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KINETICS OF HALOPERIDOL BINDING TO MONOCLONAL ANTIBODIES AS MEASURED BY DIRECT FLUORESCENCE QUENCHING

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Abstract—Monoclonal antibodies which bind small neurogenic ligands may mimic certain aspects of the stereospecific binding present in the natural biological receptor, and may prove useful in designing engineered protein receptors, provided their interactions are correctly understood. We report here the kinetic and equilibrium characteristics of ligand–antibody complexes of haloperidol, a dopaminergic antagonist, with three of its monoclonal antibodies as studied by fluorescence quenching techniques. These antibodies possessed moderate to high affinity constants, ranging from 10^5 to 10^9 M^{-1}, and caused fluorescence quenching of a fluorescein-labeled haloperidol as well as quenching (40–60%) of the internal tryptophan fluorescence. The dissociation rates of the ligand from the complexes were measured at different conditions of temperature, pH and ionic strength. The results provide important information regarding the kinetic and thermodynamic parameters of the binding pockets of these antibodies.

Antibodies produced against small biologically active ligands can demonstrate extraordinary stereospecific binding and this property is due, in part, to the precise stereochemical interactions with the amino acid side chain moieties which make up the binding pocket of the antibody [1]. In some instances, such stereospecific binding characteristics may closely mimic those found in the natural biological receptor. With respect to small neurogenic ligands, monoclonal antibodies to alprenolol [2–5] have been carefully studied for their ability to mimic binding site features common to neurogenic receptors. Such model systems may prove useful in the design of artificial receptors and ligands. In addition, antibodies which mimic receptor binding sites have proved useful in the production of anti-idiotypic antibodies, and these anti-idiotypes have proved to be powerful tools for the study of receptor structure and function (for review see Ref. 6).

There have been numerous experimental studies detailing the binding kinetics and energetics of ligand binding to myeloma proteins or monoclonal antibodies [7–10]. Both enthalpic and entropic factors have been found to make significant contributions to the binding energetics depending upon the size and nature of the ligand. Several studies have employed the use of fluorescent ligands, and fluorescein itself, as a tool for the study of ligand–antibody interactions [11–13].

From a number of crystallographic determinations, it has been observed that the antibody binding site, in almost all examples, is created by six peptide loops at the tip of the Fv immunoglobulin fragment. These six complementarity determining regions (CDR‖), or hypervariable loops, are generated by the folds and turns of the anti-parallel β-stranded barrels or "sandwiches" of the heavy and light chain variable regions (14). Specific side chain moieties for the amino acids of the CDR provide the basis for the electrostatic, H-bonding, van der Waals, charge transfer, and hydrophobic interactions with the antigen. A sub-set of "energetic" contact residues in the CDR may account for the majority of the attractive forces involved in antigen binding, and these determinants are, of course, complemented by favorable interactive contacts in the antigen [10]. The quantitative measurement of the kinetics and driving forces involved in the ligand–antibody interactions may be more useful than less precise terms, such as "affinity", because these aspects provide some insight into the chemical nature of the binding site.

In the present study, we used sensitive fluorescence quenching techniques to examine the kinetics of ligand binding for several monoclonal antibodies which bind the neuroleptic drug haloperidol. Haloperidol, a butyrophenone, is an antagonist for the D2 receptor. We have previously generated a large collection of specific monoclonal antibodies to this drug and several related neuroleptic ligands [15, 16]. Sequencing of the variable region genes [17] and subsequent computer-aided molecular modeling of some of these antibodies [18, 19] have suggested that one of the important features of the binding site for several of the antibodies is the formation of a charge-transfer or π–π complex involving the tryptophan residue at position H:50 and the *p*-fluorophenyl ring of the ligand. Refined modeling [19] of antibody 185-6 suggests the

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‖ Abbreviations: CDR, complementarity determining regions; and FITC, fluorescein isothiocyanate.

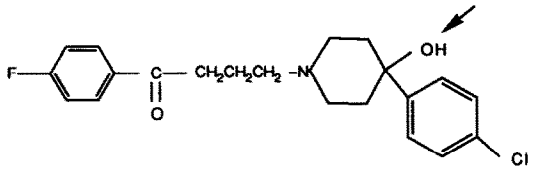

Fig. 1. Structure of the butyrophenone haloperidol (pK_a 8.3). The immunoconjugation and fluorescein derivatization were made through the position of the tertiary alcohol, as indicated by the arrow.

formation of a salt-bridge between the piperidinyl nitrogen of the ligand and H100:E and the formation of a hydrogen bond with the residue L93:Y. Some of the anti-haloperidol antibodies examined in the present study have displayed different binding kinetics when employed in radioimmunoassays at different temperatures (unpublished observations). Visual inspection of the computer-generated binding site models suggests that the observed thermal effects may be due to the relatively large number of aromatic residues, viz. tyrosines, and their ability to rotate freely in the binding sites, thereby destabilizing the binding site environment for a flexible ligand, such as haloperidol. In an effort to carefully examine the kinetics of haloperidol binding, and to determine if these antibodies are sensitive to solvent conditions, we elected to examine the binding kinetics under various pH, salt and temperature conditions.

MATERIALS AND METHODS

Preparation of monoclonal antibody. Monoclonal antibodies to the neuroleptic drugs haloperidol, ketanserin and pimozide have been developed in our laboratory, using standard procedures [3, 6]. For the study present study, we used antibodies 185(2)-1, 237(2)-1 and HAL-S-1, which were all made against haloperidol that was conjugated through the tertiary alcohol of the piperidine ring (Fig. 1). Ligand interactions with mAb 185(2)-1 have been previously studied [18] using competitive radioimmunoassays.

Preparation of the fluorescein derivative of haloperidol. The fluorescent-haloperidol was prepared by the addition of a fluorescein moiety to haloperidol hemisuccinate. The conjugation was via the tertiary alcohol of the piperidine ring (Fig. 1) using aminomethylfluorescein. The fluorescent derivative was purified by preparative HPLC on a C18 reverse plate and eluted with methanol:water:acetic acid.

Fluorescence measurements. Fluorescence measurements were carried out on an SLM 8000 Spectrofluorometer equipped with a thermostatic cell holder and a magnetic stirrer. Excitation spectra of the fluorescein derivative of haloperidol and fluorescein isothiocyanate (FITC) are shown in Fig. 2; two wavelength regions between 295 and 345 nm (panel A) and between 480 and 520 nm (panel B) are shown. Due to the conjugation of fluorescein to haloperidol, a new excitation peak appeared in the shorter wavelength region (310–330 nm) and the peak in the longer wavelength region (480–510 nm) showed a red-shift from 493 nm (for FITC) to 499 nm (for the ligand-derivative).

The emission band had a peak at 520 nm upon excitation at either wavelength (320 or 499 nm) (Fig. 2, panel C). No emission band shift was observed for the ligand–antibody complex. Because the shorter excitation band is specific for the derivatized ligand, excitation and emission at 320 and 520 nm, respectively, were used for the kinetic and binding experiments. This choice also avoided the use of an excitation wavelength close to the emission wavelength, thereby eliminating potential interference by scattered light due to the red-shift of the longer wavelength excitation band.

Emission spectra of the fluorescent-haloperidol bound to antibody at different time intervals are presented in panel D of Fig. 2. Curve 1 shows the spectra for the fully saturated antibody–ligand complex, and subsequent increases in emission due to dissociation of the bound ligand (curves 2–7) as initiated by the addition of non-fluorescent haloperidol during the observed time period. The infinity reading (maximal emission) was obtained either by adding haloperidol to the antibodies prior to the addition of the fluorescent haloperidol derivative or by measuring the fluorescence intensity after a long time period (estimated to be about ten to fifteen half-lives). It was experimentally determined that both methods gave the same value. All measurements were done in triplicate and the mean values were used for the computational analysis. Because of the large changes in the fluorescence intensity, the kinetic measurements were very accurate and the standard errors of three sequential measurements were usually within 1–3% of the average values. The relative fluorescence intensities were obtained as the ratio of a fluorescent beam to a straight-through beam, in order to compensate for any fluctuation in the lamp intensity. The data were collected, stored, and manipulated using a microcomputer dedicated to SLM 8000.

Kinetic calculations. The binding of the monoclonal antibodies to the fluorescein derivative of haloperidol caused about 40–60% quenching of the ligand fluorescence depending on the antibody used. The subsequent addition of unlabeled haloperidol resulted in a time-dependent reversal of the fluorescence intensity. The increase in the intensity followed a first order kinetics in the initial phase (more than three half-lives) as expected for a simple binding scheme as follows:

$$\text{Antibody (Ab)} + \text{Ligand (L)} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} \text{Complex (Ab–L)}$$

with the forward rate association constant (k_{+1}) being several fold higher than the reverse dissociation rate constant (k_{-1}). The data from a typical kinetic analysis are presented in Fig. 3. The dissociation rate constants and half-lives were calculated using an algorithm developed in our laboratory for the Macintosh IIx which utilizes the standard relaxation equation [20–23]. In these experiments the forward rates (k_{+1}) were observed to be much faster than the reverse rates (k_{-1}). The relaxation time is

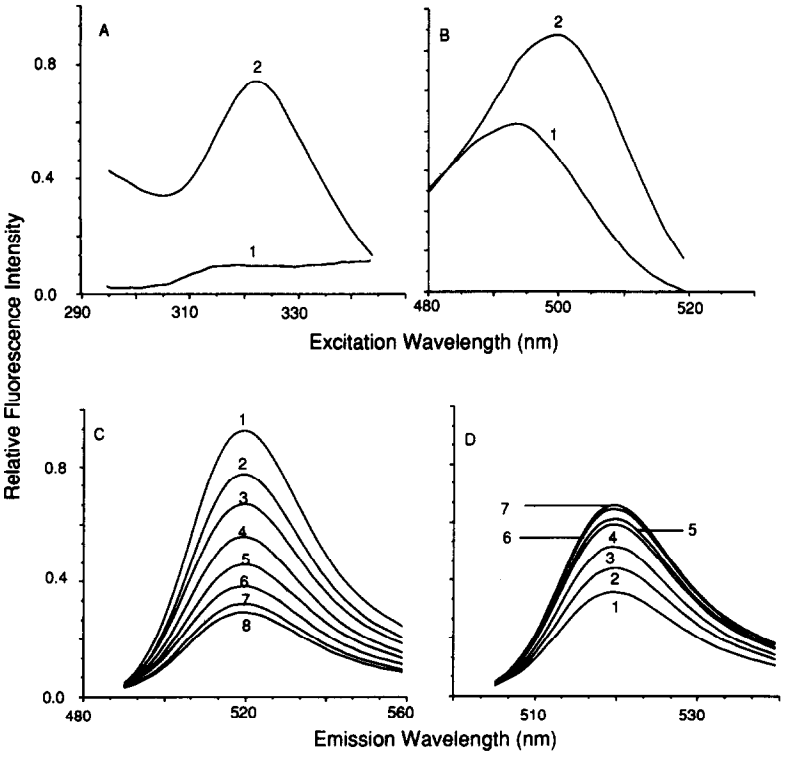

Fig. 2. Excitation and emission spectra of the fluorescein derivative of haloperidol in 0.15 M sodium chloride, 10 mM sodium phosphate, pH 7.6, temperature 20°. Panel A: Fluorescence spectra of FITC (1) and the fluorescein conjugate of haloperidol (2) in the excitation wavelength range between 295 nm and 345 nm with emission at 520 nm. Panel B: Fluorescence spectra of FITC (1) and the fluorescein conjugate of haloperidol (2) in the excitation wavelength range between 480 and 520 nm with emission at 520 nm. Panel C: Fluorescence spectra of the fluorescein conjugate of haloperidol in the emission wavelength range between 480 and 560 nm during the course of a titration with the antibody 185(2)-1. Curve 1 corresponds to that of the conjugate alone at a concentration of 100 nM. Curves 2 through 8 are the spectra obtained by the successive addition of the antibody (stock concentration = 400 nM) at volumes of the titrant of 50, 100, 150, 200, 250, 350, and 500 μL, respectively. Panel D: Curves 1 through 7 are the spectra obtained at time intervals of 5, 10, 20, 30, 40, 60 and 120 min, after the addition of 100 μL of unlabeled haloperidol (20-fold in excess of the fluorescein derivative, Stock concentration = 30 μM).

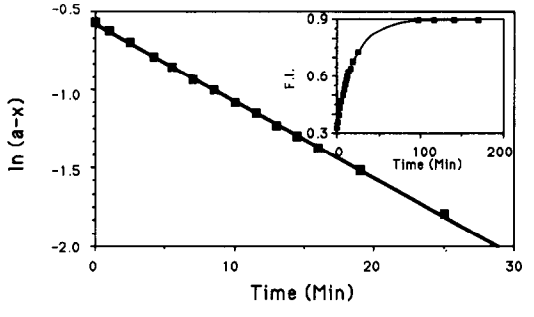

Fig. 3. First order kinetics of the enhancement of fluorescence. Measurements were made by adding 2 μM haloperidol to a mixture of the fluorescein derivative of haloperidol (100 nM) and the monoclonal antibody 185(2)-1 (100 nM) in 0.15 M sodium chloride and 10 mM sodium phosphate at pH 7.6 and 20° (regression coefficient = 1.000). Inset: The corresponding kinetic trace. The relative fluorescence intensity at 520 nm is plotted against time in minutes. Excitation wavelength was 320 nm.

independent of reactant concentrations and the inverse of the relaxation time is equal to the reverse rate constants.

Binding calculations. Binding of haloperidol or the fluorescein derivative caused quenching of internal tryptophan fluorescence in the range of 40–50% for the monoclonal antibodies in this study. The internal tryptophan fluorescence quenching is located in a different spectral region than that observed for the fluorescein derivative; the excitation and emission maxima are 280 and 320 nm, respectively. Either tryptophan or fluorescein quenching could be used for binding studies, but the fluorescein derived fluorescence data were preferred because of the high emission intensity. Studies to examine tryptophan fluorescence quenching kinetics with non-fluorescent ligands with these and other antibodies are currently underway. We have observed that not all monoclonal antibodies have a tryptophan residue in or near the ligand binding site and, therefore, the assessment of tryptophan quenching may be of limited usefulness in certain cases.

For high affinity antibodies, the maximum ligand

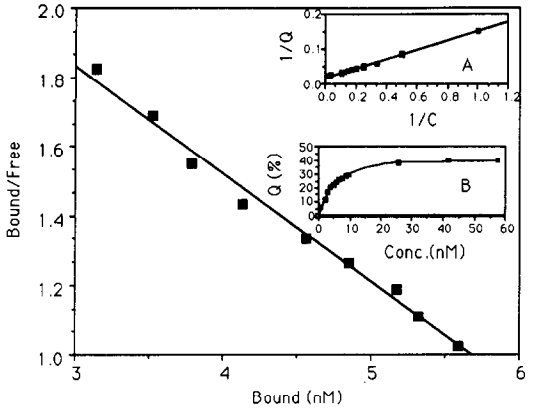

Fig. 4. Scatchard plot of the binding of the fluorescein derivative of haloperidol to the monoclonal antibody 237(2)-1 in 0.15 M sodium chloride and 10 mM sodium phosphate at pH 7.6 and 20°. The ratio of the concentration of the bound ligand to its concentration in the free form is plotted against the concentration of the bound ligand (regression coefficient = 0.989, slope = 0.312 per nM). Inset A: Double inverse plot of corrected fluorescence quenching versus the concentration of the added antibody in nM (regression coefficient = 0.996, intercept = 1.99×10^{-2}). Inset B: The titration curve corrected for fluorescence quenching versus concentration of the added antibody. Excitation was at 320 nm and the emission was followed at 520 nm.

fluorescence quenching (Q_{max}) was obtained by titration with excess antibody, but for low affinity antibodies the Q_{max} value was obtained from the extrapolation of the double inverse plot of the concentration versus extent of quenching (Fig. 4). Simultaneous titrations were performed using a non-reactive monoclonal antibody (anti-hemocyanin) which served as a negative control. The fluorescence intensities were corrected for attenuation and dilution using equations described earlier [24–26]. Association constants were obtained from Scatchard analysis (Fig. 4), and the values of bound/free and bound ligand concentrations were calculated from the extent of quenching. Standard free energy ($\Delta G°$) of binding was calculated using the equation: $\Delta G° = -RT \ln K_A$ (for review see Ref. 23), where R is the gas constant, T, is the absolute temperature, and K_A is the affinity binding constant.

pH Dependence. The pH dependence of the dissociation rate of the complex between the monoclonal antibodies and the fluorescent derivative of haloperidol was studied in the range between 4 and 10. The acidic pH buffers (pH 4–7) contained sodium acetate (10 mM) and phosphate (10 mM) while the alkaline buffers (pH 8–10) contained Tris (10 mM) and phosphate (10 mM); in addition all the buffers contained 0.15 M NaCl.

Temperature dependence. The temperature dependence of the dissociation rates was studied in the range between 5 and 36°. The temperature was maintained within ± 0.5° of the desired temperature in a thermostatic cuvette holder. The enthalpy ($\Delta H^{\#}$), free energy ($\Delta G^{\#}$) and entropy($\Delta S^{\#}$) of activation were calculated according to the following equations [27]:

$$\frac{d(\ln k_{-1})}{d\left(\frac{1}{T}\right)} = -\frac{\Delta E}{R}$$

$$k_{-1} = \frac{kT}{h} e^{-\Delta G^{\#}/RT}$$

$$\Delta H^{\#} = \Delta E - RT : \Delta G^{\#} = \Delta H^{\#} - T\Delta S^{\#}$$

where k is the Boltzman's constant, h is Plank's constant and ΔE is the measured energy of activation and was obtained from the slope of the plot of

$$\ln(k_{-1}) \text{ vs } \frac{1}{T}.$$

Ionic strength dependence. The effect of the ionic strength of the buffer on dissociation rates was studied by adding sodium chloride (up to 1 M) in addition to that present in the normal saline (0.15 M). The pH of the phosphate buffer changes with the ionic strength; hence, the observed rates were corrected for the changes in pH due to the salt effect. To examine the influence of any charged species in ligand binding, the kinetic data were analyzed using the following equation [28]:

$$\log(k_I) = \log(k_0) + 3.65 \times 10^6 (D)^{-\frac{3}{2}} (T)^{-\frac{3}{2}} (z_A z_B)\sqrt{I}$$

where k_I and k_0 are the rate constants at ionic strengths I and 0, respectively, D is the dielectric constant of the solvent, and $z_A z_B$ is the product of the ionic charges of the reacting species. From the slope of the plot of $\log(k_I)$ versus $\sqrt{I}$ the product of charges ($z_A z_B$) was obtained.

RESULTS

The dissociation kinetics of haloperidol from the three monoclonal antibodies 185(2)-1, 237(2)-1 and HAL-S-1 were studied under various conditions of pH, temperature and ionic strength. The initial dissociation rates for all the antibodies followed first order kinetics. The rate constants, half-lives, binding constants and free energies of the complexes are presented in Table 1. These monoclonal antibodies bound haloperidol with different affinities and the dissociation rates also differed considerably. Antibodies 185(2)-1 and 237(2)-1 bound haloperidol with a higher affinity than HAL-S-1. The dissociation rate for HAL-S-1 was almost the same as that for 185(2)-1; the dissociation rate observed for 237(2)-1 was two orders of magnitude faster.

Temperature dependence. Dissociation rates of the fluorescein derivative of haloperidol from the antibodies were measured at a number of different temperatures. Arrhenius plots produced straight lines as shown in Fig. 5. The thermodynamic parameters, e.g. free energy, enthalpy and entropy of activation, were calculated using the equations presented above and these values are presented in Table 2. The free energies of activation of the complexes with 185(2)-1 and HAL-S-1 were almost the same, but their enthalpies of activation differed due to the difference in the positive entropic

Table 1. Kinetic and thermodynamic parameters of the complexes of haloperidol with its monoclonal antibodies*

Clones	Dissociation rate (sec^{-1})	Half-life (min)	Assoc. const. (M^{-1})	Δ° free energy (kcal/mol)	Q_{max} (%)
185(2)-1	$8.55 \pm 0.17 \times 10^{-4}$	13.5 ± 0.26	$1.13 \pm 0.26 \times 10^{9}$	-12.2 ± 0.11	49
237(2)-1	$3.19 \pm 0.08 \times 10^{-2}$	0.36 ± 0.01	$3.12 \pm 0.47 \times 10^{8}$	-11.5 ± 0.10	42
HAL-S-1	$7.92 \pm 0.13 \times 10^{-4}$	14.6 ± 0.21	$1.58 \pm 0.46 \times 10^{5}$	-7.01 ± 0.19	60

* Experiments were performed in the phosphate saline buffer (0.15 M sodium chloride and 10 mM sodium phosphate) at pH 7.6 at 20° except for the dissociation rate for the complex with 237(2)-1 , the value for which was obtained from the extrapolation of the results in the lower temperature range (5° to 16°) in the same buffer. The mean and standard errors (SEM) were calculated for 3 or 4 measurements.

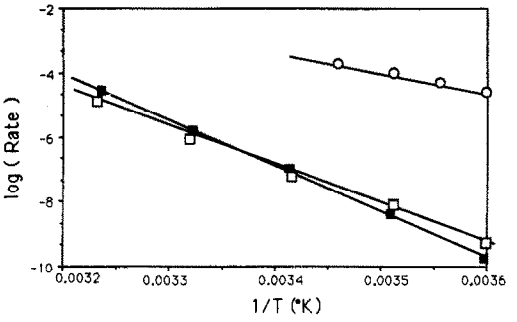

Fig. 5. Natural logarithm (base e) of the dissociation rate constants. Release of the fluorescein derivative of haloperidol from the complexes with the monoclonal antibodies is plotted against the inverse of absolute temperatures in 0.15 M sodium chloride and 10 mM sodium phosphate at pH 7.6. Key: (□) 185(2)-1, slope = -1.44×10^{4}, regression coefficient = 1.00; (■) HAL-S-1, slope = -1.21×10^{4}, regression coefficient = 0.989; and (○) 237(2)-1, slope = -6.73×10^{3}, regression coefficient = 0.931.

contributions. The complex with 237(2)-1 showed the least enthalpic barrier, and a negative entropic contribution, yielding a free energy of activation lower than those of the other two antibody–ligand complexes. As a result, the dissociation rate for the complex with 237(2)-1 was about two orders of magnitude faster than those for the other antibodies.

pH dependence. The pH dependence of the dissociation rates of the fluorescein derivative of haloperidol was measured between pH 4 and 10 (Fig. 6). The dissociation rates of the complexes with the monoclonal antibodies 185(2)-1 and HAL-S-1 steadily increased in the pH region between pH 6 and 9 while the off rates from the complex with 237(2)-1 showed a steady decrease in the pH range between 5 and 8. The dissociation rates for all the complexes increased sharply from pH 9 to 10. The dissociation rates for the complex with 185(2)-1 increased sharply from pH 6 to 4. Antibody 237(2)-1 showed a sharp increase in the off rate below pH 5. The dissociation rate for the complex with HAL-S-1 was too slow to be measured precisely below pH 6. The fluorescence intensity of the fluorescein derivative decreased sharply below pH 6, but the off rates from the complexes with 185(2)-1 and 237(2)-1 could be followed accurately because the fractional enhancement of fluorescence intensities were the same as those observed in the higher pH range.

Ionic strength dependence. The effects of ionic strength on the off rates of the fluorescent derivative of haloperidol from the complexes with all three monoclonal antibodies were studied with sodium chloride concentrations up to 1 M in addition to that present in normal phosphate-buffered saline (0.15

Table 2. The thermodynamic parameters of the activated complex obtained from the binding kinetics of haloperidol with the monoclonal antibodies.*

Monoclonal antibodies	Free energy ($\Delta G^{\#}$) (kcal/mol)	Enthalpy ($\Delta H^{\#}$) (kcal/mol)	Entropy ($\Delta S^{\#}$) (e.u.)
185(2)-1	21.2 ± 0.3	28.2 ± 0.4	23.9 ± 0.4
237(2)-1	19.1 ± 3.8	13.5 ± 2.7	-19.1 ± 3.8
HAL-S-1	21.3 ± 1.5	23.6 ± 1.6	7.85 ± 0.6

* Experiments were carried out in the phosphate saline buffer containing 0.15 M sodium chloride and 10 mM sodium phosphate at pH 7. 6. For complexes with 185(2)-1 andHAL-S-1 the dissociation rates were measured in the temperature range between 5 and 36° while for the complex with 237(2)-1 the temperature range was 5 to 16°. Means ± SEM are shown and were calculated from the slope of the plots of logarithm of rate constants against the inverse of absolute temperature containing 4 or 5 pairs of data points. # signifies the "activation of energy" state.

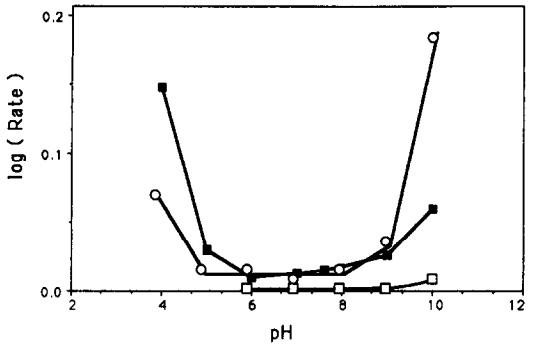

Fig. 6. Natural logarithm (base e) of the association rate constants. Change of the fluorescence by addition of haloperidol to the complexes of the fluorescent ligand–antibody is plotted against pH in 0.15 M sodium chloride, 10 mM sodium phosphate and 10 mM sodium acetate (below pH 7) or 10 mM Tris (at pH 8 and above). Key: (■) 185(2)-1 at 36°; (○) 237(2)-1 at 12° and (□) HAL-S-1 at 20°.

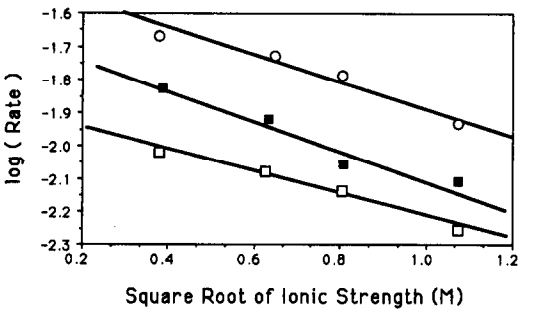

Fig. 8. Logarithm (base 10) of the rate constants of the dissociation rate of the fluorescein derivative of haloperidol from the antibody complexes versus the square root of the ionic strength of the medium in 10 mM sodium phosphate at pH 7.6. Key: (■) 185(2)-1 at 36°, slope = −0.436 and regression coefficient = 0.950; (○) 237(2)-1 at 12°, slope = −0.407 and regression coefficient = 0.956 and (□) HAL-S-1 at 36°, slope = −0.329 and regression coefficient = 0.974.

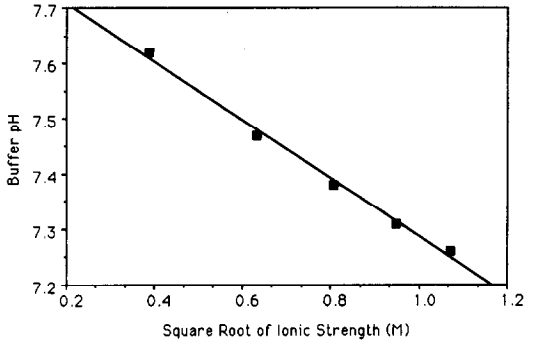

Fig. 7. The pH of a 10 mM sodium phosphate buffer with the square root of ionic strength of the medium at 20°. Slope = −0.53, intercept = 7.81 and regression coefficient = 0.995.

M). The pH of the phosphate buffer was found to be strongly dependent on the ionic strength of the medium. Figure 7 shows the linear dependence of the measured pH of phosphate buffered saline with the square root of the ionic strength. The measured values of the rates at different salt concentrations were corrected for the change in the pH of the buffer using the relation of pH with the square root of ionic strength and the dependence of the rates on pH in the range between 7 and 8. The plots of logarithm (base 10) of the corrected values of rate constants are shown in Fig. 8. The slopes of the plots for all the monoclonal antibodies are negative. The products of charges ($z_A z_B$) were calculated and are presented in Table 3. The nitrogen atom in the heterocyclic ring of haloperidol has a pK_a value of 8.3, and thus, haloperidol is positively charged in the neutral pH region. The negative value of the product of charges suggests that there is a net negative charge in the binding site of haloperidol, and this was found to be true for all three of the antibodies under study. For all the complexes, the products of the charges had fractional values, which suggests that the net effective negative charge in the vicinity of the hapten is less than one unit of charge.

DISCUSSION

The molecular interactions which take place between a ligand and the antibody binding site are predicated on the precise location and identity of the amino acids that make up the antibody CDR loops. The amino acid side chains provide the basis for the H-bonding, electrostatic, charge transfer, van der Waals and hydrophobic interactions with the ligand. The size, shape and orientation of the binding site residues provide the stereospecificity and energetics of ligand binding. In addition, the ligand possesses a set of complementary interactive contacts. It can be hypothesized that any mimicry of binding properties common to both a monoclonal antibody and the natural biological receptor may be due, in part, to homologous or analogous structures [29]. This aspect may prove very important in the design and engineering of receptor proteins. In addition, the design of new ligands based on our understanding of ligand–receptor interactions may benefit from such paradigms.

We have completed several studied of monoclonal antibody binding sites using experimental and theoretical techniques. Biophysical measurements of the ligand–antibody interactions, competitive binding studies and computer-aided modeling have proved useful in understanding the chemical make-up and intermolecular forces that participate in the reaction. The measurement of an association constant or "affinity" for an individual antibody–ligand system as derived from a binding assay does not provide adequate information regarding these aspects. Kinetic and thermodynamic analyses can often

Table 3. Effect of ionic strength on the off rates of the complexes between haloperidol and its monoclonal antibodies*

Monoclonal antibody	Slope of the the plot of $\log(k_{-1})$ vs square root of ionic strength	Product of charges ($z_A z_B$)
185(2)-1	−0.436 ± 0.072	−0.451 ± 0.074
237(2)-1	−0.407 ± 0.061	−0.373 ± 0.056
HAL-S-1	−0.329 ± 0.038	−0.341 ± 0.039

* Off rates of the fluorescein ligand from the antibody complexes were measured at pH 7.6 in 10 mM sodium phosphate at 36° for 185(2)-1 and HAL-S-1 and at 12° for 237(2)-1. Means ± SEM were calculated from the slope of the plot of logarithm of rate constants against the square root of ionic strength containing 4 pairs of data points.

provide clues regarding the enthalpic or entropic forces which are involved in the stabilization of the ligand–receptor complex [30]. Enthalpy stabilization of a ligand–receptor complex is usually associated with the formation of new bonds, e.g., hydrogen bonds and van der Waals interactions, whereas entropy stabilization is achieved with the displacement of ordered water molecules and formation of new hydrophobic interactions.

Analysis of the thermodynamic aspects of ligand–antibody interactions have proved to be useful in understanding the driving forces and the molecular features of the interaction. For the small hapten 2,4-dinitrophenyl, the binding energy to polyclonal antibodies or the myeloma protein 315 was determined to be primarily contributed by enthalpic factors [7, 8]. The analysis of van't Hoff calculations for polyclonal antibodies tended to underestimate the exothermic heats of binding as compared to direct calorimetric determinations using the myeloma proteins [31], but this was probably due to the heterogeneous nature of the polyclonal antibodies. The hapten binding energies in these systems were all large and negative. Subsequent analysis of the amino acid sequences from these antibodies revealed that several tryptophans in the binding site could be in contact with the hapten, and perhaps as much as −10 kcal/mol of enthalpy change could be ascribed to charge transfer. Magnetic resonance techniques used to refine a model of the antibody binding site revealed an "aromatic box" with π–π stacking interactions between the dinitrophenyl ligand and L93:W of the antibody [8]).

The standard free energy (ΔG°) values calculated for the antibodies in this study are consistent with those observed for anti-ligand mAb in other studies [9]. The association constant for two of the mAbs in this study have been confirmed using a competitive radioimmunoassay employing [^{3}H(G)]haloperidol [3]; the K_A values for mAbs 185(2)-1 and 237(2)-1 were $2.0 \times 10^9\,M^{-1}$ and $4.2 \times 10^8\,M^{-1}$, respectively. These values are slightly higher than those determined in this study (Table 1) but may be due to experimental error and different conditions of the assays.

The energy differences corresponding to the state

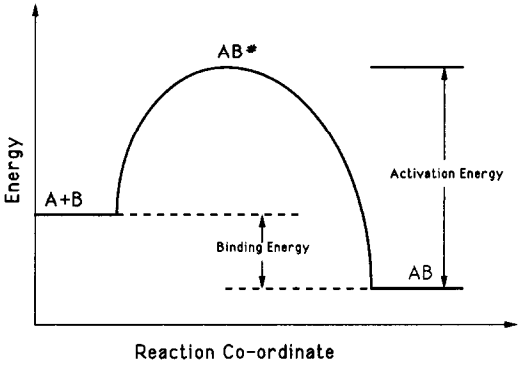

Fig. 9. Energies of activation for a simple reaction such as that observed for the formation of an antigen–antibody complex.

of activation, i.e. A + B to AB, as shown in Fig. 9, and presented in Table 2, correspond to the process of activation in the release of the ligand from the binding pocket of the antibody. These values account for the activation barrier in going from the stable complex AB to the high energy activated state AB* (Fig. 9) before breaking apart from the complex. These parameters are useful to describe the chemical environment in the immediate vicinity of the ligand binding pocket. Any change in the binding site would be reflected in the energy terms resulting in the acceleration or deceleration of the reaction velocity and, subsequently, its temperature dependence. For the antibodies 185(2)-1 and HAL-S-1, the free energies of activation (ΔG^*) are the same, but their enthalpic and entropic contributions appear to be different. The enthalpy term arises from the bonding contributions (e.g. π–π stacking, van der Waals, hydrogen bonding and other electrostatic energies) while the entropy term arises from the alteration in the extent of solvation, and the change in the rotational and vibrational freedom of the side chains of the residues. The higher enthalpy of activation of the complex with 185(2)-1 is balanced by more positive entropic contribution. Because the binding sites of the antibodies are thought to contain

numerous water molecules and known to possess conserved aromatic groups (from sequencing and modeling studies), the differences in the entropic terms would most likely arise from both solvation and aromatic side chain movements. The energy of activation for the complex with 237(2)-1 is very low, thereby creating an off rate which is about forty times faster than that observed for the other two antibodies. In this case, the process of activation is accompanied by a negative entropic effect (Table 2), implying increased solvation and/or decreased rotational or vibrational motions in the activated state.

The negative value of the product of charges ($z_A z_B$) found in these mAbs implies that in the neutral pH region the antibody binding pocket exists as an anionic environment. This negatively charged area probably contributes to the stability of the ligand–antibody complexes. We have identified the presence of aspartate at position CDR3 H:100 for most of these antibodies made to haloperidol through cDNA sequencing. We have similar findings for monoclonal antibodies which bind with other alkaloid ligands, such as nicotine and morphine [32]. The sharp rise in the dissociation rate at pH values above 9 is probably due to an increasing concentration of non-protonated species of haloperidol ($pK_a = 8.3$). The instability of the complex below pH 5 as observed by the increased off rates of the complexes with 185(2)-1 and 237(2)-1 could reflect the loss of this anionic environment due to protonation of the key aspartate residue at very low pH values.

Thermodynamic analysis of ligand binding data for several drug–receptor systems has been carried out in hopes of elucidating the nature of agonist and antagonist interactions. For the β-adrenergic receptor the binding of agonists is primarily enthalpy driven, whereas antagonists are totally entropy driven [33]. Investigators have proposed that the decrease in enthalpy associated with agonists reflects a conformational change (new bonds) in the receptor thereby transducing a signal (work). In contrast, the antagonists demonstrate binding only, with no apparent transfer of information (i.e. occupancy of the receptor). Similar studies with the dopaminergic ligands have been controversial [34, 35]. The observed entropy changes may correspond with the lipophilicity of the displacing ligands and not with any intrinsic activity [34]. The analyses of ligand binding data using an antibody based system eliminates the problems of lipids present in biological membrane preparations. We believe that the monoclonal antibodies examined in this study have some supramolecular features in common with those of the putative dopamine receptor. It has been established that these antibodies react with the putative pharmacophore of haloperidol based on QSAR analog binding studies [3]. In addition, when haloperidol is chemically conjugated via the tertiary alcohol to a protein carrier (e.g. bovine serum albumin) or fluorescein, it retains pharmacological activity, with respect to the ability to bind to putative D2 membrane receptors [3]; it is not known if *in vivo* neuroleptic activity is retained, because conjugated haloperidol does not cross the blood–brain barrier. The presence of an anionic site (H:100a:E) which forms a salt-bridge with the protonated piperidinyl nitrogen appears to be crucial to interactions with all dopaminergic ligands. The charge-transfer complex for the *p*-fluorophenyl ring may also be the key feature for the antagonist binding site. Such π–π stacking may stabilize key receptor site residues, thereby preventing the transduction of a signal. Examination of monoclonal antibody binding sites, and careful analysis of the thermodynamic properties associated with ligand binding may provide some insight in the study of antibodies as exemplars of receptors.

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