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Transfer-of-Saturation NMR Studies of Protein–Ligand Complexes. Three-Site Exchange

G. M. CLORE, G. C. K. ROBERTS, A. GRONENBORN, B. BIRDSALL, AND J. FEENEY

Division of Molecular Pharmacology, National Institute for Medical Research, Mill Hill, London NW7 1AA, United Kingdom

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Equilibrium and transient saturation transfer effects were calculated for a three-site exchange system involving a ligand binding to a protein to form a complex which can exist in two states. The calculations show that, in an experiment in which the change in intensity of a resonance of the free ligand on saturating the corresponding resonance from one of the states of the complex is monitored, substantial effects can be seen even when the form of the complex being saturated represents less than 0.1% of the total complex at equilibrium. The implications of this finding for experiments in which the saturation transfer effect is used to locate resonances of bound ligands are discussed. The time course of the change in intensity of the resonance of the free ligand on irradiation of the resonance of one of the states of the complex consists of two exponential phases, which offers a method for distinguishing two-site and three-site exchange, but it is shown that this will not be reliable in practice, since there are many circumstances under which the two phases will not be resolvable.

INTRODUCTION

Since its introduction in 1963 by Forsén and Hoffman (*1, 2*), the double-resonance transfer-of-saturation NMR experiment has been widely used to study relatively slow chemical exchange processes in organic, organometallic, and biological systems (*3–12*). Although the extension of this experiment to multisite exchange processes was described in 1964 (*13*), the vast majority of its applications have been to two-site systems. In the biological applications, exchange is often simply *assumed* to be a two-site process—e.g., for a ligand binding to an enzyme

$$E + L \rightleftharpoons EL, \qquad [1]$$

where E represents the protein and L the ligand. However, the existence of more than one form of the complex can rarely be excluded—particularly when the resonance of the bound ligand cannot be observed directly. This is a situation in which the saturation transfer experiment has been widely used (*8–11*) to locate these resonances by finding the irradiation frequencies at which transfer of saturation to the corresponding resonances of the free ligand is observed.

Our interest in the implications of multisite exchange processes for this application of the saturation transfer experiment has arisen from studies of the complex of dihydrofolate reductase with folate and $NADP^+$, where the existence of (at least)

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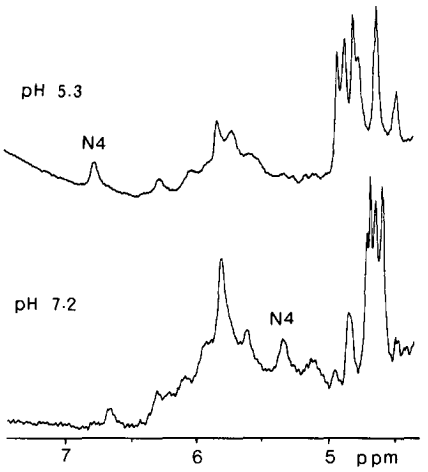


FIG. 1. A portion of the 270-MHz ^1H NMR spectrum of the *L. casei* dihydrofolate reductase–folate–$NADP^+_{d-6}$ complex at pH* 5.3 and 7.2. The signal labeled N4 is that of the nicotinamide-4-proton of the *bound* $NADP^+_{d-6}$. Enzyme, folate, and $NADP^+_{d-6}$ concentrations were each 1 m*M*, in ^2H_2O containing 500 m*M* KCl, 50 m*M* potassium phosphate, and 1 m*M* dioxane. The notation pH* denotes a meter reading uncorrected for the isotope effect on the glass electrode. The sample temperature was 11 (±1)°C. Twenty thousand transients were averaged, using quadrature detection with a spectral width of 4.2 kHz and an acquisition time of 0.513 sec. Before Fourier transformation the free-induction decay was multiplied by an exponential equivalent to line broadening by 2 Hz. Chemical shifts are given with respect to internal dioxane (3.71 ppm downfield from 2,2-dimethyl-silapentane-5-sulfonate).

two forms of the complex can be clearly demonstrated. Part of the 270-MHz ^1H spectrum of this complex is shown in Fig. 1. The signal labeled N4 is that of the 4-proton of the nicotinamide ring of the *bound* $NADP^+$; it has been assigned unequivocally by using [4-^2H-nicotinamide]$NADP^+$ (*14*). This resonance clearly has a strikingly different chemical shift at pH*[1] 5.3 (6.82 ppm) and pH* 7.2 (5.37 ppm). As the pH* is increased from 5.3 to 7.2, the intensity of the lower-field N4 signal decreases and that of the higher-field signal increases. These two resonances thus represent two different forms of the complex whose proportions are pH dependent (*14*). Exchange with free $NADP^+$ is slow, so that transfer of saturation experiments of the kind described above could be performed. Irradiation at 5.37 ppm produced a substantial saturation transfer effect on the N4 proton resonance of free $NADP^+$ throughout the pH range 5.3 to 7.2. By contrast, irradiation at 6.82 ppm had no effect at any pH in this range (*14*). Thus only one of these two states of the complex was detectable by saturation transfer and this state was readily detectable even at a pH where it represented less than 10% of the total complex present.

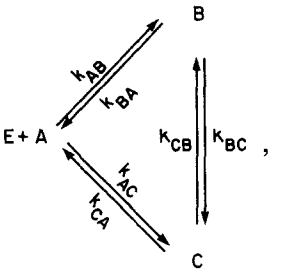
In this situation, the use of saturation transfer alone to locate the N4 proton resonance of the bound $NADP^+$ would clearly give misleading results. To understand the limitations imposed by the possibility of multisite exchange on this application of the saturation transfer experiment, we have examined the way in which

[1] pH* denotes a meter reading uncorrected for the isotope effect on the glass electrode.

the observed effects depend upon the rate constants and relative site populations in a three-site system.

THEORY

We consider a three-site exchange system defined by the scheme



[2]

where E and A are free enzyme and free ligand, respectively, and B and C are ligand bound in two forms of the complex. For the nucleus of interest A, B and C are magnetically nonequivalent states.

We define the equilibrium constants

$$K_1 = k_{AB}/k_{BA} = [B]/[A][E], \quad [3]$$

$$K_2 = k_{BC}/k_{CB} = [C]/[B], \quad [4]$$

$$K_3 = k_{AC}/k_{CA} = K_1 \cdot K_2 = [C]/[A][E], \quad [5]$$

$$K_{app} = ([B] + [C])/[A][E] = K_1 + K_3, \quad [6]$$

where K_{app} is the overall equilibrium constant between free and bound species which would be measured in an equilibrium-binding study. In addition, the pseudo-first-order rate constants, or fluxes, f_{AB} and f_{AC} are defined by

$$f_{AB} = k_{AB}[E] = k_{BA}[B]/[A], \quad [7]$$

$$f_{AC} = k_{AC}[E] = k_{CA}[C]/[A]. \quad [8]$$

The general form of the coupled differential equations describing saturation transfer in an n-site exchange system has been given by Forsén and Hoffman (*13*), based on McConnell's (*15*) modification of the Bloch equations. The equilibrium solution for a three-site system has been given by Perrin and Johnston (*4*). Since we are concerned with both the equilibrium and transient effects, we give the full equation for a three-site system (Scheme [2]) explicitly.

If a strong radiofrequency field is applied at $t = 0$ at the position of the resonance from site B in Scheme [2] so as to saturate it completely and instantaneously (or at least in a time short compared to t), then $M_B = 0, dM_B/dt = 0$. The magnetizations in A and C are described by the equations

$$dM_A/dt = (M_{A0} - M_A)\rho_A - M_A(f_{AB} + f_{AC}) + M_C k_{CA}, \quad [9]$$

$$dM_C/dt = (M_{C0} - M_C)\rho_C - M_C(k_{CA} + k_{CB}) + M_A f_{AC}, \quad [10]$$

where M_i and M_{i0} are the magnetization of site i at time t and at $t = 0$, respectively, and ρ_A and ρ_C are the spin–lattice relaxation rates of the nucleus in sites A and C. The solution of Eqs. [9], [10], describing the dependence of the magnetization in site A on the time t for which the resonance B is saturated, is given by

$$M_A(t) = M_{A0}\left\{\frac{a_2}{\gamma_1\gamma_2} - \frac{\gamma_1^2 - \gamma_1 a_1 + a_2}{\gamma_1(\gamma_2 - \gamma_1)}e^{-\gamma_1 t} - \frac{\gamma_2^2 - \gamma_2 a_1 + a_2}{\gamma_2(\gamma_1 - \gamma_2)}e^{-\gamma_2 t}\right\}. \quad [11]$$

The observed rate constants of the fast and slow phases of the decrease in magnetization, γ_1 and γ_2, respectively, are the roots (taken with negative signs) of the quadratic equation

$$\gamma^2 + \gamma(\rho_A + \rho_C + f_{AB} + f_{AC} + k_{CA} + k_{CB}) + (\rho_A + f_{AB}) \times (\rho_C + k_{CA} + k_{CB}) + f_{AC}(\rho_C + k_{CB}) = 0. \quad [12]$$

The amplitudes of the fast and slow phases are given by the preexponential terms of $e^{-\gamma_1 t}$ and $e^{-\gamma_2 t}$, respectively, the constants being given by

$$a_1 = \rho_A + \rho_C + k_{CA} + k_{CB} + k_{CA}M_{C0}/M_{A0}, \quad [13]$$

$$a_2 = (\rho_C + k_{CA} + k_{CB})\rho_A + \rho_C k_{CA} M_{C0}/M_{A0}. \quad [14]$$

We use the normalization $M_{A0} + M_{C0} = 1$, and since in addition $M_{A0}/M_{C0} = [A]/[C]$,

$$M_{A0} = [A]/([A] + [C]). \quad [15]$$

The normalized magnitude of the saturation transfer effect for $t \to \infty$ on the resonance of a site i, following saturation of the resonance of site j, $F_i(j)$, is defined by

$$F_i(j) = (I_{0i} - I_{\infty i})/I_{0i} \quad [16]$$

so that from Eq. [11]

$$F_A(B) = 1 - a_2/\gamma_1\gamma_2. \quad [17]$$

Calculations

In the calculations described below, we have examined the effect of varying the rate and equilibrium constants on the transfer of saturation to A on irradiating B. The equilibrium effect, $F_A(B)$, will be abbreviated as F_A. The parameter values used, chosen to be representative of those found in proton NMR experiments on protein–ligand systems, are given in Table 1. Of the four rate constants which enter into the calculations, three (f_{AB}, f_{AC}, and k_{CB}) were fixed in any one calculation; the fourth, k_{CA}, was calculated from

$$k_{CA} = \frac{f_{AC}}{[E]K_3} = \frac{f_{AC}(1 + K_2)}{[E]K_2K_{app}}. \quad [18]$$

It should therefore be noted that k_{CA} varies with K_2 (to maintain detailed balance), increasing with decreasing K_2 for $K_2 \leq 1$.

TABLE 1

VALUES OF THE PARAMETERS USED IN THE CALCULATIONS

Parameter	Value
A_T	$2 \times 10^{-3}\ M$
E_T	$1 \times 10^{-3}\ M$
K_{app}	$1 \times 10^{8}\ M^{a}$
ρ_A	$0.5\ sec^{-1}$
ρ_C	$10\ sec^{-1}$ (unless otherwise noted)
f_{AB}, f_{AC}, k_{CB}	0 to $100\ sec^{-1}$

[a] Essentially identical results were obtained with $K_{app} = 10^{5}\ M$.

RESULTS AND DISCUSSION

The general scheme for exchange of a ligand between free solution and two forms of a complex with a protein is shown in [2]. We assume throughout that, for the nucleus of interest, exchange between the bound and free states is slow, so that a saturation transfer experiment can be performed. If, in addition, exchange between the two forms of the complex is slow, the chemical shift of the nucleus of interest in these two sites can potentially be measured individually by a saturation transfer experiment. However, exchange with the third site (C) can have a substantial influence on the magnetization changes in the free state (A) produced by saturating the resonance from one of the bound states (B), and it is this effect with which we are concerned here. Since these magnetization changes depend in a rather complex way on the rate constants and relaxation rates, they can most readily be appreciated by numerical calculations of the saturation transfer effect as a function of the various parameters in Eq. [11].

It is useful to start with the simple two-site case for reference. Taking $\rho_A = 0.5\ sec^{-1}$, as for all subsequent calculations, the normalized magnitude of the saturation transfer effects, F_A, on resonance A when resonance B is saturated (note $F_A(B)$ is abbreviated as F_A throughout), are given in Table 2 for various values of f_{AB} in a two-site exchange system. In a system of type

$$E + A \underset{k_{BA}}{\overset{k_{AB}}{\rightleftharpoons}} B \qquad [19]$$

the A → B exchange is a second-order process so that f_{AB} is a pseudo-first-order rate constant, and will depend on the total concentrations of protein (E_T) and ligand (L_T)

$$f_{AB} = k_{AB}E_T/(L_T K + 1) \qquad [20]$$

(where $K = k_{AB}/k_{BA}$). For $L_T K \gg 1$ (which will usually be the case experimentally), f_{AB} is directly proportional to the ratio E_T/L_T; thus the value of F_A will decrease as the ratio of total protein to ligand concentration decreases.

For the three-site exchange process [2], the values of f_{AB} and f_{AC}, and hence F_A,

TABLE 2

DEPENDENCE ON THE FLUX f_{AB} OF THE NORMALIZED MAGNITUDE (F_A) OF THE SATURATION TRANSFER EFFECT ON SITE A WHEN SATURATING THE RESONANCE FROM SITE B FOR $t = \infty$ IN A TWO-SITE EXCHANGE SYSTEM

f_{AB} (sec^{-1})	F_A[a]
100	0.995
10	0.952
1	0.667
0.1	0.167
0	0

[a] For $\rho_A = 0.5$ sec^{-1}, calculated from $F_A = f_{AB}/(f_{AB} + \rho_A)$.

will clearly also vary with E_T/L_T in a similar way. To simplify the following discussion, we have held E_T/L_T fixed at 0.5, and kept f_{AB} and f_{AC} constant as a function of K_2 (allowing only k_{CA} to vary), thus eliminating any contribution from this population effect.

On considering the scheme for three-site exchange given by Eq. [2], it is apparent that a decrease in the intensity of resonance A on irradiating resonance B may

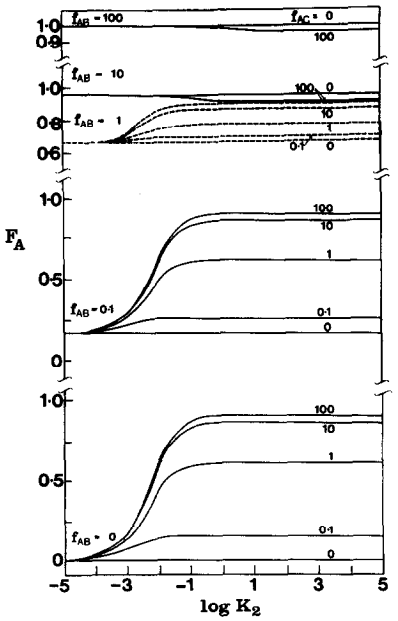


FIG. 2. The fractional change in intensity of the resonance of site A on saturation of that of site B (F_A) as a function of K_2 (=[C]/[B]). For all curves, $k_{CB} = 100$ sec^{-1}. Values of f_{AB} and f_{AC} (0 to 100 sec^{-1}) are indicated on each curve. (Dashed lines are used for $f_{AB} = 1$ sec^{-1} for clarity.)

occur in one of two ways:

(1) by the direct exchange process A → B

(2) by the indirect exchange process A ⇌ C → B via site C.

These two routes of exchange can be illustrated by the calculations shown in Figs. 2 and 3. In these figures we show the fractional change in intensity of the resonance of site A (F_A) on saturating the resonance of site B, as a function of K_2 = [C]/[B], for various values of f_{AB}, f_{AC}, and k_{CB}.

When the loss of magnetization from site A can only occur by the direct A → B exchange (i.e., $f_{AC} = 0$ sec^{-1}; see Fig. 2), the value of F_A is independent of K_2, is determined only by f_{AB} and ρ_A, and is identical to that for the two-site exchange system given in Table 2.

When the loss of magnetization from site A can only occur by the indirect A ⇌ C → B exchange (i.e., $f_{AB} = 0$ sec^{-1}; see Figs. 2 and 3) then as K_2 increases, the value of F_A increases, until $K_2 \geq 10$. For $K_2 \geq 10$ (i.e., more than 90% of the complex in form C), the fractional change in intensity of the free ligand signal, F_A, becomes independent of K_2. The larger the values of f_{AC} and k_{CB} compared to ρ_A, the larger the value of F_A at a given value of K_2. (Note that, for three-site as for two-site exchange, the magnitude of F_A is determined not by the absolute values of the exchange rates but rather by their values compared to the relaxation rate ρ_A.)

When f_{AB}, f_{AC}, and k_{CB} are all greater than zero, both direct and indirect exchange routes are possible. Then F_A depends not only on these rate constants but also on ρ_C, the spin–lattice relaxation rate of the nucleus in site C, since some of the magnetization being transferred by the indirect route from A to B is lost by spin–lattice relaxation in site C. If $k_{CB} = 100$ sec^{-1}, as in Fig. 2, then as f_{AC} increases so does F_A at values of $K_2 \geq 10^{-3}$, where the indirect exchange route can contribute. The only exception is for $f_{AB} \geq 10$ sec^{-1}, where the direct exchange is already very efficient,

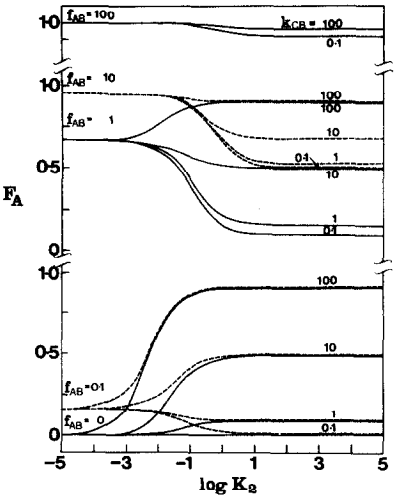


FIG. 3. The dependence of F_A on K_2 (as in Fig. 2). For all curves $f_{AC} = 100$ sec^{-1}. Values of f_{AB} and k_{CB} (0 or 0.1 to 100 sec^{-1}) are indicated on each curve. (Dashed lines are used for $f_{AB} = 10$ sec^{-1} for clarity.)

and the efficiency of the indirect route is limited by ρ_C. In the indirect route, exchange (k_{CB}) and spin–lattice relaxation (ρ_C) represent alternative fates for the magnetization in site C. Thus when $k_{CB} \leqslant \rho_C$ (see Fig. 3), exchange by the indirect route leads to a substantial loss of magnetization by relaxation and F_A decreases as K_2 increases (i.e., as the indirect route becomes more important).

In a simple two-site ligand–protein exchange, the observed transfer of saturation on irradiating the resonance of the bound ligand is of course independent of the spin–lattice relaxation rate of the nucleus in the bound state, and hence of the molecular weight of the protein–ligand complex. In the three-site case, however, the spin–lattice relaxation rate of the nonirradiated bound ligand nucleus (i.e., site C) does become important when exchange via the indirect route is significant. It therefore follows that under conditions where the indirect exchange route is important the change in intensity of the free ligand signal on irradiating resonance B for $t \to \infty$ will be least when $\omega_0^2 \tau_c^2 \sim 1$.

It is clear from the calculations summarized in Figs. 2 and 3 that a species of protein–ligand complex which has only a very small relative population can nevertheless manifest a large transfer of saturation to the free ligand, provided that *either* the direct or the indirect exchange route is efficient enough—that is, provided either f_{AB} is large, or f_{AC} and k_{CB} are large and ρ_C is small. On the other hand, the predominant species of complex can *fail* to show transfer of saturation if neither exchange route is efficient.

We can now reproduce qualitatively the observations on the dihydrofolate reductase–folate–$NADP^+$ complex described in the Introduction. We identify form B of the complex as that having a nicotinamide-4-proton resonance at 5.37 ppm, while in form C this resonance is at 6.82 ppm (cf. Fig. 1.). The equilibrium constant K_2 thus decreases from $\geqslant 10$ at pH* 5.3 to $\leqslant 0.1$ at pH* 7.2. Now if $f_{AB} = 20$ sec^{-1} (and $\rho_A = 0.5$ sec^{-1}), irradiation of the resonance from site B leads to a substantial saturation transfer effect on the resonance of the free ligand ($F_A(B) = 0.96$) which is independent of K_2 over the range 0.1 to 10. At the same time, if $f_{AC} = k_{BC} = 0.1$ sec^{-1}, there is no efficient route (either direct or indirect) for exchange between sites A and C and irradiation of the resonance from site C leads to a very small decrease in the intensity of resonance A ($F_A(C) \leqslant 0.075$), even when 90% of the complex is in form C.

It is obviously important, if errors in interpretation are to be avoided, to establish whether there is some feature of the saturation transfer experiment which can distinguish between two-site and three-site (or multiple-site) exchange when the resonances of the bound ligand cannot be observed directly. The most obvious such feature is the transient behavior—the way in which the intensity of the free ligand resonance varies with the time for which the resonance of the bound ligand is saturated—since, from Eq. [11], for three-site exchange this time course is in theory biphasic, whereas for two-site exchange (*1, 2*) it is a single exponential.

An example of the conditions under which this distinction can be made is shown in Fig. 4. Here $f_{AB} = 100$ sec^{-1}, and $F_A > 0.95$ for all values of K_2, f_{AC}, and k_{CB}. The kinetic behavior, however, depends markedly on the values of these parameters. Provided that f_{AC}, $k_{CB} \ll f_{AB}$ there is a "switch" in the kinetics as K_2 is increased, from exclusively slow phase ($Amp_2 = 1.0$) at $K_2 < 10^{-3}$ to a significant

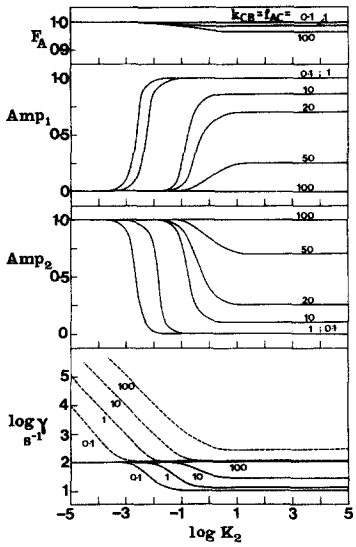


FIG. 4. Equilibrium and transient saturation transfer effects (on saturation of the resonance from site B) as a function of K_2 (=[C]/[B]). Top, the equilibrium change in intensity, F_A. Center, the amplitudes of the fast (Amp_1) and slow (Amp_2) kinetic phases. Bottom, the apparent rates of the fast (– – –) and slow (——) kinetic phases. For all curves, f_{AB} = 100 sec^{-1}. Values of $f_{AC} = k_{CB}$ are given on each curve.

contribution from the fast phase at $K_2 > 1$. The relative proportions of the two phases at $K_2 > 1$ depend upon the values of f_{AC} and k_{CB}, with an increasing proportion of the slow phase appearing as these parameters increase. Comparable amplitudes for the two phases (at $K_2 > 1$) are thus seen when the direct exchange route is dominant but there is a significant contribution from indirect exchange, and for the parameter values used in Fig. 4 the observed rates of these two phases will differ by up to a factor of 5. The existence of two phases should therefore be experimentally detectable under these conditions (f_{AB} large, $K_2 > 1$, and intermediate values of f_{AC} and k_{CB}). (It should be emphasized that, although the biphasic kinetics are most apparent when the two routes of exchange are contributing, the two phases of kinetics *cannot* be identified with these two exchange pathways because we are dealing with a coupled system.)

When the direct route of exchange dominates (f_{AB} = 100 sec^{-1}, $f_{AC} = k_{CB}$ = 0.1 sec^{-1}; see Fig. 4), the "switch" in kinetic behavior with increasing K_2 is still observed, but it is now complete so that Amp_2 = 1.0 for $K_2 < 10^{-3}$ and Amp_1 = 1.0 for $K_2 > 10^{-2}$. With the exception of this narrow range of K_2 values only a single kinetic phase will thus be observed, and in fact the observed rate will be essentially independent of K_2. (This is the situation for the parameter values used above to reproduce the behavior of the dihydrofolate reductase–folate–$NADP^+$ complex.)

When, on the other hand, the indirect route of exchange dominates ($K_2 > 1$, f_{AC}, $k_{CB} \gg f_{AB}$) biphasic kinetics are again manifest, but in a rather different way. Under these conditions the fast phase appears with a negative amplitude (i.e., representing a lag phase) of up to 16%; since the rates of the fast and slow phases

can differ by a factor of 10, this lag phase should be detectable, though perhaps with difficulty.

The observation of biphasic kinetics thus indicates the existence of at least three sites involved in the exchange process, but even when three sites are involved a simple single exponential time course can be observed under many circumstances —for example, when $K_2 \ll 1$, when f_{AC} or k_{CB} is very small, or when $f_{AC} \sim f_{AB} \sim k_{CB}$. When biphasic kinetics *are* observed, analysis of the time course will, as in the case of two-site exchange (*1, 2*), allow the rate constants for exchange to be evaluated.

CONCLUSIONS

The calculations reported here show that considerable caution is required when using saturation transfer experiments to determine the chemical shifts of nuclei in a ligand bound to a protein. Since in general one does not know whether the complex exists in only a single state or in several, the resonance position identified in the saturation transfer experiment may be that of a species representing only an infinitesimal proportion of the total complex present.

Consider the simple linear scheme

$$E + A \rightleftharpoons X \rightleftharpoons Y, \qquad [21]$$

where X and Y represent two states of the complex and A the free ligand. When the resonance of the ligand in X is saturated only the direct $A \rightarrow X$ exchange is relevant, and the observed change in intensity, $F_A(X)$, is completely independent of the population ratio [X]/[Y]. When the resonance from site Y is saturated, only indirect $A \rightleftharpoons X \rightarrow Y$ exchange is possible and this will only be efficient when site X predominates, $F_A(Y)$ reaching its maximum for $[X]/[Y] \geqslant 10$. Thus in this linear scheme one is always more likely to observe transfer of saturation from site X than from site Y, whatever their relative populations.

For example, in the dihydrofolate reductase–trimethoprim–$NADP^+$ complex, resonances from the nicotinamide protons of the bound $NADP^+$ were located by saturation transfer experiments (*11*). However, these were subsequently found to arise from the coenzyme in only one of the two approximately equally populated forms of this complex, and a second set of resonances, from the other form, were located both by direct observation and by transfer of saturation at a higher temperature (*16*).

Studies of the kinetics of the transfer of saturation provide a test for multiple-site (as opposed to two-site) exchange, but unfortunately not an infallible one. It will be easiest to resolve two kinetic phases when the indirect exchange route makes a significant contribution, but under these conditions transfer of saturation from *both* forms of the complex should in any case be detectable. The conditions under which only one form of the complex can be detected (for example, those used above to simulate the behavior of the dihydrofolate reductase–folate–$NADP^+$) are just those under which the two kinetic phases are least likely to be observed.

It is clear that saturation transfer experiments of the sort considered here pro-

vide, in isolation, only incomplete information on the nature of the complex(es) formed. Whether the resonances located by saturation transfer arise from the only form of the complex or, at the other extreme, from a form representing less than 0.1% of the total will only be clear if the resonances can be directly observed. In view of the complexity of protein spectra, direct observation of the resonances of the bound ligand is likely to require isotopic substitution—for example, deuteration of the ligand in combination with difference spectroscopy (*10*) or deuteration of the protein itself (*14* and references therein).

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