Therefore, we presently favor a direct interaction between the isobutyl side chain in MeLeu^6 with the receptor to explain the loss of biological activity reported for (MeAla)^{6}CsA. Similarly, an independent interaction between the butenyl side chain in MeBmt with the receptor may account for the dramatically reduced immunosuppressive activity of (MeLeu(3-OH))^{1}CsA.

The use of the conformational search and energy minimization strategy we have described above has provided insight into the effects of the primary structure of this peptide on its conformations and biological activity. The proposed bioactive conformation serves as a standard against which to evaluate the potential biological activity of new CsA analogues. Further studies to compare the conformational preferences of 1-position CsA analogues with the immunosuppressive activities are in progress.

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Registry No. CsA, 59865-13-3; (MeBm$_2$t)^{1}CsA, 114865-22-4; ((4*S*)-MeBmt)^{1}CsA, 122090-69-1; (MeBth)^{1}CsA, 114891-20-2; MeBmt, 59865-23-5.

Supplementary Material Available: Tables of energetic components of the total strain energy of CsA, (MeBm$_2$t)^{1}CsA, ((4*S*)-MeBmt)^{1}CsA, and (MeBth)^{1}CsA, figures of the superposition of unique conformations of those given above, and listings of coordinate files of MeBmt (conformers A and B), MeBth (conformer B), MeBm$_2$t (conformer B), and (4*S*)-MeBmt (conformer F) (34 pages). Ordering information is given on any current masthead page.

Surface-Enhanced Raman Spectroscopic Investigation of Human Immunoglobulin G Adsorbed on a Silver Electrode

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Abstract: SERS spectra of human IgG adsorbed on a silver electrode from solution concentrations of 1.0 μM IgG in pH 7.2 phosphate buffer are presented and spectral assignments made. Laser power of 50 mW was sufficient to obtain high-quality spectra. Overall enhancement was in the range of 10^4–10^5. The spectrum consisted only of vibrations from amino acid residues with functional groups that would complex with silver, including tyrosine, tryptophan, cystine, and acidic groups. No amide backbone stretches were observed. From the relative intensities of the vibrations, average conformations of the adsorbed amino acid groups were proposed. The selective enhancement, afforded to adsorbed residues, allowed monitoring of protein conformational changes at low coverage in high buffer ionic strength.

Recently, several groups have used surface-enhanced Raman spectroscopy (SERS) and surface-enhanced resonance Raman spectroscopy (SERRS) to characterize biological molecules. Koglin and Sēquaris conducted in-depth investigations of nucleic acids and DNA with silver colloids ref 1 and references therein. Several amino acids have been also studied by using silver colloids to obtain SERS.[2-6] The aromatic amino acids, tyrosine (Tyr), phenylalanine (Phe), tryptophan (Trp), and histidine (His), exhibited intense spectra[2-4] but displayed great variations in relative peak intensities and frequencies from study to study. Methods of preparation and age of the colloid, as well as amino acid concentration, greatly affected the SERS spectra. Some of these experiments used the spectrum to predict the orientation of the amino acids on the colloidal silver.[2,5,6] The researchers discovered that all of the amino acids bound to the silver through their carboxyl groups except for cystine, which was attached through a strong silver–sulfur bond.[6] It would be expected that SERS spectra for proteins would differ considerably from those of amino acids since the carboxyl groups would be incorporated in the amide bonds of the skeletal structure of the protein and become inaccessible for binding to the silver. Cotton and Van Duyne examined metal-containing biomolecules including myoglobin, cytochrome *c*,[7] and chlorophyll pigments[8] adsorbed on silver electrodes using SERRS. This technique yielded high-quality spectra, but the resonance enhancement only probed the region of the molecule surrounding the metal center. Again, nonresonance spectra would be expected to emphasize very different features.

A few attempts have been made to generate SERS spectra of amino acids[9] and the proteins bovine serum albumin and lysozyme[10] at electrodes, and the resulting spectra were rather weak and featureless. The SERS electrode spectra of leucine–isoleucine–valine binding and leucine-specific proteins have also been observed.[11] The goal of the present work was extension of SERS to the study of large proteins. In this paper, the spectrum of human immunoglobulin G (IgG) is reported and vibrational assignments made. Based on these results, adsorptive behavior is described as a function of electrode potential and ionic strength.

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(1) Koglin, E.; Sēquaris, J.-M. L. Analytical Problems. *Top. Curr. Chem.* **1986**, *134*, 1.
(2) Kim, S. K.; Kim, M. S.; Suh, S. W. *J. Raman Spectrosc.* **1987**, *18*, 171.
(3) Nabiev, I. R.; Savchenko, V. A. *J. Raman Spectrosc.* **1983**, *14*, 375.
(4) Picquart, M.; Lacrampe, G.; Jaffrain, M. *Spectroscopy of Biological Molecules, First European Conference on the Spectroscopy of Biological Molecules*; John Wiley and Sons: New York, 1985; p 190.
(5) Suh, J. S.; Moskovits, M. *J. Am. Chem. Soc.* **1986**, *108*, 4711.
(6) Curley, D.; Siiman, O. *Langmuir* **1988**, *4*, 1021.
(7) Cotton, T. M.; Schultz, S. G.; Van Duyne, R. P. *J. Am. Chem. Soc.* **1980**, *102*, 7960.
(8) Cotton, T. M.; Van Duyne, R. P. *FEBS Lett.* **1982**, *147*, 81.
(9) McMahon, J. J. Ph.D. Dissertation, University of Michigan, Ann Arbor, MI, 1982.
(10) Kisters, B. Ph.D. Dissertation, University of Köln and KFA Jülich, Germany, 1986.
(11) Nabiev, I. R.; Trakhanov, S. D.; Efremov, E. S.; Marinyuk, V. V.; Lazorenko-Manevich, R. M. *Bioorg. Khim.* **1981**, *7*, 941.

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IgG was chosen for these studies because it is known to adsorb on metal electrodes through disulfide bonds,[12,13] so that a SERS spectrum should be (and is) attainable. The primary structure of IgG has been determined[14-17] and the binding sites between IgG and its antigen have been investigated thoroughly by X-ray crystallography.[18-27] Three-dimensional models of IgG have been produced from the results of the crystallographic studies. These models depicted which amino acid residues were directly exposed to the solution and which were important in binding haptens and antigens. With this information, it is possible to assign Raman shifts in terms of molecular binding to the electrode and orientational changes with potential and ionic strength.

As is the case with resonance Raman spectra, SERS spectra of adsorbed macromolecules should not closely resemble normal Raman spectra, since only the residues near the electrode surface will be enhanced. Cotton et al. recently used SERRS to measure the distance from the electrode over which surface and resonance enhancements extend, and it was found to be approximately 16 nm.[28] Since resonance and surface enhancements are multiplicative and nearly equal in magnitude,[7] the conclusion could be reached that surface enhancement alone would extend over roughly 8 nm, assuming exponential decay of the enhancement with distance. This would mean that only amino acids bound directly to the surface of the electrode or within a space no greater than five or six residues from the surface would be enhanced.[29] Such selectivity makes SERS an extremely sensitive tool for studying changes in conformation, provided the part of the molecule involved in the rearrangement is also that which binds to the electrode.

Experimental Section

Solutions. All spectral solutions contained 1.0 μM human IgG, essentially salt free, used without further purification, (Sigma Chemical Co.) in pH 7.2 phosphate buffer (Fischer) of either 0.25, 0.5, or 1.0 M ionic strength. All solutions were prepared with water deionized with a Barnstead Nanopure system and sterilized with ultraviolet illumination from a Barnstead Organicpure. All supporting electrolytes mentioned above were reagent grade and used without further purification. Potassium iodide, (Fischer) was added to the buffer solutions at 0.01 M concentration to collisionally quench the native fluorescence of the proteins. Concentrations of KI up to 0.2 M have been demonstrated to quench native fluorescence without altering protein structure.[30] The addition of KI limited the potential window for study to potentials more negative than −0.4 V, above which adsorption of I^- became too great and the SERS signal was lost.

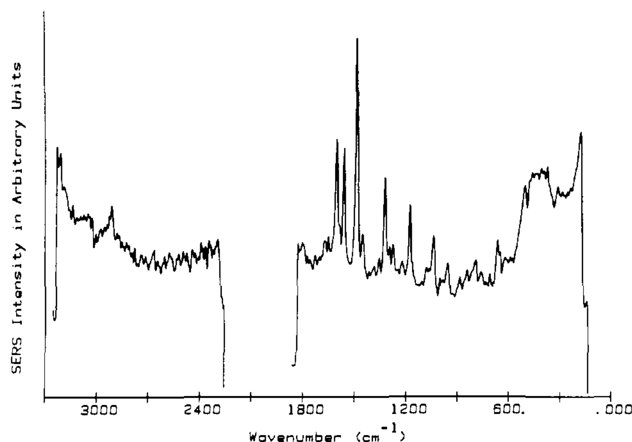

Figure 1. SERS spectrum of 1.0 μM IgG in pH 7.2, 0.25 M phosphate buffer solution. Conditions: 514.5-nm excitation, 40-mW power, electrode rotated at 1600 rpm, −0.5-V electrode potential, 128 scans of 1.00-s exposure time, gain of 5.0 on intensifier, entrance slit open 2 × 10 mm, filter slit, 6 mm, spectrometer slit, 200 μm.

Table I. Raman Bands and Assignments for 1.0 μM IgG (in 0.25 M Phosphate Buffer, pH 7.2, −0.5 V Electrode Potential)

shift,[a] cm^{-1}	intensity[b]	assignment[c]
3066	vw	ν(C—H) aromatic
2914	vw	ν(C—H) aliphatic
1728	vw	Asp, ν(C=O)
1695	vw	Asp, ν(C=O)
1673	vw	Glu, ν(C=O)
1605	s	Trp, tyr, ν(ring)
1585	w	Asp, glu, ν_a(C=O)
1562	s	Trp, tyr, ν(ring)
1487	vs	Trp, tyr, ν(ring)
1455	w	Trp or $\delta(CH_2)$
1418	vw	Asp, glu, ν_a(C=O)
1385	vw	Tyr, ν(ring)
1355	vw	Trp, ν(ring)
1326	m–s	Trp, tyr, ν(ring)
1294	w	Tyr, ν(ring)
1277	w	Tyr, δ(ring)
1225	w,br	Trp, tyr, δ(ring)
1182	m	Tyr, δ(ring)
1085	w,br	Trp, tyr, ν(ring)
1042	m	Trp, ν(ring)
978	w	Trp, tyr, δ(ring)
882	w	Trp, ν(ring)
843/815	w	Tyr, ν(ring)
792	w	Trp, ν(ring)
764	vw	Tyr, δ(ring)
707	vw	Trp?
667	w–m	Cys, ν(C—S)
622	vw	Tyr, δ(ring)
509	m	Cys, ν(S—S)

[a] Relative to the 514.5-nm argon ion excitation line. [b] vw, very weak; w, weak; m, medium; s, strong; vs, very strong, br, broad. [c] ν, stretch; δ, deformation; Asp, aspartic acid; Glu, glutamic acid; Trp, tryptophan; Tyr, tyrosine; Cys, cystine.

Spectral Parameters. The three-electrode spectroelectrochemical cell used in all SERS experiments was built according to the design of Pemberton.[31] For all spectra, the working electrode was rotated at 1600 rpm, and incident laser powers of 40 mW were used to minimize thermal damage and photodamage. Spectra were collected from a backscattering configuration. An argon ion laser (Spectra Physics Model 164) was used to excite SERS scattering with a wavelength of 514.5 nm. Spectra were obtained with a TN-1710 diode array spectrometer (Tracor Northern) with a Triplemate triple monochromator (Spex).

The electrode was roughened by an ex situ anodization procedure in 0.1 M KCl (Fischer). The working electrode was cleaned and polished before oxidation with slurries of successively finer grades of alumina (Buehler) from 1.0 to 0.05 μm. Alumina was removed from the surface of the electrode by rinsing and sonicating in deionized water. The po-

(12) Fontaine, M.; Rivat, C.; Ropartz, C.; Caullet, C. *Bull. Soc. Chem. Fr.* **1973**, *6*, 1873.
(13) Rivat, C.; Fontaine, M.; Ropartz, C.; Caullet, C. *Eur. J. Immunol.* **1979**, *38*, 697.
(14) Edelman, G. M.; Cunningham, B. A.; Gall, W. E.; Gottlieb, P. D.; Rutishauser, U.; Waxdal, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1969**, *63*, 78.
(15) Edelman, G. M. *Science* **1973**, *180*, 830.
(16) Chen, B. L.; Poljak, R. J. *Biochemistry* **1974**, *13*, 1295.
(17) Poljak, R. J.; Nakashima, Y.; Chen, B. L.; Konigsberg, W. *Biochemistry* **1977**, *16*, 3412.
(18) Poljak, R. J.; Amzel, L. M.; Avey, H. P.; Chen, B. L.; Phizackerley, R. P.; Saul, F. *Proc. Natl. Acad. Sci. U.S.A.* **1973**, *70*, 3305.
(19) Amzel, L. M.; Poljak, R. J.; Saul, F.; Varga, J. M.; Richards, F. F. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 1427.
(20) Davies, D. R.; Padlan, E. A.; Segal, D. M. *Annu. Rev. Biochem.* **1975**, *44*, 639.
(21) Poljak, R. J.; Amzel, L. M.; Phizackerley, R. P. *Prog. Biophys. Mol. Biol.* **1976**, *31*, 67.
(22) Poljak, R. J.; Amzel, L. M.; Chen, B. L.; Chiu, Y. Y.; Phizackerley, R. P.; Saul, F.; Ysern, X. *Cold Spring Harbor Symp. Quant. Biol.* **1977**, *41*, 639.
(23) Deisenhofer, J. *Biochemistry* **1981**, *20*, 2361.
(24) Amit, A. G.; Mariuzza, R. A.; Phillips, S. E. V.; Poljak, R. J. *Nature* **1985**, *313*, 156.
(25) Amit, A. G.; Mariuzza, R. A.; Phillips, S. E. V.; Poljak, R. J. *Science* **1986**, *233*, 747.
(26) Mariuzza, R. A.; Phillips, S. E. V.; Poljak, R. J. *Annu. Rev. Biophys. Biophys. Chem.* **1987**, *16*, 139.
(27) Alzari, P. M.; Lascombe, M.-B.; Poljak, R. J. *Annu. Rev. Immunol.* **1988**, *6*, 555.
(28) Cotton, T. M.; Uphaus, R. A.; Möbius, D. *J. Phys. Chem.* **1986**, *90*, 6071.
(29) Lehninger, A. L. *Biochemistry*, Worth Publishers: New York, 1975; p 74.
(30) Lehrer, S. S. *Biochem. Biophys. Res. Commun.* **1967**, *29*, 767.
(31) Pemberton, J. E.; Buck, R. P. *Appl. Spectrosc.* **1981**, *35*, 571.

tentials were generated by a Model 175 Universal Programmer (EG& G-PARC), and a combination potentiostat/galvanostat (Model 363 EG&G-PARC) was used to control the potential between the electrodes. The potential was repeatedly stepped from −0.3 to +0.25 V and back with a 2-s hold at the anodic limit for 25 cycles. To increase reduction and the formation of silver microstructures, the silver electrode was illuminated during the ORC with a defocused laser beam of 100-mW power at 514.5 nm. The electrode was then rinsed with deionized water, immersed in the spectral solution, and held at the desired potential. These surfaces consistently gave stabile, high-intensity SERS spectra.

Spectra were recorded at −0.5 and −0.8 V (vs SCE) for each solution to observe the effect of reduction of the disulfide bonds. The former potential corresponds to the reduction peak for IgG[32] and the latter to the pzc of the silver electrode, where hydrophobic molecules should have maximum adsorption.

Results

The SERS spectrum for 1.0 μM IgG in 0.25 M phosphate buffer, pH 7.2, is shown in Figure 1, and major bands are listed with assignments in Table I. The assignments for the Raman shifts were made by comparison with normal Raman studies of amino acids and proteins[33–42] and with SERS studies of amino acids.[2,3,6]

When any SERS spectrum of IgG was compared to normal Raman spectra of IgG and other proteins, several obvious differences were noted. First, from an experimental viewpoint, the SERS spectra had a much larger S/N despite the fact that solutions were $^1/_{1000}$ times less concentrated and less than one-tenth the laser power of the normal Raman studies was used in obtaining the spectra.[42] The increase in sensitivity was on the order of 10^4–10^5. The obvious advantages in this enhancement included the ability to record spectra with lower laser power, lessening thermal damage to the sample, and the option of using concentrations much closer to those found in biological fluids, diminishing the possibility of molecular interactions at the higher concentrations. Additionally, no Raman active stretches due to water were evident in the SERS spectra. These vibrations can often mask the protein vibrations in the region from 1600 to 1700 cm^{-1}, so that the use of D_2O is required to observe the amide and ring stretches.[38,41] The ability to monitor this frequency range without any additional procedures greatly simplified the experiments.

The normal Raman and SERS spectra also differed greatly in regard to the peaks observed and their relative intensities.[41,42] The most obvious difference was a lack of amide stretching bands near 1670 and 1240 cm^{-1} in the SERS spectra. These bands, usually among the most dominant bands in a protein spectrum, are due to the amide backbone of the protein and contain information about the secondary structure of the protein.[35,39] The frequency of the vibrations depends on whether the bond is part of an α helix, a β pleated sheet, or a random coil. Simulations have been developed that predict the structure of a protein based on the relative intensities and positions of these stretches.[43] The absence of these bands indicated that only the side-chain groups were being enhanced. It was unlikely that all of the peptide backbone was oriented parallel to the electrode surface or was too far away from the surface to be enhanced. However, the exclusive enhancement of the side groups may be the consequence of specific adsorption

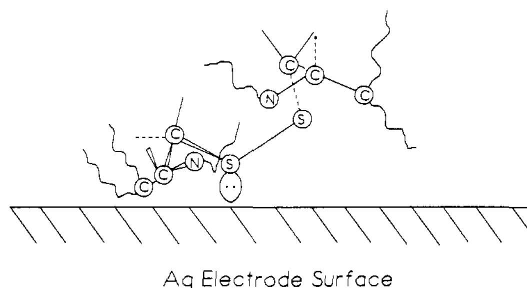

Figure 2. Probable orientation of cystine in IgG on the silver electrode surface at −0.5 V in 0.25 M phosphate buffer, pH 7.2. S, sulfur; C, carbon; N, nitrogen.

of these side groups on the silver electrode, generating a charge-transfer type of enhancement. If no such adsorption occurred between the molecules forming the amide bonds of the backbone and the silver electrode, no enhancement of this type would result, even if the backbone was in close proximity to the electrode. The result was a much simpler spectrum, representative of only the amino acid residues near the surface of the electrode.

Discussion

Assignment of Raman Bands. Proteins would be expected to bind to the electrode through those amino acids that form complexes with silver. These include sulfur-containing cystine, nitrogen-containing histidine and tryptophan, and the basic amino acids lysine and arginine at high pH, above their pK_a's. The acids, aspartic acid and glutamic acid, would also be expected to adsorb when deprotonated. At the pH used in these experiments, 7.2, the deprotonated acids would be expected to bind to the electrode, but not the bases.[29] The results in Table I confirmed this hypothesis. All major vibrations could be assigned to tryptophan, tyrosine, or cystine residues, with minor stretches originating from the acidic groups. Any differences in relative intensities of the Raman signals for the amino acids do not necessarily reflect differences in the numbers of each type of residue adsorbed, since aromatic groups display much more intense Raman scattering than aliphatic groups.[35] However, the acidic groups would not be expected to adsorb strongly, due to the repulsion from the negatively charged iodide anions, which coadsorbed on the silver. A weaker bond would result in less charge-transfer type of enhancement, which would further decrease the signal from these groups. Indeed, only the most intense bands from the acid residues are visible: the C=O stretches.

When the IgG SERS spectrum (Figure 1) was compared with a composite of normal Raman spectra of the amino acids tryptophan, tyrosine, phenylalanine, histidine, and cystine, described by Lord and Yu,[35] several significant differences in frequencies and relative intensities of the bands were noted. These differences might have resulted from (1) changes in bond strength as the residue was adsorbed on the electrode and (2) the molecular orientation of the molecule on the surface, respectively. Electromagnetic enhancement theory explains the dependence of intensity on molecular orientation by describing how the electric field at the surface of the electrode couples with the polarizability tensors.[44] Bonds that have polarizability tensors perpendicular to the electrode surface would be enhanced, while those parallel to the surface would not.[45] Those vibrations with the largest enhancements should emanate from molecular movements causing sizable variations in polarizability normal to the electrode surface. If this was the case, then orientations of the amino acids on the electrode surface could be proposed. As mentioned earlier, SERS spectra of these amino acids have been reported,[2,5,6] but all of these studies stated that the amino acids adsorbed to the silver through the COO^- or the NH_2 groups, which were used in the amide linkage of the protein backbone. Therefore, the orientations and

(32) Grabbe, E. S.; Buck, R. P.; Melroy, O. R. *J. Electroanal. Chem.* **1987**, *223*, 67.
(33) Simons, L.; Bergstrom, G.; Blomfelt, G.; Forss, S.; Stenback, H.; Wansen, G. *Commentat. Phys.-Math.* **1972**, *42*, 125.
(34) Hirakawa, A. Y.; Nishimura, Y.; Matsumoto, T.; Nakanishi, M.; Tsuboi, M. *J. Raman Spectrosc.* **1978**, *7*, 282.
(35) Lord, R. C.; Yu, N.-T. *J. Mol. Biol.* **1970**, *50*, 509.
(36) Lagant, P.; Vergoten, G.; Loucheux, C.; Fleury, G. *Polymer J.* **1979**, *11*, 345.
(37) Fasman, G. D.; Itoh, K.; Liu, C. S.; Lord, R. C. *Biopolymers* **1978**, *17*, 1729.
(38) Koenig, J. L.; Frushour, B. *Biopolymers* **1972**, *11*, 1871.
(39) Yu, N.-T. *CRC Crit. Rev. Biochem.* **1977**, *4*, 229.
(40) Parker, F. S. *Applications of Infrared, Raman, and Resonance Raman Spectroscopy in Biochemistry*; Plenum Press: New York, 1983; Chapter 3, p 83.
(41) Pezolet, M.; Pigeon-Gosselin, M.; Coulombe, L. *Biochim. Biophys. Acta* **1976**, *453*, 502.
(42) Painter, P. C.; Koenig, J. L. *Biopolymers* **1975**, *14*, 457.
(43) Williams, R. W. *Methods Enzymol.* **1986**, *130*, 311.
(44) Birke, R. L.; Lombardi, J. R. *Spectroelectrochemistry: Theory and Practice*; Gale, R. J., Ed., Plenum Press: New York, 1988; p 324.
(45) Van Duyne, R. P. In *Chemical and Biochemical Applications of Lasers*; Moore, C. B., Ed.; Academic Press: New York, 1979; Vol. 4, p 101.

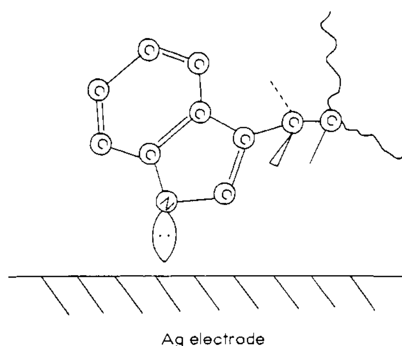

Figure 3. Probable orientation of tryptophan in IgG on the surface of the silver electrode at −0.5 V in 0.25 M phosphate buffer, pH 7.2. C, carbon; N, nitrogen.

binding mechanisms of these amino acids would be expected to vary if they were incorporated in a protein molecule. The differences in relative intensities and shifts in frequency between the spectra reported here and those of the free amino acids reflect these distinctions in adsorption mechanisms.

The strong cystine bands in the SERS spectrum at 509 and 667 cm^{-1} closely resembled those in the composite amino acid spectrum. Consequently, the disulfide bridge was believed to be bound to the surface through only one of the sulfur atoms, thereby positioning both the S–S bond and the C–S bond roughly perpendicular to the silver surface, allowing both bands to be enhanced. The S–S stretching frequency at 509 cm^{-1} suggested a gauche–gauche–gauche configuration about the dihedral angle and the two S–S–C angles. The C–S stretch at 667 cm^{-1} also denoted a gauche arrangement about the S–C–C angles.[39] The splitting of this band implied slightly different angles about these two bonds. This arrangement is illustrated in Figure 2.

Comparison of the SERS spectrum of tryptophan with the normal Raman composite revealed substantial decreases in intensity and shifts to higher wavenumbers in the "ring-breathing" modes, located at 1042 and 792 cm^{-1}, and increases in intensity for the 1605- and 1487-cm^{-1} bands. Smaller reductions in intensity were noted in the 1455- and 1355-cm^{-1} bands. By implementing the normal vibrational modes for the indole ring of tryptophan described in ref 34, some of these variations could be explained. It was postulated that tryptophan adsorbed on the electrode through the nitrogen of the indole ring, probably after replacing the hydrogen. This arrangement would cause the residue to be tilted at an angle to the surface, as diagrammed in Figure 3. In this position, the band at 1605, corresponding to the stretching of the C_4–C_9, C_8–N_1 and C_7–C_8, C_9–C_3 bonds, would have a strong component normal to the surface and would be greatly enhanced. Similarly, the 1562-cm^{-1} band, denoting stretches of the C_8–C_9 band, would lie predominantly parallel to the electrode, losing some of the enhancement effect. The breathing modes involve small stretches of the rings along every bond, radially outward. Consequently, these vibrations would have no strong components of atomic movement normal to the surface, so their stretches would receive very little amplification. The 1455-cm^{-1} band also results from small radially oriented atomic stretches.

Disparities between relative intensities of tyrosine vibrations in SERS and normal Raman spectra could be explained in a similar fashion. Tyrosine was assumed to bind to the surface through the oxygen atom side group, possibly after loss of the hydrogen atom. This end-on conformation is demonstrated in Figure 4. The SERS spectrum of tyrosine showed weak bands at 1225 and 882 cm^{-1} and much stronger bands at 1605, 1562, 1487, and 1182 cm^{-1}. When the normal modes of vibration for a para-disubstituted benzene were analyzed, the vibrations with strong components of atomic motion perpendicular to the surface received the most enhancement.[46] These included the 1487- and

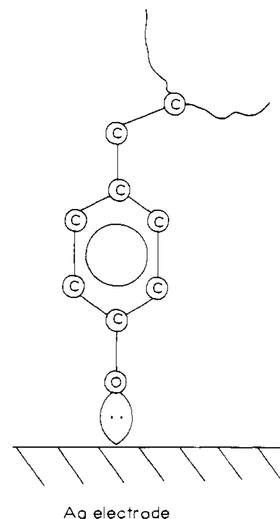

Figure 4. Probable orientation for tyrosine in IgG on a silver electrode at −0.5 V in 0.25 M phosphate buffer, pH 7.2. C, carbon; O, oxygen.

1187-cm^{-1} bands. The "ring-breathing" mode was less intense due to the small atomic movements in all directions. The combined factors of surface enhancement and natural intensity correlate reasonably well with the relative intensities of the peaks in the SERS spectrum for bands such as 1605, 1562, 1487, 1294, 1277, 1182, and 622 cm^{-1}.

Figures 3 and 4 display the tryptophan and tyrosine residues in a deprotonated state when adsorbed. The evidence for this configuration was purely spectral and would, in fact, appear unlikely, since the pH of the buffer was below the pK_a's of these groups.[29] However, lowering of the pK_a of the tyrosine side chain in iron metalloproteins has been observed and used as an indicator of the Fe–O bond strength.[47] In the spectra described in this paper, the ring stretching modes at 1605, 1562, 1487, 1182, and 1042 cm^{-1} appeared to be degenerate. Under some conditions these bands were observed to split, which was interpreted as a lowering of the symmetry of the groups due to adsorption of the residues in a protonated form.[48] Therefore, the amino acids in this study were considered to be deprotonated at the binding sites. The possibility remains that these residues were protonated, but the splitting of the peaks was below the resolution of the monochromator, approximately 5 cm^{-1}. The orientation of the molecules or the strength of the adsorption to the electrode may have resulted in an increase in splitting, beyond the limits of the resolution in the latter study.[48]

Effect of Potential. Spectra were recorded at −0.5 and −0.8 V. As stated in the Experimental Section of this paper, the first voltage corresponded to the reduction peak of the disulfide bridges and the latter to the pzc of the silver electrode. The differences between the spectra obtained at these two potentials were minimal, except at high ionic strengths and, therefore, are not illustrated. The same Raman bands were present in both spectra and with the same relative intensity, with one exception. The disulfide stretch at 509 cm^{-1} was stronger at −0.5 V, which signified some small reduction of bridges as the potential was made less positive. However, the 509-cm^{-1} band was still visible at −0.8 V, indicating that most of the S–S bonds were reduced, but some remained intact. The absolute intensity of the spectra, measured by the difference in signal between the largest peak (1487 cm^{-1}) and the base line, nearly doubled as the electrode potential was switched to −0.8 V. Previous studies using a quartz crystal microbalance (QCM) concluded that there was no increase in the amount of adsorbed IgG over this potential range,[32] so this intensity increase was taken as a feature of SERS charge-transfer enhancement, which has been previously reported.[44] Also in agreement with the QCM results was the lack of any molecular rearrangement,

(46) Dollish, F. R.; Fateley, W. G.; Bentley, F. F. *Characteristic Raman Frequencies of Organic Compounds*; John Wiley and Sons: New York, 1974; p 180.

(47) Nagai, M.; Yoneyama, Y.; Kitagawa, T. *Biochemistry* **1989**, *28*, 2418.

(48) Grabbe, E. S.; Buck, R. P., in preparation.

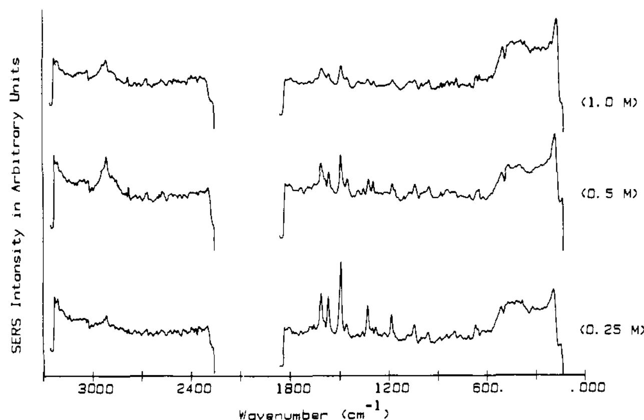

Figure 5. Variation of SERS spectra of 1.0 μM IgG with ionic strength in pH 7.2 phosphate buffer solutions. Ionic strengths of 0.25, 0.5, and 1.0 M. Conditions: 514.5-nm excitation, 40-mW power, electrode rotated at 1600 rpm, −0.5-V electrode potential, 128 scans of 1.00-s exposure time, gain of 5.0 on intensifier, entrance slit open 2 × 10 mm, filter slit, 6 mm, spectrometer slit, 200 μm.

as displayed by the constant relative intensities of the Raman peaks.

Effect of Ionic Strength. As the ionic strength of the buffer was increased to 1.0 M, the overall S/N of the SERS spectra decreased significantly, as shown in Figure 5. Additionally, the spectra began to display a potential dependence. At −0.5 V, the acid residue bands at 1637 and 1585 cm^{-1} increased in relative intensity, as did the tryptophan bands at 1455, 1080, and 1048 cm^{-1}. The cystine groups differed in several respects. The 667- and 508-cm^{-1} bands grew substantially in relative intensity and a peak representing the stretching of the S–H group appeared at 2581 cm^{-1}. Furthermore, the splitting of the 667-cm^{-1} band increased until the 650- and 667-cm^{-1} bands were of equal strength. Many other bands broadened, including 1605, 1385, 1182, and 1080 cm^{-1}, indicating a multiplicity of these states, differing slightly in bond strength. The expanded diversity but low intensity of this spectrum suggested a low surface coverage with more, but weaker, bonding contacts and more heterogeneity in the positions of the aromatic residues relative to the surface. The differences in the cystine residues implied the exposure of additional disulfide bridges, hidden on the interior of the antibody in lower ionic strength solutions. The spectra did not display any features characteristic of adsorbed phosphate,[49,50] signifying that coadsorption of the electrolyte was not the cause of these differences in the spectra.

The concentration of IgG used in this study (1.0 μM) was too dilute for saturation of mercury electrodes,[12,13] but concentrated enough for monolayer coverage of nonpolar polymer surfaces.[51] Consequently, it was not certain whether the SERS spectrum resulted from a completely covered surface. Morrissey had reported conformational changes in IgG bound to surfaces as the coverage increased.[51,52] At low surface concentrations, the IgG adsorbed in a flattened, denatured state, with many points of contact between the protein and the surface. Figure 6 illustrates this alignment. As the concentration reached saturation, the molecules formed a close-packed arrangement. In this configuration, the repulsive electrostatic forces between the molecules caused them to expand into the solution, bonding to the surface through a limited number of residues. The SERS spectra indicated that the consequence of raising the ionic strength was a decrease in surface coverage, typified by a decrease in the S/N ratio. At −0.5 V, positive of the pzc, the surface concentration became low enough to cause an apparent restructuring of the surface. As stated in the previous section, adsorption was maximized at the pzc, with electrostatic binding occurring between the negatively charged acid residues at more positive potentials. IgG was slightly negatively charged at pH 7.2.[51] The increase in ionic strength would be expected to stabilize the repulsions within the molecules, thereby raising solubility. The result would be a loss in the attraction between the molecules and the electrode, primarily at positive potentials, where the binding was chiefly electrostatic.

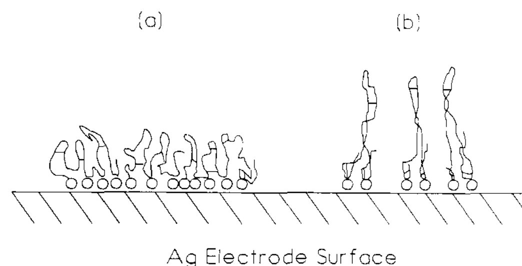

Figure 6. Conformational changes of IgG adsorbed to surfaces as surface concentration increases. (a) flat configuration at low coverage, (b) close-packed structure at high coverage.

Conclusion

The SERS spectra of IgG contained bands of the amino acid residues that were in close contact with the silver electrode, but did not include any amide backbone vibrations or solution bands. Average configurations of the adsorbed residues could be postulated, based on the relative intensities of the vibrations. Tyrosine adsorbed through the O–H of the side group in an end-on configuration. Tryptophan bonded through the nitrogen on the indole ring, which tilted the molecule. Cystine residues were bound through one sulfur of the disulfide bridges, except at high ionic strength, where S–H groups were also present near the surface. Acidic residues of aspartic and glutamic acid were attached through the COO^- group. Adsorption was maximized at the pzc and at lower ionic strengths. SERS proved capable of monitoring changes in conformation with coverage. Examining fluctuations in band width and relative intensity of the Raman shifts provided sufficient evidence to describe the molecular rearrangements. SERS studies following the binding of IgG to anti-human IgG antigens have been completed and the results are forthcoming.

Acknowledgment. We credit the Army Research Office (Durham, NC) for support under Contract DAAL03-86-K-0055.

(49) Ramakrishnan, V.; Aruldhas, G. *Curr. Sci.* **1985**, *54*, 627.
(50) Toupry-Krauzman, N.; Poulet, H.; Le Postollec, M. *J. Raman Spectrosc.* **1979**, *8*, 115.
(51) Morrissey, B. W. *Ann. N.Y. Acad. Sci.* **1977**, *283*, 50.
(52) Morrissey, B. W.; Stromberg, R. R. *J. Colloid Interface Sci.* **1974**, *46*, 152.