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Dimethyl sulfoxide binding to globular proteins: A nuclear magnetic relaxation dispersion study

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Abstract

The ^2H magnetic relaxation dispersion (NMRD) technique was used to characterize interactions of dimethyl sulfoxide (DMSO) with globular proteins. A difference NMRD experiment involving the N-acetylglucosamine trisaccharide inhibitor, demonstrated that the DMSO ^2H NMRD profile in lysozyme solution is due to a single DMSO molecule bound in the active cleft, with a molecular order parameter of 0.47 ± 0.05 and a residence time in the range 10 ns to 5 ms. With the aid of transverse ^2H relaxation data, the upper bound of the residence time was further reduced to 100 μs. A ^1H shift titration experiment was also performed, yielding a binding constant of 2.3 ± 0.3 M^{-1} at 27 °C. In contrast to lysozyme, no DMSO dispersion was observed for bovine pancreatic trypsin inhibitor (BPTI), indicating that a stable DMSO–protein complex requires a cleft of appropriate geometry in addition to hydrogen-bond and hydrophobic interactions.

Keywords: ligand binding; mixed solvents; order parameter; protein solvation; relaxation dispersion; residence time

Organic and mixed solvents are used increasingly in biochemistry and biotechnology to modulate the catalytic properties of enzymes and the stability of proteins and other biomolecules (Zaks & Russell, 1988; Klibanov, 1989; Gómez-Puyou, 1992; Koskinen & Klibanov, 1996). There is, therefore, a clear need to improve the current, rather fragmented, molecular-level understanding of organic solvent–protein interactions. Dimethyl sulfoxide (DMSO) is a powerful dipolar solvent, completely miscible with water and most organic liquids, and perhaps the most extensively used aprotic (co)solvent in chemistry, biology, and medicine (Jacob et al., 1971; Martin & Hauthal, 1975). The effects of DMSO on proteins are extremely varied; it can act as a stabilizer (Rajeshwara & Prakash, 1994; Rajendran et al., 1995), a denaturant (Bettelheim & Senatore, 1964; Hamaguchi, 1964), an inhibitor (Perlman & Wolff, 1968), an activator (Rammler, 1971; Almarsson & Klibanov, 1996), and as a cryoprotector (Lovelock & Bishop, 1959; Yu & Quinn, 1994).

While most proteins are soluble in neat DMSO (Singer, 1962; Chin et al., 1994), their tertiary and even secondary structures are usually highly disrupted at high DMSO concentrations (Broadhurst et al., 1991; Evans et al., 1991; Jackson & Mantsch, 1991; Desai & Klibanov, 1995). The denaturation of proteins by DMSO, as well as by other organic cosolvents, occurs in a sharp transition at a protein-dependent threshold concentration (Khmelmitsky et al., 1991). In the case of lysozyme, for example, the tertiary fold is disrupted at a DMSO concentration around 70% (ca. 10 M) (Hamaguchi, 1964; Fujita et al., 1982). Even at lower DMSO concentrations, however, rotatory dispersion, optical (Hamaguchi, 1964; Williams et al., 1965) and infrared spectra (Huang et al., 1995) indicate minor structural perturbations and/or DMSO binding to the active cleft and the protein surface.

Whereas strong ligand binding to biopolymers has been extensively studied, with NMR techniques contributing much of the structural and dynamic information (Jardetzky & Roberts, 1981; Craik & Higgins, 1989; Otting, 1993; Lian et al., 1994), relatively few NMR studies have been reported of weak binding, the main feature of protein–solvent interactions in mixed solvents. Recent progress in multidimensional NMR, however, has enabled structural studies of urea binding to lysozyme (Lumb & Dobson, 1992) and BPTI (Liepinsh & Otting, 1994). Another potentially useful NMR technique, nuclear magnetic relaxation dispersion (NMRD) has so far been quantitatively applied only to the problem of protein–water interactions (Koenig & Brown, 1991; Denisov & Halle, 1996). The observation of ^1H NMRD profiles for methanol in carbonic anhydrase solution (Jacob et al., 1980) and for DMSO in lysozyme solution (Bryant & Jarvis, 1984), however, suggests that the NMRD technique can be used to characterize protein–ligand interactions as well. A recent study of DMSO spin relaxation in

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; DMSO, dimethyl sulfoxide; GlcNAc, N-acetylglucosamine; $GlcNAc_3$, N-acetylglucosamine trisaccharide; HEWL, hen egg-white lysozyme; NMR, nuclear magnetic resonance; NMRD, nuclear magnetic relaxation dispersion

gelatin solutions and gels indicated strong gelatin-DMSO interactions (Hills & Favret, 1994), but the molecular details remain unclear.

In the present study, we show that the ^2H NMRD profile from deuterated DMSO in lysozyme solution is almost eliminated by addition of the inhibitor $GlcNAc_3$, which is known to strongly bind to the active site of the enzyme. This finding demonstrates that the observed NMRD is due to a single specifically bound DMSO molecule. A quantitative analysis of the dispersion yields information about the order parameter and residence time of this DMSO molecule. In contrast, we observe no NMRD from DMSO in BPTI solution, consistent with absence of specific binding sites on the surface of this protein.

Results and discussion

DMSO binding to lysozyme

Lysozyme is known to bind various small ligands, which mimic the interactions of the acetamido group of its natural substrates within the active cleft of the enzyme (Imoto et al., 1972; McKenzie & White, 1991). Comprehensive structural studies have been reported for GlcNAc oligosaccharides and urea, that bind to the active site both in crystals (Blake et al., 1967; Pike & Acharya, 1994) and in solution (Cohen & Jardetzky, 1968; Lumb & Dobson, 1992; Lumb et al., 1994). Although DMSO cannot form as many hydrogen bonds with the enzyme as GlcNAc or urea, the similarity of its structure with the acetamido group suggests that it can bind at the same site. Indeed, a neutron diffraction study of lysozyme crystals soaked in 15% DMSO solution localized six bound DMSO molecules, with the most strongly bound one in subsite C (Fig. 1) (Lehmann & Stansfield, 1989).

When a methylated molecule binds to subsite C, the methyl protons experience a ring-current shift due to the nearby aromatic residues Trp108 and Trp63, a fact used to determine the binding constants of several homologous lysozyme inhibitors (Raftery et al., 1969). Due to the nonplanar geometry of the DMSO molecule (Thomas et al., 1966), the protein discriminates between the two methyl groups. Whereas the two methyl resonances of a DMSO molecule free in solution are degenerate, the asymmetric protein environment of a bound DMSO molecule thus breaks this degeneracy, leading (under fast exchange conditions) to a splitting of the bulk DMSO resonance (Liepinsh & Otting, 1997).

^1H spectra from lysozyme solution (not shown) exhibit the DMSO methyl splitting of 0.005–0.015 ppm (concentration dependent), indicating fast exchange between the free and bound states (Fig. 2). Assuming a 1:1 binding stoichiometry (vide infra), a two-parameter fit based on Equation 5 (see Materials and methods) to the titration data in the figure yields a binding constant $K = 2.3 \pm 0.3\ M^{-1}$ and a bound state splitting $\Delta\delta = 0.66 \pm 0.08$ ppm at 27 °C. Analogous fits for the individual methyl resonances (not shown) yield the (upfield) shifts of 0.8 ± 0.2 and 1.4 ± 0.2 ppm upon binding, the latter value being a factor 2 larger than for the methyl resonance of GlcNAc (Dahlquist & Raftery, 1968). The binding constant obtained here is considerably smaller than the 40–60 M^{-1} obtained for GlcNAc by various techniques (Imoto et al., 1972; Hamaguchi, 1992), as expected because for GlcNAc both the acetamido group and the saccharide ring are involved in the binding interaction. On the other hand, DMSO binding appears to be somewhat stronger than that of urea, with reported binding constants between 0.17 M^{-1} and ca. 1 M^{-1} (Shimaki et al., 1971; Lumb & Dobson, 1992; Liepinsh & Otting, 1997). Similar binding constants were obtained for several other small ligands: acetamide (0.9 M^{-1}), ethanol (4.6 M^{-1}) (Kuramitsu et al., 1973), acetone (3.1 M^{-1}), acetonitrile (3.9 M^{-1}), dichloromethane (4.6 M^{-1}), and isopropanol (1.0 M^{-1}) (Liepinsh & Otting, 1997).

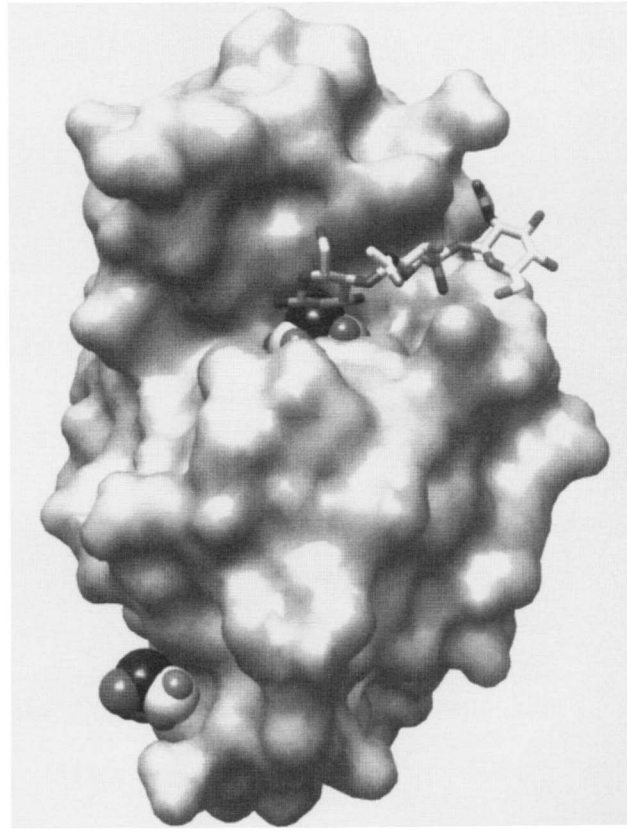

Fig. 1. Crystal structure of the lysozyme–$GlcNAc_3$ complex (Cheetham et al., 1992) with two of the six DMSO molecules in the crystal structure in 15% DMSO (Lehmann & Stansfield, 1989) superimposed. One of the DMSO molecules overlaps with the acetamido group of $GlcNAc_3$ (shown in rod representation) in subsite C of the active cleft .

It is known that the chemical shifts of several lysozyme resonances are perturbed upon binding of GlcNAc oligosaccharides (Cohen & Jardetzky, 1968; Dobson & Williams, 1975; Lumb et al., 1994), reflecting direct inhibitor contacts with residues in the active cleft as well as small conformational changes in the protein-inhibitor complex. Binding of urea similarly affects protein chemical shifts, and, additionally, perturbs resonances of several exposed residues through interactions with the protein surface (Lumb & Dobson, 1992). Although no attempt was made here to characterize the structural consequences of DMSO binding to lysozyme in solution, significant shifts of lysozyme resonances upon addition of DMSO were seen in the one-dimensional ^1H-NMR spectra. All of the 24 resonances with detected chemical shift change of more than 0.02 ppm belong to residues that are in (or close to) the active site, and which are also influenced by GlcNAc oligosaccharides (Lumb et al., 1994) or urea (Lumb & Dobson, 1992). The binding constants, obtained from the fits of Equation 5 to the protein chemical shift titration data (not shown) all fall in the range 2.2–3.2 M^{-1} and are consistent within the propagated errors with the DMSO binding constant of $K = 2.3\ M^{-1}$. Although some changes,

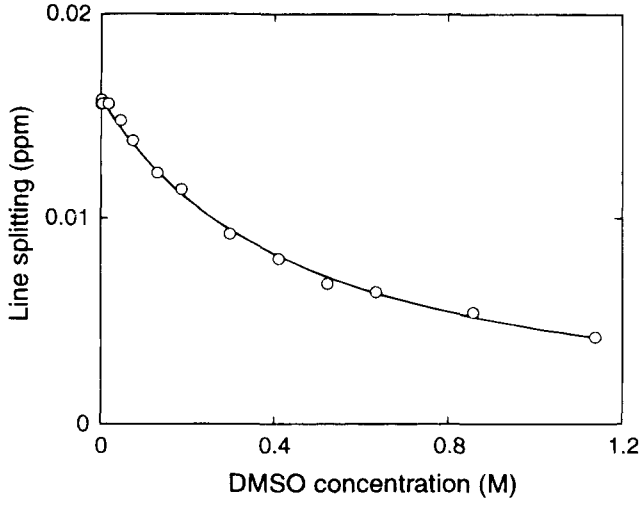

Fig. 2. Splitting of the DMSO methyl ^{1}H resonance at 500 MHz in 11 mM lysozyme in D_2O, pH* 4.16, 27 °C, as a function of total DMSO concentration. The solid curve represents a two-parameter fit based on Equation 5 to the data points. The estimated error bars are of the same size as the data symbols.

mainly line broadening, were also seen in spectral regions corresponding to the resonances of Trp123, which is in the second DMSO binding site (denoted number 3 in Lehmann & Stansfield (1989), Fig. 1), the above results indicate that DMSO binding to lysozyme in solution is essentially localised to the active cleft. The crystallographic data (Lehmann & Stansfield, 1989) also suggest that subsite C binds DMSO stronger than the second site: for the former the site occupancy is 0.88, the temperature factor is 23.0 Å^2, and the fraction exposed DMSO surface area is 11%, while for the latter the corresponding figures are 0.70, 50.1 Å^2, and 45%. The four additional DMSO molecules identified in the neutron diffraction map are located in contact regions between two or more symmetry-related lysozyme molecules. Because, in solution, 50–70% of the surface area of these four bound DMSO molecules would be accessible to the solvent, these sites are presumably significantly weaker in solution. All this indicates that the determined binding constant, $K = 2.3\ M^{-1}$, refers essentially to subsite C. Although DMSO molecules must occasionally contact almost any site at the protein surface, these interactions should be weak and the corresponding binding constants much smaller than K.

^{2}H relaxation dispersion from DMSO in lysozyme and BPTI solutions

Recent water ^{2}H and ^{17}O NMRD studies of protein solutions have established that the dispersion of the longitudinal relaxation rate R_1 in the MHz range is due to a small number of crystallographically well-defined water molecules, with residence times long compared to the rotational correlation time, τ_R, of the protein but short compared to the intrinsic relaxation time T_1 in the bound state (Denisov & Halle, 1995a, 1995b, 1995c; Denisov et al., 1995; Denisov & Halle, 1996). The R_1 dispersion is accurately described by (Denisov & Halle, 1995b)

$$R_1(\omega_0) = R_{bulk} + \alpha + \beta\tau_R\left[\frac{0.2}{1 + (\omega_0\tau_R)^2} + \frac{0.8}{1 + (2\omega_0\tau_R)^2}\right], \quad (1)$$

where ω_0 is the Larmor frequency and R_{bulk} the bulk solvent relaxation rate. The dispersion amplitude β originates from the long-lived water molecules, while α is the frequency-independent contribution from short-lived water molecules at the protein surface and from fast internal motion of long-lived water molecules. The same theoretical framework should also describe the R_1 dispersion of the cosolvent DMSO, as investigated here, provided that the parameters α and β are interpreted accordingly (vide infra).

Figure 3 shows the ^{2}H relaxation dispersion profiles from solutions of HEWL, HEWL inhibited with $GlcNAc_3$, and BPTI (for sample compositions, see Table 1). The upper two curves resulted from a four-parameter fit of Equation 1 to the combined HEWL and HEWL-$GlcNAc_3$ data points (with common α and τ_R for the two samples). Because there is no significant dispersion for BPTI, we set $\beta = 0$ in Equation 1 and determine α from the data. The values of the parameters α, β, and τ_R derived from the fits are collected in Table 2.

The lack of dispersion from the BPTI solution demonstrates that there are no DMSO molecules associated with BPTI with residence times longer than τ_R, i.e., 6–7 ns under the present conditions (Szyperski et al., 1993; Denisov & Halle, 1995b). This finding is consistent with the lack of well-defined ligand binding sites on the surface of BPTI (Wlodawer et al., 1984). Furthermore, it suggests the general conclusion that hydrophobic or hydrogen bond interactions alone are not sufficient to produce a long-lived DMSO–protein complex. The strong dispersion seen for HEWL must, therefore, be due to a small number of long-lived DMSO molecules bound to specific site(s) of the required geometry. The correlation time for this dispersion, $\tau_R = 7.5 \pm 1.0$ ns, is consistent with the rotational correlation time of 7.1 ± 0.3 ns, obtained for the lysozyme molecule in aqueous solution under similar conditions (Denisov & Halle, 1996), taking into account the ca. 10% difference in solvent viscosity (Aminabhavi & Gopalakrishna, 1995). The residence time of the bound DMSO molecule(s) must, therefore, be considerably longer than 7.5 ns.

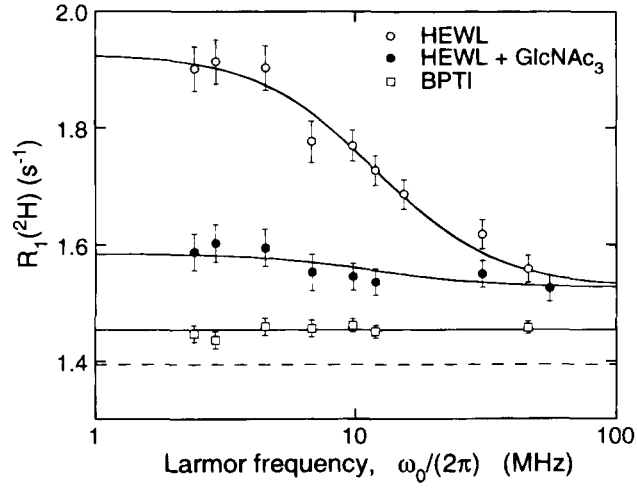

Fig. 3. Dispersion of DMSO ^{2}H longitudinal relaxation rate from 90% H_2O/10% DMSO-d_6 solutions (27 °C) of (open circle) HEWL, (closed circle) HEWL inhibited with $GlcNAc_3$, and (open square) BPTI. The BPTI data are scaled to the same molar protein concentration, as for HEWL, assuming that the excess relaxation rate, $R_1 - R_{bulk}$, is proportional to $1/N_{DMSO}$. The solid curves represent fits using Equation 1 as described in the text. The dashed line refers to the ^{2}H relaxation rate of the bulk solvent with the same H_2O/DMSO molar ratio as in the protein solutions.

Table 1. *Sample compositions (solvent—H_2O/DMSO-d_6)*[a]

Protein	pH	C_p (mM)	N_{aq}	N_{DMSO}	$N_{GlcNAc3}$
BPTI	4.54	14.2	3,373	91	—
HEWL	4.18	7.0	6,814	184	—
HEWL-$GlcNAc_3$	4.17	7.0	6,814	181	1.2

[a]Solution composition is represented by the protein concentration (C_p), the total number of molecules (per protein molecule) of water (N_{aq}), DMSO (N_{DMSO}), and $GlcNAc_3$ ($N_{GlcNAc3}$).

The specific lysozyme inhibitor $GlcNAc_3$ binds to subsites A–C of the active cleft (Blake et al., 1967; Imoto et al., 1972; Cheetham et al., 1992) with a binding constant $K = 9.6 \times 10^4\ M^{-1}$ at pH 4.2 and 25 °C (Banerjee & Rupley, 1973), and should thus completely displace the much more weakly bound DMSO molecule from subsite C (cf. Fig. 1). Although some small structural perturbations were observed in remote regions of the protein upon $GlcNAc_3$ binding, the residues forming the second DMSO site are not affected (Cheetham et al., 1992; Lumb et al., 1994). A comparison of the two upper NMRD profiles in Figure 3 thus shows that subsite C, although not fully occupied, is responsible for at least 85% of the dispersion from HEWL solution. With the binding constant of 2.3 M^{-1} obtained from the chemical shift data (neglecting any H-D isotope effects), the site occupancy (in the absence of $GlcNAc_3$) is 75% at the concentrations used. The ca. 2% of the protein molecules with DMSO not replaced by $GlcNAc_3$, contribute to the small (barely significant) dispersion step seen for the HEWL-$GlcNAc_3$ solution.

Specific DMSO binding

In the case of ^2H relaxation, the dispersion amplitude β can be expressed as (Halle & Wennerström, 1981; Denisov & Halle, 1995c):

$$\beta = \frac{N_\beta}{N_{DMSO}} \frac{3}{2} (\pi \chi S_{CS} S_{Me})^2 \qquad (2)$$

where N_β is the number of long-lived DMSO molecules that give rise to the dispersion and χ is the ^2H quadrupole coupling constant in the DMSO CD_3 groups. In Equation 2, the orientational order parameter for the C–D bond has been factorized into a product of order parameters for the independent rotation of the methyl group, $S_{Me} = (3\cos^2\theta - 1)/2$, and (librational) motion of the C–S bond, S_{CS}, Because the methyl groups in the crystal structure of DMSO are of nearly tetrahedral geometry (Thomas et al., 1966), with the average angle, θ, between the CS bond (the symmetry axis for methyl group rotation) and the CD bond (the principal direction of the electric field gradient tensor) of 70.4° [a slightly different value of 71.35° was reported for DMSO in the gas phase (Feder et al., 1969)], we can set $S_{Me} = -1/3$. The quadrupole coupling constant for the methyl deuterons in DMSO-d_6 is taken to be, $\chi = 175 \pm 5$ kHz. This value is consistent with NMR data for neat DMSO (Zeidler, 1965), DMSO intercalated in clays (Duer et al., 1992; Hayashi, 1995), and for methyl groups in other small molecules (Emsley & Lindon, 1975; Hansen & Jacobsen, 1980; Lickfield et al., 1984). From the difference between the HEWL and HEWL-$GlcNAc_3$ dispersion amplitudes β (Fig. 3 and Table 2), we then obtain with the help of Equation 2, $N_\beta S_{CS}^2 = 0.16 \pm 0.03$. With the DMSO occupancy difference of 73% (vide supra), we deduce the order parameter $S_{CS} = 0.47 \pm 0.05$ for DMSO bound to subsite C. The small amplitude of the dispersion from the HEWL-$GlcNAc_3$ solution corresponds to $N_\beta S_{CS}^2 = 0.03 \pm 0.01$. Because the occupancy of any nonrandom binding site should be larger than the volume fraction of DMSO in the bulk solvent (corresponding to binding constants $K > 0.07$–$0.08\ M^{-1}$ in 1.2 M DMSO), this indicates the absence of sites with order parameters S_{CS} approaching that of subsite C. The fact that no DMSO binding site, other than subsite C, was detected by NOESY (Liepinsh & Otting, 1997), also supports this conclusion.

Figure 4 shows the ^2H relaxation dispersions from the lysozyme solution in H_2O/DMSO-d_6 (Table 1) at three different temperatures, along with the corresponding bulk solvent relaxation rates (dashed lines). The parameters α, β, and τ_R derived from the fits

Table 2. *Parameters derived from fits to the ^2H NMRD data*

Sample	T (°C)	R_{bulk} (s^{-1})	α (s^{-1})	β ($10^7\ s^{-2}$)	τ_R (ns)
BPTI	27	1.39	0.12 ± 0.01	—	—
HEWL	27	1.39	0.14 ± 0.01	5.3 ± 0.6	7.5 ± 1.0
HEWL-$GlcNAc_3$	27	1.39	0.14 ± 0.01	0.8 ± 0.3	7.5 ± 1.0
HEWL	4	2.43	0.34 ± 0.05	5.7 ± 0.9	18.1 ± 3.8
HEWL	60	0.783	0.06 ± 0.04	4.3 ± 2.9	3.1 ± 1.5

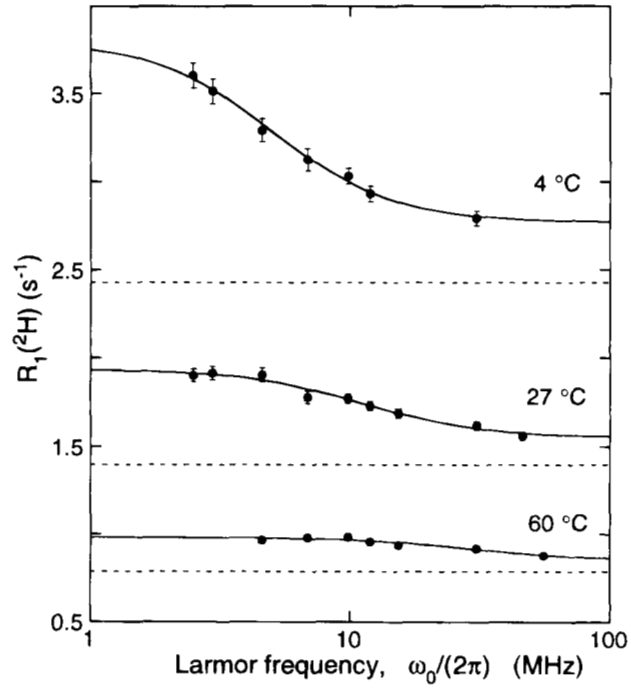

Fig. 4. Dispersion of DMSO ^2H longitudinal relaxation rate from HEWL solution in 90% H_2O/10% DMSO-d_6 at 4, 27, and 60 °C. The solid curves represent fits of the parameters α, β, and τ_R in Equation 1 (see Table 2) to the data points. The dashed lines refer to the ^2H relaxation rates (at the three temperatures) of the bulk solvent with the same H_2O/DMSO molar ratio as in the protein solution.

(using Equation 1) to these data are collected in Table 2. Because the quadrupole coupling constant should be independent of temperature, the variation of the dispersion magnitude β reflects the reduction of $N_\beta S_{CS}^2$ at higher temperature, presumably due to the decrease of the binding constant. Equating N_β with K[DMSO]/(1 + K[DMSO]), and neglecting any temperature dependence in the order parameter S_{CS}, we find that the temperature variation of β corresponds to a (van't Hoff) standard binding enthalpy of between −10 and −17 kJ mol^{-1}. A negative binding enthalpy is also consistent with the slightly smaller binding constant of 1.75 M^{-1}, obtained for DMSO binding to subsite C at 36 °C (Liepinsh & Otting, 1997).

Nonspecific DMSO binding

The frequency-independent contribution, α, to the relaxation rate can be expressed as

$$\alpha = \frac{N_\alpha}{N_{DMSO}}(\langle R_\alpha\rangle - R_{bulk}) + \frac{N_\beta}{N_{DMSO}}\left\{\frac{3}{2}(\pi\chi)^2\tau_{int} - R_{bulk}\right\} \quad (3)$$

where N_α is the number of short-lived DMSO molecules associated with the protein surface and $\langle R_\alpha\rangle$ their average intrinsic relaxation rate. The second term in Equation 3 accounts for internal motion of the long-lived DMSO molecule in subsite C, with an effective correlation time τ_{int}. If methyl group rotation (correlation time τ_{Me}) is much faster than C–S bond libration (τ_{CS}), one can show that $\tau_{int} = S_{Me}^2(1 - S_{CS}^2)\tau_{CS} + (1 - S_{Me}^2)\tau_{Me}$ (Dayie et al., 1996). For the order parameters deduced above, $\tau_{int} = 0.087\tau_{CS} + 0.89\tau_{Me}$.

Because the HEWL dispersion (with N_β = 0.75) does not approach a larger high-frequency R_1 plateau (= R_{bulk} + α) than the HEWL-GlcNAc$_3$ dispersion (with N_β = 0.02), the internal motion contribution to α must be negligible. Unless τ_{CS} or τ_{Me} are significantly larger than ca. 100 ps or 10 ps, respectively, which is unlikely (Huntress, 1970; London et al., 1977; Kowalewski & Kovacs, 1986; Nicholson et al., 1992; Zhang et al., 1996), the second term in Equation 3 is indeed negligible compared to the measured α. We thus obtain from Equation 3, $N_\alpha(\langle R_\alpha\rangle/R_{bulk} - 1)$ = 19 ± 3 for HEWL and 7.8 ± 0.6 for BPTI at 27 °C. These numbers are smaller by factors 100 and 150, respectively, than the corresponding values for water, derived from water ^{2}H and ^{17}O NMRD (Denisov & Halle, 1996), the latter scaling roughly with the solvent accessible surface areas of the two proteins (6550 and 3990 Å^2). If uniformly distributed in the solvent, ca. 10 DMSO molecules are expected to be in contact with the HEWL surface (at the present DMSO concentration), which is only a factor 40 smaller than the corresponding number of water molecules. This comparison of DMSO and water suggests a preference for water solvation in protein solutions, as previously found (Kita et al., 1994; Rajeshwara & Prakash, 1994; Rajendran et al., 1995) and/or a slightly smaller dynamic perturbation for DMSO. The (average) effective correlation time for the surface DMSO molecules is thus within an order of magnitude of the bulk solvent value, as also found for surface water (Denisov & Halle, 1996).

Residence time of DMSO in the active cleft of lysozyme

A ligand or water molecule exchanging between a bound site and the bulk contributes fully to the observed NMRD only if its mean residence time in that site falls in the window $\tau_R < \tau_{res} < T_1$, where T_1 is the zero-frequency intrinsic spin relaxation time in the bound site (Koenig & Schillinger, 1969; Denisov & Halle, 1996). For the present case of DMSO-d$_6$ ^{2}H relaxation, T_1 = $[3\tau_R(\pi\chi S_{CS}S_{Me})^2/2]^{-1}$ = $N_\beta/(N_{DMSO}\beta\tau_R)$, which varies from 5 ms at 4 °C to 29 ms at 60 °C (cf. parameters in Table 2). Because T_1 and τ_{res} have opposite dependencies on temperature, τ_{res} < 5 ms at all temperatures studied. In the other limit, if τ_{res} approaches τ_R, the effective correlation time obtained from NMRD becomes equal to $(\tau_{res}^{-1} + \tau_R^{-1})^{-1}$. Although the obtained correlation times are of a magnitude expected for lysozyme tumbling, and decrease with temperature approximately as η/T, where η is viscosity of the mixed solvent (Cowie & Toporowski, 1961), we cannot exclude the possibility that τ_{res}, which should have a stronger temperature dependence than τ_R, approaches τ_R at 60 °C. In conclusion, for the DMSO molecule in subsite C, 10 ns < τ_{res} < 5 ms at all temperatures studied.

To obtain a more restrictive upper bound on τ_{res}, we performed additional measurements of the transverse relaxation rate, R_2, using the Carr-Purcell-Meiboom-Gill pulse sequence. For nuclear spins exhanging between sites of different chemical shift, R_2 exhibits a dispersion at 180°-pulse repetition frequencies of the order τ_{res}^{-1} (Allerhand & Gutowsky, 1964; Carver & Richards, 1972; Hills et al., 1989). As shown in Figure 5, no such dispersion was detected in the investigated pulse-frequency range. With the aid of the theoretical curves in Figure 5, we can thus reduce the upper bound on τ_{res} by a factor 50, leading to

$$10\text{ ns} < \tau_{res} < 100\ \mu\text{s}. \quad (4)$$

It is interesting to note that the water molecules that are displaced on ligand binding to subsite C (Blake et al., 1983) appear to have shorter residence times. A recent ^{1}H-NMR study of lysozyme identified weak nuclear Overhauser effects with a few water molecules residing in small pockets within the active cleft, suggesting residence times in the range 0.1–1 ns at 36 °C (Otting et al., 1997).

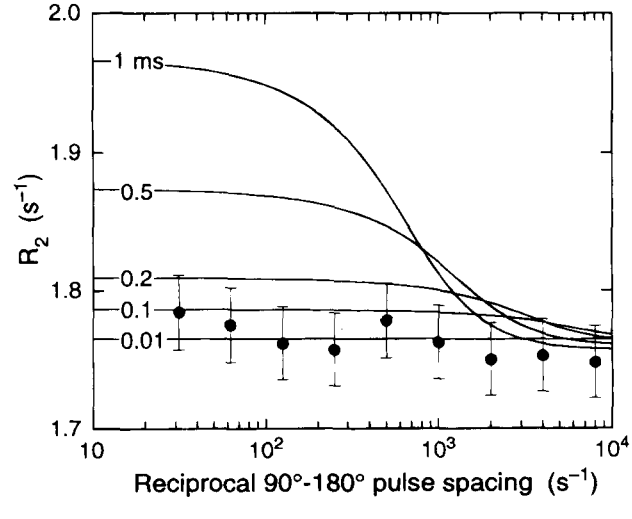

Fig. 5. Dependence of DMSO ^{2}H transverse relaxation rate from HEWL solution in 90% H_2O/10% DMSO-d$_6$ (pH 4.18) at 27 °C on the reciprocal 90–180° pulse spacing. The points represent experimental data, the curves are calculated numerically for exchange between two sites with the chemical shift difference 1.1 ppm, the bound fraction 1/184 (cf. Table 1), the resonance frequency 30.7 MHz, and the residence times indicated for each curve (in ms).

Conclusions

NMRD measurements of DMSO-d_6 deuteron longitudinal relaxation were performed for 90% water/10% DMSO solutions of lysozyme, lysozyme inhibited with $GlcNAc_3$, and BPTI, and complemented by proton chemical shift titration and transverse relaxation measurements. The fact that no significant dispersion was observed from inhibited lysozyme demonstrates that a single DMSO molecule bound in subsite C of the active cleft is responsible for the observed DMSO ^2H NMRD profile. This should also be the case for the DMSO ^1H dispersion (Bryant & Jarvis, 1984). A quantitative analysis of the present data shows that this specific DMSO binding site is characterized (at 27 °C) by a binding constant $K = 2.3 \pm 0.3\ M^{-1}$, a C–S bond order parameter of 0.47 ± 0.05, and a residence time in the range 10 ns to 100 μs.

The absence of a dispersion for BPTI suggests that hydrophobic and/or hydrogen bond interactions of DMSO with the surface of globular proteins are not sufficient to produce a stable complex with long residence time and high order parameter. For such a complex, a cleft of appropriate size is also required (Laskowski et al., 1996), as identified here for subsite C in lysozyme and also seems likely for gelatin solutions and gels (Hills & Favret, 1994). A similar mechanism (i.e., physical entrapment in clefts and pockets) was previously found to be responsible for long residence time of water in proteins (Denisov & Halle, 1995b; Denisov & Halle, 1996).

Finally, the present work illustrates the usefulness of the NMRD technique for quantitative studies of protein–ligand interactions and kinetics. If ligand dissociation is gated by the protein, such data may shed light on the concomitant protein conformational dynamics, as recently illustrated for buried water (Denisov et al., 1996). For very weakly bound ligands and/or large proteins, τ_{res} may be smaller than τ_R, in which case NMRD experiments can in principle yield τ_{res} directly from the dispersion frequency (Denisov et al., 1997), provided that the complications of low site occupancy and small order parameters can be overcome by using high protein concentration. For very strongly bound ligands, τ_{res} may be comparable to the intrinsic ^2H relaxation time, in which case it can be determined from the variation of the dispersion amplitude β with temperature (Denisov et al., 1996).

Materials and methods

Materials

Lyophilized powders of hen egg-white lysozyme, HEWL (Sigma, St. Louis, MO, L6876, M = 14,300 g mol^{-1}) and recombinant bovine pancreatic trypsin inhibitor, BPTI (a gift from Novo Nordisk A/S, Gentofte, Denmark, M = 6,504 g mol^{-1}) were dissolved in doubly distilled water. Calculated amounts of DMSO (Sigma, D8779, M = 78.13 g mol^{-1}), DMSO-d_6 (Sigma, D8511, M = 84.17 g mol^{-1}), and N-acetylglucosamine trisaccharide, $GlcNAc_3$ (Sigma, T2144, M = 627 g mol^{-1}) were added to the protein solutions at the final stage.

pH was adjusted by addition of small amounts HCl to the solutions. Protein concentrations were calculated on a weight basis, calibrated with amino acid analyses of previous sample preparations from the same protein batches. The compositions of the three different samples used in this study are collected in Table 1.

NMR measurements

The DMSO-d_6 deuteron longitudinal relaxation rate, $R_1 = 1/T_1$, was measured at several field strengths corresponding to Larmor frequencies in the range 2.5 MHz to 55.5 MHz, using four different Varian or Bruker spectrometers, and an iron magnet (Drusch EAR-35N) equipped with field-variable lock and flux stabilizer. An inversion recovery pulse sequence ($\pi - \tau - \pi/2$) with 16-step phase cycling was used for the T_1 measurements. A sufficient number of transients was accumulated to ensure a signal-to-noise ratio better than 100. Twenty values of the pulse delay τ, taken in random order in the interval $0.05T_1$ to $5.3T_1$, were used for each experiment. The relaxation time T_1 was obtained by fitting an exponential function to the 20 data points using standard spectrometer software. Relaxation rates for a bulk solution with the same water/DMSO molar ratio as in the protein samples were measured on different spectrometers and are listed in Table 2.

DMSO-d_6 deuteron transverse relaxation rates, $R_2 = 1/T_2$, were measured at 27 °C on a Bruker DMX-200 spectrometer, using the Carr-Purcell-Meiboom-Gill pulse sequence with 90–180° pulse spacing varying between 32 ms and 125 μs. The calibrated length of the 180° pulse was 20.4 μs, and the duration of 90° pulse was set to 10.2 μs. Eight acquisitions preceded by 16 dummy scans were accumulated. The echo decay envelope was obtained by repeatedly recording the echo at 20 different times in the interval $0.05T_2$ to $5.3T_2$ (random order) using a recycle delay of $5.3T_1$, Fitting the 20 data points to an exponential function using standard spectrometer software yielded the transverse relaxation time T_2,

^1H chemical shifts of lysozyme and DMSO resonances were measured at 27 °C on a GE Omega spectrometer operating at 500 MHz. Assignments of the lysozyme resonances were done with the help of published chemical shift data (Redfield & Dobson, 1988), using the residual water proton line as a reference (4.76 ppm at 27 °C). The DMSO binding constant K was determined from the concentration dependence of the DMSO methyl proton shift δ, assuming fast exchange of each of the two methyl groups in a bound DMSO with the bulk:

$$\delta = \delta_{free} - \frac{(\delta_{free} - \delta_{bound})[HEWL]}{[HEWL] + [DMSO] + K^{-1}}, \quad (5)$$

where δ_{free} and δ_{bound} are the chemical shifts in the free and bound states, [HEWL] and [DMSO] are the total lysozyme and DMSO concentrations.

The sample temperature in NMR experiments was regulated by a thermostated air flow with an accuracy better than 0.1 °C.

The fraction exposed surface of the DMSO molecules bound in the crystal (Lehmann & Stansfield, 1989) was determined by calculating the solvent accessible surface area of each DMSO atom (i.e., with standard atom radii increased by 1.4 Å) in the presence and absence of the protein. The average accessible surface area of the isolated DMSO molecules was 230 Å^2.

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