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Rapid Report

Measurement of the local translational diffusion rates of proteins by saturation transfer EPR spectroscopy

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The concentration dependence of the normalized integral of the saturation-transfer EPR spectrum of human serum albumin spin-labelled on amino groups is found to be sensitive to viscosity and to temperature in aqueous solution and in 60% glycerol. The dependence on protein concentration is consistent with theoretical predictions for a diffusion-controlled Heisenberg spin-exchange interaction and the experimentally derived bimolecular collision rate constants are in reasonable agreement with those calculated theoretically for a diffusion-controlled process. The method is therefore applicable to the determination of translational diffusion coefficients of proteins in solution and, because of the nature of the saturation transfer EPR method, should be ideally suited to the study of local translational diffusion of proteins in membranes.

The long-range translational diffusion of integral proteins in membranes has been studied extensively by photobleaching techniques (for a review see, for example, Ref. 1). In cells, the long-range diffusion has been found, in many cases, to be highly restricted (see, for example, Ref. 2). This result is not entirely surprising in view of the heterogeneous nature of biological membranes, and points to the interesting possibility of the existence of functionally differentiated membrane domains [3,4]. This spatial microheterogeneity is best studied by techniques sensitive to local or short-range translational diffusion. For technical reasons, photobleaching techniques are limited in the lower distance scale to which they may be applied, but techniques that are based on the measurement of bimolecular collision rates would seem to be ideal for such studies. Methods employing the determination of Heisenberg spin exchange frequencies are well established for studying the short-range translational diffusion of spin-labelled lipid molecules in membranes by EPR spectroscopy (for a review see, for example, Ref. 5). However, conventional EPR techniques are not sufficiently sensitive to low exchange frequencies to be applicable to the slower translational diffusion of integral proteins.

Recently, saturation transfer EPR spectroscopy has been shown to be sensitive to weak spin-exchange interactions [6,7] and this new application of a method which is normally used for the determination of slow *rotational* diffusion rates has considerable promise also for the study of slow translational diffusion. In the present paper we explore the possibility of using saturation transfer EPR for the determination of protein translational diffusion rates. To demonstrate feasibility, the experiments are performed in homogeneous solution, since it is then better able to control the protein concentration which is the essential parameter. Additionally, this also allows a direct comparison of the experimental diffusion rates with theoretical predictions because, unlike in the membrane case, the viscosity of the medium is known with certainty. In all other respects, experiments on membranes are likely to be more favourable, since the greater restriction of the rotational diffusion will give rise to higher spectral intensities [8] and hence improve sensitivity. In addition, segmental motion of the label is likely to be more restricted for integral membrane proteins, which will

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Abbreviations: HSA, human serum albumin; 5-MSL, 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxy; 5-SASL, 5-(4,4-dimethyloxazolidine-*N*-oxyl)stearic acid; Hepes, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid; STEPR, saturation transfer EPR; V_1, first harmonic EPR absorption signal detected in phase with respect to the field modulation; V_2', second harmonic absorption EPR signal detected 90° out-of-phase with respect to the field modulation.

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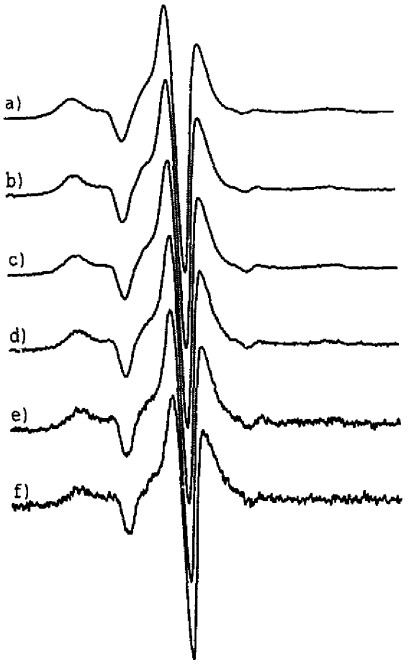

Fig. 1. Second harmonic, phase-quadrature STEPR spectra (V_2'-display) of maleimide (5-MSL) spin-labelled human serum albumin at different concentrations in 60% aqueous glycerol (buffer 0.1 M NaCl/50 mM Hepes (pH 7.0)). [HSA] in mM: (a) 1.52; (b) 0.76; (c) 0.41; (d) 0.22; (e) 0.11; (f) 0.076. Total scan range = 100 G; T = 18°C.

have a similar consequence. However, it should be noted that, unlike the situation for rotational diffusion measurements [6,9], the absence of segmental mobility is not essential to the method; it simply enhances sensitivity.

The saturation transfer EPR spectra (V_2'-display) of spin-labelled human serum albumin (HSA, Sigma, St. Louis, MO) at various concentrations in 60% aqueous glycerol (buffer: 0.1 M NaCl/50 mM Hepes (pH 7.0)) are given in Fig. 1. A high degree of labelling of surface residues was achieved (about 7.5 spin labels/protein as determined by the double integrated intensity of the conventional V_1 EPR spectrum) by reacting with the maleimide spin label (5-MSL, Syva, Palo Alto, CA) at high pH to modify amino groups (cf. Ref. 10). This was done in order to maximize the likelihood of spin-exchange on collision of the protein molecules. STEPR spectra were recorded under the standardized conditions and spectrometer set-up described in Refs. 11 and 12. The spectral lineshapes in Fig. 1 are not particularly sensitive to protein concentration, as is to be expected, since the concentrations used are below those giving rise to protein aggregation [13] and Heisenberg spin-exchange interactions do not cause much change in STEPR lineshape, only in the STEPR

TABLE I

Rotational correlation times

Rotational correlation times (μs) deduced from the low-field (L''/L), central (C'/C) and high-field (H''/H) diagnostic STEPR line-height ratios (V_2'-display) of spin-labelled stearic acid (5-SASL, see Ref. 14) bound to human serum albumin at different protein concentrations, c, in 60% aqueous glycerol (buffer: 0.1 M NaCl/50 mM Hepes (pH 7.0)) at 18°C. Rotational correlation times are obtained from the calibrations given in Refs. 8 and 15.

c (mM)	τ_R (μs)		
	L''/L	C'/C	H''/H [a]
0.252	0.31	0.28	< 0.1
0.511	0.30	0.30	< 0.1
0.746	0.30	0.31	0.13
1.10	0.27	0.27	≈ 0.1
1.46	0.29	0.28	0.11
2.27	0.37	0.29	0.10
4.55	1.4	0.83	0.42

[a] Values for H''/H are somewhat uncertain because of low signal strength in this spectral region.

intensity [7]. The lack of an appreciable effect of increasing viscosity of the solution with increasing protein concentration is seen further from the STEPR spectra of spin-labelled stearic acid (5-SASL) bound to HSA. This label exhibits little segmental motion rela-

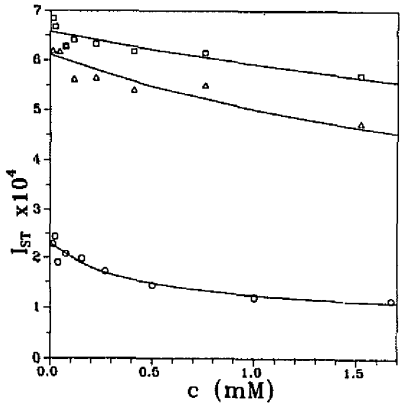

Fig. 2. Dependence of the normalized integral intensity, I_{ST}, of the STEPR spectra (V_2'-display) of maleimide (5-MSL) spin-labelled human serum albumin on protein concentration, c. ○, in water at 18°C; □, in 60% aqueous glycerol at 18°C; △, in 60% aqueous glycerol at 35°C. In each case, the aqueous component of the solution contains 0.1 M NaCl/50 mM Hepes (pH 7.0). The solid lines are non-linear least-squares fits of the data to Eqn. 1, with the following optimized fitting parameters: $T_1^\circ k_{ex} = 2.7 \cdot 10^3$ M^{-1}, $I_{ST}^\circ = 2.3 \cdot 10^{-4}$, $Z = 0.36$ in water at 18°C; $T_1^\circ k_{ex} = 0.18 \cdot 10^3$ M^{-1}, $I_{ST}^\circ = 6.6 \cdot 10^{-4}$, $Z = 0.33$ (fixed) in 60% aqueous glycerol at 18°C; $T_1^\circ k_{ex} = 0.38 \cdot 10^3$ M^{-1}, $I_{ST}^\circ = 6.1 \cdot 10^{-4}$, $Z = 0.33$ (fixed) in 60% aqueous glycerol at 35°C.

tive to the protein as compared to the labelled surface amino groups and the corresponding rotational correlation times are given as a function of protein concentration in Table I. The rotational correlation time hardly varies up to protein concentrations of 2 mM in 60% aqueous glycerol.

The normalized saturation transfer integral intensities, I_{ST}, calculated according to Ref. 8 are given as a function of protein concentration in Fig. 2 for maleimide spin-labelled HSA in water and in 60% aqueous glycerol (buffer: 0.1 M NaCl/50 mM Hepes (pH 7.0)) at temperatures of 18 and 35°C. In each case, the integral intensity decreases with increasing protein concentration, i.e., with increasing spin label concentration. It should be noted that an increase in rotational correlation time due to protein aggregation or to protein concentration dependent changes in viscosity would have just the opposite effect (cf. Ref. 8). The steepness of the concentration dependence decreases with increasing viscosity of the medium and increases with increasing temperature, as would be expected if it were determined by translational diffusion of the protein.

The theoretical dependence of the saturation transfer integral on spin concentration, c, assuming that the former is linearly proportional to the effective spin lattice relaxation time [16], is given by (Ref. 7):

$$I_{ST} = I_{ST}^{\circ}(1 + Z \cdot T_1^{\circ} k_{ex} \cdot c)/(1 + T_1^{\circ} k_{ex} \cdot c) \qquad (1)$$

where I_{ST}° and T_1° are the saturation transfer integral and intrinsic spin-lattice relaxation time in the absence of spin exchange, $1/Z$ is the redistribution factor for saturation transfer throughout the spectral lineshape, and k_{ex} is the rate constant for spin-exchange. Nonlinear least-squares fits of this expression to the experimental data are given in Fig. 2 and are seen to describe the concentration dependences reasonably well. The fitted value of the redistribution factor for the protein in water is $1/Z \approx 3$, which is what would be expected simply for redistribution between the three ^{14}N hyperfine lines [17]. This result is consistent with the relatively high mobility of the protein-attached labels in water, whose conventional V_1 EPR spectrum contains a relatively high proportion of a near-isotropic three-line component (data not shown). For the protein in 60% glycerol, the experimental scatter combined with the smaller concentration dependence precluded an accurate determination of Z. Therefore, this was maintained constant at a value of $Z = 1/3$ (i.e., close to that obtained in water) for fitting the concentration dependence. If Z is small, an approximately linear dependence of $1/I_{ST}$ on c is predicted from Eqn. 1. This was found also to approximate the smaller concentration dependence of the protein in 60% glycerol reasonably well, but not that in water. In the former case, values of $T_1^{\circ} k_{ex} = 0.1$ and $0.2 \cdot 10^3$ M^{-1} were obtained from linear regression at 18 and 35°C, respectively.

The second-order rate constant for spin-exchange is related to the bimolecular collision rate constant, k_{coll}, by the intrinsic probability of exchange on collision, p_{ex}, and the normalized cross-section for collision between the spin-labelled groups on the protein, σ:

$$k_{ex} = p_{ex} \sigma k_{coll} \qquad (2)$$

where $p_{ex} = \frac{1}{2}$ for strong exchange [18] and $\sigma \leq 1$. Assuming a value of $T_1^{\circ} \approx 1$ μs in the STEPR regime (cf. Ref. 16), the collision rate constants are therefore given by: $k_{coll}\sigma = 5.4 \cdot 10^9$, $0.36 \cdot 10^9$ and $0.75 \cdot 10^9$ M^{-1} s^{-1} for the protein in water at 18°C, and in 60% glycerol at 18 and 35°C, respectively.

The theoretical expression for the second-order diffusion-controlled rate constant for collision is [19]:

$$k_2^{diff} = 16\pi N_A 10^{-3} r_{NN} D_T \qquad (3)$$

where D_T is the translational diffusion coefficient of the protein, r_{NN} is the effective collision radius for the spin label groups on the protein and N_A is Avogadro's number. The translational diffusion coefficient is related to the viscosity, η, of the medium by the Stokes-Einstein relation:

$$D_T = kT/(6\pi\eta F_T R_0) \qquad (4)$$

where k is Boltzmann's constant, R_0 is the radius of a sphere of volume equal to that of the protein and F_T is the frictional ratio for translational diffusion [20]. Since the shape of the protein is prolate with an axial ratio of close to 3.5 [10], the frictional ratio is $F_T \approx 1.15$ [20]. Combining Eqns. 3 and 4 yields values of $k_2^{diff}(R_0/r_{NN}) = 5.8 \cdot 10^9$, $0.47 \cdot 10^9$ and $0.96 \cdot 10^9$ M^{-1} s^{-1} for the protein in water at 18°C, and in 60% glycerol at 18 and 35°C, respectively.

By comparison with the results of the STEPR measurements, it is seen that the experimental and theoretical values for the collision rate constant are in quite good agreement if it is assumed that $\sigma \approx 1 \approx R_0/r_{NN}$, as might be expected because of the high degree of protein modification. The comparison of the two sets of estimates involves some uncertainties, particularly with respect to the exact value of T_1°. However, since the experimental determinations clearly provide the correct order of magnitude and more importantly, also yield the expected dependence on solvent viscosity and temperature, there is little doubt that the concentration dependence of the experimental data is faithfully reflecting the translational motion of the protein.

Taking $r_{NN} = R_0$, $\sigma = 1$ and $k_2^{diff} = k_{coll}$ from the above results allows an estimation of the experimental

diffusion coefficients from Eqn. 3. The equivalent spherical radius of the protein is given by:

$$R_0 = (3M\bar{v}/4\pi N_A)^{1/3} \quad (5)$$

where $\bar{v} = 0.733$ ml g^{-1} is the partial specific volume of the protein and $M = 66.5$ kDa is its molecular mass. This then yields values of $D_T = 6.6 \cdot 10^{-7}$, $0.44 \cdot 10^{-7}$ and $0.93 \cdot 10^{-7}$ cm^2 s^{-1} for the protein in water at 18°C, and in 60% glycerol at 18 and 35°C, respectively. For comparison, a value of $D_T = 6.5 \cdot 10^{-7}$ cm^2 s^{-1} has previously been obtained for HSA in water at 20°C by intensity fluctuation spectroscopy [13].

The translational diffusion coefficients of integral proteins in fluid lipid bilayers are typically in the range of $(0.2–0.4) \cdot 10^{-7}$ cm^2 s^{-1} [1], suggesting that the method should be applicable also to these systems and to the study of local translational diffusion in biological membranes. In fact, because of the reduction in dimensionality, both the collision rate constants and effective protein concentrations will be higher than in homogeneous solutions. This will therefore give rise to higher observable exchange rates in membrane systems, for the same values of the diffusion coefficient.

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