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Ultraviolet Resonance Raman Spectra of Insulin and α-Lactalbumin with 218- and 200-nm Laser Excitation†

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ABSTRACT: Ultraviolet resonance Raman (RR) spectra, with 200- and 218-nm excitation from a H_2-shifted quadrupled Nd:YAG laser, are reported for insulin and α-lactalbumin in dilute aqueous solution, at pH values known to produce differences in the exposure of the aromatic residues to solvent. At 200 nm, the spectra are dominated by tyrosine bands, whose intensity is lowered somewhat in protein conformations in which tyrosine is exposed to solvent. The expected shift in the relative intensities of the components of the ~850-cm^{-1} tyrosine doublet is difficult to discern because the higher energy component shows much greater resonance enhancement and the lower energy component appears as a weak shoulder. The peptide vibrations, amides I, II, and III, are also enhanced at 200 nm. The infrared active amide II mode is particularly prominent, although it is not observed in Raman spectra with visible excitation. In addition, the amide I band is quite broad in the 200-nm RR spectra, and the peak frequency is lower than that seen in visible excitation Raman spectra and is close to the infrared frequency. It appears that 200-nm excitation produces resonance enhancement of the infrared-active components of both amide I and amide II. Excitation at 218 nm enhances tryptophan modes strongly. The 876-cm^{-1} band, assigned to a deformation mode of the five-membered ring, shows a measurable upshift upon exposure of tryptophan to solvent, attributable to N–H hydrogen bonding. The broad band seen at ~1360 cm^{-1} in visible excitation spectra is shown to be a doublet, the two components of which alter their relative intensities upon tryptophan exposure to solvent. When tryptophan is absent in the protein, tyrosine and phenylalanine modes are seen in the 218-nm spectrum.

Since the pioneering studies of Edsall and co-workers in the 1950s (Edsall et al., 1950; Garfinkle & Edsall, 1958a–c; Garfinkle, 1958), there has been continual interest in applying Raman spectroscopy to elucidate the structure of proteins (Lord & Yu, 1970; Spiro & Gaber, 1977). The responsiveness of the vibrational spectrum to molecular geometry gives wide scope to the technique, but with the usual visible light excitation, there are serious limitations of low sensitivity and the overlap of numerous vibrational bands. Accordingly, there is much current interest in the use of ultraviolet excitation, which promises large enhancements of vibrational modes of aromatic residues, and of the peptide bonds, via the resonance Raman (RR) effect (Carey, 1982). The attendant gain in sensitivity and selectivity for these chromophores may permit much more discriminating application of Raman spectroscopy to protein structural problems than has heretofore been possible.

We have recently reported Raman spectra for tryptophan and tyrosine with 218- and 200-nm excitation, obtained with the fourth harmonic of the Nd:YAG laser and a H_2 Raman shift cell (Rava & Spiro, 1984). High quality spectra were obtained in dilute (~1 mM) aqueous solution, and almost complete selectivity was observed, with tryptophan dominating the 218-nm spectrum and tyrosine dominating the 200-nm spectrum. In this study we examine UV RR spectra, with 200- and 218-nm excitation, for two small proteins, insulin and α-lactalbumin, which have previously been studied by nonresonance Raman (Yu et al., 1972, 1974; Nakanishi, 1974) and other spectroscopic techniques (Muszkat et al., 1984; Sommers & Kronman, 1980). Insulin contains no tryptophan (Trp) but has four tyrosine (Tyr) and three phenylalanine (Phe) residues. Bovine α-lactalbumin has four each of Trp,

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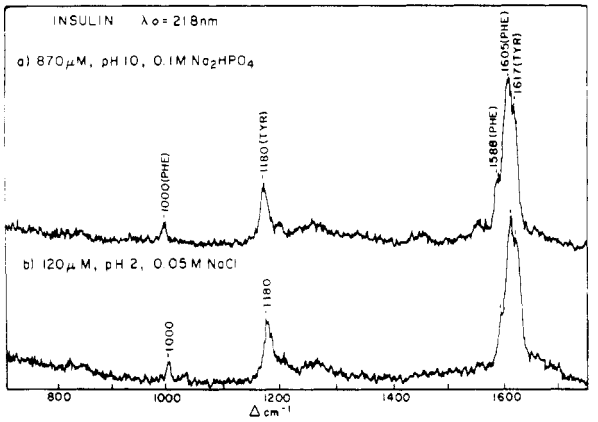

FIGURE 1: Resonance Raman spectra of insulin with 218-nm excitation. (a) Solution I (870 μM, pH 10, 0.1 M Na_2HPO_4) contains predominantly monomers. (b) Solution II (120 μM, pH 2, 0.05 M NaCl) contains monomers and dimer.

Tyr, and Phe. Bands from these aromatic residues are readily discerned with 218- and 200-nm excitation. For Trp and Tyr, frequency and intensity changes can be observed, which are associated with exposure of the residues to solvent. In addition vibrational modes localized on the peptide bonds are seen with 200-nm excitation. A novel feature of these spectra is enhancement of amide II (Harada et al., 1975; Sugawara et al., 1978), which is normally seen only in infrared spectra. These enhanced bands may prove useful in the characterization of protein secondary and tertiary structure.

Experimental Procedures

The experimental apparatus for ultraviolet resonance Raman spectra has been described (Fodor et al., 1985). Insulin (bovine) and α-lactalbumin (bovine) were obtained from Sigma Chemical Co. (I5500 and L5385, respectively) and used as supplied. A spectral range of 700–1700 cm^{-1} required 20 min to scan, and the spectra shown are from 6 (218 nm) to 16 (α-lactalbumin; 200 nm) scans. In order to guarantee sample integrity, solutions of protein were changed every three or four scans. With 10 mL of solution in the flow system and average laser powers estimated at $\lesssim$0.5 mW, no sample degradation was detected by differences in Raman spectra with successive scans or changes in the ultraviolet absorption before and after irradiation.

Results and Discussion

Insulin. Insulin is a small (5.7 kilodalton) protein with two chains, A and B, linked by two disulfide bridges. The bovine protein has two Tyr on the A chain (A14 and A19) and two on the B chain (B16 and B26), while all three Phe residues are on the B chain (B1, B24, and B25). Raman spectra with visible excitation have been reported (Yu et al., 1972, 1974) for crystalline and lyophilized insulin, as well as for concentrated solutions (100 mg/mL) in native and denatured states.

At lower concentrations insulin is known to exist in different aggregation states, monomer, dimer, tetramer, and hexamer, depending on its concentration, pH, Zn^{2+} binding, and ionic strength (Blundell et al., 1972). For this study we chose two dilute solutions, in which the composition is known from photo-CIDNP (chemically induced dynamic polarization) studies (Muszkat et al., 1984) to be relatively simple: solution I, with 0.87 mM protein in 0.1 M Na_2HPO_4, pH 9.9, contained predominantly monomers, while solution II, with 0.12 mM protein in acidified 0.05 M NaCl, pH 2.2, contained a 50:50 mixture of monomers and dimers. The photo-CIDNP study suggested that Tyr-A14, Tyr-B16, and Tyr-B26 are more

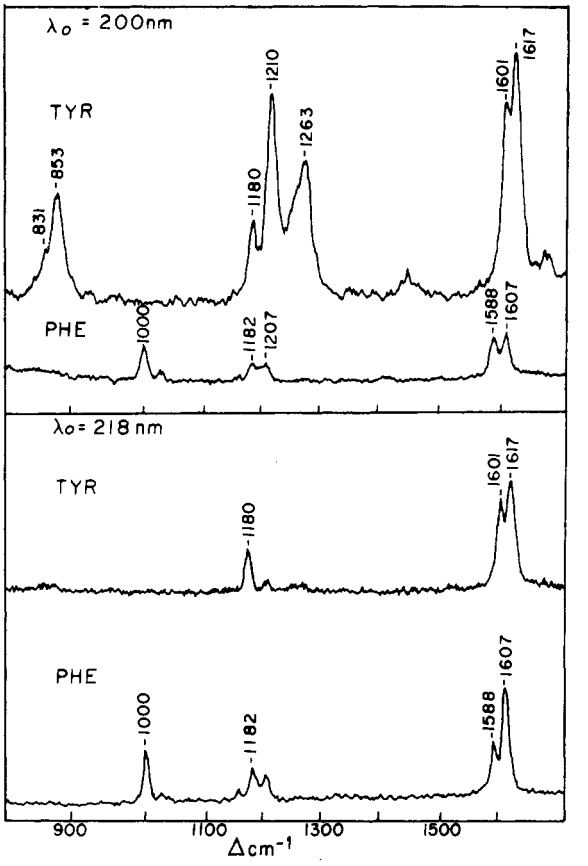

FIGURE 2: Resonance Raman spectra aqueous solutions (pH 7, 1 mM) of the individual amino acids tyrosine and phenylalanine irradiated at 200 and 218 nm. The spectra have been scaled to the relative enhancements of the amino acids at these wavelengths (Fodor et al., 1985).

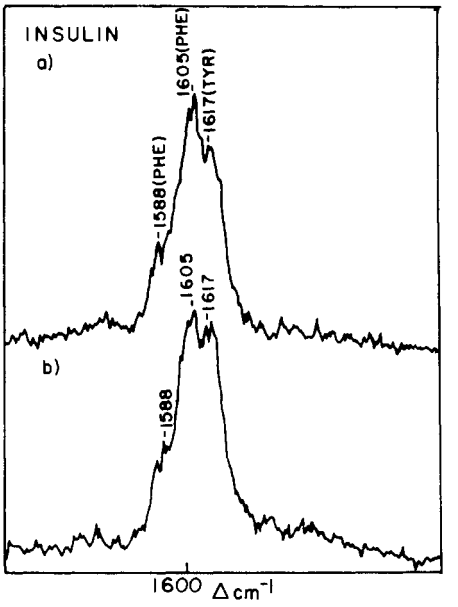

FIGURE 3: Higher resolution trace of the 1600-cm^{-1} region of insulin using the same conditions as in Figure 1.

accessible to solvent in the monomer than in the dimer.

Figure 1 compares RR spectra of solutions I and II taken with 218-nm excitation. The few bands seen at this wavelength are all attributable to Tyr (1617 and 1180 cm^{-1}) or Phe (1605, 1588, and 1180 cm^{-1}), which give comparable scattering, as shown in Figure 2. No frequency shifts are seen between the two solutions, but there are discernible differences in the relative intensities. This is illustrated in Figure 3, in which

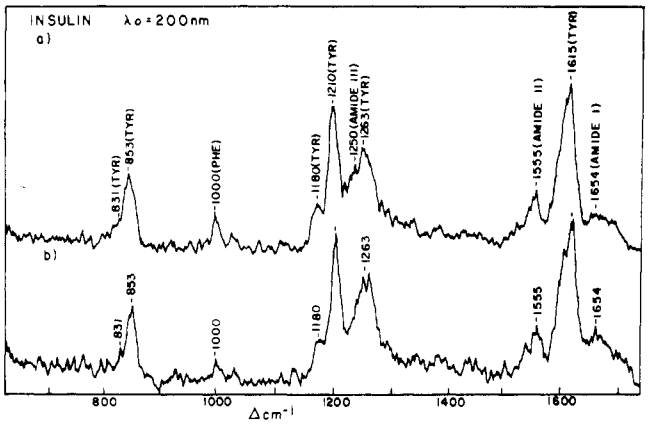

FIGURE 4: Resonance Raman spectra of insulin with 200-nm excitation. (a) Solution I; (b) solution II.

the 1600-cm^{-1} region is expanded. In solution I the 1617-cm^{-1} Tyr band clearly loses intensity relative to solution II when compared to the 1605-cm^{-1} Phe band. Thus, the Tyr signal is weaker for the monomer than for the dimer.

Figure 4 compares RR spectra of the same solutions taken at 200 nm. At this wavelength the scattering from tyrosine is particularly strong, as shown in Figure 2, and the dominant bands, at 1615, 1263, 1210, 1180, and 853 cm^{-1}, are attributed to Tyr. The only Phe contribution is the 1000-cm^{-1} band.

Additional bands are seen, however, at 1654, 1555, and 1250 cm^{-1}, which are assignable to amide I, II, and III modes, respectively, of the peptide groups in the protein backbone (Carey, 1982). We have observed these bands at comparable intensities in Raman spectra of polylysine (not shown), taken at 200 nm. The first absorption band of the peptide group maximizes at ~190 nm, and the peptide vibrational modes show substantial enhancement at 200 nm. A point of considerable interest is the prominence of the amide II mode, which is largely N–H bending coupled to C–N stretching in character; it is not observed in Raman spectra obtained with visible excitation. The enhancement of this mode in preresonance Raman spectra, obtained with 257 nm excitation, has previously been noted in simple amides and homopolymers (Harada et al., 1975; Sugawara et al., 1978). Also, Hudson and co-workers have reported the enhancement of this mode in *N*-methylacetamide excited with 213-nm excitation and suggest an enhancement mechanism (Hudson & Mayne, 1984). Amide II gives rise to a strong band in protein infrared spectra and is known to be sensitive to the peptide conformation (Krimm, 1983; Suzuki et al., 1966). Its prominence in RR spectra may prove helpful in analyzing protein secondary structure.

Another point of interest is the unusual breadth of the amide I band and its maximization at 1655 cm^{-1}, lower than the amide I frequencies seen for insulin with visible excitation, 1663 (assigned to α-helix) with a shoulder at 1680 cm^{-1} (assigned to random coil) (Yu et al., 1972, 1974). In the infrared spectrum, however, the amide I band center of insulin is at 1655 cm^{-1} (Yu et al., 1972); the frequency shift relative to the Raman bands is believed to reflect coupling among the amide local modes, with different infrared and Raman selection rules for the coupled modes (Carey, 1982). The selection rules may be relaxed in the resonance Raman spectrum with intensification of the infrared component, perhaps by a mechanism analogous to that responsible for the enhancement of the infrared-active amide II mode. This would explain the breadth of the amide I band, all components becoming enhanced to varying degrees. It is also notable that the amide II RR band maximum, 1555 cm^{-1}, is at a higher frequency than that reported (Ambrose & Elliott, 1951) for the infrared band, 1538 cm^{-1}. The latter frequency, however, corresponds to a shoulder observable on the side of the 1555-cm^{-1} RR band. Again the band shape may result from variable enhancements of the differently coupled amide II local modes.

As in the 218-nm RR spectra, the only difference between solutions I and II in the 200-nm RR spectra is a relative weakening of the Tyr contribution for solution I. This is seen most easily in the 1250-cm^{-1} region, where the amide III band shows up as a shoulder on the 1265-cm^{-1} Tyr band for solution I but is not readily discerned for solution II. We attribute the intensity decreases for the Tyr contribution to the increased solvent exposure of the Tyr residues in monomeric insulin (Muszkat et al., 1984; Blundell et al., 1972), which apparently lowers the Tyr enhancement factors somewhat.

The different tyrosine environments in the two solutions might also have been expected to be reflected in the bands at 831 and 853 cm^{-1}. This doublet has been assigned to a Fermi resonance between the tyrosine ring breathing mode and the overtone of an out-of-plane bending mode of the ring (Siamwiza et al., 1975). This resonance and the relative intensities of the two components have been shown to be sensitive to tyrosine hydrogen bonding. Exposed Tyr is expected to have a higher relative intensity for the low frequency component of the doublet. Unfortunately, the effect of resonance enhancement is to greatly intensify the ring breathing fundamental, relative to the overtone contribution (Rava & Spiro, 1985). Consequently, only a weak shoulder is seen at 831 cm^{-1}, and its change in relative intensity between the two solutions cannot reliably be determined.

Finally, we searched for enhancement of the disulfide stretch in the 200-nm RR spectra. Only a very weak band, 500–510 cm^{-1}, ~30–40 times less intense than the Tyr 853-cm^{-1} band, was detected.

α-Lactalbumin. α-Lactalbumin (14.2 kilodaltons) is the smaller of the two protein components of the lactose synthetase complex. The similarity of its amino acid sequence to that of egg white lysozyme suggests a similar three-dimensional architecture (Hill & Brew, 1975). α-Lactalbumin has two Ca^{2+} binding sites, and lowering the pH to 4 induces a change from the native (N) conformation to another one (U), in which one of the calcium ions is lost (Kronman et al., 1981). the bovine protein contains four each of Trp (at positions 26, 60, 104, and 118), Tyr (positions 18, 36, 50, and 103) and Phe (positions 9, 31, 58, and 80). In the N conformation the Trp are largely inaccessible to solvent, as determined from fluorescence measurements (Sommers & Kronman, 1980). The U conformation apparently involves a significant unfolding, resulting in a looser structure with partial exposure of the Trp residues (Sommers & Kronman, 1980). Raman spectra with visible excitation have been examined for highly concentrated (100 mg/mL) solutions at different pHs (Yu, 1974). There were changes in the peptide vibrational frequencies, particularly in the amide III region, which were interpreted in terms of backbone unfolding. A Trp band at 1364 cm^{-1} was observed to decrease in intensity in the U conformation.

Figure 5 shows RR spectra excited at 218 nm for α-lactalbumin at pH 6.6 and 2.1 at a concentration of only 0.04 mM. (We remark that similar sensitivity can be obtained for insulin; the higher concentrations used in this study were selected for comparison with the previous spectroscopic work.) At this wavelength Trp scattering is very strong and dominates the spectrum (Rava & Spiro, 1984, 1985). As indicated in the figure, all of the bands are due to tryptophan except the

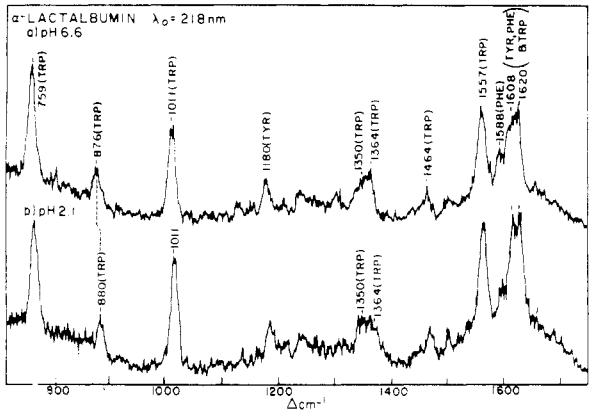

FIGURE 5: Resonance Raman spectra of 40 μM α-lactalbumin with 218-nm excitation. (a) At pH 6.6, protein exists in the N conformer; (b) at pH 2.1, protein exists in the U conformer.

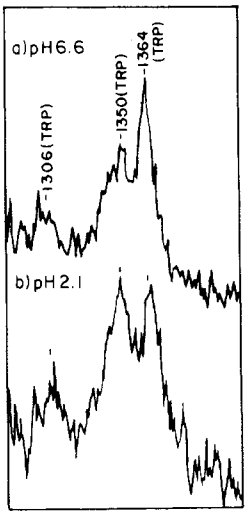

FIGURE 6: Higher resolution trace of the 1350-cm^{-1} region of α-lactalbumin using the conditions of Figure 5.

1183-cm^{-1} Tyr mode and the complex of bands at ~1615 cm^{-1}, which are due to a combination of Trp, Tyr, and Phe. The 1364-cm^{-1} band, observed in the visible Raman spectra, is seen to be a pair of bands in the RR spectra. This is shown clearly in Figure 6 which is an amplification of the 1350-cm^{-1} region of the spectrum. The decrease in the 1364-cm^{-1} band at pH 2.1, attributed to exposure of Trp to solvent, is actually accompanied by an increase in the intensity of the 1350-cm^{-1} band. Another point of interest is the upshift in the 876-cm^{-1} Trp band to 880 cm^{-1} at pH 2.1. This band has been assigned to a deformation mode of the five-membered ring, with some bending of the N–H bond (Hirakawa et al., 1978). Its upshift upon exposure of the Trp is attributable to an increase in the force constant due to H bonding. In Raman spectra with visible excitation for other proteins this band has been reported to decrease if intensity upon Trp exposure (Kitagawa et al., 1979; Itoh et al., 1982). However, with visible excitation the 880-cm^{-1} band appears as a shoulder on the 853-cm^{-1} Tyr band, and the shift in frequency at low pH might have produced an apparent loss of intensity.

Figure 7 compares RR spectra at 218 and 200 nm for α-lactalbumin in the N state. At 200 nm the spectrum is completely different than at 218 nm. The Trp modes are gone, and the spectrum is dominated by tyrosine, as indicated in the figure. As in the case of insulin, a Phe mode is seen at 1000 cm^{-1}, and amide contributions are apparent, with a prominent amide II mode at 1556 cm^{-1}; a broad amide I band is centered at 1663 cm^{-1}. Figure 8 compares 200-nm spectra for the N

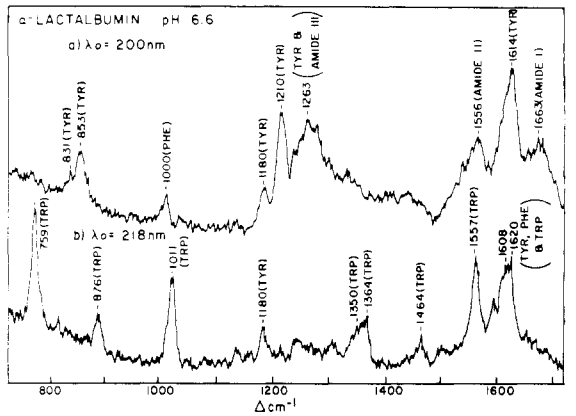

FIGURE 7: Comparison of the resonance Raman spectra of the N conformer of α-lactalbumin obtained by using 200- and 218-nm excitation.

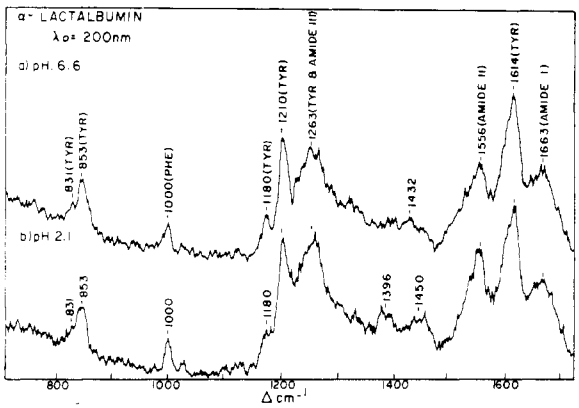

FIGURE 8: Resonance Raman spectra of α-lactalbumin (40 μM) with 200-nm excitation using the conditions of Figure 5.

and U states. The partial unfolding of the protein seems to reduce the intensity of the tyrosine bands relative to the amide bands, an effect similar to that seen for insulin monomers with exposed tyrosine. For α-lactalbumin the tyrosine doublet at 853/831 cm^{-1} also shows evidence for tyrosine exposure, the 831-cm^{-1} shoulder becoming slightly more intense at low pH.

Bands are also observed in the pH 2.1 spectrum at 1396 and 1450 cm^{-1} in the U state. We have observed similar bands for poly(L-lysine) at pH 12 and tentatively assigned them to C–H bending modes (Yu et al., 1973). In the pH 6.6 system of α-lactalbumin, these bands appear to coalesce into a single band at 1342 cm^{-1}, perhaps reflecting the change in backbone conformation.

CONCLUSIONS

In this study we have shown that it is possible to obtain good quality UV RR spectra for proteins in dilute (tens of μM) solutions. At 200 nm, the spectra are dominated by tyrosine modes, although peptide bands are also apparent. The amide II mode is seen strongly, in contrast to Raman spectra with visible excitation, and the amide I mode is broad and maximizes at a frequency lower than the amide I band seen with visible excitation. This is attributed to resonance enhancement of the infrared components of amide I. At 218 nm, tyrosine modes can also be observed, as can modes of phenylalanine, but if tryptophan is present, its bands dominate the spectrum.

Two Trp bands are sensitive to solvent exposure, at 876 and ~1360 cm^{-1}. The 876-cm^{-1} band shifts up a few wavenumbers on solvent exposure; this is attributable to H bonding of the Trp N-H. In nonresonance Raman spectra this upshift appears as an intensity loss, due to interference from the nearby 858-cm^{-1} Tyr band. The ~1360-cm^{-1} band, which has been

reported with visible excitation to decrease upon Trp exposure, is actually a doublet; the 1365-cm^{-1} component loses intensity, while the 1355-cm^{-1} component gains intensity when tryptophan is exposed to solvent. Exposure of tyrosine to solvent appears to produce a general lowering of the tyrosine band intensities relative to phenylalanine, or the amide bands. The low-energy component of the ~850-cm^{-1} doublet is expected to increase with tyrosine H bonding, but this effect is more difficult to discern in the UV RR spectra, because the high-energy component is much more strongly enhanced.

Registry No. Trp, 73-22-3; Tyr, 60-18-4; Phe, 63-91-2; insulin, 9004-10-8.

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