Electron spin echo decay as a probe of aminoxyl environment in spin-labeled mutants of human carbonic anhydrase II †

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Genetically-engineered human carbonic anhydrase II mutants have been prepared with cysteine introduced at selected locations and spin-labeled with an aminoxyl (formerly known as nitroxide) radical. Two-pulse electron spin echo data have been obtained for samples in 1 : 1 water–glycerol employing a Bruker ESP380E spectrometer. Data obtained at 11 and 40 K are fitted to the function $Y(\tau) = Y(0)\cdot \exp[-(2\tau/T_m)^x]$. T_m = 4.4 to 4.1 μs with $x > 2$ for labels near the surface, but the decay shape changes to T_m = 2 μs, $x = 1$ for a label buried in a hydrophobic region of the protein. To identify characteristics of the spin label environment that impact T_m and x, 0.1 to 0.5 mM solutions of aminoxyls are examined in a series of glassy solvents. At these spin label concentrations spin echo dephasing is dominated by interaction with solvent protons. For solvents that do not contain methyl groups $1/T_m$ increases as solvent proton concentration increases. The smallest values of x and of T_m are observed for solvents with the least sterically hindered methyl groups. In samples of spin-labeled engineered proteins the aminoxyl-probe is generally used to explore local motions near room temperature. The data presented here indicate that the shape of the echo decay obtained at low temperature is a sensitive indicator of the proton environment of the spin-label. The combination of lineshape studies at room temperature and spin echo studies at low temperature provide complementary information in spin labeling studies of protein folding and protein–protein interaction.

Introduction

Spin-labeling has been used widely to study the structure and motion of proteins and membranes.[1] Information concerning local structure and dynamics can be obtained through analysis of the aminoxyl‡ continuous wave (CW)–EPR lineshape or saturation transfer techniques. The utility of spin labeling has been greatly increased by the rapid development of protein engineering techniques that permit introduction of spin labels at virtually any selected location in a protein.[2–4] In our studies of protein folding of carbonic anhydrase, cysteines have been introduced at interior and exterior sites of the folded protein.[5] Analysis of CW lineshapes reveals differences in mobility for probes at various sites in the protein structure. In studies of protein structure it is important to map the surfaces or regions of the protein that are exposed to, for example, a polar solvent. This can be done by introducing a fluorescent label in the appropriate position since there is a marked dependence of the energy-shift of the emitted photon, relative to the absorbed one, on the polarity of the local environment surrounding the probe.[6] It would be useful to have additional information about the local environment of the spin-probe. The aminoxyl nitrogen hyperfine splitting is sensitive to the polarity of the surrounding medium,[7] however in the slow tumbling regime the observed value of A_{zz} depends both on the mobility of the probe and on the limiting value of the hyperfine coupling in the slow motion limit.

Time domain EPR has the potential to provide information concerning the interaction between an unpaired spin and neighboring electron and nuclear spins. Much of the emphasis in the electron spin echo literature has been on analysis of nuclear modulation to characterize nuclear spins within a few Ångstroms of the unpaired electron. In this report we examine the shape of the aminoxyl spin echo decay curve. Data for spin-labeled carbonic anhydrase mutants demonstrate a dependence of the echo decay shape upon the location of the label in the protein. To examine solvent characteristics that impact the echo decay, data were collected for selected aminoxyl radicals in a variety of glassy solvents.

Experimental

Materials

TEMPONE (2,2,6,6-tetramethyl-4-oxopiperidin-1-yloxyl) and 3C-PROXYL (3-carbamoyl-2,2,5,5-tetramethylpyrrolidin-1-yloxyl) were purchased from Sigma and used as received. Engineered mutants of human carbonic anhydrase II (HCA II) were prepared, spin-labeled with 3-(2-iodoacetamido)-2,2,5,5-tetramethylpyrrolidin-1-yloxyl (3I-PROXYL) at the cysteine position and purified at Linköping University, following procedures reported previously.[4] The solvents used to prepare solutions of TEMPONE and PROXYL were reagent grade and used as received. Solvents were selected that give good glasses to avoid problems with locally-high radical concentrations that can occur when solvent crystallizes. Decalin refers to a mixture of *cis*- and *trans*-isomers and xylene refers to a mixture of isomers. Nujol (Plough, Inc., Memphis, TN) is a mixture of hydrocarbons for which the $CH_3 : CH_2$ ratio was obtained by proton NMR. Deuteriated solvents, D_2O and [$^2H_8$]glycerin (Cambridge Isotope Laboratories), were 99.9 and >98% enriched, respectively.

For electron spin echo (ESE) measurements freshly-prepared

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‡ Formerly known as nitroxide.

Table 1 Spin-echo decay data for spin-labeled mutants of HCA II at 40 K

Sample	Label position	T_m/μs	x (Exponent)	Mobility/Polarity
in 1 : 1 H_2O–glycerol				
W97C/C206S	deeply buried	2.0	1.0	slow/non-polar[a]
W123C/C206S	buried	3.7	1.5	slow/intermediate[a]
S56C/C206S	at/near surface	4.4	2.1	medium/polar[a]
HCA II (C206)	internal, near active site	4.1	2.0	slow/intermediate[a]
F176C/C206S	at/near surface	4.1	1.9	medium/polar[a]
I59C/A174C/C206[b]	at/near surface	4.4	2.1	n.d.[c]
in 1 : 1 D_2O–[$^2H_8$]glycerol				
W123C/C206S	buried	6.0	1.2	n.d.[c]
F176C/C206S	at/near surface	5.5	1.0	n.d.[c]

[a] Data taken from Table 2 and Figs. 3(*c*) and 3(*d*) in ref. 5. [b] This is a doubly spin-labeled mutant. The distance between the β-carbons of the two labeled cysteines is approximately 5 Å, based on the crystal structure of wild type HCA II. EPR spectra do not show resolved splittings so the distance between unpaired electrons of the two individual spin labels is >*ca.* 12 Å. [c] Not determined.

protein solutions (*ca.* 4–20 mg ml^{-1} in Tris-H_2SO_4 buffer, pH 7.5) were mixed with an equal volume of glycerol. The resulting solutions were *ca.* 0.1 to 0.3 mM in spin label. Solutions of TEMPONE, 3C-PROXYL or 3I-PROXYL were freshly prepared with concentrations between 0.1 and 0.5 mM. 150–200 ml of solution was pipetted into 4 mm OD quartz EPR tubes. Air/oxygen contained in the solvent was removed by repeated freeze–pump–thaw cycles. Tubes were back-filled with a partial pressure of helium and sealed with a torch or back-filled with 1 atm helium and placed directly into the cryostat.

Protein samples in deuteriated solvent were prepared by repeated concentration with D_2O in a Centriprep 10 tube (Amicon). 1 ml of the spin labeled protein was concentrated to *ca.* 0.1 ml, and was diluted 10-fold with D_2O (99%). The sample in D_2O was concentrated again to *ca.* 0.1 ml. This procedure was then repeated giving 97–99% replacement of H_2O with D_2O in the samples. Perdeuteriated glycerol ([$^2H_8$]glycerol) was used to prepare the EPR samples.

Electron spin echo (ESE) experiments

Data were obtained on a Bruker ESP380E spectrometer at the University of Denver that is equipped with a Bruker split-ring resonator and an Oxford ESR935 liquid helium cryostat. The temperature was 11 or 40 K and microwave frequencies were between 9.3 and 9.7 GHz. Most of the two-pulse echo decays were obtained with 40 and 80 ns pulses to reduce excitation of proton modulation. Data were recorded at the magnetic field that gave the most intense echo, which occurs near the center of the aminoxyl spectrum. At this field there are contributions from radicals with a wide range of orientations with respect to the external magnetic field. At 40 K data were also recorded at 20 G higher field. Using this magnetic field spin-labels with an orientation intermediate between the aminoxyl z-axis and the x,y-plane are projected along the magnetic field which determines the resonance condition.

To describe the envelope of the two-pulse echo-decay it is customary to fit experimental data to a stretched exponential [eqn. (1)], where $Y(\tau)$ is the intensity of the echo as a function

$$Y(\tau) = Y(0)\exp[-(2\tau/T_m)^x] \quad (1)$$

of τ, the time between the two pulses. $Y(0)$, echo intensity extrapolated to time zero, and $Y(\tau)$ are in arbitrary units that depend upon the concentration of the sample, resonator Q and instrument settings. The parameters x and T_m describe the shape of the echo decay and are discussed in the following paragraphs. ESE data were fitted to eqn. (1) using a Levenberg–Marquardt algorithm. In analyzing decays with echo modulation, the fit was based on the maxima in the modulation pattern. The echo modulation can introduce a bias toward smaller apparent values of T_m.

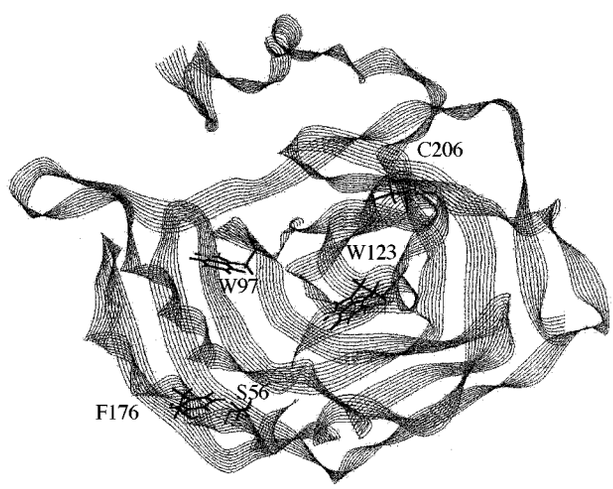

Fig. 1 Schematic picture of HCA II based on coordinates from X-ray diffraction.[11] The location of selected residues that were replaced by cysteines and spin-labeled are indicated along with the associated abbreviation for the mutated protein.

For each sample a series of turning angles was examined by varying the microwave power at constant pulse length, and thereby provide a test for the impact of instantaneous diffusion.[8–10] For the radical concentrations used in this study only a minor dependence of echo decay on turning angle was observed for the protiated solvents, which indicates that the contribution to dephasing from instantaneous diffusion was negligible. The echo-decay parameters reported in the text and tables were all obtained at a small turning angle, typically 11 or 22° for the first pulse. For samples in protiated solvents the estimated uncertainties at low turning angle are ±0.1 μs for T_m and ±0.1 for the exponent. The uncertainties are larger for data obtained in deuteriated solvents as discussed below.

Results

A series of spin-labeled mutants of genetically-engineered human carbonic anhydrase II were selected for study. The locations of amino acids that were replaced by cysteines and subsequently spin-labeled are shown in Fig. 1. The drawing is based on coordinates obtained by X-ray crystallography of HCA II.[11] Several of these mutants have been studied previously at room temperature using both an aminoxyl spin-label and a fluorescent label (AEDANS)[5] and selected results are summarized in a column in Table 1. Based on the analysis of the CW–EPR spectra the mobility of the aminoxyl spin probe of the folded native protein was classified as *slow* or *medium*.[5] *Slow* denoted a rotational correlation time similar to that of the protein as a whole, which indicates that the spin label is tightly

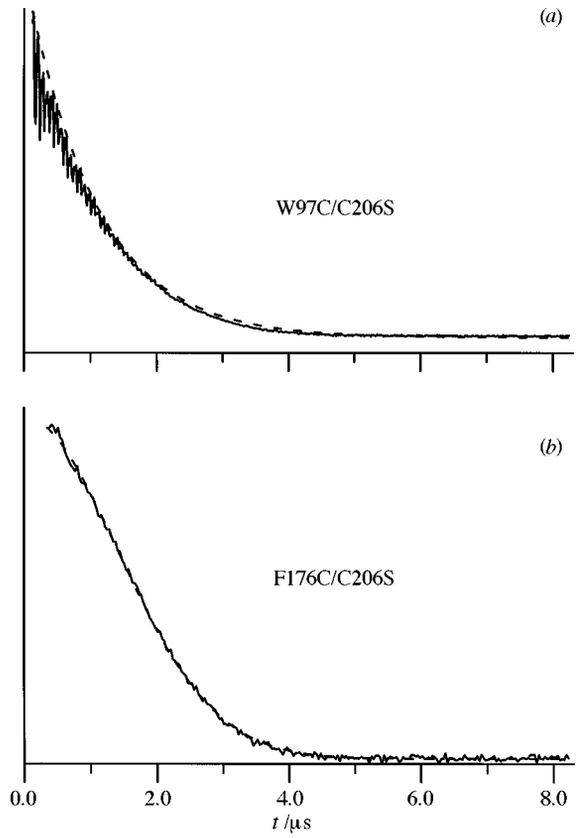

Fig. 2 (*a*) ESE decay for spin-labeled W97C/C206S (0.35 mM) in glassy 1 : 1 H_2O–glycerol at 40 K. Nominal pulse lengths were 16 and 24 ns and the turning angle for the first pulse was 90°. The dashed curve is the fit to eqn. (1) with $x = 1.0$ and $T_m = 2.1$ μs. (*b*) ESE decay for spin-labeled F176C/C206S (0.10 mM) in glassy 1:1 H_2O–glycerol at 40 K. Nominal pulse lengths were 40 and 80 ns and the turning angle for the first pulse was 22°. The dashed curve is a fit to eqn. (1) with $x = 2.0$ and $T_m = 4.0$ μs.

locked within the protein. *Medium* denoted increased mobility as expected for label positions at peripheral regions of the protein, albeit considerably slower than for a spin label in a denaturated and unfolded protein, for which the mobility was referred to as *rapid*. The λ_{max} of the fluorescence from the AEDANS label reflects the polarity of the environment. The designations in Table 1 are: *nonpolar*, $\lambda_{max} < 475$nm; *intermediate*, λ_{max} *ca.* 475–480 nm; and *polar*, $\lambda_{max} > 480$ nm. A more complete discussion of these data can be found in the original report.[5]

Electron spin echo decays for spin-labeled HCA II mutants

Two-pulse electron spin-echo decays for samples of spin-labeled HCAII mutants W97C/C206S and F176C/C206S recorded at 40 K are shown in Figs. 2(*a*) and 2(*b*). The echo decay trace in Fig. 2(*a*) was obtained with 16 and 24 ns microwave pulses and a microwave power that gave a turning angle of 90° for the first pulse. Under these conditions there is substantial excitation of proton echo envelope modulation, which makes it more difficult to determine the shape of the echo decay curve. The data in Fig. 2(*b*) were obtained with 40 and 80 ns pulses and a microwave power that gave a turning angle of 22° for the first pulse. The longer pulses [Fig. 2(*b*)] excite much less proton modulation than the shorter pulses [Fig. 2(*a*)], which gives a smoother decay curve and facilitates analysis of the shape of the decay. Most of the data reported in this paper were obtained with 40 and 80 ns pulses. In addition to the differences in the modulation there are substantial differences in the overall shapes of the decays for the probes at the two locations on the protein. These differences are the focus of the ensuing discussion. The decays in Fig. 2(*a*) and 2(*b*) are representative of two extremes of decay shape observed for the spin-labeled proteins. ESE curves at 11 and 40 K were fitted to eqn. (1) and the resulting values of T_m and x at 40 K are given in Table 1. Values obtained at 11 K for the spin-labeled HCA II mutants agreed with those obtained with the sample at 40 K within experimental uncertainty.

The values of T_m for the spin labels bound to HCA II (Table 1) in proton-containing solvents ranged from 2.0 to 4.4 μs and the exponent x ranged from 1.0 to 2.1. The smallest values of T_m (2.0 μs) and x (1.0) were observed for W97C/C206S. Room temperature AEDANS fluorescence data indicated that this probe is in a non-polar environment.[5] Large values of T_m (4.1 to 4.4 μs) and x (1.9 to 2.1) are associated with sites that fluorescence data had identified as polar, S56C and F176C. Intermediate values of T_m (3.7 μs) and x (1.5) were observed for W123C, for which the environment had been identified as intermediate in polarity, based on fluorescence data. The similarity in trends for the fluorescence data and for T_m and x suggests that the shapes of the echo decays reflect characteristics of the probe environment.

For most of the HCA II samples the prior CW–EPR lineshape and fluorescence studies indicated a correlation: (*a*) low probe mobility and low polarity environment for buried sites, or (*b*) high probe mobility and high polarity for sites near the surface.[5] In view of this classification the data for C206 were somewhat enigmatic because the fluorescence and CW–EPR lineshape data indicated low mobility but intermediate polarity at this particular site. The spin echo data for C206 indicate an environment similar to that for F176C, S56C and I59C/A174C. Thus, from these data combined with spin-label and AEDANS data obtained at room temperature, the spin-label as well as the fluorescence probe appear to be fixed in the protein structure but due to the relatively short distance from the attachment site to the surface the probes are nevertheless near the surrounding solvent phase.

The interiors of globular proteins are hydrophobic, particularly for proteins such as HCA II that are rich in β-pleated sheet structure. In contrast, the water–glycerol solvent is rich in polar hydrogen bonds. To seek a physical picture to explain the origin of what appears to be a solvent effect on the ESE data for the spin-labeled proteins, data were examined for aminoxyl spin probes in various glassy solvents.

Electron spin echo decays for aminoxyls in glassy solvents

To examine solvent characteristics that impact ESE dephasing, data were obtained for TEMPONE, 3C-PROXYL and 3I-PROXYL in glassy solvents. Fewer solvents were used with 3C-PROXYL and 3I-PROXYL than with TEMPONE because of their poor solubility in non-polar solvents. The ESE data were fitted to eqn. (1) and the resulting values of T_m and x at 40 K are summarized in Table 2. Values of T_m and x at 11 K were the same as at 40 K, within experimental uncertainty, except that in toluene or xylenes the values of T_m at 11 K were about 20% smaller than at 40 K. The echo decays were approximately independent of radical (Table 2). The shapes of the echo decays were found to be strongly dependent upon the solvent environment (Table 2). For example, for 3C-PROXYL in 1:1 water–glycerol $T_m = 4.4$ μs and $x = 2.1$ but in 9:1 toluene–THF $T_m = 4.5$ μs and $x = 1.4$. For TEMPONE in 9:1 propan-2-ol–methanol $T_m = 2.1$ μs and $x = 2.2$, but in 9:1 propan-1-ol–methanol $T_m = 0.7$ μs and $x = 0.8$. Thus, the change from a branched to a linear alcohol caused a dramatic increase in the rate of echo dephasing.

The aminoxyl phase memory decay rate, T_m^{-1} as a function of total proton concentration in the solvent is plotted in Fig. 3. In solvents that do not contain methyl groups, there is a linear correlation between T_m^{-1} and total proton concentration. Points for solvents that contain methyl groups deviate from this line. The relationship between the exponent and T_m^{-1} also depends upon the type of protons in the solvent (Fig. 4). Solvents that contain aromatic methyl groups cause a decrease in x

Table 2 Spin-echo decay data for aminoxyls in glassy solvents at 40 K[a]

Solvent	Spin-probe	[H][b]	[H_{Me}][c]	T_m/μs	x (Exponent)
No methyls					
Conc. H_2SO_4	Ion radicals[d]	38	—	10.5	2
OTP[e]	TEMPONE	49	—	8.5	2.5
19:1 OTP–decalin	TEMPONE	64	—	7.9	2.5
3:1 OTP–decalin	TEMPONE	75	—	6.7	2.3
1:1 OTP–decalin	TEMPONE	89	—	6.3	2.2
1:1 H_2O–glycerol	TEMPONE	110	—	4.6	2.3
1:1 H_2O–glycerol	3I-PROXYL	110	—	4.5	2.2
1:1 H_2O–glycerol	3C-PROXYL	110	—	4.4	2.1
Glycerol	3C-PROXYL	110	—	4.8	2.2
Decalin	TEMPONE	120	—	4.2	2.1
Hindered methyls					
2-MeTHF	TEMPONE	90	10	2.4	1.9
4:1 EtOH–MeOH	TEMPONE	102	19	1.7	1.8
9:1 Pr^{i}OH–MeOH	TEMPONE	104	26	2.1	2.2
Aromatic methyls					
9:1 toluene–THF	TEMPONE	78	8.5	4.8	1.4
Xylene–decalin	TEMPONE	86	14	3.2	1.2
9:1 toluene–THF	3C-PROXYL	78	8.5	4.5	1.4
Less hindered aliphatic methyls					
9:1 Pr$''$OH–MeOH	TEMPONE	106	14	0.7	0.8
1:1 Bu$''$OH–Pr$''$OH	TEMPONE	108	12	1.0	1.0
Nujol	TEMPONE	142	12	1.7	1.2
Sucrose octaacetate	TEMPONE	71	15	2.8	0.9

[a] Aminoxyl concentrations were between 0.1 and 0.5 mM. [b] Total proton concentration/mol dm^{-3}. [c] Concentration of methyl groups/mol dm^{-3}. [d] Data from ref. 9. [e] OTP is *o*-terphenyl.

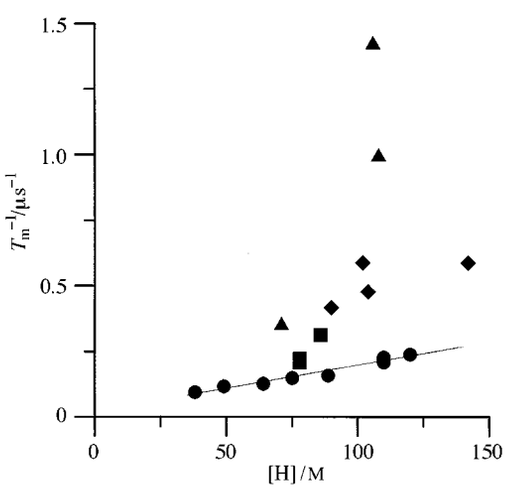

Fig. 3 Dependence of aminoxyl phase memory rate on total proton concentration in the solvent (data from Table 2). Solvents are identified by type of protons: (●) no methyl groups, (■) aromatic methyl, (▲) less hindered aliphatic methyl, (◆) hindered methyl. The solid line is a linear fit to the data in solvents with no methyl group and gives T_m = 45 μs when extrapolated to zero-concentration of proton.

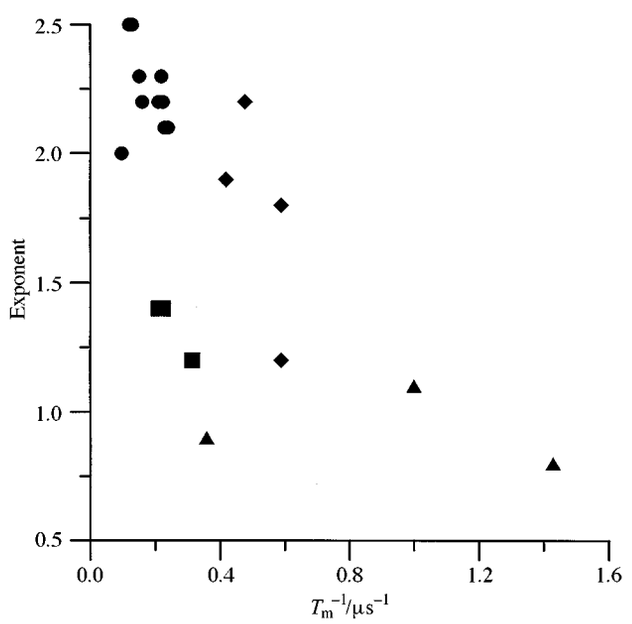

Fig. 4 Dependence of exponent x in eqn. (1) on T_m^{-1} for data collected in Table 2. Solvents are identified by type of protons: (●) no methyl groups, (■) aromatic methyl, (▲) less hindered aliphatic methyl, (◆) hindered methyl.

in eqn. (1) with little change in T_m^{-1}, whereas aliphatic protons typically tend to decrease x and increase T_m^{-1}.

Deuteriated solvents

To further examine the role of solvent protons in the echo dephasing, data were obtained in deuteriated solvent. For a 0.1 mM solution of TEMPONE in 1:1 D_2O–[$^2H_8$]glycerol the effect of solvent deuteriation on echo dephasing was dramatic. In the deuteriated solvent, unlike the protiated solvent, the value of T_m was strongly dependent upon pulse turning angle, which indicated that in these solvents with very low solvent proton concentration dephasing was dominated by instantaneous diffusion. At small pulse turning angles T_m became so long that the echo amplitude did not decay to baseline within the 128 μs time window that is the maximum available on the ESP380E. T_m for TEMPONE in 1:1 D_2O–[$^2H_8$]glycerol is ⩾100 μs at 11 and 40 K, which is an increase by a factor of >25 over that which was observed in the analogous protiated solvent.

Data were obtained for W123C/C206S and F176C/C206S in D_2O–[$^2H_8$]glycerol (Table 1). In the echo decays for these samples there was significant deuterium modulation even when 40 and 80 ns pulses were used, which results in increased uncertainty in fitting the shape of the decay curves. For W123C/C206S, which has the spin label buried in the protein, replacement of solvent protons and exchangeable protons by deuterons caused T_m to increase by about a factor of two. For F176C/C206S, which has the spin label near the surface of the

protein, T_m also was longer in deuteriated solvent. The much smaller effect of solvent deuteriation on the echo decay for the spin-labeled HCA II samples than for TEMPONE emphasizes the important role of the protein protons in echo dephasing for labels located at both surface and buried locations.

Discussion

In describing two-pulse spin echo dephasing it is customary to refer to the spins that are excited by the microwave pulses as A spins and all other spins as B spins.[12] Echo dephasing results from processes that change the resonance frequency of an A spin such that it is not refocused by the second pulse. Depending upon sample and experimental conditions, a variety of processes can contribute to the dephasing time constant, T_m. The conclusion in the literature, based on many observations, is that fluctuations of nuclear spins typically dominate echo dephasing for radicals in proton-containing solvents at low temperatures and low concentrations.[9,10] The following observations demonstrate that a variety of possible relaxation mechanisms are not the dominant contributions for spin labels under the conditions of our experiments and support the conclusion that solvent protons dominate the echo dephasing. (1) The values of T_m and x are strongly solvent dependent and independent of aminoxyl. (2) At high radical concentrations or in the absence of protons, instantaneous diffusion can dominate dephasing.[10] The contribution from instantaneous diffusion increases with increasing microwave B_1. The small dependence of T_m on pulse turning angle for our samples in proton-containing solvents demonstrated that the effects of instantaneous diffusion were minimized by working at low radical concentration. (3) The effects on T_m due to rotation of the aminoxyl methyl groups or to softening of the glass that have been observed at temperatures above 90 K[13] are negligible at 11 and 40 K. (4) The value of T_m for an aminoxyl in ethanol glass at 77 K was found to depend upon position in the spectrum due to the effects of low-amplitude molecular motions.[14] For the samples examined in this study values of T_m at 40 K were approximately the same in the center of the spectrum and at a magnetic field that corresponds to an intermediate orientation of the radical with respect to the external field. This indicates that molecular librations do not dominate the echo decay at 40 K. Librations are expected to contribute less at 11 K than at 40 K. (5) Electron–electron spin–spin interaction dominates dephasing at electron spin concentrations of the order of 5 to 50 mM.[10] For the 0.1 to 0.5 mM aminoxyl samples in proton-containing solvents T_m was independent of concentration, which indicates that electron–electron interactions become negligibly small at the radical concentrations used in this study. (6) The aminoxyl electron spin lattice relaxation rate increases by about an order of magnitude between 11 and 40 K.[15] The negligible temperature dependence of T_m between 11 and 40 K indicates that T_m is not determined by the electron spin T_1 at these temperatures.

For a series of organic radicals with concentrations <3 mM Brown observed that at 77 K the value of T_m decreased in the order borate glass (14.6 μs) > sulfuric acid glass (10.4 μs) > MeTHF (3.2 μs), which is the order of increasing proton spin density.[9] In a borate glass most of the protons are driven off by heating but the residual protons may be the reason why T_m is shorter in the borate glass than is observed for radicals in irradiated SiO_2 (T_m *ca.* 25 μs[8,16]). The value of T_m for TEMPONE in MeTHF (Table 2) is 2.4, which is similar to the value of 3.2 μs reported by Brown for certain organic anion radicals in MeTHF.[9]

For solvents that do not contain methyl groups a linear correlation between the aminoxyl phase memory relaxation rate (T_m^{-1}) and total proton concentration was observed (Fig. 3). The values of T_m^{-1} for aminoxyls in solvents that contain methyl groups deviated from the best fit line for the other solvents, which indicates that the methyl groups cause enhanced rates of echo dephasing. The extent of deviation from the line appears to depend upon the type of methyl group. Extrapolation of the line in Fig. 3 to zero proton concentration gave $T_m = 45$ μs. In deuteriated solvents T_m at low turning angle was >100 μs. The smaller extrapolated value of T_m for aminoxyls in protiated solvents than was observed in deuteriated solvents suggests that the functional dependence of T_m^{-1} on proton concentration is more complicated than the apparent linear dependence exhibited over a relatively narrow range of proton concentrations. The radicals produced by irradiation of SiO_2 ($T_m = 25$ μs[8,16]) are likely to be less uniformly distributed than in the glassy solutions of aminoxyls so electron–electron spin–spin interactions may make T_m shorter in the irradiated solid for the same average concentration of electron spins.

In solvents without methyl groups the exponent x in eqn. (1) varied between 2.1 and 2.6 (Table 2, Fig. 4). For solvents containing methyl groups, the value of x depended upon the type of methyl group. The smaller values of x for solvents containing less sterically hindered methyl groups suggest that the value of x may depend upon the dynamics of the motion of the methyl groups.

Much of the theoretical work on ESE dephasing has focused on the effects of magnetically dilute electron spins acting as B spins.[9,10] The predicted exponent depends upon the model for the time dependence of the fluctuations of the B spins. In the limit where the correlation time for the fluctuation, τ_C, is long relative to the time between pulses, τ, a sudden jump model[12,17] predicts $x = 2$ and a Gauss–Markovian model[18] predicts $x = 1.5$. Smaller values of x are predicted if τ_C is short compared with τ.[17,18] In analyzing the effect of nuclear spin diffusion on electron spin T_m it has been proposed that there is a diffusion barrier because the resonance frequencies for nuclei in close proximity to the unpaired electron are shifted sufficiently relative to that for bulk protons that the near neighbors do not participate in spin diffusion.[10] A model for the effect of nuclear spin flip-flops on the electron spin echo decay for slow spin flip rates gave values of x between 1.75 and 3, depending on the length of the diffusion barrier.[10] These models suggest that changes in the exponent in eqn. (1) may reflect different rates of spin diffusion for various types of solvent protons. New models likely will be required to take into consideration interaction with different types of magnetically concentrated protons with distributions of characteristic motional modes and rates of motion.

Conclusions

The values of T_m and x obtained by fitting electron spin echo decay curves for spin-labeled protein samples in glassy solutions at low temperature to eqn. (1) are different for buried and external spin probes. Comparison with previous data from fluorescent labeling suggests that these parameters are related to the proximity of the probe to a polar or non-polar phase (Table 1).

For 0.1 to 0.5 mM solutions of aminoxyl spin labels in glassy solvents at 11 and 40 K T_m and x depend on total proton concentration and on the types of methyl groups in the solvent. The phase memory relaxation rate increases with total proton concentration and with the presence of sterically less hindered methyl groups in the solvent. The value of the exponent x in eqn. (1) varies between approx. 1 and 2.6. Larger values of x are observed in solvents with no methyl groups or sterically hindered methyls and smaller values are observed for solvents with less sterically hindered methyls. The number of freely rotating aliphatic methyl groups is likely to be larger within the hydrophobic interior of the protein structure than on the hydrophilic surface. This difference in types and concentrations of methyl groups may explain the apparent *solvent effect* that was suggested by the locations of the probes determined from the protein crystal structure and by comparison of the spin

echo data with data obtained for fluorescent probes. Near the surface of the protein the *solvent* environment of a spin label is a combination of water–glycerol which contains no methyl groups and the protein which contains methyl groups. The net effect is a lower average concentration of methyl groups which results in longer T_m and larger values of x for spin label near the surface than for spin label buried in the protein.

We and others[2–5] have used CW–EPR lineshape analysis of spin-labeled proteins prepared by site-directed mutagenesis to study the mobility of the probe at various sites in the protein structure. Monitoring the change of mobility of a spin probe linked to selected regions in the structure during various stages of denaturation previously allowed us to follow the successive unfolding of various substructures of the protein molecule.[5] The results presented here for the same or similar spin-labeled mutants indicate that the shapes of the ESE decays obtained for the sample at low temperature can be used to probe the proton environment of the aminoxyl spin label and give data that are complementary to the mobility information obtained from the same probe at or near room temperature.

Characterization of specific dynamic states of proteins, such as folding intermediates and various protein–protein interactions, is generally quite cumbersome, but can be facilitated by the introduction of specific spectroscopic labels into the protein. In the context of selectively spin-labeled proteins it seems particularly attractive to apply the spin-echo method presented here to follow folding kinetics at specified positions for rapidly frozen samples. Moreover, similar approaches might also be useful for studies of equilibrium folding intermediates and of the interface structure in protein–protein interactions. Work along these lines is currently in progress.

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