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The chromophore-binding site of bacteriorhodopsin. Resonance Raman and surface-enhanced resonance Raman spectroscopy and quantum chemical study

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Abstract. Surface-enhanced Raman spectra of membrane protein, located in native mem brane, bacteriorhodopsin, adsorbed by silver electrodes and hydrosols have been obtained for the first time. The distance between the retinal Schiff's base and the external side of purple membrane of Halobacteriim halobiim was shown to be 6–9 A.

The possible distribition of the point charges aroind protonated retinal Schiff's base has been proposed on the basis of the resonance Raman data and quantim chemical CNDO/S-CI calculations. Such a model contains tyrosine residue located near the retinal Schiff's base and connected with COO^- groip *via* hydrogen bond COO^- group acts as a protonated Schiff's base counterion. The distance between oxygen atoms of COO^- group and retinal Schiff's base plane is 2·5-3·0A. The hydrogen bond (O-H. . .O^-) length between oxygen atom of OH-group and oxygen atom of COO^- group has been chosen 2·7±0·1Å Tyrosine hydroxyl group is located at 2·8-3·5 A from retinal Schiff's base plane·

It was shown that in contrast to generally accepted Honig and Nakanishi model the spectral properties of Brh570, K610, L550 and M4Ï2 forms of bacteriorhodopsin photocycle as well as observed tyrosine deprotonation and COO^- group protonation during M412 formation can be explained reasonably well by the suggested charge distribution· Furthermore, such a model of bacteriorhodopsin active site microenvironment allows to explain catalyzing of photo-induced protonated retinal Schiff's base deprotonation observed in our preliminary experiments.

Keywords. Bacteriorhodopsin; Raman effect; quantum chemical study; membrane protein.

Introduction

Bacteriorhodopsin (Brh) from purple membranes of Halobacterium halobium is a unique light energy-transducing pigment which pumps the protons across the cell membrane (Oesterhelt and Stoeckenius, 1971) Following the publication of the results of the electron microscopy and diffraction to a 7 Å resolution (Henderson and Unwin, 1975) as well as the amino acid sequence (Ovchinnikov *et al.,* 1978) the three-dimensional structure of Brh has been elaborated (Engelman *et al.,* 1980; Ovchinnikov, 1982). Various general molecular schemes for Brh functioning have been recently proposed (Honig *et al.,* 1979; Kalisky, 1981) Since its discovery 15 years ago, the purple membrane of Halobacterium halobium has become one of the most studied biological membranes (Ovchinnikov, 1982; Stoeckenius and Bogomolni, 1982) At the same time a number of serious problems concerned with Brh structure and function are still unresolved. Thus the spatial location of the retinal residue and molecular mechanism of light-induced electronic-conformational transformations in the Brh active site are of particular interest.

Using a surface-enhanced Raman spectroscopy (SERS) technique we studied the location of chromophore in purple membrane Furthermore, on the basis of resonance Raman and quantum chemical CNDO/S-CI study we have proposed the model of possible charge distribution in the Brh active site· Such a model in contrast to well accepted Honig and Nakanishi scheme (Nakanishi *et al.,* 1980) allows to explain reasonably well not only the chromophore spectral properties but experimentally observed changes of the state of some protein groups included in retinal Schiff's base microenvironment.

Materials and methods

Purple membranes (PM) were isolated from Halobacterium halobium (strain R_1 M_1) as described in Oesterhelt and Stoeckenius (1971). Apo-purple membranes, free of retinal oxime, and envelopes of Halobacterium halobium cells with the external surface only were prepared according to Tokunga and Ebrey (1978) and Blaurock *et al.* (1976), respectively.

Arylpolyene aldehydes (so-called "aromatic" analogs of retinal) of the type $X–C_6H_4–(CH=CH)_n–CHO$ (n =1–4, X = MeO) were a generous gift of Dr. A. V. Rodionov. The conversion of such analogs into apo-PM was performed as described in Shkrob (1981).

Concentration of Brh, "aromatic" analogs of retinal and chromoproteins was tested spectroscopically on a "Specord M40" (GDR) and "Beckman Acta MVI" (Austria) spectrophotometers.

Resonance Raman and surface-enhanced Raman spectra were acquired by exciting with 476·5 nm or 514·5 nm lines of Ar^+-laser "Spectra-Physics", model 164-03 (USA). Other details of the Raman instrumentation, electrochemical cell, potentiostat, electrodes and sols preparations have all been described (Nabiev *et al.,* 1981, 1983).

Resonance Raman (RR) and surface-enhanced Raman spectra were reproduced in more than three independent experiments to control the stability of the experimental conditions.

Method of calculation

The CNDO/S–CI molecular orbital method (Del Bene and Jaffe, 1968) was employed in this work to calculate absorption maxima and electronic structure. The S–CI calculations included 80 lowest energy single-excited configurations and involved both δ and π orbitals. The Mataga (Mataga and Nishimoto, 1957) formula was used to obtain the two center repulsion integrals.

We approximate the protonated and deprotonated all-trans and 13-cis retinal Schiff's base (RSB) using a $H–(CH=CH)_5CH=N^+H_2$ moiety in all-E and 3-Z conformations In this case the spectral properties and electronic structure of natural chromophore and the model used were the same A planar structure and standard geometry were assumed for all of the calculations (Efremov and Nabiev, 1985).

External point charges were incorporated into the diagonal element of the Fock matrix $F_{\mu\mu}$ so that:

$$F_{\mu\mu} = F^0_{\mu\mu} + \sum_{i=1}^{N} Q_i. \quad (1)$$

$$Q_i = \int \frac{Z_{Ai}\phi_\mu(j)\phi_\mu(j)}{R_{Ai}j} d\tau_j, \quad (2)$$

Where $F^0_{\mu\mu}$ is an unperturbed Fock matrix's element, Q_i is a Coulombic interaction. In the CNDO/S approximation Q_i can be replaced by the two center integral $\Gamma_{AIB'}$ where A_i and B represent the external charge Z_{Ai} and the atom to which the atomic orbital ϕ_μ belongs, respectively R_{Aij} is the distance between the external charge A_i and electron j on the orbital ϕ_μ. We have also assumed in calculations that the point charge values are equal to net charges located on atoms of COO^- and OH-groups.

A series of polyenes and cyanine dyes have been used to select an optimal CNDO/S parameters set to achieve a best fitting with the experimental λ_{max}. The optimal set of CNDO/S parameters (Ellis *et al.,* 1972) which displays the best fitting to experimental data has been used.

Results and discussion

Purple membranes and envelopes of Halobacterium halobium cells adsorbed on Ag-electrodes

The RR-spectrum of PM water suspension (10^{-5}M) and SERS-spectrum of native PM and envelopes of Halobacterium halobium cells adsorbed on silver electrode (10^{-5} M) are shown in figure 1. The main contribution to the mechanism of the anomalously large intensity of the Raman signals of molecules near the metal surface is connected with chemisorption and adsorbate-metal complexes formation (Otto *et al.,* 1982). The electrode anodization increases the point surface defects (adatoms) density on the Ag-electrode surface. The point defects serve as an "adsorbate sites" for molecules studied by SERS (Otto *et al.,* 1982).

The overlap of the metal and adsorbate atoms wavefunctions is responsible for SERS phenomenon arising. In such a case an additional electronic level corresponding to metal-adsorbate complex appears. An anomalously enhanced Raman intensity is observed upon the Raman spectra excitation by laser line in resonance with the main electronic transition of metal-adsorbate complex.

An enhancement mechanism connected with the chemisorption is known to have short-range character Removal of the molecule in question at a distance more than 5 Å (Koglin and Sequaris, 1983) results in the SERS intensity decreasing proportional to α/R^{10},where a is a coefficient depending on medium polarization properties and R is the distance between the molecule and the metal surface (Horia, 1982).

A comparison of the spectra of water suspended and Ag-adsorbed PM allows to conclude that the chromophore vibrational bands are unenhanced upon adsorption on Ag electrodes (figure 1). The broad structureless band at 1600-1610 cm^{-1} is assigned to the contribution of carbonized CO_2 on silver (Cooney *et al.,* 1980) As is evident from

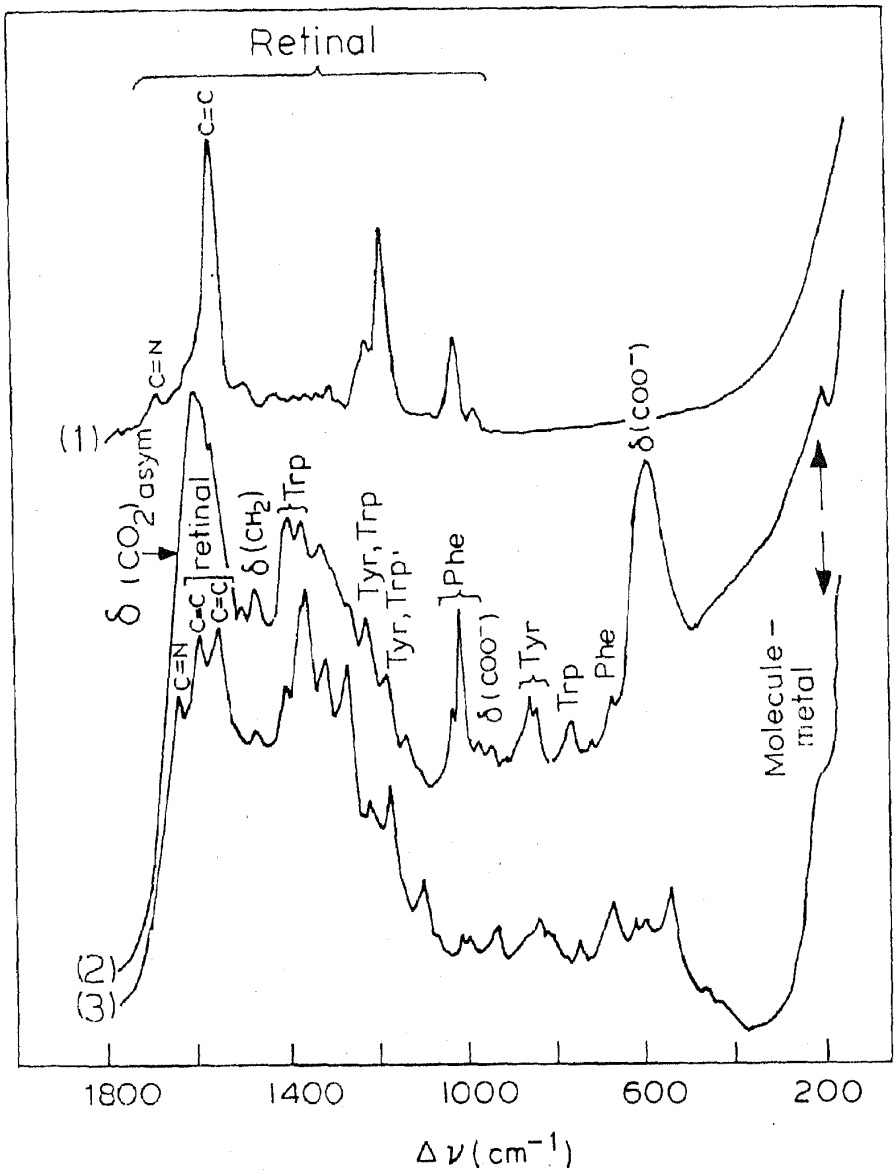

Figure 1. Resonance Raman spectrum of the purple membranes water suspension (10^{-5} M)—(1), and SERS-spectra of the purple membranes (2) and envelopes of Halobacterium halobium cells (3) adsorbed on Ag electrodes, [C]=10^{-7} M, 0·01N KCl, electrode potential is equal to -0·65V.

figure 1, a fine structure of the broad band has appeared in the SERS spectra of Halobacterium halobium cell's envelopes adsorbed on silver electrode. Such spectral features are connected with the chromophore vibrational bands enhancement upon adsorption. In this case SERS intensity of the chromophore assigned bands is increased by a factor more than 100 in comparison with the protein groups vibrational bands in the SERS spectrum of native PM.

The data obtained allow to make a conclusion that the retinal chromophore is located near the external surface of PM.

Purple membranes adsorbed on silver hydrosols

The adsorption of biomolecules on silver hydrosol is known to result in sol particles aggregation (Nabiev *et al.,* 1983).Therefore the PM external and cytoplasmic sides are believed to be in contact with metal surface.The resonance Raman spectra of the purple membrane water suspension and SERS spectra of PM adsorbed on silver hydrosol are shown in figure 2. The presence of the only chromophore-assigned bands in the SERS spectrum suggests our conclusion about near-surface topography of retinal in Brh.

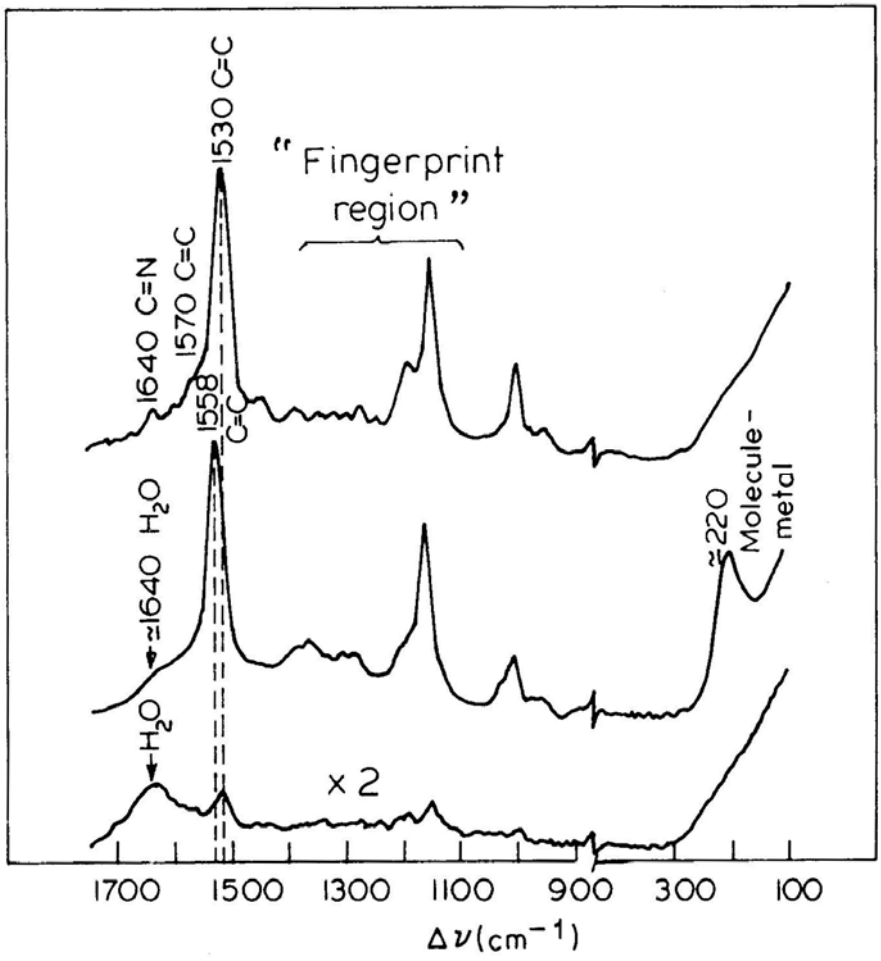

Figure 2. Resonance Raman spectra of the purple membranes water sispension:
(1): [C] = 10^{-5}M,
(3): [C] = 10^{-7} M (with a double increased sensitivity); SERS-spectrum of the purple membranes adsorbed on Ag colloid (2): [C] = 10^{-7} M.

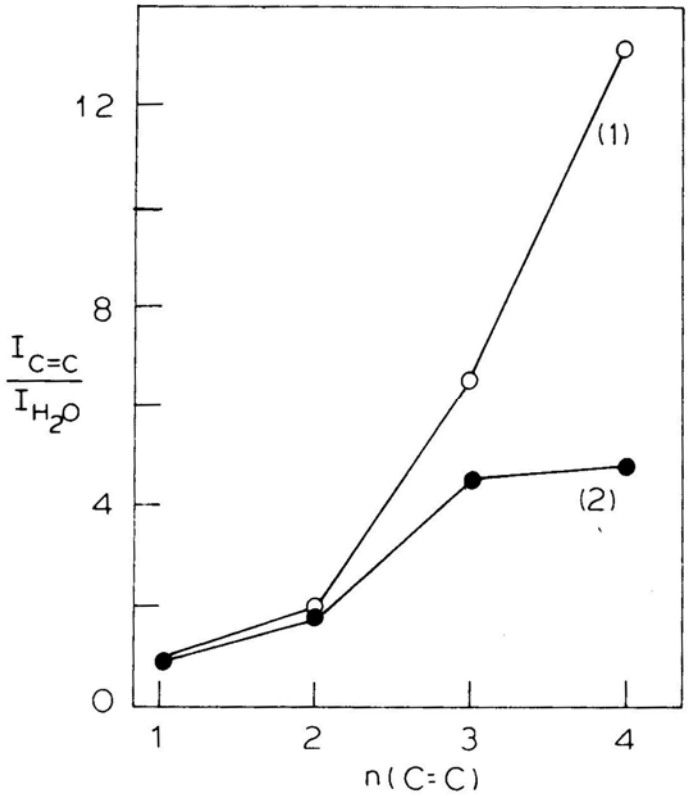

Figure 3. Plot of relative intensity of C=C vibrational bands observed in the SERS-spectra of free 'aromatic' analogs of retinal (1) and corresponding chromoproteins (2) adsorbed on Ag colloid *versus* the number of C=C conjugated bonds in the chromophore polyene chain (n).

The SERS phenomenon is known to appear if the π-electron system interaction with a metal atom includes at least a small number of π-electrons. Such an interaction is possible in the case when the π-orbitals and adatoms wave functions overlap· Thus, the presence of the only chromophore-assigned bands in SERS spectrum of PM proves the partial penetration of π-electron system into the double electrical layer whose depth is estimated to be about 5 Å (Koglin and Sequaris, 1983).

It should be noted that the 'fingerprint' regions (1100–1400 cm^{-1}) of the resonance Raman spectra of PM water suspension and the SERS spectra of adsorbed ones are quite similar. Therefore, the retinal polyene chain maintains the all-trans conformation upon adsorption on silver colloids.

SERS spectra of chromoproteins adsorbed on silver hydrosols

To determine more accurately the distance between the RSB and the membrane external side we have succeeded in obtaining the SER-spectra of several chromo-proteins reconstituted from apo-PM of Halobacterium halobium cells and "aromatic"

retinal analogs of varying chain length (Shkrob *et al*., 1981). In the chromoproteins these analogs are known to convert into the same binding site as the retinal in natural pigment. Figure 3 shows a minimal SERS intensity observed for the shorter chained analog. Such an effect is concerned with both the distance alteration between the chromophore and the metal surface and the increase of π-electrons number in the chain of C=C conjugated bonds.

To estimate the influence of the π-electron system strength alteration depending on C=C double bonds number (n) on the SERS intensity we have obtained SER-spectra of free "aromatic" analogs adsorbed on Ag colloids. It should be noted that the all "aromatic" analogs of retinal have an extended π-electronic system and its geometry of binding corresponds to minimal potential energy of the metal–adsorbate complex. For this reason we believe that for free analogs adsorbed on Ag particles the mean distance between the metal surface and adsorbate doesn't depend on the length of the polyene chain. Figure 3 shows a relative intensity of C=C vibrational bands observed in SER-spectra of chromoproteins (Henderson, 1975) and free analogs (Oesterhelt and Stoeckenius, 1971) versus the number of C=C conjugated bonds in the chromophore polyene chain (n). The curves differ greatly in appearance especially for analogs with three and four conjugated C=C bonds. At the same time as is evident from figure 3, the relative intensities as well as their changes upon $n = 1 \rightarrow n = 2$ transition are almost the same for both the chromoproteins and the free analogs adsorbed on Ag. Therefore, for shorter chained molecules the decrease of the intensity observed in SERS spectra of chromoproteins on Ag colloids is caused not only by the corresponding π-electron system alteration but the distance change between the adsorbate molecule and the metal surface too.

Thus we can conclude that in chromoproteins including analogs with one and two C=C conjugated bonds in polyene chain the chromophores are disposed farther than 5 Å from the metal.The results obtained allow to estimate the distance between RSB and the membrane external side. Since the diameter of spherical colloid particles is known to exceed ~ 150–200 Å (Nabiev *et al*., 1983) while the characteristic size of Brh trimer in PM is less than 60 Å (Stoeckenius and Bogomolni, 1982) one may suppose that the metal-adsorbate interface is the plane.

According to well-accepted model of Brh structural organization in PM the protein consists of seven transmembrane α-helical rods. Retinal residue is attached to the ε-amino group of Lys^{216} and the angle between the chromophore polyene chain and the normal to the membrane plane (θ) is estimated to be 71–78° (Rodionov *et al*., 1981; Heyn *et al*., 1977). Since available data indicate that the retinal analogs occupy about the same spatial position as the retinal residue in Brh, it is reasonable to suppose that in all chromoproteins investigated the depth of the aldimine bond from the membrane surface is the same and that the angle θ is close to its value in the native pigment.Taking into consideration all discussed above we have succeeded in calculating the distance between the aldimine bond in Brh and the external surface of PM. According to our estimations it is equal to 6-9 Å.

Figure 4 summarizes the current views including this work (Henderson and Unwin, 1975;Ovchinnikov *et al.,* 1978; Engelman *et al.,* 1980; Ovchinnikov, 1982; Rodionov *et al*., 1981; Heyn *et al*., 1977; Jubb *et al*., 1984) on the location of bacteriorhodopsin polypeptide chain and retinal in the PM.

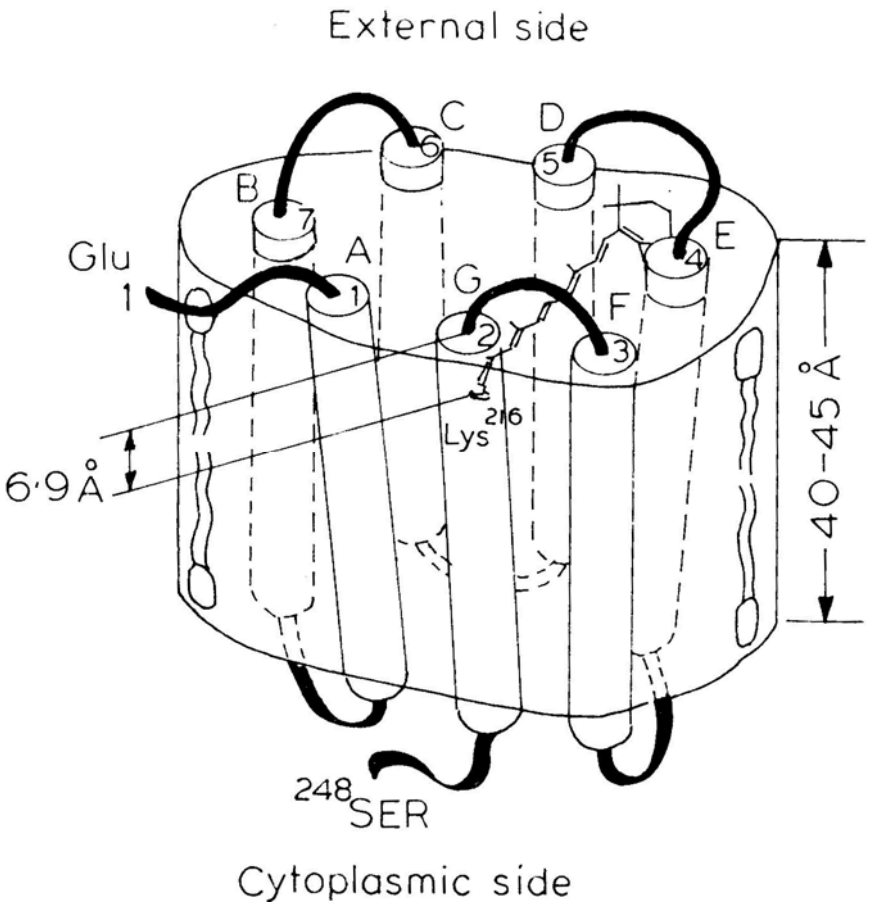

Figure 4. The scheme of BRh structural organization in the purple membrane (according to results of [2–5, 23–25] and this work).

It is remarkable that in this work we have succeeded for the first time in using a SERS phenomenon to study the membrane proteins adsorbed on silver electrodes and hydrosols.

Recently some biological molecules (nucleic acids and proteins) were investigated by means of SERS technique (Nabiev *et al*., 1981,1983; Koglin *et al.*, 1982; Cotton *et al*., 1980, 1981, 1982a, b; Copeland *et al*., 1984) not just to clarify the mechanism of the surface-enhanced Raman effect but also to demonstrate new possibilities in the use of the phenomenon in the study of molecular conformation and some kinetic processes at solution/electrode interfaces. SER spectra of nucleic acid components adsorbed at silver electrodes were presented by Koglin *et al.* (1982). Subsequently we have succeeded in obtaining the SERS spectra of some proteins and amino acids on silver electrodes (Nabiev *et al·,* 1981).Recently results for cytochrome C, myoglobin (Cotton *et al*., 1980), cytochrome Cd_1 (Cotton *et al*., 1981), some porphyrins (Cotton *et al*., 1982b) and reaction center from Rhodopseudomonas sphaeroides (Cotton *et al.,* 1982a) adsorbed on silver electrodes were published.

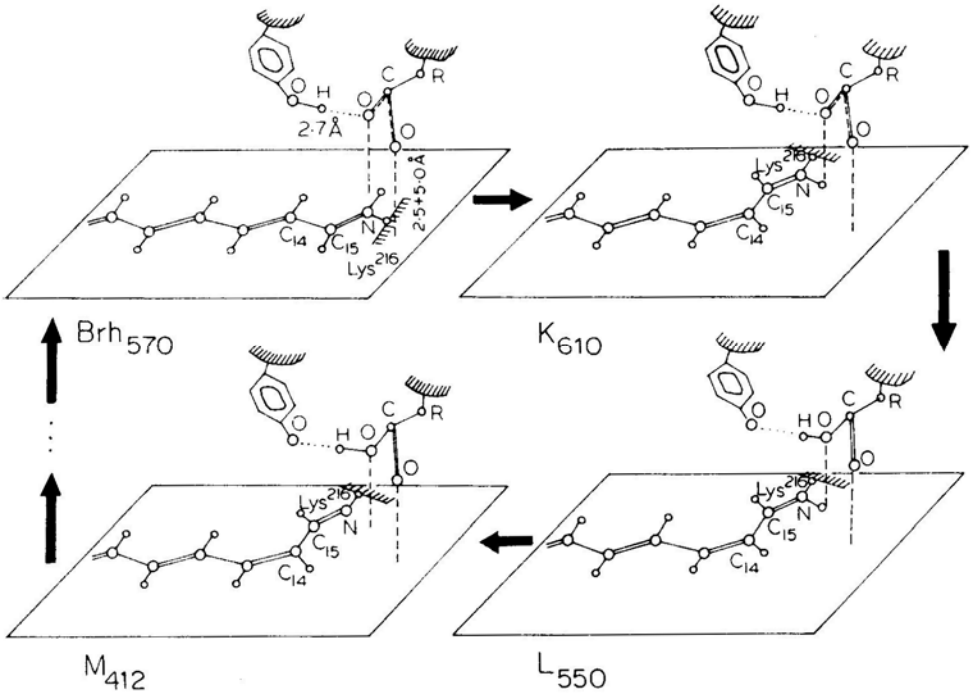

Figure 5. Proposed charge distribution in the chromophore-binding site of BRh during BRh_{57O} , K_{610}, L_{550} and M_{412} stages of the photocycle.

The fact that adsorption of biomolecules on metal surface seems to occur without significantly altering its essential conformational and chemical properties plays an important role in biological applications of SERS (Nabiev *et al*., 1981,1983). Therefore in each case it is necessary to resolve specially such a problem. Recently ,Copeland *et al.* (1984) have shown 86 % enzymatic activity of glucose oxidase on silver colloid, 95 % of the activity is recovered when the enzyme is displaced from the colloid by cyanide.

In this work we have demonstrated for the first time the possibility of SERS technique application to study such complex biological objects as membrane proteins. SERS is shown to provide unique information about membrane surface topography, the chromophore groups location within it as well as the processes on metal/membrane interface.

The chromophore-binding site model

We assume that the spectral properties of Brh are regulated by protein charged groups around the protonated retinal Schiffs base (PRSB). Approximating charged groups in chromophore microenvironment by point charges equal to net charges on atoms of these groups, we have proposed the possible distribution of external charges near PRSB. Such a model is based on the resonance Raman data (Kiselev *et al.,* 1985) and quantum chemical CNDO/S-CI calculations (Efremov and Nabiev, 1985) and contains a tyrosine

Table 1. Calculated and experimental spectral shifts (Δν) for various photoinduced transitions during Brh photocycle.

Transition	$\Delta\nu_{calc}$, cm^{-1}	$\Delta\nu_{exp}$, cm^{-1}
Protonated Schiff's base in solution → protonated Schiff's base in bacterioopsin	+3097	+(3293 ÷ 5187)
Brh570 → K610	+939	+1150
K610 → L550	−1449	−1790
L550 → M412	−5880	−6092
Brh570 → M412	−6390	−6732

residue located near RSB and connected with COO^- group *via* hydrogen bond (figure 5).

COO^- group of Asp or Glu residue forms a salt bridge with the positively charged PRSB and acts as a counterion producing a complex absorption near 470 m as is observed in solution (Honig *et al*., 1976). At the same time an isolated PRSB in vacuo would absorb at about 600 nm (Honig *et al*., 1976). Bathochromic shift of λ_{max} upon "solvent-bacterioopsin" transition is regulated by: (1) addition of positive external charge near PRSB (proton of tyrosine hydroxyl group) to COO^- group charges The distance between this proton and PRSB plane is equal to 2·8–3·5 Å, and (2) formation of a hydrogen bond with great proton polarizability (Merz and Zundel, 1983) between carboxylic group and tyrosine residue (OH. . .O^-, R = 2·7 ± 0·1 Å) Such a hydrogen bond with proton translocation OH . . . O^- ⇌ ^-O HO upon deprotonation of tyrosine residue and protonation of carboxylate group produces an efficiency redistributton of electronic density on atoms of COO^- and OH groups and results in large spectral shifts of λ_{max}. In contrast to well accepted Honig and Nakanishi scheme (Nakanishi *et al*., 1980) the model proposed (figure 5) doesn't demand an additional negative charge near *β* -ionone ring of retinal to account for red shift of λ_{max} in "solvent-bacterioopsin" transition.

The point charge distribution calculated is shown to reproduce the spectral shifts values observed for several initial stages of Brh photocycle. As is evident from table 1 the results obtained are an attractive fit to experimental data. The relative difference between predicted and experimental data for Δν is generally less than 15 %.

Figure 5 shows the retinal Schiff's base microenvironment for Brh570, K610, L550 and M412 spectral intermediates It should be noted that the theoretical predictions of λ_{max} in the model proposed are systematically less than the observed one.We suppose that an additional bathochromic shifts of λ_{max} may be induced by increasing solvent polarizability or by the presence of the water molecules in Brh chromophore-binding site (Hildebrandt and Stockburger, 1984). An efficient red shift upon Brh570→K610 transition is due to a change in charge separation caused by *trans-cis* isomerization of retinal. Deprotonation of tyrosine residue (Hess *et al*., 1979) and protonation of COO^- group (Siebert *et al*., 1982) seems to play an important role at the stage L55O The charge density redistribution thus induced results in a blue shift of $\lambda_{max.}$ Furthermore, a tyrosinate-ion appears close to aldimine bond and catalyzes photo-induced PRSB deprotonation (Kiselev *et al*., 1985; Gogel *et al*., 1981) which occurs simultaneously with the formation of M412.

We have shown that the spectral properties of Brh570, K610, L550 and M412 intermediates of Brh photocycle as well as observed tyrosine deprotonation and COO^- protonation during L550 and M412 formation could be explained reasonably well by the suggested chromophore binding site model Furthermore, such a model allows to explain catalyzing of photo-induced PRSB deprotonation observed in preliminary experiments (Kiselev *et al*., 1985; Gogel *et al*., 1981).

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