Part II: Surface-Enhanced Raman Spectroscopy Investigation of Methionine Containing Heterodipeptides Adsorbed on Colloidal Silver

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Surface-enhanced Raman scattering (SERS) spectra of methionine (Met) containing dipeptides: Met-X and X-Met, where X is: L-glycine (Gly), L-leucine (Leu), L-proline (Pro), and L-phenylalanine (Phe) are reported. Using pre-aggregated Ag colloid we obtained high-quality SERS spectra of these compounds spontaneously adsorbed on colloidal silver. Additionally, we measured Raman spectra (RS) of these heterodipeptides in a solid state as well as in acidic and basic solutions. The RS and SERS spectra of Met-X and X-Met presented in this work appear to be different. One of the most prominent and common features in the SERS spectra of all these dipeptides is a band in the 660–690 cm^{-1} range that is due to the C–S stretching, ν(CS), vibration of Met. This suggests that all the abovementioned compounds adsorb on the silver surface through a thioether atom. On the other hand, the SERS spectra of X-Met show clearly that not only the S atom but also the carboxylate group interact with the colloid surface as manifested by the enhancement of bands in the 920–930 and 1380–1396 cm^{-1} regions. These bands are ascribed to the ν(C–COO^-) and ν_{sym}(COO^-) vibrations, respectively. Additionally, a SERS spectrum of Phe-Met indicates that the interaction of the thioether atom, amine group, and aromatic side chain with the silver surface is favorable and may dictate the orientation and conformation of adsorbed peptide.

Index Headings: **Surface-enhanced Raman scattering; SERS; Silver colloid; Raman spectroscopy; Heterodipeptides.**

INTRODUCTION

Raman spectroscopy (RS) has been employed as a powerful structural and analytical tool for biological molecules and materials.[1–3] It can be utilized *in situ* under physiological conditions, and when resonance Raman (RR) scattering can be applied it is extremely selective and sensitive. However, there are a few problems that can severely limit the applications of Raman spectroscopy, especially to biological science, namely, the small Raman cross-section (often it is not a problem in RR), the possibility of sample damage, or the strong fluorescence background from crude biomedical extracts, tissues, or cells.

Long efforts of several research groups have led to the development of a new Raman technique of adsorbed species that allows Raman signals to be increased by several orders of magnitude in comparison with those of nonadsorbed species.[4–15] This method has been called surface-enhanced Raman scattering (SERS) and has a few other advantages over RS or RR. For example, it enables fluorescence backgrounds to be quenched. It also provides surface selectivity, and thus information is obtained only from molecules on or very near the metal surface.[10–34] In many cases SERS signals can be collected from samples with concentration at the level of 10^{-10} M.

A wide range of experiments designed to probe the structure, topology, and composition of biological species and their model compounds using SERS spectroscopy has been performed. This includes determination of the distribution of drugs within a living cell on the cellular membrane, the selective studies of cell membrane components, and the analyses of crude biomedical mixtures and extracts.[10,11] Moreover, SERS spectra have been obtained from amino acids,[12–15] peptides,[16–19] proteins,[20–22] nucleic acids,[23,24] purines and pirymidines,[1,2,17,25,26] catecholamines,[27,28] porphyrins and chlorophylls,[29–32] flavins,[33,34] and others. However, unfortunately, SERS studies of biological materials have been plagued by poor reproducibility, which severely restricts the quantitative applications.

Recently, we reported that methionine adsorbs on colloidal silver through its thioether atom.[35] Additionally, we provided clear evidence that the carboxyl and amine functional groups are also involved in this adsorption process.[35] Surprisingly, under these conditions **Met-Met** homodipeptide interacts with the silver hydrosol by the thioether atom and *N*-terminal group only;[35] the carboxyl group is not involved in the adsorption. Thus, we have decided to study this issue further. In this study we present a Raman and SERS investigation on eight methionine-containing heterodipeptides with the general formula **Met-X** and **X-Met**, where X stands for L-glycine (Gly), L-leucine (Leu), L-proline (Pro), and L-phenylalanine (Phe). The main goal of this study is to examine the structural changes of these dipeptides that take place upon interaction with slightly positive charged silver hydrosol. We also demonstrate the utility of the SERS technique to selectively probe and characterize such changes. This can be a key point in understanding the recognition of short peptides or proteins by other biological systems.

EXPERIMENTAL

Preparation of Silver Colloid. $AgNO_3$ and $NaBH_4$ were purchased from Wako Chemical Co. (Osaka, Japan) and used without further purification. Solutions of the colloidal silver were prepared according to the standard procedure.[35,36]

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Heterodipeptides. Met-Gly and **Gly-Met**, **Met-Leu** and **Leu-Met**, and **Met-Pro** were purchased from Wako Chemical Co. (Osaka, Japan). **Pro-Met**, **Met-Phe**, and **Phe-Met** were purchased from Bachem Bioscience Inc. (Geneva, Switzerland). All samples were used without further purification. Sample solutions were prepared by dissolving the dipeptides into doubly distilled water. The concentrations of the samples before mixing with the colloid system were set at 10^{-3} M. The final sample concentrations in the silver colloid were $\sim 10^{-4}$ M.

Instrumentation. Raman and SERS spectra were obtained with a triple grating spectrometer (JASCO, NRS-2100 Raman Spectrometer). The spectral resolution of 2 and 4 cm^{-1} was set for the solid and solution samples, respectively. A liquid nitrogen cooled charge-coupled device (CCD) detector (Princeton Instruments, Model LN-CCD1100) was used in these measurements. The 514.5 nm line of an argon ion laser (Spectra-Physics, Model 2016) was used as the excitation source for the Raman and SERS measurements. The output of the laser power was set at 100 mW.

RESULTS AND DISCUSSION

The SERS spectra of amino acids and peptides have been studied quite extensively during the past two decades.[12–19] However, only a few studies have been done on short peptides that contain a sulfur atom or a disulfide bridge in their structures.[35,37] From among about twenty amino acid residues, methionine, cysteine, and cystine contain a sulfur atom (S) that gives stretching vibrations of the C–S or S–S group. These vibrations are very useful in the Raman studies of proteins because they yield strong Raman bands in the region from 500 to 550 cm^{-1} (S–S) and in the region from 630 to 720 cm^{-1} (C–S).[38] Their frequencies are very characteristic for polypeptide and protein conformations in the vicinity of the disulfide bridge(s) and the C–S bonds.[39,40] Met does not form a S–S bridge and thus, we concentrate on its characteristic ν(CS) vibrations, which have been well investigated using many model compounds with the X–CH_2–CH_2–S– group, including series of monosulfides and thiols.[37,41–43] There are several notations to describe the relationship between internal rotation about the –CH_2–CH_2– and –CH_2–S– bonds. The simplest but still often used has been proposed by Shimanouchi and co-workers[44] and Miyazawa and co-workers,[43,45] and thus we have adopted it in this work. This notation includes P_C-T, P_C-G, P_H-T, and P_H-G, where P_C and P_H refer to the two possible conformations of the –CH_2–CH_2–S– group with the carbon and hydrogen atoms at the trans position with respect to the sulfur atom, respectively, while T and G stand for *trans* and *gauche* internal rotation about the –$C(H_2)$–S– bond. The ν(CS) frequency of the –$C(H_2)$–S– group is expected in the 640–680 cm^{-1} range for the P_H and between 740–760 cm^{-1} for the P_C conformers. On the other hand, the ν(CS) of the –S–CH_3 group appears usually between 700–725 cm^{-1}.[37]

It must be mentioned that very recently up to seven optimized conformers of Met have been studied by quantum-chemical calculations.[46] These conformers correspond to calculated local energy minima on the potential energy surface and are characterized by three dihedral angles. However, we are not going to discuss this case in detail, and we do not adopt the suggested notation since the calculated rotamers have only been predicted for isolated Met molecules. In the solid state and in the solutions, Met molecules aggregate due to hydrogen bonding, and thus their structures are different from those predicted for isolated conformers.

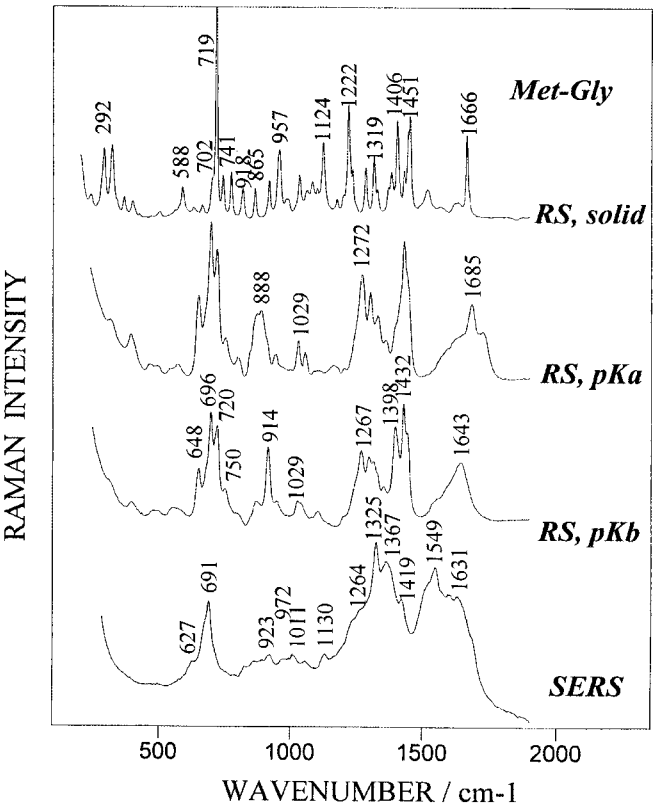

FIG. 1. Raman spectra of **Met-Gly** in a solid state and aqueous solutions at pK_a and pK_b and a SERS spectrum of **Met-Gly** adsorbed on colloidal silver in an aqueous solution of pH 8.3. Measurement conditions: sample concentrations in the silver colloid, 10^{-4} M; excitation line, 514.5 nm; power at the laser output, 100 mW.

In Figs. 1 to 8 we present the results of our SERS study on the abovementioned methionine-containing heterodipeptides, **Met-X** and **X-Met**, adsorbed on the silver colloidal particles. In order to make assignments for the SERS spectra we also measured Raman spectra of all the dipeptides in the solid state as well as in aqueous acidic pK_a (cation $^+H_3NRCOOH$) and basic pK_b (anion NH_2RCOO^-) solutions. Unfortunately, most of the studied dipeptide zwitterions precipitated out from the solution, and thus, their Raman spectra could not be recorded.

Due to the complexity of the heterodipeptide Raman and SERS spectra, it is rather difficult to make firm vibrational assignments. Thus, only assignments of the key vibrations are given below, based on earlier studies.[16,19,47–51]

Met-Gly and Gly-Met. Figures 1 and 2 show Raman and SERS spectra of **Met-Gly** and **Gly-Met**, respectively. Detailed band assignments for the Raman and SERS spectra of both amino acids were reported in our previous work.[35] Band assignments for the Raman and SERS spectra of **Met-Gly** and **Gly-Met** are made based on those for Met[49,52] and Gly.[13,53] We also include amide vibrations that are expected at around 1650 cm^{-1} (amide I), 1550

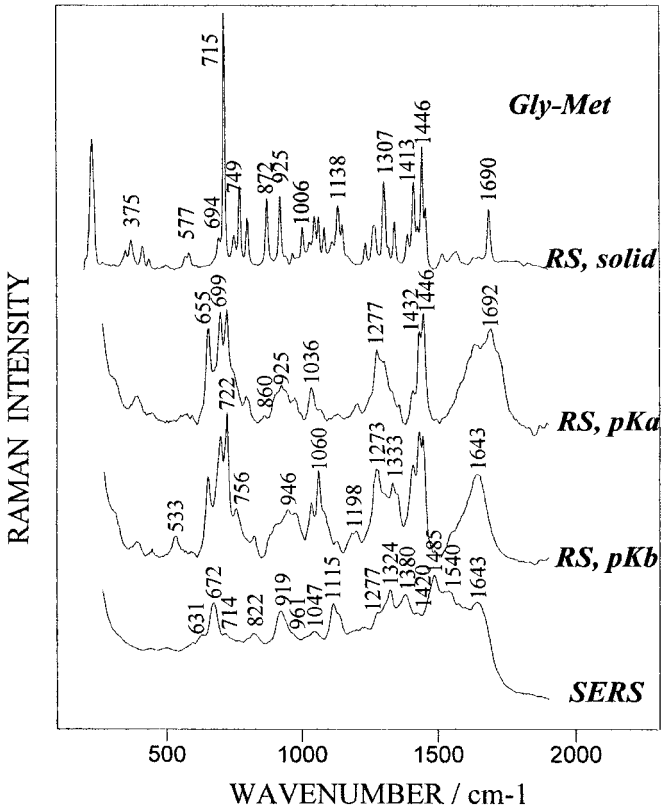

FIG. 2. Raman spectra of **Gly-Met** in a solid state and aqueous solutions at pK_a and pK_b and a SERS spectrum of **Gly-Met** adsorbed on colloidal silver. Measurement conditions are the same as those in Fig. 1.

cm^{-1} (amide II, usually not Raman active), 1280 cm^{-1} (amide III), 710 cm^{-1} (amide V), 650 cm^{-1} (amide IV), and 550 cm^{-1} (amide VI).

The most intense bands in the Raman spectra of the solid **Met-Gly** and **Gly-Met** (the top spectra in Figs. 1 and 2) appear at 719 and 715 cm^{-1}, respectively. These bands are obviously assigned to the C–S stretching mode of the P_C-T conformers. Additionally, a shoulder on the 719 cm^{-1} at 702 cm^{-1} and a weak band at 741 cm^{-1} are observed in the **Met-Gly** spectrum. They are due to the additional ν(CS) vibrations of different P_C-G conformers. The corresponding bands due to the P_C-G conformers are observed at 694 and 749 cm^{-1} in the Raman spectrum of **Gly-Met**. The Raman spectra taken from the acidic and basic aqueous solutions of both dipeptides yield four bands due to the C–S stretching vibrations. These bands at 648, 696, 720, and 750 cm^{-1} are assigned to the P_H-T, P_C-G, P_C-T, and P_C-G conformers, respectively. The 750 cm^{-1} band may not be due to the ν(CS) vibration but may also be assigned to the CH_2 rocking vibrations, as observed in Raman spectra of monosulfate model compounds.[54] It is noted that the above mentioned spectra in the C–S stretching region (Figs. 1 and 2) closely resemble previously reported spectra of Met and its homodimer.[35] It seems that bands at 696 and 720 cm^{-1} are due to vibrations of the ν(CS) of the $–S–CH_3$ bond. Thus, this result clearly shows that the $–S–CH_3$ Met fragment exists in two different rotamers in solution, in contrast to the solid-state case. It should be noted that no frequency changes were observed on raising the pH of the solution. Similar behavior is observed for **Gly-Met**, where the ν(CS) vibrations are observed at 655, 699, 722, and 756 cm^{-1}, i.e., they are up-shifted by a few wavenumbers. The assignment of these vibrations to the respective conformers is the same as above. Therefore, we conclude that in the solid state the Met residues of both **Met-Gly** and **Gly-Met** exist in the P_C-T form, but in aqueous solution three or four conformers appear.

As mentioned above, most of the bands in the studied heterodipeptides can be assigned based on the spectra of their amino acids.[13,49,52,53] However, in the solution spectra two broad Raman bands (probably due to the amide I and amide III modes) appear around 1643 and 1270 cm^{-1}, respectively. In the acidic condition the amide I band overlaps with the H–O–H bending of water and the C=O stretching of the carboxylate group at 1685 cm^{-1}. The amide III vibrations overlap with the CH_2 and CH_3 deformation modes of the $–CH_2–CH_2–S–CH_3$ moiety.

The SERS spectra of **Met-Gly** and **Gly-Met** differ considerably from the SERS spectra of their amino acids. There are not only observed shifts up to 20 cm^{-1} in band frequencies, but also significant enhancement in intensities or even disappearance of some bands from the spectra. These are typical features of SERS spectra. Such behavior is not surprising because the enhancement of a proper mode depends strongly upon the orientation of a group on the surface. Thus, there is no reason to expect that the backbone orientation or side chain conformation is the same between the dipeptides and their constituent amino acids.

The SERS spectra of both heterodipeptides show dramatic changes compared with the corresponding Raman spectra in many regions. One of these is the region where the ν(CS) is expected. For example, **Met-Gly** exhibits a strong band at 691 cm^{-1} with a shoulder at 627 cm^{-1} that are due to the ν(CS) of the P_C-G and P_H-G conformers, respectively. The shoulder at 627 cm^{-1} is assigned to the ν(CS) mode rather than the amide IV mode, since the same pattern is observed in the SERS spectrum of Met.[35] A slightly different pattern in this range is observed for the SERS spectrum of **Gly-Met**. A similar doublet of bands is observed in Fig. 2 with the frequencies of 672 (conformer P_H-T) and 631 cm^{-1}, respectively. A down shift of the ν(CS) mode from 691 to 672 cm^{-1} clearly shows that Met adsorbs on the silver particles by changing its conformation around the $–CH_2–CH_2–S$ bond from the P_C-G to P_H-T. Additionally, a weak band at 714 cm^{-1} that is not observed in the SERS spectrum of **Met-Gly** appears. This band is assigned to the P_C-T conformer of the $–S–CH_3$ bond. Also, a band due to the C–S–C asymmetric stretching vibration appears at 822 cm^{-1} in the SERS spectrum of Fig. 2. This shows clearly that Gly modifies strongly the orientation of Met molecules on the silver particles.

In the case of **Gly-Met**, strong SERS bands appear at 919 and 1380 cm^{-1} that are due to the $C–COO^-$ and COO^- stretching vibrations, respectively, demonstrating that the carboxylate group is involved directly in the adsorption process of **Gly-Met** on the silver colloid. A slightly different situation is observed for **Met-Gly** dimer, where in Fig. 1 a very weak band at 923 cm^{-1} extracted from the envelope of 4–5 bands can be assigned to the $C–COO^-$ stretching mode. Additionally, it is probable that the COO^- stretching vibration is responsible for the

appearance of a band at 1367 cm^{-1}, i.e., 13 cm^{-1} lower than in the case of **Gly-Met**. This shows that despite the fact that in the adsorption process of both heterodimers on the silver colloid the thioether atom and carboxylate group are involved, the structure of the adsorbate in each case is different. In the case of **Met-Gly** mainly the P_C-G conformer adsorbs on the silver particles. Additionally, the C–COO^- and COO^- stretching bands are weakly enhanced, indicating that the *C*-termini group either lies parallel to the surface or is sufficiently far from the surface. In contrast, the P_H-T conformer around the C–S group is present on the surface and the carboxylate group is also strongly involved in the adsorption of **Gly-Met**.

Additionally, some SERS bands associated with the NH_2 group can be traced in the spectra of both dipeptides, indicating that the NH_2 group is also in close proximity to the surface. Features around 1631, 1325, and 1011 cm^{-1} in the SERS spectrum of **Met-Gly** are assigned to the NH_2 deformation, C–NH_2 stretching, and C_α–N stretching modes, respectively. **Gly-Met** yields the corresponding bands at similar positions in the SERS spectrum (1643, 1324, 1115, and 1047 cm^{-1}), although their relative frequencies and intensities are slightly changed. These frequency shifts and intensity alterations depend on the orientation of the NH_2 group and the distance between it and the metal surface. The emergence of the bands attributed to the vibrations of the NH_2 group rather than the NH_3^+ group indicates that the aqueous heterodipeptides are deprotonated on the surface.

It must be noted that a band around 1130 cm^{-1} in the SERS spectra of **Met-Gly** and **Gly-Met** corresponds to a weak band at 1129 cm^{-1} in the Raman spectrum of the aqueous **Met-Gly** and one at 1106 cm^{-1} in the Raman spectrum of the aqueous **Gly-Met**. We have assigned this band to the asymmetric $C_\alpha CN$ stretching mode. The intensity of this mode suggests that it does not involve a significant polarizibility change in the direction perpendicular to the surface and also that this portion of the molecule is not very close to the surface. The enhancement of this mode and the presence of the NH_2 twisting mode in the SERS spectrum of **Met-Gly** are good evidence for the close interaction of the amino group with the surface. If the amine group interacts directly with the surface, the C_α–C bond could be oriented nearly parallel to the surface. In this case we would expect the mode to be observed only weakly or not at all.

In the aqueous **Gly-Met** spectra, the $C_{\alpha 2}H_2$ and $C_{\alpha 1}H_2$ deformation modes occur at 1432 and 1446 cm^{-1}, respectively. Neither of these bands is present in the SERS spectra of **Gly-Gly** homodipeptide. We do, however, see medium intensity bands at 1420 and 1485 cm^{-1} assigned, respectively, to the $C_{\alpha 2}H_2$ and $C_{\alpha 1}H_2$ deformation vibrations of Met residues in the SERS spectra of **Gly-Met**. In the case of **Met-Gly** only a weak band at 1419 cm^{-1} is observed and is assigned to the $C_{\alpha 2}H_2$ deformation mode.

The amide I vibration[55] is only very weakly enhanced in the solid Raman spectra of **Met-Gly** (Fig. 1) and **Gly-Met** (Fig. 2). Strong bands at 1666 and 1690 cm^{-1} are due to the ν(C=O) of the carboxylate moiety. In the Raman spectra of the basic aqueous solutions of these heterodimers a band at 1643 cm^{-1} is due to the amide I vibrations, overlapping with the H–O–H deformation mode of water molecules. In the SERS spectrum of **Met-Gly** a band at 1631 cm^{-1} can be assigned to the amide I mode, down-shifted by 12 cm^{-1}, whereas the amide I band does not shift in the SERS spectrum of **Gly-Met**. Amide II is not usually observed in Raman spectra of aqueous solutions of peptides and proteins. However, this mode can be activated in SERS spectra when the structure of the amide bonds is changed.[56–58] Thus, we postulate that a medium intensity band at 1549 cm^{-1} in the SERS spectrum of **Met-Gly** and a weak band at 1540 cm^{-1} (**Gly-Met**) are assigned to the amide II vibrations. Alternatively this band could be associated with the asymmetric stretching vibration of the COO^-. Amide III is normally seen in Raman spectra in the 1250–1310 cm^{-1} region.[56–58] Again, it is very weakly enhanced in the Raman spectra of the solid-state **Met-Gly** and **Gly-Met**. In the aqueous solution spectra it is easily seen around 1270 cm^{-1}. In the SERS spectra weak bands at 1264 and 1277 cm^{-1} are associated with the amide III vibrations. Amides IV and V are not enhanced in the SERS spectra and are not present in the aqueous solution Raman spectra. The amide IV vibration arises mainly from a C=O–N in-plane bending mode and is expected to be enhanced only if the group is not parallel to the surface.[38,42] The same is true for the amide VI vibration (C=O out-of-plane bending). Their weak or zero enhancements in the SERS spectra show that the geometries of these two amides exclude these vibrations from being SERS active.

To conclude, both amino acids that form the heterodimers take part in the adsorption process on the colloidal silver. They adsorb by the thioether atom and carboxylate and amine groups. Also the amide I, II, and III modes are enhanced in the SERS spectra. However, the frequencies and intensities of modes involved in adsorption are frequently very different, showing that the structures of **Met-Gly** and **Gly-Met** after adsorption are substantially different. In other words, Gly strongly modifies the structure and activity of Met and vice versa.

Met-Leu and Leu-Met. Figures 3 and 4 depict the Raman and SERS spectra of **Met-Leu** and **Leu-Met**, respectively. Because of the complexity of the heterodipeptides, it is rather difficult to make firm vibrational assignments for all spectral features. Preliminary assignments for the SERS spectra of **Met-Leu** and **Leu-Met** are given in Tables I and II, respectively. We followed the band assignments for L-Leu[38,59] and L-luecine nitrate,[60] as well as our previous work on amino acids and homodipeptides.[35]

As expected, the ν(CS) vibration appears as the strongest band in the Raman spectra of **Met-Leu** and **Leu-Met** in the solid state. In the case of **Leu-Met** its intensity is the second strongest in the spectrum (Fig. 4). However, with no doubt, its intensity is much larger than that of the 1455 cm^{-1} band. In **Met-Leu** (Fig. 3) we observe up to four C–S conformers, while in the case of **Leu-Met** at least five conformers exist. It should be noted that the strongest intensity band of the C–S conformer is observed at 718 cm^{-1} in **Met** and at 719 and 715 cm^{-1} in **Met-Gly** and **Gly-Met**, respectively, i.e., Gly did not influence the C–S bonds of Met. In the case of **Met-Leu** the strongest band at 693 cm^{-1} that is obviously assigned to the ν(CS) of the conformer P_C-G is down-shifted by about 25 cm^{-1} from that observed in Met, **Met-Gly**, and

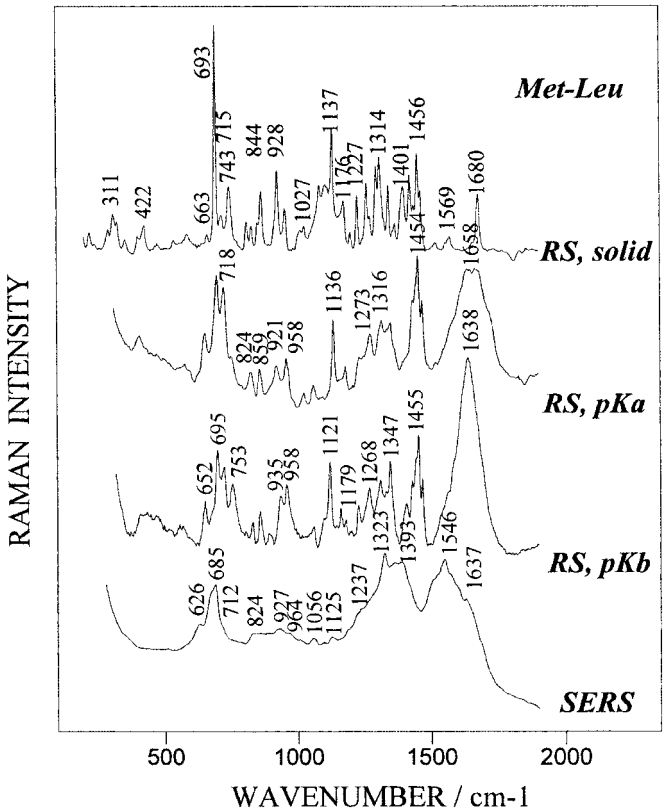

FIG. 3. Raman spectra of **Met-Leu** in a solid state and aqueous solutions at pK_a and pK_b and a SERS spectrum of **Met-Leu** adsorbed on colloidal silver. Measurement conditions are the same as those in Fig. 1.

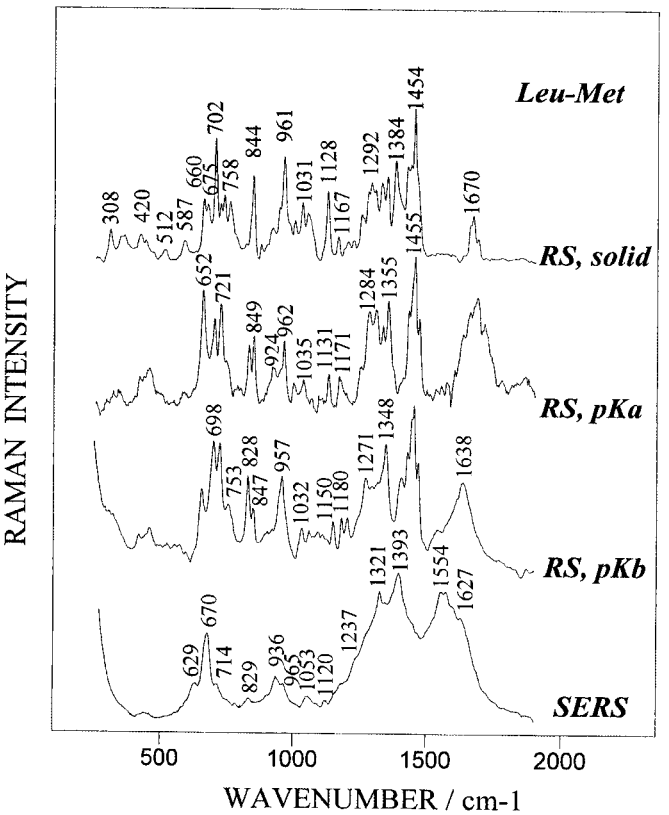

FIG. 4. Raman spectra of **Leu-Met** in a solid state and aqueous solutions at pK_a and pK_b and a SERS spectrum of **Leu-Met** adsorbed on colloidal silver. Measurement conditions are the same as those in Fig. 1.

Gly-Met (conformer P_C-T). The Raman spectrum of solid-state **Leu-Met** shows the strongest ν(CS) band at 702 cm^{-1} that can be ascribed to the appearance of the P_C-G conformer. The second strongest band in **Met-Leu** arises from the P_C-G conformer (743 cm^{-1}), while a band due to the P_C-T conformer of **Leu-Met** is observed at 758 cm^{-1}. Somewhat surprisingly, in the aqueous solution (both acidic and basic) Raman spectra of **Met-Leu** the ν(CS) pattern of existing conformers is very similar to that observed for **Met-Gly**. They show only small changes, often negligible, in the frequencies and intensities. On the other hand, the ν(CS) pattern of **Leu-Met** in the aqueous solution follows that of **Gly-Met**. In this case, changes of pH cause changes in the intensity pattern of the C–S conformers. However, in all cases only four conformers are observed in the solutions.

Adsorption of **Met-Leu** on silver colloid causes the appearance of a strong ν(CS) band at 685 cm^{-1}. This band, as in the other cases discussed here, is accompanied by two other conformers: P_H-G (626 cm^{-1}) and P_C-T (712 cm^{-1}). The SERS spectrum of **Leu-Met** shows that the P_H-T conformer has the highest population on the silver surface (band at 670 cm^{-1}). This conformer is accompanied by two others with the same structure as discussed

TABLE I. Band assignments for SERS spectra of Met-X (X = Met, Gly, Leu, Pro, and Phe) dimers.

Assignment	Frequencies [cm^{-1}] in the SERS spectra of			
	Met-Gly	Met-Leu	Met-Pro	Met-Phe
ν(C–S)	627	626	629	626
ν(C–S)	691	685	677	670
ν(C–S)	⋯	712	706	688
ν_{as}(C–S–C)	826	824	853	829
ν(C–COO^-)	923	927	915	922
ν(C_αN)	1056	1056	1054	1059
τ(NH_2)	⋯	1095	1100	⋯
ν_{as}(C_αCN)	1130	1125	1156	1142
δ(CC_αH)	1264	1237	1277	1261
ν(C–NH_2)	1325	1323	1321	1323
ω($C_\alpha H_2$)	⋯	1363	⋯	1356
ν_s(COO^-)	1367	1396	1379	1396
δ($C_{\alpha 2}H_2$)	1419	⋯	1423	⋯
δ($C_{\alpha 1}H_2$)	⋯	⋯	1465	1460
amide II (δ(NH) + ν(CN))	1549	1546	1548	1522
δ(NH_2) and amide I (ν(C=O) + ν(CN) + δ(NH))	1631	1637	1630	1628

TABLE II. Band assignments for SERS spectra of X-Met (X = Met, Gly, Leu, Pro, and Phe) dimers.

Assignment	Frequencies [cm^{-1}] in the SERS spectra of			
	Gly-Met	Leu-Met	Pro-Met	Phe-Met
ν(C–S)	631	629	632	623
ν(C–S)	672	670	670	664
ν(C–S)	⋯	714	712	⋯
ν_{as}(C–S–C)	822	829	822	811
ν(C–COO^-)	919	936	926	917
ν(C_αN)	1047	1053	1036	1036
τ(NH_2)	1115	1091	1090	1116
ν_{as}(C_αCN)	1129	1120	⋯	⋯
δ(CC_αH)	1277	1237	1278	1293
ν(C–NH_2)	1324	1321	1324	1316
ω($C_\alpha H_2$)	⋯	⋯	⋯	1361
ν_s(COO^-)	1380	1393	1385	1396
δ($C_{\alpha 2}H_2$)	1420	⋯	1420	1410
δ($C_{\alpha 1}H_2$)	1485	⋯	⋯	1486
amide II (δ(NH) + ν(CN))	1540	1554	1550	1551
δ(NH_2) and amide I (ν(C=O) + ν(CN) + δ(NH))	1643	1627	1628	1635

above for **Met-Leu** (bands at 629 and 714 cm^{-1}). These results suggest that the investigated heterodipeptides are present with the three C–S conformers on the colloidal silver. Thus, not only one specific orientation of Met residue is induced by the interaction of the thioether atom and carboxylate group with the silver surface. The most abundant conformer is strictly dependent upon the composition of the investigated dipeptides. Thus, the SERS spectra of these dipeptides indicate clearly the different pattern of interaction between the dimer and colloidal silver particles.

The interactions between the thioether atom and the silver particles get some support from the additional interaction of the carboxylate group with the surface. This is easily observed by the strong enhancement of bands at 936 and 1393 cm^{-1} (**Leu-Met**) that are assigned to the C–COO^- and COO^- stretching vibrations, respectively. These vibrations are also seen at 927 and 1393 cm^{-1} in the SERS spectrum of **Met-Leu**. Of particular note is that the intensities of the ν(C–COO^-) and ν_{sym}(COO^-) bands are much weaker in the SERS spectrum of **Met-Leu** than in that of **Leu-Met**. The relative intensity of ν(C–COO^-) is greater in the SERS than in the Raman spectra, indicating that the C–C bond does not lie parallel to the silver surface.[47] Additionally, some SERS bands associated with the NH_2 group can be observed in the spectrum, indicating that this group is also in close proximity to the silver surface. We assigned features around 1627, 1321, 1120, and 1053 cm^{-1} in the SERS spectrum of **Leu-Met** to the NH_2 deformation, C–NH_2 stretching, NH_2 twisting, and C_α–N stretching modes, respectively. The presence of these bands associated with the NH_2 group indicates that this dipeptide is deprotonated on the surface. The bands due to the vibrations of the NH_2 moiety with similar frequency appear in the SERS spectrum of **Met-Leu**.

The Leu residues in **Met-Leu** and **Leu-Met** yield a characteristic strong Raman band at around 1347 and 1348 cm^{-1}, respectively, in their aqueous solution spectra. This band is associated with the CH deformation mode.[38,59,61] The C–C stretching modes of leucine residues are known to mix with the methyl rocking mode[62] and give rise to bands around 1179 or 1180, at 1121 or 1150, and at 935 or 957 cm^{-1} in the Raman spectra of **Met-Leu** or **Leu-Met**, respectively. The appearance of bands at around 1121 and 935 cm^{-1} is the result of this mixing. The band at around 1179 cm^{-1} is not observed since a band due to the NH_3^+ rocking mode also lies in this region.

In the 1400–1200 cm^{-1} region bands due to different vibrations of the methylene groups, especially wagging deformation $\omega(CH_2)$ and twisting deformation $\tau(CH_2)$, are expected at around 1347, 1348, 1268, and 1271 cm^{-1} in the Raman spectra of **Met-Leu** and **Leu-Met**. Moreover, in the 1425–1445 cm^{-1} region we observe bands arising from the CH_2, CH_3, and $NC_\alpha H$ deformation modes.

From the discussion above one can see that there is every indication that **Met-Leu** adsorbs on the colloidal silver through the thioether atom; however, the terminal groups (COOH and NH_2) are also in close proximity to the surface. Thus, we conclude that the thioether atom and carboxyl group as well as the NH_2 group are involved in the absorption of **Leu-Met** on the silver particles.

As in the cases of **Met-Gly** and **Gly-Met**, Raman spectra of **Met-Leu** and **Leu-Met** solutions display bands that are obviously associated with the amide I and III vibrations. Amides II, IV, and V are too weak to be observed clearly in the spectra. The amide I band in the acidic solution, as in previously discussed cases, appears as a broad band at around 1650 cm^{-1}. The broadness of this band arises from its splitting into two subbands, which, according to Asher and co-workers,[63] is probably derived from the coupling of the amide I vibration with the H–O–H bending motion due to formation of the hydrogen bonding between the carbonyl group of the peptide and proton from the water molecules. There is also a possibility that this broadness is a result of geometrical overlap of two bands: the amide I and deformation modes of water molecules.

All the SERS spectra presented in Figs. 3 and 4 display the enhancement of the amide I, II, and III bands. It is noted that the amide I and II bands are observed at 1637 and 1546 cm^{-1}, respectively, for **Met-Leu**, while these bands are much lower in the case of **Leu-Met**, i.e., 1627 and 1502 cm^{-1}. In particular, the down shift of the amide II band in **Leu-Met** is not clearly understood at this moment. Amide II is not active in Raman but in many cases can be clearly observed in SERS.[57] On the other hand, the amide III vibration is not strongly enhanced in the

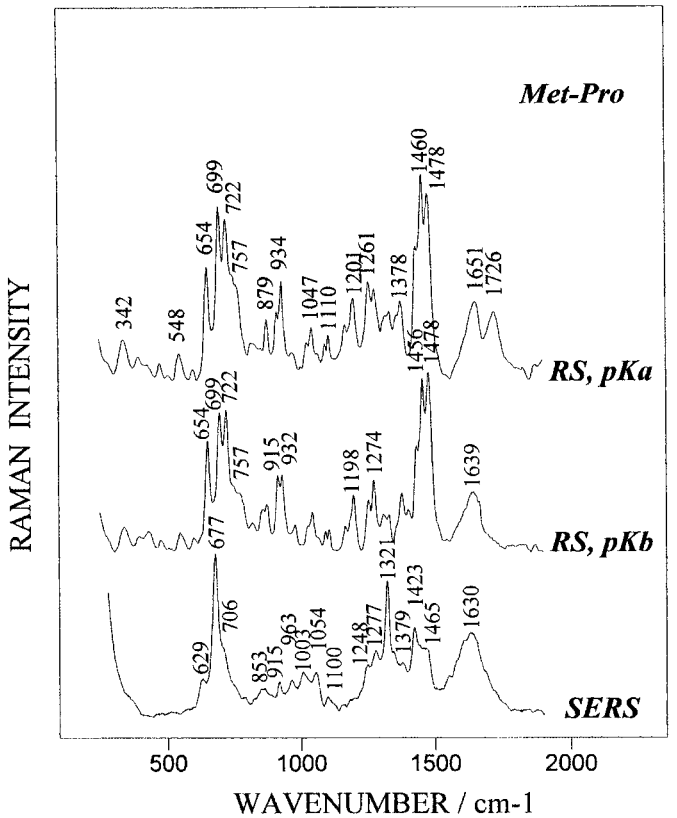

FIG. 5. Raman spectra of **Met-Pro** in a solid state and aqueous solutions at pK_a and pK_b and a SERS spectrum of **Met-Pro** adsorbed on colloidal silver. Measurement conditions are the same as those in Fig. 1.

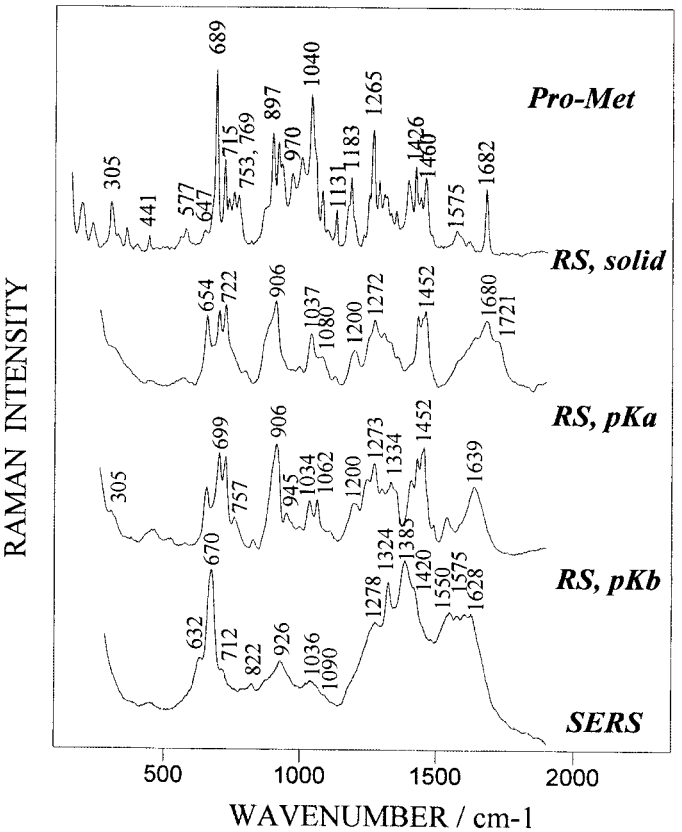

FIG. 6. Raman spectra of **Pro-Met** in a solid state and aqueous solutions at pK_a and pK_b and a SERS spectrum of **Pro-Met** adsorbed on colloidal silver. Measurement conditions are the same as those in Fig. 1.

SERS spectra of both dipeptides and as a result is not clearly observed since it is hidden under the envelope of bands between 1200–1400 cm^{-1}. However, a shoulder at around 1237 cm^{-1} may be safely assigned to the amide III vibration. On the other hand, it is noted that this frequency is lower than that of the amide III vibration observed in the solution Raman spectra (around 1270 cm^{-1}) and the SERS spectra of **Met-Gly** and **Gly-Met** (*vidè supra*).

Met-Pro and Pro-Met. Figures 5 and 6 display Raman and SERS spectra of **Met-Pro** and **Pro-Met** dipeptides. Analyses of these Raman spectra were made based on previous work by Garfinkel[38] and theoretical calculations of normal mode vibrations of proline dipeptide,[52] as well as our previous assignments for amino acids and their homodipeptides.[35] As is seen in Fig. 5 the Raman spectrum of **Met-Pro** in the solid state is missing. We were not able to measure obtained sample, as the solid state of **Met-Pro** forms an amorphic phase.

Comparison of these two figures shows immediately that proline makes distinct alterations in the Raman and SERS spectra between **Met-Pro** and **Pro-Met**. The most distinctive feature in the Raman spectra of the aqueous solutions of **Pro-Met** is a band at 906 cm^{-1} due to the C–C stretching ring mode of the Pro residue. The spectral features at 305, 1034, 1062, and 1334 cm^{-1} are assigned to the ring coordinates, δ(CNC) + δ(CCN), ν(NC) + δ(NCC), ν(CC) + ν(CC), and δ(CCH), of the Pro residue, respectively. This C–C stretching ring mode appears at 932 cm^{-1} in the Raman spectrum of **Met-Pro** in the solution. The second most intensive feature in the **Pro-Met** spectrum is a band at 1452 cm^{-1} assigned to the deformation vibration of the ring methylene groups, $\delta(CH_2)$ (Fig. 6). Moreover, in the 1425–1445 cm^{-1} region bands arising from the S–CH_2, S–CH_3, and $NC_\alpha H$ deformation modes are expected. The region from 1000 to 400 cm^{-1} is of interest because the methylene rocking, skeletal C–S, and C–S–C vibrations appear in this region.

The Raman spectrum of solid **Pro-Met** shows several bands in the 650–750 cm^{-1} region due to the C–S stretching vibrations. The strongest bands appear at 689 and 715 cm^{-1}. The band at 689 cm^{-1} is associated probably with the P_C-G conformer, whereas that at 715 cm^{-1} belongs to the P_C-T conformer. For the aqueous solutions similar patterns of four C–S stretching bands at 654, 699, 722, and 757 cm^{-1} are observed for both **Met-Pro** and **Pro-Met**. This shows the coexistence of the P_H-G, P_C-G, P_H-G, and P_C-T conformers in the solutions. It must be emphasized that very similar patterns of Raman spectra are observed for all the dipeptides in the solutions investigated in this work. The SERS spectra of both dipeptides, **Met-Pro** and **Pro-Met**, show only one strong band due to the C–S stretching vibration at 677 and 670 cm^{-1}, respectively. This shows that the P_H-T conformer is preferably adsorbed on the silver particles. Additionally, Met interacts with the silver colloid as the P_H-G (629–632 cm^{-1}) and P_C-G (706–712 cm^{-1}) conformers. These findings show clearly that both peptides, as in the cases of others studied so far, interact with the silver particles through the thioether atom, forming three Met conformers. Some SERS bands associated with the NH_2 group

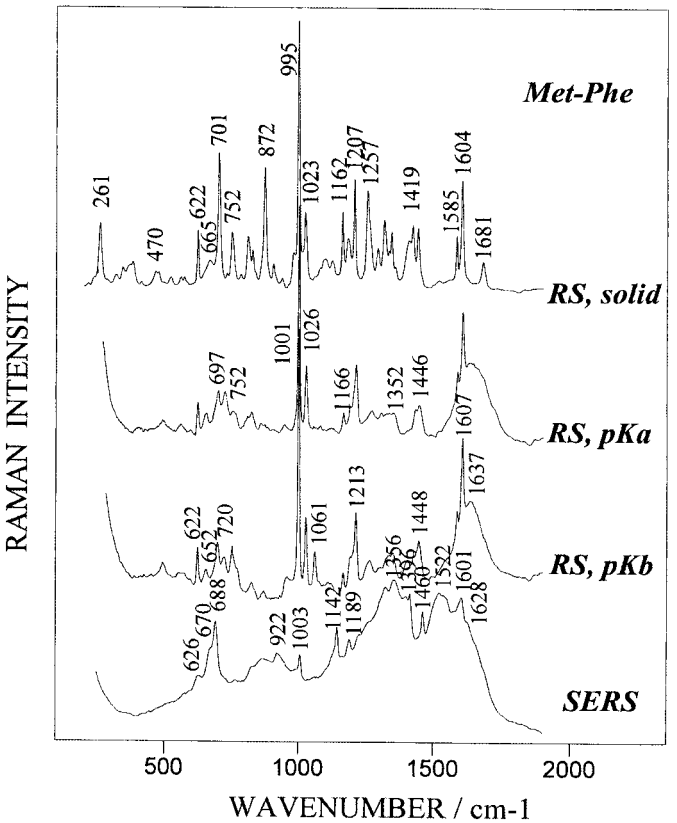

FIG. 7. Raman spectra of **Met-Phe** in a solid state and aqueous solutions at pK_a and pK_b and a SERS spectrum of **Met-Phe** adsorbed on colloidal silver. Measurement conditions are the same as those in Fig. 1.

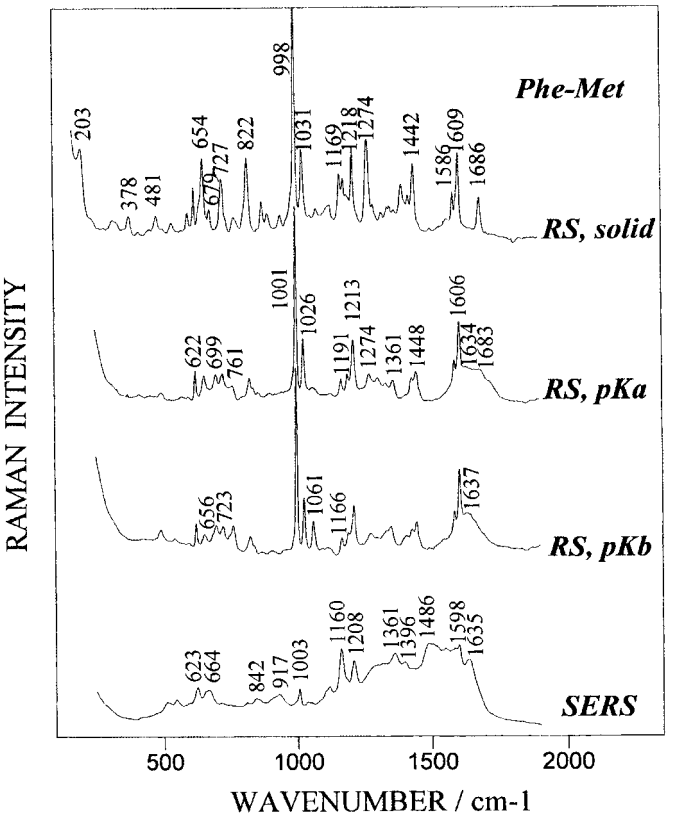

FIG. 8. Raman spectra of **Phe-Met** in a solid state and aqueous solutions at pK_a and pK_b and a SERS spectrum of **Phe-Met** adsorbed on colloidal silver. Measurement conditions are the same as those in Fig. 1.

can also be identified in the spectra, indicating that this group either interacts directly with or is in close proximity to the surface. Features around 1630, 1321, 1100, and 1054 cm^{-1} in the SERS spectrum of **Met-Pro** are assigned to the NH_2 deformation, C–NH_2 stretching, NH_2 twisting, and C_α–N stretching modes, respectively. We have noted a surprising role of the carboxylate group in the interaction of these two dipeptides with the silver colloid. Namely, in the case of **Pro-Met** two strong bands at 926 and 1385 cm^{-1} appear that are assigned to the ν(C–COO^-) and $\nu_{sym}(COO^-)$, respectively. This shows that the carboxylate group is directly involved in the adsorption process. These bands are absent in the case of **Met-Pro**. Based on the above findings, we conclude that the thioether atom and NH_2 group of **Met-Pro** are involved in its adsorption on the silver particles and that the thioether atom as well as the carboxyl and NH_2 groups of **Pro-Met** interact with the silver surface.

Additionally, as we discussed above for other dimers, amides I, II, and III are enhanced in the SERS spectra. Again, the behavior of **Pro-Met** is different from that of **Met-Pro**. In the case of the SERS spectrum of **Pro-Met** (Fig. 6) all three amides are enhanced and seen as broad bands centered at 1628 (amide I), 1550 (amide II), and 1278 cm^{-1} (amide III). Amides I and III are enhanced in the SERS spectra of **Met-Pro** and are seen at 1630 and 1277 cm^{-1}, respectively, i.e., at virtually the same frequencies as for the former dimer. What is important is the fact that the amide II vibration is missing in the SERS spectrum of **Met-Pro**.

Also, it is worth noting that the CH_2 and CH_3 deformation modes are present in the SERS spectrum of **Met-Pro** as two bands at 1423 and 1465 cm^{-1}, but they are absent (or hidden under other strong bands) in the SERS spectrum of **Pro-Met**.

Met-Phe and Phe-Met. Figures 7 and 8 show Raman and SERS spectra of **Met-Phe** and **Phe-Met**, respectively. The band assignments for both Raman and SERS spectra of **Met-Phe** and **Phe-Met** are not straightforward because a number of bands due to the ring mode vibrations appear. Here, we point out that in an ordinary Raman spectrum of Phe the most intense and characteristic feature is a band at 1002 cm^{-1} assigned to the ''breathing'' mode of the phenyl ring accompanied by a mode at 1033 cm^{-1}. This characteristic pattern of two bands is also observed at slightly lower frequency, i.e., 995 and 1023 cm^{-1} for **Met-Phe** and 998 and 1031 cm^{-1} for **Phe-Met**. However, in the SERS spectra of Phe as well as its methionine-containing heterodipeptides the corresponding band at 1003 cm^{-1} is very weak. It has been believed that the weak enhancement is due to direct participation of the phenyl ring π-system in the complex formation with the silver particles[19,48] The phenyl rings of **Met-Phe** and **Phe-Met** may directly interact with the surface. According to Lee et al.,[47] who compared relative Raman intensities of the ring modes of Phe measured in an aqueous solution to those observed in the SERS spectrum, the weak enhancement of some ring modes has to be taken as evidence for the tilted orientation of the ring in adsorbed Phe.

Phe has a number of aromatic ring vibrations that are

marked out by a number of Raman measurements.[19,63,64] These characteristic bands are also seen in the Raman spectra of the **Met-Phe** and **Phe-Met** aqueous solutions at 622, 1001, 1026, 1191, 1213, 1585, and 1606 cm^{-1}. These bands are assigned to the in-plane ring deformation (ν_{6b}), symmetric ring breathing (ν_{12}), in-plane CH bending (ν_{18a}), combination of in-plane CH bending (ν_{9a}) with ring stretching, phenyl-C stretching (ν_{7a}), and two in-plane ring stretching vibrations (ν_{8b} and ν_{8a}), respectively. A band at 1166 cm^{-1} and bands in the region of 1300–1500 cm^{-1} are assigned to the $\nu_{as}(C_{\alpha}CN)$ and $\delta(S–CH_2)$ and $\delta(S–CH_3)$ vibrations, respectively. The Raman spectra of **Met-Phe** and **Phe-Met** are very similar to each other since both spectra are dominated by Phe ring vibrations. On the other hand, we note distinctive differences between the SERS spectra of these two heterodipeptides.

From the presented Raman spectra it is not easy to isolate bands associated with the C–S vibrations. In the solid state of **Met-Phe** it is rather obvious that bands at 665, 701, and 752 cm^{-1} represent the P_H-G, P_H-T, and P_H-G conformers, respectively. If this is true, Phe modifies strongly the structure of heterodipeptides, particularly the conformation around the C–S–C bonds. In solution spectra the pattern of the ν(CS) is practically the same for both dipeptides. Again, a surprising result is obtained in the SERS measurement. Here, **Met-Phe** adsorbs on the silver particles by the thioether sulfur forming three conformers with the characteristic ν(CS) at 626, 670, and 688 cm^{-1}, where the band at 688 cm^{-1} is the strongest. On the other hand, it seems that only one or two conformers are formed during the interaction of **Phe-Met** with the silver colloid (bands at 623 and 664 cm^{-1} that are characteristic for the P_H-T and P_H-G conformers).

In the SERS spectrum of **Met-Phe** or **Phe-Met**, apart from the characteristic bands due to the phenyl ring vibrations, enhanced signals due to the C–COO^- stretching and COO^- symmetric stretching modes are observed at 922 or 917 cm^{-1} and 1396 cm^{-1}, respectively. Thus, we conclude that **Met-Phe** adsorbs on the silver surface through both the thioether atom and carboxyl functional group but **Phe-Met** binds on the silver surface probably mainly through the carboxyl functional group; however, very weak enhancement of the ν(CS) is also noted. Also, as in the previous cases, amides I, II, and III are probably enhanced in the SERS spectra of both dimers. However, their frequencies are difficult to determine as they overlap with other broad bands.

CONCLUSION

Binding of L-glycine (Gly), L-leucine (Leu), L-proline (Pro), and L-phenylalanine (Phe) to L-methionine (Met) strongly influences the structure and bonding of Met. It has been demonstrated that in the investigated dimers Met exists in four to six conformers that are characterized by their ν(CS). However, only three of them are formed on the silver surface, as is clearly seen from their SERS spectra. On the other hand, the SERS spectra of **X-Met** and **Met-X** show that not only the thioether atom interacts with the silver particles but also, in most cases, the carboxylate group takes part in the adsorption process. This is manifested by the enhancement of the ν(C–COO^-) and ν(COO^-) stretches observed in the 920–930 and 1380–1396 cm^{-1} regions, respectively. Additionally, SERS spectra indicate that the *N*-termini group is involved in these processes, and the amide I, II, and III vibrations can be enhanced in some cases and observed in the SERS spectra. Only in the case of **Phe-Met** does the SERS spectrum indicate that the thioether atom probably does not take part in the adsorption process, or its interaction with the silver surface is very weak. Instead, the aromatic side chain interacts with the silver surface and this may dictate the orientation and conformation of the adsorbed dipeptide.

ACKNOWLEDGMENTS

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