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Defined Analyte–Enzyme Conjugates as Signal Generators in Immunoassays

Se-Hwan Paek,* Leonidas G. Bachas,† and Willfried Schramm*,‡

Reproductive Sciences Program and Bioengineering Program, University of Michigan, 300 N. Ingalls, Ann Arbor, Michigan 48109; †Department of Chemistry, University of Kentucky, Lexington, Kentucky 40506; and ‡BioQuant, Inc., 1919 Green Road, Ann Arbor, Michigan 48105

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We investigated the synthesis of progesterone–horseradish peroxidase (P–HRP) conjugates and products purified by affinity chromatography. The obtained preparations were characterized with an immobilized monoclonal antibody in solid-phase immunoassays. Three homogeneous P–HRP conjugates were isolated. Two preparations were identified to contain a single progesterone ligand on the enzyme molecule. A third preparation contained two progesterone ligands. We postulate that conjugation can occur at two different positions on the enzyme, and that the different microenvironment of the protein structure surrounding the ligand contributes to different binding constants of the conjugates with immunoglobulin. By comparing the effective binding constants derived from affinity chromatography and from Scatchard analysis, we have demonstrated that the divalent conjugate binds to antibody immobilized on planar surfaces only by a single attachment due to steric restriction. Dose–response curves for progesterone using the isolated P–HRP conjugates have been investigated and compared.

In the development of competitive enzyme immunoassays (EIA)[1] for small molecules, we face a distinct problem: the preparation of defined analyte–enzyme conjugates as indicators for analyte concentration in samples. The quality of analytical methods based on the recognition of this conjugate by a binding protein as a measure of analyte concentration depends, however, on the defined synthesis and reproducible purification of such analyte–enzyme conjugates.

The majority of traditional radiolabeled derivatives can be synthesized such that the tracer molecule contains a known amount of analyte (usually one). This is not so with enzymes as signal generators. Most of the radiolabeled analyte derivatives (e.g., tyrosine methylester, tyramines, or histamines) can be purified after synthesis by HPLC, which usually separates nonreacted analyte molecules from mono- and diiodinated derivatives (1). Such a convenient and efficient purification method is usually not available for analyte–enzyme conjugates.

For the synthesis of enzyme-labeled derivatives, the ϵ-amino groups of lysine residues of the protein molecules are often used as functional groups to conjugate activated analyte molecules. The majority of enzymes, however, contains several amino groups suitable for conjugation. Two problems arise:

(i) These amino groups may be positioned at different locations of the protein structure, and may extend to different distances from the surface of the enzyme molecule. Since binding proteins (e.g., antibodies) used for analytical systems are highly selective for the analyte, changes in the electrostatic environment surrounding the analyte ligand and accessibility to the conjugate (steric hindrance) can impair their binding affinity to the ligand (2,3).

(ii) In the synthesis of the conjugate, the number of analyte molecules reacting with the enzyme cannot be easily controlled. Multiple analyte ligands on the enzyme, however, have the potential of binding to multiple

[1] Abbreviations used: ^{125}I-BHR, ^{125}I-labeled Bolton–Hunter reagent (monoiodinated, *N*-succinimidyl-3-(4-hydroxy-3-[^{125}I]iodophenyl) propionate); ^{125}I-P, ^{125}I-labeled progesterone derivative; DMSO, dimethyl sulfoxide; DSS, disuccinimidyl suberate; EIA, enzyme immunoassay; Gel–PBS, phosphate-buffered saline containing gelatin; Gel–PBS–thimerosal, PBS containing gelatin and thimerosal; HRP, horseradish peroxidase; HRP-Ab, antibody to HRP; NS-Ab, antibody nonspecific to progesterone and HRP (antibody to urease); P-Ab, antibody to progesterone; P-CAD, *N*-(4-aminopentyl)-4-pregnen-11α-ol-3,20-dione succinyl amide; P-NHS, progesterone-11α-*N*-hydroxy succinimide; P-TME, progesterone-11α-hydroxy hemisuccinyl tyrosine methylester; TMB, 3,3′,5,5′-tetramethylbenzidine.

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recognition sites of one or more binding proteins thus changing the conditions for competing with the analyte in the sample.

Upon reaction of an activated derivative of the steroid hormone progesterone with the enzyme horseradish peroxidase (EC 1.11.1.7), we obtain reproducibly different distinct products after affinity chromatography. We have investigated the hypotheses (a) that in these preparations progesterone is bound to different sites of the enzyme molecule, and (b) that the number of progesterone molecules per enzyme differs.

In the absence of methods to determine the number and position of ligands on the enzyme molecule directly, we have made an attempt to characterize the analyte–enzyme conjugates by comparison of Scatchard analysis and affinity chromatography of the antibody–conjugate complexes.

MATERIALS AND METHODS

Reagents

Horseradish peroxidase (HRP, type VI, 300 units/mg solid), 1,5-diaminopentane (cadaverine), and poly-L-lysine hydrobromide (M_r 421,000, determined by a viscosity test as specified by manufacturer) were purchased from Sigma (St. Louis, MO). Disuccinimidyl suberate (DSS) and *N*-succinimidyl-3-(4-hydroxy-3-[^{125}I]iodophenyl) propionate (^{125}I-Bolton–Hunter reagent, monoiodinated; ^{125}I-BHR) were obtained from Pierce (Rockford, IL) and NEN Research Products (DuPont Co., Wilmington, DE), respectively. Progesterone and progesterone-11α-hemisuccinate (4-pregnen-11α-ol-3,20-dione hemisuccinate) was purchased from Steraloids (Wilton, NH). The following progesterone derivatives were synthesized and purified by HPLC (1): progesterone–11α-*N*-hydroxy succinimide (P–NHS); *N*-(4-aminopentyl)-4-pregnen-11α-ol-3,20-dione succinyl amide (P–CAD) by reacting P–NHS with excess cadaverine; and progesterone–11α-hydroxy hemisuccinyl tyrosine methylester (P–TME). The P–TME was iodinated (^{125}I-P) with the carrier-free ^{125}I (NEN Research Products, DuPont Co.) and purified as described elsewhere (1). P–CAD was purified by HPLC under the same conditions.

Substrate for HRP. The substrate solution for HRP contained: 10 μl of 3% (v/v) H_2O_2 in water; 100 μl of 10 mg/ml 3,3′,5,5′-tetramethylbenzidine (TMB) in dimethyl sulfoxide (DMSO); and 10 ml of 0.05 mol/liter acetate buffer, pH 5.1.

Production of Monoclonal Antibodies

Antibodies were raised in mice by immunizing with a conjugate of progesterone–11α-hemisuccinate and bovine serum albumin (molar ratio of steroid:albumin, 30:1). Clones were screened against a conjugate of progesterone–ovalbumin with the same linkage. Details of the procedures are described elsewhere (1). We used antibodies to progesterone (P-Ab) with a high affinity constant (BQ.1, subisotype IgG_{2bk}; $K_a = 1.1 \times 10^{11}$ liter/mol for P–TME; 3×10^9 liter/mol for native progesterone) and with a low affinity constant (4C10, subisotype IgG_{2bk}; $K_a = 9.0 \times 10^8$ liter/mol for P–TME), and antibodies to two enzymes, HRP (HRP-Ab, 6A5; $K_a = 7.4 \times 10^9$ liter/mol) and urease (as nonspecific antibody to progesterone and HRP; NS-Ab). The affinity constants of two antibodies to progesterone were evaluated in a solid-phase assay (1), and the affinity constant of antibody to HRP was evaluated with free HRP by determining the enzyme activity of the binding complex in a solid-phase colorimetric assay (see below). All antibodies were precipitated by ammonium sulfate (4), redissolved in 0.01 mol/liter phosphate buffer, pH 7.0, containing 0.14 mol/liter NaCl (PBS) containing 0.02% (w/v) thimerosal (PBS–thimerosal) to a concentration of 10 mg/ml, and aliquots were stored at −20°C.

Immobilization of Antibody in Microtiter Wells

Three monoclonal antibodies were immobilized separately on the surface of microtiter wells (Immulon II, Dynatech Inc., Alexandria, VA): P-Ab (BQ.1), HRP-Ab, and NS-Ab. The immobilization was accomplished by the following modified version of the sodium periodate method (5): (a) incubate 200 μl of 10 μg/ml poly-L-lysine in 0.5 mol/l carbonate buffer, pH 9.6, (b) incubate 200 μl of the antibody in PBS–thimerosal, and 3×10^{-4} mol/l $NaIO_4$ (1 μg/ml antibody for radioimmunoassay or 2.5 to 10 μg/ml for colorimetric assays), and (c) incubate 300 μl of PBS–thimerosal containing 0.1% (w/v) gelatin (gel–PBS–thimerosal) and 1×10^{-3} mol/l $NaCNBH_3$. For each step, the preparations were incubated overnight in a sealed polypropylene box with 100% humidity at room temperature. After decanting the liquid, the wells were washed 3 times with deionized water. The treated wells were dried under vacuum and stored in the presence of silica gel as desiccant at 4°C.

Conjugation of Progesterone to HRP

The progesterone derivative, P–CAD, was chemically reacted with the enzyme (HRP) via DSS (6,7) as a cross-linking reagent. P–CAD and DSS were dissolved in anhydrous DMSO. The conjugates were synthesized by: (a) adding dropwise the solution of P–CAD to the solution of cross-linking reagent with 2 *M* excess of cross-linking reagent, (b) incubation for 30 min on a shaker, (c) adding dropwise this mixture to enzyme solution in PBS with 20 *M* excess of P–CAD, and (d) incubation for 2 h on a shaker. All steps were performed at room temperature.

The reaction mixture of progesterone–HRP conjugate was dialyzed for 1 day at 4°C in PBS–thimerosal.

The dialyzed mixture was then purified by exclusion chromatography on Bio-Gel P-30 (Bio-Rad, Richmond, CA). The enzyme product was eluted with PBS–thimerosal. Each eluted fraction was analyzed for progesterone by a solid-phase radioimmunoassay and for enzyme by a colorimetric assay (see below). The fractions containing the analyte–enzyme conjugate were pooled and stored at −20°C, after adding an equal volume of glycerol, until being used for immunoaffinity purification.

Determination of Reactive Amino Groups on HRP

To determine the number of amino groups on HRP that are acylated with *N*-succinimidyl derivatives under the conditions applied with the conjugation of progesterone to HRP, we reacted ^{125}I-BHR with the enzyme. After spiking nonlabeled BHR with ^{125}I-BHR, the solution in DMSO was added dropwise to HRP in PBS with a 1000-fold molar excess of BHR. After reaction for 2 h, the mixture was purified by dialysis and size-exclusion chromatography (Bio-Gel P-30). We determined the enzyme concentration in the eluate with the highest radioactivity by means of oxidation of TMB against a dose–response curve with free HRP (see below), estimated the specific radioactivity from measurement of γ-radiation, and calculated the number of ligands bound to HRP.

Immunoaffinity Chromatography of Progesterone–HRP Conjugates

Preparation of gel. The monoclonal antibody to progesterone, 4C10, was selected for the preparation of the immunoaffinity gel because of its low affinity constant to the antigen. The antibody was immobilized on an activated agarose gel (CNBr-activated Sepharose 4B; Pharmacia Fine Chemicals, Inc., Piscataway, NJ) by following a protocol recommended by the manufacturer. Briefly, the dry gel was suspended in and washed with 1 mmol/liter HCl. The antibody was dissolved in 100 mmol/liter bicarbonate buffer, pH 8.3, containing 140 mmol/liter NaCl and was mixed with the washed gel (5 mg antibody per 1 g of dry gel). Lysine residues on the antibody molecule were reacted with functional groups from the gel for 2 h at room temperature. After coupling the antibody, excess antibody was washed off with the bicarbonate buffer, and remaining reactive sites on the gel were hydrolyzed with 100 mmol/liter Tris–HCl buffer, pH 8. The gel was then washed by alternating acidic and alkaline conditions to remove the nonspecifically adsorbed antibody on the solid matrix.

Chromatography. Before the conjugation mixture was applied to the immunoaffinity gel column (1.1 × 37 cm, 20 ml bed volume), the gel was equilibrated with PBS–thimerosal. Then, the mixture containing about 16 nmol protein was loaded and subsequently washed with PBS–thimerosal to separate the unbound enzyme from the bound conjugate which was eluted without changing the buffer. The eluate from the column was delivered to a fraction collector (Model 1200 Pup, ISCO, Lincoln, NE) by a peristaltic pump (Type 4912A, LKB, Stockholm, Sweden). The elution rate was 2.3 ml/h and the fraction volume was 3.5 ml.

After purification, the column was extensively washed with 0.1 mol/l sodium acetate buffer, pH 4.5, containing 0.5 mol/l NaCl, 5% (v/v) Tween-20, and 0.02% (w/v) thimerosal until no progesterone eluted from the column. The eluted fractions were analyzed for total enzyme and conjugated enzyme by colorimetric assays (see below). Fractions containing the conjugate were diluted with the same volume of gel–PBS–thimerosal, and stored at 4°C. The enzymatic activity of the conjugates was preserved without loss for more than 1 year.

Determination of purified conjugate concentrations. The enzyme activity of the conjugate is not affected by the analyte ligand on the enzyme if lysine residues are utilized under the mild conditions employed for the conjugation (8). This was confirmed by comparing the specific enzymatic activity of the starting material of HRP and the progesterone–HRP conjugates which were not different (oxidation of TMB per unit amount of protein; the protein concentration was measured by the method described by Bradford (9)).

The concentration of the affinity purified HRP conjugate in solution was determined by comparing its activity in a solid-phase assay with standard concentrations of free HRP. Antibody to HRP was immobilized on microwells and incubated with different concentrations of HRP (standard solutions). The wells were washed and the oxidation of TMB was measured at 450 nm by absorbance. Solutions of affinity-purified progesterone–HRP conjugate were incubated in the microwells under the same conditions and color development with TMB was compared with standard curves. This method was reproducible and provided a variation that was within ±1.5% of the average concentration of triplicate determinations. Free enzyme and progesterone–HRP conjugate bound to the immobilized antibody with the same binding constant as determined by Scatchard analysis (see below).

Binding constants. The binding constants of affinity-purified HRP conjugates to the high affinity P-Ab were determined by Scatchard analysis (10) by use of the solid-phase colorimetric assay. The Scatchard relation was expressed by plotting the ratio of bound over free conjugate concentration against the bound at equilibrium. The concentration of the bound conjugate was determined by a standard regression line that was prepared by the liquid-phase colorimetric assay with free HRP diluted in gel–PBS without preservative.

The enzyme reaction on a solid surface might initially be limited by diffusion of the substrates, H_2O_2 and chromogen TMB, which might cause an inaccuracy in matching the concentration of the conjugate in a solid-phase with that of HRP in a liquid phase. We tested the effect of diffusion by varying the concentrations of the two substrates. When the substrates were used in higher concentrations, the enzyme reaction on the solid surface was not significantly enhanced if the reaction proceeded for at least 5 min.

To test if the catalytic activity of the binding complex between HRP and the antibody immobilized on solid surface is different from enzyme in solution, we compared color development with TMB with identical amounts of HRP in wells without and with immobilized antibody on polypropylene discs as described above. We did not find a difference in enzymatic activity beyond a variation of 5% in color development.

Assays

The following assays were used for analysis of the eluted fractions from the chromatography columns. If not otherwise mentioned, all assays were performed in duplicate, and the means were used for the plots. Nonspecific binding measured by NS-Ab was subtracted from total binding.

Solid-phase radioimmunoassay. This assay was performed to identify fractions of progesterone–HRP conjugates and free progesterone from eluates of the immunochromatography column. A total of 200 μl of radiolabeled ^{125}I-P (40,000 counts/min; 13.8 pg) and sample in gel–PBS–thimerosal was added to microtiter wells containing the immobilized high affinity P-Ab, and incubated for 1.5 h on a shaker. After washing, the remaining radioactivity was measured by using a gamma counter (GammaTrac 1290, Tm Analytic, Elk Grove Village, IL).

Colorimetric assays. We performed two solid-phase colorimetric assays to measure enzyme activities of the conjugated HRP and of the total enzyme activity in eluate from the columns. For the quantitative determination of the enzyme components containing analyte, microwells were prepared with the immobilized high affinity P-Ab (coat with 200 μl per well of a solution of 2.5 μg/ml of antibody). For the evaluation of the total enzyme activity, microwells with immobilized HRP-Ab were prepared (coat with 200 μl of 10 μg/ml of antibody). Aliquots of eluate from the affinity columns (10 μl of a dilution 1:20 in buffer) were incubated in a total volume of 200 μl in the wells for 5 h on an orbital shaker. We washed the wells and measured the remaining enzyme activity by a modification of the method described by Bos *et al.* (11): (a) add 200 μl of the substrate solution prepared just before use, (b) develop the colored TMB derivative, (c) add 50 μl of 0.5 mol/liter of sulfuric acid to stop the enzymatic reaction, and (d) measure the absorbance at 450 nm with a spectrophotometer (Titertek Multiscan, Type 310C; Eflab Oy, Finland).

Dose–response curves. Dose–response curves were obtained by competitive immunoassay with the immobilized high affinity P-Ab. A constant concentration of labeled progesterone (e.g., affinity-purified progesterone–enzyme conjugate) and different concentrations of progesterone in gel–PBS–thimerosal were added to the wells (total volume 200 μl) and incubated for 5 h on a shaker. After washing the wells, the signal from the labeled progesterone was measured as described above. The dose–response curves were analyzed by the log-logit transformation (12).

RESULTS AND DISCUSSION

The most commonly used method for the quantitative determination of small analytes by means of antibodies as binding proteins is the competitive immunoassay. If this assay uses an enzyme as a signal generator, a tracer, i.e., the conjugate of the enzyme and the analyte, needs to be synthesized. The properties of this tracer substantially affect the quality of the analytical method.

Some of the properties that determine the binding energy between an antibody and a labeled analyte that functions as signal generator are similar for radiolabeled tracer and enzyme labeled derivatives, e.g., bridge group recognition. Others are specifically related to the enzymes, such as diffusion rates and variations in the attachment of the small ligand molecule to the large protein as outlined below.

We immobilized immunoglobulins via a polylysine coat on the solid surface to minimize the probability of dissociation of antibody during incubation (13). The polymer of lysine is bound to surfaces with an energy that is not unlike a chemical bond. Immunoglobulins can be bound to ε-amino groups of lysine residues through the carbohydrate groups on the F_c region of IgG which leaves the paratope of the antibody accessible for complex formation with antigens.

Bridge group recognition. Incorporation of the chemical link between the analyte component and the tracer moiety can contribute to the interaction with the binding site of the antibody (bridge group recognition) and is a concern with radiolabeled tracers (14–16) as well as with enzyme-labeled signal generators (17–20). The specific binding characteristics to the antibodies of the preparations isolated in these studies may be determined in part by the recognition of the group linking the steroid to HRP. However, bridge group recognition alone cannot explain the different properties of the antibody–antigen complexes that are formed with the tracers we have isolated.

Effect of functional groups on the enzyme. Typical reactive sites in enzymes for the chemical reaction of

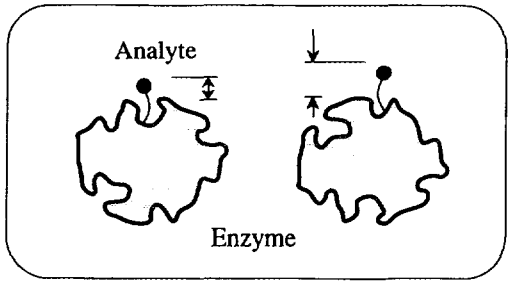

FIG. 1. Protrusion of small conjugated analyte molecules in variable distances from the periphery of an enzyme molecule.

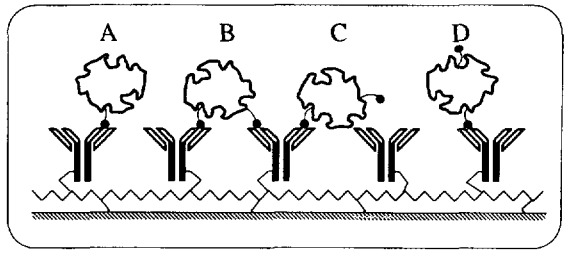

FIG. 2. Different hypothetic binding patterns of analyte-enzyme conjugates to immobilized antibody. Binding of the conjugate with one ligand (A) and the conjugate with multiple ligands at different positions (B to D).

activated ligands are often ϵ-amino groups of lysine residues, sulfhydryl groups, or carbohydrate moieties in glycoproteins. Since most proteins have more than one of the functional groups available for reactions, a defined synthesis as to the site of ligand attachment or as to the number of ligands per enzyme molecule is not straightforward. The preparations we have isolated indicated that the ligand is bound to the enzyme at different sites and that we have different analyte-to-enzyme ratios.

Electrostatic environment around the analyte ligand. The enzyme is a protein that is composed of the defined sequence of amino acids that can create a multitude of different electrostatic environments. The different charge distributions surrounding the analyte molecules conjugated to the enzyme can result in different ionic interactions between the analyte and the binding site of the antibody thus affecting the enthalpy of the binding complex.

Protrusion of analyte ligand. The proteinacious nature of the enzyme provides a comparatively large three-dimensional structure. The attached analyte may protrude from the protein periphery with a variable distance according to the positions of the different conjugation sites (Fig. 1) and, therefore, provide varying bridge lengths between the two conjugated molecules. The resulting accessibility of the analyte ligand to the antigen binding domain of the antibody is another contributing factor to the magnitude of the binding constant.

Multiple analyte ligands. An enzyme conjugated with one ligand binds to the antibody by single attachment (Fig. 2A). The intrinsic affinity (K) of the conjugate is determined by the interaction of one antibody binding site with one ligand attached to the enzyme. If the conjugate has two ligands, these may occupy two binding sites of the antibody at the same time (Fig. 2B) and contribute to a higher effective binding constant, K_a, which may approach αK^2 where α has the dimension of concentration and is equal to $\rho[(2\pi/3)r_{ab^2}]^{-3/2}$ (21,22). The factor ρ reflects the effective concentration of the second ligand for the volume through which it can sweep when tethered through the first binding site to the antibody. The term in brackets is a statistical factor for the multiple binding, and r_{ab} the distance between two antibody binding sites (23). Even at dense packing of antibody molecules, α is unlikely to be greater than 10^{-3} mol/liter.

Binding enhancements with the divalent conjugate are further reduced by steric hindrance of the conjugate, freezing out of segmental flexibility of antibody, and added ring strain introduced with the second binding event (21,22) which may prevent the simultaneous binding of both ligands at the same time (Figs. 2C and 2D). If the conjugate binds to antibody via one ligand, the question arises as to the expected effective binding constant. Suppose that two conjugates, one with a single ligand and the other with two ligands as shown in Fig. 3, encounter the binding site of an antibody. This will happen as a result of diffusion of the conjugate toward the binding site of the immobilized antibody. All diffusional processes are independent of potential or actual binding reactions (assuming the difference in size of the conjugates is negligible). However, the divalent conjugate (Fig. 3A) has a higher probability than the monovalent conjugate (Fig. 3B) to meet an antibody binding site during diffusion and form an encounter complex which can lead to the formation of the binding complex (24). If two ligands on one enzyme molecule can contribute to the formation of the binding complex, the effective bind-

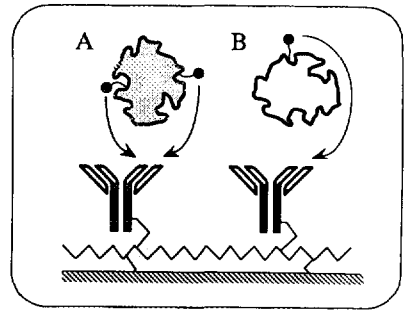

FIG. 3. The effect of multiple ligands (A) versus monoligand (B) on the effective binding constant.

ing constant for this conjugate as a whole is higher than that for the monovalent conjugate.

Separation of Conjugates

When using amino groups of lysine residues as functional groups for ligand conjugation, HRP has the advantage that of a total of six groups (25) only two are available for chemical conjugation under mild reaction conditions (26,27) as applied in this investigation. The remaining four amino groups can react only under strong acylating conditions with anhydrides (25) and dithioesters (8) whereupon the protein might be partially denatured (28).

We confirmed these findings by reacting HRP with radiolabeled BHR under the same mild conditions as with the progesterone derivative. We found that an average of 1.8 ligands are bound to HRP with an excess of BHR reacted. This approaches the maximal number of two ligands. That it is <2 may be related to the shorter sequence of the linker molecule in BHR which for progesterone is much longer, i.e., the succinimidyl group can more easily reach the amino group on the protein.

In order to minimize the maximal number of analyte (progesterone) molecules bound to a HRP, the conjugation was carried out under mild conditions by using a homobifunctional, activated *N*-hydroxysuccinimide cross-linking reagent. Under these conditions, a maximum of two amino groups react on a HRP molecule. The number of reactive functional groups is larger in most enzymes. For example, more than 13 lysine residues are available for reaction on a glucose oxidase molecule (29) and maximum 116 on β-galactosidase (30). The larger the number of analyte ligands per enzyme molecule, the more complicated is the isolation of defined conjugate preparations. Purification methods based on molecular size, charge, or density are not suitable while affinity chromatography is very effective.

Immunoaffinity purification. After low molecular weight components were removed by size-exclusion chromatography, the progesterone–HRP conjugates were purified on an affinity gel column with an antibody of relatively low-affinity (4C10, $K_a = 9.0 \times 10^8$ liter/mol) to progesterone. The fractions eluted from the gel column (Fig. 4) were analyzed in two solid-phase colorimetric assays with different antibodies immobilized on microwells: antibody to HRP (HRP-Ab) and to progesterone (P-Ab). HRP-Ab captured the HRP in both free and conjugated molecules, therefore reflecting the total HRP activity (Tot. Act.) in the eluent, and P-Ab captured the progesterone in the conjugated molecules, i.e., the specific HRP activity (Spec. Act.).

Four fractions with increased HRP activity were isolated (Fig. 4, peaks a to d). In fraction a, the total activity was much higher than the specific activity. This peak

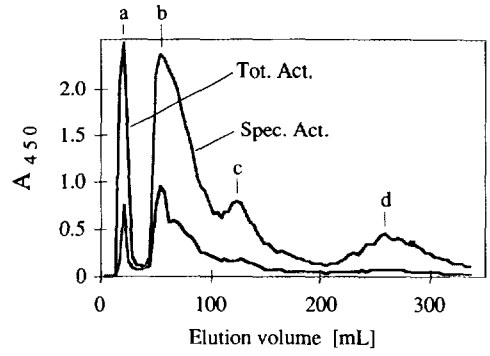

FIG. 4. Purification of progesterone–horseradish peroxidase (HRP) conjugates by immunoaffinity chromatography. The dark curve represents the total activity (Tot. Act.) of HRP and the gray curve the specific activity (Spec. Act.). Peak a contains free HRP as major component, and peaks b to d represent three different preparations of progesterone–HRP conjugates. The *y*-axis refers to absorbance at 450 nm of the oxidized substrate as explained under *Colorimetric assays.*

contained mainly free HRP and only 5% accounted for specific binding. We assume that some of the free HRP formed aggregates that had enzyme molecules with the analyte enclosed so that no interaction with the affinity gel could occur. Upon dilution, the analyte–enzyme conjugate dissociates from the aggregates and is detected as specific binding in wells containing the immobilized antibody to the analyte. This assumption was supported by the observation that we obtained a similar profile as shown in Fig. 4 if we rechromatographed combined fractions from peak a on an immunochromatography column.

Three fractions of conjugate in peaks b to d were retained by the affinity column and showed significant specific activities in colorimetric progesterone assays. These fractions represent three different species of progesterone–HRP conjugates. The retention volumes of these conjugates were measured by subtracting the elution volume of the free enzyme from that of the conjugates (Table 1).

Characterization of the Conjugates

Homogeneity of purified preparations. After pooling fractions from each peak, the homogeneity of the separated conjugates was tested by Scatchard analyses (Fig. 5). According to the slopes of the regression lines, each preparation of the three conjugates was homogeneous, but the affinity constants to the antibody were different. The binding constants (K_a) determined from the slopes are listed in Table 1. Conjugates retained longer on the affinity column had higher binding constants. It is noteworthy that the binding constants of the three purified conjugates b to d were higher than that of native progesterone (3×10^9 liter/mol). Likewise, the iodinated pro-

TABLE 1

Comparison of the Retention Volumes, Equilibrium Binding Constants (K_a), and ED_{50} of the Affinity-Purified Progesterone–HRP Conjugates and the Nonpurified Preparation

Progesterone–HRP conjugate	Retention volume[a] (ml)	K_a[b] (liter/mol)	ED_{50}[c] (ng/well)
b	33	9.3×10^9	0.055
c	102	2.8×10^{10}	0.076
d	238	3.5×10^{10}	0.111
Nonpurified	—	—	0.077

[a] Obtained by subtracting the elution volume of free enzyme from that of the conjugates (Fig. 4).
[b] Calculated from the Scatchard analysis (Fig. 5).
[c] Obtained from dose-response curves (Fig. 6).

gesterone derivative has a higher binding constant (1.1×10^{11} liter/mol (1)). In the case of iodinated progesterone derivative, both the enzyme conjugates and the immunogen for raising the monoclonal antibodies (1) have the same succinyl group that has been shown previously to contribute to antibody binding (31). We conclude that the bridge group between progesterone and the enzyme also participates in the interaction of the antigen with the antibody. As expected, the nonpurified mixture showed a nonlinear Scatchard relation (not shown).

Dose–response curves. We compared the three purified preparations of HRP conjugates (b to d from Fig. 4) with the nonpurified mixture in dose–response curves (Fig. 6). The conjugates b to d gave parallel dose–response curves with an increasing ED_{50} (effective dose of progesterone at 50% displacement of the bound conjugate, i.e., 50% of B/B_0) (Table 1). The ED_{50} of the preparation d was about twice as high as that of b. With increasing binding affinity, a higher concentration of free analyte is required to compete with the conjugate for binding sites of the antibody. The curve obtained with the nonpurified mixture did not parallel those of the purified preparations as expected from its nonhomogeneous composition.

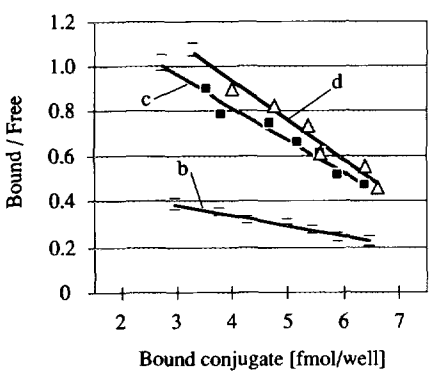

FIG. 5. Scatchard plots of three affinity-purified progesterone–HRP conjugates (b to d from Fig. 4). The error bars indicate typical standard deviations for duplicate estimates.

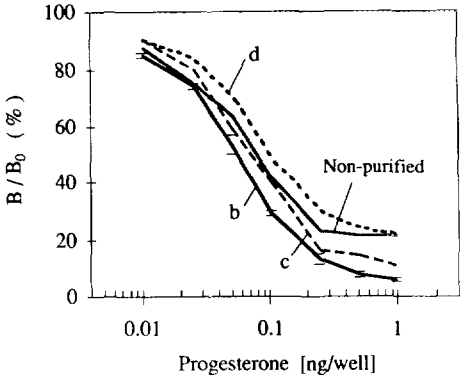

FIG. 6. Dose–response curves of three affinity-purified progesterone–HRP conjugates (b to d from Fig. 4) and nonpurified HRP conjugate mixture. Contrary to the nonhomogeneous mixture, the dose–response curves of the purified preparations were parallel to each other. The total enzyme activity of tracers was the same for all dose–response curves. Typical standard deviations for duplicate estimations are shown for curve b.

Comparison of Binding Constants

Binding constants derived from affinity chromatography. The retention of molecules upon affinity chromatography can be used for the determination of binding constants between interacting molecules (32,33). The elution volume is inversely proportional to the concentration of soluble antibody and provides a means to calculate the affinity constant between immobilized antibody and antigen. The procedure has been applied for different systems of competitive (34–36) and noncompetitive binding (32). Correlation studies with equilibrium dialysis have established the validity of this method (35,37).

Under the condition of immunoaffinity chromatography, conjugates are separated according to (a) the number of ligands and (b) the affinity of the conjugated analyte. The effective binding constant (K_a) of the conjugate is a function of these two factors, and is proportional to the retention volume (V_r) in the gel column,

$$V_r = V - V_0 = K_a(V_0 - V_m)[Ab_t], \qquad [1]$$

where V is the elution volume of the conjugate, V_0 that of the free enzyme, and V_m the void volume of the column. $[Ab_t]$ is the concentration of the binding sites of the immobilized antibody on the gel. We have used a relatively slow flow rate of elution buffer (2.3 ml/h) to assure equilibrium of antigen–antibody binding although it has been claimed that the flow rate has little

TABLE 2

Comparison of the Ratios of Effective Binding Constants Determined by Affinity Chromatography and with Antibody Immobilized on Microwells

Ratio of effective binding constants of two conjugates	Affinity chromatography	Scatchard analysis[a]
$(K_a)_{conj.c}/(K_a)_{conj.b}$	3.1	3.0
$(K_a)_{conj.d}/(K_a)_{conj.b}$	7.2	3.8

[a] Derived from Table 1.

effect on complex formation (38). Flow rates as high as 400 ml/h have been used in comparable columns (39).

By applying Eq. [1] to the chromatographic data shown Fig. 4, the ratio of the effective binding constants, K_{a1}/K_{a2}, of any two purified conjugates is determined by the ratio of the retention volumes, V_{r1}/V_{r2}, since V_0, V_m, and $[Ab_t]$ are constant. According to this method, we calculated the relative effective affinity constants for the three purified conjugates (peaks b to d of Fig. 4; see Table 2). It has been shown that multivalent species can form multiple binding complexes on affinity gels and are, therefore, longer retained (36). The situation is different if antibodies are immobilized on surfaces that approach planar structures, such as microwells.

Binding constants derived from Scatchard analysis. Another method for the determination of the binding constants is the Scatchard analysis as described above. In our experiments, the analysis was carried out by utilizing microwells for the immobilization of the antibody. Contrary to the affinity gel, the surface geometry of the microwells at the molecular level can be considered as two-dimensional. The binding of the conjugate with multiple analyte ligands to the antibody on the two-dimensional surface may be subject to the steric restriction as mentioned above.

Evaluation of purified conjugates with regard to the number of ligands. The affinity gel as the solid matrix contains pores and the surface available for immobilization is three-dimensional. Under this condition, the probability that conjugates with multiple analyte ligands can simultaneously bind to more than one binding site of an immobilized antibody is proportional to the number of conjugated ligands and the density of antibody binding sites. The latter is substantially higher on the surface of the affinity gel than on the surface of the microwells. Provided a multiligand conjugate can bind to antibodies on the microwells only with one ligand at a given time, a higher effective binding constant for the conjugate with more ligands can be expected if determined on the gel as compared to that determined on microwells. For example, if the ratio of binding constants of one conjugate to the other is significantly higher if determined with the gel than that obtained on microwells, these two conjugates will be different species regarding the number of ligands on the enzyme. Based on this analysis, we could determine that conjugate d has two progesterone molecules bound to the enzyme (Table 2).

The binding constant of conjugate c was higher than that of conjugate b by about the same ratio regardless of the methods of determination. As mentioned above, the higher binding constant of conjugate c can result from: (a) a higher intrinsic affinity constant of the conjugated analyte and (b) multiple analyte molecules on the enzyme. We exclude (b) as a possibility. If conjugate c has two ligands, for sterical reasons they may not be able to bind simultaneously with antibodies immobilized on a surface such as on microwells. Binding to antibodies immobilized on a gel, however, would be facilitated because of the three-dimensional arrangement of antibodies in a gel. Since we did not find different binding constants whether measured in microwells or on the gel, we conclude that this preparation does not contain two ligands of progesterone.

Based on the same reasoning, we come to the conclusion that preparation d has multiple ligands bound to each enzyme molecule. The binding constant obtained with antibody immobilized on gel is twice as high as that measured with antibody on microwells (Table 2). In conjugate d progesterone has reacted with both of the amino groups on the enzyme that are available under the reaction conditions. These two ligands are not simultaneously available to antibody immobilized on a planar surface as described later.

Given that under the conjugation conditions employed there are a maximum of two progesterone molecules on the enzyme, in summary we conclude: (i) Conjugate b is a monoderivatized analyte–enzyme conjugate. (ii) Conjugate c is also a monoderivatized conjugate but the analyte molecule is bound to a different site on the enzyme which results in a different binding constant of the antigen–antibody complex. (iii) Conjugate d has two analyte ligands per enzyme molecule. Why does a conjugate with two analyte ligands per enzyme not behave like a heterogeneous tracer in the Scatchard analysis but follows a linear regression? This question will be investigated in the following section.

Binding of Divalent Conjugate to Immobilized Antibody

If different populations of antigen–antibody complexes can be formed, the binding reaction for each species reaches its own thermodynamic equilibrium. Consequently, instead of a linear regression between B/F vs B in a Scatchard plot, a nonlinear curve is obtained in the presence of two species of binding complexes (40).

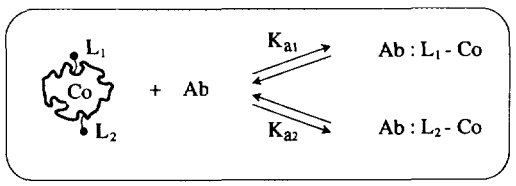

FIG. 7. Formation of two different binding complexes with an immobilized antibody and an enzyme conjugate that has two analyte ligands.

This curve represents in practice two linear regressions, one for each antigen–antibody complex.

In the beginning of systematic studies on antibody binding reactions, it had been shown that multivalent antigens can form different binding complexes in liquid-phase assays (41,42). However, the situation is different when the antibody is immobilized. As mentioned above, the small ligand in a progesterone–HRP conjugate with two analyte molecules bound to the enzyme surface may not be able to form simultaneously two different antigen–antibody complexes (Fig. 2).

The conjugate (Co) with the two ligands (L_1, L_2) can bind to the antibody binding site (Ab) via either L_1 to form the complex Ab:L_1–Co with the intrinsic affinity constant K_1 or it can bind via L_2 to form Ab:L_2–Co with K_2 (Fig. 7). The two binding constants for each ligand are expressed as a function of the concentrations of the participating components at equilibrium.

$$K_1 = [\text{Ab:L}_1\text{–Co}]/([\text{Ab}][\text{Co}]) \quad [2]$$

$$K_2 = [\text{Ab:L}_2\text{–Co}]/([\text{Ab}][\text{Co}]) \quad [3]$$

The sum of the Eqs. [2] and [3] is

$$K_1 + K_2 = ([\text{Ab:L}_1\text{–Co}] + [\text{Ab:L}_2\text{–Co}])/([\text{Ab}][\text{Co}]) = B/([\text{Ab}][\text{Co}]), \quad [4]$$

where B is equal to [Ab:L_1–Co] + [Ab:L_2–Co], i.e., the total concentration of the conjugate bound via two ligands which is measured in the Scatchard analysis. Equation [4] shows that the effective binding constant, K_a, for the divalent antigen, able to attach only with one antigen at a given time, is the sum of $K_1 + K_2$. We can formulate the Scatchard equation by using expressions [2], [3], and two supplemental equations from the law of mass action for the antibody and the conjugate,

$$B/F = -(K_1 + K_2)B + (K_1 + K_2)[\text{Ab}_t], \quad [5]$$

where [Ab_t] is the concentration of antibody.

Equation [5] can be graphically expressed in a plot of B/F vs B with the slope of $K_1 + K_2$ that determines the effective binding constant of the divalent conjugate. As expected, not only is the slope linear, but the determined effective binding constant is also equal to the sum of the two intrinsic constants of each ligand. Accordingly, the calculated affinity constant ($K_1 + K_2$, see Table 1 for the intrinsic binding constants) is 3.7×10^{10} liter/mol which is comparable with the measured value for conjugate d (3.5×10^{10} liter/mol). Therefore, we conclude that the divalent conjugate binds to the immobilized antibody by a single attachment which results in a linear slope in the Scatchard plot.

Implications on the type of interaction between the antibody and the divalent conjugate. If the antibody immobilized on the surface of microwells would be evenly distributed (no cluster formation), two ligands attached to opposite sites of a progesterone–HRP conjugate cannot simultaneously bind to the binding sites of two antibody molecules. The amount of immunologically active binding sites was about 10 fmol per 157 mm^2 of polystyrene surface inside the microwells (determined from the Scatchard plot). At this density, the average distance between the centers of two adjacent antibody molecules is approximately 160 nm. If the distance between two binding sites on a single antibody molecule is estimated as 24 nm (43,44), two binding sites of adjacent immunoglobulins molecules are on the average 136 nm apart. This distance is at least 25 times longer than the diameter of an HRP molecule (5.34 nm; (45)).

However, the steric dimensions allow simultaneous binding of the two ligands of a bivalent conjugate if antibodies are immobilized in clusters. Likewise, a circular complex involving two idiotypic sites of the same antibody molecule is possible. The hinge region can form an elbow angle between 130° to 180° (46) and the idiotypic sites can, therefore, span a distance between 4 to 25 nm (43,44). Simultaneous binding would, however, result in an increased observed affinity constant (24). We interpret the lack of an increased affinity constant as evidence that the two ligands of the conjugate in preparation d were sterically hindered from simultaneous binding.

Conclusion

This study demonstrates that it is feasible to prepare homogeneous populations of monosubstituted analyte–enzyme conjugates with horseradish peroxidase. Under the applied reaction conditions, only two amino groups on HRP can react with the activated ligand progesterone. Therefore, it was possible to characterize the three different progesterone–HRP conjugates that could be reproducibly obtained upon affinity chromatography. We demonstrated that it is advantageous to use monosubstituted rather than disubstituted (or more generally multisubstituted) conjugates in the development of enzyme immunoassays with better detection capabilities. Further, the availability of defined analyte–enzyme

conjugates along with monoclonal antibodies should enable the initiation of detailed modeling studies in enzyme immunoassays. Indeed, it has been difficult to perform such studies about the effect of ligand substitution on the properties of liquid-phase (47,48) or solid-phase immunoassays (49,50) with polyclonal antibodies (i.e., heterogeneous mixtures of immunoglobulins). It should be noted that for the standardization of solid-phase enzyme immunoassays as analytical tools, at least three requirements need to be met: (i) the availability of monoclonal antibodies as well defined binders, (ii) the controlled and reproducible immobilization of these antibodies, and (iii) the synthesis of defined analyte–enzyme conjugates. Finally, it is our intention to extend the investigations on defined analyte–enzyme conjugates to the enzyme glucose oxidase.

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