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A Kinetic Study of Protein–Protein Interactions†

Ruth Koren and Gordon G. Hammes*

ABSTRACT: Kinetic studies have been carried out of the monomer–dimer interaction of insulin, β-lactoglobulin, and α-chymotrypsin using stopped-flow and temperature-jump techniques. The pH indicators bromothymol blue, bromophenol blue, and phenol red were used to monitor pH changes associated with the monomer–dimer interaction. In all three cases a kinetic process was observed which could be attributed to a simple monomer–dimer equilibrium, and association (k_1) and dissociation (k_{-1}) rate constants were determined. The results obtained are as follows: for insulin at 23 °C, pH 6.8, 0.125 M KNO_3, $k_1 = 1.14 \times 10^8$ M^{-1} s^{-1}, $k_{-1} = 1.48 \times 10^4$ s^{-1}; for β-lactoglobulin AB at 35 °C, pH 3.7, 0.025 M KNO_3, $k_1 = 4.7 \times 10^4$ M^{-1} s^{-1}, $k_{-1} = 2.1$ s^{-1}; for α-chymotrypsin at 25 °C, pH 4.3, 0.05 M KNO_3, $k_1 = 3.7 \times 10^3$ M^{-1} s^{-1}, $k_{-1} = 0.68$ s^{-1}. The kinetic behavior of the separated β-lactoglobulin A and B was similar to that of the mixture. In the case of chymotrypsin, bromophenol blue was found to activate the enzyme catalyzed hydrolysis of *p*-nitrophenyl acetate, and a rate process was observed with the temperature jump which could be attributed to a conformational change of the indicator–protein complex. The association rate constant for dimer formation of insulin approaches the value expected for a diffusion-controlled process, while the values obtained for the other two proteins are below those expected for a diffusion-controlled reaction unless unusually large steric and electrostatic effects are present.

A number of studies have been directed toward characterizing the equilibrium properties of protein–protein interactions (cf. McKenzie, 1967; Blundell et al., 1972; Aune et al., 1971; Horbett and Teller, 1974). However, relatively little information is available concerning the dynamics of protein–protein interactions. In the work reported here, kinetic studies have been carried out of the self-association of insulin, β-lactoglobulin, and α-chymotrypsin using stopped-flow and temperature-jump techniques. Changes in pH accompanying the self-association have been used to monitor the course of the reaction.

Ultracentrifuge studies of insulin (Zn free) aggregation have been made at pH 2 (Jeffrey and Coates, 1966) and at neutral pH (Pekar and Frank, 1972). Monomer, dimer, tetramer, and higher aggregates have been used to account for the data at pH 2, while at neutral pH values monomer, dimer, hexamer and higher aggregates were found to accommodate the data better. Although a detailed study of the pH dependence of the aggregation equilibria is not available, the amount of polymerization appears to decrease with decreasing pH (Blundell et al., 1972). In the kinetic studies, attention was confined to a range of protein concentrations in which the monomer and dimer are the prevalent species.

The aggregation properties of β-lactoglobulin have been studied over a wide pH range (McKenzie, 1967; Albright

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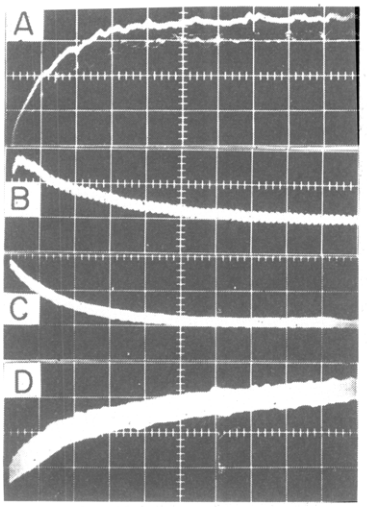

FIGURE 1: Typical kinetic traces for the monomer–dimer interaction of insulin, β-lactoglobulin AB and α-chymotrypsin. (A) Temperature-jump experiment with insulin (7.15 μM), bromothymol blue (20 μM), 0.125 M KNO_3 (pH 6.8) at a final temperature of 23 °C. The wavelength used for observation was 615 nm; the vertical scale is 10 mV/division and the horizontal scale is 50 μs/division. (B) Stopped-flow experiment with β-lactoglobulin AB (23 μM)–bromophenol blue (20 μM), 0.025 M KNO_3 (pH 3.7) at 35 °C. The wavelength used for observation was 590 nm; the vertical scale is 2 mV/division, and the horizontal scale is 0.1 s/division. (C) Stopped-flow experiment with α-chymotrypsin (84 μM), bromophenol blue (10 μM), 0.05 M KNO_3 (pH 4.0) at 25 °C. The wavelength used for observation was 590 nm; the vertical scale is 5 mV/division, and the horizontal scale is 0.5 s/division. (D) Same as C except pH 3.7.

and Williams, 1968; Visser et al., 1972; Towend et al., 1961; Timasheff and Towend, 1961; Kelly and Reithel, 1971; Zimmerman et al., 1970). In the pH range 4 to 5, β-lactoglobulin A exists in a dimer–octamer equilibrium at low temperatures, whereas β-lactoglobulin B is a dimer under similar conditions. Both proteins tend to dissociate to monomer below pH 4, and kinetic experiments were carried out under conditions where only monomer and dimer species should be present.

The dimerization of α-chymotrypsin in the pH range 2 to 6 has been studied extensively (Aune and Timasheff, 1971; Timasheff, 1969; Horbett and Teller, 1974). The standard free energy of association goes through a minimum at pH 4, and kinetic studies have been carried out in the pH range 3.7–4.3.

Rate constants characterizing the monomer–dimer interaction have been obtained for all three proteins and are compared with the rate constants for other protein–protein interactions.

Experimental Section

Materials. The Zn free porcine insulin was a generous gift of Dr. B. H. Frank of Eli Lilly. The β-lactoglobulin AB (mixture) and *p*-nitrophenyl acetate were obtained from Sigma and three times crystallized α-chymotrypsin from Worthington Biochemicals. The pH indicators bromothymol blue, bromophenol blue, and phenol red were purchased from Fisher Scientific Company. In some cases bromophenol blue was recrystallized from glacial acetic acid. However, the results obtained with the crystallized material did not differ significantly from those obtained with the commercial material. All other chemicals used were the best available commercial grades, and all solutions were prepared with distilled deionized water.

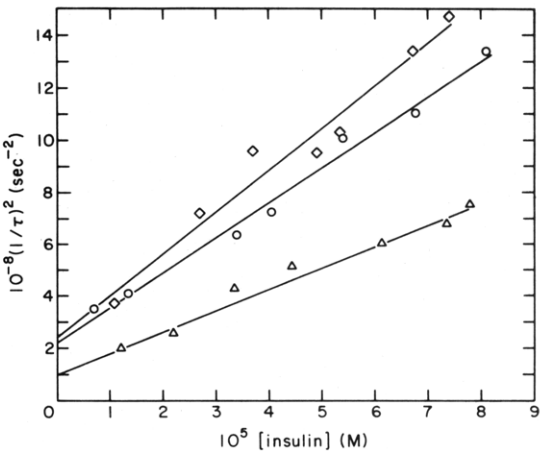

FIGURE 2: A plot of the square of the reciprocal relaxation time, $(1/\tau)^2$, vs. the total insulin concentration (calculated in terms of the monomer) in 0.125 M KNO_3: (○) 20 μM bromothymol blue, 23 °C, pH 6.8; (◇) 20 μM phenol red, 23 °C, pH 7.8; (△) 20 μM bromothymol blue, 14 °C, pH 6.8.

The A and B components of β-lactoglobulin were obtained from the commercial preparation essentially following the procedure of Piez et al. (1961) except that a DEAE-cellulose column was substituted for DEAE-Selectacell. The protein eluted at slightly higher salt concentrations than reported by Piez et al. (1961). The protein fractions of each component were pooled, precipitated with ammonium sulfate, and recrystallized (Armstrong et al., 1967).

Methods. The protein concentrations were determined spectrophotometrically using an extinction coefficient of 1.05 ml/(mg cm) at 276 nm and a molecular weight of 5800 for insulin (Frank and Veros, 1968), an extinction coefficient of 2.03 ml/(mg cm) at 282 nm and molecular weight of 24 800 for chymotrypsin (Smith and Hansch, 1973), and an extinction coefficient of 0.97 ml/(mg cm) at 280 nm and a monomer molecular weight of 18 000 for β-lactoglobulin (Span et al., 1974). The β-lactoglobulin AB solutions were dialyzed overnight against distilled water prior to use.

Difference spectra between protein–pH indicator mixtures and the pH indicator were determined with a Zeiss PMQ II spectrophotometer.

Steady state kinetic measurements of the chymotrypsin catalyzed hydrolysis of *p*-nitrophenyl acetate were carried out on a Cary 14 spectrophotometer by monitoring the reaction at 30 °C at 340 nm with a constant slit width of 1.75 mm (Hartley and Kilby, 1954; Gutfreund and Sturtevant, 1956). The difference in extinction coefficient between substrate and product was determined to be 7026 M^{-1} cm^{-1}. The reference spectrophotometer cell contained all reaction components except enzyme, thus automatically correcting for the small amount of nonenzymatic reaction.

Stopped-flow measurements were carried out on a Durrum D-10 stopped-flow spectrometer. In the stopped-flow experiments, an unbuffered protein–pH indicator–KNO_3 solution was mixed with an equal amount of unbuffered pH indicator–KNO_3 solution. The pH of the solutions was carefully adjusted with 10^{-2}–10^{-3} M KOH or HCl immediately prior to inserting them into the syringes of the stopped-flow instrument.

Temperature-jump measurements were carried out using the instrument and procedures previously described (Faeder, 1970). The temperature jump was 7.7 °C and the heat-

Table I: Rate Constants for $2A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A_2$.

Protein	pH	T (°C)	k_1 (M^{-1} s^{-1})	k_{-1} (s^{-1})	(k_1/k_{-1}) (M^{-1})
Insulin[a]	6.8	23	1.14 (±0.15) × 10^8	1.48 (±0.11) × 10^4	0.77 × 10^4
	7.8	23	1.31 (±0.31) × 10^8	1.55 (±0.23) × 10^4	0.85 × 10^4
	6.8	14	1.0 (±0.25) × 10^8	1.0 (±0.2) × 10^4	1.0 × 10^4
β-Lactoglobulin AB[b]	3.7	35	4.7 (±1.5) × 10^4	2.1 (±0.6)	2.3 × 10^4
α-Chymotrypsin[c]	4.3	25	0.37 (±0.09) × 10^4	0.68 (±0.12)	5.5 × 10^3

[a] 0.125 M KNO_3. [b] 0.025 M KNO_3. [c] 0.05 M KNO_3.

ing time of the instrument is about 8 μs. Solutions were prepared similarly to those used for the stopped-flow measurements.

The data from the stopped-flow and temperature-jump experiments were analyzed by an on-line computer system as described elsewhere (Hilborn and Hammes, 1973; Hilborn et al., 1973). Signal averaging of 5–10 kinetic traces was carried out, and the data were fit to a single exponential by a least-squares procedure.

Results

Insulin. Temperature-jump experiments on insulin–0.125 M KNO_3 solutions were carried out at pH 6.8 using bromothymol blue as a pH indicator and at pH 7.8 using phenol red as a pH indicator. The concentration range of insulin was 0.7–8.2 × 10^{-5} M. In most cases, the indicator concentrations were fixed at 2 × 10^{-5} M and the insulin concentration was varied; in some experiments the insulin concentration was fixed at 7.3 × 10^{-6} or 1.6 × 10^{-5} M and the pH indicator concentration was varied up to 10^{-4} M. A single relaxation process was observed under all conditions over the accessible time range, from 10 μs to about 500 ms. Slower kinetic processes were not observed in dilution experiments using the stopped-flow instrument. A typical kinetic trace is shown in Figure 1A. With bromothymol blue an increase in transmittance was observed at 615 nm and a decrease in transmittance at 430 nm. Since the absorbancy of the dye is significantly higher at 615 nm (the absorbance maximum of the basic form of the dye), this wavelength was generally utilized to monitor the reaction progress. With phenol red the reaction progress was monitored at 510 nm where a transmittance increase was observed.

The relaxation times calculated from the signal averaged data were found to vary significantly with insulin concentration at constant indicator concentration, but varying the concentration of indicator from 2 × 10^{-5} to 10^{-4} M did not alter the relaxation time within the experimental uncertainties (±10%). If the relaxation process is attributed to a monomer–dimer equlibrium (eq 1)

$$2A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} A_2 \qquad (1)$$

the reciprocal relaxation time, $1/\tau$, can be written as in eq 2 or 3

$$1/\tau = k_{-1} + 4k_1(\bar{A}) \qquad (2)$$

$$(1/\tau)^2 = (k_{-1})^2 + 8k_1k_{-1}(A_T) \qquad (3)$$

where $(\bar{A})$ is the equilibrium concentration of monomer and (A_T) is the total protein concentration expressed in terms of monomer. Plots of $(1/\tau)^2$ vs. (A_T) at pH 6.8 (14 and 23 °C) and pH 7.8 (23 °C) are shown in Figure 2. The scatter at pH 7.8 is somewhat greater than at pH 6.8, primarily due to the pH instability of the unbuffered solutions at pH 7.8. The pH was monitored before and after measurements, and the variation of pH was ±0.2 unit at the higher pH. A least-squares analysis of the data in Figure 2 according to eq 3 was used to obtain the rate constants in Table I. (Similar rate constants were obtained using eq 2, assuming an equilibrium constant, and using a least-squares analysis according to eq 2 to obtain an equilibrium constant from the ratio of rate constants. This new equilibrium constant was then used to recalculate the equilibrium concentrations, and the data again were analyzed according to eq 2. This process was repeated until the rate constants and assumed equilibrium constant were self-consistent.)

The concentration dependence of the relaxation times is in good agreement with the mechanism of eq 1. The possibility that a mechanism involving a dye–protein interaction is occurring seems unlikely in view of the lack of dependence of the relaxation time on dye concentration. In addition, a difference spectrum between dye–protein mixtures and dye could not be detected at pH 6.8 (5 × 10^{-5} M bromothymol blue, 2.5 × 10^{-5} or 5 × 10^{-5} M insulin, 0.1 M imidazole chloride) or pH 8 (5 × 10^{-5} M phenol red, 6.7–13.4 × 10^{-5} M insulin, 0.05 M potassium phosphate) at room temperature (23 °C). This indicates a strong indicator–insulin interaction does not occur. From the rate constants obtained at 14 and 23 °C, *approximate* Arrhenius activation energies of 2.5 and 7.4 kcal/mol can be calculated for k_1 and k_{-1}, respectively.

β-Lactoglobulin. Below pH 4, both β-lactoglobulin A and B exist in a monomer–dimer equilibrium at a conveniently accessible concentration range (McKenzie, 1967; Albright and Williams, 1968; Towend et al., 1961; Visser et al., 1972). The maximum sensitivity for studying the kinetics of the monomer–dimer reaction with relaxation processes is under conditions where approximately equal amounts of monomer and dimer are present. In addition the total protein concentration should be as large as possible relative to the hydrogen ion concentration so that the changes in pH used to monitor the reaction are significant compared with the equilibrium hydrogen ion concentration. Conditions were optimized by varying the pH, ionic strength, and temperature until a relaxation effect with an easily measurable amplitude was obtained in stopped-flow dilution experiments. The final conditions employed were pH 3.7, 0.025 M KNO_3, 35 °C, 1–10 × 10^{-5} M β-lactoglobulin AB and 2 × 10^{-5} M bromophenol blue (concentrations after mixing). Under these conditions a single relaxation process was observed in dilution experiments with the stopped-flow apparatus. (The amplitude of the relaxation effect increased with increasing temperature and decreasing ionic strength.) No relaxation processes were found in temperature jump experiments; apparently the amplitude of the

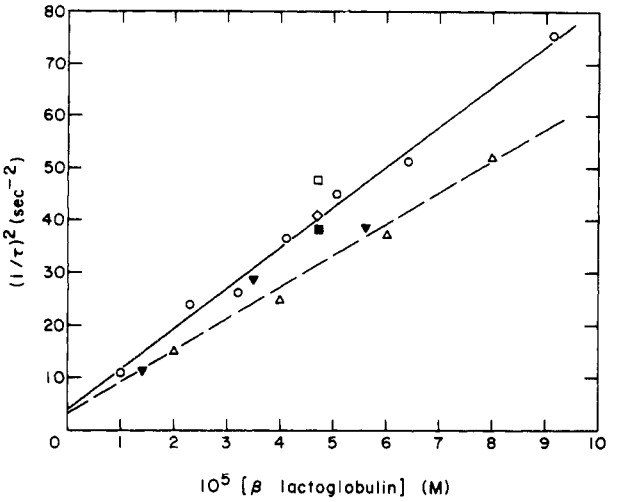

FIGURE 3: A plot of the square of the reciprocal relaxation time, $(1/\tau)^2$, vs. the total β-lactoglobulin concentration (calculated in terms of the monomer) in 0.05 M KNO_3, pH 3.7, 35 °C: β-lactoglobulin AB, 20 μM bromophenol blue (○), 12 μM bromophenol blue (□), 40 μM bromophenol blue (◇), 60 μM bromophenol blue (■); β-lactoglobulin B, 20 μM bromophenol blue (▼); β-lactoglobulin A, 20 μM bromophenol blue (△).

process observed in stopped-flow experiments is too small for observation with the temperature-jump apparatus.

A typical kinetic trace observed at 590 nm is shown in Figure 1B. Observations at 440 nm revealed a similar relaxation process with a smaller amplitude of opposite sign. The signal averaged data were used to obtain the relaxation times as previously described, and the results are summarized in Figure 3 as a plot of $(1/\tau)^2$ vs. β-lactoglobulin concentration according to eq 3. The data are consistent with the mechanism of eq 1, and the rate constants obtained by a least-squares analysis of the data are included in Table I. The relaxation time does not depend significantly on the indicator concentration: this is illustrated in Figure 3 where points at three different indicator concentrations and a fixed protein concentration are shown. Some kinetic experiments also were carried out with the separated A and B proteins, and the results obtained are included in Figure 3. The relaxation times for the separated components are systematically longer than those found for the mixture, although the differences are barely outside the experimental uncertainty. The rate constants calculated from the limited data for the separate A and B proteins are 10–20% lower than those reported in Table I for the mixture. These differences are two small to warrant a more extended study of this phenomenon.

Although altering the dye concentration has little effect on the relaxation process, some interaction between dye and β-lactoglobulin occurs since a difference spectrum is found between a protein–dye mixture and the dye (4×10^{-5} M bromophenol blue, 2.38×10^{-4} M β-lactoglobulin AB, 0.2 M potassium acetate, pH 3.7). The difference spectrum is shown in Figure 4. The bound dye has a decreased extinction coefficient at the absorption maximum of the acid form and an increased extinction coefficient at the absorption maximum of the basic form (this could be attributed to a change of the ionization constant of the dye upon binding to the protein), and the absorption maximum of the basic form is red shifted. A spectrophotometric titration of the protein with dye indicated the binding is too weak to characterize quantitatively. However, it is estimated that, under the conditions of the kinetic experiments, no more than 10% of the protein has dye bound to it.

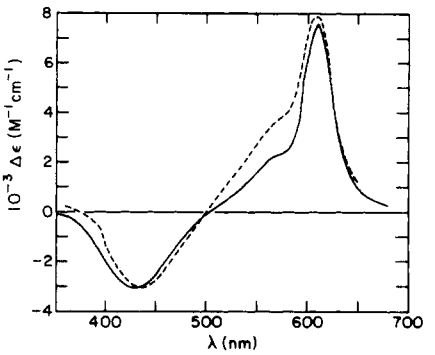

FIGURE 4: A plot of the difference extinction coefficient, $\Delta\epsilon$, vs. wavelength, λ, at 23 °C for bromophenol blue (40 μM)–β-lactoglobulin AB (240 μM) and the separate components (- - -), and for bromophenol blue (50 μM)–α-chymotrypsin (400 μM) and the separate components (—).

Chymotrypsin. α-Chymotrypsin dimerizes in the pH range 2–6, with a maximum association constant of about 10^4 M^{-1} at pH 4 (Timasheff, 1969; Horbett and Teller, 1974). Stopped-flow and temperature-jump experiments were carried out in the pH range 3.7–4.3 using bromophenol blue to monitor the pH changes. Two relaxation processes could be resolved clearly: one with the temperature jump with a relaxation time of about 100 μs, the other with the stopped flow with a relaxation time of about 0.5 s.

The fast relaxation process proceeded with an increase of transmittance at 440, 590, and 620 nm (pH 3.7, 0.125 M KNO_3, 24 °C). This process clearly cannot be due solely to a pH shift. The amplitude of this process is quite small, necessitating signal averaging of 7–10 kinetic traces. The dependence of the relaxation time on the chymotrypsin concentration at a constant bromophenol blue concentration (2×10^{-5} M) is shown in Figure 5. The fact that a limiting value of the relaxation time is reached at high enzyme concentrations indicates a conformational change of dye–enzyme complex is probably responsible for the relaxation process (cf. Hammes and Schimmel, 1970). The relaxation time was found to be independent of dye concentration over the range 3×10^{-5} to 10^{-4} M at a chymotrypsin concentration of 5.2×10^{-5} M. Since the primary focus in this work is protein aggregation, a detailed kinetic study of this relaxation process was not carried out. However, additional results were obtained in support of this interpretation. A difference spectrum was found between indicator (5×10^{-5} M)–enzyme (4×10^{-4} M) mixtures and indicator (5×10^{-5} M) in 0.2 M potassium acetate, pH 3.7. A typical difference spectrum is shown in Figure 4. Bromophenol blue also was found to influence the hydrolysis of *p*-nitrophenyl acetate by chymotrypsin ($\sim 7.5 \times 10^{-6}$ M) in 0.19 M potassium acetate–10% isopropyl alcohol, pH 3.7, 30 °C. Steady state initial velocities were determined in the presence and absence of 4.7×10^{-5} M bromophenol blue, with the substrate concentration being varied between 1 and 9×10^{-4} M. Bromophenol blue was an activator of the enzyme at all substrate concentrations; the primary effect of bromophenol blue appeared to be about a 20-fold increase in the maximal velocity. Thus compelling evidence exists that bromophenol blue binds specifically to the enzyme and that a conformational change occurs following binding which may be related to activation of the enzyme.

The slower relaxation process proceeded with a transmit-

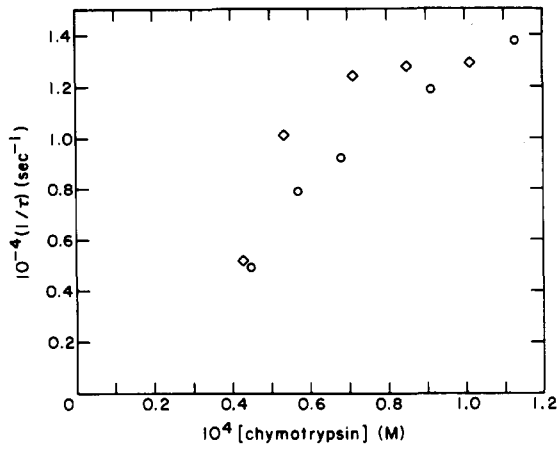

FIGURE 5: A plot of the reciprocal relaxation time determined with the temperature-jump technique, $1/\tau$, vs. the total chymotrypsin concentration (calculated in terms of the monomer) in 0.125 M KNO_3, 20 μM bromophenol blue, 24 °C; signal change monitored at 620 nm (○) and 440 nm (◇).

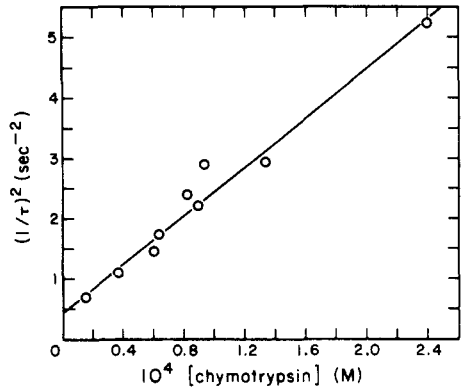

FIGURE 6: A plot of the square of the reciprocal relaxation time determined with the stopped-flow technique, $(1/\tau)^2$, vs. the total chymotrypsin concentration (calculated in terms of the monomer) in 0.05 M KNO_3, 10 μM bromophenol blue, pH 4.3, 25 °C.

tance decrease at 590 nm and an increase at 440 nm above pH 3.9. At pH 3.7 the amplitudes were reversed in sign. Some typical kinetic traces illustrating the variation of amplitude with pH are shown in Figure 1C–D. The wavelength dependence of the amplitude indicates a pH change is being monitored, and the pH dependence of the amplitude is consistent with that predicted for the monomer–dimer reaction (Horbett and Teller, 1974). The reaction kinetics were studied in detail at pH 4.3, 0.05 M KNO_3, 1×10^{-5} M bromophenol blue. A plot of the square of the reciprocal relaxation time vs. the chymotrypsin concentration according to eq 3 is shown in Figure 6. At the higher protein concentrations, the perturbation was sufficiently large so that some second-order contribution to the rate process could be observed. In these instances, only the last 50% of the observed approach to equilibrium was used to calculate the relaxation time. The results obtained are in good accord with the simple monomer–dimer mechanism of eq 1, and the rate constants obtained by a least-squares analysis of the data are included in Table I. The linearity of the plot indicates that the pH indicator is not significantly influencing the reaction kinetics.

Discussion

For all three proteins, insulin, β-lactoglobulin, and chymotrypsin, the kinetic processes observed can be attributed to a monomer–dimer equilibrium. The kinetic data provide an independent determination of the monomer–dimer equilibrium constant which can be compared with values obtained by equilibrium methods. In the case of insulin, the most recently determined value of the equilibrium constant under conditions similar to those employed in the kinetic experiments is 1.4×10^5 M^{-1} (Pekar and Frank, 1972) which is about one order of magnitude larger than the kinetically derived constant (Table I). The reason for this discrepancy is not apparent, but several possibilities exist. In our treatment of the data, the existence of aggregates higher than dimer has been neglected. If the monomer–dimer–hexamer aggregation model and associated constants of Pekar and Frank (1972) are correct, a significant amount of hexamer exists in the concentration range employed in the kinetic experiments (a maximum of 30% by weight). Two limiting cases can be considered in this regard. First the dimer–hexamer equilibrium can be assumed rapid compared with the monomer–dimer equilibrium. This possibility can be excluded because unrealistic rate constants are required, and the observed kinetic monomer–dimer equilibrium constant would be even smaller than the one calculated without the correction. A second possibility is that the dimer–hexamer equilibrium is adjusted very slowly compared with the monomer–dimer equilibrium. If the kinetic data are treated with this assumption, a plot of $(1/\tau)^2$ vs. the total concentration of monomer and dimer displays some curvature rather than the predicted linearity. The equilibrium constant obtained by drawing the best straight line through the data is about 2×10^4 M^{-1}, which is still significantly smaller than the constant derived from the ultracentrifuge data. Another possibility is that a second relaxation process exists, such as a rapid conformational change, which is too fast to observe. In this case, the observed relaxation process would be coupled to another, and the expression for the relaxation time becomes complex. Model calculations have been carried out with this assumption and, as might be expected, since four rate constants are available as parameters, the data can be fit reasonably well with the ultracentrifuge equilibrium constant. For example, one such calculation gives $k_1 \sim 8 \times 10^8$ M^{-1} s^{-1}, $k_{-1} \sim 2 \times 10^4$ s^{-1} and the rate constants associated with the conformational change are $\sim 2.5 \times 10^4$ s^{-1}. None of the above explanations are actually supported by existing data, and the possibility should not be excluded that the value of the equilibrium constant derived from the ultracentrifuge data is not correct since it is based on curve fitting to a model over a particular concentration range. Variations in the model and concentration range could alter the constant, and in fact significantly different constants have been reported by others under different experimental conditions (Jeffrey and Coates, 1966). In any event, the bimolecular rate constant is not greatly influenced by these considerations and must be about 10^8 M^{-1} s^{-1}.

In the case of β-lactoglobulin, equilibrium constants have not been determined under exactly the same conditions as reported here, but the value obtained from the kinetic data is well within the range expected from equilibrium measurements (McKenzie, 1967; Visser et al., 1972; Zimmerman et al., 1970; Kelly and Reithel, 1971). The equilibrium constant for the chymotrypsin dimerization is in fair agreement with that determined from equilibrium measurements, $\sim 1.5 \times 10^4$ M^{-1} (Horbett and Teller, 1974). The rate constant for dimer dissociation has been previously estimated

Table II: Second-Order Rate Constants for Protein–Protein Interactions.[a]

Proteins	$10^{-6}k$ (M^{-1} s^{-1})	Reference
Trypsin–turkey ovomucoid	0.97	Haynes and Feeney, 1968
Trypsin–chicken ovomucoid	2.7	Haynes and Feeney, 1968
Trypsin–lima bean inhibitor	1.4	Haynes and Feeney, 1968
Trypsin–soy bean inhibitor	8.2, 6	Haynes and Feeney, 1968; Luthy et al., 1973
Trypsin–pancreatic secretory inhibitor	6.8	Schweitz et al., 1973
Trypsin–pancreatic trypsin inhibitor	1.3	Vincent and Lazdunski, 1972
α-Chymotrypsin–pancreatic trypsin inhibitor	6.8	Vincent and Lazdunski, 1973
Insulin–insulin receptor (liver membrane)	3.5	Cuatrecasas et al., 1971
Acetylcholine receptor–cobra toxin	0.17	Fulpius et al., 1972
Acetylcholine receptor–α-bungarotoxin	0.012	Hess et al., 1975
Hemoglobin–haptoglobulin	0.7	Nagel and Gibson, 1971
Deoxyhemoglobin dimer–deoxyhemoglobin dimer	0.63	Andersen et al., 1971
Phosphofructokinase monomer–phosphofructokinase monomer	0.008	Parr and Hammes, 1976

[a] The pH was in the range 7–8 and the temperature was 20–25 °C, except for the acetylcholine receptor–α-bungarotoxin reaction where the temperature was 4 °C.

from steady state measurements (Kézdy and Bender, 1965) and the values reported are 0.023 s^{-1} for α-chymotrypsin in 0.05 M citrate, pH 2.7, and 0.1 s^{-1} for β-chymotrypsin in 0.05 M sodium acetate, pH 4 at 25 °C.

The measured second-order rate constants can be compared with the upper limit for a second-order rate constant calculated from diffusion theory. This upper limit is given by eq 4 (cf. Amdur and Hammes, 1966).

$$k = \frac{4\pi N}{1000} RDf \qquad (4)$$

In this equation N is Avogadro's number, R is the distance of closest approach of the two reactants, D is the sum of the diffusion coefficients of the reactants, and f is a factor which takes into account electrostatic interactions between reactants. For insulin, D_{12} is 30×10^{-7} cm^2 s^{-1} (Blundell et al., 1972), R is 24 Å (assuming a specific volume of 0.75 ml/g for the protein), and f can be approximated as unity at pH 6.8; the calculated diffusion-controlled rate constant is 5×10^9 M^{-1} s^{-1}. Although this value is significantly greater than the observed rate constant, some steric restrictions must exist in the dimerization process; it is not unreasonable to assume that only 2% of the total solid angle of approach of 4π leads to reaction. Thus the dimerization of insulin appears to be close to diffusion controlled. The rate constants for dimerization of β-lactoglobulin and chymotrypsin are considerably less than that for insulin. However, at the pH of the experiments, both β-lactoglobulin and chymotrypsin are highly charged (Nagasawa and Holtzer, 1971; Marini and Wunsch, 1963) so that strong repulsive interactions exist. Although current theories are inadequate to calculate f for such strong interactions, an approximate calculation using a Debye–Hückel type potential (cf. Hammes and Alberty, 1959) indicates f would be approximately 10^{-3} for β-lactoglobulin and 10^{-2} for chymotrypsin. Thus the rate constant for β-lactoglobulin dimerization is within the range which might be expected for a very sterically restricted diffusion controlled reaction, but the rate constant for α-chymotrypsin dimerization appears to be considerably too small for a diffusion controlled process.

Finally a comparison of the rate constants reported here with those found for other protein–protein reactions is of interest. A summary of rate constants observed in other systems is given in Table II. Most of the rate constants which have been reported are around 10^5–10^6 M^{-1} s^{-1}; these values are somewhat less than might be anticipated for a diffusion-controlled reaction and considerably less than found for the dimerization of insulin. Of course, it should be noted that some of the reactions listed in Table II involve covalent changes which could be rate determining. For the reaction of trypsin with lima bean inhibitor and turkey ovomucoid, activation energies of 7–8 kcal/mol were reported (Haynes and Feeney, 1968); these are higher than would be anticipated for a diffusion-controlled reaction. In addition, the reaction rate was unchanged in 20% sucrose. An activation energy of 14 kcal/mol has been measured for the reaction of α-chymotrypsin with pancreatic trypsin inhibitor (Vincent and Lazdunski, 1973) and 8.6 kcal/mol for the reaction of the inhibitor with trypsin (Schweitz et al., 1973), which indicates these reactions are not diffusion controlled.

In conclusion, the rate constants for protein–protein association which have been measured are all quite large; however, only in the case of insulin dimerization does the rate constant appear to approach the value anticipated for a diffusion-controlled process.

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Determination of the Complete Amino Acid Sequence of Bovine Cardiac Troponin C†

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ABSTRACT: The amino acid sequence of bovine cardiac troponin C has been completely determined. The protein was cleaved by cyanogen bromide and the resulting peptides were isolated. All of the 161 residues of the protein could be accounted for in 12 cyanogen bromide peptides. Overlapping peptides were generated by tryptic digestion of citraconylated troponin C and isolation of the resulting five peptides. The primary structure of cardiac troponin C was elucidated by sequential manual Edman degradation of these peptides. It consists of four homologous regions, one of which probably has lost the ability to bind calcium ions. By comparing the amino acid sequence of cardiac troponin C with the sequence of skeletal troponin C, it was found that the mutation rate of the region that does not bind calcium is almost twice as high as the mutation rate of the three homologous regions that do bind calcium.

Troponin plays an important role in the regulation of muscular contraction (Ebashi and Endo, 1968). One of its subunits (troponin C) has a strong affinity for calcium (Hartshorne and Pyun, 1971) and upon calcium binding it undergoes a large conformational change (van Eerd and Kawasaki, 1972) which triggers a set of events resulting in muscular contraction.

The almost complete amino acid sequence of rabbit skeletal troponin C has been reported by Collins et al. (1973). Troponin C is homologous to parvalbumins, a group of calcium binding proteins with a molecular weight around 12 000 (Pechère et al., 1971b, Collins et al., 1973). More recently Collins has indicated that troponin C is also homologous to the "alkali light chains" of myosin (Collins, 1974). The three dimensional structure of a parvalbumin of carp muscle has been determined by x-ray diffraction analysis,

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