Labeling Proteins via Hole Burning of Their Aromatic Amino Acids: Pressure Tuning Spectroscopy of BPTI

Markus Stübner, Christoph Hecht, and Josef Friedrich
Physik Department E14—Lehrstuhl für Physik Weihenstephan, Technische Universität München, 85350 Freising, Germany

ABSTRACT We demonstrate hole burning on a protein by using an intrinsic aromatic amino acid as a probe. The protein is bovine pancreatic trypsin inhibitor (BPTI), the labeled amino acid is tyrosine. Only one of the four tyrosines could be burned. As an application we present pressure tuning experiments from which the local compressibility around the burned tyrosine probe is determined.

INTRODUCTION

Since the first application of spectral hole burning in protein spectroscopy (Friedrich et al., 1981), a wide field of problems in protein physics has been tackled with this technique, among them phototransformation mechanisms and level structure (Reddy et al., 1993; Creemers et al., 1999, 2000; Hayes et al., 2000; Reinot et al., 2001; Zazubovich et al., 2002), the relation between structural and spectral heterogeneity (Jankowiak et al., 1993; Frauenfelder and Wolynes, 1994; Friedrich, 1995), conformational and spectral diffusion (Köhler and Friedrich, 1989; Den Hartog et al., 1996; Schlichter et al., 2001), energy and electron transfer (Köhler et al., 1988; Reddy et al., 1992; De Caro et al., 1994; Small, 1995), Stark spectroscopy and local fields (Zollfrank and Friedrich, 1992; Gafert et al., 1995; Köhler et al., 1998; Rätsep et al., 1998; Reinot et al., 2001), etc. In all investigations so far, hole burning experiments were confined to chromoproteins. In the present paper, we show that it is as well possible to use the intrinsic aromatic amino acids as hole burning probes. We demonstrate the technique with bovine pancreatic trypsin inhibitor (BPTI), a rather small protein with four tyrosines. One out of these four tyrosines undergoes a persistent phototransformation and gives rise to narrow spectral holes. The shift of these holes under external pressure changes is measured. From the respective data, the local compressibility around the probe (and, hence, the associated volume fluctuations) can be determined. At present, the nature of this reaction (photochemical or nonphotochemical) is still unknown.

EXPERIMENTAL

Materials

BPTI (purchased from Sigma–Aldrich, Deisenhofen, Germany) was dissolved in 0.1 M Tris/HCl buffer at pH 8 and mixed with glycerol (2:3, vol/vol). The final concentration of BPTI was $c = 8.79 \times 10^{-4}$ M. For comparison, a solution of free tyrosine in glycerol/water (3:2, vol/vol) was prepared. For this latter sample, the best glass quality was obtained at a pH of 1.65, which was adjusted with 1 M HCl. The respective concentration was $c = 3.9 \times 10^{-3}$ M. For pressure tuning experiments, these samples were sealed in small plastic bags to ensure hydrostatic pressure conditions.

Spectroscopy

The broad-band absorption was measured with a resolution of $\sim$10 cm^{-1}, the resolution for the emission was set to $\sim$100 cm^{-1}. Ultraviolet (UV) hole burning was performed with the radiation from a dye ring laser whose frequency was doubled in an external ring resonator. The second harmonic from the external resonator could be synchronously scanned with the fundamental. The power level of the UV radiation from the external resonator was $\sim$10 mW, the respective linewidth $<$3 MHz. In BPTI, holes could be burned only within the frequency range 34600–35700 cm^{-1}. Their depth was $\sim$2%. In the case of free tyrosine, holes could be burned everywhere within the inhomogeneous S_1 transition. Typical power levels at the sample and burning times were of the order of 0.03 W/cm^2 and several minutes, respectively.

RESULTS

Figure 1 shows the absorption and emission spectra of tyrosine and BPTI in glycerol/buffer. The tyrosine spectra are measured at pH 1.65 where the glass quality was best. We stress, however, that the respective spectra did not change up to a pH level of 7.5. Above that, they undergo a characteristic change. Absorption spectra of BPTI where measured at pH 8 and the respective emission spectra at pH 5. Within this pH range, the protein is intact.

The long-wavelength band of BPTI is associated with the four tyrosine molecules of the protein (Parkin et al., 1996). The spectrum differs considerably from the respective one of the free tyrosine molecule. It turned out that hole burning in BPTI was possible only in the range between 34600 and 35700 cm^{-1} (*hatched bar* in Fig. 1 *A*). We stress that we could not detect any significant wavelength dependence of the emission spectra.

Figure 2 shows the behavior of the holes under pressure for (*A*) the protein and (*B*) for the free tyrosine. There are a few noteworthy features: First, in all cases, the pressure shift is to the red and is perfectly linear with pressure. Second, there is no significant indication of a splitting of the hole under pressure. This is especially interesting for BPTI,

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Address reprint requests to Josef Friedrich, Vottinger Str. 40, 85350 Freising, Germany. Tel.: +49-8161-71-3294; Fax: +49-8161-71-4517; E-mail: j.friedrich@lrz.tu-muenchen.de.

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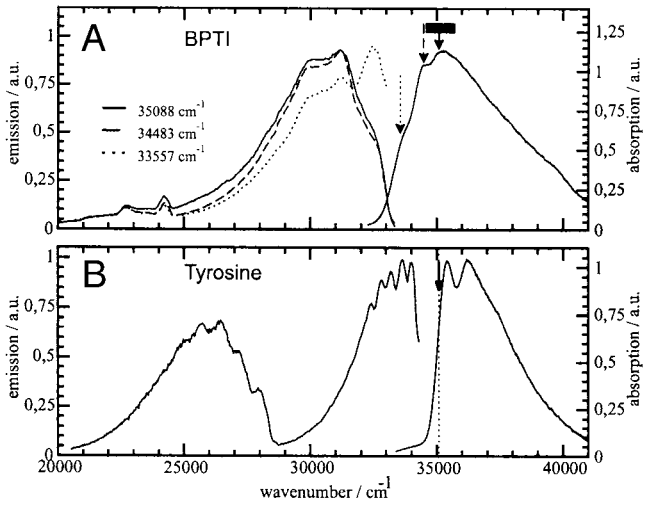

FIGURE 1 Absorption and emission spectra of (*A*) BPTI and (*B*) tyrosine in a glycerol/water mixture at 2 K. The cross-hatched bar in (*A*) shows the range where holes could be burned. Arrows indicate the wavenumbers where excitation was carried out. Note that, for the redmost excitation, the laser radiation is superimposed the emission spectrum.

because there are four tyrosines, and, if several of them were simultaneously burned, a splitting would be expected (Friedrich et al., 1994).

Figure 3 shows the pressure shift s_p of the holes as a function of burn wavenumber within the inhomogeneously broadened long-wavelength absorption band. In both cases, the pressure shift depends in a linear fashion on the burn wavenumber. However, what is more surprising is the fact that the slope of the straight line and the wavenumber ν_{vac} where the pressure shift vanishes (the so-called vacuum absorption wavenumber) are nearly the same for the protein and for the free tyrosine.

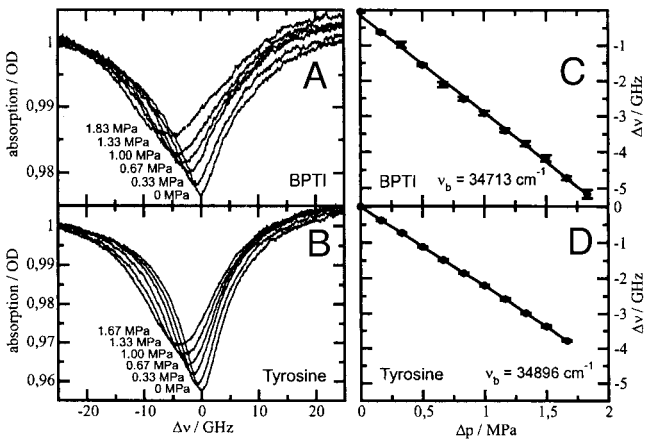

FIGURE 2 Spectral holes as they change with pressure. Samples: (*A*, *C*) BPTI and (*B*, *D*) tyrosine. Temperature: 2 K.

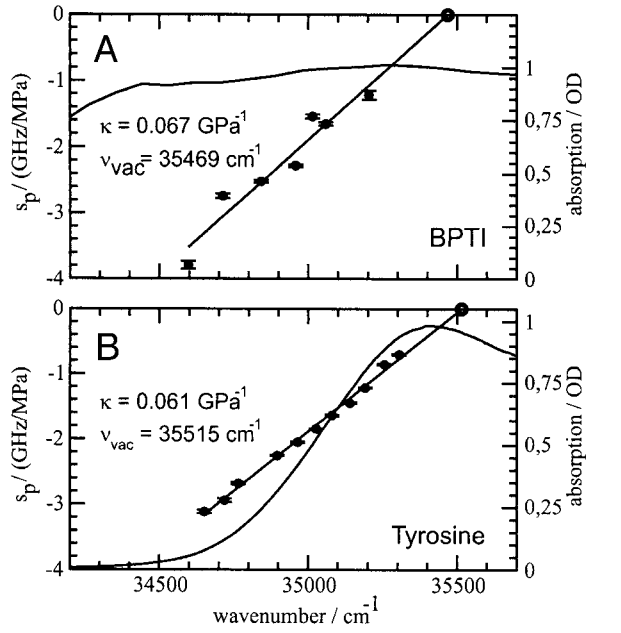

FIGURE 3 Pressure shift s_p of spectral holes over the burn wavenumber for (*A*) BPTI and for (*B*) tyrosine. The vacuum wavenumber ν_{vac} and the compressibility κ are indicated. The absorption spectrum in the respective range is also shown.

DISCUSSION

The broad band spectra

As mentioned above, the emission from BPTI does not depend on excitation energy in a significant fashion. From this we conclude that the four tyrosines in the protein are coupled via energy transfer processes sufficiently strongly so that emission from the blue-absorbing molecules is suppressed. However, the tyrosine emission from BPTI is very different from the respective one of the free amino acid (Fig. 1). The respective shape lacks the sharp features that are prominent in the free molecule. Such a behavior is indicative of a rather large coupling of the excited electronic state to the lattice or to anharmonic low-frequency large-amplitude motions. The pronounced Stokes shift of the long-wavelength shoulder ($\sim$1000 cm^{-1}, Fig. 1 *A*) supports this view. If the coupling is strong, hole burning is suppressed because of the low intensity in the zero phonon lines. This may be one reason why we did not succeed in burning holes into the long-wavelength shoulder of the BPTI absorption. However, it could as well be that the respective tyrosine is too tightly packed within the protein that photochemical and nonphotochemical burning processes are prohibited. In this context, it should be noted that, at room temperature, BPTI undergoes a destructive photoreaction if irradiated with UV laser light. Sharp holes can be burned into the BPTI absorption only within the range between 34600–35700 cm^{-1}. This range roughly coincides with the red edge of the free tyrosine absorption (Figs. 1 and 3).

Hole burning

Hole burning experiments with free tyrosine in solution are difficult to perform mostly because of the low solubility of the molecule in water containing solvents. At low pH values, however, good-quality glasses are obtained so that high-quality hole-burning experiments become feasible. Figure 2 compares the results for the protein with the respective ones for the free amino acid. Figure 3 shows the frequency dependence of the pressure shift together with the broad-band absorption. The simplest approach to interpret pressure effects on spectral holes is based on the so-called solvent shift–pressure shift model (Sesselmann et al., 1986; Zollfrank et al., 1991; Zollfrank and Friedrich, 1992; Friedrich et al., 1994). Within the frame of this model, it is assumed that pressure does nothing else than increase the solvent shift via an increase of the density of the sample. The solvent shift ν_s of aromatic molecules is usually interpreted in terms of the dispersion interaction or higher-order electrostatic interaction of the probe with its environment (Bayliss, 1950; Bayliss and McRae, 1954; Liptay, 1965). These interactions fall off with distance as R^{-6}. To be general, we write

$$\nu_s = C/R^n. \tag{1}$$

Then, we obtain

$$s_p = \left[\frac{\partial \nu_s}{\partial p}\right] = \left[\frac{\partial \nu_s}{\partial V} \times \frac{\partial V}{\partial p}\right] = \frac{n}{3}\,\kappa\nu_s, \tag{2}$$

where V is the interaction volume between probe and solvent, C is a coupling constant, and κ is the compressibility. The solvent shift ν_s for a frequency-selected ensemble of molecules is the difference between the laser wavenumber ν_L, where hole burning is carried out, and the vacuum absorption wavenumber ν_{vac}, where the isolated probe molecule has its 00-transition. Inserting these relations into Eq.2, we have

$$s_p = \frac{n}{3}\,\kappa(\nu_L - \nu_{vac}). \tag{3}$$

Hence, measuring the pressure shift s_p as a function of ν_L, one can determine the two parameters of the above equation, namely the compressibility κ and the vacuum frequency ν_{vac}, by purely optical means, given that the main contribution to the interaction that determines the solvent shift is known (Zollfrank et al., 1991; Zollfrank and Friedrich, 1992).

We want to stress a few points. First, in the above reasoning, we have neglected all angular dependencies and have considered only attractive interactions. As a consequence, such a minimal description does not account for the fact that the holes not only do shift but also broaden under pressure (Laird and Skinner, 1989), as is also obvious from Fig. 2. Second, the vacuum frequency as obtained from a pressure tuning experiment need not coincide with the respective frequency from a gas phase jet or molecular beam experiment. In frozen solutions, the probe molecule may experience certain structural changes, e.g., strong hydrogen bonds, distortions, etc., which are not influenced by pressure at the pressure level of our experiment. Hence, in this case, ν_{vac} is the vacuum frequency of the structurally changed probe. For instance, the four tyrosines in BPTI need not necessarily be characterized by the same vacuum frequency because they may not have the same detailed structure due to constraints imposed by the protein environment. Third, the compressibility is a very interesting parameter in protein biophysics because it determines the respective volume fluctuations (Zollfrank et al., 1991 Zollfrank and Friedrich, 1992; Köhler et al., 1998)

$$\Delta V/V = [k_B\kappa T/V]^{1/2}. \tag{4}$$

The volume fluctuations are a measure for the flexibility of a protein and, hence, are related to functional properties. Moreover, the volume fluctuations can be calculated by various simulation techniques (Hayward et al., 1994; Tama et al., 2000; Dadarlat and Post, 2001). The optical technique of determining the compressibility has a very nice feature with respect to protein biophysics because it measures the respective parameters via an extremely local probe, namely via the photoreactive molecule. Accordingly, the respective quantities are local quantities (i.e., comprising a few shells of neighboring molecules) and there is no need for correcting e.g., for hydration shell effects or solvent effects (Gekko and Noguchi, 1979; Gavish et al., 1983).

As to the results obtained in the present work, it is quite surprising that the two samples yield, within a rather narrow margin, the same results. For instance, in previous investigations on protein compressibilities, we found rather large fluctuations that depended strongly on the structural state of the probe molecules (Friedrich et al., 1994). From the fact that ν_{vac} is similar for the two samples, we conclude that the tyrosine probed in the BPTI must structurally be closely related to the free amino acid. From an inspection of the respective structure (Fig. 4), we feel that the most likely candidate out of the four tyrosines, is Tyr-10. It has the largest access to the solvent, namely about half of its surface and seems to be least constrained by the BPTI building blocks in line with the observation that it absorbs also in the same range as the free amino acid.

As to the compressibility, the situation is less straightforward. On the one hand, our results, if evaluated with $n = 6$ (Eq. 3), yield a compressibility of 0.06 GPa^{-1}, which is in very good agreement with experimental results obtained from ultrasound measurements (Gekko and Hasegawa, 1986) and also with recent model calculations (Dadarlat and Post, 2001). On the other hand, however, we measure almost the same result for the free tyrosine molecule in the glycerol/water glass. One immediate obvious conclusion

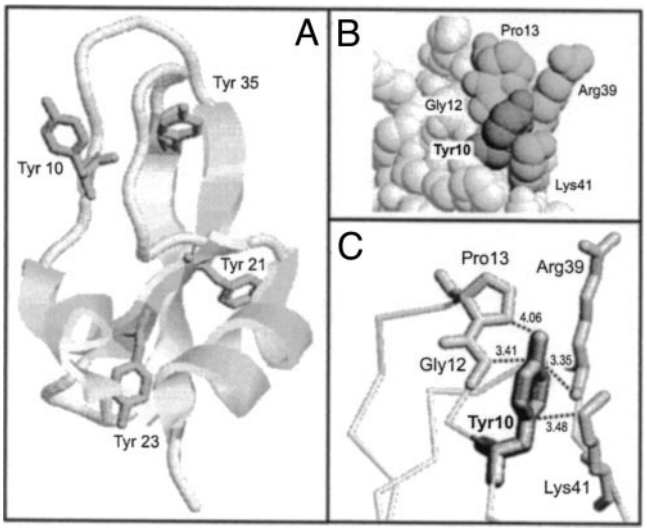

FIGURE 4 (*A*) Ribbon structure of BPTI. The ribbon diagram was adapted from the x-ray structure (Parkin et al.; 1996 (PDB-code: 1BPI) and drawn with RasMol. (*B*) and (*C*) show details around Tyr-10.

from this observation would be that solvent and protein happen to have the same compressibility. However, although possible, this conclusion does in no way offer a satisfying explanation. Because not only the κ but also ν_{vac} values are very similar in the protein and glass sample, we infer that the immediate environment of the probe must be similar in both cases. Indeed, as we mentioned above, roughly 50% of the surface of the aromatic moiety of Tyr-10 is exposed to the solvent, i.e., mostly to water molecules in the hydration shell. The polar OH group, which sticks out into the hydration shell, will polarize the surrounding water molecules. The respective interaction is a dipole-induced dipole interaction, supporting an exponent $n = 6$ (Eq. 1 and 3). Electrostatic interactions may cause red and blue shifts in the bands of the spectra depending on whether the dipole moment in the excited state strengthens or weakens the interaction in the ground state. Because the dispersion interaction is always red shifting due to the higher polarizability in the excited state, the two interactions may compensate, resulting in a vanishing solvent shift. That something like this does occur for tyrosine in glycerol water/glass is obvious from Fig. 3 *b*: ν_{vac}, the wavenumber where the pressure shift vanishes (35515 cm^{-1}, see Eq. 3) is within the inhomogeneous band very close to the band maximum. Hence, the average solvent shift, i.e., the difference between ν_{vac} and ν_0, the wavenumber of the band maximum is really very small, namely of the order of 100 cm^{-1}.

We stressed above that the pressure-tuning hole-burning technique measures a local compressibility, i.e., the compressibility of that volume around the chromophore, which is within the average range of the probe solvent interaction. As to the protein sample, a larger part of this volume is in the hydration shell, hence is built from water molecules. Consequently, the same must be true for tyrosine in the glycerol/water glass if the respective microenvironments should be roughly the same as we concluded from the very similar values for κ and ν_{vac}. If so, the tyrosine probe must obviously induce kind of a microphase separation in the sense that water molecules in the glycerol/water solution form kind of an ordered solvent cage around the probe whose lower entropy is compensated for by the formation of stronger hydrogen bonds. Because the strength of ordered hydrogen bonds is large compared to the van der Waals interaction, they may be less affected by pressure at the pressure level of our experiment, which is of the order of 1 MPa. This argument might explain the rather low value of κ as compared to other systems obtained with the same technique (Fidy et al., 1998).

To estimate the volume fluctuations, the total volume has to be known (Eq. 4). For our experiment, the respective volume is the volume spanned by the interaction range between probe and environment. If this volume is within the protein, and the optically determined compressibility is a reasonable average for the protein, the volume fluctuations of the protein can be determined in case its dimensions are known. In our case, we argued that we rather probe a local volume in the hydration shell, hence, an estimation of the volume fluctuations of the protein is not possible. However, it seems possible to estimate the dimensions of the interaction volume. Because the influence of the protein on the compressibility is marginal, the respective distances to the probe must be larger than the dimension of the interaction volume. From Fig. 4 *C*, we estimate an average protein–probe distance of ~3.5 Å. We take this as an upper limit for the linear dimension of the interaction volume and calculate a volume fluctuation of order of 3%. Note that we take the relevant volume fluctuations at a temperature of 200 K, i.e., at the glass transition temperature of glycerol water glass where these fluctuations become frozen in. This rather large value of the volume fluctuations is because the respective volume itself is small.

CONCLUSIONS

We showed that narrow-bandwidth hole-burning experiments on proteins with an aromatic amino acid as a probe molecule are possible. We performed pressure tuning experiments with BPTI and determined the local compressibility around the active tyrosine molecule, most probably Tyr-10. The respective value turned out to be rather small and is of the same magnitude as in a solution of the free amino acid in glycerol/water. We argued that this behavior is indicative of a rather strongly hydrogen-bonded solvent cage of the water molecules around the probe.

BPTI is the first protein where aromatic amino acids have been labeled by narrow-bandwidth photobleaching. We definitely feel that this line of research opens new perspectives

in protein spectroscopy, also for larger proteins. Electrostatics could be probed in different areas of the proteins via the respective Stark splitting. The same may be true for the elastic properties as obtained from pressure tuning spectroscopy. Interesting questions may also be addressed with respect to the nature of the phototransformations. Of course, there may as well be limitations, e.g., too many aromatic amino acids, prohibited phototransformation, very fast energy transfer to tryptophans or chromophores, etc. The future will show how UV hole burning of proteins evolves.

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