Binding of 4-Methylumbelliferyl α-D-Mannopyranoside to Dimeric Concanavalin A: Fluorescence Temperature-Jump Relaxation Study†

Robert M. Clegg,* Frank G. Loontiens,‡ and Thomas M. Jovin

ABSTRACT: The kinetics of saccharide binding to the dimer form of concanavalin A (con A) has been studied at pH 5.5 with the fluorescence temperature-jump method. 4-Methylumbelliferyl α-D-mannopyranoside, a fluorescent carbohydrate derivative which is quenched upon binding to con A, was used as the ligand. Three relaxation effects were seen. The major relaxation (τ = 20–400 ms) was investigated at four different temperatures. The behavior of this relaxation as a function of reactant concentrations is consistent with a simple one-step bimolecular association reaction. These conclusions result from the analysis of both the relaxation times and amplitudes, and from the comparison of the kinetically determined equilibrium parameters (K_{ass} = 3.5 × 10^4 M^{-1} at 18.5 °C, $\Delta H°$ = −(6–7) kcal/mol) to those obtained from a parallel series of equilibrium experiments (Loontiens, F. G., Clegg, R. M., and Jovin, T. M. (1977), *Biochemistry 16,* preceding paper in this issue). The association and dissociation rate constants are in the range of (6–15) × 10^4 M^{-1} s^{-1} and (1.5–5.6) s^{-1}, respectively, within a temperature range of 13.5–28.1 °C. The activation energies for the forward and reverse reactions are ~10 and ~15 kcal/mol, respectively. The two additional relaxations which are also present in the absence of saccharides result from changes in the protein fluorescence and are attributed to protein conformational changes which are not affected by the binding of saccharides. These effects were further studied using succinylated, acetylated, and demetallized con A. The faster relaxation (13 ms at 18.5 °C) was independent of the concentration of the protein and was not present in the derivatized con A samples. The two derivatized forms of con A show almost identical carbohydrate binding parameters as the underivatized protein. A limited series of stopped-flow experiments yielded results which were fully compatible with those from the relaxation measurements.

The biologically interesting effects involving concanavalin A (con A[1]) include selective agglutination of different cell types (Inbar and Sachs, 1969) and the stimulation of mitogenic activity in lymphocytes (Beckert and Sharkey, 1970). The lectin has also been used as a physical probe for studying the structure and dynamics of the cell surface (Nicolson, 1973; Edelman et al., 1973). A useful compendium of the many diversified effects and uses of con A has recently been published (Chowdhury and Weiss, 1975). These properties of con A in vivo, the utility of the protein as a membrane probe, and the widespread use of con A as an analytical tool in the laboratory appear to be related to the ability of con A to form complexes with specific saccharides.

Evidence for conformational changes in the equilibrium structure of con A has been presented (Becker et al., 1976; Pflumm et al., 1971) and kinetic observation of such a change could help clarify the effects of con A upon the membranes of cells. The mechanism of ligand binding and the values of the rate constants are important when considering the various biological effects of lectins and a comparison of the kinetic parameters of several lectins may reveal parallels between lectins possessing similar properties.

In view of the importance and widespread use of con A, a better understanding of the kinetics of saccharide binding to this lectin would be especially useful. The binding kinetics of *p*-nitrophenyl α-D-mannopyranoside have been studied by stopped-flow (Gray and Glew, 1973) and these experiments have recently been extended to several temperatures (Lewis et al., 1976). In both cases, a simple association–dissociation mechanism is consistent with the results. The results of stopped-flow NMR experiments (Grimaldi and Sykes, 1975) have been interpreted by suggesting conformational changes in the protein at pH 5.28 induced by Mn^{2+}, Ca^{2+}, and methyl α-D-mannopyranoside. The influence of the saccharide was observed by its effect on the proton relaxation enhancement of water by Mn^{2+}. A two-step mechanism for the binding of the sugar was proposed.

To increase the time resolution and the sensitivity of saccharide-binding kinetic experiments, we have applied the temperature-jump method using 4-methylumbelliferyl α-D-mannopyranoside (MUM[1]) as a ligand. Its fluorescence was reported to be quenched totally upon binding to con A and this effect is inhibited specifically by simple carbohydrates (Dean and Homer, 1973; Loontiens et al., 1977). We have studied the binding reaction of MUM to the dimer species of con A as a function of temperature and the reaction-rate parameters and thermodynamic parameters (activation and equilibrium) are presented. The results are interpreted in terms of the simplest consistent mechanism and are compared to the results of parallel equilibrium data determined by several methods (Loontiens et al., 1977; see preceding paper in this issue).

Experimental Procedure

Materials and Methods. MUM, con A, and acetylated and succinylated con A were prepared and purified as described in the preceding paper of this issue describing the equilibrium measurements (Loontiens et al., 1977). The preparations of

† From the Abteilung Molekulare Biologie, Max-Planck-Institut für Biophysikalische Chemie, D-34 Göttingen, Postfach 968, West Germany, and the Laboratory of General and Biological Chemistry, State University of Gent, B-9000 Gent, Belgium. *Received June 17, 1976.* This work was supported in part by the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek. A preliminary account of this work was delivered at the 5th International Biophysics Conference in Copenhagen, Abstract no. P-38, August 4–9, 1975.

‡ Recipient of an EMBO Fellowship.

[1] Abbreviations used are: con A, concanavalin A composed of intact polypeptide chain; MUM, 4-methylumbelliferyl α-D-mannopyranoside; RC_1, the galactose binding protein from *Ricinus communis.*

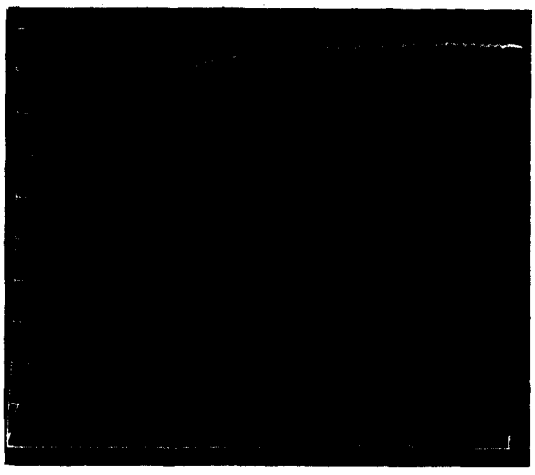

FIGURE 1: Example of the major relaxation signal observed with the con A–MUM system caused by a 3.2 °C temperature-jump. The fluorescence increases due to the dissociation of MUM from the quenched complex (excitation at 313 nm and emission measured above 360 nm). The total concentrations of con A binding sites and MUM were 123 and 3.73 μM, respectively. The pH was 5.5 and the final temperature 18.5 °C. The relaxation time is 79.2 ms and the amplitude of the signal is 5.9% of the steady-state signal.

all solutions and concentration determinations were the same as described there. Demetallized con A (Kalb and Lustig, 1968) contained <0.01 g-atom of Mn^{2+}, as determined with a Pye Unicom SP 98B atomic absorption spectrometer. The kinetic experiments with demetallized con A were carried out in doubly distilled deionized water to which only NaCl was added (0.1 M). The standard solution conditions, except where otherwise noted in the text, were 0.05 M NaOAc–HOAc, 1 M NaCl, 1 mM $NiCl_2$, 1 mM $CaCl_2$, and pH 5.5. These conditions are identical to those in the preceding paper of this issue (Loontiens et al., 1977).

The temperature-jump apparatus has been described (Rigler et al., 1974; Jovin, 1975). Fluorescence detection was used for the kinetic experiments. Some relaxations were also recorded by measuring the change in MUM absorbance upon binding. The relaxation times obtained by both methods were identical within the experimental error. Since the fluorescence measurements yielded a much larger signal, the absorbance measurements were not used in the kinetic analyses. A 200-W Hanovia 901-B Hg-Xe lamp was used as a light source. The excitation wavelength for the solutions containing MUM was 313 nm. 360-nm cutoff filters (Schott, WG 360) were placed in the two detection light paths. The excitation wavelength for the solutions containing only protein was 280 or 296 nm, and 320-nm cut-off filters (Schott, WG 320) were used. The excitation path length of the temperature-jump cell is 7 mm and the heated volume is 0.7 ml. A temperature jump of 3.2 °C resulted from an 18.3 kV discharge.

The temperature increase was determined by using 7-hydroxy-4-methylcoumarin as a fluorescence indicator to measure the temperature-dependent pH change of a tris(hydroxymethyl)aminomethane solution (Chen, 1968). The temperature increase was also calibrated using the temperature dependence of MUM fluorescence (2.2%/1 °C in the standard pH 5.5 buffer). Both calibrations agreed within 5%.

The distorting effect of lamp fluctuations is reduced by dividing the output of the detector phototubes by the output of a reference photomultiplier. The signal is amplified, digitized, stored, and analyzed with an on-line minicomputer (DEC no. PDP 11/20) (Jovin and Striker, 1977). The relaxation times and amplitudes were determined by a nonlinear iterative least-square analysis of the relaxation data (modified Gauss–Newton method). The nonlinear program fits up to two exponentials, including a sloping straight line (to include any very long relaxations). Each data record consists of 3072 sample points (consisting of two time regions) allowing accurate determination of the relaxation parameters.

The samples were made up individually rather than either titrating one component into the cell or diluting the cell content. They were allowed to stand at room temperature for several hours. Each sample was subjected to at least three temperature jumps. The con A-MUM solutions were stable to repeatedly applied temperature jumps. Each series of measurements was repeated at least twice with separately prepared stock solutions of protein and MUM. The relaxation times and amplitudes were reproducible within experimental error even with different con A preparations.

Stopped-flow experiments were performed with very similar instrumentation and data analysis system (Lehrach, 1974; Jovin, 1975). The temperature-jump cell was exchanged for a stopped-flow cell.

Results

Major Relaxation. A major relaxation effect, referred to below as the major relaxation, was observed when solutions containing MUM and con A were perturbed by temperature. Figure 1 is a relaxation curve showing the increase of the fluorescence of the solution due to the dissociation of the ligand from the complex. The relaxation times varied from 20 to 350 ms throughout the range of the reactant concentrations studied and this relaxation was absent in solutions containing only MUM or con A. The relaxation time was not changed upon increasing the concentrations of either Ni^{2+} or Ca^{2+} or both ions from 1 to 11 mM in a mixture of 3 μM MUM and 100 μM con A (these samples were allowed to stand at room temperature overnight). This finding implies the existence of saturating conditions for both ions. The relaxation was abolished by adding a large excess of methyl α-D-mannopyranoside or mannotriose, both known to be effective inhibitors of con A–polysaccharide interaction and agglutination. Thus, the signal is directly related to the binding of MUM to the sugar binding site of con A.

In the majority of experiments, the concentration of the protein was varied while keeping the concentration of the ligand constant (2–4 μM). This "titration procedure" minimizes the effect of MUM absorption on the relaxation amplitudes and also simplifies the subsequent data analysis (Jovin, 1975; Chock, 1971; Thusius, 1973).

The dependence of the inverse relaxation times on the concentrations of free ligand and protein is presented in Figure 2. The behavior of the data is consistent with a one-step mechanism of MUM binding with protein sites,

$$P + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PL;\ K_{ass} = k_1/k_{-1} \qquad (1)$$

where P = free protein sites, L = free MUM, and PL = complex, and the inverse relaxation time is a linear function of the free *equilibrium* concentrations ($\bar{P}$, $\bar{L}$), as in eq 2 (Eigen and DeMaeyer, 1963).

$$1/\tau = k_1(\bar{P} + \bar{L}) + k_{-1} \qquad (2)$$

We have calculated the rate constants and corresponding equilibrium constants from a large series of experiments using linear-regression analysis in terms of this mechanism (Table I).

TABLE I: Reaction-Rate Parameters, Activation Energies, and Equilibrium Constants Calculated from the Rate Constants Assuming a One-Step Mechanism of MUM Binding to Con A.[a]

Temp (°C)	k_{-1} (s^{-1})	$E_{act,k_{-1}}$ (kcal/mol)	$10^{-4} \times k_1$ ($M^{-1}\ s^{-1}$)	E_{act,k_1} (kcal/mol)	$K_{ass} = k_1/k_{-1}$ (mM^{-1})	$\Delta H°_{kin}$[b] (kcal/mol)
13.5	1.5 ± 0.1		6.3 ± 0.2		43	
18.5	2.4 ± 0.1		8.5 ± 0.3		35	
24.1	3.4 ± 0.1	15 ± 2	11.3 ± 0.4	10 ± 1	33	−5 ± 2
28.1	5.6 ± 0.2		15.7 ± 0.5		28	

[a] The definitions of the rate constants are given in the text (eq 1). E_{act} is the Arrhenius activation energy. [b] The $\Delta H°$ can be calculated either from the activation energies ($E_{act,k_1} - E_{act,k_{-1}}$) or from a van't Hoff plot. The $\Delta H°_{kin}$ refers to the enthalpy of reaction, which is obtained from the kinetic constants.

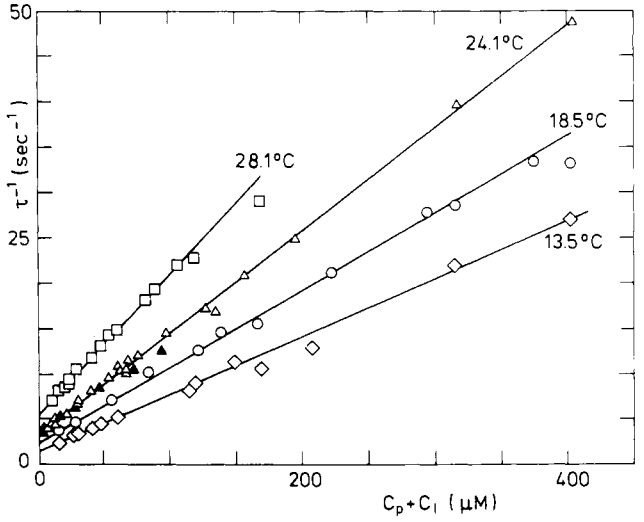

FIGURE 2: A plot of the inverse relaxation time (τ^{-1}) vs. the sum of the concentrations of free binding sites of con A (dimer) and free MUM. The free concentrations were calculated from the equilibrium constants resulting from the equilibrium measurements (Loontiens et al., 1977). The data are presented for four final temperatures: (◇) 13.5 °C, (○) 18.5 °C, (△) 24.1 °C, (□) 28.1 °C. Each symbol represents the average of at least three temperature jumps. Most of the experiments represented are kinetic "titration" series with the MUM concentration between 2 and 4 μM and varying protein concentrations. The data at 24.1 °C are for three individual protein preparations and include a series of kinetic "dilution" experiments (▲); see Methods. The MUM concentration was 7.7 μM for all protein concentrations greater than 300 μM. The straight lines represent linear fits through the data at each temperature, each point weighted by τ^4. The estimated error of the relaxation parameters is about 5% (this refers to the individual times and amplitudes).

TABLE II: Reaction Enthalpy as Determined from the Kinetic Amplitude Data.

Final Temp of Temp-Jump (ΔT = 3.2 °C) (°C)	$\Delta H°$[a] (kcal/mol)	$\Delta H°$[b] (kcal/mol)
13.5	−5.9	−5.6
18.5	−7.4	−6.4
24.1	−6.6	−6.5
28.1	−6.2	[c]
	−6.5[d]	−6.2[d]

[a] As determined from the $\Delta amp/\Delta\Phi$ vs. g^{-1} of Figure 4. [b] As determined from the relative amplitude plots of Figure 5. [c] The data at this temperature were acquired over a period of several days and the stability of the static measurements was not sufficient for a reliable analysis of this type. [d] Mean value.

The temperature-jump experiments were carried out at four temperatures (Figure 2) in order to obtain activation parameters and to compare the resulting molar reaction enthalpy changes with those determined from the equilibrium experiments (Loontiens et al., 1977). The activation energies (E_{act}) determined from Arrhenius plots are listed in Table I. The reaction enthalpy can be determined from the rate constants by defining an apparent association equilibrium constant ($K_{ass} = k_1/k_{-1}$) and constructing a van't Hoff plot. The value of $\Delta H°$ is listed in Table I.

To check for interactions between binding sites on the protein which would depend upon the extent of binding, it is important to measure the kinetics throughout a sufficiently large region of fractional binding. If the sites interact such that the kinetic constants depend upon the occupancy of neighboring sites, a kinetic "dilution" experiment can be diagnostic (in such an experiment, both reactant concentrations are varied but the ratio of their concentrations is kept constant (Jovin, 1975)). A series of dilution experiments was performed at 24.1 °C using 1:1 molar mixtures of MUM and con A in the total concentration range 1.8–250 μM. The amplitudes of these experiments were corrected for the absorption of the exciting light due to the high concentrations of MUM. The results were fully consistent with those from the titration experiments, even at total concentrations of the reactants resulting in 60% saturation of the binding sites (Figure 2). Thus, by kinetic and equilibrium (Loontiens et al., 1977) criteria, there exists no interaction between the carbohydrate binding sites of dimeric con A.

The relaxation amplitudes contain information about the reaction molar enthalpy change (Jovin, 1975; Thusius, 1972, 1973) and can be used to help decide upon a consistent mechanism (see Discussion). If the data are consistent with the mechanism of eq 1, the relaxation amplitude divided by the change in the fluorescence of a molar solution of ligand upon binding, $\Delta\Phi$, will be a linear function of $(1/\overline{PL} + 1/\overline{P} + 1/\overline{L})^{-1}$, where the symbols are the same as defined for eq 1. The intercept should be zero and the slope should be equal to the change in the log of the equilibrium constant ($\Delta\ln(K_{ass})$) caused by the temperature jump. Figure 3 is such a plot and the $\Delta H°$'s calculated from the slopes are given in Table II.

The relative amplitude is defined to be the observed amplitude normalized to the difference between the measured fluorescence of the T-jump cell and the calculated fluorescence of the solution assuming no binding (i.e., where only the *free* ligand and *free* protein sites are present in the solution) (Jovin, 1975). The calculated fluorescence (F_0) is determined by measuring separately the fluorescence of standard solutions of MUM and protein in the T-jump cell. The dependence of

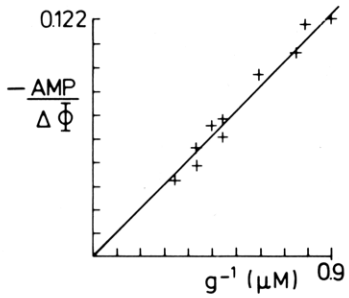

FIGURE 3: A representative plot of the fluorescence amplitude (AMP) which has been normalized to the signal change per micromolar concentration of MUM ($\Delta\Phi$) vs. the function $g^{-1} = (1/\overline{PL} + \overline{P} + \overline{L})^{-1}$. (See the text for the definitions of the barred quantities.) The data are shown for a series of intact subunit con A–MUM relaxation data at 18.5 °C. Each point is an average of at least three temperature jumps. The solid line is computed from a linear least-square analysis of the data with the intercept fixed at the origin. The free equilibrium concentrations are calculated from the association constant determined in the preceding paper of this issue (Loontiens et al., 1977). The slope is equal to $-\Delta H^\circ/RT^2$ provided that the mechanism is a bimolecular association reaction with a single step (see Table II).

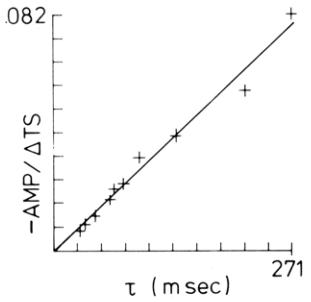

FIGURE 4: A representative plot of the relative fluorescence amplitude ($AMP/\Delta TS$) vs. the relaxation time (τ) for the intact subunit con A–MUM relaxation data at 18.5 °C. ΔTS is the difference between the total measured fluorescence signal of the solution and the calculated total fluorescence signal of the solution assuming no binding. The solid line is the linear least-square fit of the data where both the slope and the intercept are fitted parameters. (See the text for a discussion of this plot.) The slope is equal to $-k_{-1}\,\Delta\ln(K)$ for a simple one-step bimolecular binding mechanism. See Table II for a listing of the reaction enthalpies determined from this plot of the relaxation data for all temperatures studied.

the relative amplitude on the relaxation time is given in eq 3 for the kinetic model of eq 1.

$$\Delta F/(F_m - F_0) = (\Delta\ln(K_{ass}))k_{-1}\tau$$
$$\Delta H^\circ = RT^2(\Delta\ln(K_{ass}))/\Delta T \qquad (3)$$

where ΔF = amplitude, F_0 = calculated fluorescence, and F_m = measured fluorescence. The ΔH°'s determined from these *relative* amplitude plots are given in Table II. Figure 4 is a representative plot of the relative amplitudes of relaxation as a function of the relaxation time.

Measurements of the Major Relaxation with Demetallized, Succinylated, or Acetylated Con A. Several derivatives of con A were tested for binding to MUM and were also used to investigate further the other relaxation effects reported in the section below. Demetallized con A is known not to bind saccharides (Kalb and Levitzki, 1968; Agrawal and Goldstein, 1967) and accordingly does not show the major relaxation.[2] In mixtures of MUM and acetylated or succinylated con A, however, the major relaxation was observed. A few selected experiments in the concentration range of 40–250 μM of these con A derivatives, using 2 μM MUM, gave relaxation times that agreed with those calculated from the rate parameters for underivatized con A.

[2] In this case, the demetallized protein solution contained the standard buffer solution (see Loontiens et al., 1977, Methods) for pH 5.5. Only the bivalent metals were absent.

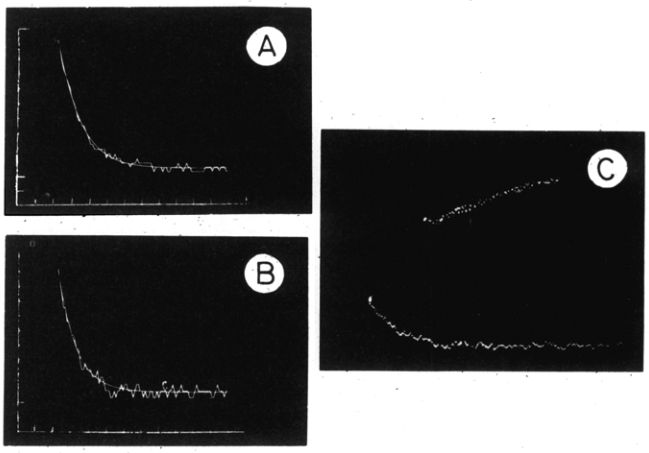

FIGURE 5: (A) Fast relaxation of con A dimer (135 μM binding sites) without added saccharide ligand, pH 5.5, 1 mM Ni^{2+}, 1 mM Ca^{2+}, T = 18.5 °C. The relaxation amplitude is −0.3% of the total fluorescence signal and the relaxation time is 12.7 ms. The smooth curve represents the computer fit of the data. Excitation = 280 nm, emission > 320 nm. (B) Fast relaxation of con A dimer (122 μM binding sites) with 1.04 mM methyl α-D-mannopyranoside, pH 5.5, 1 M NaCl, 1 mM Ca^{2+}, 1 mM Ni^{2+}, T = 18.5 °C. The relaxation amplitude is −0.3% of the total fluorescence signal and the relaxation time is 10.2 ms. The smooth curve represents the computer fit of the data. Excitation = 280 nm, emission > 320 nm. (C) Slow relaxation (top) and for comparison the corresponding fast relaxation (bottom); pH 5.5, 1 M NaCl, 1 mM Ca^{2+}, 1 mM Ni^{2+}, 122 μM con A binding sites, 1.04 mM methyl α-D-mannopyranoside, T = 18.1 °C. Excitation = 313 nm, emission > 320 nm. The relaxation time of the fast relaxation of this solution is 10.6 ms. The total time shown for the slow relaxation is 1 s.

Faster and Slower Relaxations. The relaxation curves recorded with solutions containing protein at concentrations higher than 200 μM exhibited two additional relaxation effects which became more pronounced as the protein concentration was increased (Figure 5). These additional effects could be increased in magnitude by exciting the protein fluorescence at 280 or 296 nm, and were also observed with solutions containing only protein (no MUM or other saccharides).

The magnitude of the faster relaxation, relative to the static protein fluorescence was ca. −0.3% (exciting the protein fluorescence and observing above 320 or 360 nm; ΔT = 3.2 °C). The amplitude and relaxation time (about 6 ms at 24.1 °C and 13 ms at 18.5 °C) were unaffected by the presence or absence of α-D-methyl mannopyranoside (see Figure 6), and were also independent of the protein concentration over a sevenfold range (see Figures 6 and 7).

The slower effect was much too slow to determine the relaxation parameters but could definitely be shown not to be due to cooling of the solution or photolysis. Within the time range of the relaxation measurements, the slow signal was of the same order of magnitude as the faster effect but opposite in direction (see Figure 5). Methyl α-D-mannopyranoside did not have a perceptible effect on this signal, although this was very difficult to judge due to the very long times and small amplitudes.

Both of the protein-related kinetic processes were investigated further in a series of experiments with demetallized, succinylated and acetylated con A. The pertinent information and conclusions are given in Table III.

Stopped-Flow. Most stopped-flow experiments were performed with concentrations of con A binding sites in large

TABLE III: Summary of Experiments Studying the Faster and Slower Relaxations.

Case	Protein Present	Ligand	Relaxations Obsd	Conclusions
A	Con A dimer metallized, pH 5.5.	None	4 ms faster relaxation and a very long time (5 s); the fast time is independent of protein concentration.	The fast time is probably not directly related to ligand binding (see F).
B	Con A dimer demetallized, pH 5.5 (cannot bind saccharides).	None	Same as for case A but the fast amplitude is larger.	Same as for case A; the amplitude of the fast time may be affected by Ca^{2+} or Ni^{2+} ions.
C	Same as case B.	MUM	Same as case B; no major relaxation signal due to MUM binding.	Major relaxation signal definitely due to MUM binding; faster time not affected by MUM binding.
D	Con A acetylated or succinylated, pH 5.5 (cannot form tetramers).	None	No fast relaxation seen; slow relaxation still present	Slower relaxation not due to tetramer-dimer conversion; fast relaxation either abolished or amplitude diminished.
E	Same as case D.	MUM	No faster relaxation present and the major relaxation has same kinetic parameters as case A plus MUM; slower relaxation still present.	Fast relaxation probably not related to the MUM binding (also same conclusions as D); binding kinetics same with derivatized and nonderivatized con A
F	Same as case A.	α-Methyl-mannoside (in excess)	Fast and slow relaxations still present; faster relaxation has same time and amplitude as with no saccharide present.	Faster relaxation is not related to saccharide binding

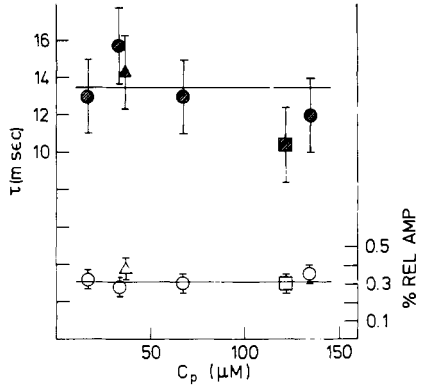

FIGURE 6: Protein concentration dependence of the fast relaxation time and amplitude in the absence of ligand (○) and the effect of adding methyl α-D-mannopyranoside (△ and □ refer to 0.31 and 1.03 mM methyl α-D-mannopyranoside, respectively), pH 5.5, T = 18.5 °C. The protein concentration (C_p) is given in terms of protomer species. The shaded symbols refer to the relaxation times and the unshaded symbols are the percent relative amplitudes (100 × amplitude/total signal). The protein fluorescence is excited at 280 nm and the fluorescence is observed above 320 nm. The error bars refer to the scatter of the fitted parameters for several temperature jumps on the same samples. The straight lines indicate the average of the relaxation times and amplitudes in the absence of ligand.

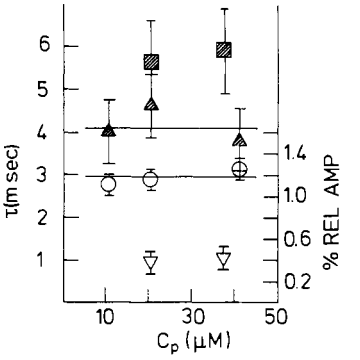

FIGURE 7: Protein concentration dependence of the fast relaxation time (▲) and relative amplitude (○) for demetallized con A at 24.1 °C. Percent relative amplitude = 100 × amplitude/total signal. For comparison, the values of the relaxation time (■) and the percent relative amplitude (▽) for the intact subunit con A without ligand, but in the presence of 1 mM Ca^{2+} and 1 mM Ni^{2+}, pH 5.5, T = 24.1 °C, are given in the same concentration range. The protein concentration (C_p) is given in terms of the protomer species. The straight lines refer to the average of the relaxation times and amplitudes for the demetallized protein. The error bars present the reproducibility of several temperature jumps on the same solution.

excess over that of MUM. These experimental conditions define a pseudo-first-order situation. The time course of a bimolecular association reaction with the mechanism of eq 1 can be represented by an exponential decay with a time constant equal to $k_1(P_0) + k_{-1}$, where P_0 is the total concentration of con A binding sites. An example of a stopped-flow experiment is shown in Figure 8 and the analysis of a concentration series is represented in the insert of this figure. The results of the stopped-flow experiments were in agreement with the kinetic constants determined from the results of the major relaxation in the temperature-jump studies.

Discussion

It has already been indicated under Results that the kinetic results for the major relaxation are qualitatively consistent with a one-step binding mechanism (see eq 1).

The thermodynamic parameters which result from the analysis of the data also attest to the completeness of the simple one-step binding model. The inner consistency of the $\Delta H°$'s derived from the data for all temperatures (Table I) and from each temperature individually (Table II) indicate that the enthalpy of reaction is constant throughout the temperature range of 13–28 °C. In addition, the value of $\Delta H°$ determined from equilibrium measurements on the same system by several methods (ca. −8 kcal/mol; see preceding paper in this issue, Loontiens et al., 1977) agree well with the values determined kinetically. This finding indicates that the major relaxation which we observe represents the total binding mechanism and that there does not exist a slower process which we are not observing but which contributes greatly to the reaction enthalpy of saccharide binding.

The forward rate constants listed in Table I are definitely not in the range to be expected for diffusion-controlled reactions (Eigen et al., 1964). Second-order rate constants for the formation of enzyme–substrate complexes are generally in the range of 10^6–10^7 M^{-1} s^{-1} (Hammes and Schimmel, 1970),

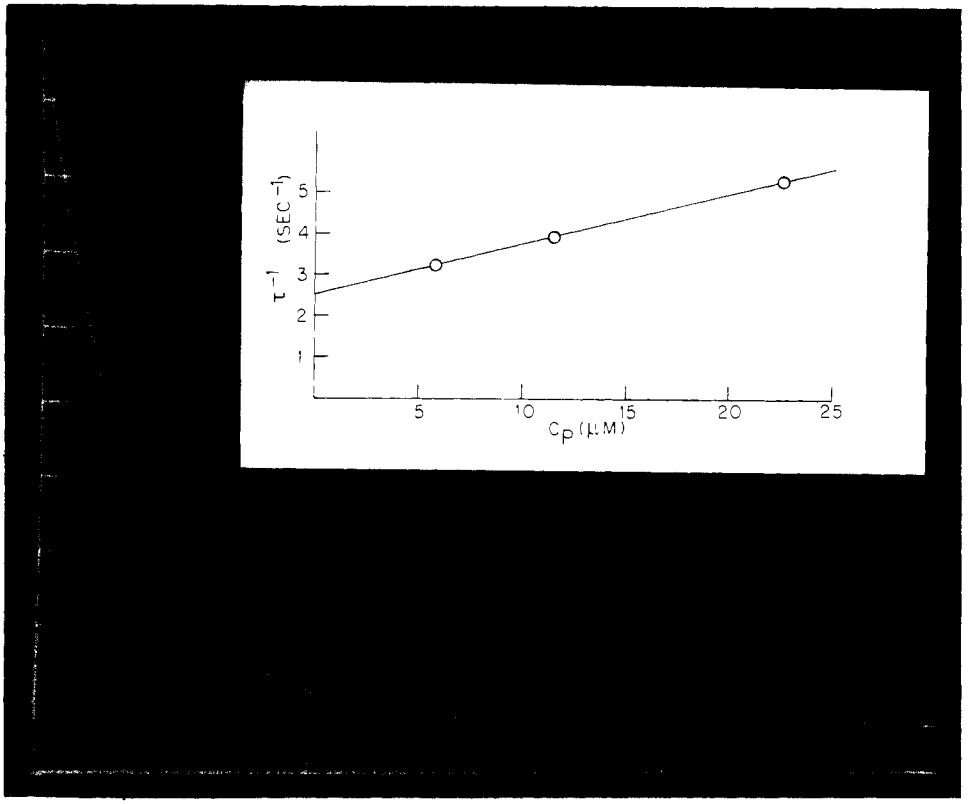

FIGURE 8: Representative stopped-flow kinetic curve. The total time shown is 2.78 s. The final protein and MUM concentrations were 11.5 and 0.5 μM, respectively, and the standard buffer conditions were used. The excitation wavelength was 313 nm and the fluorescence was collected above 360 nm. The conditions of the stopped-flow experiments were such that a pseudo-first-order approximation applied and the time constant of the reaction curve shown is 256 ms. The insert is a plot of the inverse time constant (vertical axis) of a short series of stopped-flow experiments where the protein concentration (horizontal axis) was varied, keeping the concentration of MUM constant (0.5 μM). The temperature was 21 °C. Each point represents the average of at least five individual stopped-flow experiments. The slope and intercept of the linear fit yield the forward and reverse rate constants of 1.2×10^5 M^{-1} s^{-1} and 2.5 s^{-1}, respectively, assuming the bimolecular mechanism of eq 1.

which are also less than the expected theoretical diffusion limit. The rate constant we observe is $\sim 10^5$ M^{-1} s^{-1}, which is considerably smaller than even this range of values, and this may be an indication that if our experiments were extended to higher concentrations we would observe the kinetic effect of a previous fast bimolecular step upon the major relaxation. However, higher concentrations of the protein or ligand are impractical.

The large activation energy (Table I) is also inconsistent with a diffusion-controlled step. The expected values for the activation energy of such a reaction would only be of the order of a few kilocalories at the most (Eigen et al., 1964). The apparent activation entropy (which we calculate from the effect of temperature on the forward rate constant) of ca. −3 eu is of the same order that might be expected from a bimolecular reaction where a solvent water molecule is replaced by a reactant ligand (Jencks, 1969). Assuming the validity of the calculation (Hammett, 1970; Schlager and Long, 1963), the small value of $\Delta S^{\ddagger}$ implies an energetic and not an entropic barrier for the binding reaction. Recent reports (Becker et al., 1976; Hardman and Ainsworth, 1976) indicate that the specific binding site on the con A molecule is in the vicinity of the metal binding sites, in agreement with magnetic resonance measurements (Brewer et al., 1973a), not in a deep cavity separated by 20 Å from the metal binding sites (Becker et al., 1975). This would be consistent with our results of low entropic contributions to the overall binding reaction. To investigate directly the possibility of a faster association step between MUM and con A, we searched the time regions below the millisecond time range. No absorbance or fluorescence relaxations were observed, except for the ligand-independent effects discussed under Results.

Further experiments monitoring the polarization or the anisotropy of the fluorescence showed no relaxation in any time region. It thus appears that MUM does not form a fluorescent intermediate with con A before it is quenched such that the rotational mobility of the MUM is affected to an extent detectable by polarization measurements. If such a complex would exist, it should have affected the amplitude of the MUM fluorescence to an observable extent.

The results summarized in Table III show that the faster relaxation time and amplitude seen by protein fluorescence are independent of ligand or protein concentrations. The demetallized protein also showed this effect in the same time range, but with a fourfold larger relative amplitude. Thus, the process may be affected by, but does not require, the presence of the Ca^{2+} and Ni^{2+}.

Acetylated and succinylated con A can only form dimers (Gunther et al., 1973) and despite the derivatization the equilibrium binding characteristics of these forms of con A are only slightly changed from those of normal con A (Loontiens et al., 1977; Gunther et al., 1973). The kinetic experiments with these derivatives showed only very minor variations in the kinetics of the binding process compared to the unmodified intact subunit con A. These results further substantiate the conclusions of the equilibrium experiments that the major effect of succinylation and acetylation is only to change the aggregation properties of the protein. The faster protein relaxation, however, was absent in these derivatives, suggesting that the underlying process may be localized in the contact regions

responsible for tetramer formation. However, the relaxation is not concentration dependent. Furthermore, the tetramer form of con A (pH 7.2), with no MUM, also showed this relaxation with similar times and amplitudes (to be published), indicating that this process is not due to a tetramer–dimer equilibrium. It is, of course, possible that the physical process reflected by the fast relaxation is still present in the derivatized proteins but that the amplitude (e.g., the $\Delta H°$) is diminished.

The very slow relaxation was present in all protein samples used in this study. Its presence in the case of demetallized and derivatized proteins suggests the lack of a direct relationship to ion or ligand binding or to the state of aggregation of con A.

It is interesting at this point to show the limitations that can be placed on the value of the association constant for a putative intermediate complex assuming the model of eq 4,

$$P + L \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} pl \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} PL$$

$$K_I = k_1/k_{-1}; K_{II} = k_2/k_{-2} \qquad (4)$$

where P = free protein sites; L = free MUM; pl = intermediate complex; PL = final quenched complex, under the assumption that the first step is much faster than the second. Such a mechanism is commonly proposed for a substrate binding to an enzyme (Hammes and Schimmel, 1970). This is discussed because such a complex (pl) could exist and be unobservable if there is no signal change and/or if the $\Delta H°$ is zero (or almost) for the first step. Grimaldi and Sykes (1975) propose just such a mechanism to explain their NMR results (although they did not observe the first step directly). If the first step, under all conditions, equilibrates much more rapidly than the second (i.e., the second step is kinetically uncoupled from the first), it can be shown that the observed longer relaxation time is given by

$$1/\tau = k_2(\hat{K}_I(\overline{P} + \overline{L})/(1 + K_I(\overline{P} + \overline{L}))) + k_{-2} \qquad (5)$$

The inverse relaxation-time plots will, therefore, not be linear unless $K_I \ll (\overline{P} + \overline{L})^{-1}$, in which case eq 5 reduces to

$$1/\tau = k_2(K_I(\overline{P} + \overline{L})) + k_{-2} \qquad (6)$$

Eq 6 has the same functional form as eq 2 and, thus, the measured constant would now be an apparent quantity (equal to $K_I k_2$) and the reverse rate constant would be k_{-2}. Since our $1/\tau$ plots are linear within the experimental error even at free concentrations of about 400 μM, the association constant of the first step (K_I) of the mechanism of eq 4 would have to be less than 250 M^{-1}. Grimaldi and Sykes (1975) have estimated the association constant of the first step of the mechanism in eq 4 to be 200 M^{-1} (although it must be emphasized that their solution conditions, especially with respect to the metal ion concentrations, were very different from ours). The behavior of the amplitude plots discussed in the results would be unaffected under these conditions. Subject to these boundary conditions, we cannot discard the mechanism of eq 4, but its existence would imply that either the $\Delta H°$ of the first step is zero and/or there is no spectroscopic change upon the formation of the intermediate.

X-ray crystallographic studies (Becker et al., 1976) and changes in the circular dichroic spectrum of con A upon binding of methyl α-D-mannopyranoside (Pflumm et al., 1971) suggest that a conformational change in the protein takes place upon binding of the saccharide. We find no direct kinetic evidence for conformational transitions in the protein but cannot exclude small changes concomitant with ligand binding (Loudon and Koshland, 1972). A somewhat similar situation would occur if the mechanism of eq 4 was to apply and the second step, corresponding to such a conformational change, was faster and kinetically uncoupled to the first step. Provided that there was no fluorescence or absorption signal change accompanying the second step, we would observe the same kinetic behavior as for a one-step reaction and the resulting equilibrium constants and reaction enthalpy determined from both the kinetic and equilibrium measurements would agree.

We conclude by relating our studies to others concerned with the kinetics of saccharide binding to con A. A recent report by Lewis et al. (1976), which appeared as this paper was in final preparation, has extended the experiments of Gray and Glew (1973) to several temperatures. Both of these investigations used *p*-nitrophenyl α-D-mannopyranoside as the binding saccharide and investigated the kinetics with the stopped-flow technique. No evidence was found which would indicate a more complex mechanism than a simple bimolecular reaction and the rate constants obtained were in the same range as we find with the umbelliferyl derivative. Thus, the binding process is not grossly affected by the chromophoric group, which is attached to the carbohydrate moiety. Lewis et al. (1976) reported activation parameters for the binding reaction which are in very close agreement with our data. They also estimated that if the mechanism of eq 4 is operative, K_I must be less than 350 M^{-1}, which is roughly the same upper boundary on K_I that we have been able to derive (see above). The other stopped-flow kinetic study involving a simple saccharide (Grimaldi and Sykes, 1975) is difficult to compare because the experimental conditions were much different (see the discussion above). Rate constants for the binding of methyl α-D-glucopyranoside (and also methyl β-D-glucopyranoside) to Zn- and Mn-con A have been estimated from NMR data (Brewer et al., 1973b; Brewer et al., 1975). The dissociation rate constants are a factor of 10 to 100 times larger than what we observe for the binding of MUM. The forward rate constants can be estimated from the equilibrium constants to be 5×10^4 and $2.8 \times 10^4 M^{-1} s^{-1}$, respectively, for the α and β isomers. These values are somewhat lower, but still in the range of values which we have found for MUM. Thus, the difference in the stability between these manno- and glucopyranoside moieties seems to be due mainly to the longer time of residence of the mannopyranoside on the con A. Recent results (Podder, S. K., personal communication) of temperature-jump experiments indicate that the binding of *p*-nitrophenyl β-D-galactopyranoside to the galactose binding protein from *Ricinus communis* (RC_1[1]) (Nicolson et al., 1974) has a forward rate constant of about $5 \times 10^5 M^{-1} s^{-1}$ and a dissociation rate constant of approximately 30 s^{-1} (0.05 M phosphate buffer at pH 6.8, 0.2 M NaCl). This is also certainly lower than would be expected for a diffusion-controlled reaction and is of the same order that we observe with con A and MUM.

The rate of con A binding to RC_1 (itself a glycoprotein containing mannose residues) has been measured by following the change in turbidity of the solution as the binding progresses (Podder et al., 1974). The forward rate constant, assuming a one-step bimolecular reaction, is more than an order of magnitude lower than we observe with MUM or than is found for *p*-nitrophenyl α-D-mannopyranoside (Gray and Glew, 1973; Lewis et al., 1976). This difference may be due to the more complicated nature of the ligand (a glycoprotein), which may involve other interactions in addition to the simple recognition of a carbohydrate, or it could be possible that the turbidity

measurement is sensitive to a later stage of aggregation, which would complicate the analysis of the primary binding event.

The binding of isolated fat cells to ^{125}I derivatives of con A has been studied (Cuatrecasas, 1973). From these data, Gray and Glew (1973) estimated a lower bound for the association rate constant of 10^6 M^{-1} s^{-1}. If this estimate is correct, it seems that an additional process is involved for the binding of con A to receptors in membranes, as compared to the binding to simple saccharides. The reason for the increase in the rate constant of association when con A binds to a membrane-bound receptor is not clear but may be related to the arrangement of the receptors in the membrane.

It is tempting to consider the possible correlation between the extremely slow kinetics of ligand association and dissociation and the biological properties of con A. The multivalent interactions required for the agglutination and possibly mitogenic potency should be enhanced if complexes form with long life-times during which appreciable lateral diffusion (Elson, 1976; Schlessinger et al., 1976) can take place (about 1 s for con A assuming the general validity of our kinetic constants). The discrepancies observed between binding isotherms and the concentration-dependent cellular activation (state function) (Ferber et al., 1976) might arise, therefore, from the finite probability of forming high-order interactions within the time window dictated by the dissociation rate constant. Conceivably, a lectin exchanging rapidly between free and bound states might not be as effective in inducing a cellular response as another molecule with the same specificity and thermodynamic affinity but characterized by lower rates of reaction. It will be of interest to examine the lectins other than con A for a possible correlation between mitogenic activity and kinetic properties.

In summary, we conclude that the binding reaction of MUM to con A is compatible with a simple bimolecular mechanism, represented by eq 1, in agreement with the kinetic mechanism proposed by Gray and Glew (1973) and with Lewis et al. (1976) for the binding of *p*-nitrophenyl α-D-mannopyranoside with con A. The binding sites of the con A dimer are independent and identical, and the kinetic constants of binding for the succinylated and acetylated derivatives of con A are very similar to the intact subunit nonderivatized con A. Even though the reaction can be completely described by a one-step bimolecular reaction, the rate constant for the association is not a diffusion-controlled process, and the barrier for the reaction is energetic, not entropic. The binding process shows a normal behavior upon temperature (linear Arrhenius plot), indicating no large heat-capacity effects or a major change in the protein over the temperature range studied which affects the binding mechanism. Experiments are presently in progress on the tetramer form of con A, and the preliminary results indicate that the tetramer reacts very similarly to the dimer form described in this paper.

Acknowledgments

The authors gratefully acknowledge George Striker for the development of the on-line data acquisition computer programs. We thank Dr. Sunil K. Podder for showing us his data prior to publication.

References

Agrawal, B. B. L., and Goldstein, I. J. (1967), *Biochim. Biophys. Acta 147,* 262.

Becker, J. W., Reeke, Jr., G. N., Cunningham, B. A., and Edelman, G. M. (1976), *Nature (London) 259,* 406.

Becker, J. W., Reeke, Jr., G. N., Wang, J. L., Cunningham, B. A., and Edelman, G. M. (1975), *J. Biol. Chem. 250,* 1513.

Beckert, W. H., and Sharkey, M. M. (1970), *Intern. Arch. Allergy Appl. Immunol. 39,* 337.

Brewer, C. F., Sternlicht, H., and Grollman, A. P. (1975), *Adv. Exp. Med. Biol. 55,* 55.

Brewer, C. F., Sternlicht, H., Marcus, D. M., and Grollman, A. P. (1973a), *Proc. Natl. Acad. Sci. U.S.A., 70,* 1007.

Brewer, C. F., Sternlicht, H., Marcus, D. M., and Grollman, A. P. (1973b), in Lysosyme, Osserman, E., Beychok, S., and Canfield, R., Ed., New York, N.Y., Academic Press, p 239.

Chen, R. F. (1968), *Anal. Lett. 1,* 423.

Chock, P. B. (1971), *Biochemie 53,* 161.

Chowdhury, T. K., and Weiss, A. K., Ed. (1974), *Adv. Exp. Med. Biol. 55.*

Cuatrecasas, P. (1973), *Biochemistry 12,* 1312.

Dean, B. R., and Homer, R. B. (1973), *Biochim. Biophys. Acta 322,* 141.

Edelman, G. M., Yahara, I., and Wang, J. L. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1442.

Eigen, M., and De Maeyer, L. (1963), *Tech. Org. Chem. 8,* 895.

Eigen, M., Kruse, W., Maass, G., and De Maeyer, L. (1964), *Prog. React. Kinet. 2,* 285.

Elson, E. L. (1976), in Membranes and Neoplasia, Progress in Clinical and Biological Research, Fox, F., Ed., New York, N.Y., Alan R. Liss (in press).

Ferber, E., Reilly, C. E., and Resch, K. (1976), *Biochim. Biophys. Acta* (in press).

Gray, R. D., and Glew, R. H. (1973), *J. Biol. Chem. 248,* 7547.

Grimaldi, J. J., and Sykes, B. D. (1975), *J. Biol. Chem. 250,* 1618.

Gunther, G. R., Wang, J. L., Yahara, I., Cunningham, B. A., and Edelman, G. M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 1012.

Hammes, G. G., and Schimmel, P. R. (1970), *Enzymes 2,* 67.

Hammett, L. P. (1970), Physical Organic Chemistry, New York, N.Y., McGraw-Hill, p 101.

Hardman, K. D., and Ainsworth, C. F. (1976), *Biochemistry 15,* 1120.

Inbar, M., and Sachs, L. (1969), *Proc. Natl. Acad. Sci. U.S.A. 63,* 1418.

Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology, New York, N.Y., McGraw-Hill, p 608.

Jovin, T. M. (1975), in Concepts in Biochemical Fluorescence, Chen, R., and Edelhoch, H., Ed., New York, N.Y., Marcel Dekker, p 305.

Jovin, T. M., and Striker, G. (1977), in Chemical Relaxation in Molecular Biology, Rigler, R., and Pecht, I., Ed. Springer-Verlag, West Berlin (in press).

Kalb, A. J., and Levitzki, A. (1968), *Biochem. J. 169,* 669.

Kalb, A. J., and Lustig, A. (1968), *Biochim. Biophys. Acta 168,* 366.

Lehrach, H. (1974), Dissertation, Technische Universität Carolo-Wilhelmina, Braunschweig, West Germany.

Lewis, S. D., Shafer, J. A., and Goldstein, I. J. (1976), *Arch. Biochem. Biophys. 172,* 689.

Loontiens, F. G., Clegg, R. M., and Jovin, T. M. (1977), *Biochemistry 16* (preceding paper in this issue).

Loudon, G. M., and Koshland, D. E. (1972), *Biochemistry 11,* 229.

Nicolson, G. L. (1973), *Nature (London), New Biol. 243,*

218.
Nicolson, G. L., Blaustein, J., and Etzler, M.E. (1974), *Biochemistry 13,* 196.
Pflumm, M. N., Wang, J. L., and Edelman, G. M. (1971), *J. Biol. Chem. 246,* 4369.
Podder, S. K., Surolia, A., and Bachhawat, B. K. (1974), *Eur. J. Biochem. 44,* 151.
Rigler, R., Rabl, C. R., and Jovin, T. M. (1974), *Rev. Sci. Instrum. 45,* 580.
Schlager, L. L., and Long, F. A. (1963), *Adv. Phys. Org. Chem. 1,* 1.
Schlessinger, J., Koppel, D. E., Axelrod, D., Jacobson, K., Webb, W. W., and Elson, E. L. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 2409.
Thusius, D. (1972), *J. Am. Chem. Soc. 94,* 356.
Thusius, D. (1973), *Biochemie 55,* 277.

Immunological Measurements of Conformational Motility in Regions of the Myoglobin Molecule†

John G. R. Hurrell,‡ John A. Smith,§ and S. J. Leach*

ABSTRACT: The conformational motilities of three regions of the sperm whale myoglobin molecule and of an isolated peptide of myoglobin have been examined by measuring the equilibrium constant for the native ↔ nonnative transition. The immunological approach of Furie et al. (Furie, B., Schechter, A. N., Sachs, D., and Anfinsen, C. B. (1975), *J. Mol. Biol. 92,* 497–506) was used with convenient modifications. Antibodies specific to the nonnative conformations were used in assaying for competition between the radioactively labeled peptide and native myoglobin. Labeling was by ^{125}I iodination of the peptide or its 3-(4-hydroxyphenyl)propionyl derivative, and separation of the immune complex from the free peptide was either by ammonium sulfate precipitation or by centrifugation of the antibodies immobilized on Agarose beads. For the antigenic regions of the sequence (1–55), the measured conformational equilibrium constant was 840 ± 200 at 22 °C; the value for the C-terminal region (132–153) was 280 ± 120 at 25 °C, while that for the region (66–76) adjacent to the heme group was greater than 2.5×10^6. Measurements on the isolated peptide (132–153) indicated that 1% of the molecules adopt native-type folding in aqueous solution at 36 °C.

Globular proteins in solution undergo conformational fluctuations during which their structure alternates rapidly between native and nonnative conformations (Anfinsen and Scheraga, 1975). The native structure, which normally predominates, is in equilibrium with a population of nonnative or "denatured" states that are present in concentrations below the limit of detection of physiochemical techniques.

Several approaches have been introduced to measure the overall dynamics of a protein molecule. Hydrogen–deuterium exchange experiments on insulin by Linderstrøm-Lang (1955) gave the first evidence for conformational flexibility of a native protein. The extent of this flexibility was recently estimated by Nakanishi et al. (1972) using the hydrogen–deuterium exchange method on lysozyme at 20 °C. Only 1 molecule/3×10^5 was in a nonnative conformation. Upon addition of 6.5 M LiCl, however, the number increased to 1/83, although there was no detectable change in molar ellipticity at 222 nm. Other physical methods which have been used to measure conformational equilibria in proteins usually focus on a group of chromophores (as in circular dichroism, ultraviolet difference spectroscopy, nuclear magnetic resonance, or fluorescence), and provide averaged information about their state of unfolding or flexibility. In special cases, attention can be focused on a single chromophore or chain segment. In general, however, the amount of information is limited by the sensitivity of these methods; their utility for quantitative measurements of conformational equilibria has been largely in the analysis of data acquired from transition curves in the region representing 10–90% unfolding.

The use of proteolytic enzymes as probes of protein unfolding (Rupley and Scheraga, 1963; Ooi and Scheraga, 1964; Klee, 1967; Imoto et al., 1974; and Burgess et al., 1975) has given qualitative information about local chain flexibility, and some indication of the sequence of events during unfolding. Kinetic studies using small chemical probes specific for individual protein residues are capable, in principle, of providing much quantitative information about regions surrounding the modifiable residues. Indeed, Vas and Boross (1974) studied regional conformational changes around a partially buried cysteine residue (Cys-153) of glyceraldehyde-3-phosphate dehydrogenase by a kinetic analysis of its reaction with *p*-mercuribenzoate. They concluded that 1 molecule/37 in the carboxymethylated holoenzyme at 25 °C was in a nonnative conformation, while the carboxymethylated apoenzyme had 1 in 18. This approach is restricted in its application to regions of a protein possessing unique and specifically reactive residues, but has obvious potential for active site residues in enzymes.

A highly sensitive immunological approach, which allows quantitative evaluation of the motility or tightness of folding of antigenic regions within protein molecules, but without the

† From the Russell Grimwade School of Biochemistry, University of Melbourne, Parkville, Victoria, 3052, Australia. *Received August 5, 1976.* This work was supported by a grant from the Australian Wool Corporation to S. J. Leach.
‡ Supported by the Commonwealth Serum Laboratories, Parkville, Victoria, 3052, and a Commonwealth Postgraduate Research Award.
§ Supported by a National Health and Medical Research Council (Australia) Postgraduate Medical Scholarship.