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Structure-potential dependence of adsorbed enzymes and amino acids revealed by the surface enhanced Raman effect

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Abstract. Surface enhanced Raman scattering (SERS) of some enzymes (alkaline phosphatase, horseradish peroxidase and lactoperoxidase) and some amino acids (tryptophan, tyrosine and phenylalanine) on silver electrodes has been studied. The spectral band intensities of certain amino acids and amino acid residues were determined by their orientation on the surface and depended on the electrode potential (E).

Key words: Surface enhanced Raman scattering, alkaline phosphatase, lactoperoxidase, horseradish peroxidase, tyrosine, tryptophan, phenylalanine, orientation-potential dependence

Introduction

Structural studies of proteins adsorbed on metal surfaces fall into the field of molecular biophysics. The results obtained are of great interest to physicists (biomolecular electronics), neurobiologists (models of neuron systems), electrochemists (chemotronic devices), bioengineers (biocompatible prosthesis) and others.

A 10^5-10^8-fold increase in the Raman scattering intensity, achieved in 1974, allowed the study of monolayers of organic compounds adsorbed on metal surfaces (Furtak and Roy 1985; Taniguchi et al. 1986; Campion 1985).

Johnson et al. (1986) and Caswell and Spiro (1986) studied the resonance Raman spectra and Nabiev et al. (1981) and Nabiev and Chumanov (1986) the SERS of amino acids and proteins. In these studies they assigned some spectral bands to specific vibrations of amino acid residues and investigated the dependence of their location and intensity on the wavelength of the exciting light.

However, the variation in the structure of a protein at different potentials of the electrode. i.e. upon changes in the structure of the double electric layer on the surface, is also a question of great interest.

The aim of the present work is to study the structure of proteins and amino acids on silver surfaces as a function of the electrode potential. The objects of this study are the following three enzymes – alkaline phosphatase from E. coli, horseradish peroxidase and lactoperoxidase from bovine milk. The proteins mentioned were chosen because of the difference in their molecular weight, amino acid composition and active centre structure (Table 1). The following amino acids:

Table 1. Amino acid composition of alkaline phosphatase (McComb and Bowers 1979), horseradish peroxidase and lactoperoxidase (Dunford and Stillman 1976)

Amino acid	Alkaline phosphatase	Horseradish peroxidase	Lacto-peroxidase
Lysine	50	6	33
Histidine	16	3	14
Arginine	24	20	39
Aspartic	100	46	71
Threonine	92	25	28
Serine	34	26	30
Glutamic	91	20	60
Proline	37	17	42
Glycine	86	17	41
Alanine	123	23	40
Cysteine	–	–	12
Cystine/2	8	8	–
Valine	42	18	29
Methionine	13	4	12
Isoleucine	25	12	28
Leucine	73	35	68
Tyrosine	20	6	15
Phenylalanine	16	20	31
Tryptophan	8	–	16
Total number	858	306	609
MW in kDa	89	40.5	76.5

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Abbreviations: SERS, surface enhanced Raman scattering; Trp, tryptophan; Tyr, tyrosine; Phe, phenylalanine; E, electrode potential; ORC, oxidation-reduction cycle

Trp, Tyr and Phe were also investigated. Their vibrations are clearly exhibited in the SERS spectra of proteins.

Materials and methods

Electrophoretically homogeneous alkaline phosphatase (MW 89,000) (E.C. 3.1.3.1) from E. coli (USSR) was used. The extinction coefficient (ε) at 280 nm was 61.9 $mM^{-1}cm^{-1}$ (Thomas and Kirsch 1980). Isoenzyme C of horseradish peroxidase (MW 40,500) (E.C. 1.11.1.7) ("Reanal") had an absorption ratio (RZ) of 3.15 at 403 and 280 nm (Berezin et al. 1975). Electrophoretically homogeneous lactoperoxidase (MW 76,500) (E.C. 1.11.1.7) with RZ = 0.87 (absorption ratio at 412 and 280 nm) was obtained from bovine milk as described by Razumas et al. (1985). Peroxidase concentrations were determined spectrophotometrically using $\varepsilon_{403} = 10^2$ $mM^{-1}cm^{-1}$ for horseradish peroxidase (Dunford and Stillman 1976) and $\varepsilon_{412} = 114$ $mM^{-1}cm^{-1}$ for lactoperoxidase (Morison et al. 1957). Protein preparations were subjected to dialysis against 0.1 *M* KCl solution for 24 h at 4 °C to eliminate any low-molecular weight impurities.

Amino acids were obtained from the USSR and Hungary ("Reanal").

The experiments with the enzymes and amino acids were carried out in 0.1 *M* KCl solutions prepared from bidistilled water and twice-recrystallized salt. The solutions of amino acids were centrifuged using a "Janetzki" K 23 centrifuge (Poland) for 30 min at 15,000 rpm. The concentration of amino acids was determined spectrophotometrically using $\varepsilon_{219} = 47.0$ (Trp), $\varepsilon_{222} = 8.0$ (Tyr) and $\varepsilon_{206} = 9.3$ $mM^{-1}cm^{-1}$ (Phe) (Freifelder 1976). Anaerobic solutions were prepared by bubbling nitrogen of the highest purity for 30 min.

Raman spectra were recorded on an automated spectrometer DFS-12 (USSR) equipped with a microcomputer DVK-1 (USSR). The excitation of spectra was done using an ILA-120 argon laser (German Democratic Republic). The 488 nm laser excitation line was used with a maximum power of 250 mW at the sample. The slit width was 8 cm^{-1}. The cell scheme was analogous to that described by Brandt (1985). Electrochemical measurements were carried out with a PA-3 polarograph ("Laboratorni Pristroje", Czechoslovakia).

The working electrode was a copper wire (0.4 cm in diameter) pressed in teflon on the surface of which silver was electro-deposited over the course of 25 min from a solution containing 0.19 *M* $AgNO_3$, 89 m*M* KOH and 1.84 *M* KCN at 20 °C and current density 1 A/dm^2. A saturated calomel electrode was used as reference. All potentials in the text are referenced to this electrode. The platinum electrode (surface area 12.4 cm^2) served as an auxiliary.

Before measurements the working electrode was treated for 10 s with a mixture (3:1 v/v) of concentrated HNO_3 and H_2SO_4 and for 10 s with a mixture of aqueous solutions containing 25% ammonia and 30% hydrogen peroxide (1:1 v/v) cooled to 4 °C.

Before measurements the working electrode was subject to an oxidation-reduction cycle (ORC) in the solution studied at potentials of −0.6, 0.4 and −0.6 V for 5, 2 and 5 s, respectively (Creighton 1980).

Results

Figure 1 shows the SERS spectra of alkaline phosphatase versus the Ag electrode potential. These spectra occur if the electrode pre-treatment by ORC is carried out in the enzyme solution. Spectral response shows

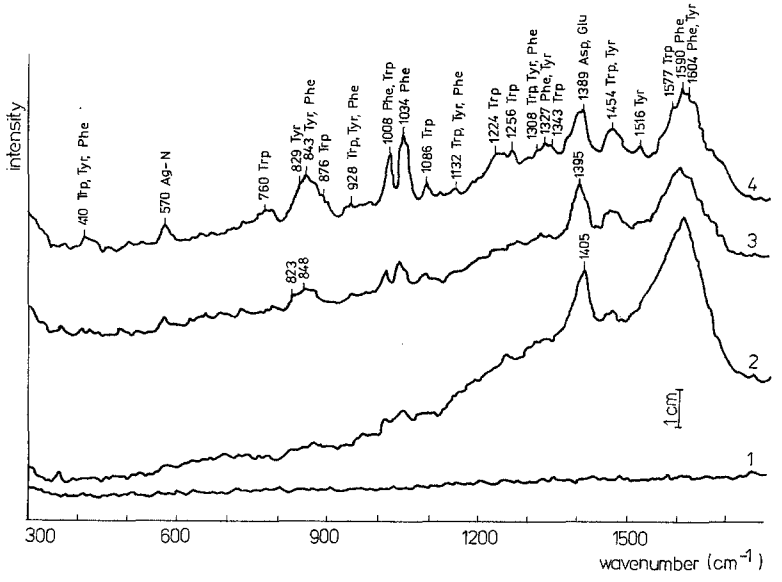

Fig. 1. SERS spectra of 0.1 *M* KCl solution (1) and with dissolved alkaline phosphatase (2–4) at the silver electrode potentials 0.0 (2), −0.6 (3) and −1.0 (4). $\lambda_{ex} = 488$ nm, enzyme concentration 6.4 μM, pH 6.3, 25 °C. Interpretation according to Nabiev et al. (1981), Nabiev and Chumanov (1986) and Rava and Spiro (1985)

little dependence on the direction of the potential change in the range from 0 to −1 V. In contrast to albumin and lysozyme (Nabiev and Chumanov 1986) the SERS spectrum of alkaline phosphatase indicates an additional intense band in the 1400 cm^{-1} region that probably arises from the vibration of ionized carboxylate groups of the aspartic and glutamic acids, the number of which is 191 (McComb and Bowers 1979). The vibration band maximum of these groups is shifted from 1405 cm^{-1} (E = 0.0 V) to 1400, 1395, 1392 and 1389 cm^{-1} at the potentials −0.2, −0.6, −0.8 and −1.0 V. This may be accounted for by the fact that the electrode is positively charged over this potential range [potential of the Ag surface at zero charge equals −0.95 V (Frumkin 1979)] and may interact electrostatically with the adsorbed enzyme carboxylate groups. In this way, with an increase in potential the vibration bands of these groups are brought towards the high-energy region. This is in accord with the data on the IR spectra of carboxylic acids (Bellamy 1963). From here it follows that an increased location of negative charge on the ionized carboxylate group (e.g. the introduction of a strong electrophilic substituent) results in a shift of the symmetric vibration band towards the low-energy region. In contrast, the formation of chelates shifts the band towards the high-energy region.

In addition to these changes an increase in the electrode positive charge decreases the intensity of some bands (Fig. 1). This is characteristic of the bands of Trp and Phe amino acid residues at 1008 and 1034 cm^{-1}. They reflect the out-of-phase skeletal vibrations of benzene and pyrrole rings in Trp and the vibrations ν_1 (1008 cm^{-1}) and ν_{12} (1034 cm^{-1}) of a benzene ring in Phe (Rava and Spiro 1985; Johnson et al. 1986). With the same potential change a decrease in the intensity of vibrations ν_3 (1224 cm^{-1}) of the benzene ring and benzene and pyrrole ring-breathing in-phase vibrations (760 cm^{-1}) in Trp is also observed. Practically, at a potential of −0.2 V the Fermi-resonance doublet of Tyr (823 and 843 cm^{-1}), that is due to interaction of the out-of-plane ring vibration overtone (~410 cm^{-1}) and its symmetric ring breathing (~840 cm^{-1}), is absent in the spectrum of alkaline phosphatase (Carey 1982). More positive values of potential lead to a band intensity decrease in the 570 cm^{-1} region. Evidently, the vibrations of the coordination bond Ag-N are likely to occur in this region (Wokaun et al. 1985). At more positive potential values the band intensities are increased in the 1500–1700 cm^{-1} region. This applies to the vibrations of benzene rings in Trp (ν_{8b}, 1577 cm^{-1}), Phe (ν_{8b}, 1590 cm^{-1}; ν_{8a}, 1604 cm^{-1}) and Tyr (ν_{8b}, 1604 cm^{-1}) (Rava and Spiro 1985; Johnson et al. 1986).

Unlike alkaline phosphatase, the SERS spectra of peroxidase exhibits few bands. In the spectrum of lactoperoxidase the vibration bands of the coordination bond Ag-N (560 cm^{-1}), carboxylate group (1385, 1380 and 1371 cm^{-1} at −0.2, −0.8 and −1.0 V) and a broad band of Trp, Tyr and Phe residues in the 1500–1700 cm^{-1} region are observed. The spectrum of horseradish peroxidase also exhibits a hand of low intensity in the 1425 cm^{-1} region, that is due to benzene ring vibrations ν_{19b} (Rava and Spiro 1985; Johnson et al. 1986) and a broad vibration band of Phe and Tyr rings in the 1500–1700 cm^{-1} region. The potential effect on the intensity of these spectral responses is analogous to that of alkaline phosphatase. The comparatively low complexity of peroxidase spectra may be accounted for by the fact that the isoelectric point of peroxidase and lactoperoxidase is located in the alkaline pH region (Righetti and Caravagio 1976). For this reason, most of the protein molecules are positively charged at pH 6.3 and their adsorption is weak in the potential range from 0 to −1 V. Besides that, these proteins contain carbohydrates which also give lower absorptive capacities.

Over the potential range studied the electrochemical reduction of four disulfide bonds of alkaline phosphatase and horesradish peroxidase (Table 1) as well as the heme iron of peroxidases may proceed on the Ag electrodes. However, the SERS spectra of the enzymes studied do not enable us to make any definite conclusion concerning such processes, since the vibrations of the groups C-C, C-S and protoporphyrin IX are not observed.

It follows from the SERS spectra studied that the intensity of some bands at various potential values may change in opposite directions. In the case of alkaline phosphatase when the potential is changed from −1.0 to −0.6 and 0.0 V the intensity decrease is observed merely for the in-plane benzene ring vibrations of Trp, Phe and Tyr residues (ν_1, ν_3, ν_{12}) and in contrast – the intensity of the out-of-plane vibrations ν_{8a} and ν_{8b} is increased (Herzberg 1945). The effect observed may be accounted for by the assumption that the enzyme amino acid residues change their orientation on the electrode surface with respect to the potential change. In going from normal to tangential vibrations the energy released by the induced dipole is decreased from 6.5 to 11 times (Campion 1985). Consequently, the ring planes of aromatic residues in adsorbed alkaline phosphatase are lined up in parallel with the surface at positive potential values. When the potential approaches the zero charge point of silver both parallel and perpendicular orientations are possible. In the case of peroxidases the SERS spectra exhibit the vibrations of parallel-oriented residues of aromatic R-groups.

To confirm the conclusions made the potential dependence of Trp, Tyr and Phe spectra was determined. Table 2 summarizes the frequences of the most intense

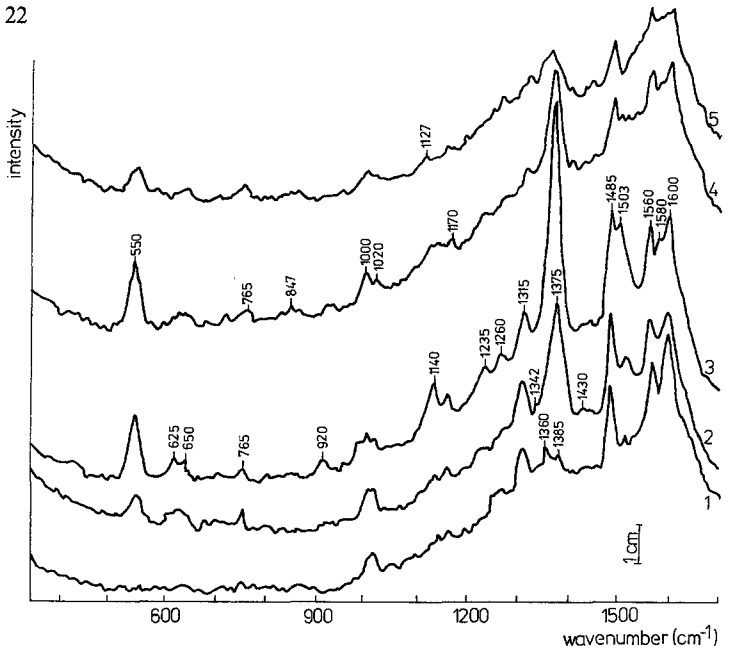

Fig. 2. SERS spectra of tryptophan in 0.1 *M* KCl at the silver electrode potentials 0.0 (1), −0.2 (2), −0.4 (3), −0.6 (4) and −0.8 (5). $\lambda_{ex} = 488$ nm, amino acid concentration 65.4 μM, pH 6.3, 25° C

Table 2. Wavenumbers and SERS spectra assignments of aromatic amino acids on the silver electrode (potential range from 0.0 to −1.4 V, 0.1 *M* KCl, pH = 6.3)

Wavenumber [cm^{-1}]	Assignment[a,b]		
	Trp	Tyr	Phe
520			Ag−N?
550	Ag−N?		
560		Ag−N?	
765	Benzene and pyrrole ring-breathing in-phase		
847	Skeletal vibration with appreciable NH pyrrole bend		
995			1 (in-plane)
1000, 1020	Benzene and pyrrole ring-breathing out-of-phase		
1030			12 (in-plane)
1127	9b (in-plane)		
1140	15 (in-plane)		
1170		9a (in-plane)	
1190			9a (in-plane)
1235	3 (in-plane)		
1265		7a (out-of-plane)	
1315	5π (in-plane)		
1342	14 (in-plane)		
1375	14π (in-plane)		
1385		19b (out-of-plane)	
1430	6π (in-plane)		
1462	19b (out-of-plane)		
1485	19 (out-of-plane)		
1500		19a (out-of-plane)	
1560	8b (out-of-plane)		8b (out-of-plane)
1600	8a (out-of-plane)		8a (out-of-plane)
1605		8a (out-of-plane)	

[a] Benzene mode numbering from Herzberg (1945)
[b] Pyrrole modes are indicated by π using numbering scheme from Herzberg (1945)

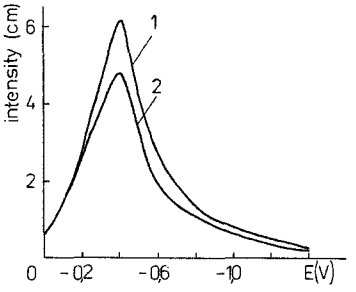

Fig. 3. Potential influence on the intensity of $\nu_{14\pi}$ band in the SERS spectrum of 65.4 (1) and 30 μM (2) tryptophan solutions in 0.1 *M* KCl, pH 6.3, 25° C. Potential shift direction from 0.0 to −1.4 V

SERS bands which are compared to the data described by Nabiev et al. (1981) and Nabiev and Chumanov (1986) and Rava and Spiro (1985).

Figure 2 presents the SERS spectrum of Trp as a function of the Ag electrode potential. Thus, on increasing the electrode positive charge, i.e. when the potential changes from −0.4 to 0.0 V, the intensity change of some bands in the SERS spectrum of Trp is not identical. For example, the signal intensity of the out-of-plane vibrations ν_{19} (1485, 1503 cm^{-1}) and ν_8 (1560, 1600 cm^{-1}) is increased. At the same time the intensity of spectral lines of the in-plane vibrations is decreased. This is particularly evident both for the benzene ring vibrations ν_{15} (1140 cm^{-1}), ν_3 (1235 cm^{-1}) and the pyrrole ring vibrations $\nu_{14\pi}$ (1375 cm^{-1}). This fact verifies the assumption made for the enzymes, i.e. with a shift of potential towards the negative region the aromatic rings of amino acid residues change their orientation from parallel to perpendicular with respect to the surface. Figure 3 shows the intensity dependence of the vibration $\nu_{14\pi}$ on the

Ag electrode potential at different amino acid concentrations. An intensity decrease of this line (and the whole spectrum) with increased negative potential values may be explained in terms of amino acid desorption at $E = -0.4$ V, which is confirmed by concentration dependence and incorporation of Ag atoms formed during the ORC into the electrode base. The latter process proceeds over the potential range from -1.2 to -1.4 V (Fleischman et al. 1974) and, evidently, determines the fact that no regeneration of the Trp spectrum occurs upon a potential change from -1.4 to 0 V. In addition, dependence of Fig. 3 may be caused by a short-range mechanism of SERS (Furtak and Roy 1985).

Conclusion

It follows from the results of SERS spectroscopy that the aromatic residues of adsorbed amino acids and enzymes change their orientation on the electrode surface as the potential changes. It was found that their planes were parallel to the surface if the electrode was charged positively, on the other hand, if the potential approached the zero change point of the silver electrode both parallel and perpendicular orientations occurred.

Orientational changes of the enzyme amino acid residues evidently reflect some conformational change of the protein, or a part of it, during the potential change. An overall manifestation of this process was revealed earlier in the electrochemical investigations carried out by Kuznetsov and Shumakovich (1984). They showed that the individual segments of a polypeptide chain in the adsorbed protein do not occupy an equivalent position – some of them are firmly adsorbed, whilst others extend in space, forming loops and branches. Changing the potential leads to some structural changes in the monolayer: some segments are removed from the surface, and others are drawn in by electrostatic field. These changes are reversible. SERS spectroscopy enables us to study these processes in more detail, at the level of individual amino acid residues.

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