a free protein, unassociated with lipoproteins (Ghiselli et al., 1986). As these unusual properties of human apo A-IV cannot be attributed to unique primary or secondary structure, it is evident that delineation of the structural basis for the biological behavior of apo A-IV will require further investigation of its conformational features.

Spectroscopy can be a useful tool in the investigation of the structure of globular proteins; such studies have yielded valuable information regarding the conformation of mammalian apolipoproteins (Morrisett et al., 1977; Osborne & Brewer, 1977). The availability of the primary amino acid sequence of human apo A-IV (Elshourgaby, 1986) allows the prediction of the secondary structure surrounding its single tryptophan residue and establishes the position and potential side-chain interactions of tyrosine residues. As the fluorescence properties of tryptophan-containing proteins are sensitive to changes in conformation-dependent interactions between tyrosine and tryptophan, we have therefore extended the spectroscopic study of human apolipoprotein A-IV to specifically investigate the exposure and electronic interaction of tyrosine and tryptophan residues. The findings demonstrate the existence of conformation-dependent intramolecular fluorescence energy transfer and quenching in human apo A-IV and suggest structural correlates which may be pertinent to an understanding of its biochemical properties.

Experimental Procedures

Isolation of Human Apolipoprotein A-IV. Human apo A-IV was isolated from fasting normolipidemic volunteer blood donors by anion-exchange chromatography in 8 M urea (Weinberg & Spector, 1984). Purified protein was concentrated to 1 mg/mL by diafiltration against 1 mM NH_4HCO_3, pH 8.6, containing 0.02% sodium azide, sterilized by passage through a Millipore 0.25-μm filter, and stored at 4 °C. Protein concentration was measured by the technique of Lowry (Lowry et al., 1951).

Ultraviolet Absorption Spectroscopy. The ultraviolet absorption of solutions of human apo A-IV was measured by using a Hewlett-Packard 8450A diode-array spectrophotom-

† This research was supported by National Institutes of Health Grant HL30897 and American Heart Association Grant-in-Aid 860956.

* Address correspondence to this author at the University of Texas Health Science Center, Department of Medicine, Box 20708, Room 4234, Houston, TX 77225. He is an Established Investigator of the American Heart Association and recipient of the American Gastroenterological Association/William H. Rorer Research Scholarship.

[1] Abbreviations: apo A-IV, apolipoprotein A-IV; GdmCl, guanidinium chloride; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/88/0427-1515$01.50/0

eter equipped with thermostated cuvettes and an on-line digital data processor. Solutions of apo A-IV in 50 mM potassium phosphate buffer, pH 7.5, were studied at 25 °C at concentrations of 10–20 μM. Differential absorption spectra were obtained by addition of aliquots of buffered 8 M GdmCl to the sample and reference cells; the changes in molar extinction from 250 to 300 nm were calculated at each GdmCl concentration by subtraction of the base-line spectrum stored in memory, with appropriate correction for dilution.

The number of tyrosine residues in apo A-IV was determined by second-derivative absorption spectroscopy of protein dissolved in buffered 6 M GdmCl, as described by Servillo et al. (1982). The tyrosine/tryptophan ratio of a denatured protein may be calculated from the second-derivative absorption spectra by the expression $(a/b - B)/[A - (a/b)C]$, where A, B, and C are empirically derived coefficients, "a" is the peak to trough distance between the second-derivative spectral minimum near 283 nm and the maximum near 287 nm, and "b" is the peak to trough distance between the spectral minimum near 291 nm and the maximum near 295 nm.

The mutual interference of the solvent-sensitive second-derivative absorption bands of tyrosine with the environmentally insensitive band of tryptophan can be utilized to estimate the exposure of tyrosyl residues in many proteins (Ragone et al., 1984). The fractional exposure can be expressed as $(r - r_a)/(r_u - r_a)$, where r is the ratio a/b measured from the second-derivative absorption spectra, r_u is the ratio for the protein dissolved in 6 M GdmCl, and r_a is the ratio calculated for a model protein solution containing the same tyrosine/tryptophan ratio dissolved in ethylene glycol to simulate residues completely buried in the protein interior. For apo A-IV, r_u was 3.99, and r_a was −0.58.

Fluorescence Spectroscopy. Fluorescence studies of human apo A-IV were performed at 25 °C using an SLM-4800 scanning spectrofluorometer equipped with a thermostated cuvette and a dedicated Hewlett-Packard computer. All studies were conducted with 1-cm quartz cells and 4-nm slits for both excitation and emission monochromators. Spectra were obtained by using excitation wavelengths of 280 and 295 nm. Fluorescence emission was monitored between 290 and 450 nm, and spectra were computed as the average of three separate scans. Solutions of 4 μM apo A-IV were prepared by dilution of the 1 mg/mL stock solution 24 h prior to study and were filtered directly into the cuvette through 0.45-μm Millipore filters. At the concentration studied, optical density was less than 0.05, and fluorescence intensity was a linear function of concentration. The spectra were excitation-corrected by reference to a Rhodamine quantum counter and corrected for Raman emission by subtraction of buffer blanks. The relative quantum yield of apo A-IV was calculated as $(A_p/A_r)(OD_r/OD_p)$, where A is the area under the complete emission spectrum, OD is the optical density at the wavelength of excitation, p is apo A-IV, and r is an equimolar reference solution of N-acetyltryptophanamide. Tyrosine emission spectra were isolated by subtracting the spectra obtained with excitation at 295 nm from the spectra obtained at 280 nm, after normalization of the two spectra at 370 nm, where tyrosine fluorescence is negligible (Weber & Young, 1964; Kronman & Holmes, 1971). The relative quantum yield of the tyrosine emission was then calculated as described above in reference to a solution of N-acetyltyrosinamide containing an equimolar concentration of tyrosine (i.e., 6 times the apo A-IV molarity).

Fluorescence excitation polarization spectra were obtained with the instrument in a T format, using 8-nm slits, calcite prism polarizers, a monochromator setting of 360 nm for the vertical photomultiplier, and a Corning 0-52, 355-nm cutoff filter for the horizontal photomultiplier. Polarization was calculated as $p = (I_{vv}/I_{vh} - G)(I_{vv}/I_{vh} + G)^{-1}$, where I is the intensity of the fluorescence emission with the first and second subscripts referring to the plane of polarization of the incident and emitted light, respectively, v is vertical, h is horizontal, and $G = I_{hv}/I_{hh}$.

The efficiency of energy transfer between tyrosine and tryptophan was determined by analyzing the wavelength dependence of the relative tryptophan quantum yield (Eisinger, 1969; Saito et al., 1981). When the minimal contribution of phenylalanine absorption is ignored, the quantum yield of a protein containing tryptophan and tyrosine residues can be expressed as

$$Q(\lambda) = Q_T[f_{Trp}(\lambda) + ef_{Tyr}(\lambda)] \quad (1)$$

where $Q(\lambda)$ is the quantum yield of the protein at wavelength λ, Q_T is the quantum yield of tryptophan in the protein, $f_{Trp}(\lambda)$ and $f_{Tyr}(\lambda)$ are the fractional absorption of light by the tryptophan and tyrosine residues at wavelength λ, and e is the efficiency of energy transfer from tyrosine residues to tryptophan. As pointed out by Saito et al. (1981), since only the wavelength dependence of the quantum yield is desired, the absolute values of the quantum yield need not be obtained, and the fluorescence emission of the protein excited at 295 nm can be taken as the protein tryptophan quantum yield. Moreover, if the fluorescence emission is monitored at a wavelength where tyrosine fluorescence is negligible, the shape of the fluorescence emission curve will be independent of the wavelength of the exciting light, and the quantum yield will be proportional to the fluorescence intensity. Substituting and rearranging, one obtains

$$[I(\lambda)/OD(\lambda)]/(I_{295}/OD_{295}) = (1 - e)f_{Trp}(\lambda) + e \quad (2)$$

where $I(\lambda)$ is the fluorescence intensity at a given wavelength in arbitrary units and $OD(\lambda)$ is the optical density of the solution subsequently measured at the same wavelength (Saito et al., 1981).

Energy transfer in native and partially unfolded apo A-IV was determined with protein solutions in buffer alone or in 0.5 M GdmCl. Fluorescence emission was monitored at 370 nm while the excitation wavelength was scanned from 260 to 295 nm. The fractional absorption of tryptophan, $f_{Trp}(\lambda)$, was obtained from model protein solutions consisting of 10 μM N-acetyltryptophanamide and 60 μM N-acetyltyrosinamide dissolved in 80% ethylene glycol or 0.5 M GdmCl. In model apo A-IV solutions, the solvent effect upon fractional tryptophan absorption at 280 nm was minimal: f_{Trp} increased from 0.40 in 80% ethylene glycol to only 0.43 in 0.5 M GdmCl. The value for e, the efficiency of energy transfer, was obtained by fitting the experimentally determined values for the left-hand side of eq 2 to curves generated by the right-hand expression for different values of e.

Quenching studies were performed by the addition of aliquots of buffered 8 M potassium iodide (containing a trace amount of $Na_2S_2O_3$ to prevent the formation of I_3^-), 8 M acrylamide, or 100 mM N-methylnicotinamide chloride to solutions of 2 μM apo A-IV and 2 μM N-acetyltryptophanamide in 50 mM phosphate buffer, pH 7.5. The excitation wavelength was 280 nm. As before, spectra were corrected for Raman and dilution effects. The spectral shape and wavelength of maximum fluorescence were unchanged by the addition of these quenchers. Quenching constants, K_q, were derived from plots of $F_0/\Delta F$ vs $[Q]^{-1}$ (Lehrer, 1971), which

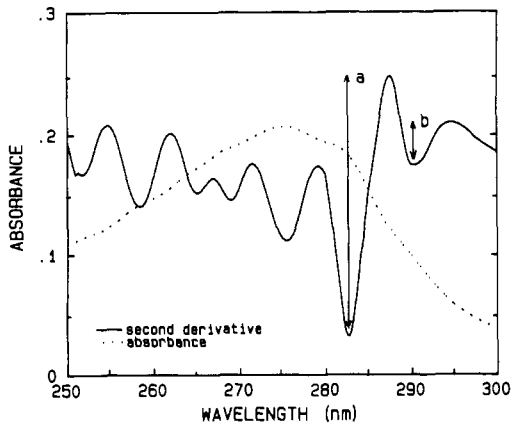

FIGURE 1: Second-derivative absorption spectrum of human apo A-IV. The absorption of a 14 μM solution of apo A-IV was determined in 6 M GdmCl and electronically differentiated. The peak-to-peak distances "*a*" and "*b*" were used to calculate the tyrosine content of apo A-IV as described under Experimental Procedures. A similar approach was used to estimate the fractional tyrosine exposure in native, partially unfolded, and denatured apo A-IV.

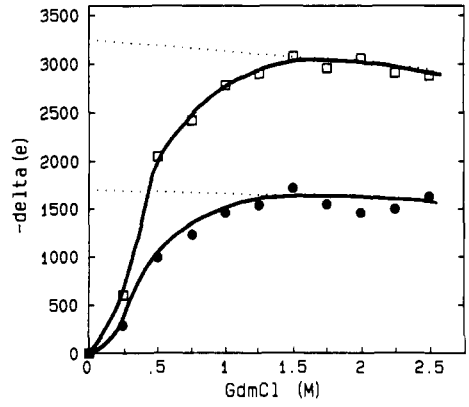

FIGURE 2: Differential absorption spectroscopy of human apo A-IV. The change in molar absorptivity at 285 nm (□) and 292 nm (●) was monitored as increasing concentrations of GdmCl were added to a solution of 20 μM apo A-IV. The dotted lines represent extrapolation of the terminal portions of the denaturation curves to the *y* axis.

were linear in all instances. The fractional exposure of tryptophan was calculated as K_q(apo A-IV)/K_q(*N*-acetyltryptophanamide) (Holmes & Robbins, 1974).

Titration of Human Apo A-IV with Tetranitromethane. Ten nanomoles of pure human apo A-IV in 1 mL of 100 mM Tris, pH 7.5, or 100 mM Tris, pH 7.5, and 0.5 M GdmCl was reacted with 2.5 μL of 84 mM tetranitromethane (Aldrich Chemical Co.) in 95% ethanol (a 20-fold molar excess). The reactions were monitored at 350 nm to detect nitroformate liberated by the nitration. At the termination of the reactions, the mixtures were dialyzed against 100 mM Tris, pH 8.0, and assayed to quantitate protein recovery (Bradford, 1976). The concentration of nitrotyrosine was then determined from the optical density at 428 nm using an extinction coefficient of 4100 (Sokolovsky et al., 1966).

RESULTS

Determination of Tyrosine Content of Apo A-IV. The tyrosine content of apo A-IV was determined by using second-derivative absorption spectroscopy. The second-derivative spectrum of 14 μM apo A-IV in 50 mM phosphate, pH 7.5, and 6.0 M GdmCl demonstrated characteristic peaks at 287 and 294 nm with accompanying troughs at 283 and 291 nm (Figure 1). As measured from the spectrum, "*a*" was 87.8 mm and "*b*" was 22.0 mm. These values yielded a tyrosine/tryptophan ratio of 5.96 using the *ABC* coefficients of Ragone et al. (1984). Since apo A-IV contains a single tryptophan residue (Weinberg & Spector, 1985a; Elshourbagy et al., 1986), it follows that apo A-IV contains six tyrosine residues.

Ultraviolet Absorption Spectroscopy. Addition of increasing concentrations of GdmCl to a solution of apo A-IV caused a decrease in the molar absorptivity over the entire wavelength range with absorption difference maxima at 279 and 285 nm, and a prominent shoulder at 292 nm, corresponding to denaturation-dependent blue shifts in the absorption of tyrosine and tryptophan residues. The curves of $\Delta\epsilon_{285}$ and $\Delta\epsilon_{292}$ vs GdmCl concentration were biphasic and demonstrated a sharp decrease in absorptivity between 0 and 0.5 M GdmCl and a more gradual change thereafter between 0.5 and 2.5 M GdmCl (Figure 2). The midpoints of the transition curves were between 0.4 and 0.5 M GdmCl. Extrapolation of the terminal portion of these curves to the ordinate gives the $\Delta\epsilon$ for the transfer of tyrosine and tryptophan residues from the hydrophobic interior of the molecule to the protein surface. Using values of 1600 for the transfer of a single tryptophan and 700 for transfer of a single tyrosine (Donovan, 1973), we determined the extrapolations are consistent with the movement of a single tryptophan and 4.6 tyrosine residues from a buried location within the hydrophobic core of the molecule. Assuming that all six tyrosine residues are completely exposed in 2.5 M GdmCl, the $\Delta\epsilon_{287}$ values are consistent with exposure of 1.4 tyrosine residues in the native form and 4.4 residues in the partially unfolded form in 0.5 M GdmCl.

The fractional exposure of tyrosine residues in native, partially unfolded, and denatured apo A-IV was also determined by second-derivative absorption spectroscopy. The peak ratio *r* was 2.13 in phosphate buffer, 3.25 in 0.5 M GdmCl, and 3.79 in 2.5 M GdmCl. These values correspond to a fractional tyrosine exposure of 0.59 for the native protein, 0.84 for the partially unfolded state, and 0.96 for the denatured protein, or exposure of 3.5, 5.0, and 5.8 tyrosine residues, respectively.

Titration of Exposed Tyrosine Residues by Tetranitromethane. To corroborate the spectroscopic estimates of fractional tyrosine exposure, human apo A-IV was titrated with tetranitromethane, a reagent which rapidly effects the nitration of exposed tyrosine residues (Sokolovsky et al., 1966). In buffer, the nitration of tyrosine, as detected by an increase in the optical density at 350 nm, was complete after 3 h. In the presence of 0.5 M GdmCl, the reaction proceeded much more rapidly. Protein recovery after these reactions was quantitative. Analysis of the nitrated proteins by sodium dodecyl sulfate/polyacrylamide gel electrophoresis demonstrated single bands which comigrated with native apo A-IV, indicating that no cross-linking reactions had occurred. Spectrophotometric assay of the nitrated apoproteins at pH 8.0 determined that 1.5 residues of tyrosine per molecule were nitrated in phosphate buffer and 4.8 residues were nitrated in the presence of 0.5 M GdmCl.

Fluorescence Emission Spectroscopy. When excited at 280 nm, solutions of apo A-IV in 50 mM phosphate buffer, pH 7.5, demonstrated maximum fluorescence at 339 nm (Figure 3A), characteristic of a single tryptophan residing in a hydrophobic environment (Longworth, 1971), and a shoulder between 300 and 310 nm, characteristic of tyrosine emission. The relative quantum yield in reference to an equimolar solution of *N*-acetyltryptophanamide excited at the same wavelength was 0.37. When excited at 295 nm to selectively excite tryptophan, no tyrosine shoulder was observed (Figure 3B). The isolated tyrosine fluorescence spectrum revealed emission between 300 and 355 nm with a peak at 307 nm

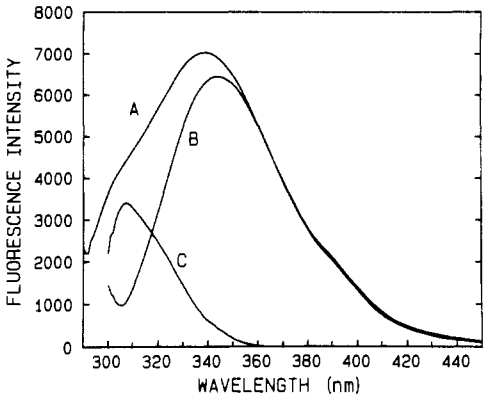

FIGURE 3: Fluorescence emission spectra of human apo A-IV. The fluorescence intensity of dilute solutions of apo A-IV was monitored from 290 to 450 nm. Fluorescence intensity is expressed in arbitrary units. Curve A, excitation at 280 nm; curve B, excitation at 295 nm; the fluorescence intensity has been normalized to curve A at 370 nm; curve C, tyrosine fluorescence with excitation at 280 nm, obtained from the difference between curves A and B.

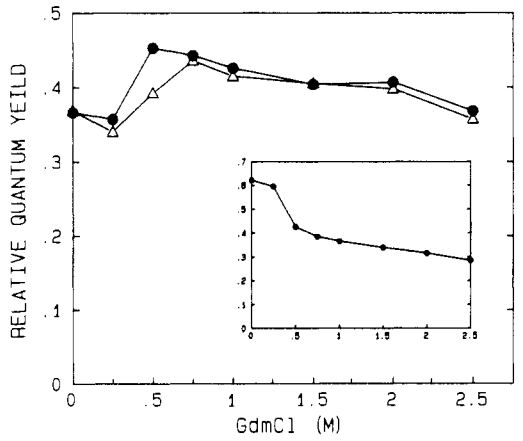

FIGURE 4: Relative quantum yield of human apo A-IV as a function of GdmCl concentration. The relative quantum yield of dilute solutions of human apo A-IV was determined in reference to an equimolar solution of *N*-acetyltryptophanamide. Excitation at 280 nm (●) and at 295 nm (△). Inset: The relative quantum yield of tyrosine fluorescence as a function of GdmCl concentration in reference to a solution of *N*-acetyltyrosinamide containing an equimolar concentration of tyrosine (i.e., 6 times the apo A-IV molarity).

(Figure 3C). The relative tyrosine quantum yield in reference to a solution of *N*-acetyltyrosinamide containing an equimolar concentration of tyrosine was 0.62.

With excitation at 280 nm, addition of increasing concentrations of GdmCl to a 4 μM solution of apo A-IV caused a 13-nm red shift in the wavelength of maximum fluorescence emission (data not shown), consistent with the exposure of the single tryptophan residue to the aqueous environment. The transition midpoint occurred at 0.5 M GdmCl. The relative quantum yield of apo A-IV increased during the early phase of protein unfolding and peaked at the denaturation midpoint at a value 24% higher than base line (Figure 4). The relative quantum yield decreased at higher concentrations of GdmCl but did not return to base line. Isolation of the tyrosine component of the total fluorescence showed that denaturation caused a small 2-nm red shift in the wavelength of maximum tyrosine emission. The tyrosine relative quantum yield dropped sharply at 0.5 M GdmCl to 69% of its base-line value and decreased in a gradual linear fashion at higher concentrations (Figure 4, inset). With excitation at 295 nm, an emission red shift was again observed during denaturation, and the increase in quantum yield, too, was similar and peaked at 18% over base line.

Table I: Fractional Exposure of Tryptophan in Apo A-IV to Charged and Neutral Quenchers at Different Concentrations of GdmCl[a]

GdmCl concn	iodide	*N*-methylnicotinamide	acrylamide
0	0.24	0.44	0.38
0.5	0.68	0.84	0.33
2.5	0.59	0.89	0.53

[a] Stern–Volmer quenching constants, K_q, were derived from least-squares plots of $F_0/\Delta F$ vs $[Q]^{-1}$. Fractional exposure is expressed as K_q(apo A-IV)/K_q(*N*-acetyltryptophanamide).

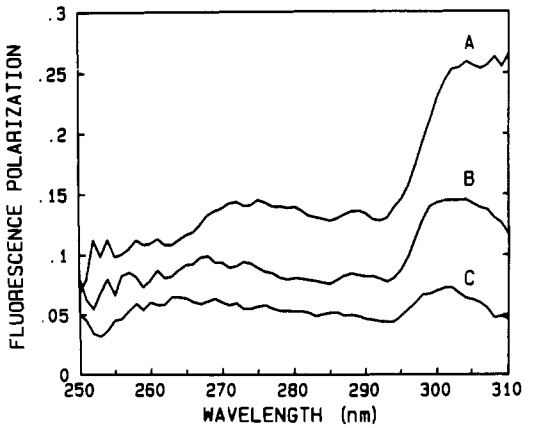

FIGURE 5: Fluorescence excitation polarization spectra of human apo A-IV. The polarization of the tryptophan fluorescence emission of apo A-IV was monitored at 360 nm while the excitation wavelength was scanned from 250 to 310 nm. Curve A, apo A-IV in phosphate buffer; curve B, apo A-IV in buffered 0.5 M GdmCl; curve C, apo A-IV in buffered 2.5 M GdmCl.

Fluorescence Quenching of Apo A-IV. Fluorescence quenching techniques were utilized to further probe the exposure and ionic microenvironment of tryptophan in apo A-IV. The values for the fractional exposure of tryptophan in native apo A-IV are indicative of a relative inaccessibility to both neutral and charged quenchers (Table I) and suggest that tryptophan is hydrophobically shielded by a domain surrounded by a relatively negative charge (Pownall & Smith, 1974; Eftink & Ghiron, 1976). In 0.5 M GdmCl, the accessibility to the charged quenchers increased over 2-fold whereas the value for acrylamide was essentially unchanged. This finding is unusual and may be a consequence of a conformation which maintains some steric shielding of tryptophan, but in which the charge density surrounding tryptophan is increased, such as would occur with disruption of hydrogen or ionic bonding. The values for the fractional exposure in 2.5 M GdmCl suggest that complete loss of tertiary structure allows equal access of iodide and acrylamide to tryptophan, and a preferential access of positively charged *N*-methylnicotinamide.

Fluorescence Excitation Polarization Spectroscopy. The polarization of the tryptophan component of the fluorescence emission of apo A-IV was monitored over an excitation wavelength range of 250–310 nm. The fluorescence excitation polarization spectrum was characterized by a broad peak which extended from 265 to 285 nm with a maximum value of 0.145 and a sharp increase to a plateau of 0.260 above 300 nm (Figure 5A). These values, particularly between 270 and 280 nm, are considerably lower than the corresponding values for tryptophan in viscous solvents at low temperature (Weber, 1960; Ghiron & Longworth, 1979) and hence indicate significant depolarization of the tryptophan emission. A small peak at 288–289 nm with an accompanying minimum at 292 nm was also observed; Konev (1967) has suggested that these features are indicative of a relatively fixed orientation of

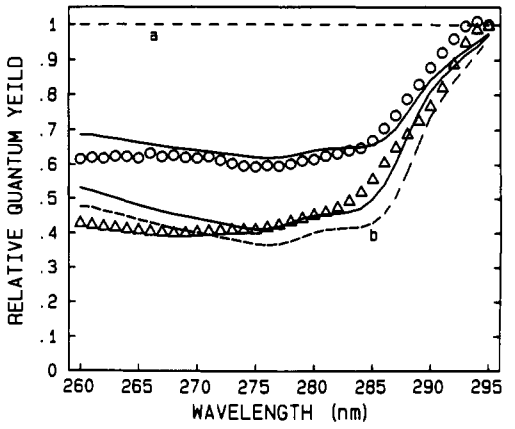

FIGURE 6: Excitation wavelength dependence of the relative tryptophan quantum yield of human apo A-IV. The relative tryptophan quantum yield of apo A-IV was determined as a function of excitation wavelength in phosphate buffer (○) and in buffered 0.5 M GdmCl (△). The solid lines are the best fits to the data and correspond to transfer efficiencies of 0.40 and 0.09. The dashed lines indicate the predicted dependence of relative quantum yield upon excitation wavelength when transfer efficiency is (a) 1.00 and (b) zero.

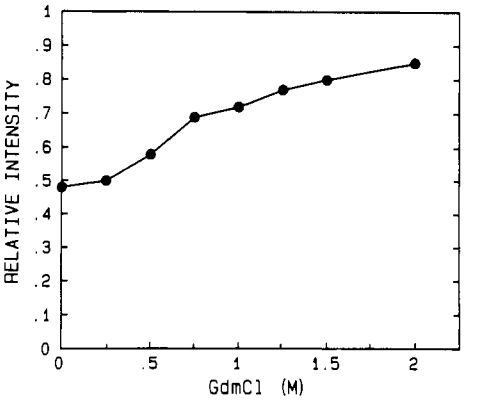

FIGURE 7: Relative fluorescence intensity of nitrated apo A-IV as a function of GdmCl concentration. The peak fluorescence intensity of a 1 μM solution of nitro-apo A-IV (nitrated at an average of 1.5 tyrosine residues per molecule) was monitored as a function of GdmCl concentration. Fluorescence intensity is expressed in reference to an equimolar solution of unmodified apo A-IV.

tryptophan within the molecular structure. The excitation polarization spectrum of apo A-IV in 0.5 M GdmCl demonstrated further depolarization, with loss of the broad peak between 265 and 285 nm and a sharp fall in the plateau between 300 and 310 nm (Figure 5B). In 2.5 M GdmCl, almost complete loss of polarization was observed, which is likely a consequence of the unrestricted rotational freedom of the tryptophan residue in the unfolded polypeptide (Figure 5C).

Tyrosine–tryptophan energy transfer in globular proteins causes a selective depolarization of tryptophan fluorescence at wavelengths shorter than 295 nm, where tyrosine absorption is significant. Hence, the ratio of polarization or anisotropy at 305 nm (where the polarization of tryptophan is at maximum) to that at 270 nm (which is near the maximum absorption wavelength for tyrosine) can be used as a qualitative measure of the efficiency of tyrosine–tryptophan energy transfer. The ratio of anisotropy at 305 nm to that at 270 nm was 2.0 for native apo A-IV, 1.6 for apo A-IV in 0.5 M GdmCl, and 1.1 for apo A-IV in 2.5 M GdmCl. Corresponding values for tryptophan immobilized in viscous solvents at low temperature range between 1.4 and 1.5 (Weber, 1960; Ghiron & Longworth, 1979). The anisotropy ratios for apo A-IV are thus indicative of tyrosine–tryptophan energy transfer in the native and partially denatured state (Weber, 1960; Ghiron & Longworth, 1979).

Excitation Wavelength Dependence of the Relative Tryptophan Quantum Yield. The quantum yield of native apo A-IV was wavelength dependent over an excitation range of 260–295 nm. The values for the relative tryptophan quantum yield were best fit by a fractional absorbance curve calculated for $e = 0.40$ (Figure 6). This transfer efficiency is the equivalent of completely efficient energy transfer from 2.6 tyrosine residues. In 0.5 M GdmCl, the transfer efficiency decreased to 0.09, indicating that the efficiency of tyrosine–tryptophan energy transfer is highly conformation dependent. The discrepancies between the theoretical curves and the experimental data are probably due to the absorption of phenylalanine at lower wavelengths and conformational-dependent wavelength shifts in the tryptophan and tyrosine absorption spectra at higher wavelengths.

Denaturation of Tetranitromethane-Modified Apo A-IV. The addition of a single nitro group to tyrosine (by reaction with tetranitromethane) quenches its fluorescence and causes a red shift of its absorption into the near-ultraviolet (Osborne et al., 1980), effectively converting it from a tryptophan fluorescence energy donor to a potent acceptor. Native apo A-IV, nitrated at 1.5 tyrosine residues, exhibited maximum fluorescence emission at 339 nm, indicating that the hydrophobic microenvironment of the tryptophan had not been altered by the chemical modification of tyrosine residues. However, the peak fluorescence intensity was 48% lower than that of an equimolar solution of unmodified apo A-IV, suggesting that the tryptophan fluorescence was partially quenched by the nitrated tyrosine residues. Yet, addition of increasing amounts of GdmCl caused an increase in fluorescence intensity with a profile similar to the denaturation-induced emission red shift (Figure 7). In 2 M GdmCl, the fluorescence intensity of denatured nitro-apo A-IV approached that of unmodified apo A-IV.

Discussion

Tyrosine Content, Exposure, and Fluorescence. Genomic sequencing of apo A-IV (Elshourbagy et al., 1986; Karathanasis et al., 1986) has established that six tyrosine residues reside at positions 14, 52, 118, 184, 206, and 290. However, Elshourbagy et al. (1986) found a seventh tyrosine at position 140 where Karathanasis et al. (1986) found a leucine residue. Although the discrepancy in the number of tyrosine residues could conceivably be due to genetic variation, our results agree with Karathanasis et al. that apo A-IV contains six tyrosines.

The denaturation-induced changes in the molar extinction coefficient at 285 nm suggest that all but one or two tyrosine residues are buried in the core of native apo A-IV. The buried location of the majority of tyrosine residues is further suggested by the finding that only 1.5 tyrosine residues in native apo A-IV were modified by tetranitromethane at concentrations which readily affect the selective nitration of exposed tyrosine residues (Sokolovsky et al., 1966). Second-derivative absorption spectroscopy, however, estimated that two additional tyrosine residues were exposed in the native apo A-IV molecule. This discrepancy may arise from the fact that the second-derivative absorption spectrum is sensitive to changes in the electronic state of buried tyrosine residues (Ragone et al., 1984). Tyrosines-52 and -206 reside within α-helical domains (Weinberg, 1987) in a 1–4 relationship with aspartic acid residues, a conformation favorable for the formation of interresidue hydrogen bonds involving the phenolic proton. Thus, these two residues, though buried, could yield second-derivative spectral bands consistent with surface exposure.

In general, very little tyrosine fluorescence is observed in the emission spectra of most globular proteins, even when there is a high tyrosine/tryptophan ratio. This has been attributed to the fact that several processes—such as energy transfer from tyrosine to tryptophan, internal conversion by vibrational coupling, intersystem crossing, and direct quenching—can contribute to the nonradiative deexcitation of tyrosine (Cowgill, 1976). Nonetheless, apo A-IV demonstrated a distinct tyrosine emission with a relative quantum yield equivalent to emission from three to four tyrosine residues. Similar tyrosine fluorescence has been observed in several well-studied proteins (Longworth, 1971; Kronman & Holmes, 1971), although the mechanisms or structural correlates of such efficient tyrosine emission have not been defined. The sharp fall in the relative tyrosine quantum yield observed during denaturation between 0.25 and 0.5 M GdmCl is consistent with exposure of buried tyrosine residues to quenching by peptide carbonyl groups (Cowgill, 1976).

Tryptophan Fluorescence. The factors which govern the fluorescence properties of single tryptophan proteins, such as apo A-IV, are complex and include not only the local environment of the tryptophan but also its interaction with distant amino acid residues capable of energy transfer and quenching (Longworth, 1980). The fluorescence emission maximum at 339 nm and the denaturation-induced emission red shift are consistent with a tryptophan residue which resides in a hydrophobic environment, shielded from the aqueous milieu (Longworth, 1980; Lackowitz, 1983). An interior location of the tryptophan residue is further suggested by the low fractional exposure of tryptophan to quenchers in the native state (Eftink & Ghiron, 1976; Holmes & Robbins, 1974). The quantum yield of tryptophan in globular proteins varies greatly over a wide range, but no consistent relationship between tryptophan exposure and quantum yield has been observed. Taking 0.130 as the quantum yield of *N*-acetyltryptophanamide (Edelhoch et al., 1968), the tryptophan quantum yield of apo A-IV is 0.048, which is on the low end of the range observed for globular proteins (Kronman & Holmes, 1971). This low value suggests that tryptophan fluorescence in native apo A-IV is significantly quenched.

The increase in the relative quantum yield of human apo A-IV during the early phases of denaturation occurs with both chemical and thermal (R. B. Weinberg, unpublished observations) denaturation and hence is an intrinsic property of the protein and not an artifact of a particular denaturation modality. We have previously established that the increase is not a concomitant of the dissociation of dimeric apo A-IV, which takes place at lower concentrations of GdmCl and is in fact accompanied by a small decrease in fluorescence intensity (Weinberg & Spector, 1985b). The minimal effect of solvent upon the fractional energy absorption at 280 nm in model apo A-IV solutions suggests that the change in quantum yield is not due to a decrease in the "inner-filtering effect" of the tyrosine residues (Longworth, 1971). As described, the fluorescence excitation studies demonstrate that the efficiency of energy transfer between tyrosines and tryptophan decreases as the protein unfolds; hence, increased intramolecular energy transfer does not play a role in the phenomenon. Indeed, the magnitude of the increase is almost as great with excitation at 295 nm, a wavelength at which tyrosine absorption is negligible. Therefore, the most plausible explanation for the early rise in relative quantum yield is a decrease in intramolecular tryptophan quenching.

Several mechanisms for such quenching can be considered. Amino acid side chains, particularly charged side chains and histidine (Lehrer, 1976), are capable of quenching tryptophan fluorescence. Since the fluorescence quenching data suggest that the ionic environment surrounding the tryptophan is predominantly negative, it is possible that vicinal acidic residues participate in tryptophan quenching in native apo A-IV and that removal of these residues during the initial stage of unfolding increases tryptophan fluorescence. Another potential quenching mechanism is the formation of tyrosinate, which is a potent quencher of tryptophan fluorescence (Edelhoch et al., 1967). As discussed above, the tyrosine residues at positions 52 and 206 are optimally situated for the formation of interresidue hydrogen bonds, which would lower their free energy of ionization (Lackowicz, 1983) and facilitate the formation of tyrosinate. In the native conformation, the proximity of such hydrogen-bonded tyrosines to the amino-terminal tryptophan would quench fluorescence; however, the partial loss of α-helical structure in the initial phase of denaturation would abolish this quenching by simultaneously disrupting tyrosine hydrogen bonds, inhibiting ionization, and increasing the distance from tryptophan.

Tyrosine–Tryptophan Energy Transfer. Intramolecular transfer of fluorescence energy from tyrosine to tryptophan is common in globular proteins, although the efficiency of transfer is highly variable (Longworth, 1971; Kronman & Holmes, 1971). Both fluorescence excitation polarization spectroscopy and the analysis of the wavelength dependence of the relative tryptophan quantum yield indicated that energy transfer between tyrosine and tryptophan residues in native apo A-IV occurs with moderate efficiency. Furthermore, the calculated efficiency of 0.40 for native apo A-IV suggests that more than one tyrosine residue participates in the process. Since only tyrosine-14 is directly within the critical energy-transfer distance of tryptophan-12, energy transfer must therefore occur as well from nonvicinal tyrosine residues which, in the native state, reside within energy-transfer range of the amino terminus. Both of these complementary excitation techniques also demonstrated that the efficiency of energy transfer in apo A-IV is conformation dependent and decreased with denaturation. Since the spectral overlap integral between tyrosine emission and tryptophan absorption is largely independent of environment (Kronman & Holmes, 1971), this decrease in transfer efficiency must therefore be caused by the loss of tertiary structure which increases the distance between nonvicinal tyrosines and the amino-terminal tryptophan.

The studies of nitrated apo A-IV support the existence of energy transfer between nonvicinal tyrosines and tryptophan and further suggest that a significant fraction of energy transfer proceeds from the exposed tyrosine residues. Addition of a nitro group to the exposed tyrosines in native apo A-IV reduced the fluorescence intensity of the folded protein by more than half, indicating that these residues were close enough to tryptophan to function as energy sinks. Nonetheless, the fluorescence intensity of nitrated apo A-IV rapidly increased with denaturation as the distance between the tryptophan and the nitrated tyrosine residues increased with unfolding (Lehrer & Elzinga, 1972).

Conclusions and Structural Hypotheses. In summary, this study documents the existence of intramolecular fluorescence energy transfer and tryptophan quenching in the native apo A-IV molecule and suggests that the amino terminus of this protein is situated in a hydrophobic environment within energy-transfer range of nonvicinal tyrosine residues. The spectral and chemical properties of the tyrosine residues further suggest that most of them are buried in the hydrophobic core

of the protein and that two of them may be hydrogen bonded to adjacent amino acid side chains.

A consideration of these findings in light of the recently elucidated amino acid sequence (Elshourbagy, 1986) suggests a hypothetical conformation for human apo A-IV. The single tryptophan in apo A-IV resides at position 12, near the amino terminus. This region is predicted to consist of short segments of random-coil and β-sheet structure (Weinberg, 1987) and does not constitute a hydrophobic domain consistent with the spectroscopic data. Thus, the hydrophobic environment of the amino-terminal tryptophan must be determined by the tertiary structure of the molecule. With the exception of tyrosine-14, the tyrosine residues in apo A-IV are located exclusively within sequential 22-residue repeat units of high amphipathic α-helical potential (Elshourbagy, 1986; Karathinasis et al., 1986). Therefore, enfolding of the amino terminus in a domain formed by a confluence of tyrosine-containing amphipathic α-helical segments not only would provide a hydrophobic environment for the tryptophan and tyrosine residues but also would situate nonvicinal tyrosine residues within energy-transfer range of tryptophan.

An important physiological consequence of this hypothetical configuration is that if the hydrophobic faces of the constituent amphipathic segments were oriented toward the interior of the protein (Doolittle, 1981), then these segments would not be available for interaction with the surface of plasma lipoproteins. Such a configuration could well explain a major paradox of the biophysical behavior of apo A-IV, namely, why, despite containing over 50% α-helical structure, native apo A-IV has the lowest lipoprotein affinity of any apolipoprotein and yet why, when apo A-IV is denatured prior to interaction with lipid, it can readily integrate into lipid monolayers to form stable complexes (Steinmetz & Utermann, 1985).

ACKNOWLEDGMENTS

I thank Cathy Matthews for use of the SLM-4800 spectrofluorometer, Barry Nall for valuable discussions and use of the HP-8450A spectrophotometer, William Mantulin for technical advice, Paul Horowitz for review of the manuscript, and Nancy Cook for document design and preparation.

REFERENCES

Beisiegel, U., & Utermann, G. (1979) *Eur. J. Biochem. 93*, 601–608.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Cowgill, R. W. (1976) in *Biochemical Fluorescence: Concepts* (Chen, R. F., & Edelhoch, H., Eds.) Vol. 2, pp 441–486, Marcel Dekker, New York.

Donovan, J. W. (1973) *Methods Enzymol. 27*, 497–525.

Doolittle, R. F. (1981) *Science (Washington, D.C.) 214*, 149–159.

Edelhoch, H., Brand, L., & Wilchek, M. (1967) *Biochemistry 6*, 547–559.

Edelhoch, H., Bernstein, R. S., & Wilchek, M. (1968) *J. Biol. Chem. 243*, 5985–5992.

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

Eisinger, J. (1969) *Biochemistry 8*, 3902–3907.

Elshourbagy, N. A., Walker, D. W., Bogusky, M. S., Reardon, C. A., Levy-Wilson, B., Jefferson, L. S., Gordon, J. I., & Taylor, J. M. (1986) *J. Biol. Chem. 261*, 1998–2002.

Ghiron, C. A., & Longworth, J. W. (1979) *Biochemistry 18*, 3828–3832.

Ghiselli, G., Krishnan, S., Beigel, Y., & Gotto, A. M. (1986) *J. Lipid Res. 27*, 813–827.

Green, P. H. R., Glickman, R. M., Saudek, C. D., Blum, C. B., & Tall, A. R. (1979) *J. Clin. Invest. 64*, 233–242.

Green, P. H. R., Glickman, R. M., Riley, J. W., & Quinet, E. (1980) *J. Clin. Invest. 65*, 911–919.

Holmes, L. G., & Robbins, F. M. (1974) *Photochem. Photobiol. 19*, 361–366.

Karathanasis, S., Yunis, I., & Zannis, V. I. (1986) *Biochemistry 25*, 3962–3970.

Konev, S. V. (1967) in *Fluorescence and Phosphorescence of Proteins and Nucleic Acids*, pp 93–96, Plenum, New York.

Kronman, M. J., & Holmes, L. G. (1971) *Photochem. Photobiol. 14*, 113–134.

Lackowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, pp 359–363, Plenum, New York.

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

Lehrer, S. S. (1976) *Biochemical Fluorescence: Concepts* (Chen, R. F., & Edelhoch, H., Eds.) Vol. 2, pp 515–544, Marcel Dekker, New York.

Lehrer, S. S., & Elzinga, M. (1972) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 31*, 1627.

Longworth, J. W. (1971) in *Excited States of Proteins and Nucleic Acids* (Steiner, R. F., & Weinryb, I., Eds.) pp 319–483, Plenum, New York.

Longworth, J. W. (1980) in *Time Resolved Fluorescence Spectroscopy in Biochemistry and Biology* (Cundall, R. B., & Dale, R. E., Eds.) pp 651–686, Plenum, New York.

Lowry, O. H., Rosebrough, N. J., Farr, A. C., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Morrisett, J. D., Jackson, R. L., & Gotto, A. M. (1977) *Biochim. Biophys. Acta 472*, 93–133.

Osborne, J. C., & Brewer, H. B. (1977) *Adv. Protein Chem. 31*, 253–337.

Osborne, J. C., Powell, G. N., & Brewer, H. B. (1980) *Biochim. Biophys. Acta 619*, 559–571.

Pownall, H. J., & Smith, L. C. (1974) *Biochemistry 13*, 2590–2593.

Ragone, R., Colonna, G., Balastrieri, C., Servillo, L., & Irace, G. (1984) *Biochemistry 23*, 1871–1875.

Rifici, V. A., Eder, H. A., & Swaney, J. B. (1985) *Biochim. Biophys. Acta 834*, 205–214.

Saito, Y., Tachibana, H., Hayashi, H., & Wada, A. (1981) *Photochem. Photobiol. 33*, 289–295.

Servillo, L., Colonna, G., Balestrieri, C., Ragone, R., & Irace, G. (1982) *Anal. Biochem. 126*, 251–257.

Sokolovsky, M., Riordan, J. F., & Vallee, B. L. (1966) *Biochemistry 5*, 3582–3589.

Steinmetz, A., & Utermann, G. (1985) *J. Biol. Chem. 260*, 2258–2264.

Utermann, G., & Beisiegel, U. (1979) *Eur. J. Biochem. 99*, 333–343.

Weber, G. (1960) *Biochem. J. 75*, 335–345.

Weber, G., & Young, L. B. (1964) *J. Biol. Chem. 239*, 1424–1430.

Weinberg, R. B. (1987) *Biochim. Biophys. Acta 918*, 299–303.

Weinberg, R. B., & Scanu, A. M. (1983) *J. Lipid Res. 24*, 52–59.

Weinberg, R. B., & Spector, M. S. (1984) *J. Lipid Res. 26*, 26–37.

Weinberg, R. B., & Spector, M. S. (1985a) *J. Biol. Chem. 260*, 4914–4921.

Weinberg, R. B., & Spector, M. S. (1985b) *J. Biol. Chem. 260*, 14279–14286.

Weisgraber, K. H., Bersot, T. P., & Mahley, R. W. (1978) *Biochem. Biophys. Res. Commun. 85*, 287–292.