Photocontrolled Uptake and Release of Photochromic Haptens by Monoclonal Antibodies. Evidence of Photoisomerization Inside the Hapten-Binding Site

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Monoclonal antibodies against a tetrapeptide carrying a photochromic azobenzene moiety (Glu–azoAla–Gly–Gly, azoAla=L-*p*-phenylazophenylalanine) were prepared under conditions where the azobenzene moiety was in the *trans* form. The binding of the hapten peptide to the antibody was investigated by fluorescence quenching of the antibodies by the hapten peptide. The results indicated that the antibodies bind the hapten peptide when the azobenzene moiety is in the *trans* form, but release the peptide in the *cis* form. The mechanism of photoreversible binding and release was studied using a pulsed laser light. Photoisomerization of the hapten peptide was found to occur inside the binding site, indicating that the latter is flexible enough to allow the *trans–cis* photoisomerization within a few ten picoseconds.

Monoclonal antibodies can be prepared as tailor-made hosts against a variety of guest molecules. In this sense, they are much more widely applicable than other host molecules commonly used in chemistry, such as cyclophanes, cyclodextrins, crown ethers, and cyclic peptides. Recent success of catalytic antibodies (abzymes) exemplifies this characteristic feature. Antibodies may also be applicable in drug deliveries, immunosensings, and other diagnostic uses. One of drawbacks of antigen-antibody systems is that the affinity to the guest molecules is often too high and the guest molecules that are captured cannot be released under mild conditions. In this article we show that monoclonal antibodies prepared against a photochromic molecule can bind and release the hapten under photoirradiations at different wavelengths. Preliminary results on photoswitching have been reported.[1] To the authors' knowledge, this system is the first example of photoreversible antigen-antibody reactions. Since the onset of antigen-antibody reactions when coupled with complementary proteins induces a variety of immunological responses,[2] photocontrol of the antigen-antibody reaction may open a way to switch those responses on and off. Similar studies on a photochromic inhibitor/enzyme system[3] and a photochromic effector/receptor system[4] have been reported by Erlanger et al.

As a hapten molecule that carries a photochromic group, we have chosen a tetrapeptide containing a nonnatural amino acid carrying an azobenzene moiety (Scheme 1). The azobenzene moiety is known to isomerize from the thermally stable *trans* form to the *cis* form under irradiation with ultraviolet light.[5,6] The *cis* form returns to the *trans* form under irradiation at wavelengths longer than 430 nm or by heat. The photoisomerization cycle can be repeated more than a thousand times.

The hapten peptide was designed to show high immunogenicity when linked to a carrier protein (BSA). The glycylglycine unit is a spacer between the carrier protein and the azobenzene unit. The glutamic acid unit was introduced to provide hydrophilicity for the peptide. Otherwise, the hydrophobic azobenzene moiety would be buried inside the inner hydrophobic region of BSA.

Experimental

Synthesis of the Hapten Peptide (I). The tetrapeptide was synthesized by the conventional liquid-phase method. All intermediates were checked for purity by TLC, ^{1}H NMR (90 MHz), and elemental analysis. The photochromic amino acid carrying the azobenzene unit was synthesized according to Goodman and Kossoy.[7] The following abbreviations are used below: azoAla, L-*p*-phenylazophenylalanine; Boc, *t*-butoxycarbonyl; OSu, *N*-hydroxysuccinimide ester; OEt, ethyl ester; OBu^t, *t*-butyl ester; DCC, dicyclohexylcarbodiimide; DCU, *N*,*N*′-dicyclohexylurea; Boc_2O, di-*t*-butyl dicarbonate; TEA, triethylamine; HOBT, 1-hydroxybenzotriazole; DOX, 1,4-dioxane; BSA, bovine serum albumin.

Boc–Gly–Gly–OH. Boc–Gly–OH (2.5 g, 14.3 mmol) and Gly–OEt hydrochloride (2.0 g, 14.3 mmol) were dissolved in chloroform (60 mL) and cooled with ice. TEA (3.7 mL, 26.4 mmol) was added followed by DCC (2.97 g, 14.3 mmol). The mixture was stirred for 3 h on ice and then stirred overnight at room temperature. The precipitated DCU was filtered off and the solvent was removed by evaporation. The residual solid was redissolved in ethyl acetate and the DCU that appeared again was filtered off. The solution was washed successively with 10% citric acid, water, 4% $NaHCO_3$, and water. The organic layer was dried over $MgSO_4$ and evaporated to an oil. Yield 2.51 g (67%).

H–Gly–Gly–OEt Hydrochloride. The above oil (0.66 g, 2.5 mmol) was dissolved in DOX containing 3 M dry hydrochloric acid (1 M=1 mol dm^{-3}). The mixture

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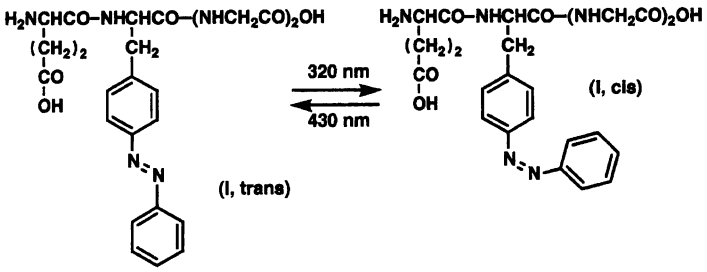


Scheme 1. Structure of the *trans* and *cis* haptens.

was allowed to stand at room temperature for 30 min, then concentrated. Ether was added to precipitate the product. Yield 0.5 g (99%), mp 173—175 °C. Anal. Calcd for $C_6H_{13}N_2O_3Cl$: C, 36.64; H, 6.68; N, 14.25; Cl, 18.03%. Found: C, 36.57; H, 6.85; N, 13.94; Cl, 18.92%.

Boc–azoAla–Gly–Gly–OEt. Boc–azoAla–OH (0.6 g, 1.6 mmol) and H–Gly–Gly–OEt (0.34 g, 1.7 mmol) were dissolved in chloroform (8 mL) and cooled with ice. TEA (0.43 mL, 3.1 mmol), HOBT (0.22 g, 1.6 mmol), and DCC (0.33 g, 1.6 mmol) were added to the solution in that order and the mixture was stirred for 3 h on ice and for 24 h at room temperature. The mixture was then concentrated and the residual oil was redissolved in ethyl acetate. The soluble part was washed as above, and the crystals that appeared after cooling the concentrated solution in a refrigerator were collected. Yield 0.22 g (27%), mp 155—157 °C. Anal. Calcd for $C_{26}H_{33}N_5O_6$: C, 61.03; H, 6.51; N, 13.69%. Found: C, 61.61; H, 6.72; N, 13.65%.

Boc–Glu(OBu^t)–azoAla–Gly–Gly–OEt. The above tripeptide (0.2 g, 0.38 mmol) was dissolved in DOX containing 3 M hydrochloric acid and the mixture was allowed to stand for 30 min at room temperature. The solvent was evaporated and the hydrochloride was precipitated by adding excess ether. The solid was washed with ether and dried under vacuum. ^{1}H NMR showed that the Boc group was completely removed. The hydrochloride (0.14 g, 0.31 mmol) was mixed with Boc–Glu(OBu^t)–OH (95 mg, 0.31 mmol) in chloroform (1.1 mL). The coupling reaction was carried out using DCC as described above. The product was crystallized from ethyl acetate. Yield 0.13 g (60%), mp 151—152 °C. Anal. Calcd for $C_{35}H_{48}N_6O_9$: C, 60.33; H, 6.94; N, 12.06%. Found: C, 60.25; H, 7.15; N, 12.00%.

Boc–Glu(OBu^t)–azoAla–Gly–Gly–OH. The fully protected tetrapeptide (0.1 g, 0.14 mmol) was dissolved in methanol (0.7 mL) and aqueous NaOH (1 M, 0.18 mL) was added. The solution was left standing at room temperature for 2 h, then neutralized with 10% citric acid solution and extracted with ethyl acetate. The extract was dried over $MgSO_4$ and concentrated. Hexane was added and the mixture was kept in a refrigerator. A crystalline product was obtained. Yield 67 mg (70%), mp 151—152 °C. Anal. Calcd for $C_{33}H_{44}N_6O_9+H_2O$: C, 57.70; H, 6.76; N, 12.24%. Found: C, 58.10; H, 6.60; N, 12.24%.

H–Glu–azoAla–Gly–Gly–OH. THe above free acid (50 mg, 0.09 mmol) was dissolved in DOX containing 3 M dry HCl. The mixture was left standing at room temperature for 40 min and the solvent was then evaporated. The solid product that appeared after adding excess ether was washed with ether. ^{1}H NMR indicated complete removal of the *t*-butoxy groups.

Attachment of the Tetrapeptide onto Carrier Proteins. Boc–Glu(OBu^t)–azoAla–Gly–Gly–OH (100 mg, 0.15 mmol) was dissolved in chloroform (1.1 mL) and cooled with ice. *N*-Hydroxysuccinimide (18 mg, 0.16 mmol) and DCC (31 mg, 0.15 mmol) were added and the mixture was stirred under cooling overnight. The chloroform was then evaporated and the oil was redissolved in ethyl acetate. The mixture was kept in a refrigerator to precipitate DCU. The filtrate was washed with 5% $KHSO_4$ and water and dried over $MgSO_4$. The ethyl acetate solution was concentrated, hexane was added to the oil, and the mixture was kept in a refrigerator to precipitate the tetrapeptide-OSu activated ester. The latter (89 mg, 0.12 mmol) was dissolved in DOX(2 mL) containing 3 M dry HCl. The mixture was left standing at room temperature for 30 min and then concentrated under vacuum. The oil was redissolved in water (6 mL). The aqueous solution of the unprotected tetrapeptide activated ester was added dropwise to BSA (160 mg) solution in water (10 mL) under cooling with ice. The pH was kept at 7 by adding 1 M NaOH during the addition of the peptide solution. The solution was kept in a refrigerator overnight and fractionated with a Sephadex G-100 column to remove unreacted peptides. The average number of peptides bound to a BSA molecule was calculated from the ratio of the absorption intensities of the azobenzene moiety ($\varepsilon_{326}=1.9\times10^4$, $\varepsilon_{280}=0.66\times10^4$) and the protein ($\varepsilon_{280}=3.4\times10^4$). The average number was 20 in the present case. By a similar procedure, the tetrapeptide–casein conjugate was also prepared. In this case the pH was kept at 8 during the reaction.

Preparation of Monoclonal Antibodies. Mice (BALB/c) were immunized against the hapten–BSA conjugate by a standard procedure using Freund's complete ajuvant. The immunization was repeated three times every two or three weeks. Three days after the third immunization, a small amount of blood was taken from the tail of the mouse and the titer of the serum was examined by an ELISA technique. The ELISA experiment was conducted using a 96-well plate (Sumilon) coated with the peptide–Casein conjugate and peroxidase-labeled anti-mouse IgG antibody with *o*-phenylenediamine as a substrate. The enzyme activity was measured by the absorbance at 492 nm.

At this stage, a preliminary experiment on the photoresponsiveness of the hapten–antibody reaction was carried out. For this purpose, the hapten peptide was mixed with the serum in the above ELISA experiment. Before photoirradiation, almost all the azobenzene moieties in the peptide were in the *trans* form. The azobenzene moieties changed to the *cis* form after irradiating the stock solution of the hapten peptide at 360 nm (Xe–Hg lamp with a bandpass filter 360±15 nm). Under these conditions, about 82% of the azo-

benzene moieties were in the *cis* form. The inhibition was efficient when hapten peptide was in the *trans* form, but was significantly suppressed when the *cis* form was predominant. The reduced inhibition indicated that the serum antibodies could not bind the *cis* peptide.

After the antibody titer became high enough, a final immunization was carried out. Lymphocytes from the spleen of the immunized mice were taken out and fused with mouse myeloma cells (PAI). The hybridoma were screened by ELISA. A portion of the hybridoma (about 10^7 cells) was injected intraperitoneally into mice. After two weeks, ascites fluid was taken out, centrifuged, and precipitated with saturated ammonium sulfate. The precipitate was redissolved in PBS buffer, dialyzed, and lyophilized. HPLC (hydroxyapatite column in phosphate buffer, linear gradient from 0.01 M to 0.3 M in 30 min) showed a single peak.

Four kinds of monoclonal antibodies were obtained in this manner and named Z1H01 to Z1H04. The subclass of the antibodies was examined by using an isotyping kit (Amersham). All four antibodies were found to be IgG1 with κ light chains.

Results and Discussion

Fluorescence Quenching Experiment and Binding Constants. The binding capability of the antibodies was followed by a fluorescence quenching experiment using the hapten peptide or the amino acid ester as a quencher. Figure 1 shows the fluorescence quenching curve of Z1H01 by the hapten peptide in the *trans* form. The sharp drop of antibody fluorescence intensity is attributed to energy transfer from the excited states of tryptophan and/or other aromatic units in the antigen-binding site[8] to the *trans* azobenzene moiety.[9] The quenching curve was fitted to Eq. 1.[10]

$$(I - I_{\min})^{-1} = K[\mathrm{H}]_0/(I_0 - I) - K[\mathrm{Ab}]_0/(I_0 - I_{\min}) \quad (1)$$

In the above equation, $[\mathrm{H}]_0$ is the concentration of feed hapten and $[\mathrm{Ab}]_0$ is that of the antibody binding site. Since the antibody is of the IgG type, the concentration of the binding site is twice the concentration of the antibody molecules. I is fluorescence intensity. I_0 is the intensity in the absence of the antibody and $I_{\min}$ is the limiting value when an excess amount of the antibody was added. A plot of $(I-I_{\min})^{-1}$ against $[\mathrm{H}]_0/(I_0-I)$ is shown in Fig. 2. From the linear relation the binding constant of Z1H01 was obtained. The binding constant as well as those of other antibodies are listed in Table 1. The validities of the binding constants were confirmed by agreements of the $[\mathrm{Ab}]_0$ values obtained from the intercepts of Fig. 2 with the experimental values calculated from the absorption of antibody at 280 nm using $\varepsilon_{280}=2.1\times10^5$.

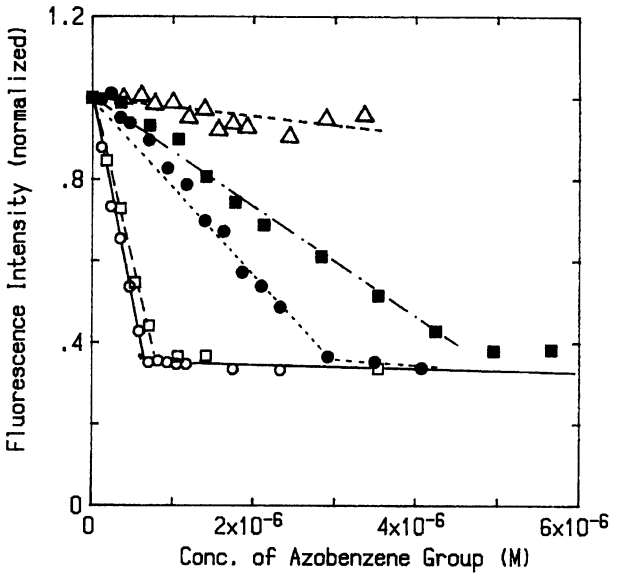


Fig. 1. Fluorescence quenching curves of Z1H01 by the hapten peptide (○, *trans*; ●, 82% *cis*) and azo-Ala–OMe (□, *trans*; ■, 82% *cis*) in PBS(-) solution (pH=7.0) at 25 °C. Open triangles are the extrapolated values to 100% *cis* form of the hapten peptide. Concentration of the antibody=2.6×10^{-7} M (A_{280}=0.05, λ_{ex}=280 nm, λ_{em}=340 nm.)

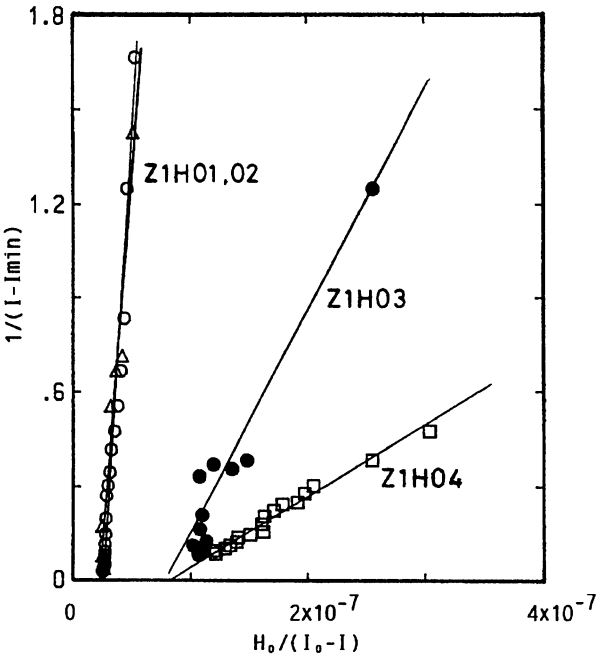


Fig. 2. Plot of Eq. 1 for the fluorescence quenching curves of the four monoclonal antibodies by the hapten peptide in the *trans* form.

Table 1. Subclass and the Type of Light Chain of Monoclonal Antibodies and Binding Constants against the Hapten Peptide (K_p) and L-*p*-Phenylazophenylalanine Methyl Ester (azoAla–OMe) (K_a) in *trans* Form (in M^{-1}at 25 °C)

Antibody	Subclass	Light chain	K_p	K_a
Z1H01	IgG1	κ	4.8×10^7	4.2×10^7
Z1H02	IgG1	κ	5.4×10^7	8.1×10^6
Z1H03	IgG1	κ	7.2×10^6	
Z1H04	IgG1	κ	2.2×10^6	

When the stock solution of the hapten peptide was irradiated with 360 nm light, the quenching curve shifted markedly to the right, indicating inefficient binding of *cis* haptens (filled circles in Fig. 1.) The ^{1}H NMR spectra of the stock solution showed that the percentage of the *cis* form was 82% under the photostationary state. The quenching efficiencies extrapolated to 100% *cis* form indicated that the binding of the *cis* hapten by Z1H01 is almost negligible (open triangles in Fig. 1). The results indicate that the antibody raised against *trans* haptens does not bind *cis* haptens.

An alternative interpretation of the results in Fig. 1 may be that the *cis* hapten remains inside the binding site but cannot accept excitation energy from the tryptophyl groups of the antibody. Indeed, a model quenching experiment using tryptophan as a fluorophore (energy donor) and *p*-phenylazophenylalanine methyl ester as an energy acceptor showed that the quenching constant $[K_q = k_q \times$(fluorescence lifetime of tryptophan)] of the *cis* form was smaller than that of the *trans* form by a factor of 1/4.1. However, the experimental quenching curve of Fig. 1 cannot be interpreted by the small quenching constant. If the *cis* peptide remains inside the binding site, but does not work as a quencher, the fluorescence quenching curve would be shifted upward from that of the *trans* form. This was not actually the case. Therefore, the experimental quenching curve for the *cis* peptide is reasonably interpreted in terms of the inability of the antibody to bind *cis* haptens.

As shown in Fig. 1, the binding behavior of Z1H01 against the *trans* and *cis* hapten peptide was similar to that against *trans* and *cis* azoAla–OMe (L-*p*-phenylazophenylalanine methyl ester). This indicates that antibody Z1H01 specifically recognizes the azobenzene moiety and possibly, its close neighbors in the tetrapeptide.

The situation was different in the case of antibody Z1H02 which showed similar binding behavior against the *trans* hapten peptide as shown in Fig. 3 and Table 1. In contrast to Z1H01, Z1H02 showed a considerable affinity to the *cis* peptide (Fig. 3). While Z1H01 showed similar affinities to the *trans* tetrapeptide and to *trans* azoAla–OMe (Fig. 1, Table 1), Z1H02 showed a significantly smaller affinity to *trans* azoAla–OMe than to the *trans* tetrapeptide (Table 1). This result indicates that Z1H02 recognizes not the *trans* azobenzene moiety, but the total structure of the tetrapeptide. As a consequence, the effect of photoisomerization of the azobenzene moiety on the binding behavior of Z1H02 is less clear than that of Z1H01.

A mixture of the antibody (Z1H01 or Z1H02) and the hapten peptide was alternately photoirradiated with UV (360 nm) and visible (>450 nm) light and the change of fluorescence intensity of the antibody was followed (Fig. 4). Before adding the hapten peptide, the fluorescence intensity was not affected by the alternate photoirradiation. After the addition of *trans* hapten that reduced the fluorescence intensity markedly, the alternate photoirradiation induced an alternate change in the fluorescence intensity, indicating that the uptake and release of the hapten were occurring alternately. Photoreversible binding was also observed for

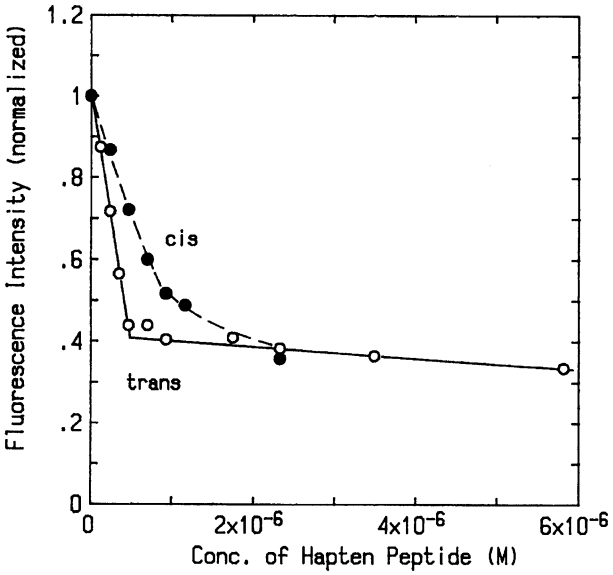


Fig. 3. Fluorescence quenching curves of Z1H02 by the hapten peptide (○, *trans*; ●, 82% *cis*) in PBS(-) solution at 25 °C. Other conditions are the same as those in Fig. 1.

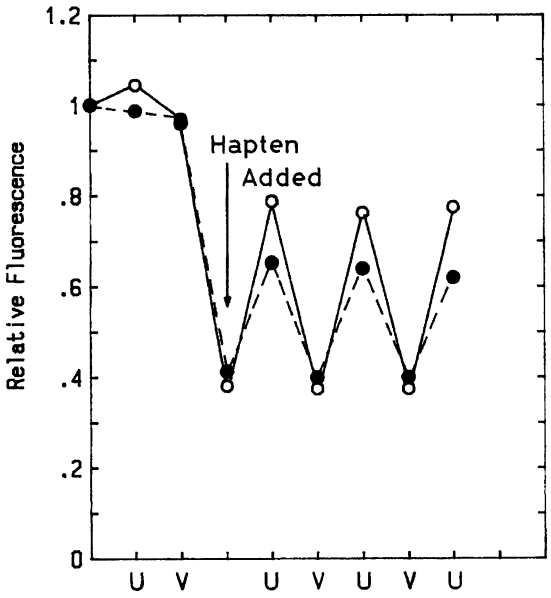


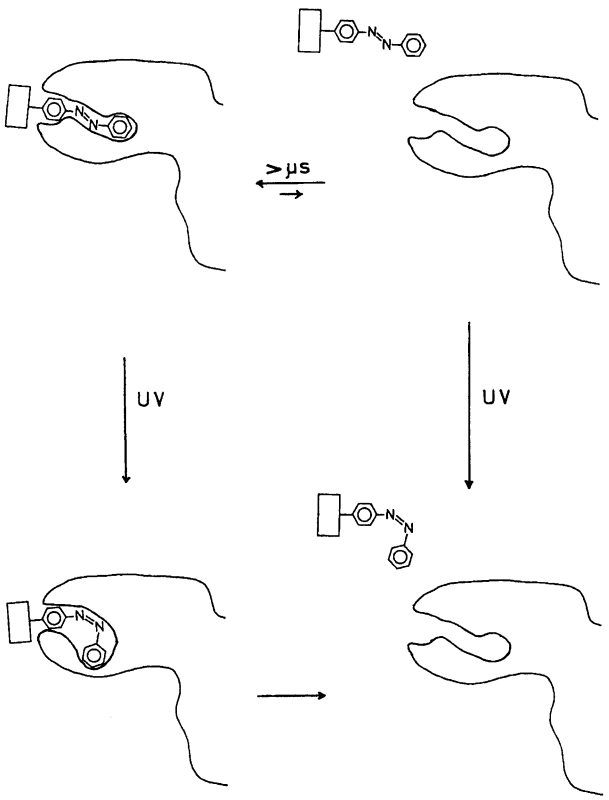
Fig. 4. Fluorescence intensity from a mixture of antibody (Z1H01 or Z1H02, 2.6×10^{-7} M) and the hapten peptide (7.0×10^{-7} M) in PBS(-) solution at 25 °C. The solution was alternately irradiated by UV light (U, 360 nm) and visible light (V, 430 nm) and the fluorescence intensity at 340 nm was monitored. The intensity was normalized by that before photoirradiation.

the Z1H02–hapten pair, but with a smaller difference. Photoreversible uptake and release of the hapten has also been confirmed by HPLC analysis of the antibody (Z1H01)–hapten peptide mixture.[1)]

Mechanism of *trans*-to-*cis* Photoisomerization of the Hapten Peptide in the Presence of the Antibody. There are two possible alternative pathways for the *trans*-to-*cis* photoisomerization of the hapten peptide that induces photoreversible uptake and release (Scheme 2). In the first pathway, the isomerization occurs inside the binding site of the antibody. In this case, the binding site that is designed against the *trans* azobenzene moiety must be flexible enough to allow the transient change of the bound hapten to the *cis* form. It should be noted that the lifetime of the excited state of the azobenzene chromophore is on the order of a few ten picoseconds or less. Therefore, if the binding site is very rigid and does not allow the conformational change within a few ten picoseconds, the azobenzene moiety cannot photoisomerize at all. In the second possible mechanism, only free haptens in solution that are in equilibrium with the bound ones photoisomerize. The equilibrium then shifts to increase the free haptens during the photoirradiation. In this case, the rate of the increase of *cis* haptens is governed by the dissociation rate of *trans* haptens that is known to be on the order of a microsecond or slower.[11)]

Therefore, the two pathways may be discriminated by using pulsed UV light that is shorter than the time required for the dissociation of *trans* haptens from the binding site (microseconds). If the pulsed irradiation still induces the photoisomerization, the first pathway prevails. According to the second pathway, only a very small amount of free haptens can photoisomerize. Therefore, the amount of *trans*-to-*cis* photoisomerization by a pulsed laser (365 nm, 12 ns) was examined in a mixture containing 7.1×10^{-6} M hapten and 9.7×10^{-6} M antibody (1.94×10^{-5} M binding sites). Under the above conditions only 0.2% of the hapten peptide remained in solution. Figure 5 shows the amount of *cis* haptens that were produced by the pulsed irradiation, plotted against the laser power. Since the laser power was not high enough to produce a large amount of the *cis* form even in solution, the *cis* contents accumulated after 30 laser shots were plotted. Prior to the pulsed irradiations, each mixture was irradiated with visible light to assure 100% *trans* form. It is clear that the pulsed irradiation induced a substantial amount of photoisomerization in the bound haptens. However, the quantum yield of the isomerization for the bound haptens was smaller than that for the free haptens in solution. This result strongly indicates that the photoisomerization occurs inside the binding site with a smaller quantum yield due to steric restriction.

A similar experiment with a single laser shot at the



Scheme 2. Two possible pathways for the photoinduced release of the hapten peptide.

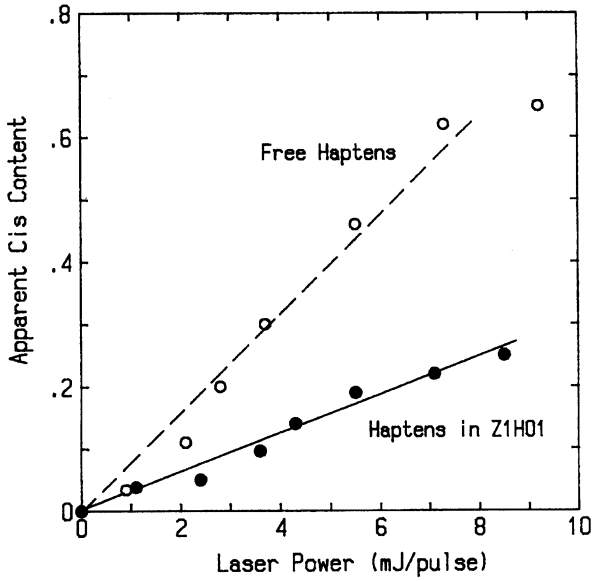


Fig. 5. The apparent isomerization yield $(A_0 - A)/(A_0 - A_\infty)$, after 30 pulsed irradiations with 355 nm laser light (pulse duration=12 ns) plotted against the laser power. A_0, A, and A_∞ are absorbances at 315 nm before irradiation, after 30 pulsed irradiations, and after irradiation with 330 nm light to the photostationary state (about 82% *cis* form). Concentration of the hapten peptide=7.1×10^{-6} M, concentration of Z1H01=9.7×10^{-6} M (concentration of the binding site=19.4×10^{-6} M).

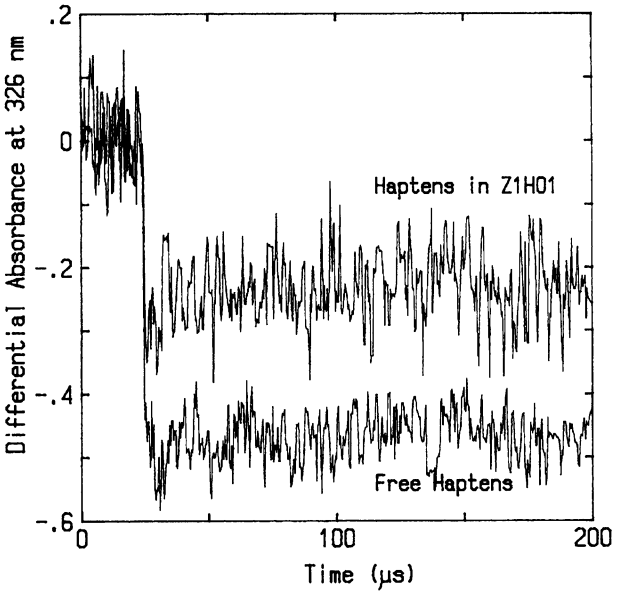


Fig. 6. Change in absorbance at 326 nm after irradiation with a pulsed laser (355 nm, 12 ns). Concentration of the hapten peptide=4.1×10^{-5} M, concentration of Z1H01=3.1×10^{-5} M.

highest power was also carried out in a more concentrated hapten/antibody mixture. Figure 6 shows the decrease of absorbance of *trans* azobenzene moieties at 326 nm after a single laser shot. Photoisomerization was definitely observed in both bound and free haptens. But the quantum yield of the isomerization was smaller for the bound haptens, indicating that steric and other constraints in the binding site retard the isomerization.

It is concluded that the antibody that recognizes the hapten peptide at the azobenzene moiety (Z1H01), allows *trans*-to-*cis* photoisomerization of the azobenzene moiety inside the binding site. After the photoisomerization, the antibody releases the improperly shaped hapten. It is somewhat surprising that the binding site of the antibody is flexible enough to allow the isomerization in a very short period on the order of several ten picoseconds.

Conclusions

Antibody Z1H01 recognizes the *trans* azobenzene moiety of the hapten peptide and releases the hapten when it is photoisomerized to the *cis* form. The *trans*-to-*cis* photoisomerization can occur inside the binding site and the improperly shaped hapten is released. In contrast to the case of Z1H01, antibody Z1H02 recognizes the peptide chain of the hapten and does not show a sharp susceptibility to photoirradiation. The photoreversible uptake and release of the hapten peptide may find wide applications in biological and chemical fields.

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