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A Hydrogen-Exchange Study of Lysozyme Conformation Changes Induced by Inhibitor Binding†

R. R. Wickett,* G. J. Ide, and A. Rosenberg

ABSTRACT: Precise data on lysozyme hydrogen-exchange kinetics have been obtained at pH 6.8 in presence and absence of the lysozyme inhibitor *N*-acetyl-D-glucosamine. The action of ligand resembles that of temperature influencing the whole exchange curve, which is the sum of individual rates, uniformly. An analysis of the apparent activation energy profile of the exchange demonstrates that the inhibitor binding effects most, if not all, of the observable exchange rates. The influence of the bound inhibitor molecule is thus propagated throughout the protein structure.

Hydrogen-isotope-exchange kinetics of polypeptides and proteins are extremely dependent upon conformation (Hvidt and Nielsen, 1966; Woodward and Rosenberg, 1971a,b; Hvidt and Wallevik, 1972). Hydrogen-exchange techniques are sufficiently sensitive to observe changes occurring when proteins bind metal ions (Emery, 1969) or ligand (Di Sabato and Ottesen, 1965; Englander and Mauel, 1972; Nakanishi *et al.*, 1973). Unfortunately, the analysis of such changes is greatly complicated by the wide distribution of exchange rates. Even oxidized ribonuclease in 6 M guandine-HCl has exchange rates distributed over at least two orders of magnitude (Woodward and Rosenberg, 1970) and the distribution in native proteins is much broader.

Because rate constants cannot be obtained specifically, the interpretation of differences in exchange curves is difficult. A difference between exchange curves in the presence and absence of ligand may result from large changes in a few rates (Englander and Mauel, 1972; Englander and Rolf, 1973) or small changes in many or all rates (Benson *et al.*, 1973).

We have obtained very precise data on hydrogen–tritium exchange from lysozyme at a variety of temperatures. The precision of the data has allowed us to obtain an activation energy profile for exchange from the lysozyme molecule. In the presence of *N*-acetyl-D-glucosamine (AcGlcn)[1] this profile is altered over the entire range of observable hydrogens. Furthermore, the rank order[2] of the exchange does not change upon inhibitor binding. These facts allow us to conclude that most of the observable exchange rates change when inhibitor is bound.

Experimental Section

Materials. Medium grade G-25 Sephadex was supplied by A. B. Pharmacia. Tritiated water was supplied by International Chemical and Nuclear Corporation. A Beckman Model L 200 scintillation counter was used. Sigma Chemical Company Grade I lysozyme and Worthington LYSF, salt-free, lysozyme were used interchangeably with no difference in results noted. *N*-Acetyl-D-glucosamine was obtained from Sigma. Protein concentrations were determined at 280 nm with a Cary 118C spectrophotometer. Scintillation fluid was prepared according to the method outlined by Bray.

In-Exchange of Protein. Protein was dissolved in pH 8, 0.05 M tricine buffer to form a solution of concentration 20 mg/ml. An equal volume of tritiated water (0.5 Ci/l.) was added to the protein solution. The resulting solution was incubated for 16 hr at 40°. The level of in-exchange represents the maximal obtainable with pH, temperature, and time as variables.

Out-Exchange. The two-column separation described by Englander (1963) was used with some modifications.

The in-exchanged protein solution (cooled to 1°) (4 ml) was filtered on a column of known dead volume equilibrated to pH

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[1] Abbreviation used is: AcGlcn, *N*-acetyl-D-glucosamine.

[2] The rank order of the exchange is determined by the relative magnitudes of the rate constants, $k_1 \ldots k_n$, associated with hydrogens $H_1 \ldots H_n$. Given a set of experimental conditions under which $k_1 > k_2 > k_{n-1} > k_n$ and a change in those conditions, the rank order is invarient to the change if $k_1' > k_2' \ldots k_{n-1}' > k_n'$ still holds for the new rate constants $k_1' \ldots k_n'$.

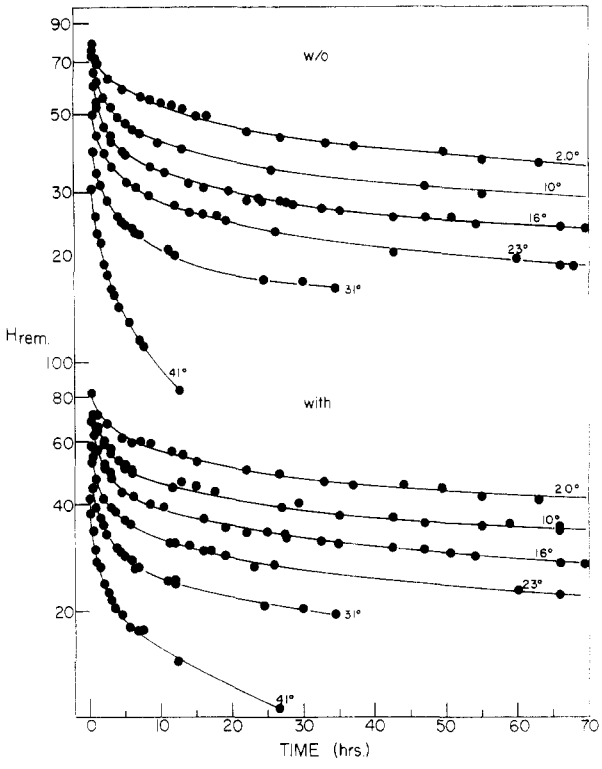

FIGURE 1: Lysozyme out-exchange curves at pH 6.80: "with" curves are in the presence of 0.20 M *N*-acetyl-D-glucosamine. Temperatures as marked on figure.

6.8 (0.05 M phosphate buffer) and contained in a 1° room; 10 ml of the eluted protein peak was collected and AcGlcn dissolved in those samples to be out-exchanged with inhibitor present. Out-exchange was begun immediately by pouring the solution into a large flask preequilibrated at the out-exchange temperature. Out-exchange temperature was maintained

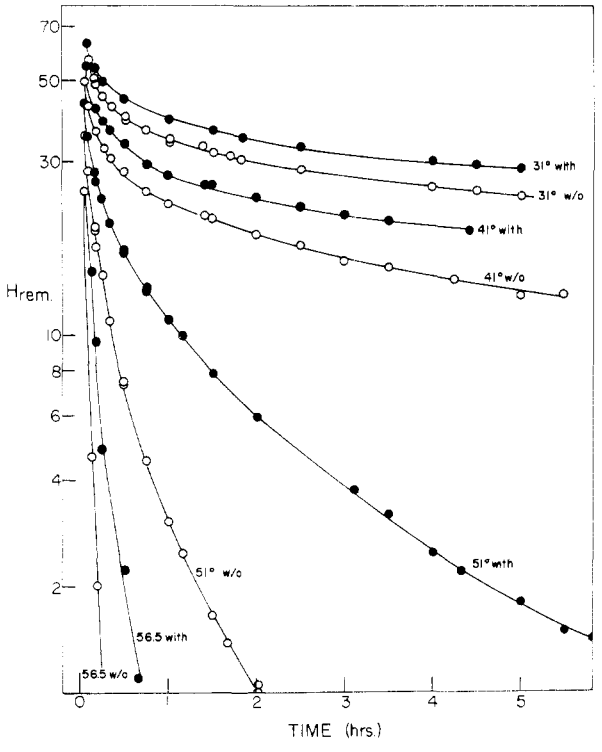

FIGURE 2: Direct comparison of with and without (w/o) AcGlcn curves at higher temperatures: "with" or w/o and temperatures as marked on figure.

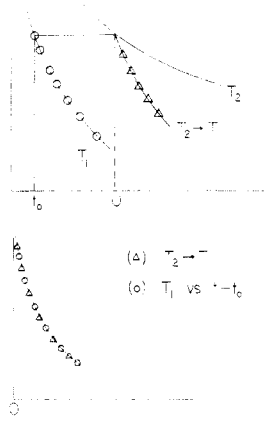

FIGURE 3: Illustration of the temperature jump procedure used to test the constancy of the rank order.

±0.02°. Out-exchange assays were done on columns having dead volume clearances less than 1 min, which were homogeneously packed with Sephadex G-25, medium. Out-exchange assays of 2, 10, 16, and 23° were done on 1° columns; assays at 31 and 41° on 6° columns; and assays at 51 and 56.5° on 23° columns. This cooling of the assays effectively quenched out-exchange at the time the sample was put on the column. All columns were equilibrated to pH 6.8 with 0.05 M phosphate buffer. Protein peak tubes were collected and H_{rem} was calculated as described before (Rosenberg and Chakravarti, 1968) using an ϵ of 3.87×10^4 at 280 nm.

Results

Figures 1 and 2 show out-exchange curves for lysozyme at pH 6.8 in the presence and absence of 0.20 M *N*-acetyl-D-glucosamine. In every case the first-order plots show marked curvature over the entire range of observable hydrogens. Because of the curvature of these plots, rate constants cannot be obtained explicitly and we must resort to other means of analysis.

Woodward and Rosenberg (1971a) showed that the apparent activation energy of exchange, E_{app}, could be obtained using two exchange curves at different temperatures and the ratio of times needed to achieve equal values of H_{rem}, from the formula

$$E_{app} = \frac{T_1 T_2 R}{T_1 - T_2} \ln t_2/t_1 \qquad (1)$$

When the activation energy remains constant over the entire exchange curve the ratio t_2/t_1 does not change. If, however, more than one mechanism is operative the situation becomes more complicated and the formula may not necessarily be correct, as the rank order of the exchange may change.

Fast temperature change experiments may be used to establish the invariance of the rank order to temperature (Woodward and Rosenberg, 1971a). We compare the out-exchange from two samples at an identical number value of ln H_{rem}. In one case we follow the out-exchange at a given temperature, T_1. In the other case we allow the exchange to occur for a period of time at a different temperature, T_2. Then the temperature is rapidly changed to T_1 (in 10 sec). The time of temperature change is then considered to be zero time. By a double filtration at the time of temperature change we determine the value of ln H_{rem} at zero time, H_0. To compare the two curves we find $H_{rem} = H_0$ on the first curve. In this case H_0 is associated with a time, t_0, and we shift the origin of the curve by plotting ln H_{rem} *vs.* $t - t_0$ for the rest of the out-exchange curve. This procedure is specifically illustrated in Figure 3.

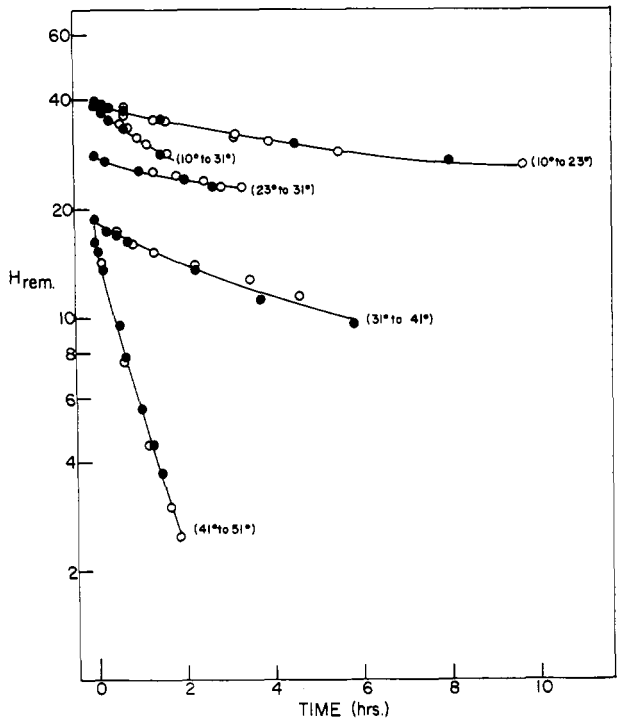

FIGURE 4: Temperature jump curves: (O) out-exchange curves at the higher temperatures, with ordinate shifted to zero time at a value of H_{rem} equal to that at the lower temperature; (●) at the time of T jump, which is also taken to be zero time.

The two curves generated by this procedure have a common origin. Although the numerical value of ln H_{rem} is the same for both curves (H_0), that value was reached through different paths. If the distributions of remaining hydrogens are identical the two exchange curves will be superimposable from the new origin on. The two samples can have identical distributions of hydrogens only if the rank order of the exchange is identical at the two temperatures used to reach $H_{rem} = H_0$. The data in Figure 4 demonstrate that the rank order of the exchange is unchanged over a wide variety of H_{rem} and temperature.

Because the rank order is constant we may use eq 1 to determine the activation energy of the exchange. In Figure 5 the activation energy is plotted not as a function of temperature as in previous work from this laboratory (Woodward and Rosenberg, 1971a,b) but as a function of H_{rem}. The closed circles are in the presence of 0.20 M AcGlcn.

Because of the low-binding constant of AcGlcn (Rupley *et al.*, 1967; Bjurulf *et al.*, 1970) the concentration noted (0.20 M) is sufficient to saturate the binding sites to 95%. This level is insensitive to any changes of binding constant that occur with temperature. A control experiment at 51° showed that 0.20 M glucose has no effect on lysozyme hydrogen exchange.

The apparent activation energy in the absence of inhibitor is approximately 25 kcal for 40–45 of the observable hydrogens and rises steeply to 80 kcal over the range of the last 20 hydrogens. The last 20 hydrogens are not accessible to observation at temperatures below 41° (Figure 1) and the high activation energy for their exchange may represent some contribution from thermal unfolding. In the presence of inhibitor the apparent activation energy for the exchange is 5–10 kcal higher over the entire range of observable hydrogens.

To determine if the rank order is the same with or without inhibitor a slightly more complex experiment is required. Because there is only a small difference between curves at a given temperature, it means little to simply add or remove inhibitor

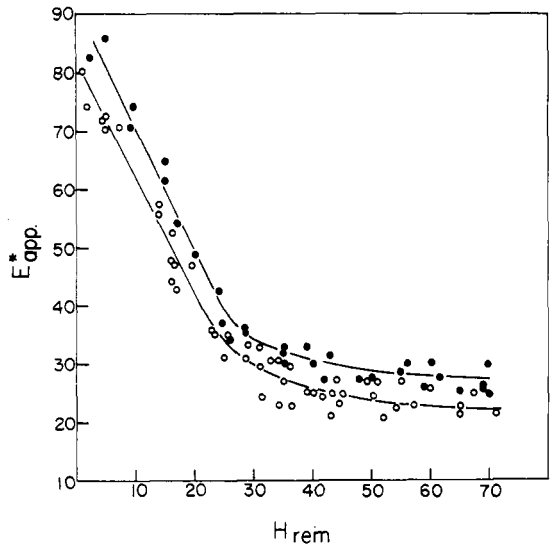

FIGURE 5: Apparent activation energy *vs.* H_{rem} at pH 6.80, with (●) and w/o (O) 0.20 M *N*-acetyl-D-glucosamine.

at some point and follow the new curve. To overcome this difficulty we exchange a sample without inhibitor to a given value of H_{rem} and then simultaneously add inhibitor and jump the temperature. Because the rank order does not change with temperature, the new curve should be parallel to the inhibitor curve at the higher temperature. This procedure provides a much more rigorous test of the rank order.

Figure 6 shows the results of these experiments. In addition to this, at 51° there is a considerable difference between with and without AcGlcn curves and one experiment was done by adding AcGlcn to a sample that had already out-exchanged to

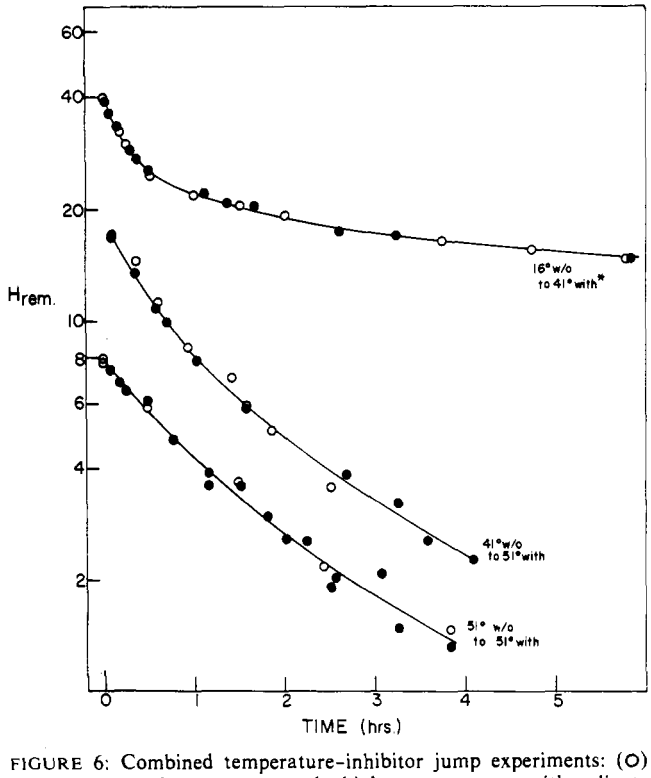

FIGURE 6: Combined temperature-inhibitor jump experiments: (O) inhibitor out-exchange curves at the higher temperature, with ordinate shifted to zero time at a value of H_{rem} equal to that at the time of temperature jump; (●) simultaneous temperature jump and inhibitor addition. Temperature goes from low to high as marked on figure. * Multiply time scale by 2.0.

H_{rem} = 8.0 at 51° without AcGlcn, and maintaining constant temperature. The new curves are parallel to the sample with AcGlcn out-exchange curves. This demonstrates that the rank order remains essentially unchanged with AcGlcn present.

Discussion

At pH 6.8 approximately 80 hydrogens are observed to exchange within the time and temperature limits of the experiment (Figures 1 and 2). Plots of ln H_{rem} *vs.* time show definite curvature at all temperatures and times studied. Since the rank order of the exchange is constant we must be gradually observing slower and slower hydrogens as the temperature is increased. This makes a different slice of the distribution observable at each temperature. We do not see any one group of hydrogens which may be singled out as slowly exchanging, nor do we observe linear first-order plots under any conditions. This is in marked disagreement with the conclusions of Nakanishi *et al.* (1972, 1973), who observed apparent first-order behavior for 44 of the lysozyme hydrogens, which they identify as internally hydrogen bonded. We have studied hydrogen–tritium exchange data on lysozyme at several other pH's between 2.0 and 9.5 and have never observed first-order kinetics (R. R. Wickett, G. J. Ide, and A. Rosenberg, manuscript in preparation) under any conditions. Linear semilog plots for any protein seem unlikely in the light of the primary structure effects discussed by Woodward and Rosenberg (1970) and Molday *et al.* (1972).

The data on *N*-acetyl-D-glucosamine demonstrate that inhibitor binding affects most of the protein molecule. A direct comparison of exchange curves (Figures 1 and 2) in the presence and absence of AcGlcn shows that the amount of difference between the two curves increases with time at the higher temperatures. This is evidence for a true kinetic difference and not merely the "protection" of four or five hydrogens by hydrogen bonds to inhibitor.

The following analysis of the results shown in Figures 4–6 further supports this point.

Formally, we consider the exchange process as a sum of exponential terms

$$H_{rem} = A_0e^{-k_0t} + A_1e^{-k_1t} + A_2e^{-k_2t} + \ldots + A_ne^{-k_nt} + B_1 \quad (2)$$

where $A_1 \ldots A_n$ represent the number of hydrogens exchanging with each rate constant $k_1 \ldots k_n$ and B is the number of hydrogens which are too slowly exchanging to observe during the course of the experiment. There are also some hydrogens which exchange too rapidly to observe and do not contribute to H_{rem}. They are denoted by A_0.

The derivative of eq 2 at definite H_{rem} is

$$\frac{dH_{rem}}{dt} = k_1[A_1e^{-k_1t}] + k_2[A_2e^{-k_2t}] + \ldots + k_n[A_ne^{-k_nt}] \quad (3)$$

We may replace $A_ie^{-k_it}$ by H_i where H_i is the number of hydrogens exchanging with rate k_i at time t. The rank order of the exchange has been proven constant (Figure 4) so these H_i's will be the same at a given value of H_{rem} at any temperature. This is true even though the time required to reach the specified value of H_{rem} is different at different temperatures.

Obviously

$$\sum_i H_i = H_{rem} \quad (4)$$

and

$$H_i = H_{rem}f_i \quad (5)$$

where

$$f_i = H_i/(H_1 + H_2 + \ldots + H_n) \quad (6)$$

is the fraction of remaining hydrogen which exchange with rate k_n.

Substituting into (3) above we get

$$\frac{dH_{rem}}{dt} = H_{rem}(k_1f_1 + k_2f_2 + \ldots + k_nf_n) \quad (7)$$

Thus at any H_{rem} we may define an apparent rate constant K_{app}

$$K_{app} = (k_1f_1 + k_2f_2 + \ldots + k_nf_n) \quad (8)$$

Because the rank order of the exchange is constant, the apparent activation energy obtained from eq 1 and plotted in Figure 5 is

$$\frac{E_{app}}{R} = \frac{d(\ln K_{app})}{d(1/T)} = \frac{d(\ln (k_1f_1 + \ldots + k_nf_n))}{d(1/T)} \quad (9)$$

$$\frac{E_{app}}{R} = \frac{f_1k_1E_1}{f_1k_1 + f_2k_2 + \ldots + f_nk_n} + \frac{f_2k_2E_2}{f_1k_1 + f_2k_2 + \ldots + f_nk_n} + \ldots + \frac{f_nk_nE_n}{f_1k_1 + f_2k_2 + \ldots + f_nk_n} \quad (10)$$

where E_1 to E_n are the individual activation energies associated with rates k_1 to k_n.

A plot of E_{app} *vs.* H_{rem} (Figure 5) is a function of the individual rate constant and activation energies.[3]

Now consider exchange from the same H_{rem} sequence in the presence of ligand (AcGlcn). If only a limited number of hydrogens are shifted from very fast to very slow (*i.e.* "protected"), the effect is a shift from A_0 to B (eq 2). If these hydrogens were originally slow enough to measure they shift out of the distribution and into the constant B. In either case this change would produce a corresponding change in rank order which would easily be detected in Figure 6. We can conclude that no hydrogens are shifted from very fast to very slow.

From the form of eq 10 and the results in Figures 5 and 6, we can conclude that most, if not all, of the observable exchanging hydrogens are affected by inhibitor binding. The apparent activation energy is a weighted average, but if E_1 changes and E_2 does not, the change not only effects E_{app} but also alters the rank order of the exchange since k has an exponential dependence on E. The data are not precise enough to rule out some small shifting of order within the distribution, but are certainly good enough to rule out major changes. If, for example, half of the activations energies changed and the other half did not, the resulting rank order change would be observable. Also, it is difficult to believe that such a change could produce the smooth curves and nearly constant differences observed in Figure 5. It seems most probable that all of the activation energies and, therefore, all of the rates are affected by inhibitor binding.

The increase in activation energy could possibly result from the ability of AcGlcn to inhibit lysozyme dimerization (Hampe, 1972). However, at pH 5.0, no lysozyme dimerization occurs (Sophianopoulos and Van Holde, 1964), and the appar-

[3] The inhibitor off and on rates are very fast compared to hydrogen exchange; thus the contributions to exchange from ligand-bound and ligand-free protein are proportional to the equilibrium concentrations of the two forms. Under the conditions for the with inhibitor experiments ~95% of the protein is in the liganded form. The temperature dependence of the equilibrium is not large enough to influence this fraction significantly and the enthalpy of inhibitor binding will not appear in the measured quantities.

ent activation energy for hydrogen exchange is still increased by AcGlcn (R. R. Wickett, G. J. Ide, and A. Rosenberg, manuscript in preparation). We thus conclude that the conformational changes affecting hydrogen exchange probably occur in the monomer.

In this analysis we have made no reference to any mechanism beyond the general one of exponential exchange. Whatever the mechanism of exchange, all rates are affected by inhibitor binding. However, to understand the implications for the protein structure, we must have some information about the mechanism of exchange.

The activation energy profile shown in Figure 5 demonstrates that 55 of the observable hydrogens exchange with an activation energy of less than 40 kcal. This activation energy is too low for the exchange step to involve complete thermal unfolding (Woodward and Rosenberg, 1971a,b). Furthermore these hydrogens are observed to exchange at a temperature of 31° or lower; this is at least 45° below the temperature for lysozyme thermal unfolding at pH's above 5.0 (Sophianopoulos and Weiss, 1964). Exchange from native protein is affected by inhibitor binding.

The exchange of hydrogen from the interior of a protein at temperatures far from thermal unfolding must involve some type of dynamic fluctuations of the native structure, permitting local accessibility to solvent. The binding of AcGlcn to lysozyme increases the energy required for these fluctuations making the energy of the activated complex relatively higher by 5–10 kcal.

It is apparent from a comparison of the observed increase in activation energy to the decrease in K_{app} that there is also a considerable increase in the entropy of the activated complex in the presence of AcGlcn. While the activation energy increase varies from 5 kcal over most of the range to 10 kcal at very low H_{rem}, the rates only decrease by a factor of 2–3 (Figure 2). A simple calculation from absolute rate theory shows that the increased entropy of the activated complex is approximately 15 cal/(mol deg) for most H_{rem}. Partially compensating entropy and enthalpy changes are a very commonly observed property of protein–small molecule systems (Lumry and Rajender, 1970).

While the only AcGlcn induced conformational changes large enough to appear in the X-ray results are a 0.75 Å shift of the tryptophan-62 side chain and small shifts of some of the neighboring residues (Blake *et al.*, 1967), exchange results present compelling evidence for the propagation of the effects of AcGlcn binding throughout the entire protein structure.

This effect on the dynamic conformation of the protein may be an example of the redistribution of free energy required for "rack" mechanisms in protein function (Eyring *et al.*, 1954, Lumry and Biltonen, 1969), in which the strength of many secondary bonds is utilized in enzyme action or ligand binding. The results are also consistent with an "R" to "T" transition of the type proposed by Hopfield (1973) for hemoglobin action. This transition also involves the distribution of ligand binding energy throughout the protein structure.

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