Molecular Probes: What Is the Range of Their Interaction with the Environment?

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ABSTRACT We performed pressure-tuning hole-burning experiments on a modified cytochrome *c* protein in a glycerol/buffer glass. The shift and the broadening of the holes were investigated for various frequencies within the inhomogeneous band. On the basis of a simple model, we were able to estimate the interaction range between chromophore and protein. It is ~4.5 Å. The parameters that enter the model are the compressibility, the static mean-square displacement, the inhomogeneous width, and the average spectral shift per pressure. From this result and from our experiments on pressure-induced denaturing, we conclude that water molecules have to be brought very close to the chromophore during the denaturation process.

INTRODUCTION

Much of the physics of condensed phases has been revealed through the interaction of molecular probes with the environment in which they are embedded. For instance, most of the photophysics of molecular crystals such as exciton trapping and detrapping, energy transfer, electron-phonon coupling, etc., was extracted from the spectroscopy of molecular probes (Agranovich and Hochstrasser, 1983). A similar situation holds for glasses, where it was especially the high resolution techniques in optical spectroscopy, such as fluorescence line narrowing and hole burning, which enabled detailed studies of the specific features of the amorphous phase. Examples are the low temperature dynamics of glasses that is reflected in spectral diffusion, or their specific behavior in electronic dephasing (Friedrich and Haarer, 1986; Jankowiak and Small, 1987). The ultimate limit of probing the physics of a condensed phase is via single molecule spectroscopy. With this approach, the dynamic, the electrostatic, as well as the elastic properties of many local environments can be probed just by selecting different reporter molecules (Moerner and Basché, 1993; Moerner and Orrit, 1999; Tamarat et al., 2000; Orrit, 2002).

Optical spectroscopy of reporter molecules in biological macromolecules is highly developed and widely used. Intrinsic aromatic amino acids can serve as such reporters. Tryptophan, for instance, is the most frequently used probe in protein spectroscopy. It is widely used in reporting on folding and unfolding processes, on binding and aggregation events as well as on conformational dynamics (Cohen et al., 2002; Demchenko, 1988; Lakowicz, 1999). However, the most sensitive reporter molecules are dye molecules. Due to their extended π-electron system, they are highly polarizable; hence, they have a strong dispersion interaction with the respective environment. In addition, their electronic transitions are usually characterized by strong oscillator strengths, and their fluorescence quantum yields are quite often rather high. Last but not least, the respective range of their long wavelength absorption bands often lies in a frequency range that is very conveniently accessible to laser spectroscopy (Frauenfelder, 1984; Friedrich, 1995; Jankowiak et al., 1993). Since most proteins do not have native chromophores, it is also quite common to attach artificial dye probes to the biomolecule under investigation and use them as reporters. The most important question in context with the spectroscopy of reporter molecules is what the reporter can see and what range of depth it can probe.

This question came up recently when we investigated the stability diagram of a cytochrome *c*-type protein, in which the native iron heme group was substituted by the respective zinc analog (Lesch et al., 2002). We measured the unfolding under temperature and pressure variation by observing the first and second moment of the vibrationless fluorescence transition. The interesting observation was that the initial variations of both experimental parameters, pressure and temperature, caused a red shift of the fluorescence. However, in a certain parameter range, the behavior of the solvent shift changed: It turned from a red shift into a blue shift. This change signaled a change in the probe solvent interaction. Since the pioneering investigations on molecular probe solvent interactions by Bayliss, McRae, and Liptay (Bayliss, 1950; Bayliss and McRae, 1954; Liptay, 1974) it is well known that the dispersive interaction shifts always to the red, whereas the main electrostatic interaction (dipole-induced dipole) can as well cause shifts to the blue. On the basis of this situation, the blue shifts could be straightforwardly interpreted: Upon unfolding of the protein, being it thermally or pressure induced, water molecules from the hydration shell come close to the chromophore and induce the observed blue shift through a significantly increased electrostatic interaction via their large dipole moments (Lesch et al., 2002). Consequently we took the blue shifting phase as an indicator for protein unfolding. Although we are

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convinced that this interpretation is correct, the question addressed above remained open: How close must the water molecules approach the chromophore so that significant changes can be detected? This question is directly related to the range of the main interactions of the chromophore with its environment. To understand what is going on during an unfolding process, it is very important to know how far a reporter molecule can see. In the following, we present a pressure-tuning hole-burning experiment on Zn-substituted cytochrome *c* (Zn-Cc) from which this interaction range can be estimated.

A simple model

We consider a chromophore at the center of a sphere with radius R. The chromophore is approximated by a point dipole. The radius R of the sphere is specified as the average interaction radius (i.e., averaged over the probe-solvent interaction) of the chromophore with its environment. Within this approximation, the chromophore interacts with the molecules inside the sphere but it does not see much from the outside world. Our goal is to determine R.

An important quantity in our consideration is the compressibility κ. For the above sphere we can write

$$|\kappa| = |V^{-1}\Delta V/\Delta p| = (3/R)|x/\Delta p|. \quad (1)$$

x is the change of this radius under a pressure variation Δp.

The compressibility can be measured in an optical pressure-tuning hole-burning experiment by assuming that the pressure induced spectral shift s_p is proportional to the solvent shift and that the main interactions fall off with distance r as r^{-n}. For the dispersion and the higher order electrostatic interaction, n has a value of 6. Within this frame of approximation, s_p is given by

$$s_p = 2\kappa(\nu - \nu_{vac})\Delta p, \quad (2)$$

where ν is the hole burning wavenumber and ν_{vac} the vacuum absorption wavenumber, i.e., the wavenumber where the isolated probe absorbs. Accordingly, $\nu - \nu_{vac}$ is the solvent shift of the frequency selected ensemble of probe molecules (Köhler et al., 1998). Plotting $s_p/\Delta p$ as a function of the hole-burning wavenumber ν, one obtains a straight line whose slope is determined by the compressibility. Note that s_p vanishes at ν_{vac}. For probe molecules in a polar environment, it is possible that ν_{vac} lies within the inhomogeneous band. In this case it can be measured directly.

Next, we consider inhomogeneous broadening. It is caused by structural disorder. A suitable parameter to describe structural disorder is the static mean-square displacement $\langle x_0^2\rangle$, which characterizes the mean deviation of an individual protein building block (e.g., an amino acid) from the average structural position. We perform our hole-burning experiments at 2 K, hence $\langle x_0^2\rangle$ is indeed static and solely due to conformational disorder. The conformational contribution exceeds the respective contributions from zero-point vibrations by far. It is straightforward to show that the inhomogeneous width σ_0 is directly proportional to $\langle x_0^2\rangle^{1/2}$ (Schlichter et al., 1999).

$$\sigma_0 = |f_0|\langle x_0^2\rangle^{1/2}. \quad (3)$$

The proportionality factor $|f_0|$ depends on the probe-environment interaction, on the structure, and on the number of interacting species. σ_0 is easily measured at 2 K, unless hidden transitions complicate the problem (as they do, for instance, in the present investigation). $\langle x_0^2\rangle$ can be measured by x-ray scattering experiments. For some heme proteins (e.g., myoglobin), low-temperature data exist. So it is possible to determine $|f_0|$. $|f_0|$ may be considered as a transformation factor that transfers a length scale into a spectral scale.

A hole under pressure does not just shift. It also broadens. The nature of this pressure broadening σ_p is inhomogeneous as well. It is due to the fact that the application of pressure to a structurally disordered system changes the local configurations of solvent molecules around the chromophore so that the degeneracy of a molecular ensemble at zero pressure is lifted and gives rise to the observed pressure broadening. At this point it should be stressed that the pressure broadening effect at the pressure levels of our experiments ($\sim$1 MPa) is observed in disordered materials only. For instance, in molecular crystals, pressure broadening is practically absent, although the shift s_p is of the same order of magnitude as in disordered systems. We take this observation as a strong indication that it is not only the density that is changed in disordered materials but also the local structures. It is quite interesting that σ_p in proteins is almost as large as in glasses, an observation that implies that the structural correlation between the building blocks of a protein on the scale of 0.001 Å, the typical displacements at the pressure levels of our experiments, is as low as in an amorphous solid.

Along these lines of reasoning, we interpret σ_p in an analog fashion to Eq. 3 and write

$$\sigma_p = |f_p|\langle x_p^2\rangle^{1/2}. \quad (4)$$

$\langle x_p^2\rangle$ is the mean-square displacement of an amino acid due to pressure. It characterizes the scale by which an amino acid deviates from the position where it was expected if pressure just changed the density. Since inhomogeneous broadening and pressure broadening have the same roots, the coupling factors $|f_0|$ and $|f_p|$ have to be the same.

The next step in our analysis concerns the relation of the pressure-induced displacement x with the associated spectral shift $\langle s_p\rangle$, the pressure shift averaged over the inhomogeneous band. As stated above, the factor $|f_0|$ transforms length scales into frequency scales; hence, we can write

$$|f_0|x = \langle s_p\rangle. \quad (5)$$

Here, we have assumed that this transformation factor is the same irrespective whether the displacement is random or not. This seems to be a reasonable assumption in case that line

broadening and line shift originate from the same type of interaction.

We express x through κ (Eq. 1) and solve for R:

$$R = [3/\kappa][\langle x_0^2\rangle^{1/2}/\sigma_0][\langle s_p\rangle/\Delta p]. \quad (6)$$

All the quantities on the right-hand side of Eq. 6 can be taken from the experiment and, hence, R can be determined.

EXPERIMENTAL

Sample preparation

Cytochrome *c* from horse heart was obtained from Sigma Chemical (St. Louis, MO). Iron was removed and zinc was incorporated as previously described (Vanderkooi et al., 1976).

Spectroscopy

Spectral hole burning has been performed at 2 K in a He bath cryostat with a single-frequency dye laser system (rhodamine 6G, 17,000–17,450 cm^{-1}). The spectral width of the laser was of the order of 1 MHz. Burning power and burning times were ~0.5 mW and 5 min, respectively. The holes were detected in transmission and showed a Lorentzian width of ~5 GHz and a depth between 0.04 and 0.1 optical density (OD). The scanning range was ± 15 GHz. During the pressure-tuning experiments, the sample was kept at 2 K. Pressure was transmitted and regulated via He gas with an accuracy of 10^{-3} MPa and varied up to 2.0 MPa. The protein sample (8 mg/ml in a 10 mM phosphate buffer (pH 7) and glycerol mixture (1:1.5 v/v)) was sealed in a small plastic bag to ensure isotropic pressure. The pressure cell was immersed in liquid helium.

Data evaluation

The evaluation of the pressure shift is straightforward. As to the compressibility, one has to be careful in the event that transitions from different conformations are hidden under the inhomogeneous band. In our case this seems to be true (see Results).

In the present instance the most problematic parameter is the inhomogeneous width σ_0 due to hidden conformations. In addition to the hidden conformations, the near degeneracy of the Q_x and Q_y transitions (Balog et al., 1997; Kaposi et al., 1993) causes a further problem. We tried a series of decomposition procedures into Gaussians. To this end we subtracted the base-line contribution from the neighboring vibrational band. The various procedures yielded σ_0 values around 100 cm^{-1}. This is a very reasonable value. For example, in the free-base analog of the present protein where σ_0 is more easily accessible, a value of 109 cm^{-1} was obtained (Schlichter et al., 1999) In free-base horseradish peroxidase, the respective value is 69 cm^{-1} (Schlichter et al., 2001).

RESULTS

Fig. 1 shows the inhomogeneous absorption spectrum of Zn-Cc with the Q_x and Q_y (0,0) transition range ~17,300 cm^{-1} and the respective range of the associated vibrational band ~18,500 cm^{-1}. The dotted line serves for the base-line correction in the (0,0) range due to the overlap with the vibrational band. In addition, the color effect in the normalized pressure shift $s_p/\Delta p$ is shown. There are small deviations from linearity in the red and blue range of the band pointing to hidden conformations. We took a pragmatic approach: An overall least-squares fit rather accurately follows the data in the center range of the whole data set to which we restricted our analysis. The respective slope yielded a compressibility of 0.05 GPa^{-1}, a value which fits reasonably well into the scenario known from small proteins: for instance, the respective value for bovine pancreatic trypsin inhibitor, a 54 amino acid protein, is 0.06 GPa^{-1} (Stübner et al., 2002; Dadarlat and Post, 2001), for myoglobin values between 0.07 and 0.1 GPa^{-1} are reported (Gafert et al.,1993; Yamato et al., 1993).

Fig. 2 *a* shows as an example the pressure effect on the hole burned at 17,271 cm^{-1}. Fig. 2 *b* demonstrates that the shift s_p is to the red and is perfectly linear with pressure. In Fig. 2 *c*, the respective behavior of the pressure-induced line broadening σ_p is shown. It is also perfectly linear with pressure. As can also be seen from the data, the shift per pressure is of the same magnitude as the broadening per pressure, namely ~1 GHz/MPa.

Fig. 3 shows how we decomposed the Q-band into a series of Gaussians due to the overlap of the Q_x and the Q_y transition and contribution from an additional conformation to get an estimation of the inhomogeneous width σ_0 of a single component. The inhomogeneous line is base-line corrected (see Fig. 1). σ_0 is ~100 cm^{-1}.

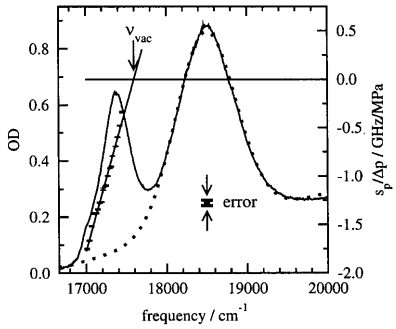

FIGURE 1 Absorption spectrum of Zn-cytochrome *c* at 4 K. The dotted line shows the part of the vibrational band that is subtracted to get the (0,0) transition. In the long wavelength band, pressure-tuning hole-burning experiments were performed. The respective values of the shift per pressure are shown. The slope of the fit line determines the compressibility. The wavenumber where s_p vanishes is the so-called vacuum absorption wavenumber ν_{vac}.

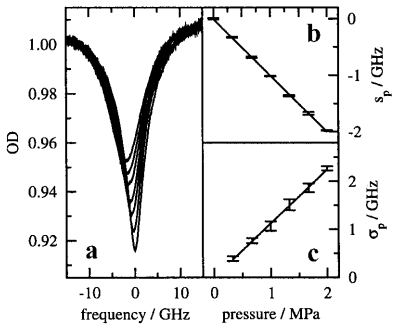

FIGURE 2 Pressure tuning of a hole at 17,271 cm^{-1} between 0 and 2 MPa. (*a*) The shape of the holes shows both a shift to the red and a broadening. (*b*) The shift of the center of the hole follows a linear pressure dependence. (*c*) Broadening of the hole under pressure.

DISCUSSION

The interaction range of the chromophore

We estimate the interaction range of the Zn-porphyrin chromophore with its protein environment on the basis of Eq. 6: taking for the compressibility the respective value as obtained from our experiments, namely 0.05 GPa^{-1}, for $\langle x_0^2 \rangle$ a value of 0.05 $Å^2$ as obtained from x-ray scattering experiments on myoglobin (Parak et al., 1987), for σ_0 the above quoted value of 100 cm^{-1} (3000 GHz), and for the average shift per pressure, $\langle s_p \rangle / \Delta p$, a value of 1 GHz/MPa from Fig. 3, we estimate an interaction range R of ~4.5 Å.

Although this value seems to represent a rather reasonable range, we are prone to believe that it represents a lower estimate of the interaction range R. In any case, we learn that the interaction range of the chromophore in Zn-Cc is much smaller than the radius of the protein. It mainly comprises the range of the protein pocket. Hence, if the fluorescence blue shift observed in pressure and temperature denaturing experiments is really due to an increased electrostatic interaction of the water molecules, the respective water molecules must be brought very close to the chromophore during the denaturing process. Whereas this is not surprising in the case of thermal unfolding, we feel it is an important observation in case of pressure-induced denaturation.

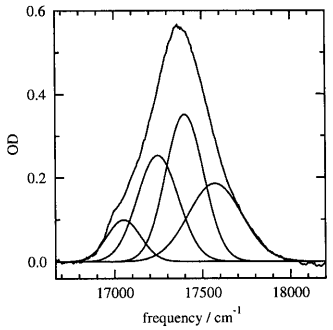

FIGURE 3 Decomposition of the electronic origin band into subbands. The Gaussian widths of the subbands σ_0 are ~100 cm^{-1}.

Obviously, the water molecules must be pressed into the interior of the protein pocket to get into close contact with the chromophore, as has also been suggested in the literature (Hummer et al., 1998a,b). The action of the water molecules with respect to protein unfolding is not quite clear but a straightforward assumption is that they break internal hydrogen bonds to form new ones thereby changing the structure sufficiently to denature the protein. Note that from fluorescence-unfolding experiments we do not get details on structural changes of the protein. We rather get information on how these structural changes might be brought about, namely by pressing water into the protein. In the case of cytochrome *c*, pressing water into the heme pocket may have an effect on the global structure of cytochrome *c*, because the polypeptide chain-porphyrin macrocycle is required for the folding of the protein. On the other hand, an infrared-unfolding experiment yields definitive information on a specific structural change, e.g., the breaking of an amide hydrogen bond, but no information on how this breaking is brought about.

Comments on the limitations of the model

The basic idea of the present work is to probe the chromophore protein interaction via pressure-tuning experiments. If this interaction falls off sufficiently quickly, then a local volume can be associated with each chromophore, the determination of whose size is the goal of this paper. There are three major assumptions in the present analysis, namely 1), the point dipole approximation that neglects any size effects of the molecules involved, 2), the assumption that line broadening and line shifts are due to the same interactions, and 3), that the length scale of the interaction is reflected in the relation between inhomogeneous broadening and static conformational mean-square displacement. If so, then it is only the absolute magnitude of the interaction that is needed to get the proper scale. This absolute magnitude is reflected in the absolute solvent shift that is obtained from pressure tuning as is directly seen by inserting Eq. 2 into Eq. 6. The detour by using the compressibility is convenient but not necessary.

A few comments on the above assumptions and special situations seem to be in order: First, Eq. 3, which is at the heart of the present analysis, is not limited to a certain type of interaction. All what is assumed is that the displacements due to static conformational disorder are small as compared to the average distances between atoms, an assumption that is always valid. Second, although line shifts and line widths may arise from different main interactions, this would only be expected if certain symmetry restrictions exist. For instance, a dipole-dipole interaction in an isotropic solvent has no contribution to the first moment of a spectral band, but can contribute to the second moment. In a protein it seems that such special situations do not play a general role. Third, counteracting interactions do exist and they may have

a significant influence on the interaction volume. For instance, polar probes may experience comparable red-shifting dispersion forces as well as blue-shifting electrostatic forces. In such a case the vacuum absorption frequency may lie within the inhomogeneous band and can be identified from the fact that s_p vanishes. Hence, if ν_{vac} is close to the band maximum ν_0, the average pressure shift and, accordingly, the interaction volume are to vanish. This, however, cannot happen, and, as a consequence, the average pressure shift has to be evaluated in an appropriate fashion, i.e., by evaluating the square average:

$$\langle s_p \rangle / \Delta p = [4\kappa^2 \langle (\nu - \nu_{vac})^2 \rangle]^{1/2}. \quad (7)$$

Averaging has to be performed over the inhomogeneous line shape. For a Gaussian Eq. 7 reduces to $2\kappa(\nu_0 - \nu_{vac})$ if the inhomogeneous width is small as compared to the average solvent shift, in agreement with Eq. 2. ν_0 is the wavenumber of the maximum of the inhomogeneous band. It is clear that the closer ν_{vac} to ν_0, the smaller the pressure shift and, accordingly, the smaller the interaction volume. Compensation of interaction terms reduces the environmental depth sampled by the chromophore. In our case, this may be partly responsible for the rather short range of 4.5 Å that we have estimated. ν_{vac} falls into the blue edge of the experimental inhomogeneous band, and hence, is already rather close to the band maximum.

The pressure broadening σ_p does not seem to be affected by ν_{vac}. However, it is strongly affected by structural order as is the case, for instance, in a crystal. Correlated displacements due to compression do not lead to line broadening. Accordingly, the correct scale of the interaction range cannot be obtained from pressure broadening. Note that the conformational displacement as obtained from line broadening is usually smaller than the respective one as obtained from the line shift. It can, in any case, never exceed it. In the present case it is roughly of the same magnitude due to the fact that ν_{vac} and ν_0 are rather close. If both frequencies coincide, pressure shift and broadening lead to the same length scale.

Uncertainty in the estimated interaction range

The uncertainty in the interaction range rests with the uncertainty of the parameters that enter Eq. 6, as well as with the validity of the approximations used. As to κ, its determination is based on the ''pressure shift solvent shift'' model. Hence, the absolute value of κ may deviate from those obtained with other techniques, although the agreement between the respective experiments is not bad. For the problem considered in the present article, it is not the thermodynamic quantity that plays a role. Rather, it is the compressibility of the local volume around the chromophore, and this is exactly the quantity that is probed in our experiment. What could cause more problems in this context are hidden conformations. They may not only have different κ-values, but also different vacuum frequencies. In the present case it seems that these uncertainties are tolerable.

The biggest problem in the present case is caused by the most trivial parameter, namely σ_0. Different conformations and different close lying transitions may come together to severely mask the correct width. As to Zn-Cc, the overall width of the red band is a superposition of two quasidegenerated states Q_x and Q_y, which are split by the protein crystal field by $\sim 100\ cm^{-1}$ (Manas et al., 2000). In addition to these two states, there are at least two different conformations, possibly more. Hence, the decomposition of the whole band into at least four subbands is mandatory. Yet, this is not all because the four states have also phonon wings. Our decomposition procedure yields values of the order of 100 cm^{-1}, which is very reasonable. Additional bands would reduce this value. This is one reason why we rather consider the 4.5 Å result a lower limit. A smaller σ_0 would increase this value.

Concerning the validity of the approximations of the model, the point-dipole approximation deserves special attention. As stressed above, it does no take into account the dimensions of the interacting molecules involved. However, that finite size effects do play a role is seen in pressure broadening. The size of the interacting molecules also puts constraints on the interaction volume. On the other hand, since the probe is a planar molecule, we consider the relative amount of space that is excluded from the interaction volume because of structural constraints due to finite size effects as small compared to the remaining two half spaces. Nevertheless, there is some interaction with the solvent molecules in the excluded volume, and this is another reason why R may be somewhat larger than determined from our experiments.

We finally compare our result with other techniques that measure interaction distances, mainly with Förster energy transfer experiments. Förster radii are much larger than the interaction range determined in our experiments, namely of the order of several tens of Angstroms. However, one has to keep in mind that resonance energy transfer is based on the interaction of transition dipoles of high oscillator strengths between similar molecules that may be an order of magnitude larger than the dipole difference vectors (or polarizability differences) that govern the solvent shift. Hence, we conclude that large Förster radii do not contradict to what we have found in our experiments.

CONCLUSIONS

In this article, we demonstrate that it is possible to estimate an effective size of an interaction volume between chromophore and protein from pressure-tuning experiments. The size of such a volume is a very important quantity in interpreting pressure-induced protein unfolding experiments via the respective behavior of the fluorescence spectrum. We found that the interaction volume is much smaller than the

volume of the protein. From this finding, we concluded that if water is pressed into the protein during pressure unfolding, the water molecules must come very close to the chromophore.

In some cases it might be difficult to determine the necessary parameters with sufficiently high accuracy, and we discussed the limiting aspects. Also, the assumptions inherent to the model may put some bounds on the accuracy of the estimated interaction range. We applied the model to a protein for which we investigated the stability phase diagram to get insight into the processes that trigger denaturing under high pressure. We stress, however, that the technique can be applied to any probe solvent system, e.g., doped glasses or polymers, even crystals. As to unfolding processes of proteins, it should be mentioned that a pressurized or thermally stressed protein is a different system as compared to the unstressed protein since water is forced close to the probe and, hence, the interaction and concomitantly the interaction volume may be different. The relevant physical parameter to estimate the respective effects is the ratio of the induced changes in the solvent shift (e.g., through the approaching water molecules) as compared to the absolute magnitude of the solvent shift. In our case, this is still a small number so that the order of magnitude value of the interaction range seems to be rather reasonable.

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