Magnetic Cross-Relaxation among Protons in Protein Solutions†

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ABSTRACT: The magnetic spin–lattice relaxation rates of solvent water nuclei are known to increase upon addition of diamagnetic solute protein. This enhancement of the relaxation rate is a function of magnetic field, and the orientational relaxation time of the protein molecules can be deduced from analysis of the field-dependent relaxation rates. Although the nature of the interactions that convey information about the dynamics of protein motion to the solvent molecules is not established, it is known that there is a contribution to the relaxation rates of solvent protons that plays no role in the relaxation of solvent deuterons and ^{17}O nuclei. We show here that the additional interaction arises from a cross-relaxation process between solvent and solute protons. We introduce a heuristic three-parameter model in which protein protons and solvent protons are considered as two separate thermodynamic systems that interact across the protein–solvent interface. The three parameters are the intrinsic relaxation rates of each system and a cross-relaxation term. The sign of the latter term must always be positive, for all values of magnetic field, in order for magnetization energy to flow from the hotter to the cooler system. We find that the magnetic field-dependence of the cross-relaxation contribution is much like that of the remaining solvent proton relaxation, i.e., about the same as the deuteron relaxation field dependence. This finding is not compatible with the predictions of expressions for the cross-relaxation that have been used by other authors, but not applied to data over a wide range of magnetic field strength. The model predicts that the relaxation behavior of both the protein protons and the solvent protons is the sum of two exponentials, the relative contributions of which would vary with protein concentration and solvent isotopic composition in a fashion suggestive of the presence of two classes of protein protons, when there is in reality only one. This finding has immediate implications for the interpretation of published proton relaxation rates in complex systems such as tissues; these data should be reexamined with cross-relaxation taken into account.

It has been known for some time that the presence of diamagnetic solute protein molecules increases the magnetic spin–lattice relaxation rate $1/T_1$ of solvent water protons. Daszkiewicz et al. (1963) attributed the effect, first observed by them in ovalbumin solutions, to an increase in the rotational correlation time of a small fraction of water molecules bound irrotationally to protein and in rapid exchange with solvent. Though they observed the enhancement of solvent proton relaxation rates at only one value of magnetic field (corresponding to a proton Larmor precession frequency[1] of 14 MHz), their theoretical interpretation of the data required that the relaxation rates be a function of magnetic field, decreasing smoothly as the field increases, and with a point of inflection at a value of magnetic field corresponding to a Larmor precession frequency roughly equal to the orientational relaxation rate of the solute protein molecules.

The explicit dependence of the relaxation rate $1/T_1$ of solvent protons on magnetic field was first demonstrated by Koenig & Schillinger (1969) for solutions of apotransferrin, and at about the same time by Kimmich & Noack (1970) for solutions of serum albumin and gelatin. Comparable data have since been obtained for solutions of many other globular proteins; the results of a systematic survey of the dispersion (i.e., magnetic field-dependence) of $1/T_1$ for solutions of proteins ranging over three decades in molecular weight have been recently reported (Hallenga & Koenig, 1976). These dispersion data (including temperature, molecular weight, and shape dependence) can be represented quite well by a two-state model of exchanging water molecules. However, subsequent data indicate that the mechanism of relaxation enhancement by solute protein is more subtle than that of a simple two-state model and, indeed, somewhat elusive. For example, Koenig et al. (1975) have argued, from results of dispersion measurements on solvent deuterons and ^{17}O nuclei, that no two-state model that incorporates the concept of a well-defined residence time (or distribution of times) for a class of exchanging solvent molecules in association with the protein molecules can be correct. They suggest that the mechanism of relaxation enhancement involves a long-range hydrodynamic interaction that superposes onto the (rapid) Brownian rotational motion of solvent molecules a small component proportional to the relatively slow motion of the solute molecules.

Any model which attributes the relaxation enhancement to an alteration of the time-averaged rotational motion of solvent molecules, whether the alteration results from chemical exchange averaging or less specific hydrodynamic effects, would predict that solvent deuterons and ^{17}O nuclei have a relaxation dispersion much like that of protons. It is indeed the case that, at least to first order, the dispersions of $1/T_1$ of protons, deuterons, and ^{17}O nuclei are quite similar, when expressed in units of their respective protein-free solvent relaxation rates (Koenig et al., 1975; Hallenga & Koenig, 1976). However, quantitatively, the relative enhancement of proton relaxation rates is larger, typically by a factor of about two, than for other solvent water nuclei. Such models of relaxation would, in addition, predict that the enhancement of the relaxation rate of solvent protons would decrease in proportion to the dilution of protons by deuterons, since protons are relaxed by magnetic dipolar interactions between neighboring protons on the same and adjacent water molecules. On the other hand, deuteron and ^{17}O relaxation rates would be independent of dilution, since their

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[1] We measure the magnetic-field intensity in units of the Larmor precission frequencies of protons or deuterons in that field, as well as in Oersteds. For protons, the conversion is 4.26 kHz = 1 Oe = 1 G; for deuterons, 0.654 kHz = 1 Oe.

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relaxation results from interactions of the electric quadrupole moments of these nuclei with internal electric field gradients in the solvent molecules. However, an early measurement of the dilution effect in solutions of apotransferrin (Koenig & Schillinger, 1969) indicated that dilution did not reduce the relaxation enhancement to the extent expected. Similarly, Kimmich & Noack (1970) reported almost no dilution effect for serum albumin solutions. More recently, dispersion data for both deuterons and protons in samples of lysozyme, carbonmonoxyhemoglobin, and hemocyanin for a limited range of proton-deuteron ratio (Hallenga & Koenig, 1976) make it quite clear that solvent protons in protein solutions must relax by an interaction in addition to their interaction with other solvent protons; the removal of protons by dilution does not reduce the solvent proton relaxation rates sufficiently.

We now report data for the dispersion of $1/T_1$ of solvent protons and deuterons, as a function of the proton-deuteron ratio of the solvent, for solutions of three diamagnetic proteins: bovine carbonic anhydrase (29 000 daltons), human carbonmonoxyhemoglobin (67 000 daltons), and *E. coli* alkaline phosphatase (85 000 daltons). The results demonstrate that, while the magnitude and form of the deuteron relaxation dispersion are essentially independent of proton fraction, the proton relaxation dispersion of $1/T_1$ depends in a complex way on the interactions of solvent protons with other solvent protons, and with protein protons as well.

We interpret our results in terms of an empirical, linear, macroscopic cross-relaxation model; the ensemble of solvent protons is regarded as one thermodynamic system coupled to the ensemble of protein protons, regarded as a separate thermodynamic system. A cross-relaxation term is introduced with the requirement that, at equilibrium, each ensemble come to its own equilibrium magnetization at all protein concentrations. Values for the several empirical parameters of the model are obtained from a least-squares comparison of the data with the predictions of the model. These values are then compared with estimates that can be made from current understanding of the microscopic mechanisms of relaxation of protein protons.

Experimental Procedures

Materials. Proteins used in this study were bovine carbonic anhydrase, *E. coli* alkaline phosphatase, and human hemoglobin. Deuterium oxide (99.9%) guaranteed low in paramagnetic impurities was obtained from Aldrich. All glassware and pipettes were immersed in 0.1 M sodium ethylenediaminetetraacetic acid solution for at least 1 day and rinsed with deionized water before use.

The procedure for the preparation of protein solutions having different solvent proton-deuteron ratios was similar for all three proteins, with the exception that alkaline phosphatase was always handled in 0.25 M Tris buffer, pH 7.5, rather than in deionized water as was the procedure for hemoglobin and carbonic anhydrase. Deuterated stock solutions of carbonic anhydrase and alkaline phosphatase were made by dissolving lyophilized protein in D_2O, letting it stand overnight at 5 °C, lyophilizing the material, and redissolving it in D_2O. This process was repeated a second time. Deuterated hemoglobin solutions were obtained by ultrafiltration, as described earlier (Hallenga & Koenig, 1976). The concentrations of stock solutions of deuterated and undeuterated protein were equalized by appropriate dilutions, using measurements of optical absorbance and the appropriate extinction coefficient. Samples with the desired proton-deuteron ratios for each protein were prepared by mixing appropriate volumes of these stock solutions.

E. coli alkaline phosphatase was provided as lyophilized powder by Dr. D. P. Hollis, who purified the enzyme by a modification of the procedure of Torriani (1966). Stock solutions of 0.6 mM enzyme in 0.25 M Tris (pH 7.58 and 7.50 for deuterated[2] and protonated samples, respectively) were made using $A_{1\,cm}^{1\%} = 7.7$ at 280 nm (Rothman & Byrne, 1963). For a study of the effect of protein concentration on the relaxation rates, samples of 0.3 mM enzyme, derived by twofold dilution of 0.6 mM protein samples were also measured; the proton–deuteron ratios of these samples were determined by calculation, using the volume percent occupied by protein, H_2O, and D_2O in each sample.

Bovine carbonic anhydrase obtained from Worthington as a lyophilized powder was chromatographed twice on a DEAE-cellulose column by the method of Kandel et al. (1970). The purified enzyme was dialyzed against deionized water and lyophilized. It was found to be homogeneous by analytical polyacrylamide gel electrophoresis. Stock solutions of 4.1 mM enzyme in 100% H_2O (pH 7.32) and in 100% D_2O (pH 7.28) were made using $A_{1\,cm}^{1\%} = 18$ at 280 nm (Lindskog, 1960).

The hemoglobin A data reported in this study are unpublished results obtained during an earlier study by Hallenga & Koenig (1976). Stock solutions having concentrations of 115 mg/mL were made using $A_{1\,cm}^{1\%} = 8.4$ at 540 nm (Chiancone et al., 1970).

Relaxation Measurements. Relaxation rates of solvent protons and deuterons were measured using apparatus and procedures that differed only slightly from that described previously (Hallenga & Koenig, 1976; Brown et al., 1977).

Data Reduction. Following the procedures used by Hallenga & Koenig (1976), we represent the magnetic relaxation dispersion by analogy with dielectric relaxation dispersion (Cole & Cole, 1941):

$$1/T_1 = (1/T_{1w}) + D + A\mathrm{Re}[1/(1 + (i\nu/\nu_c)^{\beta/2})] \quad (1)$$

$$= (1/T_{1w}) + D + \frac{A(1 + (\nu/\nu_c)^{\beta/2}\cos(\pi\beta/4))}{1 + 2(\nu/\nu_c)^{\beta/2}\cos(\pi\beta/4) + (\nu/\nu_c)^{\beta}} \quad (2)$$

Here $1/T_{1w}$ is the relaxation rate of the water nuclei in the protein-free buffer, ν the Larmor precession frequency of the nuclei at magnetic field H_0, and D, A, ν_c, and β are parameters to be determined from a fit of eq 2 to the data. "Re" stands for "the real part of" the expression that follows it.

For $\beta = 2$, eq 2 reduces to a constant plus a Lorentzian dispersive term:

$$1/T_1 = (1/T_{1w}) + D + A/(1 + (\nu/\nu_c)^2) \quad (3)$$

The dispersive part of the Cole–Cole expression, like the Lorentzian, drops to half its maximum value of A at $\nu = \nu_c$, the frequency at which the curve inflects. However, for $\beta < 2$, the Cole–Cole expression has a slower variation with ν than the Lorentzian. A simple two-site model of water exchange between bulk solvent and protein (shown to be inadequate by Koenig et al., 1975) predicts a Lorentzian contribution to the relaxation (Koenig & Schillinger, 1969), whereas the experimental data are known to vary more slowly with ν (Fabry et al., 1970; Lindstrom & Koenig, 1974). The Cole–Cole expression should be regarded as a heuristic equation that represents the relaxation dispersion data very well (Hallenga & Koenig, 1976). While it has no a priori validity, it is a convenient way of characterizing the data with a small number of parameters.

No correction was made for the small contribution of dis-

[2] pH values for samples in partially deuterated solvents are the uncorrected meter readings.

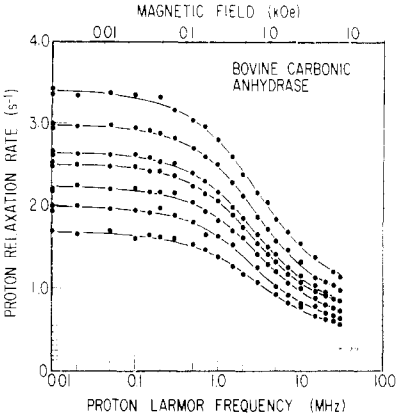

FIGURE 1: Dispersion of solvent proton spin–lattice relaxation rates at 5 °C for solutions of bovine carbonic anhydrase, for values of proton fraction f of the partially deuterated solvent of 1.0, 0.80, 0.60, 0.50, 0.40, 0.25, and 0.15. Samples were 4.1 mM protein in distilled water, pH 7.3. The relaxation rates of the protein-free buffers, for the same values of f, are indicated by horizontal lines at the lower left of the figure; greater rates correspond to greater values of f for both protein and protein-free samples. The magnitude of the relaxation contribution of dissolved O_2 for samples in equilibrium with air is indicated by the line at the lower right. The solid lines through the data points result from a least-squares comparison of the data with eq 2.

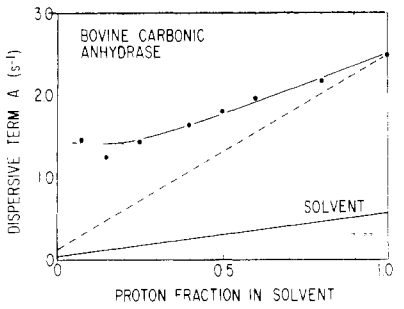

FIGURE 2: A, the magnitude of the dispersive term of eq 2 (obtained from the carbonic anhydrase data of Figure 1 and additional data not illustrated), as a function of the proton fraction f of the partially deuterated solvent. The open and closed data points are for samples obtained from two separate protein preparations. The lower line is the variation with f of the relaxation rate of the protein-free solvent (on the same scale) derived, by interpolation, from the data of Figure 4. The solid line through the data points results from a least-squares comparison of the data with the cross-relaxation model, eq 4–12. The dashed line indicates the linear variation expected in the absence of cross-relaxation.

solved oxygen to the observed relaxation rates. Typical values are of the order of 0.1 s^{-1} for solutions under 1 atm air, and fluctuations in this value are considered small enough to be disregarded in the present work. In addition, removal of O_2 introduces problems due to foaming and solvent evaporation.

Results

Data for bovine carbonic anhydrase were taken on samples derived from two different preparations of the enzyme. One preparation was used to span the entire range of values of f, the mole fraction of solvent protons; the other was used to fill in data for lower values of f. The dispersions of solvent proton relaxation rates, at 5 °C, for values of f ranging from 0.15 to 1.0, are shown in Figure 1. Also indicated are the contributions to the rates of the protein-free buffer for each value of f, as well as the expected small contribution due to the paramagnetism of dissolved O_2. Data for $f = 0.05$, 0.075, and 0.1, after correction for the buffer contribution, are indistinguishable from the results for $f = 0.15$, within the uncertainty of the data.

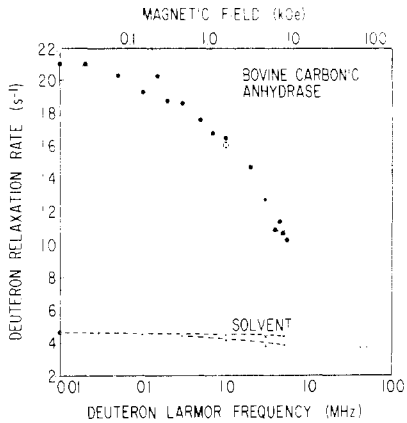

FIGURE 3: Dispersion of solvent deuteron spin–lattice relaxation rates at 5 °C for several of the bovine carbonic anhydrase samples included in Figure 1. The solvent deuteron fraction values $1 - f$ are 0.93 (●); 0.85 (○); 0.60 (□); 0.50 (+); and 0.20 (△). Also shown are deuteron dispersion data for the protein-free solvent, for two values of deuteron fraction, 1.0 (×) and 0.5 (+).

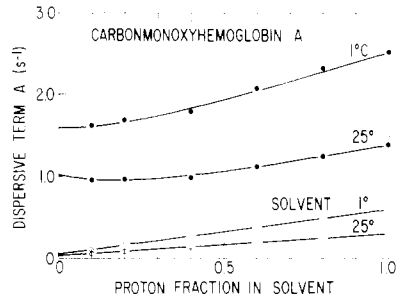

FIGURE 4: A, the magnitude of the dispersive term of eq 2, for (diamagnetic) human carbonmonoxyhemoglobin A at 1 and 25 °C, as a function of solvent proton fraction. The samples were 1.7 mM protein in distilled water, pH 7.5. The solid lines through the data points result from a least-squares comparison of the data with the cross-relaxation model, eq 4–12. Also indicated (on the same scale) are relaxation rate data for deoxygenated protein-free solvent. The straight lines through the solvent data represent the anticipated linear behavior.

The lines through the data points result from a least-squares fit of eq 1 to the data; the values obtained for the parameters A, D, ν_c, and β are given in Table I. It should be noted from Figure 1 that data were not obtained at fields sufficiently high for the D term of eq 1 to dominate the relaxation rate. Consequently, the uncertainty in D may be rather large, but this point is of little consequence here.

The dependence of A, the magnitude of the dispersive part of the data, is plotted against f in Figure 2. Results for samples made from the two different protein preparations are distinguished by different symbols; it is seen that the data for equivalent samples are in good agreement. The relaxation rate of the protein-free buffer is indicated by the lower solid line. The dashed line indicates the linear variation of A expected if the relaxation of protons were due only to intramolecular solvent proton–proton interactions (see below), whereas the line through the data points results from a least-squares comparison of the data with the heuristic model of cross-relaxation discussed below.

The dispersion of solvent deuteron relaxation rates, at 5 °C, for several of the samples of Figure 1, is shown in Figure 3. Relaxation rates are also indicated for protein-free buffer for two values of f. The signal-to-noise ratio for deuterons is less favorable than for protons and, because of the lower magnetic

TABLE I: Results of a Least-Squares Comparison of the Cole-Cole Expression (Equation 2) with Solvent Proton and Deuteron Relaxation Dispersion Data.

protein	concn (mM)	temp (°C)	nucleus	proton fraction	A (s^{-1})	D (s^{-1})	β	ν_c (MHz)
carbonic anhydrase B	4.1	5	^1H	1.00	2.49	0.37	1.44	3.0
				0.80	2.17	0.36	1.51	2.9
				0.60	1.96	0.35	1.48	2.8
				0.50	1.80	0.41	1.51	2.6
				0.40	1.64	0.35	1.49	3.0
				0.25	1.44	0.37	1.52	2.6
				0.15	1.24	0.33	1.44	2.9
				0.33	1.59	0.36	1.44	2.3
				0.20	1.38	0.45	1.45	2.2
				0.15	1.35	0.46	1.44	2.3
				0.13	1.42	0.44	1.35	2.2
				0.10	1.40	0.48	1.46	2.3
				0.075	1.40	0.49	1.44	2.3
				0.05	1.47	0.49	1.46	2.4
hemoglobin A (CO)[a]	1.7	1	^1H	1.00	2.51	0.39	1.64	1.7
				0.80	2.33	0.46	1.58	1.6
				0.60	2.07	0.49	1.62	1.4
				0.40	1.79	0.44	1.58	1.4
				0.20	1.70	0.44	1.63	1.2
				0.10	1.63	0.40	1.63	1.2
		25		1.00	1.38	0.19	1.54	2.6
				0.80	1.25	0.27	1.56	2.7
				0.60	1.13	0.24	1.55	2.3
				0.40	1.98	0.26	1.58	2.0
				0.20	0.98	0.25	1.58	1.9
				0.10	0.97	0.20	1.50	1.9
alkaline phosphatase	0.6	5	^1H	1.00	5.55	1.56	1.54	1.4
				0.80	5.20	1.66	1.54	1.4
				0.70	5.09	1.63	1.51	1.5
				0.60	4.92	1.59	1.57	1.5
				0.50	4.66	1.63	1.46	1.6
				0.40	4.52	1.57	1.42	1.8
				0.30	4.35	1.54	1.39	2.0
				0.20	4.54	1.46	1.35	2.2
				0.15	4.50	1.40	1.32	2.5
				0.10	4.40	1.30	1.25	3.2
				0.075	4.60	1.30	1.24	3.5
				0.046	5.41	1.31	1.27	3.6
				0.026	5.76	1.97	1.36	3.3
	0.3	5	^1H	1.0	2.93	0.84	1.58	1.4
				0.80	2.49	0.87	1.49	1.5
				0.65	2.36	0.86	1.52	1.6
				0.5	2.18	0.90	1.52	1.7
				0.35	2.11	0.81	1.42	1.9
				0.20	1.92	0.83	1.46	2.0
				0.10	1.93	0.83	1.39	2.5
				0.068	2.51	0.61	1.37	3.4
	0.3	5	^2H	*b*	9.56	0.76	1.73	0.91

[a] The data for hemoglobin are from Hallenga & Koenig (1976) and from associated data not included in that publication. [b] The deuteron results are averages of samples with solvent deuteron fractions: 0.93, 0.80, and 0.50.

moment of the deuteron, the maximum Larmor frequency obtainable is about one-sixth that for protons. Nonetheless, it is quite clear that the deuteron relaxation rates are independent of deuteron fraction $(1 - f)$.

Data for the magnitude of A for solvent protons in solutions of carbonmonoxyhemoglobin, at 1 and 25 °C, as a function of f, are shown in Figure 4. The data for deoxygenated protein-free solvent at 0.02 MHz are also shown. (The line in Figure 2 was interpolated from these data.) Again, the lines through the data points are the results of a least-squares comparison of the data with the model of cross-relaxation discussed below.

Representative proton dispersion data, at 5 °C, for several values of f, and for two concentrations of alkaline phosphates are shown in Figure 5. The lines through the data points result from a fit to eq 1. The resulting values of the parameters for these and other samples of alkaline phosphatase are included in Table I. It should be noted that, unlike the case of carbonic anhydrase, the form of the proton dispersion curve varies as f changes; as f is reduced, the high field part of the curve tends

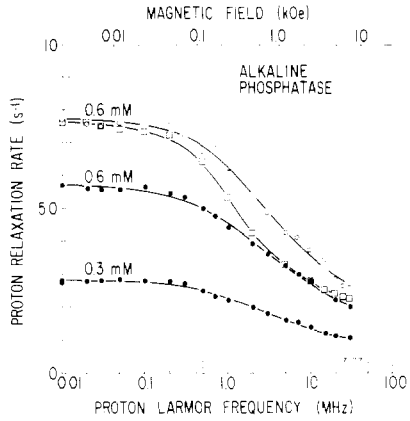

FIGURE 5: Dispersion of solvent proton spin-lattice relaxation rates at 5 °C for solution of *E. coli* alkaline phosphatase for values of solvent proton fraction f of 1.0 (□); 0.10 (●); and 0.02 (○). The samples were either 0.3 or 0.6 mM protein, as indicated, in 0.25 M Tris buffer, pH 7.5. The solid line through the data points results from a least-squares comparison of the data with eq 2.

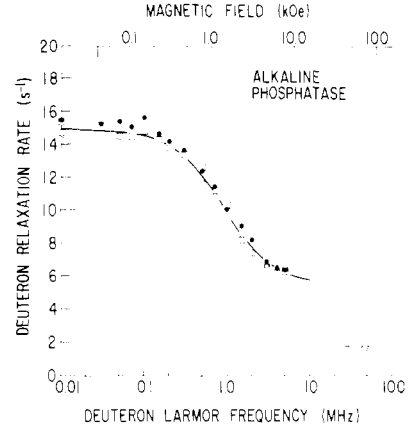

FIGURE 6: Dispersion of solvent deuteron spin-lattice relaxation rates for three samples of *E. coli* alkaline phosphatase, 0.3 mM, at 5 °C. The values for solvent fraction $1 - f$ are 0.93 (●); 0.80 (○); and 0.50 (□).

to become more extended.

The dispersions of solvent deuteron relaxation rates, at 5 °C, for representative samples of alkaline phosphatase, are shown in Figure 6. As for carbonic anhydrase, the data are essentially independent of the deuteron fraction $(1 - f)$.

The dependence of A, eq 1, on proton fraction is shown in Figure 7. What is most notable here is that, as f is decreased, A decreases only slowly, then goes through a shallow minimum and rises steeply below $f \simeq 0.1$. The experimental protocol is important to note here: the samples with the lowest f values were prepared and measured first; then appropriate amounts of protonated ($f = 1$) material were added, resulting in a decrease in A as f was increased. This order of measurement eliminates any possibility that the increase in A as $f \rightarrow 0$ could be due to contamination by some adventitious relaxing agent. Again, the lines through the data points result from the fit to the theory discussed below.

Cross-Relaxation Model and Data Analysis

Most considerations of the influence of diamagnetic solute protein on the relaxation rates of solvent protons (cf. Hallenga & Koenig, 1976) regard the protein as influencing the time-averaged motion of solvent water molecules and, thereby, the magnetic dipolar interaction of a given solvent proton with its neighboring proton. Whether this influence arises from ex-

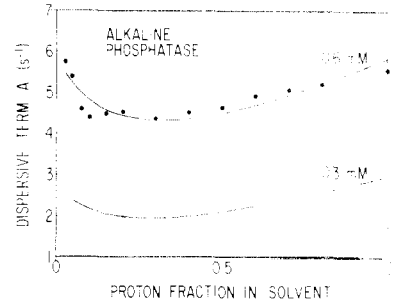

FIGURE 7: A, the magnitude of the dispersive term of eq 2, for *E. coli* alkaline phosphatase at two concentrations, as a function of solvent proton fraction. The solid lines through the data points result from a least-squares comparison of the data with the cross-relaxation model, eq 4–12.

change of solvent molecules between the bulk solvent and special sites on or near the protein molecules, as in earlier models, or whether long-range hydrodynamic effects are considered important (Koenig et al., 1975; Hallenga & Koenig, 1976), the predictions of all such interaction models do not agree with the present data. Rather, it is necessary to include, for proton relaxation though not for deuteron relaxation, interactions between solvent protons and some relaxing agents or entities in or on the protein molecules. For, as the data show, it is possible to reduce the proton fraction in the solvent, thereby reducing the number of neighbors generally regarded as causing relaxation, and not reduce the solvent proton relaxation rates proportionately. The inference that we draw, and which is the basis for the model below, is that solvent protons interact magnetically with protein protons, which are known to have a relatively high relaxation rate. When the solvent protons are sufficiently diluted, their relaxation rate is determined by the protein proton relaxation and the cross-relaxation rates.

We present a model in which we consider the solvent and protein as forming two independent thermodynamic systems, with magnetizations given at any time by S and P, respectively. We let S_0 and P_0 be the magnetization of the respective systems at thermal equilibrium; $S_{00} = S_0/f$ is the equilibrium magnetization of the solutions in undiluted solvent; and we define M, the relative equilibrium magnetizations of the two systems, by $M = S_{00}/P_0$. The following rate equations then determine the time courses of the magnetizations of the two systems:

$$\frac{d}{dt}(S - S_0) = -fR_S(S - S_0) + fMR_T(P - (S/fM))$$

$$= -(fR_S + R_T)(S - S_0) + fMR_T(P - P_0) \quad (4a)$$

$$\frac{d}{dt}(P - P_0) = -R_P(P - P_0) - fMR_T(P - (S/fM))$$

$$= -(R_P + fMR_T)(P - P_0) + R_T(S - S_0) \quad (4b)$$

Here R_S and R_P are the respective solvent and protein proton spin-lattice relaxation rates in the absence of cross-relaxation, and R_T is the rate of transfer of magnetization from the solvent to the protein. It is an assumption of the model that the cross-relaxation term is linear in the difference in magnetization between the two systems.

The reasonableness of eq 4a,b is readily argued. Each system, in the absence of cross-relaxation (i.e., when $R_T = 0$), relaxes at its own intrinsic rate, fR_S for the solvent and R_P for the protein, to its respective equilibrium magnetization value, S_0 or P_0. On the other hand, were R_T to dominate all relaxation, a steady-state defined by $S/P = fM$ would be reached, a condition clearly satisfied at equilibrium also. (The steady-

state is not necessarily an equilibrium state because the spin-lattice relaxation terms are ignored in this extreme, and these terms are the channels for communicating the temperatures of the bath to the two proton systems.)

Before solving these equations, several observations are in order. First, R_T is an intrinsically positive quantity because energy must flow from the hotter to the cooler of the two thermodynamic systems (and magnetization the opposite way). Second, the mean time for a solvent water molecule to diffuse a distance comparable to the protein-protein separation can be estimated to be about 10^{-7} s; this time is so short compared with a typical value observed for T_1 that any solvent proton has ample time to sample the protein-solvent interface many times in a relaxation time. Thus the solvent system may indeed be regarded as a homogeneous thermodynamic system in contact with protein. Third, the analogous argument can be made for the protein protons, though it is more subtle, and will be justified in the Discussion section. However, it should be noted here that P and P_0 need not refer to the complete magnetization of the protein protons, but rather those components which persist for times at least as long as $1/R_T$. Finally, it should be emphasized that only S is detected in our experiments, though the nature of the experiments imposes boundary conditions on both S and P at an initial time t which we will define as $t = 0$.

It is convenient to use variables which express the fractional deviation of the magnetization from its equilibrium, for both thermodynamic systems

$$\delta S = (S - S_0)/S_0 \tag{5a}$$

$$\delta P = (P - P_0)/P_0 \tag{5b}$$

In terms of these variables, the rate equations, eq 4a,b, become

$$\frac{d(\delta S)}{dt} = -(fR_S + R_T)(\delta S) + R_T(\delta P) \tag{6a}$$

$$\frac{d(\delta P)}{dt} = fMR_T(\delta S) - (R_P + fMR_T)(\delta P) \tag{6b}$$

These equations can be solved straightforwardly by assuming solutions for δS and δP of the form

$$\delta S = B\exp(-\lambda_+ t) + C\exp(-\lambda_- t) \tag{7a}$$

$$\delta P = E\exp(-\lambda_+ t) + F\exp(-\lambda_- t) \tag{7b}$$

and substituting these equations into eq 6a,b. The results are

$$\lambda_\pm = \tfrac{1}{2}(\lambda_P + \lambda_S) \pm \tfrac{1}{2}[(\lambda_P - \lambda_S)^2 + 4fMR_T^2]^{1/2} \tag{8}$$

where

$$\lambda_S = fR_S + R_T \tag{9a}$$

$$\lambda_P = R_P + fMR_T \tag{9b}$$

It is a matter of simple algebraic manipulation to show that both λ_+ and λ_- are always positive for all $R_T > 0$ and $f > 0$. $R_T > 0$ is required by the thermodynamics of the model; $f > 0$ is true by definition. Thus all solutions decay to equilibrium, i.e., to $\delta S = \delta P = 0$, for all initial conditions. The initial conditions of the experiments correspond to

$$B + C = E + F = 1 \tag{10}$$

which from eq 6a,b and 7a,b give

$$B = \frac{fR_S - \lambda_-}{\lambda_+ - \lambda_-};\ C = \frac{\lambda_+ - fR_S}{\lambda_+ - \lambda_-} \tag{11}$$

TABLE II: Results of a Least-Squares Comparison of the Cross-Relaxation Theory (Equations 4a,b) with the Proton Dilution Data in Figures 2, 4, and 7.[a]

protein	concn (mM)	temp (°C)	R_S (s^{-1})	R_P (s^{-1})	R_T (s^{-1})
carbonic anhydrase B	4.1	5	2.05	17	1.42
hemoglobin A (CO)	1.7	1	1.75	34	1.60
	1.7	25	1.03	13	1.03
alkaline phosphatase	0.6	5	4.47	42	5.74
	0.3	5	2.43	15	2.75

[a] The value $M = 20$ has been assumed.

$$E = \frac{R_P - \lambda_-}{\lambda_+ - \lambda_-};\ F = \frac{\lambda_+ - R_P}{\lambda_+ - \lambda_-} \tag{12}$$

In the limit of no cross-relaxation, i.e., $R_T \to 0$, B and $F \to 0$, C and $E \to 1$, $\lambda_+ \to R_P$, $\lambda_- \to fR_S$, and as expected

$$\delta S \to \exp(-fR_S t) \tag{13a}$$

$$\delta P \to \exp(-R_P t) \tag{13b}$$

In the limit of infinite dilution of solvent protons, i.e., $f \to 0$, $\lambda_+ \to R_P$, $\lambda_- \to R_T$, and

$$\delta S \to [R_P\exp(-R_T t) - R_T\exp(-R_P t)]/(R_P - R_T) \simeq \exp(-R_T t),\ \text{for } R_P \gg R_T \tag{14a}$$

$$\delta P \to \exp(-R_P t) \tag{14b}$$

In this limit, the protein protons relax exponentially at their intrinsic rate R_P, and the few solvent protons relax exponentially at the rate R_T when it is rate limiting.

The solid lines through the experimental values of A, Figures 2, 4, and 7, result from a least-squares comparison of these values with the predictions of eq 7-12, treating R_S, R_P, and R_T as the unknown parameters. The comparison was made for several values of M ranging from 5 to 50. The calculated fit for A was essentially independent of M, as were the results for R_S and R_T, whereas the results for R_P scaled roughly linearly with M, a fact that can be demonstrated from the structure of eq 8 and 9. The values for R_S, R_P, and R_T, obtained using $M = 20$, are in Table II. ($M = 20$ implies, roughly, that the number of protein protons is $\frac{1}{20}$ the number of solvent protons for $f = 1$.) Note that in all cases the values of the relaxation rates in the limit $f \to 0$ approach R_T, indicating that the cross-relaxation becomes the rate-limiting process for solvent proton relaxation. Note also that, since the forms of the dispersion curves do not change grossly, if at all, in this limit, the dispersive behavior of R_T must be very much like that of R_S.

The least-squares comparison gives $C \gg B$ in almost all circumstances considered, so that δS, eq 7a (which is the experimentally observed quantity), is dominated by the exponential term $\exp(-\lambda_- t)$. Thus, the decay of the solvent magnetization is predicted to appear exponential, as observed, though it is nonetheless a function of R_T. On the other hand, the decay of δP is not given by a single exponential. The variations of δS and δP with time, for two representative values of f, are shown in Figure 8 for the more concentrated alkaline phosphatase sample; here the essence of the cross-relaxation effects can be seen. The two upper dashed lines, labeled δS, show the expected decay of δS in the absence of cross-relaxation, i.e., for $R_T = 0$, for $f = 0.1$ and $f = 1$. The analogously labeled solid lines show the decay of δS for R_T equal to the value derived from the least-squares comparison. The differ-

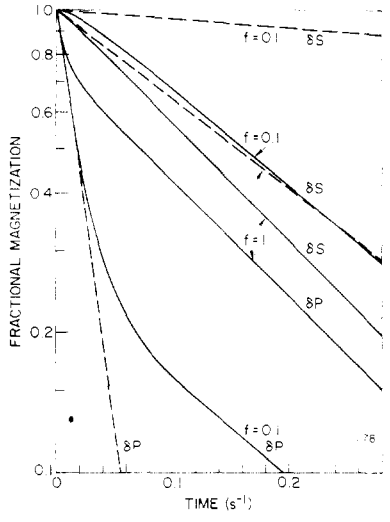

FIGURE 8: The recovery of the normalized solvent proton magnetization δS and the protein proton magnetization δP to their respective equilibrium values after perturbation, as predicated from eq 7a, b. The calculations are for the 0.6 mM alkaline phosphatase sample, using the results obtained from fitting the data to the cross-relaxation model (cf. Table II). The solid lines are the calculated nonexponential recovery paths for two values of proton fraction f, as indicated. The dashed lines represent the exponential behavior expected in the absence of cross-relaxation.

ence between the dashed and solid lines is greater for $f = 0.1$ than for $f = 1$, but in both cases the cross-relaxation increases the rate of decay of δS without altering its exponential form observably. The same is not true for the effect of cross-relaxation on the decay of δP, which decays rapidly at first and, after a time of order $1/\lambda_+$, parallels δS in its decay. However, δP does not contribute to our measurements because of instrumental limitations, even though for small values of f the magnitude of δP at $t = 0$ may become comparable to δS. First, we cannot make measurements at times as short as $1/\lambda_+$; and second, the T_2 contribution of δP, which is even shorter than $1/\lambda_+$, is too short to contribute to the spin–echo amplitude that we observe.

Discussion

We have presented data for the dispersion of solvent proton and deuteron relaxation in solutions of three different proteins, carbonic anhydrase, carbonmonoxyhemoglobin, and alkaline phosphatase, as a function of the relative proton–deuteron composition of the solvent. In contrast to the behavior of the deuteron relaxation dispersion which, for both protein solutions and protein-free solvent, is independent of proton fraction (cf. Figures 3 and 6), the behavior of the proton relaxation is not. The bulk of the data, therefore, represents a study of the dependence of solvent proton relaxation dispersion on proton fraction, for a variety of experimental conditions. The unambiguous conclusion from the data is that solvent protons relax not only by interaction with other solvent protons, but by interaction with solute protein protons as well. We are not the first to invoke the existence of interactions between protein proton and solvent protons. Kimmich & Noack (1970, 1971), for example, presented a schematic view of solvent proton relaxation dispersion in protein and polymer solutions that included cross-relaxation, but they did not model the system in any quantitative sense. Even earlier, Kruger & Helcké (1967) argued that cross-relaxation interactions must be invoked to understand solvent proton relaxation in single crystals of lysozyme, in which the water content is high. Hsi & Bryant (1977a,b) and Hilton et al. (1977) have investigated related problems in greater detail and reached similar conclusions. Edzes & Samulski (1977) have investigated hydrated collagen at one value of magnetic field (30 MHz) and found that cross-relaxation to the water is the major relaxation mechanism for the collagen protons. Their treatment, also heuristic, is very similar to that used here. More recently, Eisenstadt & Fabry (1978) demonstrated the existence of cross-relaxation between protein and solvent in suspensions of red blood cells, also at one value of magnetic field (20 MHz). They also modelled the system, but used equations (to be discussed below) that do not predict the proper magnetic field dependence of the cross-relaxation effects.

Here, for the first time, we have demonstrated the existence of cross-relaxation effects over a range of magnetic field that spans the major part of the dispersion of solvent proton relaxation, for solutions of proteins of three different molecular weights, at various pH values and temperatures, and for the entire range of solvent proton fraction. Furthermore, we analyzed the data in terms of a heuristic model that regards both classes of protons (solute and solvent) as belonging to separate, homogeneous macroscopic thermodynamic systems mutually coupled at the protein–solvent interface, and showed that values for the three parameters of the model can be obtained that give excellent agreement between model and observations. These three parameters are the intrinsic relaxation rates of both systems, R_S and R_P, and the cross-relaxation rate R_1. Before discussing their derived values in greater depth, there are two general points to be made that are, perhaps, the more significant implications of the present work.

Implications of Cross-Relaxation. First, the fact that the forms of the proton dispersion curves are little changed by alteration of the proton fraction of the solvent indicates that the cross-relaxation term has essentially the same dispersive behavior as R_S. This conclusion, noted above, follows directly from considering the limit $f \to 0$ and eq 14a. The immediate inference is that most previous models of cross-relaxation have to be reconsidered; this point will be amplified below.

Second, though we only observe the solvent proton relaxation, the model predicts that the decay of the protein signal, if it could be observed directly, would be the sum of two exponentials. The relative contribution of each would depend on the value of the proton fraction; only in the limit $f \to 0$ would the decay of δP be dominated by one of the exponential terms. Such behavior, seen naively, could be interpreted in two ways: either as indicating a single class of protein protons with a complex relaxation behavior; or as evidence for two compartmentalized classes of protein protons, one exchangeable upon deuteration of the solvent, and the other not. This latter interpretation, which is incorrect for protein solutions, might nonetheless be argued the more strongly (because of the behavior upon deuteration) in more complex samples. This observation is particularly relevant to studies of tissue samples, where questions of interpretation are hotly disputed (cf. Forster et al., 1976; Chang & Woessner, 1977; Resing et al., 1977), but where cross-relaxation effects have not, for the most part, been considered in the competing interpretations. For example, Resing & Forster (1976), in a more extended discussion of aspects of the data (for barnacle muscle) of Forster et al. (1976), note that the proton relaxation rate does not decrease as rapidly as expected upon deuteration of the muscle. This is a clear indication of the existence of proton–proton interactions between solvent and tissue macromolecules. To the extent that they have been considered previously, such intermolecular relaxation contributions have usually been regarded as additive (cf. Woessner & Snowden, Jr., 1970; Grosch & Noack, 1976). However, the cross-relaxation effects that we are considering are nonadditive and require the solution of the coupled equa-

tions, eq 6a,b. We suspect that similar effects will have to be analyzed before proton relaxation in tissue samples can be understood. The same remarks apply to somewhat less complex systems, such as water proton relaxation in polysaccharide gels (Ablett et al., 1976), in starch solutions (Lechert & Hennig, 1976), and in protein powders (Hilton et al., 1977) and frozen protein solutions (Hsi & Bryant, 1975). We note further that much data in the literature were taken at high fields where the D term, eq 1, contributes significantly or even dominates the observed relaxation behavior. Inspection of Table I shows that D is even more affected by cross-relaxation than A, further complicating the analysis of solvent protein relaxation. It is not our intention to reexamine the published data on solvent proton relaxation in macromolecular systems, but rather to call attention to the nature of the cross-relaxation phenomena that must be considered in the interpretation of such data.

Other Models of Cross-Relaxation. Our treatment of the solute and solvent proton systems as separate thermodynamic systems in thermal contact, recognizing that this defines the sign of R_T, is new. It results in a mathematical formulation identical with cross-relaxation problems considered by others, in particular Hsi & Bryant (1977a,b), Kalk & Berendsen (1976), Campbell & Freeman (1973), and Albert & Gutowsky (1973). However, the underlying physical principles of the two models differ in a fundamental way, and it is important that the distinction be clear at this point. These authors consider models in which the entire sample is regarded as a *single* thermodynamic system comprised of an ensemble of pairs of unlike, interacting spins: e.g., neighboring protons in a protein molecule, each in different local environments, such as methyl protons interacting with neighboring methylene protons. The underlying physical view, in their model, is that the dipolar interaction that couples the pairs of unlike spins, and that produces cross-relaxation effects (including Overhauser enhancement), also is responsible for the spin–lattice relaxation of both spin species, as discussed originally by Solomon (1955). In this case, the form of the interaction responsible for relaxation is known explicitly, and the magnitudes of what correspond to our R_T, R_S, and R_P can be computed directly. The result is that the cross-relaxation term is not restricted to being positive as is required in our model, and indeed R_T changes sign at a value of magnetic field comparable to that at which significant dispersion of R_S and R_T occurs. This generates the possibility of fairly complex transient behavior, including both Overhauser enhancement and deenhancement as R_T changes sign. However, the explicit form of the dipolar interaction limits the magnitude of R_T with respect to R_S and R_P in such a way that the magnetization of the ensemble of pairs always approaches thermal equilibrium, for all values of magnetic field, independent of the sign of the cross-relaxation term.

For the cases considered by Kalk & Berendsen (1976) and by Campbell & Freeman (1973), this model would appear to be a reasonable representation of their systems. Indeed, Sykes et al. (1978) have recently substantiated many of the ideas of Kalk & Berendsen. However, we question the applicability of such a model for hydrated lysozyme powders (Hilton et al., 1977) and lysozyme crystals (Hsi & Bryant, 1977a). Finally, Eisenstadt & Fabry (1978), who demonstrated cross-relaxation between solvent protons and hemoglobin molecules, treat cross-relaxation using the model used by Kalk & Berendsen (1976) which, we argue, is inappropriate for protein solutions. Nonetheless, they are able to observe both δS and δP, and these data, though limited to one value of magnetic field, supplement our present work very nicely in that they substantiate the predictions of our model regarding the complex decay of δP (cf. Figure 8).

The major differences in the underlying physical basis between the model used, for example, by Kalk & Berendsen (1976) and that used here are: (1) in our model there is no a priori relation that can be established between the magnitudes of R_T and either R_S or R_P, whereas in their model the three rates come directly out of the single expression for the dipolar interaction between the spin pairs; (2) in our case, R_T is found to disperse very much like R_S, whereas any model which derives R_T from the dipolar interaction that gives rise to R_S and R_P must give an R_T that disperses much differently from R_S; and (3) in our systems (i.e., in protein solutions, and probably in tissue and crystalline matrices as well), the magnetization of each part of the system is uniform and becomes established in a time short compared with R_T. In the solvent phase, as noted, internal equilibrium is established rapidly by spatial diffusion of solvent molecules. In the protein phase, which remains to be discussed, the internal equilibrium is established by spin diffusion. Thus our model represents two homogeneous thermodynamic systems in thermal contact, each with uniform magnetization and each therefore characterizable by its own temperature. The (positive) sign of the cross-relaxation must be imposed such that the cooler system cools the hotter one. The model used by Kalk & Berendsen (1976) represents a single homogeneous thermodynamic system of spin-pairs, and therefore thermodynamics puts no limitation on the sign of the cross-relaxation interaction.

The Intrinsic Solvent Relaxation Rate R_S. It is well-established that the dispersive behavior of R_S results from a protein–solvent interaction that conveys to the solvent molecules information regarding the rotational Brownian motion of the solute macromolecules (at least for A, the dominant dispersive term in eq 1). In essence, in the presence of solute protein, the angular orientation–autocorrelation function of the solvent molecules gains a small additional term with a correlation time equal to the orientational relaxation time of the protein molecules. Put more descriptively, in the presence of solute protein, the rotational Brownian motion of the solvent molecules, which normally has a fast component that makes the angular orientation of the solvent molecules average out to be isotropic in a very short time (of order 10^{-11} s), now has a small bias that makes the average motion follow the much slower (of order 10^{-7} s) rotational motion of the protein molecules. It is the Fourier spectrum of this Brownian motion that causes relaxation. Though the phenomena are well-documented (cf. Hallenga & Koenig, 1977), the mechanism of interaction is not understood. A simple picture of two-site exchange has been shown experimentally to be invalid (Koenig et al., 1975), and it has been suggested that the interaction involves a subtle, long-range hydrodynamic effect (Hallenga & Koenig, 1977). However, attempts to demonstrate this interaction from fundamental theoretical considerations of the statistical mechanics of mixed fluids (Montgomery & Berne, 1977; Montgomery et al., 1977; Wolynes & Deutch, 1977) have not been successful.

Deuteron relaxation is known to arise from the interaction of the detueron electric quadrupole moment with the gradient of the internal electric field at the positions of the deuterons in the solvent molecules. This interaction is so much stronger than the intramolecular solvent proton–deuteron interaction (and the proton–proton interaction, as well) that nearby protons cannot observably influence the deuteron relaxation rates. Rather, it is solely contributions to the dynamic history of the solvent molecules as influenced by the presence of solute protein that enhance the relaxation rate of solvent deuterons and produce its dispersion (in the diamagnetic systems considered here). Because of this, the deuteron dispersion should be in-

TABLE III: The Net Enhancement (NE $\equiv AT_{1w}/[C]$) of the Relaxation of Deuterons Compared with NE and $R_S T_{1w}/[C]$ for Protons for the Samples and Conditions Indicated.

protein	temp (°C)	nucleus	NE (M^{-1})	$R_S T_{1w}/[C]$ (M^{-1})
carbonic anhydrase B	5	^1H	1090	890
		^2H	750[a]	
hemoglobin A (CO)[b]	1	^1H	2600	1800
	25	^1H	2500	1900
	25	^2H	1900	
alkaline phosphatase	5	^1H	1700	1300[c]
	5	^1H	1700	1500
	5	^2H	690	

[a] A value for A was obtained assuming $D = 0.1 \times A$ since D could not be obtained for the deuteron dispersion (see text). [b] The data for hemoglobin are from Hallenga & Koenig (1976) and from associated data not included in that publication. [c] For the 0.6 mM sample.

dependent of solvent deuteron fraction, as observed (Figures 3 and 6). Finally, the absence of dependence of the deuteron relaxation rates on deuteron fraction shows that exchange with surface imino groups, as discussed by Kimmich (1977), plays no role here.

The net enhancement (NE) of the relaxation rate may be defined, for both protons or deuterons, in terms of the parameters of eq 1 as

$$NE = AT_{1w}/[C] \tag{15}$$

where [C] is the molar concentration of protein, and $1/T_{1w}$ is the appropriate nuclear relaxation rate in protein-free buffer. Values of NE for several protein solutions are given in Table III. In the absence of cross-relaxation effects, and assuming no special average alignments (no anisotropy of tumbling) of the solvent molecules with respect to the protein molecules, the NE of protons and deuterons should be essentially the same, for all values of solvent proton fraction. This is because protons are relaxed mainly by intramolecular magnetic dipolar interactions with their neighbor on the same water molecule, an interaction that has the same symmetry as the deuteron quadrupolar interaction. (This argument does not quite hold for a smaller translational contribution to proton relaxation, neglected in this discussion.) The NE for solvent protons will then depend only on the dynamic history of the solvent protons, as is the case for deuterons. (As the solvent proton fraction is decreased, both A and $1/T_{1w}$ would both decrease, but their ratio would remain fixed.) The NE for protons is compared with that for deuterons in Table III, and it is seen that the values for protons are systematically greater than for deuterons. This is another way of exhibiting the cross-relaxation contribution to solvent relaxation rates that must be considered for protons.

The last column in Table III lists the values of R_S for protons (from Table II) divided by $1/T_{1w}$ and [C]; these entries should approximate the NE values for protons with cross-relaxation effects removed. It is seen that these values compare well with NE for deuterons, for both carbonic anhydrase and hemoglobin, but not for alkaline phosphatase. The cross-relaxation interactions are less for the first two proteins, as judged both from the values of R_T and R_S, Table II, and from the fact that the forms of the proton dispersion curves of alkaline phosphatase do vary with solvent proton fraction. Thus, whereas we do not know the mechanism by which solute protein enhances the relaxation rates of solvent nuclei, we have demonstrated quite clearly that there are similar dynamic contributions to both solvent proton and deuteron relaxation rates, plus an additional contribution (of magnitude comparable) to the proton relaxation rate. This contribution arises from cross-relaxation between protein and solvent protons.

The Proton Cross-Relaxation Rate R_T. From Table II, it is seen that the magnitudes of R_T and R_S at zero field are about equal for each of the three different proteins, and that they vary similarly with temperature, protein concentration, and protein molecular weight. Moreover, as emphasized earlier, R_T and R_S disperse similarly with magnetic field. Though understanding of the microscopic processes that determine the magnitude of R_T is secondary to the main purpose of the present work, which is to establish the existence of R_T and estimate its magnitude for a range of sample parameters, nonetheless, it is constructive to conjecture what the responsible underlying physical mechanism might be.

It is known that R_S does not arise from tightly bound or strongly hindered water molecules that exchange with solvent (Hallenga & Koenig, 1976; Koenig et al., 1975) since the majority of the waters of hydration are too mobile and exchange too rapidly to convey to the solvent the long orientational relaxation time of the protein molecules. Moreover, titratable protons on ionizable groups on the surfaces of the protein molecules were shown to exchange too slowly to contribute observably to solvent relaxation if they were about as near to other protein protons as the interproton distance (1.5 Å) in water (Koenig & Schillinger, 1969). However, if these protons are significantly farther from nonexchangeable protein protons, then the earlier arguments fail, and this smaller proton–proton interaction may give rise to R_T.

Unlike R_S, R_T involves mechanisms that must occur at the solvent–protein interface. If R_T is ascribed to dipolar interactions between titratable protons and nonexchangeable protons within the protein, say about 2.5–3 Å distant, then the net interaction per proton (which varies as the inverse sixth power of distance) is smaller by a factor 25 to 100 than the interproton interaction in a water molecule bound to the protein; therefore, to obtain a value of R_T about equal to R_S, the number of such exchangeable protons would have to approximate the number of water molecules in the first hydration shell (cf. Koenig & Schillinger, 1969), which is indeed the case for protein surfaces. The smaller interaction of a larger number of protons would obviate the problems associated with exchange time (Koenig et al., 1975), and could produce a cross-relaxation rate that would satisfy, at least qualitatively, the observed properties of R_T, and in particular its dispersive properties. The above, then, is a suggested mechanism for the source of R_T. Implicit in the mechanism is a pH dependence of R_T; it would probably be minimum at a pH between neutral and the isoelectric point of the protein, and increase toward either pH extreme much like the protein titration curve. Data showing an otherwise unexplained increase in A toward high pH for carbonic anhydrase solutions, with no variation of ν_c (Wells et al., 1977), could possibly be due to this suggested cross-relaxation mechanism.

Finally, there is another interaction that may contribute to R_T so as to alter the shape of the dispersion curve by increasing R_T in a broad region around 4 MHz, as is observed for the alkaline phosphatase samples. The effect would result from interactions of solvent protons with protein nitrogen nuclei, which have quadrupolar splittings of about 4 MHz. The result would be analogous to that considered by Voigt & Kimmich (1976) at higher fields in solutions of chlorine-containing polymers. This has yet to be investigated.

The Intrinsic Solute Proton Relaxation Rate R_P. The

spin–lattice relaxation rates of protons in solute protein, particularly at the lower values of magnetic field considered here, have not been measured. However, one estimate of the rates can be obtained from data for the spin-spin relaxation rates ($1/T_2$) of protons in immobilized proteins. For example, Edzes & Samulski (1977) find a spin–spin relaxation rate of 6×10^4 s^{-1} for protons in solid collagen. Krüger & Helcké (1967) find a rate $\simeq 5 \times 10^4$ s^{-1} in lysozyme crystals, and Forster et al. (1976) find (and quote others as finding) values of about 5×10^4 s^{-1} for $1/T_2$ in various tissues. These are "solid-state" rates and indicate the full strength of the proton–proton interactions in the protein; for proteins in solution, the rate would be "motionally narrowed" (cf. Solomon, 1955) by a factor of (approximately) the product of this rate and the orientational correlation time of the protein. The result, taking 5×10^{-8} s as a representative correlation time, is 180 s^{-1}. Because the proteins are in solution, $1/T_1$ would equal this value at zero field; thus we obtain an estimate for the zero-field value of R_P of 180 s^{-1}, or a T_1 of about 6 ms. Another estimate may be obtained from the work of Sykes et al. (1978), who report values of T_2 for protein protons that cluster around 10 ms at high fields. Dividing by three to approximate the zero-field value gives a T_1 of about 3 ms.

A similar estimate may be obtained by quite independent considerations. We assume the protein protons to be uniformly distributed with a concentration of 100 M, about the same as for water. The average proton–proton separation is then 2.5 Å which, when compared with the 1.5-Å separation of the two protons in a water molecule, gives a pair interaction lower by a factor $(2.5/1.5)^6 \simeq 20$. On the other hand, the orientational relaxation time for the protein is of order 5×10^{-8} s, to be compared with 5×10^{-12} s for a water molecule at 25 °C; this difference increases the relaxation rate significantly. Taking the value 4 s for T_1 protons in pure water and applying the above correction factors, and another factor of 6 to account (approximately) for the number of equivalent near-neighbors of a protein proton, gives a value for T_1 of the proton proteins at zero field of 2 ms.

The estimates of 2, 3, and 6 ms (we will use the average, 4 ms) together with the proton-proton separation of 2.5 Å allows us to estimate the spin diffusion constant $D_P \simeq (2.5 \times 10^{-8})^2/4 \times 10^{-3} = 1.5 \times 10^{-13}$ cm^{-2} s^{-1}. In a time of order 0.1 s, the protein magnetization can diffuse about $\sqrt{(1.5 \times 10^{-13} \times 10^{-1})}$ cm, or about 12 Å, which is about the radius of a small protein molecule.

Thus, from the above, it is seen that in a time somewhat less than the observed solvent relaxation time in a typical experiment, the proton magnetization in the protein molecules can almost diffuse from the inside to the surface of the molecules. This is relevant to our assumption, made earlier, that the protein molecules could be considered as a thermodynamic system in internal equilibrium, interacting with solvent across the protein–solvent interface. The estimated values of R_P and D_P show that this assumption is not too bad, but suggests that there is an internal bottleneck in the transport of protein magnetization to the protein surface. This could be the reason that the derived values of R_P in Table II, though they are estimates that depend on the value of M chosen, are systematically less (by an order of magnitude) than the rate 250 s^{-1} estimated above.

A remark is in order concerning the relation between the value of R_P at high fields, at low fields, and the role of methyl protons in the protein. The high-field situation has been considered in some depth by Kalk & Berendsen (1976) for proteins in solution, high being defined such that the proton Larmor frequency is well above the orientational relaxation rate of the protein molecules, and comparable (within an order of magnitude) to the orientational relaxation rate of methyl groups about their axes of symmetry. In this situation, the spin–lattice relaxation rates for all protein protons except the mobile methyl protons have dispersed away; relaxation of a typical protein proton then occurs by diffusion of its magnetization to the methyl protons. These protons, which have relatively short T_1 values, therefore serve as sinks for the magnetization of the protein molecules. Experimental justification for this process can be found, for example, in the early work of Connor (1966) on polyethylene oxides with methyl end groups, in the work of Andrew et al. (1976) which shows that proton spin–lattice relaxation times are very long in solid polyamino acids which contain no methyl groups and short otherwise, and in the work of van Putte (1971) which demonstrates the existence of spin diffusion from methylene to methyl protons in solutions of partially deuterated ketones. However, these high-field results do not apply directly to the present work since, at low fields, the spin–lattice relaxation rates would not be dispersed away and all solute protons can be expected to relax at comparably rapid rates.

Summary

We have demonstrated that the dispersion of the spin–lattice relaxation rate of solvent protons in protein solutions contains a contribution from cross-relaxation processes between solvent and solute protons. This contribution is not simply additive; rather, the solute and solvent must be regarded as two thermodynamic systems of comparable scale interacting in a manner such that the dynamics of the magnetization of one system influences the dynamics of the other. We have developed a heuristic model with three parameters that fits the many experimental observations very well. What is new is the finding that R_T, the cross-relaxation rate, disperses as a T_1 process, and not as a T_2 process as has been considered by others. We suggest that R_T may arise from dipolar interactions between solvent protons and nonexchangeable protein protons reasonably distant from exchangeable protons on titratable groups on the surfaces of the protein molecules.

Though the experiments only measure the behavior of solvent protons, the model predicts that the relaxation behavior of protons in the protein will be influenced by cross-relaxation and therefore by the proton–deuteron ratio of the solvent. The implications of these results are particularly germane to the interpretation of the relaxation behavior of water protons in tissue and protein protons in solution. With regard to the latter, it should be noted that high resolution nuclear magnetic resonance measurements of proteins are traditionally done using deuterated solvents. We would expect that the relaxation rates of the protein protons would be a function of the solvent isotopic composition.

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