NMR Studies of Protein Surface Accessibility*

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Characterization of protein surface accessibility represents a new frontier of structural biology. A surface accessibility investigation for two structurally well-defined proteins, tendamistat and bovine pancreatic trypsin inhibitor, is performed here by a combined analysis of water-protein Overhauser effects and paramagnetic perturbation profiles induced by the soluble spin-label 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl on NMR spectra. This approach seems to be reliable not only for distinguishing between buried and exposed residues but also for finding molecular locations where a network of more ordered waters covers the protein surface. From the presented set of data, an overall picture of the surface accessibility of the two proteins can be inferred. Detailed knowledge of protein accessibility can form the basis for successful design of mutants with increased activity and/or greater specificity.

Interactions of proteins with other molecules can ultimately be ascribed to their surface features. Direct studies of protein surface accessibility are emerging as a new dimension of structural studies of proteins, particularly because repeated observations in either solution (1, 2) or crystal state (3, 4) have pointed out that proteins have regions where small and uncharged organic molecules, even those different from their physiological ligands, preferentially approach the molecular surface and also account for allosteric disruption of substrate binding (5, 6). We have shown that these “hot spots” of the protein surface can be easily mapped by a surface survey based on paramagnetic perturbation of conventional NMR spectra (7, 8).

The surface properties are dictated by the relative position and specific features of exposed residues, but even detailed knowledge of the protein architecture may not be sufficient for a thorough description of surface properties because of the intrinsic disorder of these residue side chains. The complex properties of the protein surface are modulated by a variety of factors (e.g. electrostatics, hydrophobicity, and hydrogen bond ability) but share a common unifying feature: hydration. The blanket of water covering the protein surface is the actual interface between the solution environment and the underlying modulations. The possibility of exploiting the blanket resides mainly on two of its features, namely, the variable thickness of the water layers and the fact that residence times of water molecules vary from point to point (9). Since the pioneering studies on protein hydration by Wüthrich’s and co-workers (10, 11), it has been well established that NMR is a reliable technique with which to detect water molecules bound to proteins (12). The early approaches were burdened by delicate hardware requirements, but now, thanks to developments in gradient-controlled sequences (12–16), intermolecular nuclear Overhauser effects between water and protein molecules can be routinely measured and correlated to overall protein hydration.

It has recently been proposed that the ability of surface mapping to reveal hot spots on the protein surface relies on the hindrance of bound water molecules to the approach of suitable paramagnetic probes to the protein (7). A combined use of paramagnetic perturbation and water-protein Overhauser spectroscopy based on 1D and 2D ePHOGSY (13, 14) can prove this link and may thus yield a faithful description of the static and dynamic properties of the water blanket. The attenuation of a specific set of hydrogen resonances in the NMR spectrum in the presence of a soluble paramagnetic probe reflects the accessibility of the corresponding locations on the protein surface (17). On the other hand, ePHOGSY experiments trace out different types of water-protein interactions by means of an initial intermolecular magnetization transfer step due to chemical exchange or dipolar interactions or both processes. Provided some prior knowledge of the protein structure (which is necessary anyway for result interpretation) is available, coupling of the two techniques should enable one to characterize the solvent modulation of the protein surface accessibility. Experimental assessment of different “modes” of water molecules on the protein surface can explain subtle phenomena that are difficult to reveal even by sophisticated molecular dynamic simulations because of the still approximate nature of available force fields.

BPTI and tendamistat, two paradigmatic proteins that are well characterized both structurally (18–21) and with respect to interactions with the paramagnetic probe 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO), represent an ideal benchmark to test this novel surface survey approach.

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† The abbreviations used are: 1D, one-dimensional; 2D, two-dimensional; BPTI, bovine pancreatic trypsin inhibitor; ePHOGSY, enhanced protein hydration observed through gradient spectroscopy; NOE, nuclear Overhauser effect; ROE, rotating frame Overhauser effect; TEMPO, 4-hydroxy-2,2,6,6-tetramethyl-piperidine-1-oxyl; TOCSY, total correlation spectroscopy. FT, Fourier transform; FID, free induction decay.
EXPERIMENTAL PROCEDURES

Sample Preparation—Tendamistat (a gift from Hoechst AG) and BPTI (obtained from Fluka) were purified by high pressure liquid chromatography, whereas TEMPOL (obtained from Sigma) was used without any further manipulation.

NMR Spectroscopy—Tendamistat and BPTI samples were prepared by dissolving the proteins in H2O/D2O (90:10, v/v) to make 2 and 6 mM solutions, respectively, adjusted to pH 3.15 and 3.5. The paramagnetic NMR samples contained an optimal TEMPOL concentration, which was achieved at a nitroxide/protein ratio of 10:1. This condition was reached by adding a few microliters of a 2 mM TEMPOL solution directly to the NMR tube.

NMR measurements were obtained at 309 and 323 K, respectively, for BPTI and tendamistat with a Bruker Avance 600 spectrometer to reproduce the experimental conditions of the original structural studies (19, 21). Data processing was performed with the Bruker software, and the spectral analysis was performed with NMRView (24). The 1H-13C heteronuclear single quantum coherence spectra have been referenced on trimethylsilylpropionic 2,2,3,3-d4 acid sodium salt at 0 ppm.

Resonance assignments for both proteins, which were available from the original structural work (19, 21), were checked anew under our experimental conditions using the scheme of sequential assignment described by Wuthrich (25). According to this scheme, a conventional set of 2D spectra was recorded: correlated spectroscopy (26), TOCSY (27), and nuclear Overhauser enhancement and exchange spectroscopy (28). Water resonance was attenuated using a DANTE presaturation pulse. In the specific sequence, Additional experimental parameters were the same as those described previously (7, 17). A total of 470 increments were collected in 2D spectra to match the spectral dimensions of 256 increments. Each increment was 2048 data points and 4096 scans, whereas the 2D ePHOGSY-NOE spectrum was recorded with 2048 data points. After zero-filling, the final matrix was 4096 x 2048 x 1024 data points.

1D ePHOGSY was performed with NOE and with ROE to discriminate between Overhauser and exchange effects, and 2D ePHOGSY-NOE-TOCSY (13, 14) was performed for a quantitative analysis of the effects. In all 1D ePHOGSY experiments, spectra were acquired with 16,384 data points and 4096 scans, whereas the 2D ePHOGSY-NOE-TOCSY spectra were obtained with 256 increments and 320 scans over 2048 x 512 data points. After zero-filling, the final matrix was 4096 x 512 data points. In all ePHOGSY experiments, the mixing time to build up the intermolecular Overhauser effects was 200 ms, and the H2O-selective 180° Gaussian pulse between the first two pulsed field gradients had a duration of 50 ms and 60 db attenuation (13, 14). Water suppression was achieved through an excitation sculpting (29) module appended to the sequences (13, 14). The experimental conditions of TOCSY, nuclear Overhauser enhancement and exchange spectroscopy, and 1H-13C heteronuclear single quantum coherence spectra have been described elsewhere (7, 17). The decrease of peak intensities caused by the added TEMPOL was evaluated from a comparison of ePHOGSY-TOCSY with NOE spectra obtained in the presence and absence of the paramagnetic probe and performed and processed with the same parameters.

Attenuation Calculations—As reported previously (17), paramagnetic effects were measured by comparing autocorrelated cross-peak attenuation figures (Ai, paramagnetic attenuation) defined as:

\[ A_i = \left[ 1 - \left( \frac{V_{i,d}}{V_{i,p}} \right) \right] \]  

(Eq. 1)

(\text{i.e. the individual deviations from the average of the cross-peak autocorrelated values, }V_{i,p}, \text{ the latter of which is defined as:})

\[ V_{i,p} = V_{i,p}(1/n)(\sum V_{i,p}) \]  

(Eq. 2)

where \( n \) is the number of measured cross-peak volumes, and \( V_{i,d} \) and \( V_{i,p} \) are the protein individual cross-peak volumes measured in the absence and presence of the spin probe, respectively.

The individual Ai values are plotted versus protein sequence position, and the values above or below the average attenuation level (unitary by construction because \( \langle V_{i,p}(dH) \rangle = 1 \) ) correspond to high or low spin probe accessibility levels, respectively (17). With this representation, it is easy to compare experiments performed under different conditions (temperature, protein and paramagnetic probe concentration, and solvents) because any specific effect is included in the mean value and the observed attenuations.

Model Building of Hydrated BPTI and Tendamistat—A reference BPTI model was built by fitting all the backbone atoms of the solution and crystal structures found in the Protein Data Bank (Protein Data Bank code 1PIT and 1BPI, respectively). The best fit (root mean square deviation = 0.728 Å) was achieved by comparing the fifteenth structure of 1PIT with 1BPI. In the hydrated solution structure, the coordinates of the four buried water molecules W111, W112, W113, and W122, according to the nomenclature of the crystallographic Protein Data Bank file 1PIT, were incorporated together with W105, W110, W138, and W139, conserved in all the available BPTI x-ray data. The reference tendamistat model was built by fitting all the backbone atoms of the solution and crystal structures of the Protein Data Bank (Protein Data Bank code 4AIT and 1HOE, respectively) with a root mean square deviation = 2.22 Å. Then, the coordinates of the water molecules W84 and W85, according to the numbering of the crystal structure, were added to the solution structure. These waters, which are indeed partially buried and well conserved in the available crystal structures 1HOE and 1BVN, should be considered structural ones. Both models were refined with 500 steps of energy minimization by the steepest descent method. The resulting structures were placed in a truncated octahedral box of equilibrated water. Ten steps of energy minimization with the steepest descent method followed by another 490 steps with the conjugate gradient method were carried out. Afterward, the equilibration of the BPTI/water and tendamistat/water systems was done in three stages by a molecular dynamic approach at increasing temperatures. In all runs, the temperatures of the protein and solvent were separately coupled to an isothermic bath. The pressure was kept constant by coupling to an isobaric bath at 1 bar. Bonds were always kept constrained with SHAKE methods with a relative tolerance of 10−8 Å. The length of the molecular dynamic elementary time step was 0.002 ps in all runs. During the first 2 ps trajectory at 100 K, the constants for coupling to the temperature and the pressure bath were 0.05 fs. For the subsequent 2 ps, the temperature was increased to 200 K. Finally, a run of 6 ps at 277 K was performed with temperature and pressure equilibrium constants increased to 0.1 and 0.5 ps, respectively. All displayed structures derived from the above-mentioned protocols were generated with the program MOLMOL (30).

RESULTS

In Fig. 1, the relative signal intensities measured in 2D ePHOGSY-NOE-TOCSY spectra (13, 14) of BPTI and tendamistat are reported with bar heights referring to the sum of the intensities of the cross-peaks measured in the \( \omega_d \) dimension. Sizeable water-protein Overhauser effects arising from intermolecular contacts shorter than 4–5 Å report on direct interactions between the solute and water molecules in its first hydration shell (12); hence, the number of the observed water-protein NOEs should be, at first approximation, proportional to the protein accessible surface. This is not the case for the two proteins here investigated. The models built by using Protein Data Bank 4AIT and 1PIT entries, corresponding respectively to hydrated solution structure of tendamistat and BPTI, indicate that tendamistat has a water accessible surface of 4699.2 Å2, i.e. 11% larger than that of BPTI. Furthermore, in agreement with the different number of amino acid residues of the two proteins, BPTI exhibits 328 resolved proton signals, as compared with the 391 signals of tendamistat. In principle, both of these features should lead to a larger number of ePHOGSY signals for the latter protein. On the contrary, 121 and 86 protons of BPTI and tendamistat (31% and 22%, respectively, of the corresponding resolved NMR protons) yield ePHOGSY signals. This observation might reflect, in part, the difference in temperature of the data collection in the two structure determinations. However, the complex nature of the water blanket covering the two surfaces plays a major role (vide infra).

ePHOGSY spectra report on protein hydrogen connectivities to water via all sorts of magnetization transfer effects, including the direct chemical exchange with bulk water that involves side chain or backbone labile protons. By comparing the peak signal of 1D ePHOGSY-NOE and ePHOGSY-ROE, direct information on the contributions from different magnetization-transfer time scales can be retrieved (13, 14). Thus, the intensities of the peaks arising from protons undergoing chemical exchange are positive in the 1D ePHOGSY-ROE (Fig. 2), i.e. opposite to any Overhauser effects in the rotating frame. In-
stead, in 1D ePHOGSY-NOE, the intensity of the water-protein Overhauser effects, which depends on the length and reorientation rate of the specific intermolecular internuclear vector, most commonly has the same sign as the chemical exchange effects as a consequence of the low frequency motional regime of macromolecules (negative Overhauser effect). It should be noted that both these mechanisms could contribute at the same time to the observed intensity of an individual ePHOGSY signal. From inspection of the histogram heights of Fig. 1, it is apparent that the exchange signals found in the 1D ePHOGSY-ROE are among the peaks with the highest intensity. Indeed, several protons of both proteins appear to be involved in chemical exchange processes. This is the case for tendamistat amide protons within the active site, i.e. S17, R19, and Y20, and for several other hydrogens highlighted in the same figure. However, according to previous observations (12), all ePHOGSY peaks of serines, threonines, and tyrosines could be partly due to relayed transfers via the exchanging OH groups of these residues. This could partially explain the very high intensity of ePHOGSY signals of Y10 H₆ and Hₑ of BPTI and, in general, the enhancement observed in both proteins for any of the three OH-bearing residues.

Because of the small size of the proteins, the high temperature, and the short mixing time used in the experiment, we can safely confine the chemical exchange contribution to only the protons in direct contact with the exchangeable protons. Almost all the detectable water-protein NOEs are negative (corresponding to positive peaks in Fig. 2), but a few positive Overhauser effects can be observed, both in the 1D ePHOGSY and 2D ePHOGSY-TOCSY spectra with NOE of BPTI and tendamistat. In the case of BPTI, positive effects are observed for L6 H₆/H₉253, A16 H₆/H₉251, I19 H₆/H₉252, K26 Hₑ/H₉280, and L29 H₆/H₉252, all nuclei belonging to aliphatic moieties located on the surface of the protein. This feature may suggest that local high flexibility is responsible for this NOE sign inversion. Water molecules bound to surface hydrogens with residence times consistent with the build up of sizeable NOEs and involved in intermolecular dipolar interactions with mutual reorientation faster than 3 × 10⁻¹⁰ s (the sign inversion limit at the operative Larmor frequency) should indeed give positive Overhauser ef-
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Effects. A similar pattern is found for tendamistat because positive NOE effects (negative ePHOGSY peaks) are detected for the surface-exposed aliphatic side chain protons T2 Hγ, T3 Hγ, V4 Hγ, and L44 Hδ, as shown in Fig. 2b. However, some care must be exerted before ascribing such results exclusively to the local flexibility of the macromolecules. Although positive NOEs between water and a structured macromolecule can always be considered as true dipolar effects, the same outcome would also be expected if the local mobility were due to bound but locally reorienting water molecules or to rapidly diffusing ones (12). Additional independent measurements, such as NMR relaxation or diffusion-filtered determinations, are necessary to safely attribute the local mobility to the macromolecule rather than water or some combination of individual motions.

Once the characteristics of all the peaks found in ePHOGSY-ROE and ePHOGSY-NOE spectra are analyzed in detail, and Overhauser effects and exchange processes are properly recognized, possibly by extending the experimental data set with diffusion-filtered determinations (13, 14), it is possible, in principle, to interpret the NOE intensities in terms of internuclear distances and/or molecular dynamics, but such a calculation is far from trivial. Apart from possible difficulties with overlap of NOEs and relayed exchange contributions, even for genuine negative NOEs, no a priori assumption on correlation times or interproton distance calibrations can be easily made, as is customarily done with intramolecular Overhauser effects. On the other hand, when trying to map the features of the overall accessibility to a protein surface, we are more interested in relative mobility than in absolute mobility. Thus, in this report, no absolute analysis of the ePHOGSY peak intensities has been performed, and only the corresponding TEMPOL-induced paramagnetic perturbations are discussed.

Additions of TEMPOL to the water solutions of BPTI and tendamistat cause, to different extents, attenuations of the 2D ePHOGSY-NOE-TOCSY peaks, in total analogy with the effects induced by the same spin label in other more conventional 2D spectra (17). Attenuations of 2D ePHOGSY-NOE-TOCSY peaks filter out the set of protein hydrogens whose accessibility is mediated by neighboring water molecules, whereas the corresponding attenuations in conventional 2D spectra (7) yield the global accessibility. A comparison between the two ensembles allows the definition of fine aspects of accessibility. TEMPOL-edited ePHOGSY intensities, in fact, enable one to classify the protein-connected water molecules as: 1) bound (dynamically bound) to exposed and flexible (rigid) protein locations, 2) bound to exposed and rigid protein locations, 3) buried, or 4) ordered in a network that prevents close approach of small uncharged ligands. However, recognition of chemical exchange interactions that do not concern the entire water molecule but only its protons still rests on the comparison of ePHOGSY-ROE and ePHOGSY-NOE peak signs.

Fig. 3 shows the comparison of 2D ePHOGSY-NOE-TOCSY peak intensities measured in the presence and absence of the paramagnetic probe. Autoscaled peak volumes of all detected signals are compared, as suggested previously (17) for comparisons involving different data sets. The signal intensities of both proteins are modulated by specific attenuations along the sequence, but it is apparent that, overall, BPTI ePHOGSY signals are much less attenuated by TEMPOL than those of tendamistat. This finding is fully consistent with the presence of the four water molecules buried in the BPTI interior (18, 19). These water molecules can yield an efficient intermolecular magnetization transfer to the inner proton environment of the protein that cannot be perturbed by the external paramagnetic probe. This situation is well illustrated by Fig. 4c, which shows a BPTI three-dimensional model in which the internal waters (highlighted) are surrounded by all the hydrogens within a 5 Å shell from the oxygen atom of each of the buried waters. It is clear that resonances of protons “illuminated” by buried water magnetization cannot be quenched by TEMPOL. Most of the BPTI ePHOGSY signals exhibiting very low TEMPOL-induced attenuations are located in the inner part of the protein. In fact, 19 of 35 ePHOGSY signals that exhibit \( A_i < 0.4 \), an already proposed upper limit for unperturbed signals (7), are related to hydrogens that have close contacts with the internal waters. The remaining 16 hydrogens with \( A_i < 0.4 \) can be divided in two groups. R1Hδ3, F4NH, Y23Hδ, Y23Hε, Q31Hδ3, Y35Hδ1, Q31Hβ3, and T54Hβ, although not completely buried, are sterically hindered with respect to the approach of a paramagnetic probe of the size of TEMPOL. Others, however, are surface hydrogens, i.e. Q31Hγ1, R39Hα, E49Hβ, C55Hα, and A58Hα, that are totally unattenuated not only when viewed in a static picture but also in molecular dynamics simulations. The analysis of the reduced accessibility of these hydrogens is less straightforward, but it discloses a new scenario with respect to previous views of the surface of BPTI. The most direct explanation is the presence of water molecules with long residence times in close proximity to the poorly attenuated surface hydrogens. The presence of many hydrogen bond donor and/or acceptor groups near the latter nuclei is consistent for an ordered network of water molecules that prevents the close approach of TEMPOL to these surface sites.

Only 7 tendamistat ePHOGSY signals exhibit paramagnetic attenuations < 0.4. The fact that this number of signals is very limited can be ascribed primarily to the absence of totally buried water molecules. However, water molecule W84 (according to the 1HOE water numbering) buried in our reference model and conserved in 1HOE and 1BVN structures accounts for the low attenuations of K34Hδ, V35NH, L44NH, and V56Hβ ePHOGSY signals (see Fig. 4b). It is worth noting that the other three hydrogens whose \( A_i < 0.4 \), i.e. S21Hβ, D58NH, and R72NH, belong or are very close to polar groups. This feature can be related to the presence of strong hydration sites, which prevent a close approach of the paramagnetic probe, in agreement with that observed for BPTI.

DISCUSSION

Previous TEMPOL-induced paramagnetic perturbation studies on tendamistat (7) and BPTI (17) yielded an overall picture of accessibility to the two protein surfaces that suggests...
that the main features connected to accessibility were directly or indirectly linked to hydration properties. The main conclusions drawn from unfiltered perturbation studies can be summarized as follows: 1) few surface patches are highly accessible, including, in both cases, the protein active sites; 2) in general, for all of the analyzed protons, a good correlation was found between exposed surface areas and paramagnetic attenuations; and 3) whenever surface-exposed hydrogens exhibited only minor paramagnetic effects, the presence of a strong hydration site was invoked.

The present water-filtered paramagnetic perturbation studies substantiate these findings and unveil additional fine aspects of surface accessibility.

Despite the high percentage of hydrated surface that is expected for proteins from static observations (22), the NMR data indicate that only a small number of water molecules are statistically close enough to protons of BPTI and tendamistat to affect their resonances. The solvent dynamics occurring at the surface of the two proteins is responsible for the limited number of water-protein interactions because too short residence times of water molecules quench the intermolecular Overhauser effects, whereas the lifetime of a close water-protein contact can be as long as $10^{-6}$ s for the internal waters of BPTI (23) or even $10^{-3}$ s at 4 °C (11).

Thus, tightly bound solvent molecules yield detectable NOEs, as in the case of all BPTI structural waters, such as the buried W111, W112, W113, and W122 and the partially buried W105, W110, W138, and W158. The same trend is observed for the NMR spectra of the structural and buried W84 of tendamistat, which is responsible for a large number of water-protein Overhauser effects (see Fig. 4b). These NOEs are essentially unaffected by TEMPOL, confirming that paramagnetic perturbation studies of ePHOGSY signal intensities may be useful in finding structural waters. Furthermore, waters experiencing long residence times and not perturbed by TEMPOL, even when located on the protein surface, must be involved in strong hydration sites that prevent the approach of TEMPOL. However, the majority of the proton ePHOGSY signals, particularly the ones of tendamistat, experience $\Lambda_i > 1$, i.e. medium to strong paramagnetic effects arising from close encounters of these hydrogens with both water and TEMPOL molecules.

The high selectivity attainable by ePHOGSY experiments in connection with paramagnetic perturbations is best exemplified by the possibility of explaining even minute discrepancies...
between expected and predicted accessible surface areas. The paramagnetic perturbation profile of the $^1$H-$^{13}$C heteronuclear single quantum coherence spectrum of BPTI was obtained for only 37 resolved heteronuclear correlations $C_{\alpha}/H_9251-H_{\alpha}$. The agreement between the paramagnetic attenuation ($A_i$) and accessible surface areas of BPTI $C_{\alpha}$ protons reported in the quoted paper was good in 24 cases. The remaining 13 discrepancies came from residues $P2$, $F4$, $R39$, $K41$, $R42$, and $T54$, whose $C_{\alpha}/H_9251-H_{\alpha}$ correlations exhibited paramagnetic attenuations smaller than the expected ones. Residues $T11$, $C14$, $K15$, $R17$, $T32$, $V34$, and $G36$, which exhibited $A_i$ values for accessible surface areas.

This finding reflects the fact that the paramagnetic perturbation profiles of the $^1$H-$^{13}$C heteronuclear single quantum coherence spectrum of BPTI were obtained for only 37 resolved heteronuclear correlations $C_{\alpha}/H_9251$. In both cases, the paramagnetic attenuation ($A_i$) is estimated by the program OLENIQ. Gray refers to atoms whose missing $A_i$ value is due to NOE absence, missing assignment, or spectral overlapping. The lack of detectable NOEs is observed only for hydrogens that are more than 4 Å distant from the water oxygen atom.

The paramagnetic perturbation profiles are not simply related to the static hydrogen topology inferred from the protein structure. A direct comparison of the exposed surface areas of hydrogens of the various residues with the paramagnetic attenuations measured both in ePHOSGY and $^1$H-$^{13}$C heteronuclear single quantum coherence spectra provides information on differential accessibilities of the protein surface. Thus, the presence of tightly bound water molecules that prevent the approach of TEMPOL to BPTI can explain the $A_i$ values observed for residues $F4$, $R39$, $K41$, $R42$, $T54$ because they have one Overhauser effect with water, at least. In the case of $P2$, which is very close to the very hydrated $F4$, an indirect shielding from the paramagnetic probe could be effective. It is interesting to note that as shown by Fig. 5a, all the residues with anomalously low attenuations are aligned on the same side of the protein surface. From Fig. 5b, it is also apparent that residues $T11$, $C14$, $K15$, $R17$, $V34$, and $G36$, which are characterized by anomalously high attenuations, define a surface region centered on the protein active site.

It is fair to conclude that an overall picture of protein surface accessibility, valid at least for the two chosen examples, is obtained by the combined use of the TEMPOL-induced paramagnetic perturbations on the 2D conventional spectra and the ePHOSGY or some other equivalent spectroscopy experiment. Thus, most of the water molecules involved in close contacts with proteins appear to be characterized by residence times short enough to prevent the onset of detectable Overhauser effects. Tightly bound water is never found near the protein active sites, which always result to be very TEMPOL accessible. This feature is of primary relevance because in this way it is experimentally confirmed that active sites are molecular regions that are very accessible by chemical species, even those that are quite different from the natural ligand. Almost all the surface-exposed regions showing NOE with water are also TEMPOL accessible, unless the solvent molecules are involved.
in the formation of strong hydration sites. Structural water molecules, which, in general, are buried and thus not at all perturbed by the paramagnetic probe, can be easily identified.

It can be concluded, therefore, that from paramagnetic attenuation profiles of protein NMR spectra with and without water-protein dipolar-interaction editing, many critical characteristics of protein surface accessibility may be obtained. Thus, provided that the protein structure, (possibly in solution) is known, a new dimension of structural biology given by the protein accessibility can be explored.

Detailed knowledge of all features of the protein surface is of utmost importance in all biological problems involving molecular recognition. As mentioned in the “Introduction,” a study of the surface of MNEI, sweet protein, by means of TEMPO revealed the presence, on its surface, of interaction points that include residues previously predicted by ELISA tests and by mutagenesis (8). If we limit the discussion to the two main examples of this study, it can be said that a better understanding of the borders and properties of the surface areas of inhibitors can lead to the rational design of mutants with tailored specificity, a vital need in the case, for instance, of viral proteinase inhibitors.

Obviously, other paramagnetic probes might be used, particularly in the case of redox protein systems, which can be affected by the weak oxidant properties of TEMPO.

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