Multiligand Interactions at the Combining Site of Anti-Fluorescyl Antibodies. Molecular Recognition and Connectivity†

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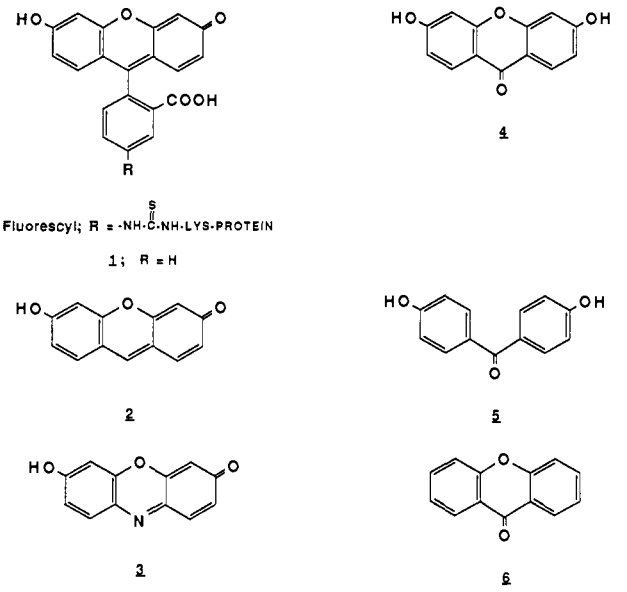
Abstract: Partitioning of binding energy among the constituent subsites in a ligand–antibody complex can be useful in assessment of bimolecular processes in the combining site. Binding affinities of five anti-fluorescyl monoclonal antibodies for fluorescein (**1**) and two compounds representing its component parts, xanthenone (**2**) and benzoate, were studied by the fluorescence quenching technique. Dissociation constants were found to be in the range 10^{-12}–10^{-10} M for **1**, 10^{-8}–10^{-7} M for **2**, and 10^{-3}–10^{-1} M for benzoate. Binding at both subsites was found to depend strongly on the recognition of polar or charged groups. The carboxylate accounts for ca. 4 kcal/mol for binding at the benzoate site whereas two hydroxyl groups contribute at least 5.5 kcal/mol toward binding of xanthene derivatives. The thermodynamic changes associated with ligand binding were described in terms of the intrinsic (ΔG^i) and the "connection" (ΔG^S) free energies. The connection free energies, estimated at 1.0–2.5 kcal/mol, represent a lower limit for reduction of the entropic energy barrier to bimolecular reactions that would ensue from binding of two reactants prior to chemical transformation. Study of this model system provides a thermodynamic rationale for the utility of monoclonal antibodies as catalysts for bimolecular processes.

Utilization of specific binding interactions between enzymes and their substrates is an essential feature of catalysis in biological systems.[1-3] Recognition of similarities between antibodies and enzymes with regard to their binding specificities has recently led to the development of methodology for the production of catalytic antibodies. Indeed, this newly emerging field has greatly benefited from the understanding of the fundamental principles of enzymology. Antibodies elicited to haptens that resemble transition-state structures have proved to be efficient catalysts for several reactions, including ester hydrolysis,[4,5] carbonate hydrolysis,[6,7] lactonization,[8] a Claisen rearrangement[9,10] and amide hydrolysis.[11,12] Broadening the spectrum of reactions catalyzed by antibodies is important for realizing their utility. The success of this endeavor will depend on exploitation of features contributing to rate enhancement in both unimolecular and bimolecular reactions. Recently, acceleration in the rate of a bimolecular amide formation by an antibody has been reported.[13,14]

A likely strategy for design of catalytic antibodies for bimolecular reactions will employ molecules representing multisubstrate assemblies as haptens. It is useful to consider the thermodynamic properties of such systems in relation to the ability to bind potential substrates. The binding of smaller ligands relative to binding of the hapten that contains these fragments provides an estimate of the magnitude of binding energy that may be used to stabilize the larger molecule or activated complex formed from two substrates bound at the combining site. Jencks' treatment of connectivity describes its relation to the entropy factor in enzyme catalysis and in the chelate effect.[1,2,15] Enzymes can bind multisubstrate analogues with considerable affinity that is greater than can be accounted for by the sum of affinities of the individual substrates. In this study we ask whether connectivity as manifested in enzymatic binding can play a role in antibody binding.

Fluorescein (**1**, Chart I) is a model compound for the combination ligand (A–B) consisting of a xanthenone (**2**) and a benzoate fragment. The anti-fluorescyl combining site is used to describe distinct subsites having affinity for ligands similar to these fragments. Binding affinities of a set of five anti-fluorescyl monoclonal antibodies for fluorescein and other fluorescent ligands was examined by fluorescence quenching. The selectivity and affinity of the benzoate binding site for nonfluorescent ligands were determined by competition binding using a fluorescent reporter ligand. The recognition of structural groups within these fragments was investigated through binding measurements with a series of related ligands. The thermodynamics of binding are analyzed in terms of the intrinsic and the "connection" free en-

Chart I



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ergies.[15] The potential for utilization of binding energy in catalysis of bimolecular processes by antibodies is discussed. The ligands used in this study also suggest possible substrates for antibody-catalyzed redox reactions.

Experimental Procedures

Materials. Fluorescein (**1**), resorufin (**3**), 4,4'-dihydroxybenzophenone (**5**), and xanthone (**6**) of high purity were obtained commercially (Aldrich).

3,6-Dihydroxyxanthone (4). Heating a portion (2 g) of solid 2,2',4,4'-tetrahydroxybenzophenone (Aldrich) to 200–220 °C for 3 h results in nearly quantitative conversion to 3,6-dihydroxyxanthone.[16] The product was purified by preparatory thin-layer chromatography on Kieselgel 60 support (1:5 CH_3OH:CH_2Cl_2, R_f 0.55): ^1H NMR (d_6-DMSO) δ 6.81 (d, J = 2.2 Hz, 2 H), 6.84 (d of d, J = 2.2, 4.1 Hz, 2 H), 7.89 (d, J = 4.1 Hz, 2 H), 10.84 (br, 2 H).

6-Hydroxy-3*H*-xanthen-3-one (2). A solution containing 100 mg of 3,6-dihydroxyxanthone in 6 mL of acetic acid and 2 mL of 6 N HCl was heated to reflux with an excess of zinc dust (1 g) for 2 h. The mixture was diluted with 10 mL of water, and the product extracted with (2 × 20 mL) ethyl acetate. The solution was washed with 5% $NaHCO_3$, dryed with sodium sulfate, and concentrated by rotary evaporation. The product was purified by preparatory thin-layer chromatography on Kieselgel 60 support (1:1 CH_2Cl_2:ethyl acetate, R_f 0.07): ^1H NMR (d_6-DMSO) δ 6.00 (s, 2 H), 6.23 (d, J = 8.7 Hz, 2 H), 7.23 (d, J = 8.7 Hz, 2 H), 7.82 (s, 1 H).

Fluorescence Quenching. Fluorescence measurements were performed on a Perkin-Elmer Model LS-5 spectrophotometer equipped with a Perkin-Elmer Model R 100 chart recorder. The following excitation and emission wavelengths (in nanometers) were used for the fluorescent ligands: **1** (490, 508); **2** (488, 501); **3** (560, 580); **4** (355, 430). Entrance and exit slits were set at 5 and 10 mm, respectively.

Fluorescein isothiocyanate (Aldrich, isomer I) was conjugated to keyhole limpet hemocyanin (KLH) and used to obtain a group of 24 hybridomas from mice (129GIX$^+$) by procedures described elsewhere.[17,18] Five purified monoclonal immunoglobulins (11G2, 31F8, 32E3, 33C5, and 66D2) were selected on the basis of their ability to bind resorufin at <0.5 mM in a competition enzyme-linked immunosorbent assay (ELISA)[19] against fluorescein isothiocyanate conjugated to bovine serum albumin. Fluorescence quenching was determined by addition of purified antibody to a 0.2 μM solution of the fluorophore. Maximal fluorescence quenching (Q_{max}) was determined at pH 7.5 by extrapolating the double-reciprocal plot (i.e., [fractional quenching]$^{-1}$ vs [antibody]$^{-1}$) to infinite antibody concentration.

For titration, 3–20-μL aliquots of the fluorophore stock solutions (2–20 μM) were added to 3 mL of buffer solution (45 mM sodium phosphate, pH 7.5 at ionic strength of 0.28 maintained with NaCl) with and without 0.1 μM antibody, and the fluorescence readings recorded. The fraction of antibody bound to fluorophore at equilibrium (i) is obtained from the difference in fluorescence intensity in the presence (F) and absence (F_0) of an anti-fluorescyl antibody ($\Delta F = F_0 - F$), eq 1, where ΔF_{max} is the

$$i = \Delta F/\Delta F_{max} \tag{1}$$

maximum fluorescence difference determined from the y intercepts of the ΔF^{-1} vs [fluorophore]$^{-1}$ plots. The concentration of free ligand at equilibrium is determined from the total ligand concentration multiplied by the fraction of free ligand, X_0, which is readily calculated from the observed quantities (eq 2).

$$X_0 = 1 - \Delta F/F_0 Q_{max} \tag{2}$$

All fluorescence measurements were done at 23 °C.

Determination of Dissociation Constants. The dissociation constant for an antibody–fluorophore complex (K_{Fl}), eq 3, where [Ab], [Fl], and

$$K_{Fl} = [Ab][Fl]/[Ab\cdot Fl] \tag{3}$$

[Ab·Fl] are the concentrations of free antibody, free fluorophore, and antibody–fluorophore complex at equilibrium, respectively, can be calculated by utilizing the Scatchard equation[20] (eq 4). The value of K_{Fl}

$$i/[Fl] = 1/K_{Fl} - i/K_{Fl} \tag{4}$$

was determined from the slope of the Scatchard plot. Values for the dissociation constants are obtained from the linear regression fit of the data points.

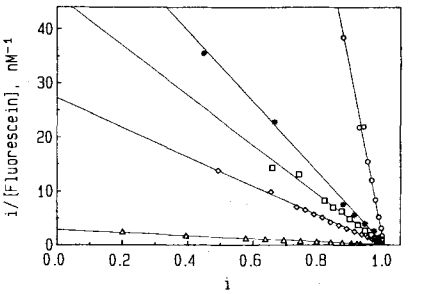
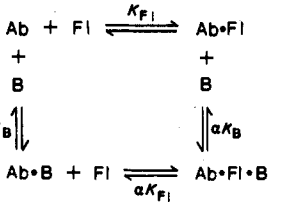


Figure 1. Scatchard plots for the binding of fluorescein to purified immunoglobulins from hybridomas 11G2 (□), 31F8 (◇), 32E3 (○), 33C5 (△), and 66D2 (*). The lines drawn represent the linear least-squares fit to the data points. The conditions of assay are described in the legend to Table I.

For an antibody with a binding pocket capable of accommodating a second ligand (B), the following scheme applies:



(5)

where α is the factor by which the dissociation constant for one ligand is altered by binding of the other ligand.[21] The corresponding Scatchard equation can be derived:

$$i/[Fl] = C/K_{Fl} - iC/K_{Fl} \tag{6}$$

where

$$C = (K_B + [B]/\alpha)/(K_B + [B])$$

The magnitude of α can describe situations where ligand binding is antagonistic ($\alpha > 1$), cooperative ($\alpha < 1$), or independent ($\alpha = 1$).

If α is not equal to 1, the dissociation constant for the second (nonfluorescent) ligand, K_B, can be evaluated from the dependence of the apparent dissociation constant of the fluorophore ($K_{Fl}^{app} = K_{Fl}/C$) on [B]. For the case where binding of the two ligands is competitive and the term $[B]/\alpha$ in eq 6 is much smaller than K_B, the ratio of dissociation constants for the fluorophore in the presence (K_{Fl}^{app}) and absence (K_{Fl}°) of the second ligand will be a linear function of [B], eq 7, with the slope equal

$$K_{Fl}^{app}/K_{Fl}^{\circ} = 1 + [B]/K_B \tag{7}$$

to $1/K_B$. This analysis was applied by using both **1** and **2** as indicator ligands and several nonfluorescent displacing ligands.

Results

A set of 24 monoclonal antibodies were obtained by immunization with a fluorescein isothiocyanate–KLH conjugate. Five of these were selected for detailed binding studies because of their efficient cross-reactivity with **3** in competition ELISA and quenching of fluorescence on binding **1**. The values of Q_{max} ranged from 0.950 to 1.00, thus providing an excellent handle for binding studies. A nonspecific antibody of the same isotype (γ_{2b}) did not perturb fluorescence of these compounds. Other anti-fluorescyl antibodies showed varying efficiency of fluorescence quenching (Q_{max} 0.498–0.988) as previously noted.[22,23] Dissociation constants for these ligands were obtained by fitting of the fluorescence measurements to the Scatchard equation (Figure 1). All five antibodies showed strong binding for **1** (K_d = (3–330) × 10^{-12} M) and somewhat weaker (K_d = (1–11) × 10^{-8} M) binding of **2** (Table I).

Binding of a small ligand that is complementary to **2** in filling the binding site was also investigated by its ability to displace the fluorescent ligand from the antibody. Under conditions of constant ionic strength, an increasing concentration of benzoate resulted

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Table I. Dissociation Constants for Ligands Binding to Selected Anti-Fluorescyl Antibodies[a]

antibody	K_F,[b] pM	K_X,[b] nM	K_B,[c] mM
11G2	22.2 ± 0.8	89.7 ± 1.5	16.7 ± 0.6
31F8	36.7 ± 0.4	95.4 ± 0.2	14.9 ± 0.3
32E3	3.25 ± 0.11	12.6 ± 0.3	1.3 ± 0.09
33C5	331 ± 2.0	94.8 ± 0.8	239 ± 28
66D2	15.4 ± 0.3	112 ± 2	0.83 ± 0.02

[a] Binding studies were performed in 45 mM phosphate buffer, pH 7.5 at 23 °C. Ionic strength was maintained at 0.28 with NaCl. Values of the dissociation constants were determined by the fluorescence quenching technique as described in the text. Subscripts F, X, and B refer to fluorescein, xanthenone and benzoate, respectively. [b] Evaluated from the linear regression fit to the data points in Scatchard plots (Figure 1). [c] The values were obtained from the plots of $K_F^{app}/K_F°$ vs the concentration of benzoate according to eq 7 (Figure 2a).

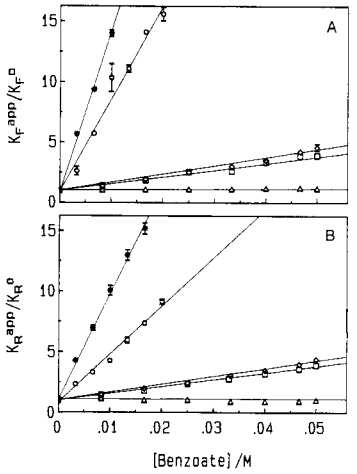

Figure 2. Representative replots of $K^{app}/K°$ for fluorescein (A) and resorufin (B) vs the concentration of benzoate. The symbols are the same as those given in Figure 1. The ionic strength of all buffer solutions was maintained constant at 0.28 with NaCl.

in weaker apparent binding of fluorescein (K_F^{app}). The binding was competitive, and the dissociation constant for benzoate K_B is calculated from a replot of the $K_F^{app}/K_F°$ versus the concentration of benzoate (Figure 2a).

The similar experiment was performed by using **3** as the competed ligand (Figure 2b). In this case, where there is the opportunity to form a ternary complex, a complete description of the system is given by the thermodynamic cycle (eq 5).[21] From the dependence of K_R^{app} on benzoate concentration the energetics of this cycle may be described. Four of the five antibodies examined showed antagonistic interaction between the two ligands, while binding of the ligands to the fifth antibody (33C5) seemed to occur in a completely independent fashion ($\alpha = 1$). The binding constants for benzoate (K_B) measured by these experiments were in good agreement with those obtained with **1** as the indicator ligand. Binding of other nonfluorescent ligands was also determined by this technique.

The dissociation constants for a representative antibody 66D2 complexed with a series of related ligands is shown in Table II. Within the benzoate subsite the largest variation ($\Delta K = 10^3$ M) is between nicotinate and pyridine, which differ only by the carboxyl substituent. Binding at the complementary subsite was investigated with xanthene and benzophenone derivatives (Table II). While **3** and **4** have affinities in the range comparable to that of **2**, binding of **5** is weaker by approximately 3 orders of magnitude. **6** does not appear to bind up to its solubility limit of about 50 μM, and therefore its dissociation constant can only be estimated to be greater than ca. 4×10^{-4} M. At both subsites the order of increasing affinity for the series of ligands is in agreement with the predicted structural recognition of the respective fluorescyl fragment.

From the thermodynamic quantities for the individual binding of the complementary ligands **2** (A) and benzoate (B) and the

Table II. Dissociation Constants for Binding of Ligands to Anti-Fluorescyl Antibody 66D2[a]

benzoate ligand[b]	K,[c] mM	xanthenyl ligand	K, μM
acetate	26.6 ± 0.8	**2**	0.112 ± 0.002[d]
benzoate	1.20 ± 0.03	**3**	0.160 ± 0.002[d]
3-acetamidobenzoate	0.301 ± 0.012	**4**	0.036 ± 0.001[d]
nicotinate	0.236 ± 0.008	**5**	82 ± 7[e]
pyridine	257 ± 100	**6**	>400[e,e]

[a] Conditions are described in the legend to Table I. [b] Carboxylic acid ligands are present as their sodium salts. [c] The values were calculated from the $K^{app}/K°$ (for reporter ligand **3**) vs the concentration of the second (displacing) ligand plots according to eq 7. [d] Determined directly by fluorescence quenching. [e] Because of limited solubility of xanthone in aqueous media, only a lower limit for the dissociation constant of this compound could be obtained from the $K^{app}/K°$ vs [xanthone] plot.

Table III. Free Energies of Binding for Fluorescein (**1**), Xanthenone (**2**), and Benzoate to Anti-Fluorescyl Antibodies in kcal/mol[a]

antibody	$\Delta G_F°$	ΔG_F^{i}[b]	$\Delta G_X°$	ΔG_X^{i}[b]	$\Delta G_B°$	ΔG_B^{i}[b]	ΔG^S[c]
11G2	−14.4	−15.6	−9.5	−12.0	−2.4	−3.6	2.5
31F8	−14.1	−15.7	−9.5	−11.7	−2.5	−4.1	2.1
32E3	−15.6	−16.9	−10.7	−11.7	−3.9	−5.2	1.0
33C5	−12.8	−15.5	−9.5	−12.0	−0.84	−3.5	2.5
66D2	−14.6	−15.9	−9.4	−10.5	−4.2	−5.5	1.0

[a] The observed free energies of binding were calculated from the dissociation constants in Table I ($\Delta G° = RT \ln K°$). [b] The intrinsic free energies; $\Delta G^i = \Delta G° - \Delta G^S$. [c] The connection free energies; $\Delta G^S = \Delta G_X° + \Delta G_B° - \Delta G_F°$.

binding of fluorescein as a model for the connected molecule (A–B), we may arrive at an estimate for the intrinsic binding energies of the fragments A and B and the "connection free energy" ΔG^S for the system[15] (Table III). The connection free energy is estimated at 1.0–2.5 kcal/mol for this set of antibodies. The quantity α, which is a measure of the "destabilization free energy", $\Delta G_{1,2}$[15,21] between the bound ligands A and B cannot be estimated. For the antibody 33C5, the two sites show no interaction, while for the remaining four antibodies this quantity is too large to be determined with the ligand concentrations that are accessible (i.e., the ternary complex is not significantly populated).

Discussion

A description of the free energy changes of binding to a protein of two ligands, A and B, in relation to that of a compound in which the two components are covalently attached, A–B, can provide a context for assessing the "entropy trap" hypothesis of bimolecular processes at the binding site.[1-3] It is well-known that the observed free energies for binding of the two ligands, $\Delta G_A°$ and $\Delta G_B°$, seldom add up to give $\Delta G_{A-B}°$.[15,24,25] The free energy difference between $\Delta G_A + \Delta G_B$ and ΔG_{A-B} has been termed the "connection free energy", ΔG^S, and can be regarded as an empirical parameter representing mainly the reduction in translational and rotational entropy on binding.[1,15] A complementary empirical parameter is the intrinsic binding energy, ΔG^i, defined as the free energy change for binding of a ligand in the absence of strain, desolvation, and the probability factors represented in ΔG^S (eq 8). In effect,

$$\Delta G^i = \Delta G° - \Delta G^S \quad (8)$$

the parameters ΔG^i and ΔG^S represent an empirical measure of binding forces and probability terms, respectively (analogous to ΔH and $T\Delta S$).

Tighter binding of **1** to anti-fluorescyl antibodies relative to the combined affinity for **2** and benzoate is a measure of the loss of entropy on ligand binding. The range of values for ΔS^S in this system is 1.0–2.5 kcal/mol, which corresponds to K_F/K_XK_B of 5–70 M. Antibodies that catalyze bimolecular amide synthesis show effective molarities in the range 10–20 M.[13,14] Values of effective molarities in enzymes of up to 10^5 M have been reported, and it

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has been estimated that the catalytic advantage from the ΔG^S term can be as large as 10^8 M.[1,2,15] The modest quantities found here for antibodies may represent a fundamental limit to connectivity available in antibody–antigen binding. Greater effective molarities might be found as a consequence of actual values for a catalyzed reaction being weaker than those measured here, which may contain nonproductive binding modes. However, the ΔG^S term represents only the contribution to potential rate accelerations arising from the entropic factors associated with binding of the reactants prior to chemical transformation.

It is relevant to note that binding of carboxylates to four of the five antibodies is competitive with the binding of a complementary ligand. This suggests the possibility for mutually destabilizing interactions between the two ligands in the ternary complex due to steric hindrance, additional entropy loss, or conformational changes in the protein.[2,15] Relief of such destabilizing interactions, as in the formation of a product (or a transition state), can, in principle, provide a further driving force for the hypothetical reaction.[26] Consequently, the estimates from the ΔG^S term represent a lower limit for the contribution of binding forces to catalysis of bimolecular processes.

The affinities of anti-fluorescyl antibodies for **1** are in the same range of "high affinity" as reported by others.[22,23] The large favorable free energy of fluorescein binding (>12 kcal/mol) is useful for considering its partitioning among the constituent subsites of the complex. Binding of **2** and **3** to the antibodies was expected due to their analogy with the structure of the xanthenyl fragment of **1**. The high affinity for **2** by all the antibodies examined may be considered a reflection of the immunodominant position of the respective fragment in the fluorescyl hapten. In contrast the affinity for benzoate makes a smaller contribution to the fluorescein affinity. Moreover, the weaker binding of fluorescein by antibody 33C5 than the others in this set can be explained primarily as reduced affinity in the benzoate binding site. This is in agreement with a model for the maturation of the anti-fluorescyl immune response that suggests a mechanism for the sequential expression of antibodies of highest affinity.[27]

The specificity of binding at each subsite was explored for one of the antibodies by determining the affinity for related ligands that might occupy the same site. Evidence for specific binding at each of the subsites defined is found in the order of increasing affinities that is in accord with the increasing structural analogy with the corresponding fragment of the fluorescyl hapten. From the large difference in affinity between nicotinate and pyridine an estimate is possible for the free energy of binding due to recognition of the carboxylate group. A value of 4.1 kcal/mol is typical as compared with values found in other systems.[28] A variation in affinities over 2 orders of magnitude is found for carboxylates with differing aryl or alkyl groups. Polar substituents also play a dominant role in binding at the xanthenyl subsite. Comparison of binding affinities of **4** and **6** indicates that appropriately positioned hydroxyl groups contribute at least 5.5 kcal/mol toward binding at this locus. On the other hand, variations in the ligand structure at the central position of the fluorescyl hapten can be tolerated without a significant compromise in binding efficiency as evidenced by similar affinity of 66D2 for **2**, **3**, and **4**. A difference of 4.5 kcal/mol in binding of **5** versus **4** may be attributed in part to hydrogen bonding to the bridging oxygen, as well as differences in rotational degrees of freedom that need to be lost upon binding. Thus the subsite specificity of monoclonal antibodies is similar, with regard to apparent binding of substrates, to many enzymatic systems. The present study represents a thermodynamic characterization of the antibody combining site that had only previously been attempted with purified polyclonal serum.[29]

Fluorescein serves as a useful ligand to probe the thermodynamics and mechanism of antibody combining site–ligand interactions.[22,23] The antibody combining site is capable of significant specificity and affinity for ligands representing fragments of the hapten. Dissociation constants were found to be in the 10^{-8}–10^{-7} and 10^{-4}–10^{-1} M ranges for the two subsites defined. This is adequate to recommend study of the interaction between the subsites as a model for enzyme chemistry. Very tight binding of **2** to the anti-fluorescyl immunoglobulins is encouraging from the standpoint of design of an antibody with a cofactor recognition region. Using the dissociation constants for **2** from Table I and 1 μM concentration of **2**, the calculated fraction of occupied sites ranges between 90 and 99%. Clearly, covalent attachment of a cofactor would not be necessary in this case. The ability to bind **2** and other similar ligands makes anti-fluorescyl antibodies attractive as proteins modulating the redox properties of these substrates.[30] The probable existence of a proximate anion (carboxylate) binding pocket creates several possibilities for recognition of substrates for redox chemistry or other bimolecular processes. More importantly, however, this study provides an additional impetus for further elaborations on the design of similar and related multisubstrate systems.

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