Correction. In the article "Kinetic evidence for hapten-induced conformational transition in immunoglobulin MOPC 460" by D. Lancet and I. Pecht, which appeared in the October 1976 issue of Proc. Natl. Acad. Sci. USA 73, 3549–3553, the authors have requested the following changes. On p. 3550, right-hand column, second line from bottom, and p. 3551, left-hand column, fourth line from the top, "Fig. 2" should be "Fig. 1A." In the legend of Table 2, third line, note (f) should read "AG = -RTlnK." On p. 3553, left-hand column, third paragraph, fifth line, "ko" should be replaced by "Ko."

Correction. In the article "Amino-terminal sequences of two polypeptides from human serum with nonsuppressible insulin-like and cell-growth-promoting activities: Evidence for structural homology with insulin B chain" by E. Rinderknecht and R. E. Humbel, which appeared in the December 1976 issue of Proc. Natl. Acad. Sci. USA 73, 4379–4381, a symbol was misprinted. In the legend of Fig. 1 on pp. 4380–4381, a symbol was misprinted. In the legend of Fig. 1 on pp. 4380–4381, the third and fourth lines should read "_. automatic Edman degradation and gas chromatography."

Correction. In the membership list of the National Academy of Sciences that appeared in the October 1976 issue of Proc. Natl. Acad. Sci. USA 73, 3750–3781, please note the following corrections: H. E. Carter, Britton Chance, Seymour S. Cohen, E. A. Doisy, Gerald M. Edelman, and John T. Edsall are affiliated with the Section of Biochemistry (21), not the Section of Botany (25).

Correction. In the Author Index to Volume 73, January–December 1976, which appeared in the December 1976 issue of Proc. Natl. Acad. Sci. USA 73, 4781–4788, the limitations of computer alphabetization resulted in the listing of one person as the author of another’s paper. On p. 4786, it should indicate that James Christopher Phillips had an article beginning on p. 128, and James Charles Phillips had an article beginning on p. 3820.

Correction. In the article "Studies of human myasthenia gravis: Electrophysiological and ultrastructural evidence compatible with antibody attachment to acetylcholine receptor complex" by J. E. Rash, E. X. Albuquerque, C. S. Hudson, R. F. Mayer, and J. R. Satterfield, which appeared in the December 1976 issue of Proc. Natl. Acad. Sci. USA 73, 4584–4588, a printer’s error deleted the first author of ref. 1. The correct reference is:

Kinetic evidence for hapten-induced conformational transition in immunoglobulin MOPC 460

(antibody function/chemical relaxation/allostery mechanism)

Doron Lancet and Israel Pecht

Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel

Communicated by Manfred Eigen, June 24, 1976

ABSTRACT  The kinetics of hapten binding to the homogeneous immunoglobulin A secreted by the murine plasmacytoma MOPC 460 was investigated by the chemical relaxation method. Two distinct relaxation times were observed in the binding equilibrium with three different haptens. A detailed concentration dependence analysis of relaxation times and amplitudes was performed with the hapten e-N(2,4-dinitrophenyl)-l-lysine (Dnp-Lys). The results support a mechanism in which two interconvertible conformational states of the protein bind the hapten with different association constants. Hapten binding shifts the equilibrium towards the better binding state. These observations form kinetic evidence for a conformational transition induced in the immunoglobulin by ligand binding to its antigen binding site, and are in line with the allosteric hypothesis for the initiation of physiological functions by antigen-antibody association.

The binding of antigen to its specific site is known to induce several physiological activities at other remote sites on the antibody molecule. Conformationally mediated interaction (allostery) is one of the models proposed to account for this phenomenon (1). Evidence for such antigen-induced conformational transitions arose mainly from static measurements (1–3), while kinetic studies failed to resolve more than a single (association) step in hapten–immunoglobulin equilibria (4, 5). Here we report a study of the interaction between MOPC-460, a homogeneous immunoglobulin A, and its specific nitroaromatic haptens. This system has been previously characterized and shown to constitute an adequate model for antibody–antigen interaction (6–8). The method of chemical relaxation (9) was used to resolve the binding mechanism. Two relaxation times, of which one was bimolecular and the other monomolecular, were observed. The results form unequivocal kinetic evidence for protein isomerization in a hapten–immunoglobulin equilibrium.

MATERIALS AND METHODS

The reduced and alkylated protein 460 monomer was prepared from the ascites fluid of tumor-bearing BALB/c mice according to Goeztel and Metzger (10). Measurements were carried out in 0.01 M sodium phosphate buffer containing 0.15 M NaCl at pH 7.4. Temperature-jump experiments were done on an apparatus described elsewhere (11). The discharge voltage was 20 kV, causing a temperature jump of 5.2°C from an initial temperature of 20.0 ± 0.1°C. Concentration changes were monitored via the quenching of the protein intrinsic fluorescence by the haptens, and in the case of 4-(e-N-L-alanine)-7-nitrobenz-2-oxa-1,3-diazone (NBD-Ala), also via the quenching of the hapten fluorescence as well as changes in its absorption. Relaxation curves were converted into digital values with a Bio-Rad model 802 transient recorder. Recordings were made at two different sweep rates and a sum of 6 to 8 jumps was used for the calculations. Relaxation times and amplitudes were fitted by the method of Grinvald and Steinberg (13). The observed amplitude ΔFobs (in arbitrary units) depends on the amplification factor which may change from one concentration point to another, and is given by the general expression:

$$\Delta F_{\text{obs}} = \sum a_i \cdot \Delta F_i,$$

where ΔFi are molar fluorescence changes of the steps in the arbitrary units, and the coefficients ai include equilibrium concentrations and molar enthalpies. To provide a consistent concentration dependence analysis, the amplitude is normalized by dividing the above equation by P1Fp (P1 is total protein concentration and Fp is the molar fluorescence of the protein in the arbitrary units). For each jump, the total fluorescence signal (F) at the high temperature equilibrium state is also measured at the same amplification. F is given by $F = F_p P_1 (1 - q)$ where q is the fractional quenching of fluorescence at the particular hapten concentration, as obtained from titrations. The normalized amplitude is then given by:

$$A = \Delta F_{\text{obs}} (1 - q) / F = (1 / P_1) \sum a_i \cdot \Delta f_i,$$

and is independent of instrumental setting. The first expression contains only measurable quantities, and the second (in which $\Delta f_i = \Delta F_i / F_p$) is theoretical and yields the formulae in rows H and I of Table 1. The expressions for the concentration dependence of times and amplitudes were derived according to Castellan (14) and Jovin (15), respectively. The experimental concentration dependences were fitted to those predicted by different mechanisms using the algorithm of Powell (16).

RESULTS AND INTERPRETATION

Overall equilibrium parameters

Parameters were obtained by fluorometric titrations and calorimetric measurements which will be described in detail elsewhere. For e-N-(2,4-dinitrophenyl)-l-lysine (Dnp-Lys) these were: overall association constant (compare Table 1) $K = 6.5 \pm 0.5 \times 10^4$ M$^{-1}$; overall fluorescence change $\Delta f = -0.48 \pm 0.03$; and overall molar enthalpy change $\Delta H = -14.5 \pm 1.5$ kcal/mol; all are in agreement with ref. 7. The corresponding values for NBD-Ala were $K = 3.5 \pm 0.2 \times 10^5$ M$^{-1}$, $\Delta f = 0.45 \pm 0.05$, and $\Delta H = -9.3 \pm 1.0$ kcal/mol (1 kcal = 4.184 kJ). The interaction of NBD-Ala with protein 460 and other Dnp-binding immunoglobulins will be fully described in a separate manuscript.

Temperature-jump experiments

The relaxation spectrum for the system of Dnp-Lys and protein 460 is composed of two relaxation times that are well separated over the whole concentration range. The concentration de-

Abbreviations: Dnp-Lys, e-N-(2,4-dinitrophenyl)-l-lysine; NBD-Ala, 4-(e-N-L-alanine)-7-nitrobenz-2-oxa-1,3-diazone; $\tau_p, \tau_s$ slow and fast relaxation times; $A_p, A_s$ slow and fast normalized relaxation amplitudes.
Table 1. Possible mechanisms and their kinetic behavior

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Formulæ are referenced by the column number and the row letter.
Symbols denote species as well as their equilibrium concentrations: $H_i$, hapten; $R_0$ and $R_i$, free and bound protein in the better binding state; $T_0$ and $T_i$, free and bound protein in the worse binding state. $P_i$, total protein concentration.
The overall association constant is defined as $K = (R_1 + T_1) / (R_0 + T_0)$. Individual equilibrium constants are defined as $K_i = k_i / k_{-i}$ and the direction of $k_i$ is defined to be from reactants to products.
The associations are assumed to be much faster than the isomerizations. For mechanism 3 the association to the $T$ state is assumed to be much faster than that to the $R$ state (see text).

$k_{ij}$ is the element of the matrix $g$ on the $i$-th row and $j$-th column. $q_{ii}$ is the principal partial matrix of order $i$ derived from $g$, and $|g_{ij}|$ is its determinant.

$\Delta \ln K_i = \Delta H_i \Delta T/R T_i$, where $\Delta T$ is the temperature difference in the jump, $\Delta H_i$ is the molar enthalpy change of the $i$-th step, $R$ is the gas constant, and $T$ is the absolute temperature. $\Delta f_i$ is the normalized molar fluorescence change of the $i$-th step (see Materials and Methods).

Pendences of the fast time ($\tau_F$) and slow time ($\tau_S$) are shown in Fig. 1A and B, respectively. The protein concentration is held constant at about 1.6 μM (sites) and the reciprocal relaxation times are plotted versus the increasing total Dnp-Lys concentration ($H_T$). $1/\tau_F$ increases linearly from 1·10³ to 4·10³ sec⁻¹, while $1/\tau_S$ decreases from 120 sec⁻¹ to a plateau value of about 50 sec⁻¹. This behavior is consistent with a reaction scheme that includes a fast bimolecular hapten-protein association and a slow monomolecular protein isomerization, most probably a conformational transition. The following observations confirm this tentative interpretation.

1. The fast association is kinetically and thermodynamically uncoupled from the slow isomerization. Also the hapten concentration is, to a good approximation, buffered (9) for all but the first three points of Fig. 2. Under these two conditions $\tau_F$ may be approximated by $1/\tau_F = k_{on} H_T + k_{off}$ independent of
and Dnp-Lys-bound the Thus, in res. Also, the process respectively (compare with agree amplitude fluorescence a method. In free haptens displayed nent the slow similar relaxation and the association step systems immunoglobulin-hapten line drawn kon X of group, uM and 3. Variation of TAXATION 1. Variation of the inverse fast (A) and slow (B) relaxation times on total Dnp-lysine concentration. The broken and solid lines are calculated using the best fit parameters for mechanisms 2 and 3, respectively. The concentration of protein 460 was 1.5 - 1.7 µM sites. The bars denote approximate standard deviation in the group of jumps represented by one point.

the detailed mechanism. (Ht is total hapten concentration and k on and k off are the rate constants for the association and dissociation, respectively.) The slope and intercept of a straight line drawn through the last nine points of Fig. 2 yield k on = 1.1 x 10^9 M^-1 sec^-1 and k off = 850 sec^-1. The equilibrium constant characterizing the association step is K as = k on/k off = 1.3 x 10^5 M^-1. The rate constants are similar to those found for other immunoglobulin-hapten systems (5) and the equilibrium constant is of the same order of magnitude as the overall constant from titrations, all in agreement with τ f representing the hapten–protein association.

2. (a) Preliminary experiments with two other haptens, (NBD-Ala and 2,4-dinitronaphth-1-ol) were found to yield similar relaxation spectra with an identical limiting value for the slow time. τ s must therefore be due to a common component of all three systems, namely the protein. (b) None of the free haptens displayed a chemical relaxation in any detection method. In contrast, the free protein exhibited in its intrinsic fluorescence a relaxation with 1/τ = 110 ± 30 sec^-1 and a normalized amplitude A = 1.5 ± 0.5 x 10^-3 which roughly agree with the extrapolation to H t = 0 of 1/τ s and A p, respectively (compare Figs. 1 and 2). This further confirms that the slow process is due to the protein.

3. Variation of the protein concentration from 1.6 to 30 µM at a constant hapten concentration did not change the value of τ s. Also, no oligomeric forms higher than the monomer were found in sedimentation velocity measurements of both the free and Dnp-Lys-bound protein at a concentration of 60 µM sites. Thus, the possibility that τ s reflects protein–protein association is excluded, and the slow step is proven to be monomolecular.

4. This monomolecular transition in the protein must be coupled to the binding of the hapten since: (a) τ f is found to change with hapten concentration (Fig. 1). (b) In the case of NBD-Ala the slow time is observed not only in the protein fluorescence but also via changes in the hapten fluorescence and light absorption.

Two minimal mechanisms that conform with this scheme are 1 and 2 of Table 1. In mechanism 1 the protein–hapten complex isomerizes, while in mechanism 2 it is the free protein. Mechanism 1 is ruled out for the following reasons: (i) The decrease of 1/τ s with increasing hapten concentration is not compatible with Eqs. 1D, 1E, and 1F*. (ii) The kinetically determined K as is identified with K f, and according to Eq. 1B, K > K f must hold, in contrast with the observed K < K as. Mechanism 2 qualitatively agrees with the observed behavior, since Eqs. 2D, 2E, and 2F predict a decreasing 1/τ s and K f = K as > K is consistent with Eq 2B. An attempt is therefore made to examine its quantitative fit to all the data. In the fitting procedure the overall association constant (K), overall molar enthalpy change (ΔH), and maximal quenching (Δf) are fixed at the above measured values.

First, k 0 and k -0 are determined by fitting the slow time data (Fig. 1B) to Eq. 2D. These are the only free parameters, since knowledge of k 0 and k -0 enables the calculation of K 0 (=k 0/ k -) and K R (using Eq. 2B and the value of K). The fast time data (Fig. 1A) are then fitted to Eq. 2C with K R as the only free parameter (k -0 is calculated as k R/K R). The broken lines in Fig. 1 represent these respective fits and the resultant rate and equilibrium constants are listed in Table 2.

The relaxation amplitudes (Fig. 2) are then analyzed. The equilibrium constants obtained above are used to calculate the elements of the g matrix (Eq. 2G) and the values of ΔH f and Δf i (molar enthalpy change and normalized fluorescence change of the i-th step, respectively) serve as free parameters.

* Equations denoted by a number and a letter appear in the corresponding column and row of Table 1.

---

**FIG. 1.** The dependence of the inverse fast (A) and slow (B) relaxation times on total Dnp-lysine concentration. The broken and solid lines are calculated using the best fit parameters for mechanisms 2 and 3, respectively. The concentration of protein 460 was 1.5 - 1.7 µM sites. The bars denote approximate standard deviation in the group of jumps represented by one point.

**FIG. 2.** The dependence of fast relaxation amplitude (upper curve) and slow relaxation amplitude (lower curve) on total Dnp-Lys concentration. The broken and solid lines are calculated using the best fit parameters for mechanisms 2 and 3, respectively. All other details are the same as in Fig. 1.
The fast amplitude (Eq. 2H) fits well (upper broken-line curve in Fig. 2) even though only the multiplying factor \( \Delta f_R, H_R \) is free and the shape of the curve is predetermined. In contrast, the slow amplitude (Eq. 2I) does not give a satisfactory agreement, either when \( \Delta f_R, H_R \) is fixed to the value obtained above, or even when both \( \Delta f_R \) and \( \Delta H_R \) are left free: in both cases the lower broken-line curve of Fig. 2 represents the best fit. Thus, mechanism 2 agrees with the behavior of three out of the four relaxation parameters, but is excluded by the slow amplitude data.

In view of this, a third mechanism (no. 3 in Table 1) is examined. Here both the free and the bound protein isomerize, and both the \( T \) and \( R \) forms bind the hapten with finite association constants (\( K_R > K_T \) by definition). Only three of the four steps are thermodynamically independent, as reflected in the relation:

\[
K_T/K_R = K/J/K_1
\]

A maximum of three relaxation times may therefore be observed, and when the isomerizations are much slower than the associations (borne out by experiment) one slow monomolecular time and two fast bimolecular times are expected (14).

The slow time is unequivocally identified with the observed \( \tau_{1R} \) and the data of Fig. 1B may thus be fitted to Eq. 3D. Using the known value of \( K \) and \( E \), four free parameters are left which are chosen as \( q = k_0 + k_{-0} \), \( p = k_1 + k_{-1} \), \( r = k_2/k_1 \), and \( s = k_{-2}/k_{-1} \). This choice of parameters is decisive for successful convergence. Also, an initial guess for \( q \) and \( p \) is available according to Eqs. 3E and 3F. While the values of the first three parameters are uniquely determined (\( q = 125.5 \pm 1 \sec^{-1}, p = 52 \pm 2 \sec^{-1}, \) and \( r = 0.68 \pm 0.03 \)), \( s \) is found to be indeterminate in the sense that values from 2.8 through 10 yield the same fit (solid line in Fig. 1B). Even higher values up to \( s = 60 \) never give worse fit than the broken line of this figure (\( s \rightarrow \infty \) corresponds to mechanism 2, see Discussion). Only when the slow relaxation amplitude is fitted to Eq. 3I it is possible to resolve this degeneracy: the best fit is obtained with a value of \( s = 7.4 \) (lower solid-line curve in Fig. 2). The fit parameters (slow rate constants, all equilibrium constants and \( \Delta f_R, \Delta H_R \)) are given in Table 2.

The fast relaxation data may now be treated in terms of two association steps having \( K_T = 1.9 \times 10^8 \M^{-1} \) and \( K_R = 2.2 \times 10^9 \M^{-1} \), respectively. \( \tau_T \) may represent either one of the two expected fast times or a combination of both. To decide between these possibilities the fast amplitude is fitted to a total amplitude expression (cf. Eq. 20 of ref. 15) for a scheme that includes only the two ligand-coupled associations (time separation implies that during the fast relaxation the slow steps may be ignored). Computer simulations show that at the concentrations used and with the values of \( K_R \) and \( K_T \), ligand-coupling is negligible and the total amplitude (\( A_T \)) assumes the form:

\[
A_T = \frac{\Delta f_R \Delta f, K_T}{s_0 - \varphi} + \frac{\Delta f_R \Delta f_0 \Delta f_R}{s_0 - \varphi}
\]

The first and second terms are the independent amplitude expressions for the associations to the \( T \) and \( R \) states, respectively, and have different functional shape. The attempted fit shows that the functional shape of the experimental fast amplitude is very close to that of a pure second term, indicating that the fast relaxation represents solely the association to the \( R \) state. The observed \( \tau_T \) may thus be identified with \( \tau_{1R} \) and \( A_T \) (Table 1), respectively, and the corresponding fits yield the full line in Fig. 1A and the upper full line in Fig. 2 with the parameters listed in Table 2 (see note \( \S \) of this table). Assuming that \( K_R \) and \( k_T \) are essentially diffusion controlled and therefore have similar values (cf. ref. 5), \( \tau_{1R} \) is expected to be shorter than \( \tau_T \) by the factor \( K_R/K_T \approx 10 \). Attempts to resolve a second fast relaxation time shorter than 100 \mu sec lead to inconclusive results due to experimental difficulties. Nevertheless, mechanism 3 appears to be the simplest scheme that is consistent with the behavior of all the observable relaxation parameters.

In preliminary experiments with the Fab fragment the same relaxation spectrum was observed. The values and the general concentration dependence of the relaxation times were similar to those obtained with the intact protein. Still, the possibility of a quantitative difference in the relaxation behavior was not excluded. Similar relaxation phenomena were also observed in a non-reduced and alkylated preparation which contained the oligomeric forms of protein 460.

**DISCUSSION**

The assignment of mechanism 3 to the reaction of protein 460 with haptens is significant in two ways: (a) Hapten binding to an immunoglobulin is kinetically found to involve a conformational transition. The implications of this finding for the understanding of antibody function are discussed below. (b) It is an unequivocal kinetic assignment of mechanism 3 to an experimental system. This mechanism forms the basic element of the generalized allosteric scheme proposed by Eigen (17), and is actually the model of Monod, Wyman, and Changeux (18), as applied to an "allosteric monomer" (\( n = 1 \)). On the other
hand, it includes the kinetically simpler mechanisms of Table 1 as limiting cases with $K_R \to \infty$, $K_O \to 0$, for mechanism 1, and $K_T \to 0$, $K_1 \to \infty$ for mechanism 2. The studied system may therefore serve also as a useful model for ligand–protein interactions in general.

The results of this study show that when only relaxation times are considered, the data may be consistent with a wide range of parameters for mechanism 3, including the limit of one of the simpler mechanisms. All three mechanisms of Table 1 may thus be taken as different numerical cases of one thermodynamically general scheme. Analysis in terms of this scheme, using relaxation amplitudes in addition to relaxation times to resolve the appropriate set of parameters, seems therefore advisable.

The relative simplicity of mechanism 3 enables its full kinetic and thermodynamic characterization without simplifying assumptions (Table 2). The value of $K_T/K_R = K_0/K_1$ (in the Monod–Wyman–Changeux formalism) is found to be 0.088. The difference between $k_0$ and $K_1$ is mostly due to a difference between $k_0$ and $K_1$ ($s = k_0/K_1 = 7.4$), while $k_0$ and $K_1$ are only slightly different ($r = k_0/K_1 = 0.66$). This result resembles the situation previously introduced as an assumption (19), namely, that the rate constants for all $T_i$ to $R_i$ isomerizations in an allosteric oligomer are equal.

A value of $\Delta(\Delta G) = \Delta G_R - \Delta G_T = 1.4$ kcal/mol is calculated from Table 2. This difference in free energy of binding to the states $T$ and $R$ is due to roughly equal and relatively small enthalpic and entropic contributions. The two states of the protein are thus seen to have quite similar interactions with the hapten. Still, the value of $\Delta(\Delta G)$ (also equal to $\Delta G_1 - \Delta G_0$) is sufficient to invert the position of the conformational equilibrium. $R$, the state function (18), changes from $K_0/(1 + K_0) = 0.23$ to $K_1/(1 + K_1) = 0.78$ with hapten saturation. This is possible since $\Delta G_0$ is only $+0.7$ kcal/mol, corresponding to $L = 1/K_0 = 3.3$ in the Monod–Wyman–Changeux notation.

The hapten-induced conformational transition in protein 460 is consistent with the allosteric model for the initiation of physiological events by antigen–antibody complexes. This model assumes conformationally mediated heterotropic interactions between the antigen-binding site and effector sites in other domains of the antibody (1, 2). For immunoglobulins of the M class this is assumed to be sufficient, presumably since their pentameric structure eliminates the necessity for aggregation (1, 20). Protein 460, an immunoglobulin of the A class, is also oligomeric in its native form, and may behave similarly. It is noteworthy that antigen binding to immunoglobulin A is known to trigger the alternative complement pathway via a site on the Fab region (21), in line with the observed similarity of the relaxation behavior between the Fab fragment and the intact monomer or oligomer.

The binding of low-molecular-weight haptens to immunoglobulins is rarely found to have a physiological outcome, or to induce conformational phenomena. It seems that the physiologically active state of the immunoglobulin is attained via a major conformational change ($\Delta G_0$ large and positive), which involves many residues in the antigen-binding site and probably has to do with the relative position of the light and heavy chains (2). $\Delta(\Delta G)$ for the binding of a small hapten may usually be insufficient to compensate for this large $\Delta G_0$. Protein 460 may be a case where $\Delta G_0$ is small, or where the pathway towards the full transition includes a conformational intermediate with a small $\Delta G_0$. As a result, hapten-binding studies in this well-defined model system are able to yield at least partial information about the dynamics and energetics of the postulated antigen-induced conformational transition.

We thank Dr. M. Eigen, Dr. T. Jovin, and Dr. R. Rigler for their invaluable advice in theoretical and experimental matters. We are also indebted to Y. Blatt and M. Goldberg for reviewing this manuscript and R. Zhidovovsky for carrying out the calorimetric measurements.