Ultraviolet Resonance Raman and Fluorescence Studies of Acid-Induced Structural Alterations in Porcine, Bovine, and Human Growth Hormone

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Abstract: Resonance Raman and fluorescence spectroscopies have been used to study acid-induced structural alterations in growth hormones from three species. Resonance Raman data for porcine (pGH), bovine (bGH), and human (hGH) growth hormones using 222-nm laser excitation show strong enhancement of the aromatic residues phenylalanine (Phe), tryptophan (Trp), and tyrosine (Tyr). Proline (Pro) is also enhanced. Resonance Raman data indicate that no significant Pro cis → trans isomeric transition occurs in the pH 8–2 range. Raman cross sections for the Trp vibrations observed at 756 and 1557 cm^{-1} increase dramatically when bGH or pGH is partially unfolded in acid (pH 2). Trp cross sections for hGH decrease slightly at acidic pH. The differences in the pH dependence of the Trp vibrational intensities in bGH and pGH, compared to hGH, indicate the presence of a different Trp environment in hGH. The vibrational intensities of Trp phenyl ring modes are decreased preferentially in native bGH and pGH. Fluorescence quantum yields and lifetimes from the single Trp in bGH and pGH increase at low pH in a similar manner to the Trp vibrational intensity increase, while the hGH quantum yield and lifetime do not change at low pH. The similarity of pH-dependent variations of both fluorescence and resonance Raman cross sections for bGH indicates that these spectral probes are being indirectly influenced by the same molecular interactions. A pK_a of 3.7 for both the resonance Raman and fluorescence Trp spectral changes indicates that acidic groups (aspartic or glutamic acids) are involved in the structural alterations. The possible roles of disulfide bridges, lysines, and histidines in decreasing Trp vibrational and fluorescence intensities in native bGH and pGH are examined.

Growth hormones[1] (somatotropins) are globular, single-domain proteins of approximately 191 amino acids which possess about 50% α-helical structure and a single conserved tryptophan (Trp) residue at position 86. Recently, a member of this family, porcine growth hormone (pGH), has been crystallized.[2] Preliminary analysis of the X-ray crystal structure[3] has revealed that it is composed of a cluster of four antiparallel α-helices, although the atomic coordinates were not disclosed. The helical regions found in pGH are generally consistent with previous secondary structure predictions for bGH[4] and hGH.[5] In addition, bGH and hGH have considerable sequence homology with pGH[1] (91% and 68%, respectively), particularly within the four α-helical regions known for pGH. Due to these similarities and their similar biological activities, it is reasonable to assume that the three-dimensional structures of bGH and hGH are similar to the known structure of pGH.

Recent work on bGH has shown that its helical structures possess unusual stability.[6-8] Equilibrium denaturation of bGH (using urea and guanidine hydrochloride) has been shown to be a multistate process in which at least four species have been identified: native, a monomeric folding intermediate, one or more noncovalent multimeric folding intermediates, and unfolded protein.[6-9] The single Trp residue, at position 86 of the sequence, has been used as a probe of the various structural forms of bGH. Spectral changes of the Trp moiety during bGH unfolding have been observed in second-derivative absorption, circular dichroism, and steady-state and time-resolved fluorescence spectra.[6-8] The data indicate that the single Trp residue occupies a hydrophobic environment and is proximate to a fluorescence quenching group. The quenching is reduced as bGH unfolds. The fluorescence emission of the single Trp in pGH in the native state indicates a hydrophobic environment, and the emission is quenched when compared to the fluorescence intensity of the unfolded state.[10] In hGH, the single Trp residue exists in an interior hydrophobic pocket, but this environment is up to 10 times more permeable to solvent than that in bGH,[11-13] and in contrast with the behavior of bGH and pGH, Trp fluorescence of native hGH is *not* quenched compared to that of the acid-induced partially unfolded state.[14]

Much interest has been recently generated in far-UV ($\lambda \simeq$ 190–240 nm) resonance Raman spectroscopy of biological materials. Far-UV resonance Raman investigations have typically focused on the assignment of free amino acid vibrations and calculations of their resonant scattering cross sections.[15-20] Applications to the study of the structures of protein families have been sparse. Copeland and Spiro's[21] investigation of horse and tuna cytochromes *c* is one of the few reported studies comparing members of a homologous protein family with UV resonance Raman spectroscopy. In this study, we report our resonance Raman investigations of the unfolding of three growth hormones, bGH, pGH, and hGH, using 222-nm excitation. This work describes spectral variations observed during acid-induced unfolding of bGH, pGH, and hGH, with special emphasis on investigation of the single conserved Trp residue and comparison of Trp UV resonance Raman and fluorescence emission spectra.

Experimental Section

Materials. Samples of *N*-acetyltryptophanamide (NATrpA), *N*-acetylphenylalanine ethyl ester (NAPheEE), *N*-acetylproline (NAPro),

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N-acetylhistidine (NAHis), *N*-acetylarginine (NAArg), *N*-acetylleucine (NALeu), and *N*-acetyltyrosinamide (NATyrA) were used as supplied by Sigma Chemical Co. (St. Louis, MO). Recombinantly produced bGH was obtained by DNA expression in *Escherichia coli* carrying a temperature-sensitive runaway plasmid, into which had been inserted the bGH gene sequence and a Trp promoter system.[22] The purification of recombinant bGH was done according to the procedure of Evans and Knuth.[23] Recombinant pGH was produced and purified in a similar manner. HGH, isolated from pituitary glands, was used as supplied by A. Parlow (Harbour Medical Center, UCLA). The carboxyl-terminal section of bGH (amino acid residues 180–191) was obtained through cyanogen bromide (CNBr) cleavage of the native molecule.[24] Acrylamide was purchased from Aldrich Chemical Co. (Milwaukee, WI) and was used as received.

Methods. Solutions of the amino acid analogues were prepared in either 0.05 M $NaHCO_3$ buffer (pH 8) or deionized water adjusted to pH 2 with concentrated HCl. Growth hormone solutions (1 mg/mL, 0.045 mM) were prepared in the same buffers. BGH concentrations were determined by measuring solution absorbance at 280 nm, assuming an extinction coefficient of 15 400 M^{-1} cm^{-1}.[25] HGH and pGH concentrations were determined in an analogous fashion.

(A) UV Resonance Raman. A 20-Hz Quanta Ray DCR-2A Nd:YAG equipped with a Quanta Ray PDL-1 pulsed dye laser (rhodamine 590 tetraborate, Exciton Chemical Co., Dayton, OH) and a Quanta Ray wavelength extension system (WEX) were used to produce about 60 mW at 221.6 nm. The beam was routed via prisms to the sample. Protein and amino acid analogue solutions were placed into a test tube that served as a reservoir for a closed-loop recirculating sample delivery system. The delivery system is based on a Gilson Minipuls 2 peristaltic pump running at about 2 mL/min. The pump discharges the solution through a 23-gauge stainless steel needle that is wrapped with 0.2 -in. stainless steel wire forming an elliptical loop below the needle tip. A flowing film originates at the needle tip and proceeds down the wire loop to the top of the reservoir.[20] The laser beam was focused about 1 cm beyond the laminar flow film, producing a sample illumination area of about 1 mm^2. This defocusing lowered the laser energy density at the sample, resulting in less long-term sample degradation. The Raman scattering at 90° was focused on the entrance slit of an ISA U-1000 1.0-m double monochromator equipped with 3600 groove/mm halographic gratings "blazed" at 280 nm. The spectral band-pass was 11 cm^{-1} (410-μm mechanical slit width). Laser power was monitored at the sample loop. The power level (0.5–1.5 mW) was controlled through the use of an iris.

Resonance Raman spectra were collected over a 70-min period and are a sum of 30 acquisitions. Half of each 2-min data collection period was used for background correction acquisition. The accumulated data were smoothed (15 point) before analysis according to Savitzky and Golay.[26] Spectral intensities were referenced to the water O–H stretching band. No sample was irradiated more than 40 min.[27]

(B) Steady-State Fluorescence. Fluorescence emission spectra were acquired with a SLM 8000C spectrofluorometer in the photon-counting mode using an excitation wavelength of 295 nm (to assure excitation of Trp only) and a band-pass of 4 nm in the excitation and emission monochromators. Solutions of the growth hormones at 0.3 mg/mL were contained in a 1 cm × 1 cm cell. Fluorescence quantum yields (Φ) were determined relative to a 0.014 mM solution (pH 8) of NATrpA with

$$\Phi_{sample} = \frac{F_{sample}}{A_{sample}} \frac{A_{NATrpA}}{F_{NATrpA}} \Phi_{NATrpA}$$

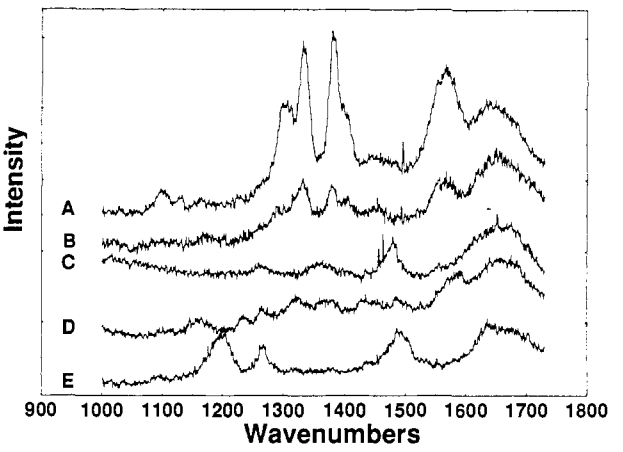

Figure 1. Resonance Raman spectra (221.6 nm) of amino acid analogues: (A) 1.3 × 10^{-3} M *N*-acetylleucine, (B) 5.9 × 10^{-4} M *N*-acetylarginine, (C) 1.3 × 10^{-4} M *N*-acetylproline, (D) 1.4 × 10^{-4} M *N*-acetylhistidine, and (E) 1.4 × 10^{-4} M *N*-acetylhistidine in water (pH 2). Unless otherwise stated, solutions contained 0.05 M $NaHCO_3$ buffer (pH 8). The molar concentrations represent 10 times those found in a 1 mg/mL bGH solution, except for *N*-acetylproline, whose concentration equals that found in bGH, pGH, and hGH.

where F is the integrated area of fluorescence emission from 300 to 450 nm, A is the absorbance at 295 nm (excitation wavelength), and Φ_{NATrpA} = 0.14. Emission maxima were determined by calculating the first derivative of emission versus wavelength.

Acrylamide fluorescence quenching experiments were performed with aliquots of a concentrated (8 M) stock solution of acrylamide which was added to a 0.3 mg/mL protein solution in a 1 cm × 1 cm fluorescence cell containing a magnetic stir bar. Fluorescence emission spectra were acquired after each addition of quencher, and the results at 350 nm were used for analysis using the Stern-Volmer equation:

$$F_0/F = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q]$$

where F_0 is the fluorescence intensity without quencher, F is the fluorescence intensity with quencher present, K_{SV} is the Stern–Volmer constant, [Q] is the concentration of acrylamide, k_q is the bimolecular quenching rate constant, and τ_0 is the fluorescence lifetime in the absence of quencher. Average lifetime values were used for τ_0. Corrections were made for dilution and for the absorbance of acrylamide at the excitation wavelength (295 nm).

Acrylamide quenching results were also analyzed with the modified Stern–Volmer equation:

$$\frac{F_0}{F_0 - F} = \frac{1}{[Q]f_aK_{SV}} + \frac{1}{f_a}$$

where the terms have the same definitions given above and f_a is the maximum fraction of tryptophan fluorescence which is accessible to acrylamide. A plot of $F_0/(F_0 - F)$ versus $1/[Q]$ has a y intercept of $1/f_a$ and a slope of $1/(f_aK_{SV})$.

(C) Time-Resolved Fluorescence. Time-correlated single photon counting was performed with a Spectra-Physics picosecond dye laser system providing excitation pulses at 295 nm.[9] Emitted light at 380 nm was detected with a Hamamatsu microchannel plate PMT. Decay curves were analyzed with the δ-function deconvolution method[28] using *p*-terphenyl and 1,1′-binaphthyl as reference fluorophores.

Fluorescence intensity decay curves [$I(t)$] were analyzed by assuming two or three components:

$$I(t) = \sum\alpha_i \exp(-t/\tau_i)$$

where τ_i is the lifetime of component i. The fraction of the fluorescence from each component (f_i) was calculated from

$$f_i = \alpha_i\tau_i/\sum\alpha_i\tau_i$$

which allowed the calculation of the average lifetime ($\langle\tau\rangle$) for each protein solution:

$$\langle\tau\rangle = \sum f_i\tau_i$$

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(27) Sample integrity was monitored by the acquisition of room temperature UV absorption and fluorescence emission spectra before and after laser irradiation. No significant band or intensity changes were observed in a comparison of the UV spectra (210–350 nm) of the native proteins and the irradiated samples. UV spectral differences appear in greater detail when the second derivative of absorption vs. wavelength is calculated. For example, in native pGH weak bands at 215 and 219 nm decreased and shifted slightly (≃1 nm) after irradiation. A very weak peak at 223 nm decreased slightly in intensity. We do not feel that these small spectral changes significantly affect the resonance Raman results but wish to emphasize the sensitivity of a second-derivative spectrum for monitoring protein integrity. Under partially denaturing conditions (pH 2), there were no second-derivative UV spectral variations observed as a result of laser irradiation. No resonance Raman bands attributed to photogenerated transients[15] were observed.
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Table I. Observed Raman Frequencies and Assignments for Aromatic Amino Acid Analogues

frequency (cm^{-1})	assignment	description
	N-Acetylphenylalanine Ethyl Ester[a]	
1003	ν_{12}	symmetric ring stretch (b_{1u})
1028	ν_{18a}	in-plane CH bend
1182	ν_{9a}	in-plane CH bend
1206	ν_{7a}	phenyl–C stretch
1587	ν_{8b}	in-plane ring stretching
1604	ν_{8a}	in-plane ring stretching
	N-Acetyltyrosinamide[b]	
1176	ν_{9a}	in-plane CH bend, C_6H_5–C stretch
1206	$\nu(a_1)$	totally symmetric stretch
1258	?	
1600	ν_{8b}	in-plane ring stretching
1615	ν_{8a}	in-plane ring stretching
	N-Acetyltryptophanamide[a]	
756		symmetric in-phase benzene/pyrrole breathing vibration
1009		symmetric out-of-plane benzene/pyrrole breathing vibration
1346		pyrrole ring vibration
1362		pyrrole ring vibration
1556	ν_{8a}	symmetric phenyl ring mode

[a] Assignments and descriptions from Asher, Ludwig, and Johnson.[17] [b] Assignments and descriptions from Ludwig and Asher.[29]

(D) UV Absorption. UV absorption spectra were acquired with a Hewlett-Packard 8450A diode-array spectrophotometer using a 1-cm cell in the near-UV (250–320 nm) and a 0.1-cm cell in the far-UV (200–250 nm). The second derivative of absorbance vs wavelength was calculated with vendor-supplied software. UV absorption difference spectra of the growth hormones were collected from a pH 2 solution of growth hormone as sample and a pH 8 solution as reference.

Results

Resonance Raman and UV Absorption Spectra of Amino Acid Analogues. The resonance Raman spectra of NATrpA, NATyrA, NAPheEE, NAArg, NAHis, NALeu, and NAPro were acquired as references for the analysis of the growth hormone data (Figure 1, Table I) and will be discussed in more detail in succeeding sections. These analogues should be better models for amino acids incorporated in a protein chain than the free acids since the amino and/or carboxyl termini are chemically blocked as they would be in a peptide backbone. We have found the aromatic amino acid analogues to have resonance Raman spectra very similar to those reported previously for the analogous free amino acids with 218-nm laser excitation.[15-19] The vibrational frequencies and assignments for the aromatic amino acids are listed in Table I. Our assignments are based on the results of previous studies of the corresponding free amino acids.[17,29]

Solutions of aromatic amino acid analogues were prepared at the same molar concentrations present in bGH. These concentrations were chosen to obtain the approximate peak intensities expected for the analogous vibrations in the growth hormones. The intensities of observed UV absorption bands in an amino acid analogue mixture, in which each amino acid component was at the same molar concentration present in the growth hormones, are similar (within a factor of 2, data not shown) to those of the analogous bands observed in the UV absorption spectra of the growth hormones.

Resonance Raman Spectra of Native Growth Hormones. The resonance Raman spectra of native growth hormones at pH 8 are shown in Figure 2A–C. On the basis of the amino acid analogue assignments (Figure 1, Table I), the growth hormone assignments are given in Table II. The resonance Raman spectra of all three native hormones possess a broad vibrational envelope centered at 1459 cm^{-1} (Figure 2A–C). Trp has a vibration at 1460 cm^{-1}, but its intensity is weak,[17] and it is not observed in the NATrpA solutions at the concentration present in the growth hormones.

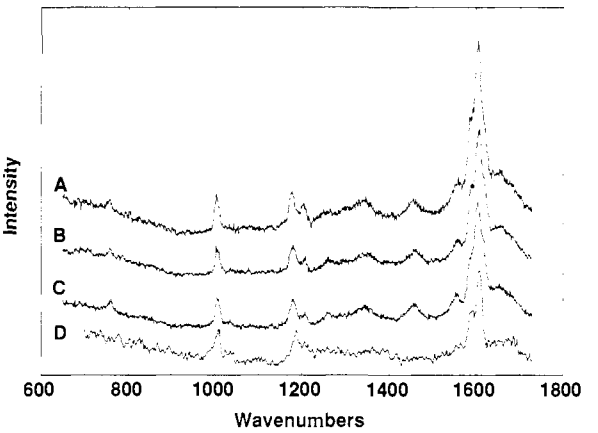

Figure 2. Resonance Raman spectra (221.6 nm) of native (A) bGH, (B) pGH, (C) hGH, and (D) bGH CNBr carboxyl-terminal fragment (residues 180–191) at 1 mg/mL in 0.05 M $NaHCO_3$ buffer (pH 8).

Table II. Resonance Raman Frequencies and Vibrational Assignments for Native Growth Hormones and the Lys-112 → Leu bGH Mutant[a]

resonance Raman frequency (cm^{-1})				
bovine	porcine	human	Lys-112 → Leu	vibrational assignment
756	754	754	754	Trp
1006	1003	1002	1001	Phe ν_{12}, Trp
1180	1176	1177	1176	Phe ν_{9a}, Tyr ν_{9a}
1206	1202	1204	1202	Phe ν_{7a}, Tyr $\nu(a_1)$
1260	1259	1259	1259	Tyr
1350	1341	1342	1339	Trp
1459	1460	1456	1455	Pro
1557	1558	1552	1555	Trp ν_{8a}
1589	1589	1583	1581	Phe ν_{8b}
1605	1606	1602	1602	Phe ν_{8a}, Tyr ν_{8b}
1618	1618	1615	1615	Tyr ν_{8a}, Trp ν_{8b}

[a] Data were acquired at pH 8 with 222-nm excitation.

On the basis of the intensities of the strongest Trp bands observed in the growth hormones and the intensities of the analogous NATrpA vibrations relative to the weak 1460-cm^{-1} NATrpA peak, we feel that the 1460-cm^{-1} Trp vibration is not observed in the growth hormones. A low-frequency shoulder on the 1460-cm^{-1} band of Trp in horse cytochrome *c* (200-nm excitation) has been assigned as a CH_2 bending mode.[21] Our investigations of aliphatic amino acids analogues, such as NALeu and NAArg, indicate no vibrations would be observed in the 1460-cm^{-1} region at the molar concentrations which they are present at in the growth hormones. Only at molar concentrations that are 10 times greater than those present in the hormones is a weak 1460-cm^{-1} band visible (Figure 1A,B). The carboxyl-terminal CNBr fragment of bGH (residues 180–191) has no prolines but many (92%) nonaromatic amino acids, and no 1460-cm^{-1} vibration was observed (Figure 2D). Resonance Raman bands have been observed in His-rich glycoprotein and ribonuclease A at $\simeq$1459 cm^{-1} and were assigned to an amide mode involving the Pro ring nitrogen, on the basis of a comparison with propanolic solutions of polyproline.[20] We observe a Pro vibrational envelope between 1455 and 1480 cm^{-1} in NAPro at molar concentrations analogous to those found in the growth hormones (Figure 1C); thus, we assign at least part of the 1459-cm^{-1} envelope to the six Pro residues in the growth hormones.

NAHis spectra were acquired to investigate the contribution of His to the width of the 1459-cm^{-1} peak in the growth hormone spectra. Resonance Raman spectra for the His analogues are similar to those that were previously reported, which show enhancement of the imidazole ring modes.[30-32] The NAHis data

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Table III. Vibrational Intensities for Native (pH 8) and Partially Unfolded (pH 2) Growth Hormones and Lys-112 → Leu bGH Mutant

Raman frequency (cm^{-1})	bovine		porcine		human		Lys-112 → Leu bGH	
	pH 8	pH 2	pH 8	pH 2	pH 8	pH 2	pH 8	pH 2
	Normalized Resonance Raman Intensity[a]							
756	5	11	5	6	8	8	5	6
1006	23	30	15	17	18	17	18	18
1180	21	23	15	14	15	14	15	16
1206	11	12	7	6	6	7	9	8
1350	7	7	5	5	7	7	6	5
1459	8	11	6	8	9	10	8	8
1557	17	26	15	19	20	17	14	15
1589	52	37	44	36	39	34	40	26
1605	94	86	78	73	79	75	78	62
1618	45	54	31	44	42	34	35	35
	Total Differential Raman Cross Sections[b]							
756	7.6	13.8	5.6	9.9	11.2	10.4	6.2	7.6
1557	9.6	14.4	5.3	7.9	10.1	7.4	7.2	8.2

[a] Data were acquired with 222-nm excitation and are normalized to the 3600-cm^{-1} water band, which has been set equal to 1000. [b] Cross-section units are 10^{-25} cm^2/(molecule sr). The cross sections were calculated according to the methods of Asher, Ludwig, and Johnson.[17]

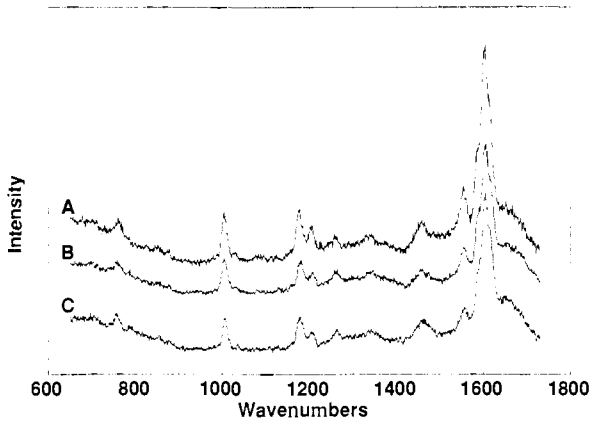

Figure 3. Resonance Raman spectra (221.6 nm) of partially unfolded (A) bGH, (B) pGH, and (C) hGH at 1 mg/mL in water (pH 2).

illustrate that resonance Raman vibrations are observed at ≃1445, 1455, and 1485 cm^{-1} at pH 8 (Figure 1D). At pH 2 the same vibrations are noted; however, the intensity at 1485 cm^{-1} increases dramatically (Figure 1E). An intensity increase of this magnitude should produce a variation in the contour of the 1459-cm^{-1} protein envelope as a function of pH. His residues account for only 1.5% of the protein composition in bGH, pGH, and hGH. No pH-dependent variations in the contours of the 1459-cm^{-1} envelopes are observed in any of the growth hormone spectra (Figures 2A–C and 3A–C). In addition, we have detected no observable resonance-enhanced imidazole vibrations in solutions in which NAHis was present at molar concentrations equal to those in the growth hormones. We feel that the breadth of the 1459-cm^{-1} peak does not derive from His vibrations.

The weak, broad resonance-enhanced envelope(s) observed in the growth hormones from 1230 to 1390 cm^{-1} (Figure 2A–C) probably contain(s) contributions from CH, CH_2, and CH_3 deformation and twisting/rocking vibrations.[33] Figure 1A shows that strong methylene vibrations occur at 1300, 1327, and 1378 cm^{-1} in NALeu. These vibrations are weak, although still observable, in NALeu at the molar concentrations present in the growth hormones. Amide III vibrations occur in this region and are contributors to the 1230–1390-cm^{-1} vibrational envelope(s).

The growth hormone resonance Raman band at ≃1557 cm^{-1} is assigned as the Trp symmetric phenyl ring mode, ν_{8a}. Vibrations in this frequency range have also been assigned as amide II protein backbone vibrations resulting from disordered structure.[34] Secondary structural estimates for bGH predict the native protein to contain a large amount of α-helix (45–60%) connected by several turns.[4,35] Amide II IR vibrations at 1560 cm^{-1} typically denote disordered structure, while vibrations associated with helices are observed around 1542 cm^{-1}.[36] Intense bands are observed at 1542 cm^{-1} in the IR spectrum of native bGH. Very little IR absorption is noted at 1557 cm^{-1}, suggesting that the 1557-cm^{-1} resonance Raman band is not an amide II vibration. In addition, amide II vibrations are extremely weak, if present, in the UV resonance Raman spectrum of the CNBr carboxyl-terminal fragment of bGH (Figure 2D). The assignment of the 1557-cm^{-1} peak to Trp is supported by the observation of other Trp vibrations in the growth hormones, specifically the doublet centered at 1350 cm^{-1}, in addition to the 1006- and 756-cm^{-1} Trp vibrations.

The resonance Raman band at 1589 cm^{-1} is assigned to Phe. The other Phe vibrations at 1180, 1206, and 1605 cm^{-1} also contain contributions from the tyrosines, while the Phe band at 1006 cm^{-1} derives, in part, from the single Trp. The Tyr band at 1618 cm^{-1} contains contributions from Trp.

Resonance Raman Spectra of Partially Unfolded Growth Hormones. Figure 3A–C shows the resonance Raman spectra of the acid-induced partially unfolded state of the three growth hormones. The data are similar to those in Figure 2 for the native conformation, with major exceptions being the Trp vibrations at 1557 and 756 cm^{-1}. The Raman cross sections (and intensities) of these peaks are greater in partially unfolded bGH and pGH than in the native states of these two hormones (Table III). There is also an increase in the width of the 1006-cm^{-1} band and a corresponding increase in the intensity of the 1618-cm^{-1} shoulder of the 1605-cm^{-1} peak of partially unfolded bGH and pGH, compared to their native conformations, which probably indicates that there is an increased Trp contribution to these peaks. There were no discernible frequency shifts in any of the vibrations for bGH and pGH as a result of changing the pH from 8 to 2. The Trp-related variations between the native and partially unfolded conformations of bGH and pGH are different in hGH. The Trp Raman cross sections in the spectrum of native hGH decrease slightly as hGH is partially unfolded (Table III).

The differences in the cross sections of the 756- and 1557-cm^{-1} vibrations observed between the folded and partially unfolded states varies among the hormones. The greatest change is noted in bGH, followed by slightly less variation in pGH and finally little change in hGH (Table III). In bGH the intensity of the 756-cm^{-1} vibration increases by 82%, while the 1557-cm^{-1} vibration grows by 50% between the native and partially unfolded forms. PGH shows analogous, but smaller, Trp band intensity increases, when going from the native to the partially unfolded form; the 754- and 1558-cm^{-1} vibrations increase 77% and 49%, respectively,

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Table IV. Fluorescence Properties of Native (pH 8) and Partially Unfolded (pH 2) Growth Hormones and Lys-112 → Leu bGH Mutant

	bovine		porcine		human		Lys-112 → Leu bGH	
	pH 8	pH 2	pH 8	pH 2	pH 8	pH 2	pH 8	pH 2
quantum yield[a]	0.03	0.14	0.04	0.19	0.14	0.14	0.03	0.07
average lifetime (ns)[b]	1.7	3.8	2.3	3.9	2.8	2.9	2.2	3.1
emission maximum (nm)	325	330	328	330	330	330	324	333
k_q(acrylamide) (10^9 M^{-1} s^{-1})[c]	0.64	0.63	0.43	0.77	0.46	0.49	0.57	0.55

[a] Based on quantum yield of NATrpA (pH 8) = 0.14. [b] Each decay curve was analyzed by assuming two or three components. [c] For each experiment $r^2 > 0.98$; estimated uncertainty = $\pm 0.02 \times 10^9$ M^{-1} s^{-1}.

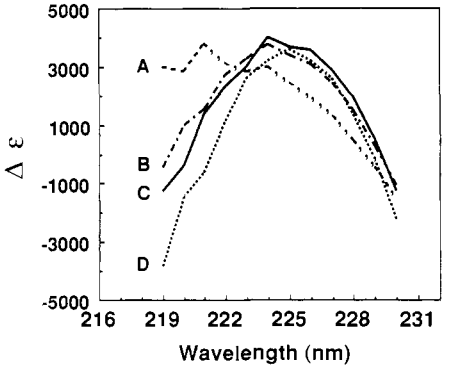

Figure 4. UV absorption difference spectra (pH 2 − pH 8) of (A) hGH, (B) bGH Lys-112 → Leu mutant, (C) bGH, and (D) pGH.

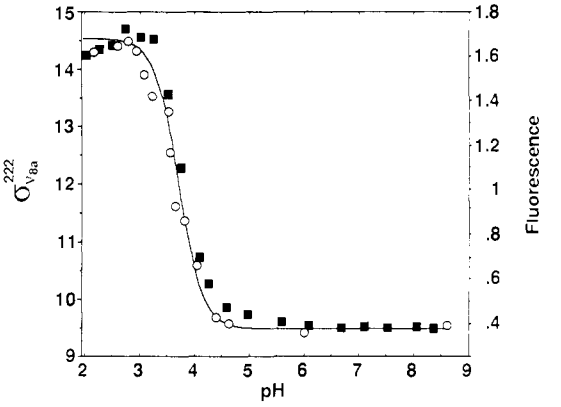

Figure 5. Variation in the Raman cross section of the 1557-cm^{-1} Trp vibration divided by that of the 1605-cm^{-1} Phe/Tyr peak (○) and fluorescence emission intensity at 350 nm (■) as a function of pH. The solid curve is calculated by assuming behavior expected for a two-proton exchange mechanism.

in the acid-induced partially unfolded state. The resonance Raman spectra for hGH exhibit 27% and 7% decreases in the intensities of the 754- and 1552-cm^{-1} vibrations, respectively.

Far-UV Absorption Spectra of Growth Hormones. Examination of the absorption difference spectrum (pH 2 − pH 8) in the 220–230-nm region of the electronic absorption spectra of the growth hormones indicates that, in the wavelength region of laser irradiation, the electronic absorbance is pH dependent (Figure 4). Variations in pH have a different effect on the intensities of the absorptions of hGH (Figure 4A) than on those of the analogous absorptions for bGH, a Lys-112 → Leu mutant of bGH, or pGH (Figure 4B–D). The pH-dependent electronic absorption changes for the bGH Lys-112 → Leu mutant (Figure 4B), native bGH (Figure 4C), and pGH (Figure 4D) are all similar to each other.

Fluorescence Properties of Growth Hormones. Tryptophan fluorescence emission spectra for the three growth hormones in the native state (pH 8) have different emission maxima, but at pH 2 they all exhibit the same emission maximum (Table IV). Fluorescence quantum yield and average lifetime measurements for the growth hormones indicate that the native-state hormones are different from one another, but like the emission maximum results, the partially unfolded states of all three proteins are similar (Table IV). Both bGH and pGH exhibit significant quenching of fluorescence in the native state (about 4-fold compared to NATrpA), while the quantum yield and lifetime of hGH is independent of pH. In the partially unfolded state (pH 2), all three growth hormones display quantum yields which are close to NATrpA, but the emission maximum for all three (330 nm) is different from that observed for NATrpA (350 nm).

Solute fluorescence quenching experiments provide a measure of the exposure of tryptophan residues in proteins.[37] We used the penetrating quencher acrylamide to study the differences in exposure of tryptophan in the growth hormones at pH 8 and 2 (Table IV). Two conclusions can be reached from the bimolecular quenching rate constants (k_q) observed for the growth hormones: (a) tryptophan exposure was similar for the three growth hormones at pH 8 and 2 as the range of k_q values was small [(0.4–0.8) × 10^9 M^{-1} s^{-1}], and (b) considerable protection from acrylamide quenching was observed in all growth hormones as the k_q values were significantly less than the reported value for quenching of NATrpA by acrylamide (6.3 × 10^9 M^{-1} s^{-1}).[38]

Results from the use of the modified Stern–Volmer equation (see Experimental Section for details of calculation) yielded f_a values that were indistinguishable from 1.0 for each growth hormone at pH 8 and 2. These results indicate that all the tryptophan fluorescence was accessible to acrylamide quenching; further, there was no detectable difference between the various growth hormones and no detectable difference between pH 8 and pH 2 solutions of the growth hormones.

Discussion

Proline Isomerization. The frequency of the 1460-cm^{-1} amide vibration of Pro is sensitive to geometric configuration and shifted from 1458 to 1466 cm^{-1} as ribonuclease A unfolded (pH 7.0 → 1.5).[20] It was proposed that the frequency shift corresponded to a Pro cis → trans conversion during ribonuclease A denaturation. No shifts of the ≃1459-cm^{-1} peak were observed in any of the growth hormones as a function of pH, indicating that no cis → trans isomeric conversion occurs in the pH 2 to pH 8 range in the growth hormones.

Variations in Tryptophan Vibrational Intensities. The increases in the Raman cross sections (and intensities) of the pH-dependent Trp vibrations at 756 and 1557 cm^{-1} (Table III) indicate that the Trp environments of native (pH 8) bGH and pGH are different than those of the partially unfolded state (pH 2). The slight decrease of analogous cross sections in hGH further indicates that the Trp environment in hGH is different than it is in bGH and pGH.

The possibility of Trp Raman vibrational intensity saturation due the overpopulation of an excited electronic state, generated as a result of high laser energy flux,[39] would lead to a decrease in the intensities of the Trp vibrations, instead of the increases observed in bGH and pGH. Raman saturation was not observed in any of the pH 8 protein samples. Mild saturation was detected in the pH 2 samples, but only at higher laser power levels (1.0–1.5 mW). We used low power levels (<1 mW) for the pH 2 protein

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Table V. Amino Acid Positions for Possible Fluorescence Quenching Groups[a]

	residue number[b]											
	19	20	30	108	111	112	125	126	163	170	175	180
bGH	Gln	His	Lys	Arg	Glu	Lys	Arg	Glu	Ser	His	Tyr	Lys
pGH	Gln	His	Lys	Arg	Glu	Lys	Arg	Glu	Ser	His	Tyr	Lys
hGH	His	Arg	Gln	Asn	Asp	Leu	Gly	Arg	Tyr	Asp	Phe	Gln
comment[c]	i	ii	i	iii	i	iv	iii	iii	i	v	i	iii

[a] List of all Ser, Tyr, Gln, Asn, Thr, Arg, His, Lys, Glu, and Asp groups in α-helices in bGH and pGH that are replaced with different amino acids in hGH. [b] Numbers refer to the bGH amino acid sequence. [c] Comments (see text for further discussion): (i) no reasons to exclude from consideration; (ii) exclude due to exposure to solvent (see text); (iii) exclude due to location at end of α-helix; (iv) exclude due to results from bGH mutant with Leu at this position; (v) exclude due to NMR results on bGH.[45]

samples; however, it is possible that mild Raman saturation may have produced, in part, the slight Trp pH-dependent intensity decreases observed in the hGH pH 2 samples, especially since the hGH fluorescence data is pH independent.

The changes in Trp cross sections in bGH and pGH, as a function of pH, correlate with quenching of Trp fluorescence (Figure 5). Neither Trp cross sections nor fluorescence quantum yields change significantly for hGH between pH 2 and pH 8 (Tables III and IV). The correlation of Raman and fluorescence data (Figure 5) suggests that the same structural entity or molecular event is responsible for both the vibrational cross section and fluorescence intensity changes observed in bGH and pGH during protein unfolding. The Trp intensity increases in bGH and pGH may simply result from solvent exposure as the hydrophobic Trp pocket "opens" during protein unfolding. However, we have determined by far-UV CD measurements that significant α-helical structure remains at pH 2 (about 75% of the α-helical content that is present at pH 8). The lack of a large red shift in the fluorescence emission at pH 2 (Table IV) indicates that Trp is not significantly exposed to solvent (fully unfolded bGH has an emission maximum near 350 nm), a result supported by acrylamide quenching experiments (Table IV).

Vibrational intensity may not be a complete indicator of the hydrophobicity of a Trp environment. It was observed that the intensity levels of Trp vibrations may either remain constant (bovine α-lactalbumin)[40] or *decrease* slightly (horse and tuna cytochromes *c*)[21] during protein unfolding, indicating that exposure to solvent alone may not produce dramatic intensity *increases* in Trp vibrations.

If the Raman cross sections and fluorescence intensity variations encountered for bGH and pGH are due solely to the opening of the respective native Trp protein pockets to solvent, these environments must be different than the analogous pocket in hGH, and the changes may be slight, given the similarity in Trp exposure to acrylamide (Table IV). Due to the amino acid sequence homology of the three growth hormones, gross differences in the Trp protein pockets appear unlikely. It seems more reasonable that a subtle change in the Trp environment produces a decrease in both the resonance-enhanced vibrational and fluorescence intensities in the native states of bGH and pGH that is not present in hGH.

The decreased Trp Raman cross sections in the native conformations of bGH, pGH, and the Lys-112 → Leu bGH mutant appear to be, at least in part, related to intensity changes in the 220–230-nm region of the analogous electronic absorption spectra (Figure 4). The interaction of aromatic amino acids in proteins has produced hypochromism (intensity decreases) in electronic absorption spectra. Hypochromism and the related Trp vibrational intensity decreases have been observed for Trp-Tyr dimers.[41] Since the Trp environment in the native growth hormones (pH 8) is known to be hydrophobic, it is reasonable to assume that nonpolar aromatic groups are located in the same region. The interaction of Trp with a proximal aromatic residue in the native state of bGH and pGH (pH 8) would account for the decreased Trp vibrational intensities. When the growth hormones are partially unfolded (pH 2), the resulting relative movements of the residues in the Trp vicinity could result in the loss of interaction between Trp and the aromatic species. Sequence differences between bGH (or pGH) and hGH could result in a Trp environment in hGH that does not have an available interacting group.

Currently, we do not completely understand the reason(s) for the relative variations in intensities that the different bGH and pGH Trp vibrations undergo during acid-induced unfolding. These variations should not be solely the result of slight wavelength shifts in the Trp electronic absorption since it has been shown that, although the resonance Raman intensities of Trp vibrations vary dramatically as a function of laser excitation wavelength, the relative percent change in the intensities of the different vibrational modes is comparable.[17] Selective deenhancement of certain Trp vibrational modes in pH 8 bGH and pGH would require either a change in vibronic coupling parameters and/or different excitation profiles for the different Trp modes, in addition to a shift in the electronic absorption.

Possible Tryptophan Fluorescence Quenchers. Protonated amines, imidazoles, phenols, alcohols, carboxylic acids, and disulfide bonds are groups known to be efficient fluorescence quenchers in proteins. For the growth hormones, it is apparent that both static and dynamic quenching occurs in pGH and bGH as both quantum yields and lifetimes increase on changing the pH from 8 to 2 (Table IV). In order to identify the group(s) responsible for static and dynamic fluorescence quenching in the native state of bGH and pGH, we can assume that the group(s) is (are) located in a hydrophobic region near the Trp and substitutions are made in the hGH sequence so that Trp fluorescence is not quenched in hGH. It can be further assumed that the quenching group(s) must be in α-helical regions near the center of the molecule since the single Trp in pGH is centrally located in an α-helix and in a hydrophobic environment.[3] With these assumptions, the list of possible fluorescence quenchers reduces to the 13 residues in Table V.

In order to further limit the possibilities for the fluorescence quenching group(s), the residues which are located near the ends of α-helices can be eliminated from consideration because they are probably too far from the Trp residue to effectively quench fluorescence. The helical regions in the growth hormones span residues 7–34, 75–96, 106–127, and 152–183.[3] With this rationale, it is possible to exclude from consideration Asp-107, Arg-108, Arg-125, Glu-126, and Lys-180 in Table V.

It has been proposed that a Lys is responsible for the observed fluorescence quenching in bGH, on the basis of data from the native molecule which had its lysines chemically acylated.[6] The acylated bGH exhibited more Trp fluorescence than the unmodified protein while retaining native structure. Lys-112 is conserved in the primary structure of bGH and pGH, but not in hGH (Table V). In order to investigate the possibility that Lys-112 is the fluorescence quenching group, resonance Raman and fluorescence emission spectra were acquired for a site-directed mutant of bGH in which the Lys-112 residue was changed to Leu.[42] Both Trp fluorescence emission (Table IV) and Raman vibrational (Table III) intensities of the mutant at pH 8 were similar to those observed for wild-type bGH, indicating that Lys-112 is not responsible for the observed quenching of fluorescence and is not involved in the mechanism that decreases Trp resonance Raman intensity in native bGH. The reduced fluorescence quantum yield of the mutant protein at pH 2, com-

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Chart I

	20	150	170
bGH	· · · Gln–*His*–Leu–*His* · · ·	Arg · · ·	*His* · · ·
	20	150	170
pGH	· · · Gln–*His*–Leu–*His* · · ·	Arg · · ·	*His* · · ·
	20	151	171
hGH	· · · *His*–Arg–Leu–*His* · · ·	*His* · · ·	Asp · · ·

pared to that of wild-type bGH, is probably due to increased concentration of a multimeric folding intermediate in the mutant protein.[42] It has been observed that a multimeric intermediate exhibits decreased fluorescence emission compared to the monomeric folding intermediate.[9] The changes observed in far-UV absorbance between the native (pH 8) and partially unfolded (pH 2) forms of the mutant protein were very similar to the variations observed for wild-type bGH (Figure 4B,C). These results do not rule out Lys-30 as a possible fluorescence quenching group.

The growth hormones described in this study each possess three histidine (His) residues that can quench fluorescence. Due to primary sequence differences, one His is conserved (His-22 in bGH and His-21 in pGH and hGH are equivalent), and the other two differ between species (Chart I). Since His-22 in bGH is conserved as His-21 in pGH and hGH, it is unlikely that this His is responsible for the quenching of Trp fluorescence in bGH and pGH.

^{1}H NMR spectroscopy has been used to determine the pK_a values for the three histidines in hGH.[43] The pK_a (6.9) of His-151 indicated it was a surface residue. His-21 exhibited a pK_a in the normal range for histidine (6.7) but also appeared to be near an aromatic residue. His-21 also possessed the slowest deuterium exchange rate of the three histidines with some degree of hindrance to the approach of water, indicating a less polar environment than that of His-18 or His-151. His-18 had an unusually high pK_a (7.9), indicative of an ion-pair interaction with an acidic residue. His-18 also exhibited a second ionization shift at p*H* 3.6, which further supported the ion-pair hypothesis. It was proposed that an ion-pair interaction involving His-18 would decrease the "true" pK_a of His by 1 unit to $\simeq$6.9 and increase the pK_a of the interacting species by a comparable amount to $\simeq$4.6. Both Asp and Glu have pK_a's close to 4.6.[44]

^{1}H NMR studies on bGH have indicated that His-170 resides on the protein surface (pK_a = 5.7), and hence, His-170 is not likely to be an efficient quencher of Trp fluorescence.[45] However, the NMR results indicate that either His-20 or His-22 has an unusually low pK_a (4.7) and is located in a buried, hydrophobic pocket, in an unprotonated state, near one or more aromatic residues in the native bGH structure.[45] No evidence of ion pairing to an acidic group was observed for either His-20 or His-22, and the residue which does not have the unusually low pK_a exhibits a pK_a of 5.9, in the normal range of His in proteins. Since the His in the most nonpolar environment in hGH (His-21) is equivalent to His-22 in bGH and His-21 in pGH (Chart I), His-22 in bGH can be assigned as the His residue that is in a hydrophobic region near an aromatic residue and exhibits a pK_a of 4.7.

Chou–Fasman secondary structure predictions[46,47] indicate that both His-20 and His-22 reside in a helical region of bGH. These predictions are supported by the crystal structure of pGH,[3] which shows an α-helix spanning residues 7–34. By analogy with these results, His-18 and His-21 in hGH should reside in a helical region. The position of His-20 in bGH (His-19 in pGH), assuming 3.6 residues per turn, should be rotated about 160° with respect to the 7–34 helical axis relative to His-22 (His-21 in pGH). Since His-22 is in a hydrophobic region (as determined by ^{1}H NMR), it is unlikely that a 160° rotation can allow His-20 to also be in a hydrophobic region. Hence, His-20 must be exposed to solvent, should not be in close contact with the Trp residue, and can be excluded from consideration as a fluorescence quenching group in bGH.

The X-ray crystal structure of pGH[3] has indicated that Trp is about 5 Å from the disulfide bridge that connects Cys-53 to Cys-164, while the disulfide bridge connecting Cys-181 to Cys-189 is further removed from Trp.[48] It has been reported that a site-directed mutant of bGH, in which the 53–164 disulfide bridge was not present, retained native protein structure (α-helical content was 90% that of wild-type bGH), had significantly more Trp fluorescence than wild-type bGH, and exhibited a 15-nm red shift in its fluorescence emission maximum.[48] It was therefore proposed that the 53–164 disulfide bridge was a fluorescence quenching group.

If the disulfide bridge is responsible for quenching Trp fluorescence, one would expect that the 53–164 disulfide bridges in bGH and pGH would possess similar spatial orientation with respect to the Trp residue, and therefore have very similar bridge geometries. In order for significant fluorescence emission to occur in hGH, however, this cystine link would have to be further removed from Trp, which might result in a substantial geometric alteration of the disulfide bridge. It has been observed that the Raman disulfide stretching frequency [ν(S–S)] can be used to indicate the geometry of a cystine linkage.[49,50] We have observed ν(S–S) for all three growth hormones at approximately 510 cm^{-1}, suggesting that bGH, pGH, and hGH all have cystine links of similar geometry.[51] We feel that a large variation in cystine link geometry would be needed to move the 53–164 disulfide bridge a sufficient distance from the hGH Trp residue to significantly reduce fluorescence quenching in native hGH. If the 53–164 disulfide bridge does not contribute to Trp fluorescence quenching, it is possible that the fluorescence results for the site-directed mutant of bGH, which does not contain this bridge,[48] can be interpreted as indicating a partial unfolding of bGH when this bridge is not present, resulting in a structure for the mutant protein that resembles the bGH monomeric folding intermediate. It has been shown that this intermediate had increased fluorescence emission and a slight red shift in fluorescence emission maximum compared to native bGH.[9] Presently, we are not, however, completely rejecting the possibility that the disulfide bridge is a Trp fluorescence quencher in native bGH and pGH, since subtle structural differences between the growth hormones could result in a relative movement of the disulfide bridge with respect to Trp in hGH.

The discussion above has allowed the list of likely Trp fluorescence quenching groups in bGH and pGH to be reduced to five: Gln-19, Lys-30, Glu-111, Ser-163, and Tyr-175. Of these five residues, Tyr-175 would rate the highest in its ability to affect the resonance Raman intensity of Trp vibrations. However, it is not necessary for the group(s) involved in Trp fluorescence quenching to also be responsible for the observed variations in resonance Raman intensity.

Role of Carboxylic Acid Residues in Structural Alterations. It seems clear that carboxylic acid residues (Glu and/or Asp) are involved in the quenching of Trp fluorescence and the decrease in resonance Raman cross sections, due to the pK_a (3.7) of these changes (Figure 5). We do not know if an acidic group acts directly to alter the intensities or if a conformational change is triggered either by an unfavorable electrostatic free energy change resulting from the protonation of carboxylate groups or by the loss of specific carboxylate interactions. Since the pH dependencies of Trp vibrational and fluorescence intensities follow a curve that is fit best by a two-proton exchange mechanism (Figure 5), it

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seems unlikely that the protonation of a single acidic group is responsible for the observed intensity variations. As previously suggested by Burger and co-workers,[25] a conformational change triggered, at least in part, by the protonation of acidic groups alters the Trp environment enough to vary fluorescence, resonance Raman, and UV absorption properties.

Conclusions

The 222-nm resonance Raman data for bGH, pGH, and hGH at pH 8 show strong enhancement of the aromatic residues Phe, Trp, and Tyr. Pro is also enhanced. The pH-dependent variations of the Trp Raman vibrational cross sections in native bGH and pGH, compared to the analogous vibrations in native hGH, indicate the presence of a different Trp environment in native hGH. The pH dependence of Trp vibrational intensity changes in bGH and pGH correlates to fluorescence emission, suggesting the same protein group or molecular event is responsible for both effects.

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Structures of Covalent Nucleoside Adducts Formed from Adenine, Guanine, and Cytosine Bases of DNA and the Optically Active Bay-Region 3,4-Diol 1,2-Epoxides of Dibenz[*a*,*j*]anthracene

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Abstract: Chemical structures of the principal adducts formed from DNA upon reaction in vitro with the four optically active 3,4-diol 1,2-epoxides derived from the *trans*-3,4-dihydrodiol enantiomers of dibenz[*a*,*j*]anthracene have been elucidated at the nucleoside level. In addition to the structures of deoxyadenosine (dA) and deoxyguanosine (dG) adducts, complete chemical characterization of a deoxycytidine (dC) adduct formed from a diol epoxide is reported for the first time. The site of covalent attachment is between the benzylic C-1 of the diol epoxide moiety and the exocyclic amino group of the base in all of these adducts, as deduced by chemical stability considerations and pH titration (pK_a = 2.1 and 9.3 of a dG adduct, pK_a = 2.2 of a dA adduct, and pK_a = 2.6 of a dC adduct). Upon acetylation, these adducts gave pentaacetates, as determined by their mass spectra. Since the purine or pyrimidine substituent must be pseudoaxial, conformational flexibility of the tetrahydro benzo ring is limited. Thus, coupling constants between methine protons on this ring of the adducts (as their pentaacetates), as well as effects of the purine on the chemical shift of the C-3 proton, when these two substituents are cis and pseudodiaxial, are diagnostic of the relative stereochemistry of the adducts (cis vs trans opening of the epoxide). All the dibenz[*a*,*j*]anthracene adducts which have *S* absolute configuration at the benzylic C-1 carbon of the tetrahydro aromatic moiety exhibit CD spectra with a positive band at 270–280 nm and a negative band at 290–300 nm. Thus, assignment of cis vs trans addition for minor adducts whose NMR spectra were unobtainable due to their low level of formation could be made on the basis of their CD spectra and the known absolute configurations of the parent diol epoxides.

Covalent modification of DNA by the chemically reactive bay-region diol epoxides, which are formed upon metabolic activation of polycyclic aromatic hydrocarbons,[1] presumably constitutes the first step in the tumorigenic and mutagenic processes initiated by these metabolites.[2] Covalent adduct formation upon reaction of nucleic acids with diol epoxides is well documented, particularly in the case of the benzo[*a*]pyrene (BaP) diol epoxides,[3] but definitive chemical characterization of many of these adducts (notably those derived from DNA) is unavailable. Recently, we reported complete spectroscopic characterization of 16 adducts formed upon cis and trans addition of the exocyclic nitrogen of purine residues in DNA to the benzylic C-1 position of the four optically active 3,4-diol 1,2-epoxides derived from benzo[*c*]phenanthrene (BcPh).[4] In the present study, we describe the structural characterization of covalent nucleoside adducts[5] formed upon reaction of DNA with the four configurationally isomeric bay-region diol epoxides of dibenz[*a*,*j*]anthracene, whose structures are shown in Figure 1.

Dibenz[*a*,*j*]anthracene (DBA), one of the three possible isomeric dibenzanthracenes, is less carcinogenic than the dibenz[*a*,*h*]anthracene isomer.[2] The bay-region theory[6] predicts that the

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(5) For brevity, the deoxyguanosine, deoxyadenosine, and deoxycytidine adducts are designated as dG, dA, and dC, respectively. The subscripts c and t are used to represent adducts derived from addition of the exocyclic amino group of DNA bases at the benzylic C-1 position by either cis or trans opening of the epoxide, respectively.