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A Heterologous Immunoglobulin Chain Recombinant Carries a Distinct Site for Dinitrophenyl and Obeys the Common Hapten Binding Mechanism†

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ABSTRACT: A heterologous recombinant of the immunoglobulin α heavy chain derived from MOPC-460 and the λ light chain from MOPC-315 was prepared. This $H^{460}L^{315}$ hybrid binds N^{ϵ}-(2,4-dinitrophenyl)-L-lysine (DNPL) with an affinity of 1.6×10^4 M^{-1} (7 °C). This Ig–hapten complex exhibits an absorption spectrum which is different from those observed for each of its parent–DNPL complexes. Very small quenching is caused in the intrinsic fluorescence of the hybrid upon hapten binding, as contrasted by the large quenching of the parent molecules. Chemical relaxation kinetic measurements show that $H^{460}L^{315}$ exists in solution in two conformations which exchange with a relaxation time of 20 ms (7 °C). This transition is accompanied by a change in the fluorescence quantum yield of the protein. DNPL binds to both conformers at comparable fast, though not diffusion-controlled, rates. The equilibrium between the two conformers is shifted upon hapten binding, and the two complexes exchange at a faster rate than the free protein conformers. Thus $H^{460}L^{315}$ carries a new binding site for DNPL but follows the common mechanism of hapten binding as that observed for other immunoglobulins. These properties of the hybrid should be closely related to the interactions between its constituting domains.

The heavy and light chains constituting immunoglobulins can be separated, after mild reduction, in dissociating solvents. Upon removal of these solvents, the separated chains can regain their native conformation and reassociate. When heavy and light chains from different parental molecules are reacted, new antibodies may be formed. Thus homologous or heterologous recombinant molecules can be prepared (Nisonoff et al., 1975; Dorrington & Tanford, 1968). Extensive studies of the recombination process revealed preferential homologous reassociations and the predominant role of the V[1] domains in that preference [cf., for example, de Preval & Fougereau (1976) and Bunting et al. (1977)]. Recent studies of the Ig gene structure (Schilling et al., 1980; Early et al., 1980) suggest that this preferential association is selected already at that level. The changes in the V_H–V_L contact residues would therefore affect both the affinity between these domains and their interactions with antigens.

The hapten binding properties of homologous recombinants remained unchanged while those of heterologous recombinants

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[1] Abbreviations used: DNPL, N^{ϵ}-(2,4-dinitrophenyl)-L-lysine; H, heavy chain of immunoglobulin; $H^{460}L^{315}$, immunoglobulin recombinant composed of heavy chain from M-460 and light chain from M-315; Ig, immunoglobulin; L, light chain of immunoglobulin; L_{2cov}, light chain dimer from M-315 with disulfide bond between the chains; L_{2ncov}, light chain dimer from M-315 with reduced and alkylated disulfide bond; M-315, IgA secreted by MOPC-315 plasmacytoma; M-460, IgA secreted by MOPC-460 plasmacytoma; PBS, 0.01 M sodium phosphate buffer (pH 7.4)–0.15 M sodium chloride; V, variable region of immunoglobulin.

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varied from one case to another. While several studies failed to obtain active heterologous recombinants, others succeeded in getting hybrids with hapten binding activities similar to those of the parental molecules (Manjula et al., 1976). Furthermore, the group of heterologous recombinants, prepared from the closely related galactan-specific mouse myeloma proteins, was shown to obey the same hapten binding mechanism as their parent proteins (Zidovetzki et al., 1980). This mechanism involves hapten binding linked conformational change in the immunoglobulin which was found to be the case in an increasing number of antibody–hapten systems (Lancet & Pecht, 1976; Vuk-Pavlović et al., 1978). The similarity of the hapten binding behavior of the hybrids and their parent molecules led us to suggest that the hapten binding linked conformational transition is an inherent property of the tertiary domain structure of the antibody and probably involves changes in the interactions between heavy- and light-chain domains (Zidovetzki et al., 1980).

In the present study, we have investigated the kinetics and thermodynamics of hapten binding to the heterologous recombinant of the H chain of M-460 and the L chain of M-315. Both parent proteins are secreted by murine plasmacytomas and known to bind 2,4-dinitrophenyl derivatives (Eisen et al., 1968). However, M-315 and M-460 are not closely related structurally. Although both heavy chains are of the α class and do not differ markedly in their variable region (Barstad et al., 1978), the light chain of M-315 is of λ type while that of M-460 is of κ type, and they differ extensively.

The mechanism of hapten binding to these two immunoglobulins is different. The hapten binding to M-315 is a single-step association process, as was shown by a detailed kinetic analysis of the binding of more than 50 different DNP derivatives used for the kinetic mapping of the combining site (Pecht et al., 1972; Haselkorn et al., 1974). In contrast, M-460 exhibits two well-resolved relaxation processes which were interpreted in terms of a hapten binding linked conformational transition (Lancet & Pecht, 1976).

Spectroscopic and thermodynamic properties of the $H^{460}L^{315}$–DNPL complex reported here suggest that H^{460}–L^{315} association results in the formation of a new hapten binding site, which differs from that of either M-460 or M-315. The behavior of the complementary hybrid $H^{315}L^{460}$ unfortunately could not be examined since rather poor yields of it were obtained and essentially no measurable relaxation could be monitored upon hapten binding. The temperature jump–chemical relaxation study of the hapten binding to $H^{460}L^{315}$ has shown that it involves a hapten binding induced conformational change of the type found in the parent protein M-460. However, the profound differences in the hapten binding parameters between these proteins emphasizes that $H^{460}L^{315}$ possesses a new binding site. The present data illustrate further the generality of the hapten binding linked conformational transitions in immunoglobulins and the universality of the heavy–light chain recognition and interactions.

Materials and Methods

Proteins 315 and 460 were purified from the ascitic fluid of BALB/c mice bearing MOPC-315 or MOPC-460 tumor according to Goetzl & Metzger (1970). During this procedure, the proteins are reduced by dithiothreitol (10 mM, 1 h) and alkylated by iodoacetamide (22 mM) in the ascitic fluid. This was sufficient to cleave inter-heavy-chain disulfide bonds. Both M-315 and M-460 are IgA's and have therefore part of the molecules with a disulfide bridge joining their light chains (Abel & Grey, 1968). The inter-light-chain's disulfide bridge of protein 460 is less susceptible to cleavage. Therefore, protein

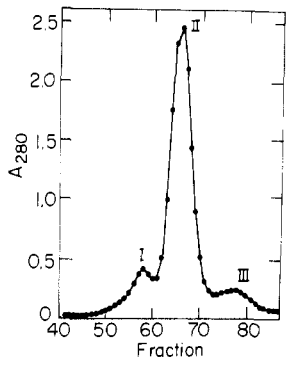

FIGURE 1: Elution pattern showing the separation of the $H^{460}L^{315}$ recombinant (II) from the heavy chain aggregates (I) and light chain (III). The Sepharose 6B column (3 × 90 cm) was eluted by PBS and 0.02% NaN_3 at room temperature.

460 was reduced and alkylated again under the same conditions prior to applying it to gel chromatography. H and L chains of protein M-460 or M-315 were separated by chromatography on a Sephadex G-150 column (5 × 95 cm) equilibrated with 6 M urea and 0.1 M acetic acid, pH 4.5. The separated chains were pooled, and then H^{460} was mixed with L^{315} in a 1:1 molar ratio. The solutions were extensively dialyzed against PBS, concentrated by ultrafiltration (membrane UM10, Amicon, Lexington, MA) to about 5 mg/mL, and then applied to a Sepharose 6B column (3 × 90 cm) equilibrated with PBS. The latter chromatography was done in order to separate the heterologous recombinants from the aggregated heavy chains and the residual nonrecombined light chains. A sample elution pattern is given in Figure 1. Each preparation was checked for purity by sodium dodecyl sulfate (gradient 5–20%)–polyacrylamide gel (Laemmli, 1970), and its sedimentation coefficient was measured on a Beckman Model E ultracentrifuge. The sedimentation coefficient of the hybrid was found to be 6.6 ± 0.1, the value characteristic for immunoglobulins composed of two heavy and two light chains.

All experiments were performed in PBS. DNPL was purchased from Sigma. Equilibrium dialysis measurements were carried out at 7 °C as described by Schepers et al. (1978). Spectrophotometric measurements were done on a Cary 118 spectrophotometer at 7 °C with a matched pair of tandem cells as described by Licht et al. (1977). Fluorescence titrations were carried out on a Perkin-Elmer MPF 44A spectrofluorometer as described by Schepers et al. (1978).

A temperature jump spectrofluorometer (Rigler et al., 1974) was employed for kinetic measurements. The temperature of the solution was raised during the jump by 5.2 °C from 2 ± 0.1 °C. The collection of the data and its analysis were done as previously described (Zidovetzki et al., 1980).

Results and Interpretation

Difference absorption titration of protein $H^{460}L^{315}$ with DNPL at 7 °C is shown in Figure 2. The spectrum of the bound hapten displays marked hypochromicity, and its lower peak is red shifted. These features can be attributed to the interaction of the DNP ring with an indole ring, forming a charge-transfer complex (Little & Eisen, 1967). The shape of the spectrum of the $H^{460}L^{315}$–DNPL complex is significantly different from those of either of its parent proteins, MOPC-315 and MOPC-460 (Figure 2A, insert). Moreover, a different shape is also displayed by the complexes of DNPL with different light-chain derivatives of protein 315: light-chain dimer with the reduced and alkylated inter-light-chain disulfide bond, L_{2ncov} (Lancet et al., 1977), light-chain dimer with this disulfide bond intact, L_{2cov} (Zidovetzki et al., 1979), and the dimer of

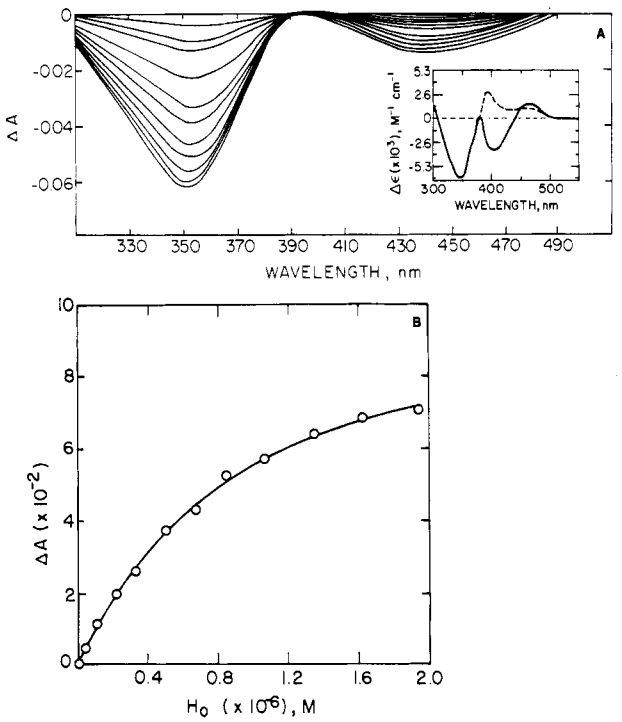

FIGURE 2: (A) Difference absorption titration of the protein $H^{460}L^{315}$ with DNPL vs. free DNPL at 7 °C. The protein concentration was 1.56×10^{-5} M sites. DNPL concentration was increased up to 2×10^{-4} M. The changes in absorption are expressed per 1-cm light path, although the titration was performed in a light path of 0.439 cm. The dilution at the end of the titration is 4.4%. (Insert) Difference absorption spectra between M-315–DNPL complex and free DNPL (Licht et al., 1977) (solid line) and between M-460–DNPL complex and free DNPL (Jaffe et al., 1971) (dashed line). (B) Saturation curve of the $H^{460}L^{315}$ with DNPL. The data are taken from the titration shown in (A). H_0 is the total DNPL added. ΔA at 354 nm is corrected for dilution. The solid line is drawn according to the best-fit parameters of $\Delta\epsilon_{354} = -6800$ M^{-1} cm^{-1} and $K = 1.3 \times 10^4$ M^{-1}.

the variable domains of the light chain (Zidovetzki et al., 1981a,b). The saturation curve for the $H^{460}L^{315}$–DNPL titration is given in Figure 2B. The best-fit parameters of the binding are $K = 1.3 \times 10^4$ M^{-1} and $\Delta\epsilon_{354} = -6800$ M^{-1} cm^{-1}. This value of K is 10-fold smaller than the one reported earlier by Bridges & Little (1971) for the same system, yet it is in satisfactory agreement with $K = 3.0 \times 10^4$ M^{-1} reported by Zeldis et al. (1979). Both the latter studies employed the equilibrium dialysis method for measuring the binding constants. Our own measurements using this method yielded $K = 1.1 \times 10^4$ M^{-1} and 1.95 binding sites per molecule of the hybrid. In light of the low accuracy of the equilibrium dialysis method for measuring affinities lower than 10^5 M^{-1}, the agreement between our results and those of Zeldis et al. (1979) is reasonable. Spectroscopic methods are more sensitive for determining affinities of this order of magnitude. Difference absorption titration ($K = 1.3 \times 10^4$ M^{-1}) and kinetic measurement (see below) monitoring the change in the protein fluorescence ($K = 1.6 \times 10^4$ M^{-1}) gave consistent results. The reason for the higher constant found by Bridges & Little (1971) is unclear.

Fluorescence titration of $H^{460}L^{315}$ with DNPL was indistinguishable from the reference titration of the routinely used normal mouse IgG performed under the same conditions. Strong nonspecific quenching of protein fluorescence was observed, which did not allow the detection of the rather small specific quenching (7% as measured by kinetic methods; see below). The nonspecific quenching is caused by the inner-filter effect due to the high absorption of DNPL in the 340-nm spectral region at the concentration which had to be used. In our case, the relatively low affinity required the high concentration of the hapten (up to 10^{-3} M) in order to achieve the desired high saturation of the protein by the hapten.

Kinetic Measurements. Free protein $H^{460}L^{315}$ in solution showed upon temperature jump perturbation a single relaxation of 20 ± 2 ms at 7 °C, detected via the intrinsic protein fluorescence (Figure 3A). Its time and amplitude were found to be independent of the protein concentration over the range 3.4×10^{-7}–3.2×10^{-6} M (Figure 4). Such a behavior is characteristic of a monomolecular reaction where the equilibrium between two conformers of the protein is temperature dependent. Upon addition of hapten, the temperature jump perturbed system exhibited two relaxation times separated by a factor of at least 20 (Figure 3B). Measurements of the same reaction, but via the absorbance monitored at 354 or 440 nm, also exhibited two relaxation steps in the same time ranges as those observed by fluorescence. The respective amplitudes measured at 354 nm were about twice those at 440 nm, as expected from the static difference absorbance titration (Figure 2). Since these amplitudes were rather small and measurements in the absorbance mode require a large amount of protein, detailed kinetic measurements were done in fluorescence mode. The concentration dependence of the fast ($1/\tau_f$) and slow ($1/\tau_s$) reciprocal relaxation times is shown in Figure 5. The reciprocal fast time increased linearly with the concentration of DNPL while the slow one increased from the minimal value of the free protein (50 s^{-1}) to a plateau at about 100 s^{-1}. If only one of the two conformers binds the hapten, the reciprocal slow time, which represents the isomerization, would decrease with increasing concentration (Lancet & Pecht, 1976; Vuk-Pavlović et al., 1978). Therefore, in the present case, the behavior of the reciprocal relaxation times is consistent with the mechanism where *both* conformers of the protein interact with the hapten:

$$\begin{array}{ccc} T_0 + H & \underset{k_{-T}}{\overset{k_T}{\rightleftharpoons}} & T_1 \\ k_0 \downarrow\uparrow k_{-0} & & k_1 \downarrow\uparrow k_{-1} \\ R_0 + H & \underset{k_{-R}}{\overset{k_R}{\rightleftharpoons}} & R_1 \end{array} \qquad (1)$$

where T and R are the two conformers of the protein in the free, T_0 and R_0, or hapten- (H-) bound T_1 and R_1 forms. This mechanism has been found to fit the behavior of several other antibody–hapten systems (Lancet & Pecht, 1976; Vuk-Pavlović et al., 1978; Zidovetzki et al., 1980).

Three relaxation modes are expected on the basis of that mechanism. The simplest explanation for the observation of only two relaxations is that the fast one represents both association steps of the hapten with the two conformers. When the association rate constants observed for other systems where DNP binds to its specific site are taken into account, these two times are expected to be very similar and therefore nonseparated (Haselkorn et al., 1974; Pecht & Lancet, 1976) (see Discussion). A similar conclusion has been reached for other antibody–hapten systems where only two relaxations were observed, although three were expected. In one of these cases (Lancet & Pecht, 1976), it was found that the contribution of the T association is negligible and that the fast time represents solely the association of the hapten with the R state. In other cases (Vuk-Pavlović et al., 1978; Zidovetzki et al., 1980), we had to assume that the spectral change of the R conformer is negligible and the fast relaxation represents the association of the T isomer with the hapten.

In the present case, the fast associations of mechanism 1 can be treated separately from the slow isomerization because

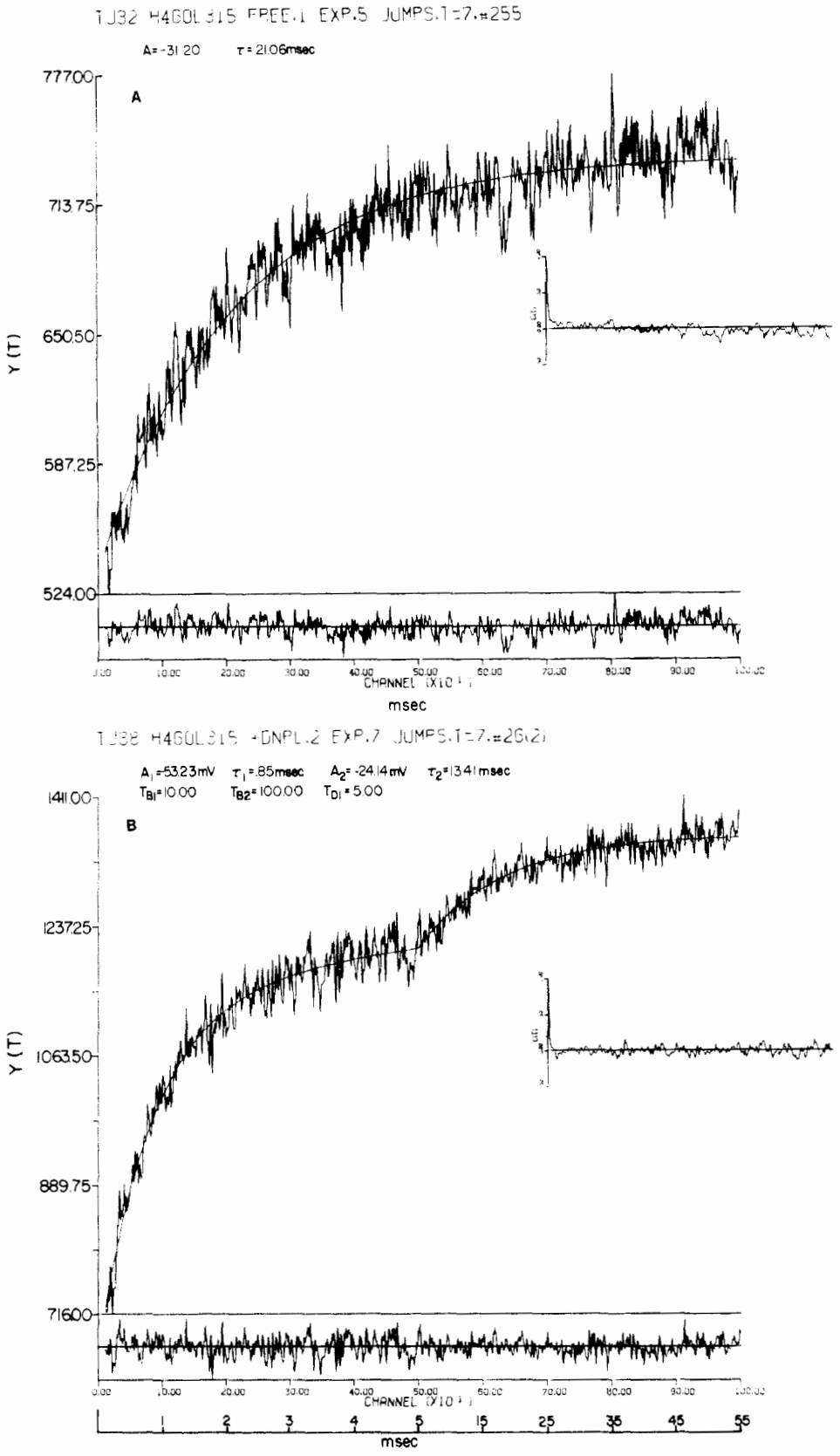

FIGURE 3: (A) Temperature jump–relaxation trace of free protein $H^{460}L^{315}$ in PBS. The final temperature was 7 °C, and the jump was of 5.2 °C. The protein was excited at 280 nm and the fluorescence signal collected through a cutoff filter at 320 nm. The experimental trace is an accumulation of five individual jumps. The protein concentration was 1.3×10^{-6} M sites. The data were fitted to the single exponent drawn as the smooth line. The autocorrelation function is shown in the right top insert and the difference between experimental data and the fitted function is given in the lower insert. For more details, see Materials and Methods and Zidovetzki et al. (1980). (B) Temperature jump–relaxation trace of a solution containing protein $H^{460}L^{315}$ (2.4×10^{-6} M sites) and DNPL (4.4×10^{-5} M). The experimental trace is recorded in two time ranges: the first 500 channels are filled at a rate of 10 ms/1000 channels, and after 5 ms, the rate is switched to 100 ms/1000 channels. The trace represents data accumulated from seven jumps and fitted to a sum of two exponents as shown by the smooth line (parameters shown in the upper left corner).

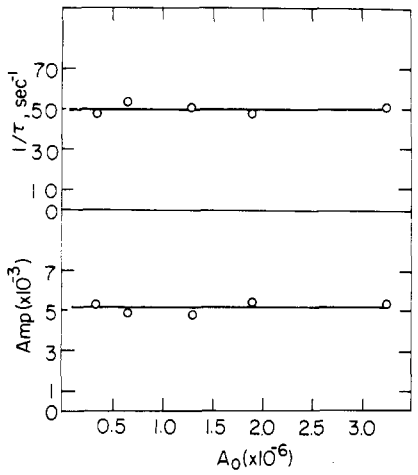

FIGURE 4: Relaxation time (upper) and amplitude (lower) of the hapten-free $H^{460}L^{315}$ hybrid at different protein concentrations. For further details, see text.

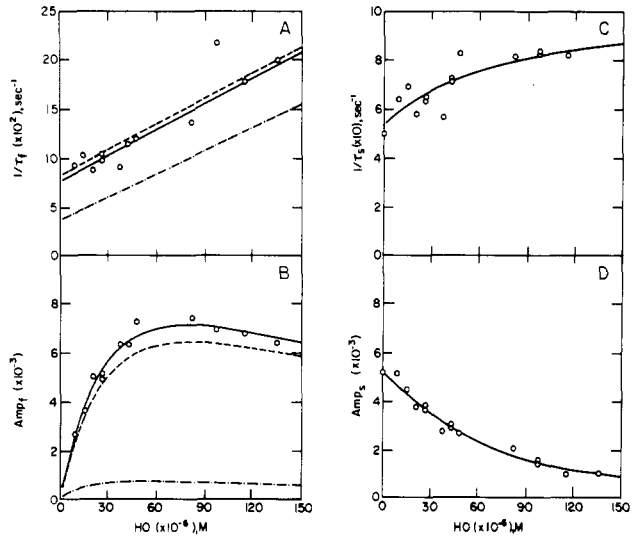

FIGURE 5: Dependence of fast (A, B) and slow (C, D) reciprocal relaxation times and amplitudes of $H^{460}L^{315}$ on total DNPL concentration at 7 °C. Solid lines in (C) and (D) are drawn by using the best-fit parameters given in Table I. (---) Best-fit parameters for the T association, Table I, and (-·-·) parameters for the R association. (—) (A and B) "Overall" values for the fast relaxation, which are weighted sums of contributions from T and R associations. The initial protein concentration is 2.37×10^{-6} M sites.

they are kinetically and thermodynamically uncoupled from the latter. Therefore, we treated the fast time as a combination of the two times in a way given by Schwarz (1968):

$$\frac{1}{\tau_f} = \left(P_1 \frac{1}{\tau_1} + P_2 \frac{1}{\tau_2} \right) \Big/ (P_1 + P_2) \qquad (2)$$

where τ_1 and τ_2 are the two times for the two associations and P_1 and P_2 are their respective amplitudes. To calculate these times and amplitudes, one has to solve the two linearized differential equations (Eigen & DeMaeyer, 1974; Guillain & Thusius, 1970). The relaxation times have been shown to be the eigenvalues of the **r**·**g** matrix, where **r** is the diagonal square matrix of the exchange rates of the reaction steps and **g** is the symmetric square matrix given below (eq 3) (Eigen & DeMaeyer 1974; Castellan, 1963). The amplitudes have been pointed out to be related to the eigenvectors of the same matrix (Jovin, 1975; Thusius, 1977). Solving this problem is expressed by a completely decoupled set of "normal" advancements which are functions of concentrations and rate constants (Eigen & DeMaeyer, 1974; Jovin, 1975; Thusius, 1977). We used the more direct approach formulated by Lancet (1978), which is oriented toward the use of computers. The **g** matrix for our mechanism is

$$\begin{vmatrix} \frac{1}{[T_0]} + \frac{1}{[H]} + \frac{1}{[T_1]} & \frac{1}{[H]} & \frac{1}{[T_0]} \\ \frac{1}{[H]} & \frac{1}{[R_0]} + \frac{1}{[H]} + \frac{1}{[R_1]} & -\frac{1}{[R_0]} \\ \frac{1}{[T_0]} & -\frac{1}{[R_0]} & \frac{1}{[T_0]} + \frac{1}{[R_0]} \end{vmatrix} \qquad (3)$$

where $[T_0]$, $[T_1]$, $[R_0]$, $[R_1]$, and $[H]$ are the equilibrium concentrations of the respective species. The reciprocal fast relaxation times $1/\tau_1$ and $1/\tau_2$ are the eigenvalues of the $r_2 \cdot g_2$ (submatrix of $r_3 \cdot g_3$) and are the solution of the quadratic equation

$$\left(a_{11} - \frac{1}{\tau_{1,2}} \right) \left(a_{22} - \frac{1}{\tau_{1,2}} \right) - a_{12}a_{21} = 0 \qquad (4)$$

where $a_{11} = r_1 g_{11}$, $a_{12} = r_1 g_{12}$, $a_{21} = r_2 g_{12}$, and $a_{22} = r_2 g_{22}$; $r_1 = k_T[T_0][H]$, $r_2 = k_R[R_0][H]$, and g_{ij} are the elements of the **g** matrix [for explicit formulas, see Guillain & Thusius (1970)]. Knowing that τ's, one has to find the four chemical amplitudes X_{ij} which are related to $P_{1,2}$ by

$$P_1 = X_{11}\Delta F_T + X_{21}\Delta F_R$$
$$P_2 = X_{12}\Delta F_T + X_{22}\Delta F_R \qquad (5)$$

where $\Delta F_{T,R}$ are the fluorescence spectral changes in the respective step. To find X_{ij}, one has to solve a system of four equations:

$$\left(a_{11} - \frac{1}{\tau_1} \right) X_{11} + a_{12}X_{21} = 0$$
$$\left(a_{11} - \frac{1}{\tau_2} \right) X_{12} + a_{12}X_{22} = 0$$
$$X_{11} + X_{12} = (g_{11}\Delta H_T + g_{12}\Delta H_R)\Delta T/RT^2$$
$$X_{21} + X_{22} = (g_{21}\Delta H_T + g_{22}\Delta H_R)\Delta T/RT^2 \qquad (6)$$

where $\Delta H_{T,R}$ are the enthalpy changes of the corresponding steps, R is the gas constant, and T and ΔT are the absolute temperature and its change during the temperature jump. The solution of this system of equations results in an explicit expression for eq 2, which is of course very complex. However, the explicit expression is not needed when computers are used, and the program becomes relatively simple.

The amplitude of the fast time is treated as the total amplitude of the two fast association steps and is given by (cf. Jovin, 1975; Thusius, 1977)

$$A_f = [\Delta F_T \Delta H_T g_{22} - (\Delta H_T \Delta F_R + \Delta H_R \Delta F_T) g_{12} + \Delta H_R \Delta F_R g_{11}]\Delta T/(|g_2| A_0 RT^2) \qquad (7)$$

where $|g_2|$ is the partial principal determinant of the 2×2 upper left **g** matrix (eq 3) and A_0 is the total protein concentration ($[T_0] + [R_0] + [T_1] + [R_1]$). The amplitudes were normalized according to Lancet & Pecht (1976) and Vuk-Pavlović et al. (1978).

The slow times and amplitudes were calculated according to Castellan (1963) and Jovin (1975)

$$\frac{1}{\tau_s} = (k_1[T_1] + k_0[T_0])|g_3|/|g_2| \qquad (8)$$

$$A_s = (Q_{13}\Delta F_T + Q_{23}\Delta F_R + \Delta F_1) \times (Q_{13}\Delta H_T + Q_{23}\Delta H_R + \Delta H_1)|g_2|\Delta T/(RT^2|g_3|A_0) \qquad (9)$$

where $Q_{13} = (g_{12}g_{23} - g_{13}g_{22})/|g_2|$ and $Q_{23} = (g_{12}g_{13} -$

Table I: Kinetic and Thermodynamic Parameters of DNPL Binding to $H^{460}L^{315}$ [a]

i	K_i	k_i	k_{-i}	ΔH_i	ΔF_i	ΔG_i	ΔS_i
overall	1.6×10^4			−10.4	−0.07	−5.39	−17.9
T	1.1×10^4	8.8×10^6	826	−15.1	−0.011	−5.19	−35.4
R	2.2×10^4	8.0×10^6	365	−4.9	−0.03	−5.57	2.4
0	0.81	24.0	29.6	−9.21	−0.07	+0.12	−33.3
1	1.66	66.0	39.8	0.97	0.01	−0.28	4.5

[a] Measurements were done at 7 °C in PBS. Units: k_T and k_R, M^{-1} s^{-1}; all other k_i and k_{-i}, s^{-1}; $K_{overall}$, K_T, and K_R, M^{-1}; K_0, K_1, and ΔF_i, dimensionless; ΔH_i and ΔG_i, kcal/mol; ΔS_i, cal/(mol·deg).

$g_{11}g_{23})/|g_2|$. ΔH_i and ΔF_i are the enthalpy and fluorescence changes of the i step, and $|g_3|$ is the full determinant of the g matrix (eq 3). During the fitting procedure, functional and boundary constraints were imposed on the parameters as described in Zidovetzki et al. (1980).

The results of the analysis are presented in Table I, and the simulated curves are shown in Figure 5. The contribution of the two association steps to the observed fast time and amplitude is shown in Figure 5A,B. The slow relaxation times and their amplitudes are presented in Figure 5C,D.

Discussion

The hybrid immunoglobulin $H^{460}L^{315}$ is constructed by substituting the native light chain of protein 460, which is of the κ type, by a λ type light chain. The spectroscopic properties and the kinetic data presented above define the nature of the newly constructed combining site on that immunoglobulin molecule and allow a comparison between the structure and activity of this hybrid with those of its parent molecules. Affinity labeling and model building studies have provided evidence that hapten contact residues in the binding site of protein 315 stem primarily from its light chain (Givol et al., 1971; Haimovich et al., 1972; Padlan et al., 1976). Indeed, the dimers of the light chains of this protein were shown to bind nitroaromatic haptens with the stoichiometry of two hapten molecules per dimer and the same fine specificity as the parent protein (Schechter et al., 1976; Licht et al., 1977). In view of this, it is noteworthy that the absorption spectrum of the $H^{460}L^{315}$–DNPL complex (Figure 2) is significantly different from those exhibited by the complexes of DNPL with the light chain dimers or with either of its two parent proteins (Lancet et al., 1977; Zidovetzki et al., 1979). All the spectra mentioned above show a charge-transfer band considered as a characteristic feature due to the interaction of the DNP ring with an indole group (Trp-93 of L^{315}; Padlan et al., 1976; Zidovetzki et al., 1981b) in the binding site. However, there are considerable differences in the details among the spectra. The spectrum of DNPL bound to $H^{460}L^{315}$ has a major negative peak at 354 nm and another negative extremum at 440 nm. The position and the sign of this longer wavelength band are the main differences between $H^{460}L^{315}$ and the other proteins discussed. The negative band of the L_{2ncov}–DNPL complex is located at 410 nm, thus showing a smaller red shift than that exhibited by the $H^{460}L^{315}$–DNPL complex. The spectra of DNPL complexes with L_{2cov} and M-315 have an additional positive peak around 470 nm, an indication for an even stronger red shift. The M-460–DNPL, on the other hand, has a positive peak at 390 nm and a shoulder at 460 nm. Thus, we see that the interaction between DNPL and the indole in the site is different in all five proteins. These differences are further emphasized by the finding that the specific quenching of the protein intrinsic fluorescence by DNPL is much stronger for M-315 (−69%) (Eisen et al., 1968) and for M-460 (−48%) (Lancet & Pecht, 1976) than for $H^{460}L^{315}$ (ΔF = −7%; Table I). Hence, although residues of the light chain probably contribute most of the hapten contacts in the hybrid site, the detailed architecture is significantly modulated by the association with either H^{460} or H^{315} or with another L^{315}.

The kinetics and thermodynamics of DNPL binding to $H^{460}L^{315}$ differ significantly from those of the binding to either of its parent proteins (Pecht et al., 1972; Lancet & Pecht, 1976). The clearest difference is between the hybrid and protein 315. The latter has been studied most extensively, and its reaction with a large number of different DNP derivatives has been found to yield a single relaxation only. This was shown to correspond to the association–dissociation step with the hapten. Thus, there is no kinetic evidence that this protein undergoes a conformational change upon hapten binding (Pecht et al., 1972; Haselkorn et al., 1974). In contrast, protein 460 does exhibit a conformational change, which was also described in the terms of mechanism 1 by Lancet & Pecht (1976). The kinetic behavior of $H^{460}L^{315}$ is therefore of the same general type as that of protein 460, in spite of the fact that H^{315} and H^{460} are much more closely related than L^{315} and L^{460}, and the spectrum of the $H^{460}L^{315}$–DNPL complex resembles more that of M-315 and its light chains derivatives. The hapten association steps with $H^{460}L^{315}$ are characterized by similar values of k_T and k_R of 8.8×10^6 and 8.0×10^6 M^{-1} s^{-1}, respectively (Table I). It has been suggested that the hapten association steps with both M-315 and M-460 may be taken to be diffusion controlled (Pecht & Lancet, 1977). Therefore, it is of interest to compare the association rates obtained for the two latter proteins with that of $H^{460}L^{315}$. The three proteins were studied at different temperatures, M-315 at 21 °C (Pecht et al., 1972), M-460 at 25 °C (Lancet & Pecht, 1976), and $H^{460}L^{315}$ at 7 °C. (Our choice of low temperature in the present case was dictated by the relatively low affinity of $H^{460}L^{315}$ for DNPL.) Some calculation is therefore required in order to allow for the comparison. This is based on the following simple considerations. (1) The association rate constant in a diffusion-controlled reaction is proportional to the sum of the diffusion coefficients of the reactants (Smoluchowski, 1917). (2) The diffusion coefficient of the antibody is negligibly small as compared with that of the hapten (Pecht & Lancet, 1977). (3) The diffusion coefficient is proportional to both the absolute temperature and the reciprocal of the solvent viscosity at the corresponding temperature. Thus, taking the viscosity of our buffer as equal to that of water, using its values at 7, 21, and 25 °C (Lange, 1961), we calculated k_{on} values at 7 °C of 7.5×10^7 M^{-1} s^{-1} for M-460 and 8.4×10^7 M^{-1} s^{-1} for M-315 and found them to be an order of magnitude higher than the values of k_T and k_R for the hybrid (Table I). Thus, the association rate constants of DNPL with $H^{460}L^{315}$ indicate that these steps are not diffusion controlled and imply a significant activation barrier for that step (Pecht & Lancet, 1977). This constitutes a further distinction between the kinetic behavior of that hybrid and its parent proteins. Noteworthy is the fact that the association rates, although slower than those determined for DNP binding antibodies, are still significantly faster than those

found for the binding of oligosaccharides to their respective antibodies (Zidovetzki et al., 1980; Pecht & Lancet, 1977).

The thermodynamic parameters of hapten binding to the T and R conformers of $H^{460}L^{315}$ show a clear difference between them. The favorable free energy of the T association is due to a contribution of a relatively large enthalpy change (ΔH_T = −15.1 kcal/mol), while the entropy change in this step is negative (ΔS_T = −35.4 eu). This negative value is that expected due to loss of rotational and translational entropies of the hapten molecule upon binding (Jencks, 1975). On the other hand, the enthalpy change due to the R conformer–hapten association is much smaller (ΔH = −4.9 kcal/mol), and thus the positive entropy (ΔS_R = 2.4 eu) of this step also contributes to the free energy change. The positive ΔS_R suggests that the binding site of the R conformer forms hydrophobic bonds with DNPL. It may mean that the binding site of the R state is deeper, so that it is able to interact with the lysyl's methylenes (Halsey et al., 1975). In contrast, the thermodynamic parameters of both R and T associations for the parent M-460 are very similar, and both of them have negative values of ΔS of about −20 eu (Lancet & Pecht, 1976). All the above data clearly show that the $H^{460}L^{315}$ hybrid possesses a binding site with properties which are different from those of both its parent proteins. Still this hybrid follows the same general reaction path as that found to be common for many antibodies hitherto examined. Significantly we have recently shown that the hybrids produced from a group of homogeneous galactan binding immunoglobulins also react with their haptens according to mechanism 1 as did their parental proteins (Zidovetzki et al., 1980).

This raises the question of the nature of the conformational transitions that are an intrinsic part of the common mechanism. These most probably involve the interactions between the domains constituting the combining site rather than changes in the immunoglobulin fold of each one of them (Schiffer et al., 1973). One possibility is that the hapten binding induced conformational transition causes changes in the relative positions of the domains (Lancet & Pecht, 1976; Zidovetzki et al., 1980). That problem of interdomain interactions must be related to the process of preferential association between H and L chains. A clear preference of autologous association of H and L chains has been established by kinetic and thermodynamic studies (de Preval & Fougereau, 1976; Bunting et al., 1977; Klein et al., 1979; Alexandru et al., 1980). This preference stems therefore from the V domains of the molecules. The group of homogeneous, galactan-binding immunoglobulins from which several galactan-binding heterologous recombinants have been prepared constitutes an instructive case (Manjula et al., 1976). By use of the known sequences of these proteins, hypothetical, three-dimensional structures for some of them were constructed (R. Feldman, personal communication). Examination of these structures revealed that several of the more important substitutions among these proteins are at the V_H–V_L contact areas. More specifically, some of the residues that determine the contacts between the domains are subject to a large extent of variation as they belong to the J and D segments of the immunoglobulins. Thus, the domains' contact forming residues are selected at the gene level by the recombination process of the J_H and D segments to V_H and of J_L to V_L (Weigert et al., 1980). This early selection of preferential H–L association would affect not only the affinity between the chains but also their mode of antigen binding and the structural rearrangements that are induced by it. On the other hand, the formation of a hybrid which maintains the hapten-binding capacity suggests that the substitutions are such that they allow for the structural and functional requirements of the newly formed site. The $H^{460}L^{315}$ hybrid constitutes a further illustration for that case since a new binding site has been formed, which maintains binding capacity for DNP derivatives in spite of the extensive differences between the respective parental chains. Still this hybrid molecule also obeys the common hapten-binding reaction mechanism which has been found to be followed by an increasing number of immunoglobulins.

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Kinetics of Head–Tail Joining in Bacteriophage T4D Studied by Quasi-elastic Light Scattering: Effects of Temperature, pH, and Ionic Strength†

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Appendix: Calculation of the Steric Factors Affecting the Head–Tail Joining Reaction

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ABSTRACT: We have determined the effects of temperature, pH, and ionic strength on the kinetics of attachment of purified T4D heads and tails, with the aim of understanding some of the mechanistic details of this viral assembly reaction. To produce heads for the reaction, we used an osmotic shock resistant mutant deficient in tails and tail fibers ($10^-/18^-/19^-/34^-/36^-/37^-$); for tails, a $13^-/23^-$ mutant was used. We conjecture that the mutation in gene 13 was necessary to prevent connectors from being attached to both heads and tails, thus blocking their joining. To follow the reaction, we used quasi-elastic light scattering (QLS) [Aksiyote-Benbasat, J., & Bloomfield, V. A. (1975) *J. Mol. Biol. 95*, 335–357], in effect measuring the decrease in average translational diffusion coefficient of the heads as tails are attached. The head and tail concentrations were in the range 6×10^{10} and 1.2×10^{11} particles/mL, respectively, corresponding to about (1 or 2) $\times 10^{-10}$ M. This extreme dilution, which is possible because of the high scattering power of the heads, slows down the very rapid bimolecular reaction to $t_{1/2} \approx 400$ s. We were able to measure the QLS autocorrelation function once every 50 s. Over a temperature range from 10 to 37 °C, the second-order rate constant k increased from 0.79×10^7 M^{-1} s^{-1} to 1.46×10^7 M^{-1} s^{-1}. An Arrhenius plot of ln k vs. $1/T$ was linear, yielding E_a = 4.06 kcal/mol, as expected for a diffusion-controlled reaction, and $\Delta S^* = -12.6$ cal/(mol·deg), corresponding to a steric factor of $^1/_{567}$. This is consistent with our previous estimate based on the deviation from von Smoluchowski's diffusion-controlled reaction theory. The pH dependence of k was measured between pH 5.4 and 8.3. It reached a maximum of 2×10^7 M^{-1} s^{-1} at pH 5.8. The falloff in more acid conditions was due to destabilization of the heads. Over the range 5.8–8.3, the pH dependence was fit well by the ionization of a single group with a pK_a of 6.8, such as an imidazole. The ionic strength dependence was measured by maintaining $[MgCl_2]$ = 0.01 M and varying [KCl] from 0 to 0.20 M. Below 0.02 M KCl, k fell sharply, perhaps due to destabilization of the tails. The rate also decreased above 0.02 M KCl, reaching a plateau above 0.10 M. The decrease in rate constant with increasing ionic strength suggests that the reaction brings together groups of opposite charge.

The assembly of bacterial viruses is of interest in its own right and also serves as a model for the assembly of other subcellular structures that involve protein–protein and protein–nucleic acid interactions. While much has been learned about phage assembly by in vitro complementation experiments with crude or semipurified extracts (Casjens & King, 1975), relatively little work has been done on purified systems, where detailed chemical questions can be investigated. Thus, we understand little about the forces that govern the assembly of bacteriophage and which confer on the structure considerable stability under the stresses that arise during the infection process. It

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