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Structure of Poly(Ethylene Glycol)-Modified Horseradish Peroxidase in Organic Solvents: Infrared Amide I Spectral Changes upon Protein Dehydration Are Largely Caused by Protein Structural Changes and Not by Water Removal Per Se

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ABSTRACT Fourier transform infrared (FTIR) spectroscopy has emerged as a powerful tool to guide the development of stable lyophilized protein formulations by providing information on the structure of proteins in amorphous solids. The underlying assumption is that IR spectral changes in the amide I and III region upon protein dehydration are caused by protein structural changes. However, it has been claimed that amide I IR spectral changes could be the result of water removal per se. Here, we investigated whether such claims hold true. The structure of horseradish peroxidase (HRP) and poly(ethylene glycol)-modified HRP (HRP-PEG) has been investigated under various conditions (in aqueous solution, the amorphous dehydrated state, and dissolved/suspended in toluene and benzene) by UV-visible (UV-Vis), FTIR, and resonance Raman spectroscopy. The resonance Raman and UV-Vis spectra of dehydrated HRP-PEG dissolved in neat toluene or benzene were very similar to that of HRP in aqueous buffer, and thus the heme environment (heme iron spin, coordination, and redox state) was essentially the same under both conditions. Therefore, the three-dimensional structure of HRP-PEG dissolved in benzene and toluene was similar to that in aqueous solution. The amide I IR spectra of HRP-PEG in aqueous buffer and of dehydrated HRP-PEG dissolved in neat benzene and toluene were also very similar, and the secondary structure compositions (percentages of α-helices and β-sheets) were within the standard error the same. These results are irreconcilable with recent claims that water removal per se could cause substantial amide I IR spectral changes (M. van de Weert, P.I. Haris, W.E. Hennink, and D.J. Crommelin. 2001. *Anal. Biochem.* 297:160–169). On the contrary, amide I IR spectral changes upon protein dehydration are caused by perturbations in the secondary structure.

INTRODUCTION

Analyzing the structure of proteins in the amorphous dehydrated state (Prestrelski et al., 1993a; Griebenow and Klibanov, 1995; Griebenow et al., 1999a; Dong et al., 1995) and after exposure of such dehydrated protein samples to neat organic solvents (Griebenow and Klibanov, 1996, 1997; Griebenow et al., 1999b, 2001; Vecchio et al., 1999; Sirotkin et al., 2001; Santos et al., 2001) by Fourier transform infrared (FTIR) spectroscopy has become an important tool to address questions of pharmaceutical and biotechnological relevance. For example, it has recently been demonstrated that dehydrated enzymes are most active in organic solvents when their structure and molecular mobility are most similar to that in water (Griebenow et al., 2001). The versatility of FTIR spectroscopy in the development of solid protein formulations (Carpenter et al., 1998; Griebenow et al., 1999a) and in the stabilization of proteins during encapsulation and release from biocompatible polymers (Griebenow et al., 1999c; Pérez et al., 2002) has recently been reviewed extensively. This period of increasing knowledge on the structure of dehydrated protein powders by FTIR spectroscopy has been initiated by the pioneering work of Prestrelski et al. (1993a). Their findings and arguments lead to the conclusion that changes of amide I IR absorbance upon protein dehydration are caused by protein structural distortions. Additional support for this notion emerged from a study by Griebenow and Klibanov (1995), who investigated the amide III region of the model protein bovine pancreatic trypsin inhibitor. They showed that the degree of structural distortions found by FTIR spectroscopy qualitatively agreed with results from ^{1}H/D-exchange NMR spectroscopy (Desai et al., 1994). It seemed that these three articles solved a longstanding dispute. Before this, several research groups postulated that protein dehydration does not cause substantial structural distortions. Changes in the IR spectrum (amide I) were attributed solely to water removal (Careri et al., 1979; Rupley et al., 1983; Rupley and Careri, 1991). Others disputed this notion with results mainly based on Raman spectroscopic investigations and argued in favor of structural changes to occur upon protein dehydration (Yu and Jo, 1973; Yu, 1974; Baker et al., 1983; Poole and Finney, 1983a,b, 1984), in agreement with theoretical expectations (Kuntz and Kauzmann, 1974). Recently, van de Weert et al. (2001) reinvestigated this issue. Their results led them to claim that IR spectral changes are not necessarily indicative of protein structural perturbations but may also be caused by the removal of water per se. They argue the amide I absorbance depends on the protein's hydration level because of the hydrogen bonding sensitivity of the

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peptide carbonyl bond. This led them to recommend that highly dry protein samples should not be used in the analysis of protein structure, unless specific lyoprotectants are used.

In view of the high importance of FTIR spectroscopy for the analysis of protein structure the above issue has to be finally resolved. FTIR spectroscopy is among the few established (but sometimes disputed) and technically simple methods to analyze protein structure in the amorphous solid state because it is insensitive to scattering. Other methods not based on vibrational spectroscopy, such as ^{13}C and ^{15}N solid-state magic angle spinning NMR (Burke et al., 1989, 1992), ^{1}H/D-exchange NMR (Desai et al., 1994; Desai and Klibanov, 1995; Wu and Gorenstein, 1993), and EPR spectroscopy (Affleck et al., 1992), which also have been used occasionally to analyze protein structure after dehydration, either allow only for a quite localized view of structural changes after labeling (Affleck et al., 1992; Burke et al., 1992) or are indirect in nature (Desai et al., 1994; Wu and Gorenstein, 1993; Desai and Klibanov, 1995). The latter fact sometimes led to quite opposing interpretation of the data collected for lyophilized enzymes (see e.g., the interpretation of ^{1}H/D-exchange NMR data by Wu and Gorenstein (1993) and Desai et al. (1994)).

It is evident that it is of utmost importance to finally overcome the dilemma by finding an example that directly and indisputably shows whether amide I IR spectral changes are caused by protein structural changes or by water removal per se. Only by means of such direct evidence can it be decided whether or not dehydration per se causes changes to the amide I spectrum. An ideal tool for this purpose would be a protein that exhibits very similar structures in the dehydrated and hydrated state. Fortunately, Mabrouk (1995) and Mabrouk and Spiro (1998) have provided a test case. Based on resonance Raman and EPR experiments they showed that poly(ethylene glycol) (PEG)-modified and dehydrated horseradish peroxidase (HRP-PEG) has a native-like structure when dissolved in the organic solvent benzene. Because HRP-PEG can be dissolved in benzene up to high concentrations required for FTIR experiments, it is an ideal system to check whether or not the amide I absorbance is significantly affected by changes of the solvent. Our results unambiguously show that the secondary structure of HRP-PEG is similar in aqueous solution and (after lyophilization) in toluene and benzene. We further elaborate the theoretical expectations for the effect of water on amide I protein spectra by invoking the current knowledge on amide I excitonic coupling (Torii and Tasumi, 1992a,b) and amide I-water coupling in small model peptides (Chen et al., 1994; Sieler and Schweitzer-Stenner, 1997; Han et al., 1998). This analysis corroborates the notion that the removal of water does not obfuscate the structure analysis of proteins by means of the amide I profile in IR spectra.

MATERIALS AND METHODS

Chemicals

Peroxidase (type II) from horseradish (HRP) (Reinheitszahl 2), 1 N HCl, and sodium bicarbonate buffer salts were purchased from Sigma-Aldrich (St. Louis, MO). M-PEG-succinimidyl propionate (mPEG) with a molecular weight of 5000 was purchased from Shearwater (Huntsville, AL). Benzene was purchased from Fisher Scientific (San Juan, PR). Fluorescamine and 2,4,6-trinitrobenzenesulfonic acid (TNBSA) were purchased from Pierce (Rockford, IL).

Preparation of HRP-PEG

HRP was covalently modified with mPEG as described by Mabrouk (1995). HRP (200 mg) and mPEG (203 mg) were dissolved in 20 ml of 0.1 M sodium borate buffer (pH 9.2) to achieve an approximate molar ratio of 1:3 (solvent-accessible lysine residues in HRP-to-mPEG) and stirred for 3 h at 4°C. The reaction was quenched by the addition of 20 ml of 0.1 M potassium phosphate buffer (pH 7.0). Nonreacted mPEG and buffer salts were removed by dialysis of the reaction mixture in bags with an exclusion cutoff of 6000–8000 from Spectra Medical Industries (Laguna Hills, CA) three times against 1 L of nanopure water (18 MΩ resistance) for 4 h. Subsequently, HRP-PEG was lyophilized.

Lyophilization

Dilute aqueous solutions of HRP-PEG were rapidly frozen in liquid nitrogen and lyophilized for 3 days using a 6-L lyophilizer (model 77530, Labconco, Kansas City, MO) at a condenser temperature of −45°C and a pressure of <60 μm of Hg. Lyophilized protein powders were kept at −20°C until used in the experiments over activated molecular sieves. Before use, the powders were allowed to equilibrate to room temperature to avoid moisture sorption upon opening of the storage vessels.

Determination of the extent of mPEG modification

The average number of mPEG-modified amino groups in HRP was determined with 2 ± 1 when using the fluorescamine method (Stocks et al., 1986; Karr et al., 1994) and with 4 ± 1 when using the TNBSA method (Habeeb, 1966). Thus, the amount of mPEG modification was approximately three residues per HRP molecule.

The fluorescamine method was performed as follows: mPEG-modified and nonmodified HRP was dissolved in 1.5 ml of 0.2 M borate buffer, pH 9, to achieve concentrations between 0 and 0.3 mg/ml. To each vial, 0.5 ml of fluorescamine solution (0.3 mg/ml in acetone) was added followed by vortexing for 5 min. Then the solutions were allowed to rest for 1 min before the fluorescence emission intensity at 475 nm ($\lambda_{exc} = 390$ nm) was determined with a computerized Cary Eclipse fluorescence spectrophotometer (Varian, Walnut Creek, CA) in a quartz cuvette with 10-mm pathlength. All fluorescence intensities were corrected with those obtained with buffer. Then, for both sets of samples (HRP and HRP-PEG), fluorescence intensity values were plotted versus the HRP concentrations. The percentage of modification was calculated using the formula [1 − (slope of HRP-PEG/HRP)] × 100 and was 49.6% ± 1.4% in three trials. Considering that four amino groups of seven present in HRP are solvent accessible, approximately two amino groups were PEG modified.

We also used the TNBSA method introduced by Habeeb (1966) to estimate the amount of PEG modification. This method has the advantage that the protein is unfolded in the procedure by HCl and SDS, and thus all seven amino groups are solvent accessible in the test. The assay was performed as follows: HRP and HRP-PEG were dissolved in 1 ml of 0.2 M sodium bicarbonate buffer (pH 8.5) to achieve concentrations between 0.1

and 0.8 mg/ml. To these solutions and buffer blanks 0.5 ml of 0.01% TNBSA (w/v), 0.5 ml of 10% SDS solution (w/v), and 0.25 ml of 1 N HCl were added. The mixtures were incubated at 37°C for 2 h, and subsequently their absorbance at 335 nm was determined with a computerized Shimadzu 160 UV-Vis spectrophotometer using 10-mm path-length quartz cuvettes. All absorbance values obtained were corrected with those obtained using buffer blanks and plotted versus the protein concentration. The percentage of PEG modification was calculated using the formula [1 − (slope of HRP-PEG/HRP)] × 100, and the extent of modification was 53% ± 7% corresponding to 4 ± 1 modified amino groups.

UV-Vis spectroscopy

UV-Vis spectra were recorded at room temperature using a computerized spectrophotometer (160, Shimadzu, Columbia, MD) and quartz cells with 10-mm path-length. Approximately 1 mg/ml HRP was dissolved in potassium phosphate buffer at pH 7 and pH 12. To obtain spectra in benzene and toluene, HRP-PEG lyophilized from pH 7 and pH 12 was dissolved in the solvents to achieve a concentration of 1 mg/ml.

FTIR spectroscopy

FTIR spectra were measured using a Magna IR 560 optical bench (Nicolet, Madison, WI) as described (Carrasquillo et al., 2000). The optical bench was purged with dry N_2 gas to reduce water vapor IR interference, and for each spectrum 256 scans at 2-cm^{-1} resolution were averaged. Solutions of HRP in phosphate buffer (pH 7) and in D_2O were measured at a concentration of 50 mg/ml (~1 mM) using a 6-μm spacer in a liquid cell equipped with CaF_2 windows at room temperature. Measurements of HRP-PEG in toluene and benzene were performed by dissolving 20–30 mg of lyophilized HRP-PEG in the respective solvent by sonication in an ultrasonication bath for 1 min in a CaF_2 cell with a 25-μm spacer. For suspensions in organic solvents, lyophilized HRP was subjected to 1 min of sonication in an ultrasonication bath and measured in a CaF_2 cell with a 50-μm spacer. Powders of HRP-PEG and HRP were measured as KBr pellets using 1 mg of HRP per 200 mg of KBr. The protein-KBr mixture was homogenized using a mortar and pestle and then pressed into the pellet as described (Prestrelski et al., 1993a; Griebenow and Klibanov, 1995). When necessary, spectra were corrected for the background in an interactive manner.

Secondary structure content was determined by Gaussian curve fitting of the spectra after resolution enhancement by Fourier self-deconvolution in the amide I (Griebenow and Klibanov, 1996, 1997) and using the original protein-vibrational spectra in the amide III (Griebenow and Klibanov, 1995) with the program GRAMS/32 (Galactic Industries, Salem, NH). The number of components and their peak position were determined by second derivation and used as starting parameters (Griebenow and Klibanov, 1995). Second-derivative spectra were smoothed with an 11-point smoothing function (10.6 cm^{-1}). Each sample was measured at least five times. The determined peak wavenumbers in the second-derivative spectra, as well as those and the areas of the fitted Gaussian bands, were averaged, and the standard deviations were calculated. The secondary structure content was calculated from the areas of the assigned Gaussian bands. The thus obtained amide I bands were primarily assigned according to the literature (Holzbaur et al., 1996; Griebenow and Klibanov, 1996). For the aqueous solution, the bands at ~1659 cm^{-1} and at 1650 cm^{-1} were assigned to α-helices, bands at 1627 cm^{-1} and 1690–1697 cm^{-1} to β-sheet, and all other bands to other secondary structures, such as β-turns, nonrepetitive (random coil) secondary structure, and extended chains. The amide I band assignment has been verified and refined within this paper. Amide III bands were assigned according to Griebenow and Klibanov (1995). Bands with peak frequencies from 1229 to 1237 cm^{-1} were assigned to β-sheet, those from 1248 to 1290 cm^{-1} to other (β-turns, random coil, and extended chains), and those from 1290 to 1337 cm^{-1} to α-helix secondary structures.

Overall structural perturbations occurring upon protein dehydration or upon dissolving the protein in different solvents were quantified by calculating the spectral correlation coefficient (SCC) from the amide I second-derivative spectra. The SCC value is a measure for the degree in difference between two spectra: for identical spectra the value is 1; for those with nothing in common it is 0. The second-derivative spectra used were stored as ASCII *xy*-pair data sets, and the SCC values were calculated by using the program Sigma Plot (Jandel Scientific, Chicago, IL) as described by Prestrelski et al. (1993a) and Griebenow et al. (1999a) for the individual spectra of each sample with respect to the spectrum of native HRP in aqueous buffer at pH 7.0 (Griebenow and Klibanov, 1995).

Raman spectroscopy

Raman spectra were measured in backscattering geometry with 457.9 nm from an argon ion laser (Lexel 95, Cambridge Laser Laboratories, Freemont, CA). The laser beam was focused onto a sample in a quartz cell mounted in a macro-chamber at room temperature. The scattered light was collimated and collected by an imaging lens system, dispersed by a triple-grating spectrometer (Jobin-Ivon, Edison, NJ), and recorded by a liquid-nitrogen-cooled CCD camera (CCD3000 from Jobin-Ivon). Four samples were used in the measurements: 1) native HRP in potassium phosphate buffer (pH 5.6) at a concentration 40 mg/ml, 2) HRP-PEG in potassium phosphate buffer (pH 6.5) at a concentration 58 mg/ml, 3) HRP-PEG in benzene at a concentration 55 mg/ml, and 4) HRP-PEG in toluene at a concentration 60 mg/ml. The spectra were usually taken with a laser power of 5 mW and an acquisition time of 10–15 min. Spectral resolution was 3.8 cm^{-1}. The spectra were calibrated using the 1605-cm^{-1} line of benzene with an of accuracy 1 cm^{-1}. Using pure solvents as reference, all solvent bands were subtracted from the spectra.

RESULTS

In a recent paper van de Weert et al. (2001) questioned the common practice of interpreting changes of amide I IR absorption upon protein dehydration as being largely caused by protein structural perturbations. The authors argued that the spectral changes could also be caused by the removal of water per se. Thus, the authors tried to reinitiate the 20-year-old discussion, in which some groups argued that changes in the IR amide I spectra upon dehydration are largely caused by protein structural changes while others interpreted the spectral changes strictly as being the result of changes in the physicochemical environment because of the removal of water. The primary goal of this work was to find a direct proof for one of the two conflicting interpretations. A direct proof of the structural perturbation hypothesis would have been achieved if one could have shown the similarity of amide I IR spectra for a protein that has been proven to have a similar structure hydrated in water and dehydrated in organic solvents by independent experiments. In the following we show that HRP meets this criterion.

UV-Vis spectra of HRP and HRP-PEG

HRP was modified with PEG-5000 following an established procedure (see Materials and Methods). HRP-PEG is soluble (up to 1 mg/ml) in many organic solvents, such as tetrahydrofuran (THF) and dioxane. However, concentra-

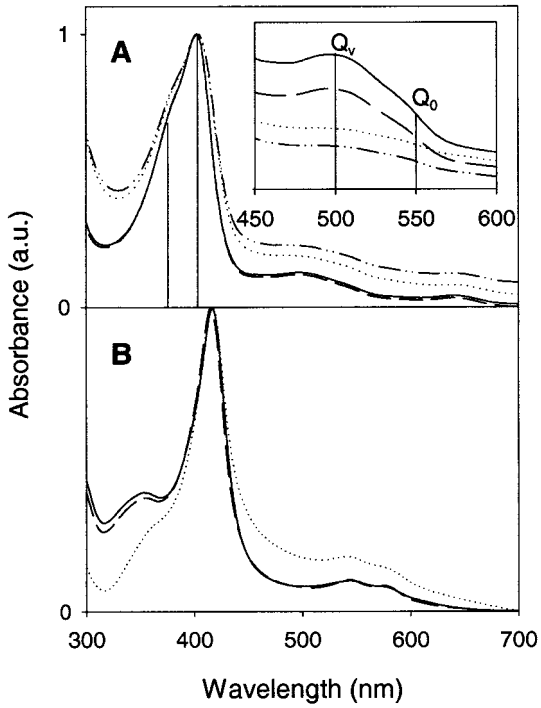

FIGURE 1 (*A*) UV-Vis absorption spectra of HRP (——) and HRP-PEG (— — —) in water at pH 6.5 and of HRP-PEG lyophilized from water at pH 6.5 and dissolved in toluene (· · ·) and benzene (— · · —). The inset shows the region of the Q-absorption bands. (*B*) UV-Vis spectra of HRP (——), HRP-PEG (— — —) in water at pH 12 and of HRP-PEG lyophilized from water at pH 12 and redissolved in benzene (· · ·).

tions of at least ~10 mg/ml required for FTIR measurements could be achieved only in toluene and benzene. UV-Vis spectra were acquired for HRP in buffer and for HRP-PEG in buffer, toluene, and benzene to investigate whether changes in the coordination, spin, and redox state of the heme iron occurred upon PEG modification and dissolution in the organic solvents (Fig. 1 *A*). The spectra are remarkably similar except for some scattering that led to a baseline offset for the spectra of HRP-PEG in organic solvents. The maximum of the Soret absorption band was found at 402.5 nm for HRP and HRP-PEG in aqueous buffer at pH 6.5. The maximum was slightly shifted to 404 nm for HRP-PEG lyophilized from water, pH 6.5, and dissolved in toluene and benzene. A similar red-shift was earlier obtained for a HRP C complexed with benzohydroxamic acid (Howes et al., 1997). This suggests that the shift may be caused by binding of benzene and toluene in the heme-binding pocket where the delocalized ring systems of the aromatic molecules and the heme group can electronically interact (Mabrouk and Spiro, 1998). Such small absorption shifts also accompanied the exchange of residues in the heme-binding pocket by point mutation (Howes et al., 1997) and thus could also be indicative of slight changes in the heme environment. A shoulder in the spectra at ~380 nm is also visible for all samples. An absorption increase at ~380 nm has sometimes been attributed to the presence of unfolded HRP (Smulevich et al., 1997). Because there is no increase of the shoulder in benzene and toluene it must be concluded that HRP does not unfold upon exposure to the organic solvents. In addition, the maxima of the Q-absorption bands appear at nearly the same wavelength for all samples. However, the spectra of HRP-PEG in benzene and toluene in this region appear somewhat broadened. The same observation was made when switching the iron state to hexacoordinated low spin by changing the pH to 12. This causes the binding of the strong ligand OH^-, which stabilizes the iron's low spin state. This gives rise to significant changes in the UV-Vis spectrum (Fig. 1 *B*). The maximum for HRP and HRP-PEG was at 416 nm at pH 12. We also measured the UV-Vis spectra of HRP-PEG lyophilized from pH 12 upon dissolving it in benzene and toluene. The spectra were very similar to that of HRP in water at that pH (Fig. 1 *B*). Thus, our optical spectra suggest that dissolving HRP-PEG in benzene and toluene does not cause any changes of the iron's oxidation, spin, and coordination states, in agreement with earlier work by Mabrouk (1995) and Mabrouk and Spiro (1998). Our results are also consistent with what is known as molecular memory upon protein dehydration and exposure to organic solvents (Klibanov, 1995; Mishra et al., 1996; Costantino et al., 1996).

Raman spectroscopy on HRP and HRP-PEG

As an additional check of the active site, we measured resonant Raman spectra of HRP and HRP-PEG in buffer (Fig. 2, *A* and *B*, respectively) and of HRP-PEG in benzene (Fig. 2 *C*) and toluene (Fig. 2 *D*) with 457.9-nm excitation. To allow for comparison, the spectra were all scaled onto the same peak intensity of the ν_4 band at 1373 cm^{-1}. The spectrum of native HRP in aqueous buffer (pH 5.6) is shown in Fig. 2 *A*. The assignment of the Raman bands is based on the work of Howes et al. (2001). The ν_4 band is an oxidation marker. The observed wavenumber is diagnostic for the Fe^{3+} state (Spiro, 1985). The two bands at 1492 and 1499 cm^{-1}, assignable to the spin marker ν_3, represent coexisting pentacoordinated highspin (pc-hs) and quantum-mixed-spin (qms) states, respectively (Smulevich et al., 1994; Howes et al., 2001). Obviously, the band at 1499 cm^{-1} is the more intense one, indicating that the qms state is predominant (Q. Huang, M. Laberge, J. Fidy, R. Schweitzer-Stenner, submitted). The wavenumber positions of other spin marker bands indicated in Fig. 2 *A* (ν_{11}, ν_{10a}, ν_{10b}, and ν_{10c}) are also consistent with the coexistence of pc-hs and pc-qms states. A comparison of all Raman marker bands in the high-frequency region (1350–1700 cm^{-1}) between HRP (Fig. 2 *A*) and HRP-PEG (Fig. 2 *B*) in aqueous solution does not reveal significant differences between both wavenumbers and intensities. The spectra in Fig. 2, *A* and *B*, are almost eclipsing when overlapped onto each other. This shows that PEG modification does not cause even subtle

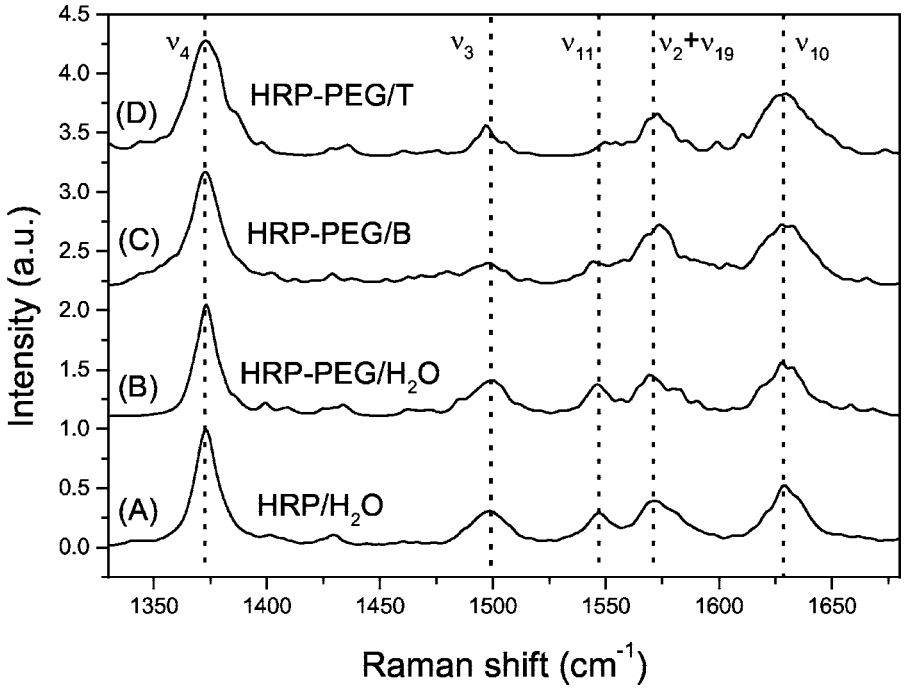

FIGURE 2 Raman spectra (1350–1700 cm^{-1}) of native HRP in water (pH 5.6) (*A*) and HRP-PEG in water (pH 6.5) (*B*), benzene (denoted as HRP-PEG/B) (*C*), and toluene (denoted as HRP-PEG/T) (*D*). In organic solutions, the Raman bands from solvents have been subtracted. For comparison of relative intensities, all spectra are plotted with intensity of ν_4 normalized to 1.

changes of the heme pocket structure. All marker bands of native HRP were found at almost the same positions in the spectra of HRP-PEG in benzene and toluene. The overall band profiles are quite similar, although some variations of the half-widths and intensities are detectable: ν_4 and ν_{10}, for instance, appear slightly broadened, and the intensities of ν_3 and ν_{11} bands are somewhat reduced. Preliminary measurements of the depolarization ratios of these lines suggest that the heme group is more asymmetrically distorted for HRP-PEG in the applied organic solvents (Q. Huang, W. Al-Azzam, K. Griebenow, R. Schweitzer-Stenner, submitted). This could be brought about by slight reorientations of the proximal histidine (Schweitzer-Stenner, 1989). Any major structural changes, however, are ruled out by the observed Raman spectra.

FTIR spectra of HRP and HRP-PEG in H_2O and D_2O: assignment issues

FTIR spectra were collected for HRP and HRP-PEG in aqueous buffer and in D_2O at pH/pD 6.5. The spectra acquired were corrected for the solvent background, subjected to resolution enhancement by Fourier self-deconvolution, and analyzed by Gaussian curve fitting for their secondary structure content. The results of the spectral analysis by second-derivative calculation and Gaussian curve fitting are assembled for all samples in Table 1. The aqueous spectrum of HRP (Fig. 3 *A*) shows one remarkable feature that deserves some attention: two bands at ~1660 and 1650 cm^{-1} have to be assigned to α-helix secondary structure to allow for a reasonable correlation of their relative intensities with the secondary structure content estimated from the x-ray crystallographic data (Henriksen et al., 1999) deposited in the Protein Data Bank (entry 6ATJ; Berman et al., 2000) by means of the algorithm of Kabsch and Sander (1983) (Tables 1 and 2). To confirm this band assignment, FTIR spectra were also analyzed in the amide III spectral region. This strategy has been successfully used before to ascertain the correct assignment of amide I IR bands (Griebenow et al., 1999a; Carrasquillo et al., 2000). By using the band assignments of Griebenow and Klibanov (1995) (Table 1) we found that the secondary structure composition determined from the relative amide III intensities is identical to that obtained from the above analysis of amide I (Table 2) and also agrees with the analysis of the x-ray structural data. This strongly suggests that the two amide I bands have to be assigned to α-helix secondary structure.

Interestingly, a marked difference between the two bands was found when the protein was dissolved in D_2O. Whereas the α-helix band at 1660 cm^{-1} did not show any frequency shift and no notable change in the area contribution (Table 1), the band at 1652 cm^{-1} in H_2O shifted notably to lower frequencies by 5 cm^{-1}. It also substantially increased in area, and thus vibrational modes from other secondary structures than just α-helix must contribute to this band in D_2O.

To find an explanation for the spectroscopic findings, the x-ray structural coordinates were scrutinized using the visualization programs RasMol and Protein Structure Explorer. The structural data were analyzed with respect to 1) the length of the α-helices, 2) the number of hydrogen bonds formed in them per residue, and 3) the number of

TABLE 1 Band position and areas of the component bands resolved by Gaussian curve fitting and second derivatization in the amide I and III region for representative HRP samples

Sample/state	Second-derivative band position (cm^{-1})	Gaussian curve-fitting		Assignment*
		Band position (cm^{-1})	Area (%)	
HRP in D_2O, pD 6.5	1682 ± 1	1682 ± 1	8 ± 1	Other
	1673 ± 1	1673 ± 1	7 ± 1	Other
	1659 ± 1	1659 ± 1	27 ± 1	α-Helix
	1647 ± 1	1647 ± 1	39 ± 2	α-Helix
	1635 ± 2	1635 ± 1	13 ± 2	Other
	1627 ± 1	1629 ± 1	8 ± 1	β-Sheet
HRP in buffer, pH 6.5	1683 ± 2	1685 ± 2	9 ± 5	Other
	1673 ± 2	1675 ± 2	13 ± 4	Other
	1658 ± 3	1660 ± 1	31 ± 2	α-Helix
	1652 ± 2	1650 ± 1	20 ± 3	α-Helix
	1638 ± 2	1638 ± 1	17 ± 1	Other
	1626 ± 1	1626 ± 2	10 ± 1	β-Sheet
HRP in buffer, pH 6.5, amide III	1338 ± 1	1337 ± 1	5 ± 1	α-Helix
	1320 ± 1	1319 ± 1	23 ± 1	α-Helix
	1305 ± 1	1306 ± 1	10 ± 2	α-Helix
	1290 ± 2	1292 ± 1	11 ± 2	α-Helix
	1270 ± 2	1273 ± 4	20 ± 6	Other
	1255 ± 2	1256 ± 7	7 ± 2	Other
	1248 ± 2	1246 ± 2	15 ± 4	Other
	1237 ± 2	1235 ± 1	10 ± 1	β-Sheet
HRP-PEG in buffer, pH 6.5	1682 ± 1	1682 ± 1	14 ± 1	Other
	1671 ± 3	1672 ± 1	9 ± 2	Other
	1660 ± 1	1660 ± 1	30 ± 3	α-Helix
	1650 ± 1	1649 ± 1	24 ± 2	β-Helix
	1641 ± 1	1638 ± 1	15 ± 1	Other
	1625 ± 1	1627 ± 1	8 ± 1	β-Sheet
HRP-PEG in benzene	1682 ± 1	1686 ± 2	10 ± 3	Other
	1676 ± 1	1676 ± 1	13 ± 3	Other
	1658 ± 1	1661 ± 1	29 ± 3	α-Helix
	1650 ± 1	1649 ± 1	26 ± 3	α-Helix
	1637 ± 1	1637 ± 1	15 ± 2	Other
	1629 ± 1	1627 ± 1	7 ± 2	β-Sheet
HRP-PEG in toluene	1682 ± 1	1684 ± 1	14 ± 1	Other
	1675 ± 2	1673 ± 1	15 ± 2	Other
	1658 ± 1	1658 ± 1	32 ± 3	α-Helix
	1651 ± 1	1648 ± 1	18 ± 1	α-Helix
	1637 ± 2	1637 ± 1	15 ± 2	Other
	1630 ± 1	1629 ± 2	6 ± 1	β-Sheet
HRP-PEG powder	1694 ± 1	1696 ± 2	7 ± 1	β-Sheet
	1684 ± 1	1685 ± 1	14 ± 2	Other
	1675 ± 1	1674 ± 1	16 ± 1	Other
	1659 ± 1	1659 ± 1	35 ± 3	α-Helix
	1648 ± 3	1646 ± 2	13 ± 2	α-Helix
	1635 ± 2	1635 ± 2	11 ± 2	Other
	1627 ± 1	1629 ± 2	3 ± 1	β-Sheet
HRP powder	1695 ± 1	1697 ± 1	8 ± 1	β-Sheet
	1685 ± 1	1685 ± 1	14 ± 1	Other
	1672 ± 1	1672 ± 1	17 ± 2	Other
	1654 ± 1	1655 ± 1	40 ± 3	α-Helix
	1638 ± 1	1642 ± 1	6 ± 1	Other
	1630 ± 1	1632 ± 1	16 ± 1	β-Sheet
HRP powder in KBR amide III	1337 ± 1	1338 ± 1	2 ± 1	α-Helix
	1329 ± 1	1330 ± 1	5 ± 2	α-Helix
	1315 ± 2	1318 ± 2	9 ± 2	α-Helix
	1300 ± 4	1307 ± 3	13 ± 2	α-Helix
	1290 ± 2	1293 ± 4	16 ± 2	α-Helix
	1273 ± 4	1278 ± 5	12 ± 3	Other
	1263 ± 1	1264 ± 3	12 ± 1	Other

(*Continued*)

TABLE 1 (*Continued*)

Sample/state	Second-derivative band position (cm^{-1})	Gaussian curve-fitting		Assignment*
		Band position (cm^{-1})	Area (%)	
	1248 ± 2	1250 ± 2	12 ± 2	Other
	1237 ± 1	1238 ± 1	13 ± 1	β-Sheet
	1229 ± 2	1227 ± 1	10 ± 1	β-Sheet
HRP powder in benzene	1700 ± 2	1699 ± 1	2 ± 1	β-Sheet
	1684 ± 1	1685 ± 1	13 ± 2	Other
	1675 ± 2	1675 ± 1	12 ± 2	Other
	1656 ± 1	1656 ± 1	53 ± 1	α-Helix
	1639 ± 1	1639 ± 1	11 ± 1	Other
	1631 ± 1	1631 ± 1	9 ± 1	β-Sheet
HRP powder in THF	1692 ± 2	1692 ± 4	7 ± 1	β-Sheet
	1683 ± 2	1684 ± 1	12 ± 4	Other
	1676 ± 1	1673 ± 1	14 ± 2	Other
	1655 ± 1	1656 ± 1	37 ± 2	α-Helix
	1643 ± 2	1644 ± 1	17 ± 2	Other
	1630 ± 1	1631 ± 1	13 ± 1	β-Sheet

*Other secondary structure elements include turns and nonrepetitive secondary structures (a.k.a. random coil or unordered). Because the assignment of amide I and III IR bands to turn secondary structure is uncertain, no such assignment has been done in this work.

water molecules in hydrogen-bonding distance of up to 3.5 Å per residue (Table 3). It became immediately apparent that there are basically two groups of α-helices in the molecule: long ones and short ones (Fig. 4). When defining long α-helices as having at least 10 residues, they contribute to 34% of the secondary structure of HRP. This relates closely to the area contribution of the α-helix amide I IR band at 1660 cm^{-1} (31%). Shorter α-helices with less than 10 residues contribute to 14% of the structure of HRP. This seems to indicate that the low-frequency amide I band at 1650 cm^{-1} (20%) is to a major extent assignable to short helices, whereas the high frequency results mostly from longer helical segments. This interpretation, however, is at

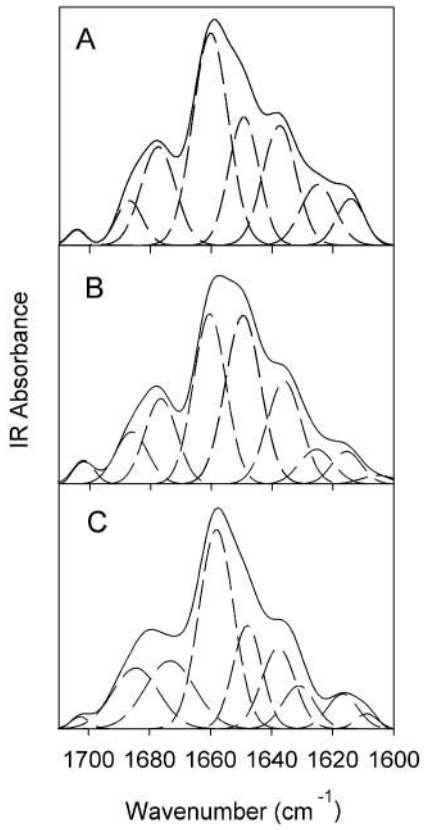

FIGURE 3 Gaussian curve fitting of the resolution-enhanced amide I spectra of HRP in water, pH 6.5 (*A*) and HRP-PEG in benzene (*B*) and toluene (*C*). The solid lines represent the original spectra overlaid with the results of the fits, which practically coincide; the broken lines are the individual Gaussian bands fitted to the spectra.

TABLE 2 Secondary structure of HRP under different conditions

Sample	Secondary structure* (%)			SC†
	α-Helix	β-Sheet	Other	
HRP crystal structure‡	45	2	53	NA
HRP in buffer				
Amide I	50 ± 5	10 ± 1	40 ± 3	0.96 ± 0.03
Amide III	49 ± 2	10 ± 1	41 ± 2	ND
HRP powder				
Amide I	40 ± 2	23 ± 2	37 ± 1	0.80 ± 0.01
Amide III	41 ± 2	22 ± 1	38 ± 2	ND
HRP suspension in THF	36 ± 2	18 ± 4	47 ± 3	0.76 ± 0.01
HRP suspension in benzene	53 ± 1	11 ± 1	36 ± 1	0.86 ± 0.01
HRP-PEG in buffer	54 ± 2	10 ± 1	36 ± 1	0.93 ± 0.03
HRP-PEG powder	48 ± 3	10 ± 1	42 ± 3	0.85 ± 0.05
HRP-PEG solution in benzene	54 ± 2	10 ± 2	36 ± 2	0.94 ± 0.01
HRP-PEG solution in toluene	48 ± 2	8 ± 1	43 ± 1	0.95 ± 0.01

NA, not available; ND, not done.

*Secondary structure was caculated from the individual Gaussian bands fitted to the amide I and III IR spectra. For the band assignment, see Table 1.

†The SCC was calculated for the amide I second-derivative spectra of HRP in buffer versus the second-derivative spectra of HRP under the various conditions.

‡Obtained from sequence details for crystal structure 6ATJ at the Protein Data Bank.

TABLE 3 Analysis of the α-helices found in the x-ray structure of HRP

α-Helix	Residues	Number of residues	Contribution to secondary structure (%)	H-bonds/ residue*	H_2O/residue†
1	14–28	15	4.6	0.9	1.5
2	32–44	13	4.3	0.7	0.8
3	77–90	14	4.7	0.7	1.2
4	97–111	15	4.9	0.7	0.7
5	131–137	7	2.4	0.4	2.2
6	145–154	10	3.3	0.6	1.8
7	159–166	8	2.6	0.6	1.0
8	181–184	4	1.3	0	1.3
9	199–208	10	3.3	0.6	1.2
10	232–238	7	2.4	0.4	0.9
11	245–252	8	2.6	0.6	1.8
12	257–268	11	3.7	0.8	1.6
13	270–284	15	4.9	0.7	1.2

Data were obtained from the Protein Data Bank (Berman *et al.,* 2000) and were calculated for entry 6ATJ using the algorithm from Kabsch and Sanders (1983) for the automatic determination of protein secondary structure composition.

*Hydrogen bonds were visually counted using the program RasMol, which is freely available on the Internet.

†The water molecules were counted manually using the program Protein Structure Explorer from Eric Martz (freely available on the Internet). It was assumed that water molecules in a van der Waals distance of up to 3.5 Å to the C=O or N–H peptide backbone groups could potentially form a hydrogen bond with those groups.

odds with the well-established fact that the amide I frequency decreases with increasing helix length (Torii and Tasumi, 1992a). However, the situation is more complicated because short helices are unlikely to exhibit a single amide I band. On the contrary, because of lesser excitonic coupling, a broad, asymmetric distribution with several maxima is displayed (Torii and Tasumi, 1992b). In longer helices, excitonic coupling focuses the most intensity into a single band assignable to an overall in-phase (A-type) combination of individual amide I vibrations (Torii and Tasumi, 1998). In view of this theoretical background we propose that the high-frequency amide I band is predominantly such an A-type mode of the long helices in HRP. The low-frequency amide I is most likely composed of some E-type contributions from the long helices and of the low-wavenumber band of the spectra of the short helices. In fact, calculations of Torii and Tasumi (1992b) suggest that this low-wavenumber band is the most intense one in the short-helix spectra. The frequencies of the localized modes of the short helices are certainly more susceptible to a mixing with water-bending modes (Chen et al., 1994; Sieler and Schweitzer-Stenner, 1997). It is therefore not surprising that only the low-frequency amide I band downshifts upon H/D exchange.

No physically meaningful relationships could be established between the number of hydrogen bonds per residue in the α-helices or the number of water molecules in a distance of 3.5 Å and the area of the two amide I IR bands (data not shown).

In conclusion, it was verified that the band assignment used in the literature by assigning two amide I IR bands to α-helix secondary structure is realistic. Furthermore, a likely explanation for this consists in the observation that there are two groups of α-helices with varying lengths.

FTIR spectra of HRP-PEG in benzene and toluene

FTIR spectra were acquired for HRP in buffer and HRP-PEG in toluene and benzene (Fig. 3). The resolution-en-

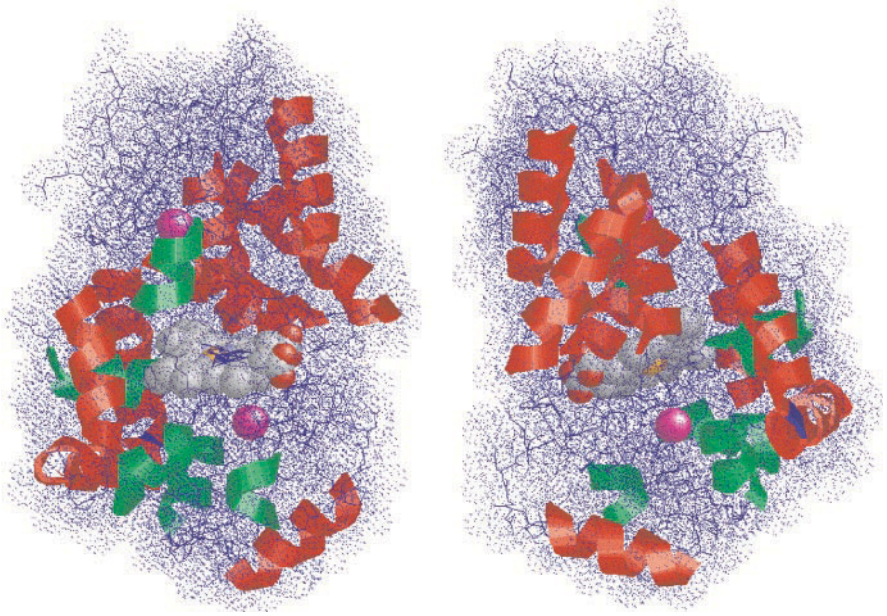

FIGURE 4 Display of the tertiary structure of HRP produced with the program RasMol from the atomic coordinates deposited in the Protein Data Bank (Berman et al., 2000) for entry 6ATJ. The locations of the α-helices were determined using the algorithm from Kabsch and Sanders (1983). α-Helices with at least 10 residues are shown in red, shorter ones in green. The heme group and the two Ca^{2+} ions are shown with their van der Waals radii in space-fill mode. To allow for an approximate appreciation of the location of these elements in the molecule, the amino acids are shown with blue lines including their respective van der Waals radii.

hanced amide I spectra of HRP in potassium phosphate buffer at pH 6.5 (Fig. 3 *A*) and HRP-PEG lyophilized from pH 6.5 and dissolved in benzene (Fig. 3 *B*) and toluene (Fig. 3 *C*) were remarkably similar with respect to the number of components and their frequencies (Table 1), but some changes in the area contributions for some Gaussian bands were noted. For example, the band at 1651 cm^{-1} has an increased area contribution when compared with the other α-helical band at 1658 cm^{-1} for HRP-PEG in benzene. However, the spectra of HRP in water and HRP-PEG in toluene are remarkably similar also with respect to this feature (Fig. 3, *A* and *C*). Because HRP-PEG was dehydrated in both solvents and the physicochemical properties of toluene and benzene are very similar, the spectral variations in the case of HPR-PEG in benzene must be because of some minor structural differences, presumably in the short helices that are caused by the solvent. This is not surprising because the IR-intensity distribution of short helices is very structure sensitive (Torii and Tasumi, 1992b). The secondary structure compositions determined from the areas of the Gaussian bands fitted to the amide I spectra for HRP in aqueous solution and HRP-PEG in benzene and toluene were also very similar (Table 2). This is in direct contrast to yet another warning published recently (Grdadolnik and Maréchal, 2000) that amide I bands of proteins under nonaqueous conditions should not be assigned to secondary structure. In contrast, the frequencies of the bands typical for the elements of secondary structure (e.g., α-helix and β-sheet) did not show any substantial shift when HRP in water is compared with HRP-PEG in benzene and toluene (Table 1). This finding has been published for many dehydrated proteins repeatedly in the past (e.g., Griebenow and Klibanov, 1995, 1996, 1997; Carrasquillo et al., 1998, 1999, 2001a,b; Griebenow et al. 1999a,c).

To perform an additional model-independent check of the comparability, a strictly mathematical method was used to compare the amide I spectra of HRP in water and HRP-PEG in toluene and benzene. The SCCs were calculated from the inverted second-derivative spectra for the samples in the organic solvents versus HRP in aqueous solution (Fig. 5). Under both circumstances, the SCC values were quite high (0.95 and 0.94), comparable to the difference between HRP and HRP-PEG in buffer at pH 6.5 (Table 2). It is evident that amide I spectral changes inflicted by the combined effect of removal of water per se and nonaqueous solvent cannot possibly be larger than the very minor structural deviations mentioned above. This clearly demonstrates that HRP-PEG can be dehydrated by lyophilization and dissolved in toluene and benzene without inducing substantial spectral changes. Thus, the argument of van de Weert et al. (2001) that water removal per se could induce substantial spectral changes in the amide I is clearly nonsubstantiated because if this were the case a dehydrated protein powder dissolved in a nonaqueous solvent could not possibly have a native-like appearance.

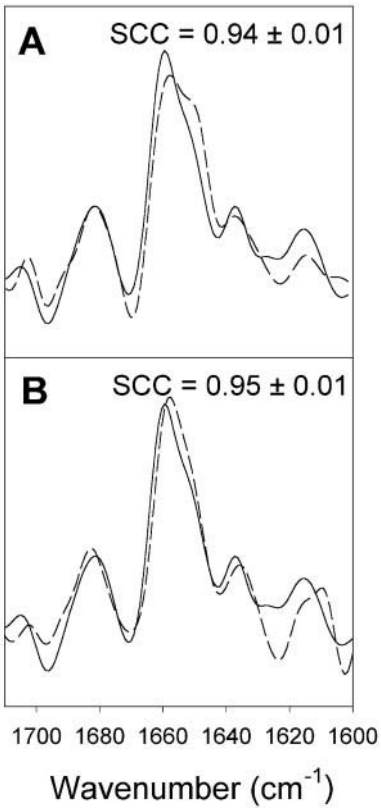

FIGURE 5 Inverted second-derivative amide I spectra of HRP-PEG in water at pH 6.5 (——) and HRP-PEG in benzene (*A*, — — —) and toluene (*B*, — — —). Also shown are the values calculated for the SCC for the spectra.

However, the advocatus diaboli could still argue that the results presented above are coincidental and are simply caused by spectral changes upon exposure of the dehydrated HRP powder to toluene and benzene. Thus, we lyophilized HRP and HRP-PEG from aqueous solution, pH 6.5, and acquired the spectra for both protein powder samples as KBr pellets. The amide I spectrum of HRP showed substantial changes (Fig. 6 *A*) when compared with that of HRP in aqueous buffer (Fig. 3 *A*). The two bands for α-helix, found in aqueous solution and in benzene and toluene, do not appear separated but coincide. This is likely a result of the general broadening of spectral components. The other components have similar frequencies as those found in aqueous solution (Table 1), and one new component appears at $\sim$1695 cm^{-1}. Because this component is not found in the spectra of dehydrated HRP-PEG in benzene and toluene, it is unlikely that it represents a free amide I group (in first-order approximation the C═O stretching vibrations of non-hydrogen-bonded amide groups) as suggested (Grdadolnik and Maréchal, 2000). It seems more likely that this band is associated with formation of β-sheets because also the area of the band at $\sim$1630 cm^{-1} increases substantially upon HRP lyophilization (Table 1). To confirm the amide I as-

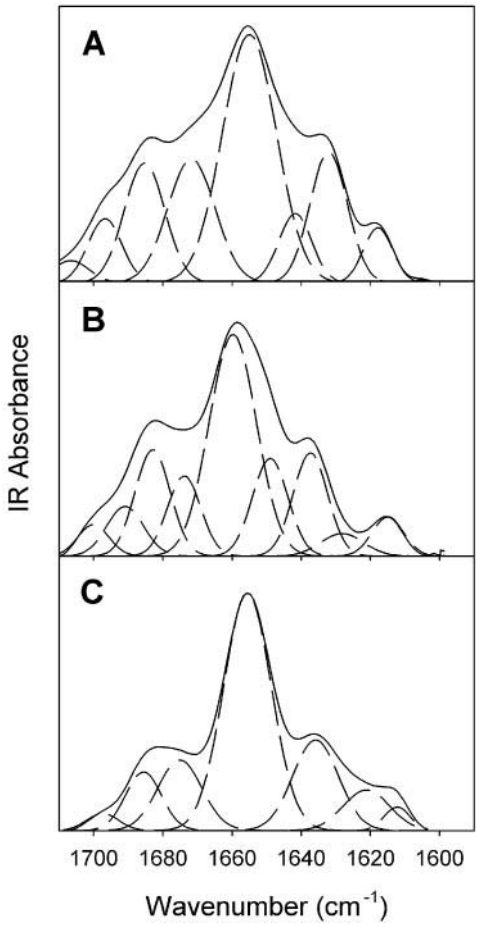

FIGURE 6 Gaussian curve fitting of the resolution-enhanced amide I spectra of HRP lyophilized from water, pH 6.5 (*A*), HRP-PEG lyophilized from water, pH 6.5 (*B*), and lyophilized HRP suspended in benzene (*C*). The solid lines represents the original spectra overlaid with the results of the curve fit, which practically coincide; the broken lines are the individual Gaussian bands fitted to the spectra.

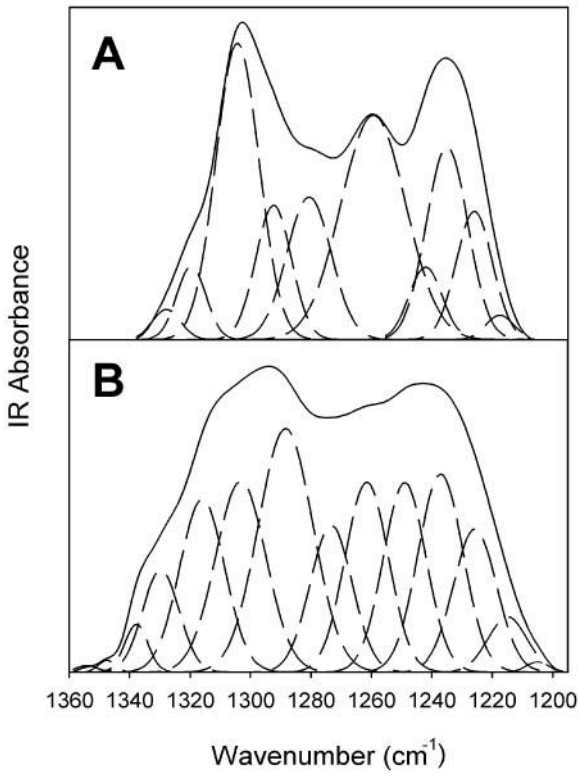

FIGURE 7 Gaussian curve fitting of the amide III spectra of HRP in water, pH 6.5 (*A*) and HRP lyophilized from water, pH 6.5 (*B*). The solid lines represent the original spectra overlaid with the results of the curve fit, which practically coincide; the broken lines are the individual Gaussian bands fitted to the spectra.

signments we also analyzed the spectra of aqueous and lyophilized HRP in the amide III region. The amide III region offers the advantage that the bands arising from different elements of the secondary structure are better separated (Griebenow and Klibanov, 1995), and thus spectral analysis by Gaussian curve fitting can be performed without previous resolution enhancement of the spectra. The individual bands were assigned according to the literature (Griebenow and Klibanov, 1995) and are summarized in Table 1. Qualitative analysis of the spectra (Fig. 7) revealed that lyophilization caused spectral changes in the amide III region. The spectrum appeared broadened, which is indicative of structural changes upon dehydration. Gaussian curve-fitting results agree with those in the amide I: the α-helix content dropped from 49% $\pm$ 2% to 41% $\pm$ 2%, and the β-sheet content increased from 10% $\pm$ 1% to 22% $\pm$ 1% (Table 2). Thus, analysis of two spectral regions resulted in a consistent picture, as previously demonstrated and reviewed for other proteins (Griebenow et al., 1999a).

The situation is different for HRP-PEG after lyophilization (Fig. 6 *B*); the spectrum appears to be more similar to that of HRP in aqueous solution. In agreement with this, frequencies of individual components in the amide I, areas of Gaussian bands, and secondary structure were more similar to those of HRP and HRP-PEG in aqueous buffer (Tables 1 and 2). The drop in the α-helix content was less pronounced, and there was no increase in the β-sheet content. However, the drop in the SCC value compared with the aqueous samples revealed some structural changes occurring after dehydration (Table 2). Nevertheless, PEG modification of HRP was efficient in reducing the magnitude of structural perturbations upon dehydration by lyophilization. In contrast to established lyoprotectants such as polyhydric alcohols and carbohydrates (Carpenter et al., 1998; Griebenow et al., 1999a), PEG cannot donate hydrogen bonds to C═O backbone groups and thus cannot replace water in this function. It is also unlikely that the few PEG molecules bound to HRP influence the distribution of water around the protein substantially (Belton and Gill, 1994) because PEG is amphiphilic. Thus, that PEG modification of HRP minimizes dehydration-induced structural changes constitutes another (though indirect) argument against the hypothesis

that water removal per se could lead to substantial amide I spectral alterations.

Next, HRP was suspended in benzene, and the IR spectra were acquired (Fig. 6 *C*). It was noted that the amide I spectrum of HRP in the suspended powder did change notably, an effect not found when lyophilized protein powders were suspended in many organic solvents and no major changes to the amide I spectra occurred (Griebenow and Klibanov, 1996, 1997; Carrasquillo et al., 1998, 1999; Griebenow et al., 1999a,c). With the exception of the finding that only one α-helix band was found instead of two as in native HRP, the band frequencies and areas were similar to those of HRP in water (Table 1), and the secondary structure and the SCC value (Table 2) were also more similar. Thus, we must conclude that suspension of HRP powder in benzene leads to a somewhat more native-like secondary structure than in the lyophilized state. This finding makes sense with respect to data reported on the catalytic activity of HRP in organic solvents. HRP was substantially more active in benzene and toluene than in any other organic solvent tested (Kazandjian et al., 1986). Because native-like enzyme structure is important for optimal activity in organic solvents (Griebenow et al., 2001), these data support that HRP powder suspended in organic solvents has a more native-like structure than in other solvents. However, the amide I spectrum was still less similar to that of HRP in buffer than that of HRP-PEG after dissolving it in benzene. Suspension in benzene was not able to completely reverse the spectral and thus structural changes occurring upon HRP lyophilization.

To investigate whether this event was a result of effects of benzene on protein secondary structure or might have been caused by the suspension itself, HRP was suspended in dry THF. Some spectral changes were also noted in this case, but neither the components in the Gaussian fit (Table 1) nor the secondary structure composition (Table 2) were significantly different from that of the lyophilized powder with the exception of a notable increase in unordered secondary structure. In addition, the SCC value remained low, similar to the value found for the lyophilized powder in the dry state. These results indicate that there is something special about benzene (and toluene), which is also reflected in the fact that only in these two solvents was a drastic solubility increase for HRP-PEG observed when compared with all other solvents tested (e.g., dry THF, dioxane, and acetonitrile). It remains to be investigated what makes benzene and toluene special solvents in this context, but a likely explanation might consist in their interaction with charged surface groups.

DISCUSSION

In this work it has been ultimately shown that changes in the amide I spectra of proteins upon dehydration are not caused by water removal per se but at least largely caused by protein structural perturbations. The experimental data on the structure of HRP and HRP-PEG under various experimental conditions allow only one conclusion, i.e., that changes in the amide I spectra of proteins are relatively insensitive to changes in the physicochemical environment but are very sensitive toward changes in the secondary structure. The results are in complete agreement with the manifold of arguments derived from other observations, some of which have been reviewed by Griebenow et al. (1999a). For example, if amide I spectral changes significantly reflected the removal of water per se, similar changes would be expected for proteins belonging to similar structural classes. However, quite the opposite has been reported in the literature. Spectral changes vary from very little to very significant (Prestrelski et al., 1993a; Griebenow and Klibanov, 1995). Even more important, there are examples where the spectral changes are quite small for proteins belonging to different structural classes. In their initial work, Prestrelski et al. (1993a) found that the spectra of aggregated poly-L-lysine (pH 12.0, heating at 75°C for 30 min) were very similar before and after lyophilization. This indicates that for such intermolecular β-sheet structures, amide I spectral changes upon dehydration are very small. In accordance with this notion the lyophilization-induced spectral changes in the amide I and III spectra are very small for recombinant humanized immunoglobulin G (Costantino et al., 1997). A similar result was obtained for granulocyte-colony-stimulating factor, a mostly α-helical protein (Prestrelski et al., 1993a; Dong et al., 1995). All these results strongly indicate that the amide I of regular secondary structures are mostly insensitive to changes of the hydration state.

Another line of arguments stems from the fact that amide I and III spectral changes can be largely prevented when the protein is co-lyophilized with a lyoprotectant, such as a polyhydric alcohol or carbohydrate (Prestrelski et al., 1993a; Griebenow and Klibanov, 1995; Carpenter et al., 1998). For example, amide I spectral changes occurred upon lyophilization of two enzymes, lactate dehydrogenase and phosphofructokinase, which were 50% (lactate dehydrogenase) or completely (phosphofructokinase) irreversibly inactivated by the process (Prestrelski et al., 1993b). Co-lyophilization with various lyoprotectants not only minimized amide I spectral changes, but also resulted in a direct correlation between the degree the spectrum appeared native (as expressed by the spectral correlation coefficient) and the amount of recovered enzyme activity upon redissolving the lyophilized powder in aqueous buffer. Similarly, Desai et al. (1994) found that co-lyophilization of bovine pancreatic trysin inhibitor (BPTI) with sorbitol reduced the magnitude of ^{1}H/D exchange in the solid state, an indisputable argument supporting that BPTI structure was more preserved by the presence of sorbitol during the lyophilization process. Amide III FTIR data indeed showed that BPTI structure was more native-like when BPTI was co-lyophilized with sorbitol (Griebenow and Klibanov, 1995). It has

also been shown that the amount of protein aggregates relates to the FTIR spectra of the proteins in the solid state (Allison et al., 1996; Castellanos et al. 2002). However, one might still argue that lyoprotectants are efficient by concentrating water around the protein molecule during the dehydration process (Belton and Gill, 1994). This would cause the protein to be simply more hydrated so that the IR spectrum becomes more native-like. However, this view is irreconcilable with recent experimental data because they show that the lyoprotectant (e.g., a sugar) forms hydrogen bonds with the protein (Sarciaux and Hageman, 1997; Costantino et al., 1998b; Allison et al., 1999). Thus, the sugars replace water molecules on the protein surface, and there is neither a need for a hydration shell nor an argument left to support the water-concentration hypothesis proposed (Belton and Gill, 1994).

Another argument in favor of the interpretation of amide I spectral changes upon protein dehydration being largely caused by changes in the secondary structure of proteins is provided by co-lyophilization of proteins with chaotrophic salts that destabilize protein structure. Such experiments have clearly demonstrated that 1) chaotrophs increase the amide I spectral changes and 2) the degree of changes is related to irreversible aggregation (Dong et al., 1995; Prestrelski et al., 1993a; Allison et al., 1996).

In addition, results obtained by analyzing the protein spectra in two distinct spectral regions, namely, the amide I and amide III, are in quantitative agreement (Griebenow and Klibanov, 1995, 1996, 1997; Carrasquillo et al., 2000; Griebenow et al., 1999a). It seems unlikely that this would be the case as a result of changes other than structural because these two amide modes have very different normal-mode compositions (Krimm and Bandekar, 1986).

Furthermore, interpretation of protein amide I spectral changes upon encapsulation in bio-erodable polymers (which includes their dehydration) as structural changes has led to the development of rational encapsulation strategies. Many publications demonstrated that minimization of amide I spectral changes upon protein encapsulation prevented their aggregation and inactivation upon release from polymer microspheres (Carrasquillo et al., 2001b; Castellanos et al., 2001, 2002; Pérez et al., 2002). If amide I spectral changes were merely caused by physical changes in the environment, e.g., removal of water per se, this would be coincidental, a fact that seems quite impossible because the results were obtained using quite different encapsulation strategies and proteins (Pérez et al., 2002). Similarly, improved enantioselectivity of suspended enzyme powder in organic solvents has been linked to the degree the structure is native-like (Griebenow et al., 1999b). Lastly, optimal enzyme activity for dry films in organic solvents recently has been linked to those formulations that display the most native-like properties, namely, structure and molecular mobility (Griebenow et al., 2001). Thus, even before the data presented in this work, many arguments supported the view that amide I and III spectral changes upon dehydration and exposure to organic solvents are largely caused by structural changes. Claims that IR spectral changes are largely caused by the removal of water per se, on the other hand, have received little, if any, experimental support.

One notable exception has to be brought up, however, as pointed out by us already (Griebenow and Klibanov, 1997; Costantino et al., 1998a): dehydration of proteins leads to the formation of protein-protein contacts because of the removal of the bulk solvent water. The protein contacts might constitute one of the driving forces leading to protein structural perturbations upon dehydration, but protein molecules might also simply start interacting and thus satisfy the hydrogen-bonding potential of backbone groups. One observation that supports this interpretation has been made with cross-linked enzyme crystals in organic solvents. FTIR investigation of such crystals in the dehydrated form and suspended in organic solvents revealed an unchanged α-helix content but increased β-sheet content, even though the x-ray structure was similar to that in aqueous solution (Griebenow and Klibanov, 1997; Vecchio et al., 1999). Thus, protein-protein contacts seem to lead to structures that absorb at IR frequencies typical for β-sheet secondary structure in the amide I and III, namely, $\sim$1630 cm^{-1} and 1215–1245 cm^{-1}. This has sometimes led to the statement that perhaps only the α-helix content should be used to quantify dehydration-induced structural perturbations occurring to proteins upon dehydration (Griebenow and Klibanov 1997; Costantino et al., 1998a).

Finally, we like to invoke some basic physical arguments with respect to a possible influence of the solvent on intensity and frequency of amide I. It has been argued by van de Weert et al. (2001) that "adding water molecules to the amide bond could alter the vibrational characteristics of the amide band." This statement is somewhat elusive. We suppose that the authors believe that the normal-mode composition of amide I can be affected by water molecules hydrogen bonded to the peptide group. It has indeed been shown by spectroscopic and computational investigations of small model peptides that hydrogen bonding of water to the carbonyl as well as to the amide group decreases the amide I frequency (Wang et al., 1991; Mirkin and Krimm, 1991; Torii et al., 1998). Moreover, the water-bending vibration mixes with amide I, most likely because of transition dipole coupling (Chen et al., 1994; Sieler and Schweitzer-Stenner, 1997; Han et al., 1998), but this coupling disappears for D_2O because of the much lower intrinsic frequency of the bending mode (Sieler and Schweitzer-Stenner, 1997). Hence, if this mixing effect had a significant impact on the amide I of the secondary structures of a protein dissolved in water, it could be eliminated by choosing D_2O as solvent. There is no evidence, however, for the notion that spectral changes obtained by dehydrating proteins disappear in D_2O. Moreover, it is still not clear whether and to what extent the above results on small peptides can be transferred to long

peptides and proteins. A single peptide group can form hydrogen bonds to three water molecules (Chen et al., 1995). In helical and sheet structures, only one such hydrogen bond can be formed per peptide (i.e., to the carbonyl group). Although experimental (Sieler and Schweitzer-Stenner, 1997) and computational results (R. Schweitzer-Stenner, unpublished) strongly indicate that amide I can also vibrationally interact with non-hydrogen-bonded water molecules of the hydration shell, one has to keep in mind that the number of water molecules in close proximity is certainly much lower for a peptide group in a protein (cf. Table 3 for HRP) compared with the hydration shell of a single peptide in water. Knapp-Mohammady et al. (1999), for instance, used up to 14 water molecules to simulate the hydration shell of dialanine in a density functional theory calculation. All these facts suggest that amide I-water coupling should not have a strong impact on frequencies and intensities of amide I modes of long helices and extended sheet structures because the (comparatively weak) local water-amide I coupling is unlikely to provide a significant perturbation of the corresponding delocalized excitonic states of amide I (Torii and Tasumi, 1992a,b). The situation might be somewhat different for short helices and extended structures, which are not stabilized by hydrogen bonding.

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