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Hydrogen Exchange and Macromolecular Motility†

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ABSTRACT: Only rarely have traditional studies of the rates of hydrogen exchange in macromolecules led to precise values of "motility". We show that if there are not too many participating conformers, if their concentrations are monitored, and if the rates of exchange and conformational interconversion are not too different, rate constants defining motility may be obtainable. Clearly, some substrates would not be appropriate for such studies. On the other hand, it is possible to manipulate the exchange rates widely (by choice of conditions) so that many macromolecules should qualify. The applicability of rate expressions in a variety of limiting situations is examined in detail.

The kinetics of peptide hydrogen exchange has often been used as a probe of the internal motions in complex molecules, e.g., proteins, nucleic acids, and polypeptides. The variety of descriptive terms given to these conformational changes, motility, (un)folding, breathing, fluctuation, flexibility, segmental mobility, opening/closing, and helix/coil transition, testifies to the breadth of interest in both the kinds of molecular motions and their types (Barksdale & Rosenberg, 1982; Woodward & Hilton, 1979; Englander et al., 1972; Woodward et al., 1982; Hvidt & Nielsen, 1966; Ghélis & Yon, 1982). Though the reviews just cited survey 3 decades of hydrogen exchange, the expectation that "standard data" (Altona, 1982) on conformational populations or relative stabilities ($\Delta H°$ and $\Delta S°$) *and* on barrier heights ($\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$) would be forthcoming has rarely been realized. Since observations on systems undergoing both hydrogen exchange and conformational interconversion may be difficult to relate to the individual processes, it seems useful to display both exact and approximate solutions of the kinetics that could be applied to the selection, design, and interpretation of experiments in the field.

A schematic representation of a flexible macromolecule containing an exchangeable proton is given in Figure 1. With respect to this proton, closed (C), partly closed (PC), and open (O) forms are indicated. In a real case, there may, of course, be several different exchangeable protons and many more conformations.

To simplify the treatment of the rate data, workers in the field normally catalyze the exchange, H for D or H for T, at constant pH. If the concentrations of the different conformations involving a single exchangeable hydrogen are written

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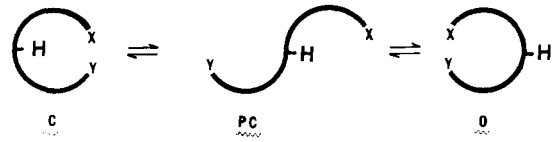

FIGURE 1: Representative conformations of a macromolecule with respect to an exchangeable hydrogen site, i.e., closed, partly closed, and open.

as A_1, A_2, ..., A_j and the corresponding products of exchange are B_1, B_2, ..., B_j, then eq 1 may be used to describe the

$$A_i \overset{k_{ij}}{\rightleftharpoons} A_j \qquad i \neq j;\ j = 1, 2, \ldots \qquad A_j \xrightarrow[k_{jj}]{} B_j \qquad (1)$$

collection of elementary processes. The k_{ij}'s ($i \neq j$) are first-order rate constants characteristic of the internal motions, while the k_{jj}'s are pseudo-first-order constants associated with the exchange at constant pH. Equilibrium constants, $K_{ji} = k_{ij}/k_{ji}$, relate to conformational populations. Alternate approaches and complications will be touched on later.

Standard techniques lead to integrated equations of the time dependence of the concentrations of all A_i's and B_i's of eq 1 (Fleck, 1971; Wei & Prater, 1962). We have found the operator method (with accompanying tables of transforms) to be particularly convenient for solving this coupled set of first-order rate processes (Rodiguin & Rodiguina, 1964; Capellos & Bielski, 1972).

It will suffice to examine the two-conformation case ($j = 2$), since the kinds of terms that appear are similar to those in larger systems. (While the theoretical complexity of a model with more conformations is no greater, the evaluation of the interesting k's may become impossible—see Appendix.) Using standard expressions for the initial concentrations, $A^0 = A_1^0 + A_2^0$, $K_{21} = k_{12}/k_{21}$, and the combinations of rate constants (eq 2 and 3), one can derive eq 4 and 5. It is useful to rewrite

$$\gamma_1 + \gamma_2 = k_{11} + k_{21} + k_{12} + k_{22} \qquad (2)$$

$$\gamma_1\gamma_2 = k_{11}k_{22} + k_{11}k_{21} + k_{12}k_{21} \qquad (3)$$

$$A_1 = \frac{1}{(\gamma_2 - \gamma_1)}[(A_2^0k_{21} + A_1^0k_{21} + A_1^0k_{22} - A_1^0\gamma_1)e^{-\gamma_1 t} - (A_2^0k_{21} + A_1^0k_{21} + A_1^0k_{22} - A_1^0\gamma_2)e^{-\gamma_2 t}] \qquad (4)$$

$$A_2 = \frac{1}{(\gamma_2 - \gamma_1)}[(A_2^0k_{11} + A_2^0k_{12} + A_1^0k_{12} - A_2^0\gamma_1)e^{-\gamma_1 t} - (A_2^0k_{11} + A_2^0k_{12} + A_1^0k_{12} - A_2^0\gamma_2)e^{-\gamma_2 t}] \qquad (5)$$

eq 4 and 5 in the forms in which they would emerge as the result of data fitting to sums of exponential terms:

$$A_1 = x_{11}e^{-\gamma_1 t} + x_{12}e^{-\gamma_2 t} \qquad (4a)$$

$$A_2 = x_{21}e^{-\gamma_1 t} + x_{22}e^{-\gamma_2 t} \qquad (5a)$$

Provided that precise "best fit" expressions 4a and 5a can be obtained for at least *two* runs with different initial A^0's, then the x_{ij}'s and γ's allow the evaluation of all k_{ij}'s.

Since the preceding analysis either does not apply or has not been applied to real systems, it is worthwhile to examine the limitations on hydrogen exchange studies. Initially, we impose defined constraints on the system of eq 1 and look at the consequences in terms of evaluation of the k_{ij}'s as well as on the interpretation of temperature effects on hydrogen exchange. Later, we consider how reported data for different molecules might resemble one or other of these constrained systems.

Case 1. Suppose that A_1 and A_2 are at equilibrium at $t = 0$. Now, $A_1^0k_{12} = A_2^0k_{21}$ and

$$A_1 = \frac{A_1^0}{(\gamma_2 - \gamma_1)}[(\gamma_2 - k_{11})e^{-\gamma_1 t} - (\gamma_1 - k_{11})e^{-\gamma_2 t}] \qquad (6)$$

$$A_2 = \frac{A_2^0}{(\gamma_2 - \gamma_1)}[(\gamma_2 - k_{22})e^{-\gamma_1 t} - (\gamma_1 - k_{22})e^{-\gamma_2 t}] \qquad (7)$$

Best fits to eq 6 and 7 for a *single* run permit the evaluation of *all* k_{ij}'s.

Case 2. Suppose that only one of the conformations undergoes exchange, i.e., $k_{11} = 0$. This condition would emerge as data for runs with different A^0's were fitted to eq 4a and 5a. If, in addition, eq 6 and 7 were applicable, the indication of $k_{11} = 0$ would perhaps be more obvious. In either case, all of the k's can be evaluated, and the system may be regarded as completely determined. Incidentally, our expression for B_2 or $A^0 - A_1 - A_2$ is identical with one given by Hvidt (1964) and is consistent with others from standard sources, provided that uniform boundary conditions are imposed (Capellos & Bielski, 1972).

Case 3. Suppose that conformational equilibrium is maintained during the course of exchange, i.e., $A_j/A_i = A_j^0/A_i^0 = K_{ji}$ or

$$k_{ij}A_i = k_{ji}A_j \qquad (8)$$

The change in A_j takes the simple form

$$-\mathrm{d}A_j/\mathrm{d}t = k_{jj}A_j \qquad (9)$$

or

$$A_j = A_j^0e^{-k_{jj}t}$$

Now $\kappa \equiv k_{jj} = k_{ii} = k_{11}$, etc.; and while K_{ji} are known but different for each A_j, the conformational interconversion rates remain undetermined.

Cases 4a–c. Suppose that A or B are monitored but the individual concentrations A_1 and A_2 are not known (case 4a). Since $A = A_1 + A_2$, the sum of eq 4 and 5 must be used to treat the data. This sum is a two-term equation from which nothing simpler than γ_1 and γ_2 can be obtained.

If the constraint that led to eq 6 and 7 applies here as well (case 4b), then combinations $k_{11} + k_{22}$ and $k_{21} + k_{12}$, but not the individual k's, can be calculated. Furthermore, temperature effects on the exchange rates will be difficult to interpret, since Arrhenius activation energies cannot be obtained from sums of rate constants.

A third situation of some practical interest is one in which A_j's are in equilibrium but only $A = \sum A_j$ is known (case 4c). Using eq 8 and 9, we deduce

$$A = \sum A_j = A^0e^{-\kappa t} \qquad j = 1, 2, \ldots \qquad (10)$$

Obviously, a fit of the experimental data to eq 10 yields κ but is otherwise not too informative.

Case 5. Suppose one of the A's predominates, e.g., $A_2 \ll A_1$. A single two-term equation, i.e., eq 4a, is obtained from which γ_1, γ_2, and the combinations $k_{21} + k_{22}$ and $k_{11} + k_{12}$ can be calculated. It is probable that the temperature dependence of these pairs will be difficult to interpret, unless it is known that one of the k's in each pair is relatively small.

Case 6. Suppose $A_2 \ll A_1$ and $k_{22} \ll k_{11}$; that is, exchange is strongly favored in the predominant isomer. The appropriate rate expression is not especially interesting:

$$A = A^0e^{-k_{11}t} \qquad (11)$$

The Arrhenius equation should be obeyed. The difficulty here, however, arises from the problem of distinguishing this case from others in which there is one (or one dominant) expo-

nential term for the dependence of A. See cases 7 below.

Cases 7. By way of introduction to the steady-state cases, we begin with the general eq 1 and impose successive constraints. Assume first that exchange occurs only in a subset of A_j, namely, A_θ, and all A_θ are subject to the steady-state condition, i.e., $A_\theta \simeq 0$ and $dA_\theta/dt \simeq 0$. The sense here is to divide the conformations into available (PC and Q) vs. non-available (C) sites pictured in Figure 1. This leads to case 7a or

$$\frac{dB_\theta}{dt} = \frac{k_\theta \sum k_{j\theta} A_j}{k_\theta + \sum k_{\theta j}} \qquad j \text{ or } \theta = 1, 2, \ldots \tag{12}$$

Since each A_j can be represented by a sum of exponential terms as in eq 4, eq 12 can be recast to

$$B_\theta = \sum D_\theta e^{-\gamma_\theta t} \tag{13}$$

in which D and γ are constants. Even if each A_j (or B_θ) is monitored, these relations can only yield composite rather than individual rate constants.

It is not surprising, therefore, that the simplest steady-state model in which $j = 1$ and 2 and $\theta = 2$ has been so attractive (case 7b). The condition $dA_2/dt \simeq 0$ leads to

$$\frac{dB_2}{dt}\frac{1}{A_1} \simeq \frac{k_{12}k_{22}}{k_{21} + k_{22}} \equiv k_{ss} \tag{14}$$

The further constraint $k_{22} > k_{21}$ reduces eq 14 to eq 15 (case

$$\text{EX1:} \quad k_{ss} \simeq k_{12} \tag{15}$$

7c) or the classic EX1 limit; in the other extreme of $k_{22} < k_{21}$, eq 14 simplifies to eq 16 (case 7b) or the classic EX2 limit

$$\text{EX2:} \quad k_{ss} \simeq k_{12}k_{22}/k_{21} = K_{21}k_{22} \tag{16}$$

(Hvidt & Nielsen, 1966). It is worth emphasizing that while the conformational rate constants are embedded in eq 14 and 16, k_{ss} does not directly reflect "exposure", "availability", motility, etc. as is sometimes suggested—see Wüthrich's comment (Englander et al., 1980). On the other hand, eq 15 yields one of the two rate constants revelant to the pertinent conformational change.

In passing, we note that another steady-state eq 14a is often

$$\frac{dB_2}{dt}\frac{1}{A_0 - B_2} = \frac{k_{12}k_{22}}{k_{12} + k_{21} + k_{22}} \tag{14a}$$

given and then applied in the forms of eq 14–16 (Hvidt & Nielsen, 1966; Woodward & Hilton, 1980). The reduction of eq 14a to 14 by the plausible assumptions $k_{12} \simeq 0$ and $A_2 \simeq 0$ is, however, less elegant than the direct route to eq 14.

Rate constants k_{ss} for eq 14 should not obey the Arrhenius equation; however, rate data may appear to have an Arrhenius dependence, if the temperature range is small, the data are scattered, $E_1 \simeq E_2$, etc. In the absence of extrakinetic information, eq 14 will not normally be distinguishable from eq 15 or 16!

A didactic conclusion is in order here. To avoid the deficiencies of cases 7, it is eminently worthwhile to adjust conditions (if possible) so that case 2 applies.

Possible Problems and Complications. Hydrogen exchange experiments are normally carried out at constant pH, a condition that precludes pH-dependent conformational changes and simplifies the processing of the kinetic data. Nevertheless, the pH dependence must be determined (Barksdale & Rosenberg, 1982; Wagner & Wüthrich, 1979a; Woodward & Hilton, 1980), if the pseudo-first-order constant k_{jj} is to be converted to a genuine second-order rate constant. Incidentally, the ubiquitous minimum in the dependence of log k (exchange) on pH for most peptide hydrogens is quite different from other property variations with pH found in macromolecules, e.g., the ^{1}H NMR chemical shifts in the tripeptide Ser-Pro-Pro (London et al., 1978).

Measured activation parameters may be composite not only because of several steps along each of the exchange paths catalyzed by H^+, OH^-, or H_2O but also because of other processes, e.g., involvement with coordinating species. Now, acid- and base-catalyzed exchange in simple amides provides a standard of $\Delta H^\ddagger \simeq 20 \pm 4$ kcal/mol and $\Delta S^\ddagger = 27$ cal deg/mol for "open" sites, while activation parameters for the pH-independent exchange may be quite different, e.g., $\Delta H^\ddagger \simeq 30$ kcal/mol (Barksdale & Rosenberg, 1982; Richarz et al., 1979). Conformational barriers begin somewhat larger than energies for local motions and librations (ca. 0.5 kcal/mol) and extend up to major unfolding (ca. 70–80 kcal/mol) (McCammon & Karplus, 1983; Lotan et al., 1972; Gurd & Rothgeb, 1979). The range of barriers to diffusion of ions and solvent into channels within folded macromolecules appears to be unknown. Finally, the formation (or dissociation) of complexes between the macromolecule and ubiquitous species, i.e., ions, solvent, or other macromolecules, could involve a range of enthalpies (Shreier & Baldwin, 1976; Brewer et al., 1983). Depending on the specific conformation involved in the exchange process, these ancillary ΔH's and ΔS's may serve to increase or decrease the overall exchange parameters. Not surprisingly, the possible separate contributions to the activation quantities may be difficult to sort out.

A recent controversial issue on the details of the exchange process has been that of access or approach to an internal site. Does such a hydrogen exchange after the macromolecule unfolds, or does the attacking species "penetrate" the structure through an open door or window and diffuse along corridors and stairwells to the reaction site? While most rate studies cannot of themselves resolve this question, there may be special cases in which such data may be indicative.

Consider one particular model for penetration: the solvated OH^- (or H^+) diffuses into a channel to reach an exchangeable hydrogen, and all exchangeable sites (A) are in channels. We use subscripts o and i to designate reagent outside the channel or inside at the site. If k'_{12} and k'_{21} are in- and out-diffusion constants of OH^-, k'_{22} is the exchange rate constant, and the steady-state condition holds for $[OH^-]_i$, then one can obtain eq 17. Certainly, the necessary condition in this treatment,

$$v_{\text{exchange}} = \frac{k'_{12}k'_{22}A[OH^-]_0}{k'_{21} + k'_{22}A} \tag{17}$$

i.e., $[OH^-]_i \ll [OH^-]_{\text{total}}$, can be set by the experimentalist.

Although eq 17 differs from eq 14, it can be reduced to an analogue of eq 16, if $k'_{21} \gg k_{22}A$. Clearly, kinetic studies cannot distinguish between the EX2 mechanism and this penetration mimic! Arguments favoring one mechanism over the other might be possible on structural grounds or if rate parameters for both conformation and penetration barriers are known and significantly different (Gregory et al., 1982; Woodward & Hilton, 1980).

In the limit of $k'_{21} \ll k_{22}A$, the rate constant for exchange is diffusion controlled, $k_{\text{obsd}} = k'_{12}$. This is different from eq 15. Of necessity, barriers to such diffusion would have to be higher than those for exchange, i.e., $\Delta H^\ddagger \gg 20$ kcal/mol, and would involve exchangeable sites that were deeply buried and/or tightly bound "inside" of a macromolecule. This and other putative models require investigation.

We turn now to the practical problem of fitting data to an equation of the general form of eq 18 (Lanczos, 1956;

$$A = \sum C_j e^{-\beta_j t} \quad (18)$$

Barksdale & Rosenberg, 1982). In the context of eq 1 and Figure 1, Laiken & Printz (1970) point out that an equation with two or three exponential terms will usually suffice to describe systems in which the number of conformations or "kinetic classes" containing a given exchangeable hydrogen exceeds two. The insights and recommendations of these workers on fitting such equations are still pertinent.

In exchange studies of large molecules with many labile hydrogens, a wide spectrum of exchange rates is typical. Experimental techniques such as IR, UV, tritium exchange, etc., which are "blind" to individual hydrogens (Barksdale & Rosenberg, 1982), compelled the researcher to lump the hydrogens into a small number of classes in order to obtain tractable kinetics. Inevitably, this led to expressions such as eq 18 or to versions of the steady-state eq 14–16 (Hvidt, 1973; Hvidt & Pedersen, 1974; Segal et al., 1967; Laiken & Printz, 1970; Nakanishi & Tsuboi, 1976; Englander et al., 1980). Of necessity, either "composite" rate constants or activation energies were used directly, or the "contributing" factors were assigned "reasonable" values in order to measure motility (Woodward & Hilton, 1980; Schreir & Baldwin, 1976; Nakanishi & Tsuboi, 1976).

More recently, the kinetics of collective or multiproton exchange has become more sophisticated in that distributions of hydrogen types are analyzed (Gregory, 1983; Barksdale & Rosenberg, 1982). Although this approach refines the classification of exchanging hydrogens, the relation of derived rate constants to elementary or group conformational motions remains complex and obscure. Admittedly, multiproton exchanges studies were necessary and informative at one time. But if the objective is to assess motility, then experimental techniques that "see" individual exchange sites are indispensable: the ^{1}H NMR technique is eminently suited to this purpose (Narutis & Kopple, 1983).

Applications. A quick review of the cases we discussed earlier may be useful at this point. The reader will find that the A_j's are known in cases 1–2. Therefore, the number of exponential terms is also known. The question becomes not so much the identity as the precision of the derived constants. Again, the fewer the conformations, the easier it should be to attain precise rate and/or equilibrium constants.

In the remaining cases 3–7, only A (or B) is known as a function of time. Suppose that a fit to eq 13 has been obtained. If several exponential terms are present, one of the cases 3–6 apply, but identification of which one is not possible. If one exponential form is present, either a limiting form of one of cases 3–6 or one of cases 7 applies; but identification of which one again is not possible. *In the absence of independent information on which case applies, we must conclude that studies of the motility of macromolecules that fall into one of the categories of cases 3–7 may be useless!*

Let us examine some simple literature examples first, namely, those in which the rates of conformational change and hydrogen exchange are vastly different. In some "small" molecules, e.g., gramicidin S (Krauss & Chan, 1982) or a cyclic dodecapeptide (Narutis & Kopple), conformational changes are much faster than exchange steps. In bradykinin, for example, the rotamer populations of non-proline side chains and the rates of base-catalyzed hydrogen exchange are known from independent experiments; since one rate constant was obtained for each exchanging residue, it is not clear whether this should be assigned to one rotamer or as a weighted value to all three (Denys et al., 1982). *In such examples, the conformational rate constants remain undetermined.*

By way of contrast, molecules may essentially remain in, and exchange from, a single conformation. In poly(DL-alanine), the rate constants and activation energies for hydrogen exchange in water are comparable to those of "open" dipeptides, e.g., Ala-Ala: therefore, a random coil rather than a helix structure was surmised (Barksdale & Rosenberg, 1982). On the other hand, human carbonic anhydrase B has a compact, tightly folded conformation from which the exposed hydrogens can be readily exchanged (Zâvodsky et al., 1975). While these examples may be attractive for measurements of conformation *or* exchange that might then be extrapolated into an overlapping time zone, they clearly do not allow estimates of motility directly from exchange rates.

Next, we consider examples of the EX1 type. Certain hydrogen exchanges in a 12-mer double helix are relatively insensitive to pH and show low activation energies (ca. 15 kcal/mol). Quite plausibly, the rate-determining step was identified with k_{12} (eq 15) for partial opening (unzippering) of the double helix (Pardi et al., 1982). In the case of the helix–coil interconversion(s) of *E. coli* tRNA, Hurd & Reid (1980) were able to manipulate conditions of temperature and pH to elicit exchanges at specific sites: the helix opening constant (k_{12}) is 35, 16, 12, 5, 6, and 67 s^{-1} for successive G-C pairs at pH 7 and 58 °C, and E_{act} = 49 kcal/mol for exchange at GC-3. A number of other systems, e.g., DNA, RNA, lysozyme, and polynucleotides, have been examined in this way (Pardi & Tinoco, 1982; Segawa et al., 1981; Kallenbach et al., 1980). *In these cases, the rates of "opening up" or "exposing" the exchange site were determined; the rates of "closing down" or "shielding" the site from exchange were not or could not be evaluated.*

By far the most frequently applied mechanism is EX2. If the pH dependence is normal and the Arrhenius plot is linear, then necessary conditions for eq 16 to hold have been met. Additional supporting indicators are often present: the value of k_{obsd} is substantially lower and E_{act} is substantially higher than corresponding values for simple peptides; the value of k_{obsd} increases, if reagents that promote unfolding are included in the medium; the exchanging hydrogen is known from structural data to be "nonexposed" or perhaps strongly hydrogen bonded. As indicated earlier, we regard the multiproton exchange examples, e.g., lysozyme (Gregory et al., 1982), basic pancreatic trypsin inhibitor (Pershina & Hvidt, 1974), α-lactalbumin (Takesada et al., 1973), and chymotrypsinogen A (Rosenberg & Enberg, 1969) as less useful by current standards. Among the single proton exchange examples are basic pancreatic trypsin inhibitor (Wagner & Wüthrich, 1979a; Woodward & Hilton, 1980), cow colostrum trypsin inhibitor (Wagner et al., 1978), soybean leghemoglobin *a* (Kong et al., 1982), lysozyme (Wedin et al., 1982), and poly(rA)·poly(rU) (Kallenbach et al., 1980).

Apart from recognition and validation, there is the problem of how to dissect both k_{ss} and related activation parameters. If $K_{21} = k_{12}/k_{21}$ is available from separate experiments so that k_{22} of eq 16 can be estimated, the hydrogen exchange experiment provides no new conformational data (Denys et al., 1982). Wedin et al. (1982), for example, took the value of k_{ss} at 72 °C for the slowly exchanging tryptophan units in lysozyme from tryptophan itself and combined it with K_{12} from unfolding experiments and k_{ss} in eq 14 to deduce a value for k_{21}. More traditionally, researchers have assumed model values of k_{22} and/or the corresponding activation parameters and have given estimates of K_{21} and/or the corresponding thermochemical quantities. Nakanishi & Tsuboi (1976) took k_{22} from polyalanine and deduced K_{21} for subtilisin inhibitor.

Using the same model, Gregory et al. (1982) estimated fairly crude values of ΔH^0_{21} = 38 kcal/mol and ΔS^0_{21} = 88 cal deg/mol for the unfolding of lysozyme. Woodward & Hilton (1980) assumed $E_{act} \simeq 20$ kcal/mol for k_{22} of bovine pancreatic trypsin inhibitor (BPT 1) and deduced that the conformations involved in several single proton exchanges fell into two broad categories with $\Delta H^0_{21} \simeq 20$ and 0–15 kcal/mol (Hilton & Woodward, 1979). For the same protein, Richarz et al. (1979) used $\Delta H^{\ddagger}_{22} \simeq 17$ kcal/mol and $\Delta S^{\ddagger}_{22} \simeq 27$ cal deg/mol for their model and deduced specific values for individual residues undergoing exchange in the range $\Delta H^0_{21} \simeq$ 0–23 kcal/mol and $\Delta S^0_{21} \simeq$ −25–43 cal deg/mol. If, as seems likely, the EX2 mechanism applies to the histidylimidazole residue in a ligated leghemoglobin (metLbCN) at pH 8.56, then $\Delta H^{\ddagger}_{ss}$ = 11.6 kcal/mol and $\Delta S^{\ddagger}_{ss}$ = 6.7 cal deg/mol (Kong et al., 1982); from these combined with model values of $\Delta H^{\ddagger}_{ss} \simeq 17$ kcal/mol and $\Delta S^{\ddagger}_{22} \simeq 27$ cal deg/mol, one would estimate $\Delta H^{\circ}_{21} \simeq -5$ kcal/mol and $\Delta S^{\circ}_{21} \simeq -20$ cal deg/mol. *In such examples, one should not lose sight of the fact that the dissection of the EX2 steps is an exercise that depends on models, sometimes of uncertain validity: at best, the estimates of the conformational rate constants are uncertain.*

Where eq 14 is demonstrably applicable by a fit of k_{ss} to an appropriate function of the catalyst concentration C, the results can be fruitful. Of course, only molecules whose conformations are insensitive to the exchange conditions qualify here, and the nature of the catalysis, say $k_{22} = f(C)k_{ex}$, should be known. By way of example (still rare), k_{ss} was measured for poly(rA)·poly(rU) as a function of base concentration. Both k_{12} and k_{22}/k_{21} could be evaluated conveniently from eq 14 in reciprocal form (Kallenbach et al., 1980). But, to obtain k_{21} and/or k_{22}, measurements by an independent method would be required.

Concluding Remarks. If the evaluation of conformational rate and equilibrium constants in a macromolecule is set as the goal of a hydrogen exchange study, then successes are rare. Indeed, the examples in which k_{12} was evaluated at the EX1 limit (eq 15) seem to be the lot. Thirty years of effort in the area of hydrogen exchange has been crucial to the development of knowledge in the field of large molecules (Barksdale & Rosenberg, 1982), but it has produced few measures of motility. NMR techniques can yield data on conformational populations or on barriers, independent of hydrogen exchange. Where the time scales of these processes overlap, eq 4 and 5 and their analogues can be used to estimate rate constants appropriate to both of them. This approach to macromolecular motility is accessible now and should not be neglected.

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Appendix

Concerning any model, we wish to know its limitations. Overly drastic assumptions may produce simple but uninteresting solutions: for example, (the assumption of) rapid equilibration among the conformers relative to hydrogen exchange precludes the evaluation of conformational barriers. On the other hand, "excessive" detail may yield rigorous but experimentally inaccessible equations: the inclusion of too many species and their interconversions may make it impracticable to extract individual rate constants. In this context, "too many" turns out to be rather small.

In the most general scheme involving j conformations of A, the analogue of eq 4′ becomes

$$A_i = x_{i1}e^{-\gamma_1 t} + x_{i2}e^{-\gamma_2 t} + \ldots + x_{ij}e^{-\gamma_j t} \quad (19)$$

The γ_j's and x_{ij}'s are complex expressions containing the k's and A^0_j's. Quite characteristic for such a scheme are the relations for a three-conformation model

$$-(\gamma_1 + \gamma_2 + \gamma_3) = \sum_{ij} k_{ij} \quad (9 \text{ terms}) \quad (20)$$

$$2(\gamma_1\gamma_2 + \gamma_2\gamma_3 + \gamma_3\gamma_1) = \sum_{i \neq j} k_{ij}k_{mn} \quad (24 \text{ terms}) \quad (21)$$

$$-\gamma_1\gamma_2\gamma_3 = \sum k_{ij}k_{mn}k_{op} \quad (16 \text{ terms}) \quad (22)$$

$$\begin{aligned} A_1 = \frac{A^0_1}{(\gamma_1 - \gamma_2)(\gamma_2 - \gamma_3)(\gamma_3 - \gamma_1)}[&(\gamma_3 - \gamma_1)e^{-\gamma_1 t}\{\gamma_1^2 + \\ &\gamma_1(k_{21} + k_{22} + k_{23} + k_{31} + k_{32} + k_{33}) + \\ &(k_{21}k_{31} + k_{21}k_{32} + k_{21}k_{33} + k_{22}k_{31} + \\ &k_{22}k_{32} + k_{22}k_{33} + k_{23}k_{31} + k_{23}k_{33}) + \\ &\frac{A^0_2}{A^0_1}(\gamma_1 k_{21} + k_{21}k_{31} + k_{21}k_{32} + k_{21}k_{33} + k_{31}k_{23}) + \\ &\frac{A^0_3}{A^0_1}(\gamma_1 k_{31} + k_{21}k_{31} + k_{31}k_{32} + k_{22}k_{31} + k_{23}k_{31})\} + \\ &(\gamma_1 - \gamma_3)e^{-\gamma_2 t}\{\quad\} + (\gamma_2 - \gamma_1)e^{-\gamma_3 t}\{\quad\}] \end{aligned} \quad (23)$$

In eq 23, the blanks within the braces indicate terms similar to those in the preceding braces. Although formal solutions for the rate equations of the three-conformation, or even j-conformation, model are possible, access to the values of the nine k_{ij}'s is unlikely!

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Substrate Specificities and Structure–Activity Relationships for Acylation of Antibiotics Catalyzed by Kanamycin Acetyltransferase†

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ABSTRACT: Antibiotic resistance caused by the presence of the plasmid pMH67 is mediated by the aminoglycoside acetyltransferase AAC(6′)-4, also known as kanamycin acetyltransferase. Bacteria harboring the plasmid are resistant to the kanomycins plus a broad range of other deoxystreptamine-containing aminoglycosides but not to the gentamicins XK62-2 and C_1 which are substituted at the 6′-position. Substrate specificity studies on the purified enzyme, however, now show that the enzyme acetylates an even broader range of aminoglycosides, including the gentamicins XK62-2 and C_1. The enzyme also accepts several acyl-CoA esters, which differ in nucleotide as well as in acyl chain length. Application of the method of analysis of structure–activity data developed earlier for gentamicin acetyltransferase [Williams, J. W., & Northrop, D. B. (1978) *J. Biol. Chem. 253*, 5908-5914] to the kinetic data obtained for AAC(6′)-4 shows that the turnover of the acylation reaction is limited by catalysis and not by the rate of release of either the acetylated antibiotic or CoA. Most structural changes in aminoglycosides cause changes in rates of release, and only drastic changes, near the 6′-amino group, affect catalysis. The structural requirements on aminoglycosides for enzymatic activity run parallel to the structural requirements for antibacterial activity.

Kanamycin acetyltransferase (EC 2.3.1.55) catalyzes the $N^{6'}$-acetylation of several deoxystreptamine-containing aminoglycosides (see Figure 1). It was first identified as the biochemical basis of R-factor mediated resistance to kanamycin by Okamoto & Suzuki (1965), who demonstrated a requirement for acetyl-CoA in kanamycin interaction by cell-free of *Escherichia coli* K12 containing plasmid R5. This report was the first ever of an aminoglycoside-modifying enzyme in bacteria and second only to that of penicillinases as an instance of bacterial resistance being caused by enzymatic modification of an antibiotic. Kanamycin acetyltransferase is now designated AAC(6′)-4 by the Plasmid Group nomenclature (Mitsuhashi, 1971), because unlike other 6′-N aminoglycoside acetyltransferases, it acetylates the clinically important amikacin.

The catalytic features of the modifying enzyme must be the determining characteristics of the bacterial resistance mediated

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