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Surface-enhanced resonance Raman spectroscopy of phycocyanin and allophycocyanin

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Abstract. High quality surface-enhanced resonance Raman (SERR) spectra were recorded from native and denatured phycocyanin and allophycocyanin on ascorbic acid treated silver hydrosols. The visible-excited SERR and resonance Raman (RR) spectra of the phycobiliproteins were very similar, indicating a predominantly electromagnetic surface enhancement mechanism. Investigation of pH-induced denaturation of allophycocyanin has shown that even small differences in protein/chromophore conformations are sensitively reflected by the SERR spectra. Concerning the adsorption of the protein to the metal surface, the experiments have shown that: (i) there is limited possibility for changing protein conformation during the adsorption process, (ii) there are no changes after the protein has been adsorbed onto the silver surface and (iii) for each protein an optimal activation of the silver sol has to be found for recording proper SERR spectra. The results obtained on phycobiliproteins are also discussed in connection with the interpretation of phytochrome Raman spectra.

Key words: Biliproteins – Phycocyanin – Allophycocyanin – Phytochrome – Surface-enhanced resonance Raman spectroscopy (SERRS)

Introduction

Phycobiliproteins, the light-harvesting pigments of cyanobacteria and red algae, are covalently bound open-chain tetrapyrrole-containing proteins with a structure optimized for efficient light absorption and energy transfer toward the photosynthetic reaction center (Gantt 1981). This process is primarily photo-physical and therefore requires precisely determined chromophore conformations and protein structures. Slight changes of either the chromophore conformations or the apoprotein structure strongly affect the functioning of these proteins.

In native phycobiliproteins the chromophores are in extended conformations (for phycocyanin chromophores the conformation is *Z-anti, Z-syn, Z-anti* according to X-ray studies (Schirmer et al. (1987)). Denaturing the protein (e.g. by decreasing the pH) causes folding of the chromophore because the perturbed apoprotein environment is no longer able to maintain the extended chromophore conformation (Scheer and Kufer 1977; Szalontai et al. 1987).

UV-excited resonance Raman (RR) spectroscopy of biliproteins revealed conformation-sensitive regions in the spectra (Szalontai et al. 1987, 1989). A band around 1 642 cm^{-1} in the UV-excited RR spectra was shifted to 1 625 cm^{-1} upon denaturation and its frequency could be used as a marker of folding of the chromophores. This band is complex by nature and can be decomposed into four components (A = 1 549 cm^{-1} – present only in the case of extended chromophores; B = 1 636 cm^{-1}; C = 1 621 cm^{-1} – present only in folded chromophores; D = 1 604 cm^{-1}) (Szalontai et al. 1991). The downshift of the 1 642 cm^{-1} complex band is the result of the disappearance of band A and the emergence of band C upon denaturing the protein.

Another band around 1 245 cm^{-1} lost intensity upon folding of the chromophores (Szalontai et al. 1987, 1989). This phenomenon was also observed in the coherent anti-Stokes Raman spectra (CARS) of phycocyanin (Schneider et al. 1987).

An important question in the interpretation of SERR spectra is whether the adsorbed biological compound has preserved its native conformation or not. Phycobiliproteins are ideal objects to study these problems because of their sensitive structure and because conformational changes of the chromophores of these proteins have been well characterized by RR spectroscopy as discussed above. Thus, by comparing SERR and RR spectra of phycobiliproteins it is possible to decide whether or not the chromophores preserve their original conformation during adsorption onto the silver surface.

Understanding the requirements for the SERR technique to yield reliable spectra from phycobiliproteins may also be important for another open-chain tetrapyrrole chromophore-containing protein, phytochrome. In particular, in explaining the differences between its

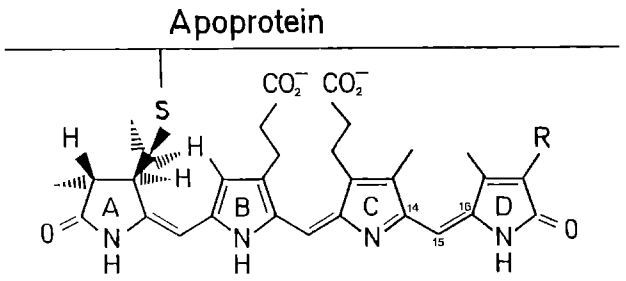

Fig. 1. Structural formula of the phycocyanobilin chromophore. $R=C_2H_5$ in phycocyanin and allophycocyanin, $R=C_2H_3$ in phytochrome

RR (Fodor et al. 1990; Tokutomi et al. 1990) and SERR (Farrens et al. 1989) spectra.

Phytochrome (P), the light sensory pigment of higher plants has two stable intermediates in its light-driven transformation cycle, the red adsorbing P_r form which is physiologically inactive and the far red-light adsorbing, physiologically active P_{fr} form. The two forms can be converted into each other using red (670 nm) and far red (730 nm) light, respectively (for a review on P see Schaffner et al. (1990)). Phytochrome is very similar to CPC and APC both in its chromophore (Fig. 1) and in certain features of the protein structure (de Kok et al. 1981). Owing to this similarity, our results obtained from the SERR spectra of phycobiliproteins may also be relevant in the study of phytochromes.

In the present communication we report the first SERR study on phycocyanin and allophycocyanin. SERR spectra were recorded in the pH range 7.0–1.6, thus studying trimer (pH 7.0), monomer (pH 4.0–3.0), and denatured (pH 2.5–1.6) phycobiliproteins. Upon monomerisation and denaturation very similar changes could be observed both in the SERR and RR spectra of phycobiliproteins. This similarity points to an electromagnetic enhancement mechanism in the SERR effect of these proteins. Interestingly, our studies show that the particular conformation that the protein had at the moment of adsorption is preserved by the protein-metal interaction.

Our primary goal was to demonstrate, by comparison of RR and SERR spectra, that SERR spectroscopy can be more reliable and informative than previously thought, so long as one finds the correct conditions for recording the SERR spectra.

Exploiting the great similarity of phycobiliproteins and phytochrome we propose assignments for certain bands in the Raman spectra of their chromophores.

Materials and methods

Phycocyanin and allophycocyanin were isolated from Synechococcus 6301 (formerly *Anacystis nidulans*) as described previously (Gombos et al. 1984). For Raman experiments the samples were prepared with dialysis at the required pH in 20 mM sodium phosphate buffer. pH values beyond the limit of the phosphate buffer were adjusted by adding the appropriate amount of 1 N HCl. Final concentrations of the RR samples were adjusted such that an OD between 30–50 was obtained at 620 nm for phycocyanin; this concentration was achieved by Centricon microconcentrators (Amicon). For SERR experiments the samples were used after dialysis without further concentration. The OD of the SERR samples was around 2.5 at 620 and 650 nm for phycocyanin and allophycocyanin, respectively.

Low temperature RR spectra were recorded as described earlier (Szalontai et al. 1987). SERR experiments were carried out on a Coderg PHO Raman spectrometer (spectral bandwidth 8 cm^{-1}, scan speed 50 cm^{-1}). For excitation the 488 nm line of an argon ion laser (Spectra Physics Stabilite 2016) was used. The laser power was 50–60 mW at the sample. Spectra were stored in an IBM XT-compatible (Schneider PC 1512) computer with 8 data point/cm^{-1} resolution. Band positions were determined after integration to 1 data point/cm^{-1}. Spectra in the figures were plotted after a further integration to 1 data point/3 cm^{-1} resolution.

SERR samples were prepared with silver hydrosols which were obtained according to the method of Hildebrandt and Stockburger (1984) by using 500 ml tridistilled water, 90 mg silver nitrate and 10 ml of a 1% solution of sodium citrate. We have previously demonstrated that this slightly alkaline hydrosol (pH 8.0–8.5) provides detailed SERR spectra from CPC (Debreczeny et al. 1989). However, with this type of sol we could not get an SERR spectra from APC. (More precisely, denatured APC yielded an SERR spectrum but native APC did not.) The SERR spectra of native APC could only be recorded when 65 µl of 1% ascorbic acid was added to 2 ml of silver sol prior to mixing it with 20 µl protein solution. The addition of this amount of ascorbic acid lowered the pH of the hydrosol to about 4.5. Both CPC and APC gave excellent SERR spectra in this sol. The final protein concentrations in these samples were in the range of 10^{-6}–10^{-7} M.

We have monitored the effect of the ascorbic acid addition to the sol by electron microscopy (Opton 902). Figures 2a–c show electron micrographs of the silver hydrosol at pH 8.0, 4.5 and 2.4, respectively. At pH 8.0 there are metal particles forming small aggregates. At pH 4.5 the particles are arranged in loose aggregates providing a much larger surface for the metal-protein interaction and probably a different distribution of the surface charges as compared to pH 8.0. In Fig. 2c the result of strong acidification is shown. On adding of 50 µl of 0.1 N HCl to 2 ml sol the colloid particles stick together and precipitate as shown by the large aggregates seen in Fig. 2c. Figure 2d shows the sol at pH 4.5 to which 20 µl CPC at pH 7.0 was added. Its structure remains loosely aggregated, as it was before adding the protein (compare Figs. 2b, d).

Results and discussion

Comparison of phycocyanin SERR and RR spectra

Figures 3 and 4 show that highly similar RR and SERR spectra can be obtained from CPC at different pH values

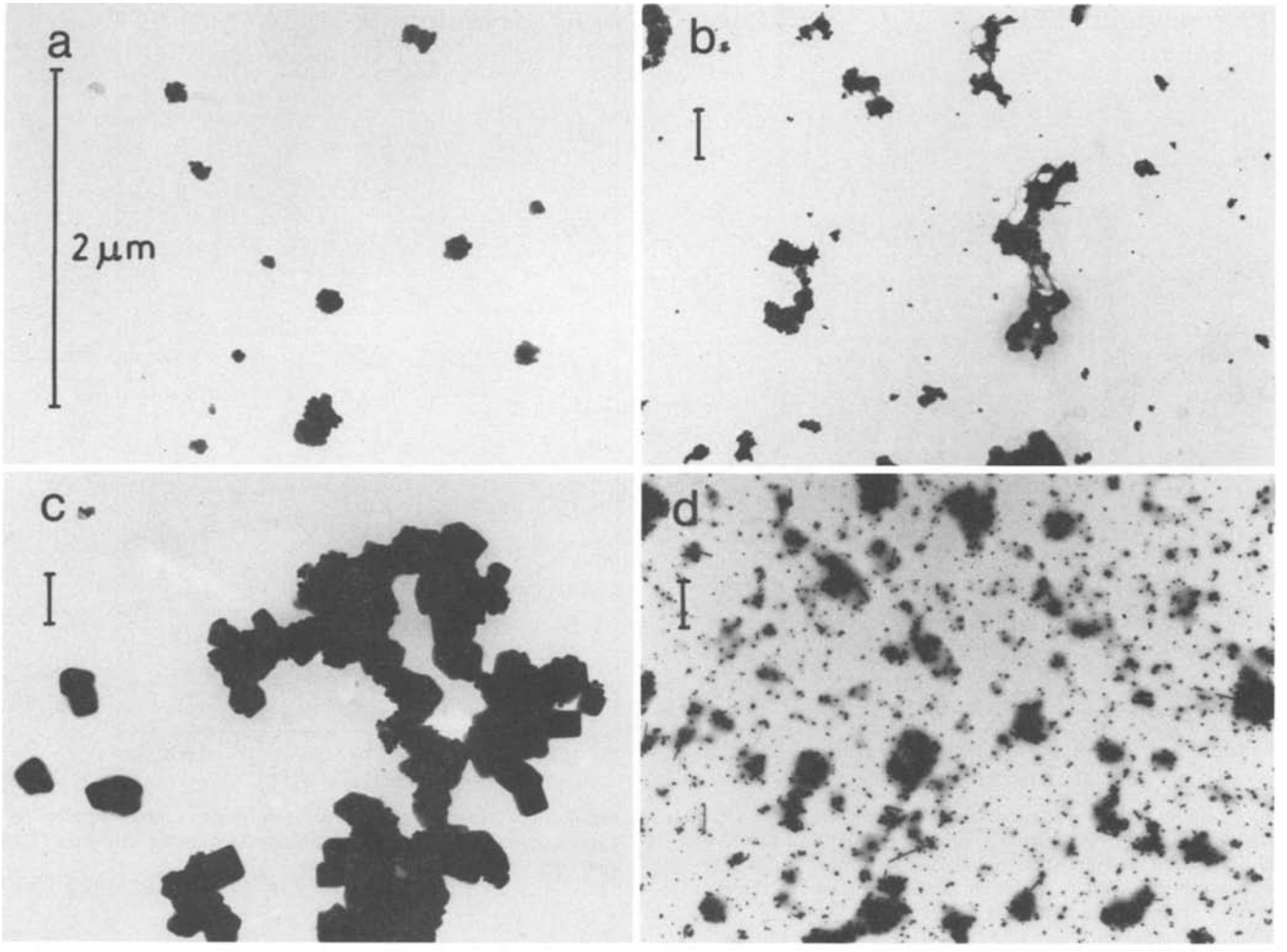

Fig. 2a–d. Electron micrographs of silver hydrosol. (**a**) – silver hydrosol at pH 8.0; (**b**) – at pH 4.5; (**c**) – at pH 2.4; (**d**) – sol at pH 4.5 when 20 µl pH 7.5 phycocyanin was added. Bars indicate 1 µm in each case. For details, see experimental section

after the silver sol has been properly prepared and activated. Both SERR and RR spectra were recorded with 488 nm excitation, at room temperature and at 30 K, respectively. Spectra at pH 7.0 are of trimeric CPC, at pH 4.0–3.0 they are of monomeric CPC, and at pH 1.5 they are of denatured CPC (Szalontai et al. 1987).

Comparing SERR (Fig. 3) and RR (Fig. 4) spectra of pH 7.0 CPC it is obvious that the same bands are present in both spectra, their frequencies agree very well. Only the relative intensities of certain bands vary. The 1 271 cm^{-1} band and the shoulder at 1 247 cm^{-1} are more intense in the RR spectra. The strongest band is around 1 647 cm^{-1} in the pH 7.0 SERR and at 1 652 cm^{-1} in the RR spectra.

A shoulder at 1 247 cm^{-1} is very weak in the SERR spectrum of pH 7.0 CPC. It is more intense in the case of monomeric APC (Fig. 5). Upon denaturation the 1 247 cm^{-1} shoulder disappears both in the SERR and RR spectra of CPC and APC (Figs. 3–5). The same phenomena were also observed in the RR spectra of CPC and APC (Szalontai et al. 1987, 1989).

The reduced intensity of the 1 247 cm^{-1} band in the CPC SERR spectra as compared to the RR spectra of CPC may indicate slight alterations in the chromophore conformations of the adsorbed protein.

A band around 1 597 cm^{-1} is the second strongest in both SERR and RR spectra of pH 7.5 CPC excited at 488 nm. This band is conformation-sensitive, it gradually loses intensity in both SERR and RR spectra with decreasing pH, i.e. upon folding of the chromophore. It has shown large isotope sensitivity (−8 cm^{-1}) on ^{14}N-^{15}N substitution (Szalontai et al. 1991 unpublished results). A strong band around the same frequency in the near infrared-excited Fourier Transform Raman spectrum of phytochrome showed downshift in deuterated samples (Hildebrandt 1991, personal communication). Taking into account both the conformation and isotope sensitivities we assign the 1 597 cm^{-1} band of the visible-excited RR and SERR spectra to ν (C=C) vibrations between rings C and D of the phycocyanobilin chromophore. Owing to its ^{15}N and ^{2}H isotope sensitivity this vibration should be coupled to the vibration of the NH group on ring D. Comparing our data later to the RR spectra of phytochromes a more specific assignment will be given, proposing that this band is characteristic for the C_{15}-*E,anti* conformation of the methin bridge.

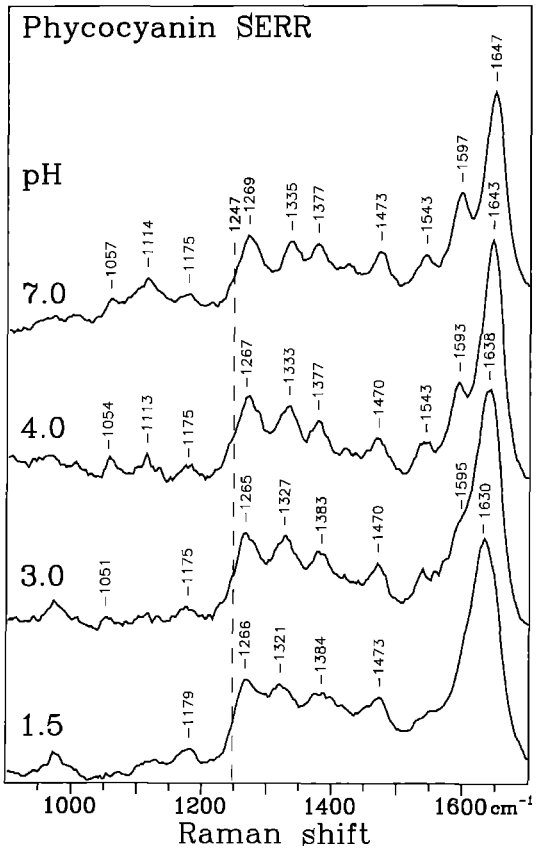

Fig. 3. Room temperature surface-enhanced resonance Raman spectra of C-phycocyanin from *Synechococcus 6301* on silver hydrosol. Excitation: 488 nm, spectral bandwidth: 8 cm^{-1} at 1 000 cm^{-1}, laser power: 50–60 mW. pH values indicate the pH of the protein solution prior to adding the pH 4 5 silver sol

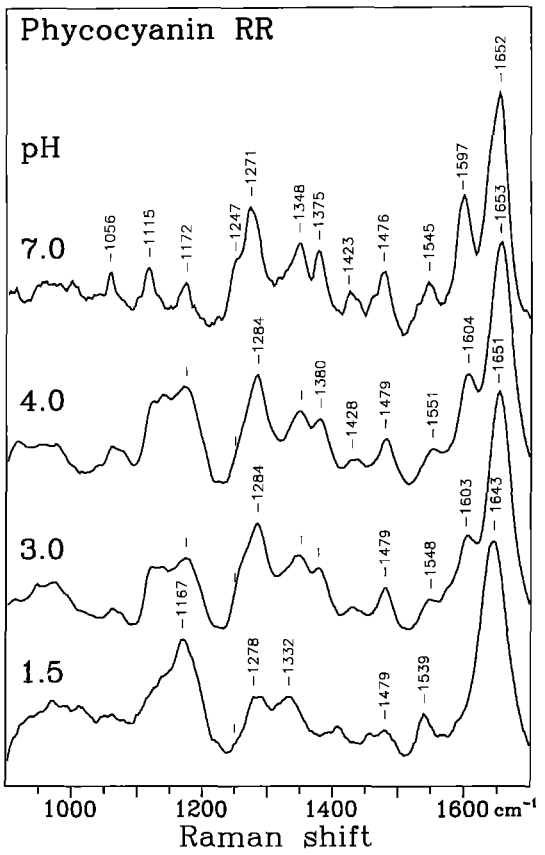

Fig. 4. Low temperature (30 K) resonance Raman spectra of C-phycocyanin. Excitation 488 nm, spectral bandwidth: 8 cm^{-1} at 1 000 cm^{-1}, laser power: 200 mW

Margulies and Toporowicz (1988) calculated a band around 1 594 cm^{-1} as a ν (C=C) vibration of the methin bridge between rings B and C of the phycocyanobilin chromophore in native phycocyanin at 488 nm excitation. Very recent experimental data on phytochrome show, however, that the ν (C=C) methin vibration at ring C is probably around 1 626 cm^{-1} (Kitagawa et al. 1991). In addition, the calculations concluded a *syn,anti,anti* molecular geometry for the phycocyanin chromophore in contrast to the *anti,syn,anti* configuration obtained from X-ray analysis (Schirmer et al. 1987).

The main bands of the SERR and RR spectra of CPC are at 1 647 and 1 652 cm^{-1}, respectively. These bands shift down upon decreasing the pH. The downshift is less in the RR (9 cm^{-1}) than in the SERR (16 cm^{-1}) spectra. We believe that in the above cases these downshifts reflect similar changes of chromophore conformations and are analogous to the downshift observed in the UV-excited RR spectra of CPC and APC (Szalontai et al. 1987, 1989).

Denaturation of allophycocyanin

The events which take place upon denaturing a phycobiliprotein are shown in higher pH resolution for APC in Fig. 5. The first spectrum at pH 3.3 corresponds to monomeric APC. At lower pH values APC is gradually denatured. This pH range was chosen because in the UV-excited RR spectra of APC the 1 642 cm^{-1} marker band was shifted down in the same pH range (Szalontai et al. 1989).

APC exhibits the same changes in its SERR spectra upon denaturation as CPC. The SERR spectra of APC (Fig. 5) changed monotonously by little alterations from spectrum to spectrum, i.e. the small variations of the protein/chromophore conformations caused by the slightly different pH in the subsequent samples are reflected in the SERR spectra. The small differences of the protein structures were also preserved in those cases when the pH of the protein solution was considerably different from the pH 4.5 of the sol at the moment of mixing.

The fact that all these spectra were stable in time shows that there were no post-adsorption modifications of the protein structures or chromophore conformations. In principle, such changes could have been initiated by the fact that pH of the medium was different from that of the adsorbed protein.

Analyzing the gradual changes of the SERR spectra of APC upon decreasing the pH (Fig. 5), it is apparent that different regions of the spectra behave differently.

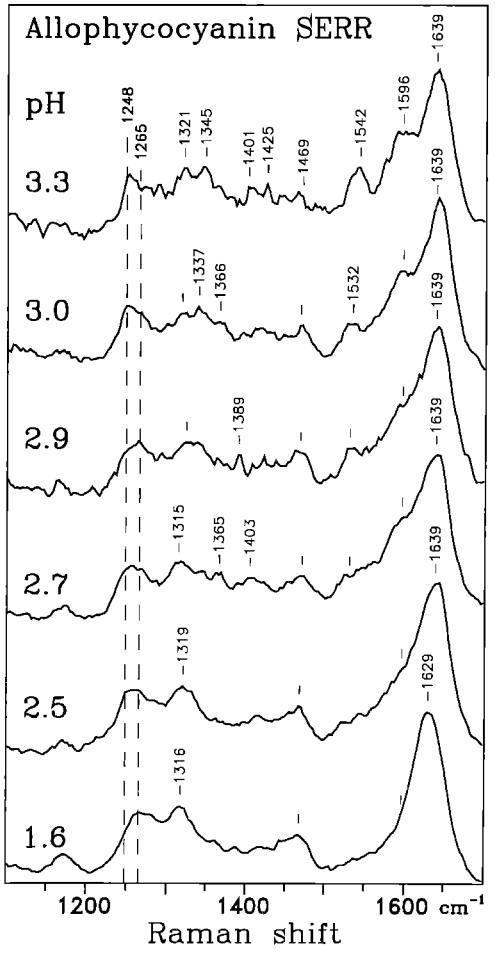

Fig. 5. Room temperature surface-enhanced resonance Raman spectra of allophycocyanin from *Synechococcus 6301* recorded on silver hydrosol in the pH range were allophycocyanin denaturation takes place. Experimental conditions as in Fig. 3

A band at 1 248 cm^{-1} has roughly the same relative intensity here as in the UV-excited RR spectrum of monomeric APC (Szalontai et al. 1989). This 1 248 cm^{-1} band loses intensity continuously over the whole pH range. It should be noted here that the 1 248 cm^{-1} band was more intense in the SERR spectrum of pH 7.0 trimeric APC (not shown) in accordance with the relative intensities seen both in the visible and UV-excited RR spectra of monomeric and trimeric APC (Szalontai et al. 1985, 1989).

A band around 1 265 cm^{-1} gains intensity step by step upon denaturing APC. The same phenomenon has also been observed in the RR spectra of APC (Szalontai et al. 1989). The band at 1 596 cm^{-1} gradually disappears as in the case of CPC. Its assignment is the same as that proposed earlier for CPC.

The downshift of the 1 639 cm^{-1} band is not monotonous. It remains constant until pH 2.5 and then shifts to 1 629 cm^{-1}. This downshift happens at somewhat lower pH values in the SERR spectra compared to what was seen in the UV-excited RR spectra (Szalontai et al. 1989). A possible explanation for this effect could be that during mixing the sol and the protein the pH 4.5 value of the sol may shift upward somewhat depending on the pH of the added protein solutions. The time for the pH effect is limited to the period of mixing because once the protein has been adsorbed to the silver surface no conformational change can take place. Thus, in the SERR spectra protein denaturation is seen at lower apparent pH values as compared to that of the RR spectra. This 'retardation' effect does not cause more than $\Delta pH = 0.5$ decrease of the apparent pH value of the marker band downshift.

Another interesting observation is that the overall signal-to-noise ratio increases considerably upon denaturation. On the other hand, several weaker bands of the spectra, especially in the 1 300–1 500 cm^{-1} region, are lost. The explanation could be that in denatured proteins the chromophores can be closer to the metal surface than in the native ones. Therefore the electromagnetic enhancement is larger. We have no evidence for chemical interaction between the chromophore and the silver surface which could be manifested by the presence of an Ag-N bond vibration for example. As regards the disappearance of the weaker bands during denaturation the same phenomenon can be observed in the RR spectra of CPC so this is not specific to the SERR spectra.

Comparison of CPC, APC and phytochrome Raman spectra

Because of the occasionally low signal-to-noise ratio and the very different experimental conditions of the published phytochrome Raman spectra only general remarks can be made on the use of our data in the interpretation of phytochrome Raman spectra. Since the way we have activated the silver hydrosol is very close to the method used by Farrens et al. (1989) our spectra can be best compared with the SERR/sol spectra of P_r and P_{fr} presented there.

Our experiments have shown that the protein structure is preserved upon adsorption to the metal surface and there are no possibilities for changes after the adsorption took place. The chances of altering the protein structure during the adsorption process are also very limited. This must be the reason why there were practically no differences between the P_r and P_{fr} electrode SERR spectra (Farrens et al. 1989) when transformation was achieved by actinic illumination of the adsorbed protein. In contrast, differences were found between P_r and P_{fr} SERR spectra when recorded from silver sol to which previously transformed P samples were added.

The major difference between P_r and P_{fr} Raman spectra is the presence of a band around 1 590 cm^{-1} regardless the excitation wavelength or the type (RR or SERR) of the experiments (Farrens et al. 1989; Tokutomi et al. 1990; Fodor et al. 1990; Kitagawa et al. 1991). We have seen this band in the Raman spectra of native CPC and APC, where chromophores have the C_{15}-*Z,anti* conformation. (See Schirmer et al. (1987) for CPC.) Upon folding the chromophore, which most probably involves a C_{15}-*Z,anti* to C_{15}-*Z,syn* transition, the band around 1 596 cm^{-1} disappears in the Raman spectra of phycobiliproteins. According to Rüdiger et al. (1983) P_{fr} has

C_{15}-*E* configuration at the methin bridge connecting rings C and D of the phytochrome chromophore but it was not specified whether the conformation of this methin bridge is *syn* or *anti*. Fodor et al. (1990) have suggested that P_{fr} should have a C_{15}-*E,anti* conformation. Their red-excited P_{fr} RR spectrum was dominated by a band around 1 599 cm^{-1}. Thus we suggest that the band found around 1 590–1 599 cm^{-1} in different Raman spectra of biliproteins should correspond to the *anti* conformation of the methin bridge connecting rings C and D of the tetrapyrrole chromophore.

In summary, from the SERR experiments on CPC and APC it can be concluded that (i) one can find experimental conditions where high quality SERR spectra can be recorded on silver hydrosol from any forms of these two proteins. The spectra are very similar to the RR spectra excited in the same electronic transition. Therefore, in native proteins the electromagnetic enhancement mechanism probably dominates. (ii) The actual protein conformation is only slightly altered during the adsorption process and it does not change further after the protein had been adsorbed onto the metal surface.

The analysis of phytochrome data suggests that earlier published (Farrens et al. 1989) SERR spectra of P_r and P_{fr} reflect genuine protein structures. The practically identical electrode SERR spectra of the two forms of phytochrome were obtained owing to the fixing of one of the forms by the metal surface which could not then be altered by actinic light. Comparison of several phytochrome and phycobiliprotein Raman spectra support the assignment of a band seen around 1 590–1 599 cm^{-1} to the *anti* conformation of the methin bridge connecting rings C and D of the chromophore.

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