

- Keniry, M. A., Gutowsky, H. S., & Oldfield, E. (1984) *Nature (London)* 307, 383-386.
- Kinsey, R. A., Kintanar, A., & Oldfield, E. (1981a) *J. Biol. Chem.* 256, 9028-9036.
- Kinsey, R. A., Kintanar, A., Tsai, M.-D., Smith, R. L., Janes, N., & Oldfield, E. (1981b) *J. Biol. Chem.* 256, 4146-4149.
- Laemmli, U. K. (1970) *Nature (London)* 227, 680-685.
- Leo, G. C., Colnago, L. A., Valentine, K. G., & Opella, S. J. (1987) *Biochemistry* 26, 854-862.
- Lewis, B. A., Harbison, G. S., Herzfeld, J., & Griffin, R. G. (1985) *Biochemistry* 24, 4671-4679.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem.* 193, 265-275.
- Lozier, R. H. (1982) *Methods Enzymol.* 88, 133-162.
- Marque, J., Kinoshita, K., Govindjee, R., Ikegami, A., Ebrey, T. G., & Otomo, J. (1986) *Biochemistry* 25, 5555-5559.
- Martin, M. L., Delpuech, J.-J., & Martin, G. J. (1980) *Practical NMR Spectroscopy*, Heyden, London.
- Oesterhelt, D., & Stoerkenius, W. (1971) *Nature (London), New Biol.* 233, 149-152.
- Oesterhelt, D., & Stoerkenius, W. (1974) *Methods Enzymol.* 31A, 667-678.
- Renthal, R., Dawson, N., Tuley, J., & Horowitz, P. (1983) *Biochemistry* 22, 5-12.
- Stoerkenius, W., & Bogomolni, R. A. (1982) *Annu. Rev. Biochem.* 52, 587-616.
- Stoerkenius, W., Lozier, R. H., & Bogomolni, R. A. (1979) *Biochim. Biophys. Acta* 505, 215-278.
- Thiault, B., & Mersseman, M. (1975) *Org. Magn. Reson.* 7, 575-578.
- Wallace, B. A., & Henderson, R. (1982) *Biophys. J.* 39, 233-239.
- White, R. H., & Rudolph, F. B. (1978) *Biochim. Biophys. Acta* 542, 340-347.
- Wray, W., Bouliskas, T., Wray, V. P., & Hancock, R. (1981) *Anal. Biochem.* 118, 197-203.

Line-Shape Analysis of NMR Difference Spectra of an Anti-Spin-Label Antibody[†]

Tom Frey,[‡] Jacob Anglister,[§] and Harden M. McConnell^{*‡}

Stauffer Laboratory for Physical Chemistry, Stanford University, Stanford, California 94305, and Polymer Department, The Weizmann Institute of Science, Rehovot 76100, Israel

Received October 14, 1987; Revised Manuscript Received March 14, 1988

ABSTRACT: Specifically deuteriated Fab fragments of the anti-spin-label antibody AN02 were prepared. NMR difference spectra were obtained, in which the spectrum of Fab with some fraction of the binding sites occupied with spin-label hapten was subtracted from the spectrum of Fab with no spin-label. The peak heights were analyzed as a function of the fractional occupation of the binding site, using a computer program that calculates a best fit to the observed spectra. This method treats all of the peaks in the spectra simultaneously. Analyzing all peaks at once allows for the interdependencies in the spectra arising from overlap of positive and negative signals from different peaks. The fitting program calculates line widths for the peaks arising from protons in the binding site region. Almost all of the line widths calculated for the spectrum of the Fab complex with diamagnetic hapten dinitrophenyldiglycine were found to be narrower than the line widths of the corresponding resonances in the spectrum of Fab with an empty binding site. The distances of the binding site region protons from the unpaired electron of the hapten were also obtained from this calculation. Two tyrosine protons were found to be close ($<7 \text{ \AA}$) to this electron. These line-width and distance results are discussed with respect to the structure and dynamics of the antibody binding site.

In an effort to improve the understanding of antibody binding site structure, we have pursued an NMR study of the anti-spin-label antibody AN02. In previous work, we used biosynthetic deuteration and protein chemistry with NMR to obtain information about the amino acid makeup of the binding site region (Anglister et al., 1984a; Frey et al., 1984) and to assign resonances to the heavy and light chains (Anglister et al., 1985). Nuclear magnetization transfer was used to identify the resonances of protons in close proximity to the hapten (Anglister et al., 1987). The effect of the paramagnetic hapten on the protein NMR spectrum was used to measure the distance of certain antibody protons from the unpaired electron of the hapten (Anglister et al., 1984b).

The effect of the hapten on the AN02 resonance spectrum was described, and a technique for extracting distance information was discussed. This technique involves titrating the binding site of the Fab with paramagnetic hapten and sub-

tracting the NMR spectrum taken at each titration point from the spectrum of the Fab with no hapten bound. The change in peak height of each resonance was analyzed as a function of fractional occupation of the binding site. Several tyrosine resonances displayed the theoretically expected behavior.

The technique presented in our earlier paper analyzed one signal at a time, required that the line width of the proton resonance be known, and assumed that the resonance signals were homogeneous and Lorentzian. Of course, this is not satisfactory for spectra with multiple, overlapping resonances. Further difficulties arise with line-width determination when resonances are very broad or very narrow, due to low signal to noise and digitization, respectively. In the present work, we find that simulation and computer fitting of the observed spectra can be used successfully to extract the desired line-width and distance information.

MATERIALS AND METHODS

The preparation and purification of the AN02 antibody have been described. Fab fragments were prepared and purified as previously described. The affinity of AN02 for spin-label

[†] This work was supported by ONR Contract N00014-86-K-0388.

[‡] Stanford University.

[§] The Weizmann Institute of Science.

has been reported to be $4 \times 10^6 \text{ M}^{-1}$ (Anglister et al., 1984a).

The Fab fragments used for the spectra in this paper were biosynthetically deuteriated in the tryptophan and phenylalanine residues, and in the 2- and 6-positions of the tyrosine rings. The only proton resonances seen in the spectral region of 6.0–9.0 ppm are therefore from the 3- and 5-protons of tyrosine and possibly from histidine. We have shown that histidine makes at most only a minor contribution to any of these difference spectra (Anglister et al., 1984b).

Three types of difference spectra were obtained. In the first type, the spectrum of Fab with spin-label bound was subtracted from the spectrum of Fab with an empty binding site. In the second type, the spectrum of Fab with spin-label bound was subtracted from Fab with dinitrophenyldiglycine (DNP) bound. In the third, the spectrum of Fab with spin-label bound was subtracted from that of Fab with chemically reduced spin-label bound. (In the reduced spin-label, the NO of the nitroxide group is replaced by the hydroxylamine group, NOH.) The chemical shifts of Fab proton resonances seen in this third type of spectrum were assumed to correspond to the chemical shifts in the Fab spin-label complex.

Two types of difference spectra titrations were recorded. In the "without minus with" titration, one subtracts the NMR spectrum of an Fab fragment in a given solution with spin-label from the spectrum of Fab in the absence of spin-label. Different difference spectra are recorded for different spin-label concentrations, corresponding to different fractional occupations of the combining site. In the "with DNP minus with spin label" titrations, one subtracts the NMR spectrum of an Fab fragment in a solution containing both spin-label and DNP-diglycine from the spectrum of a solution of Fab with (excess) DNP-diglycine from the spectrum of a solution of Fab with (excess) DNP-diglycine alone. The relative concentrations of spin-label and DNP-diglycine, together with their known binding constants, determine the fractional occupation of the binding site by each hapten. This relative fractional occupation is varied during the "with DNP minus with spin label" titration. Note that this second titration is distinct from that used earlier (Frey et al., 1984).

NMR spectra were simulated on an IBM-PC. Broadening due to the spin-label was described by the equations for paramagnetic broadening (Sternlicht et al., 1965a,b; Jardetzky & Roberts, 1981), giving an r^{-6} dependence for the effect (Solomon, 1955). Spectra for partially occupied situations were assumed to conform to the Bloch equations for one-proton, two-site exchange with an appropriate exchange rate (Gutowsky et al., 1953; McConnell, 1958). Rates found for these experiments are in a time regime intermediate between fast and slow exchange. Initial estimates for spectral parameters were improved by the Marquardt algorithm using a modification of the CURFIT routine of Bevington (1969). The programs were written in C, with time critical portions recoded in 8088 and 8087 assembly language, based on the assembly language output of the C compiler (Computer Innovations) [for further details, see Frey (1986)]. The spectra were digitized at 1-Hz intervals.

RESULTS

The best fit to the data uses an off rate of 430 s^{-1} . This gives a total squared deviation from the data of about 0.25% of the total squared amplitude. This fit is an order of magnitude better than when all distances are set equal to 10 Å and when the best-fit line widths are used. The value of 0.25% is probably set by the noise in the spectra. A further source of error may be the very low amplitude features that appear in

the high-occupancy difference spectra and that are ignored by the fit.

The fitting algorithm used finds the parameters giving a minimum in the sum of the squares of the deviations of the calculated amplitudes from the observed data. The only free parameters in the model are the line width of the signal in the Fab with an unoccupied binding site, the line width of the same resonance in the with DNP complex, and the distance of the proton from the unpaired spin in the with spin-label complex. Thus, there are 3 parameters per proton to be fit in each data set (30 for the data set discussed in this paper). There is one observed amplitude per peak per titration point in the data set (108 points). Note that peak B is a composite of 2 signals (Anglister et al., 1985) and there are therefore 9 peaks and 10 protons. The fit is still overdetermined even for this many parameters, but the derived values for the two signals from peak B are given with less confidence. Further complicating the interpretation of peak B is the possibility of a third signal forming a shoulder on the high chemical shift side in difference spectra with high spin-label occupancy.

Some assumptions are implicit in the analysis of these NMR spectra. The scaling of the spectra assumes that at least one line width in one spectrum can be measured accurately. For the present calculations, the lines labeled H and G in the without minus with 100% spin-label spectrum were used for scaling all of the without minus with spin-label spectra. These special peaks were selected on the basis of the following criteria. (i) The location of the base line is clear. In these difference spectra, the summing of negative signals in regions with many resonances can affect the shape of the base line even when any one of these negative signals is too broad to make a noticeable effect. (ii) There is no overlap with other resonances. (iii) The protons are sufficiently near the unpaired electron that the contribution of the negative signal in the difference spectrum is completely obliterated at 100% occupancy. (Note that when proton signals of different width are subtracted the width at half-height of the resulting feature is not the line width of either proton signal.) (iv) The lines are narrow enough to provide good signal to noise but broad enough to provide enough data points to minimize error from the discontinuous nature of the digital spectrum. The measured line widths should also yield relative peak amplitudes that are consistent with those observed in the spectra. Using similar criteria, we used line H to scale the with DNP minus with spin-label titration.

A second set of assumptions concerns the spectral position of resonances in the Fab spin-label complex. The chemical shifts of several tyrosine resonance change with the occupation of the binding site. Since these resonances are extremely broad in the spectrum of the Fab spin-label complex, it is not possible to determine the chemical shift directly. These shifts are therefore assumed to be the same as those observed in the complex with reduced spin-label. The chemical shifts of resonances in the reduced spin-label complex are very similar to those in the DNP complex, and so this likely to be a good approximation (Anglister et al., 1985).

Another question arises when treating the with DNP minus with spin-label spectra. The system was analyzed as a two-state system involving only the DNP complex and the spin-label complex. The concentrations of DNP and spin-label were kept high enough to saturate all the binding sites, and the concentration of free Fab was high enough to make the concentration of free Fab negligible. The Appendix shows that under these conditions a three-state NMR line-shape problem is reduced to the two-state treatment used.

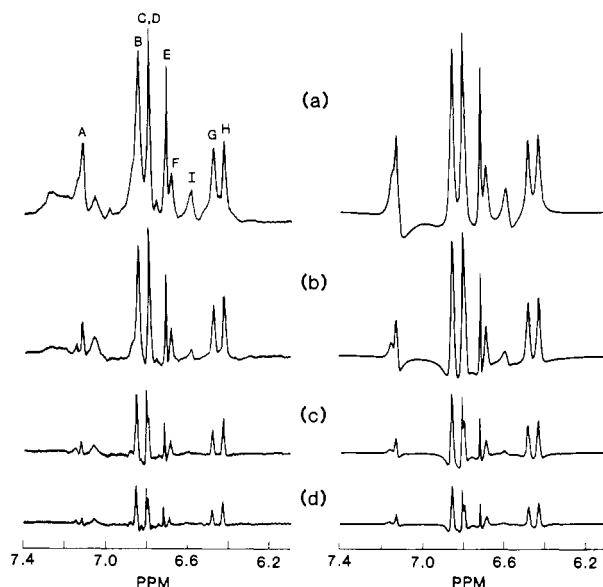


FIGURE 1: Difference spectra of without spin-label minus with spin-label. The spectra at the right are simulations, and the spectra at the left are the observed spectra. The binding site occupancies with spin-label are (a) 100%, (b) 20.03%, (c) 4.96%, and (d) 2.57%.

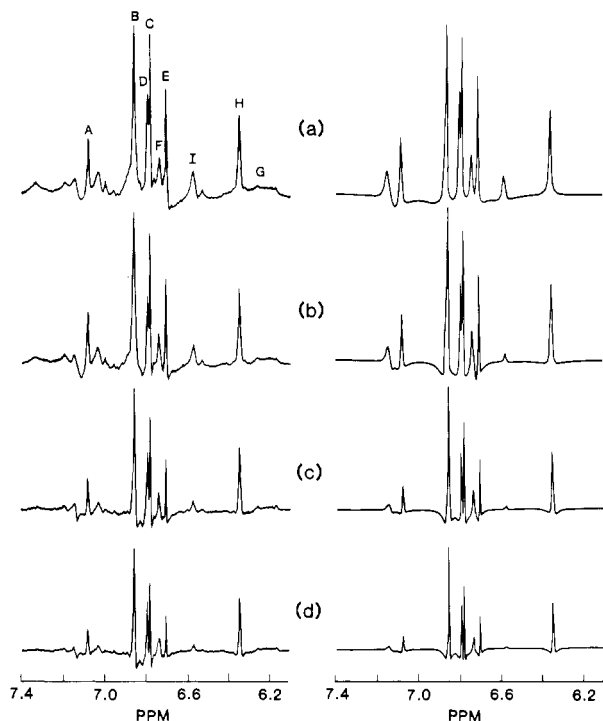


FIGURE 2: Difference spectra of with DNP minus with spin-label. The spectra at the right are simulations, and the spectra at the left are the observed spectra. The binding site occupancies with spin-label are (a) 100%, (b) 25.2%, (c) 7.52%, and (d) 3.9%.

Figure 1 shows the simulated and observed spectra for several of the difference spectra in the without minus with spin-label titration. All spectra are to the same scale. Figure 2 shows results for the with DNP minus with spin-label titration. The spectra in Figures 1 and 2 are to different scales. Only four of the six recorded spectra in each data set are shown. The spectra for the third and fifth highest occupancy are omitted in each case.

The changes in chemical shift that occur on binding can be observed by comparing Figure 1a and Figure 2a. These changes make the amplitudes of pairs of lines interdependent in several cases. The lines labeled C and D are very close to each other, and the negative signals from each of these protons

Table I: Spectral Parameters for without minus with Spin-Label Titration

proton	chemical shift ^a (ppm)	line width ^a (Hz)
A	7.126	9.4
B1	6.864	6.9
B2	6.857	7.7
C	6.810	5.1
D	6.799	8.4
E	6.722	4.8
F	6.693	12.1
G	6.487	10.8
H	6.435	10.2
I	6.595	17.0

^a The chemical shifts and line widths individually do not have any significance beyond ± 0.01 ppm and ± 1 Hz. The shapes of overlapping signals are more sensitive to the relative values of line positions and widths than these limits, as indicated by the number of significant figures used in the table.

Table II: Spectral Parameters for with DNP minus with Spin-Label Titration

proton	chemical shift ^a (ppm)	line width ^a (Hz)
A	7.082	5.8
B1	6.865	6.0
B2	6.865	2.9
C	6.784	2.8
D	6.795	4.2
E	6.706	3.3
F	6.738	8.25
G	6.135	br ^b
H	6.349	5.15
I	6.575	11.0

^a The chemical shifts and line widths individually do not have any significance beyond ± 0.01 ppm and ± 1 Hz. The shapes of overlapping signals are more sensitive to the relative values of line positions and widths than these limits, as indicated by the number of significant figures used in the table. ^b Broad.

affect the other's amplitude in one or both of the titrations. The negative feature from line F moves through the position of the positive signal from line E in the without minus with spin-label titration, thus affecting its amplitude. The fitting algorithm allows for these interdependencies. It is more difficult to model the small signal at slightly higher chemical shift than line A and its effect on the observed amplitude of line A. This is probably not a tyrosine signal (or even necessarily from a single source). Both histidine and unexchanged peptide amide protons would show signals in this spectral region. Since the number of protons giving rise to this signal cannot be determined, it cannot be scaled properly with respect to the tyrosine signals in the calculation.

Table I lists spectral parameters derived from the without spin-label minus with spin-label spectra. The chemical shifts of the protein tyrosine resonances and the line widths derived from the best fit to the titration data are shown in the table. Table II shows results for the with DNP minus with spin-label difference spectra. Identical labels for the protons in the two tables indicated that the protons are from the same amino acid. The method used to interrelate signals in the two spectra has been discussed previous (Anglister et al., 1984). Note that the line widths of a given proton are not the same in the two types of spectra and are almost always narrower in the DNP complex. Examination of the spectra in Figures 1 and 2 shows that the line shapes calculated are in most cases in good agreement with the observed spectra, so that this difference in calculated line width is not likely to be an artifact of the different scaling of the two titrations.

This narrowing of line width in the with DNP spectrum is interesting. If some paramagnetic impurity is less able to penetrate the protein when hapten is bound, a narrowing effect

Table III: Results with Relevance to Model Building

proton	change in chemical shift (without spin-label to with DNP in ppm)	distance ^a (Å)	chain
A	0.044	12.9	H
B1	-0.001	12.3	H
B2	-0.001	<7	H
C	0.026	11.5	L
D	0.004	9.0	L
E	0.016	13.7	H
F	-0.045	10.85	L
G	0.352	ND ^b	L
H	0.086	<7	H
I	0.020	16.0	L

^a The calculated distances are derived from averages of the inverse sixth power of the electron spin-proton spin distance and may represent averages over different molecular conformations. ^b Not determined.

might be observed. We have accumulated spectra in the presence and absence of dissolved oxygen and see no difference in line width, ruling out the most likely candidate for the broadening agent. A second possibility is that there is more mobility for tyrosine residues not in contact with the hapten when hapten is bound. We have previously stated that the large number of resonances showing some change in chemical shift might indicate a significant change in average conformation. On the other hand, there is clearly an immobilizing effect on the tyrosine giving rise to resonance G, along with the very large change in chemical shift seen for this resonance.

Table III shows the change in chemical shift undergone by each Fab tyrosine resonance upon DNP binding. This table also shows the calculated distance of each of these protons from the paramagnetic center of the hapten. Distances of less than 7 Å cannot be resolved because of the magnitude of the off rate of the hapten (Anglister et al., 1984), and are all labeled as <7. The change in chemical shift and the distance from the spin are the two parameters with strongest implications for structural models of the antibody. An L or H is included in the last column to indicate a light- or heavy-chain origin for the resonance (Anglister et al., 1985). The distances given here are in agreement with those reported previously (Anglister et al., 1984), except for line D. The overlap in line D with line C is likely to be a source of error in the previous calculations.

The value for the distance of line G from the paramagnetic center could not be determined. This is due to the large change in chemical shift undergone by this signal during the without minus with spin-label titration. The negative signal from the partially occupied spectrum moves out from under the positive signal at very low occupancy and is exchange broadened independent of any paramagnetic effect. A distance value for protons giving rise to signal G cannot be obtained from the with DNP minus with spin-label titration due to the extremely poor signal to noise for this line caused by its large line width.

DISCUSSION

It can be seen from the simulated spectra (Figures 1 and 2) that the computer analysis used in this work gives a good approximation to the observed spectra. The simulation of the spectra allows the analysis of overlapping signals. The rate of appearance of a resonance in a titration is dependent on the line width, but line-width measurements are not very accurate when determined directly from the spectra. Spectral simulation forces the line widths to be mutually consistent. The derived line widths also provide a quantitative confirmation of the qualitative observation that resonances in the

DNP complex are narrower than in the free Fab, as discussed under Results.

The spectra discussed here yield results with structural implications. We have previously discussed a computer model of the antibody and used it to assign likely tryptophan residues to specific resonances (Anglister et al., 1987). The assignment of tyrosine resonances is not so straightforward. There are two heavy-chain tyrosines (33 and 50) which are candidates for signals B and H, but the large number of tyrosine residues makes this assignment very provisional. Further complicating the situation is the lack of an obvious candidate for resonance G. This is the tyrosine resonance that undergoes a large change in chemical shift upon hapten binding. One reason for the lack of a candidate is that the model was computed for an empty binding site, and the change in chemical shift could be due to conformational change in the Fab. There are at least four light-chain tyrosine residues that could be candidates under these assumptions, including the three tyrosines in the second hypervariable loop of the light chain.

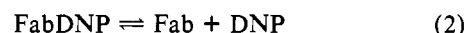
The type of analysis described here is being applied to titrations done with several different deuterations. It is hoped that a collection of these measurements will lead to an understanding of the solution structure of this antibody and, in conjunction with the crystallographic and mutagenesis studies under way, will lead to an improved understanding of antibody-hapten binding in terms of both structure and kinetics.

ACKNOWLEDGMENTS

We thank Dr. Michael Levitt for devising the computer model discussed in this paper.

APPENDIX

Magnetization Transfer Due to Chemical Exchange. The chemical components of the system are Fab, spin-label (SL), DNP-diglycine (DNP), and the complexes of Fab with spin-label (FabSL) and of Fab with DNP (FabDNP). Two reversible reactions are assumed:



Thus, we assume that FabSL can only be converted to FabDNP through the intermediate free Fab.

Let M_S be the macroscopic magnetization association with a specific proton in the Fab fragment when spin-label is bound and M_D be the magnetization of the same proton when DNP is bound. Let M_0 be the magnetization of this proton when the binding site is empty. The changes of the macroscopic magnetization due to chemical exchange can then be written as follows:

$$\dot{M}_S = -k_{S0}M_S + k_{0S}M_0 \quad (3)$$

$$\dot{M}_D = -k_{D0}M_D + k_{0D}M_0 \quad (4)$$

$$\dot{M}_0 = -k_{0S}M_0 - k_{0D}M_0 + k_{S0}M_S + k_{D0}M_D \quad (5)$$

where k_{S0} is the first-order rate constant for dissociation of the Fab-spin-label complex and k_{0S} is the pseudo-first-order rate constant for reassociation.

Assume experimental conditions where there is always an excess of spin-label and/or DNP such that the binding sites of the Fab fragments are almost always occupied. Under these circumstances, the concentration of free Fab is very small, and we may set $M_0 = 0$. We then obtain the following expression for M_0 :

$$M_0 = \frac{k_{S0}M_S + k_{D0}M_D}{k_{0S} + k_{0D}} \quad (6)$$

Substitution of this expression for M_0 in eq 3 and 4 allows these equations to be rewritten as

$$\dot{M}_S = -k_{SD}M_S + k_{DS}M_D \quad (7)$$

$$\dot{M}_D = -k_{DS}M_D + k_{SD}M_S \quad (8)$$

where

$$k_{SD} = \frac{k_{S0}k_{0D}}{k_{0S} + k_{0D}} \quad (9)$$

$$k_{DS} = \frac{k_{D0}k_{0S}}{k_{0S} + k_{0D}} \quad (10)$$

Thus, the equations describing the change of magnetization due to chemical exchange have the form of a two-site exchange reaction, as though the reaction



took place with rate constants k_{SD} and k_{DS} .

In the form of the Bloch equations described in Wien et al. (1972), the parameter τ is used, and for the present problem

$$\tau = 1/k_{SD} + 1/k_{DS} \quad (12)$$

In order to put this equation into useful form, we can express the time τ in terms of the true first-order off rate constants k_{S0} and k_{D0} and the fractional occupation of the binding site by spin-label and DNP, namely, f_S and f_D . To do this, first note that

$$k_{0S} = k'_{0S}[\text{SL}] \quad (13)$$

$$k_{0D} = k'_{0D}[\text{DNP}] \quad (14)$$

where k'_{0S} and k'_{0D} are the true second-order rate constants for the reverse reactions in eq 1 and 2. The equilibrium constant for reaction 1 is

$$K_S = \frac{[\text{Fab}][\text{SL}]}{[\text{FabSL}]} = \frac{k_{S0}}{k'_{0S}} \quad (15)$$

and for reaction 2

$$K_D = \frac{[\text{Fab}][\text{DNP}]}{[\text{FabDNP}]} = \frac{k_{D0}}{k'_{0D}} \quad (16)$$

By combining eq 15 and 16 with eq 12, we obtain the following expression:

$$\tau = \frac{1}{f_D k_{D0}} + \frac{1}{k_{S0} f_S} \quad (17)$$

or

$$\tau = \tau_{D0}/f_D + \tau_{S0}/f_S \quad (18)$$

where τ_{D0} and τ_{S0} are the lifetimes for the two haptens in the combining site. (Note that for the two-site exchange case, where there is no DNP-diglycine, $\tau = 1/k_{S0}f_S$. The effect of the DNP-diglycine is effectively to slow down the rate of entry of the spin-label into the binding site.)

Registry No. DNP-glycine, 1084-76-0.

REFERENCES

- Anglister, J., Frey, T., & McConnell, H. M. (1984a) *Biochemistry* 23, 1138-1142.
- Anglister, J., Frey, T., & McConnell, H. M. (1984b) *Biochemistry* 23, 5372-5375.
- Anglister, J., Frey, T., & McConnell, H. M. (1985) *Nature (London)* 315, 65-67.
- Anglister, J., Bond, M. W., Frey, T., Leahy, D., Levitt, M., McConnell, H. M., Rule, G. S., Tomasello, J., & Whitaker, M. (1987) *Biochemistry* 26, 6058-6064.
- Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciences*, McGraw-Hill, New York.
- Frey, T. (1986) Ph.D. Thesis, Stanford University.
- Frey, T., Anglister, J., & McConnell, H. M. (1984) *Biochemistry* 23, 6470-6473.
- Gutowsky, H. S., McCall, D. W., & Slichter, C. P. (1953) *J. Chem. Phys.* 21, 279.
- Jardetzky, O., & Roberts, G. C. K. (1981) *NMR in Molecular Biology*, Academic, New York.
- McConnell, H. M. (1958) *J. Chem. Phys.* 28, 430-431.
- Solomon, I. (1955) *Phys. Rev.* 99, 559-565.
- Sternlicht, H., Shulman, R. G., & Anderson, E. W. (1965a) *J. Chem. Phys.* 43, 3133-3143.
- Sternlicht, H., Shulman, R. G., & Anderson, E. W. (1965b) *J. Chem. Phys.* 43, 3123-3132.
- Wien, R. W., Morrisett, J. D., & McConnell, H. M. (1972) *Biochemistry* 11, 3707-3716.