Activation and Diffusion in the Kinetics of Adsorption and Molecular Recognition on Surfaces. Enzyme-Amplified Electrochemical Approach to Biorecognition Dynamics Illustrated by the Binding of Antibodies to Immobilized Antigens

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Abstract: An electrochemical method is proposed for investigating the dynamics of recognition between a biomolecule and an immobilized receptor. It involves redox labeling of the solute molecule and monitoring the binding by the electrochemical response of the electrode onto which the receptor is immobilized. With large biomolecules, as, for example, antigens and antibodies, leading to small surface concentrations, simple redox labeling may prove insufficient to obtain detectable responses. Redox enzymes are then advantageously used as labels thanks to the signal amplification offered by their catalytic properties. The applicability of the method is illustrated by the reaction of an immobilized monolayer of goat IgG antigen (or of one Fab fragment) with an antigoat antibody labeled with glucose oxidase. Particular care is taken to free the kinetic data from the effect of diffusion. The latter factor may interfere whatever the detection technique. A full account of the combination between recognition kinetics and diffusion is therefore given in terms of a kinetic zone diagram leading to diagnostic criteria and data processing procedures that allow a proper extraction of the recognition thermodynamic and kinetic constants. The theory applies as well to the dynamics of adsorption of nonbiological molecules on surfaces.

Determining the kinetic constants of recognition between biomolecules is of considerable current interest for the understanding of natural systems as well as for their use in artificial mimicking constructions. Immobilization of the receptor (e.g., an antigen) on a substrate is convenient for applications of molecular biorecognition to the detection of a target molecule (e.g., its antibody) present in solution. Monitoring the amount of immobilized antibody−antigen complex as a function of time gives access to the kinetic parameters of the antibody−antigen reaction. The equilibrium constant of the recognition reaction may be derived from the limiting value of the antigen−antibody complex, provided full saturation has not been reached. In the latter case, the backward rate constant, and hence the equilibrium constant, may be determined by exposure of the saturated surface to a pure solution. Similar approaches may be used to investigate recognition reactions in other couples of biomolecules, as, for example, the recognition of single-stranded DNA oligonucleotides by partially or totally complementary DNA strands. In this case, a known DNA strand is immobilized and subsequently recognized by the free target DNA in solution.

In all cases, the presence of the biomolecule of interest (antibodies, DNA strands, etc.) must be monitored through the measurement of a parameter bearing a definite relationship with its surface concentration. It should be sensitive enough to detect submonolayer coverages with good precision. Quartz crystal microbalance,[2] ellipsometry,[3] acoustic wave detection,[4] and surface plasmon resonance (SPR)[5,6] have been employed in this purpose. The popularity of the latter technique has recently been boosted by the availability of a commercial instrument. The antigen is immobilized into a rather thick dextran layer (1000−2000 Å). The refractive index in the evanescent field which decays exponentially within the dextran layer is measured as a function of time, giving access to the variation of the coverage with time. It is usually assumed that the two quantities are proportional. The interpretation of the data may be further complicated by the partition of the antibody between the solution and the dextran film and the interference of diffusion not only in the solution but also in the film.[8]

We propose and describe below an electrochemical approach of the dynamics of molecular biorecognition. It involves redox labeling of the solute molecule and monitoring the binding by

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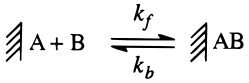
means of the electrochemical response of the electrode onto which the receptor is immobilized. The method is illustrated by its application to the kinetics of the recognition of an immobilized monolayer of goat IgG (antigen) by an antigoat antibody. Because, owing to the large size of the molecules, the surface concentrations are small, this is a typical case where simple redox labeling of the target molecule would not provide sufficient sensitivity to the attachment of the antibody onto the antigen monolayer. Labeling of the antibody with a redox enzyme obviates this difficulty thanks to the catalytic properties of the enzyme. The antibody was thus labeled with glucose oxidase. The anodic catalytic current observed in the presence of glucose and of ferrocene as the cosubsrate is directly proportional (with a known proportionality coefficient) to the amount of enzyme, and therefore to the amount of antibody, bound to the immobilized antigen.[9a] The catalytic current was recorded under steady-state conditions, using a glassy carbon disk electrode. Under these conditions, the diffusion rate may be precisely controlled by means of the rotation speed, allowing an unambiguous extraction of the kinetic parameters of the recognition reaction from the raw data after the interference of diffusion has been accounted for. Whatever the detection technique, diffusion of the target molecule toward the immobilization surface may seriously interfere in the overall response. It may even be, under certain circumstances, the rate-determining step, thus preventing the determination of any rate parameter characterizing the recognition reaction itself. Although the interference of diffusion has been emphasized in several instances,[8,10] it is still often unduly neglected in recent works.[2b,4] This is the reason that we will start with a detailed account of this problem. The same theoretical approach may be followed for the adsorption kinetics of conventional molecules, where the question of the diffusion vs activation control are current issues of discussion.[11−13] We will also examine how interactions between the bound molecules may alter the kinetics of binding in the framework of a Frumkin isotherm description of the thermodynamics of these interactions. An unambiguous evaluation of the role of mass transport is required for a sound analysis of the intrinsic factors governing the dynamics of binding. For example, it will be seen that partial diffusion control may be easily confounded with the effect of lateral interactions in the adsorbed state.

Results and Discussion

Theoretical Analysis of the Competition between Diffusion and Binding. We consider the following recognition reaction,



where A is the immobilized receptor molecule, B the target molecule, and AB the complex between them. For demonstrating the role of diffusion, it is sufficient to consider the case where the reaction obeys a Langmuirian kinetic law (more complicated mechanisms will be discussed later on):

$$\frac{d\Gamma_{AB}}{dt} = k_f C_0 \Gamma_A - k_b \Gamma_{AB} = \frac{D}{\delta}(C^b - C_0) \qquad (1)$$

where the Γ's are the surface concentrations of the subscript species. C^b and C_0 are the concentrations of B in the bulk of the solution and at the immobilizing surface, respectively. The last member of the equation expresses the diffusion flux of B toward the surface under steady-state conditions. D is the diffusion coefficient of B, and δ is the thickness of the steady-state diffusion-convection layer established by the rotation of the disk electrode. The latter parameter is inversely proportional to the square root of the rotation rate of the disk electrode, ϖ, the variation of which thus provides a convenient access to the contribution of diffusion to the overall kinetics.[14] $\Gamma_A + \Gamma_{AB} = \Gamma^S$ is the total surface concentration of immobilized receptor molecules. It is convenient to introduce the fractional coverage, $\theta = \Gamma_{AB}/\Gamma^S$, of AB on the electrode. Combination of the above equations thus leads to the following differential equation.[15]

$$\frac{d\theta}{dt} = \frac{k_f C^b(1-\theta) - k_b\theta}{1 + k_f C^b t_d(1-\theta)} \qquad (2)$$

where $t_d = \delta\Gamma^S/DC^b$ is a time that characterizes the diffusion process. We are thus led to introduce a dimensionless time normalized toward the diffusion time, $\tau = t/t_d$ Equation 2 suggests the introduction of two other dimensionless parameters, one that characterizes the adsorption equilibrium, $\kappa = k_f C^b/k_b = KC^b$ ($K = k_f/k_b$ is the binding equilibrium constant) and the other, $\lambda = k_f C^b t_d = k_f \delta\Gamma^S/D$, that compares the rate of adsorption to the rate of diffusion. Thus,

$$\frac{d\theta}{d\tau} = \lambda \frac{1 - \frac{1+\kappa}{\kappa}\theta}{1 + \lambda(1-\theta)}$$

For the adsorption process ($\theta = 0$ for $\tau = 0$), explicit integration leads to

$$\lambda\theta - \left(1 + \frac{\lambda}{1+\kappa}\right)\ln\left(1 - \frac{1+\kappa}{\kappa}\theta\right) = \frac{1+\kappa}{\kappa}\lambda\tau \qquad (3)$$

At long times the system tends toward equilibrium, i.e., $\theta = \theta_{eq} = \kappa/(1+\kappa)$, suggesting the normalization of the fractional coverage toward its equilibrium value, $\theta^* = \theta/\theta_{eq} = (1+\kappa)\theta/\kappa$. Thus,

$$\theta^* - \frac{1+\kappa+\lambda}{\kappa\lambda}\ln(1-\theta^*) = \left(\frac{1+\kappa}{\kappa}\right)^2\tau \qquad (4)$$

This closed-form equation allows the prediction of all possible coverage-time curves for systems obeying Langmuirian kinetics. To identify the respective role of diffusion and binding kinetics, it is useful to examine various limiting situations that appear as the two parameters κ and λ take large or small values. They are represented as portions of space in the "kinetic zone

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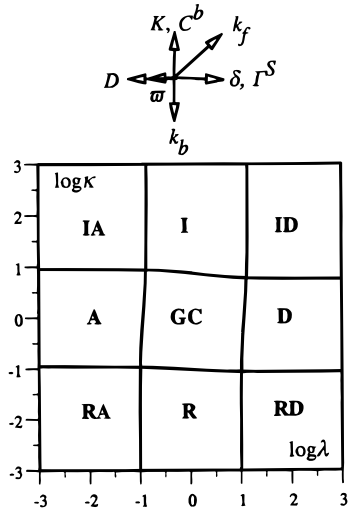
(14) (a) $\delta = 12.47D^{1/2}\nu^{1/6}(2\pi\varpi)^{-1/2}$, δ in cm, D in $cm^2\ s^{-1}$, ν (kinematic viscosity of the solvent) in $cm^2\ s^{-1}$, ϖ in rotations per minute.[14b] (b) Levich, V. G. *Acta Phys. Chim. USSR* **1942**, *17*, 257.

(15) Equation 2 is equivalent to eq 19 in ref 12b. Finite difference simulations of the adsorption−diffusion problem are not necessary thanks to the Nernst approximation, which quite reasonably assumes that the concentration profiles are linear within the diffusion−convection layer.



zone	Characteristic Equation
GC	$\theta^* - \frac{1+\kappa+\lambda}{\kappa\lambda}\ln(1-\theta^*) = \left(\frac{1+\kappa}{\kappa}\right)^2 \tau$
A	$-\ln(1-\theta^*) = \frac{1+\kappa}{\kappa}\lambda\tau = \frac{1+\kappa}{\kappa} k_f C^b t$
D	$\theta^* - \frac{1}{\kappa}\ln(1-\theta^*) = \left(\frac{1+\kappa}{\kappa}\right)^2 \tau$
I	$\theta^* - \frac{1}{\lambda}\ln(1-\theta^*) = \tau$
R	$-\ln(1-\theta^*) = \frac{\lambda}{\kappa(1+\lambda)}\tau$
IA	$-\ln(1-\theta^*) = \lambda\tau = k_f C^b t$
ID	$\theta^* = \tau$
RA	$-\ln(1-\theta^*) = \frac{\lambda}{\kappa}\tau = k_b t$
RD	$-\ln(1-\theta^*) = \frac{\tau}{\kappa}$

Figure 1. Adsorption or binding of the target molecule to the receptor. Kinetic zone diagram and characteristic equations

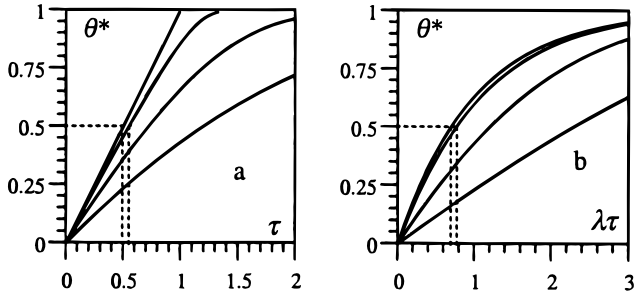


Figure 2. Passage from zone I to zone ID (a) and from zone I to zone IA (b). From left to right log $\lambda = \infty$, 1.141, 0.5, 0 (a); $-\infty$, -0.858, 0, 0.5 (b).

diagram"[16] shown in Figure 1 together with the equations characterizing each case.

I and R correspond to an irreversible and a reversible binding, respectively. A and D represent kinetic control by the binding reaction and by diffusion, respectively. Besides, the general case, GC, the two-letter labels represent the combination of the above cases.

The way in which the boundaries between each zone were determined is illustrated in Figure 2 with the example of irreversible binding (zone I). Figure 2a shows the variation of the fractional coverage θ^* with the dimensionless time variable, τ, in the vicinity of diffusion control (passage from zone I to zone ID), whereas Figure 2b shows the variation of the fractional coverage θ^* with the dimensionless time variable, $\lambda\tau$, in the vicinity of binding control (passage from zone I to zone IA). The value of λ corresponding to the passage is defined as the value for which the time for reaching half of the equilibrium coverage ($\theta^* = 0.5$) differs by 10% in the I case and in either the ID or the IA cases as represented by the dotted lines in Figure 2.

The arrows on top of the diagram in Figure 1, indicating the effect of the various experimental parameters in direction and (logarithmic) magnitude, help navigating from one zone to the other. Among these parameters, the concentration of the target molecule in solution, C^b, and the rotation rate, ϖ, may be easily varied. Varying C^b has practically no effect on the kinetic competition between binding and diffusion. Increasing C^b makes the system pass from a reversible to an irreversible behavior. The rotation rate, ϖ, is the key parameter for identification and control of the interference of diffusion. Increasing ϖ makes the system pass from kinetic control by diffusion to kinetic control by the binding reaction. A simple test that diffusion does not interfere is the observation that, upon increasing the disk rotation rate, the Γ_{AB} vs time curve ceases to depend on this parameter. The system is then in the A zone and the binding kinetics may be easily derived using the corresponding equation (Figure 1). Information on the binding kinetics may nevertheless be extracted from the experimental data even when kinetics is under mixed diffusion/binding control (zones I, GC, and R) with however less and less precision as the system is closer and closer to the boundary with zones ID, D, and RD, respectively. In other words the ability of reaching fast binding kinetics is dependent on the ability of achieving fast rotation rates. The adsorption rate constant may be determined as long as the parameter λ can be made smaller than ca. 1 (Figure 1). The upper accessible values of k_f are thus as defined by eq 5.

$$k_f \leq \frac{D}{\delta\Gamma^0} \approx 0.039 T^{2/3} r^{4/3} \rho^{-2/3} \nu^{-5/6} \sqrt{\varpi} \qquad (5)$$

(where k_f is in mol^{-1} L s^{-1}, the volumic mass, ρ, in g cm^{-3}, the solvent kinematic viscosity, ν, in cm^2 s^{-1}, ϖ in rotations per minute, Γ° in mol cm^2, r, the radius of the equivalent hard sphere, in Å). To obtain a rough estimate of the maximal k_f, the diffusion coefficient is assumed to obey the Stokes−Einstein relationship in the last member of eq 5. Ten thousand rotations per minute is about the maximal rotation speed that can be reached with a rotating disk. Thus, in water at 25 °C,

$$k_f\,(M^{-1}\,s^{-1}) \leq 81[\varpi(\mathrm{rpm})]^{1/2} r^{4/3}\,(Å) \qquad (6)$$

The maximal binding rate constant is thus on the order of 10^6, 2×10^5 and 5×10^4 M^{-1} s^{-1} for typical antibodies, for DNA strands, and for conventional organic molecules, respectively. It is seen that it is easier to observe the activation-controlled adsorption kinetics with large biomolecules than with conventional organic molecules, explaining why, in the latter case, the results are so scarce or so uncertain and also why the adsorption kinetics are considered to be under diffusion control in most circumstances.[17]

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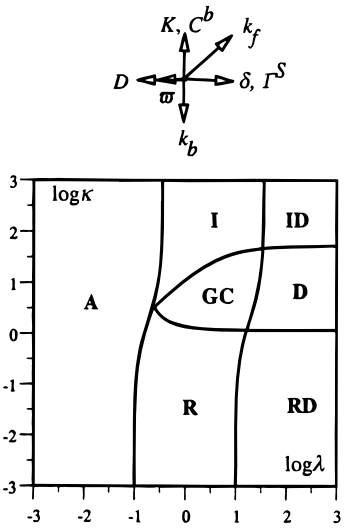

zone	Characteristic Equation
GC	$\frac{\kappa}{1+\kappa}(1-\theta^*)+\frac{1+\lambda}{\lambda}\ln(\theta^*)=-\frac{\tau}{\kappa}\left(=\frac{tD}{K\delta\Gamma^S}\right)$
A	$\ln(\theta^*)=-\frac{\lambda}{\kappa}\tau\,(=-k_b t)$
D	$\frac{\kappa}{1+\kappa}(1-\theta^*)+\ln(\theta^*)=-\frac{\tau}{\kappa}\left(=\frac{tD}{K\delta\Gamma^S}\right)$
I	$(1-\theta^*)+\frac{1+\lambda}{\lambda}\ln(\theta^*)=-\frac{\tau}{\kappa}\left(=\frac{tD}{K\delta\Gamma^S}\right)$
R	$\frac{1+\lambda}{\lambda}\ln(\theta^*)=-\frac{\tau}{\kappa}\left(=\frac{tD}{K\delta\Gamma^S}\right)$
ID	$(1-\theta^*)+\ln(\theta^*)=-\frac{\tau}{\kappa}\left(=\frac{tD}{K\delta\Gamma^S}\right)$
RD	$\ln(\theta^*)=-\frac{\tau}{\kappa}\left(=\frac{tD}{K\delta\Gamma^S}\right)$

Figure 3. Desorption or dissociation of the complex. Kinetic zone diagram and characteristic equations.

We may also examine the dissociation of the complex, after it has reached its equilibrium value, upon exposure to a pure solution. At time $t = 0$, $\theta = \theta^* = \kappa/(1 + \kappa) \times KC^b/(1+ KC^b)$, where C^b is the bulk concentration that was used during the adsorption step preceding the desorption process. Assuming that the volume-to-surface ratio is large enough for the bulk concentration of B to remain negligible throughout the experiment, the variation of the surface concentration with time obeys eq 7.

$$\frac{d\Gamma_{AB}}{dt} = k_f C_0 \Gamma_A - k_b \Gamma_{AB} = -\frac{D}{\delta} C_0 \qquad (7)$$

Using the same symbols as before, the decay of the fractional coverage with time is depicted by the equation corresponding to the general case (GC) in Figure 3. The competition between diffusion and desorption kinetics is again conveniently represented by a kinetic zone diagram. This is shown in Figure 3 together with the equations pertaining to each zone.

Kinetics of Binding Taking Account of Interactions between Adsorbates. In the preceding analysis, we have assumed that the interactions between the bound (adsorbed) molecules were negligible. At thermodynamic level, the simplest way to take into account such interactions is provided by the Frumkin isotherm:

$$\frac{\theta}{1-\theta} = K \exp(a\theta) C_0 \qquad (8)$$

where K is the binding constant when $\theta \rightarrow 0$, i.e., when the interactions vanish. $a = \Delta G_i/RT$, ΔG_i being the free energy of interaction at unity coverage. $\Delta G_i > 0$ for attractive interactions and < 0 for repulsive interactions. From a kinetic point of view, in the latter case, the interactions slow adsorption and accelerate desorption and vice versa in the former case. We may thus split ΔG_i in two fractions, $\alpha\Delta G_i$ and $(1\text{-}\alpha)\Delta G_i$ and assign the resulting free energies to the modification of the adsorption and desorption rates respectively as a result of the interactions. The kinetic law expressed by eq 9 is thus obtained.

$$\frac{d\theta}{dt} = k_f \exp[\alpha a\theta](1-\theta)C_0 - k_b \exp[(1-\alpha)a\theta]\theta \qquad (9)$$

(k_f and k_b are the adsorption and desorption constants when $\theta \rightarrow 0$). The derivation of eq 9 is similar to the establishment of the Butler−Volmer kinetic law for electrochemical electron-transfer reactions, where the symmetry factor, α, is regarded as independent from the electrode potential.[18] Likewise, in the present case, the symmetry factor, α, is assumed to be independent of the coverage, θ.

In the case of, for example, an irreversible binding reaction where the rotation rate is large enough for activation control to prevail ($C_0 = C^b$), integration of eq 9 leads to the following equation depicting how the coverage builds up with time.

$$k_f C^b t = \exp(-\alpha a)\left\{-\ln(1-\theta) + \sum_{j=1}^{\infty}\frac{(\alpha a)^j[1-(1-\theta)^j]}{jj!}\right\} \qquad (10)$$

Some representative curves are shown in Figure 4.

Application to the Recognition of an Immobilized Goat IgG by the Antigoat Glucose Oxidase Conjugate. 1. Enzyme-Amplified Electrochemical Detection. Using a glucose oxidase labeled antibody, the quantity of antibody bound to the surface antigen is determined by the catalytic current obtained at the cyclic voltammetric oxidation wave of a ferrocene mediator, P, according to Scheme 1. GL is δ-gluconolactone and $FADH^-$

(17) (a) Laviron, E. Voltammetric Methods for the Study of Adsorbed Species. In *Electroanalytical Chemistry*; Bard, A. J., Ed.; Marcel Dekker: New York, 1966; Vol. 12, pp 53−145. (b) Laviron, E.; Roullier, L. *J. Electroanal. Chem.* **1998**, *443*, 195. (c) An exception to this rule has recently been described in a case where the adsorbing surface is a chromatographic reverse phase leading to a particularly high activation barrier.[17d] (d) Hansen, R. L.; Harris, J. M. *Anal. Chem.* **1998**, *70*, 4247. (e) In the case of adsorbing substances that may be oxidized or reduced at the electrode, the adsorption kinetics may be followed by means of transient techniques such as cyclic voltammetry. The transition between activation and diffusion control should then be observed for

$$k_f = \frac{1}{\Gamma^0}\sqrt{\frac{F\nu D}{RT}}$$

where ν is the scan rate. Thus, eq 6 should be replaced by

$$k_f\,(M^{-1}\,s^{-1}) \le 7000[\nu(V/s)]^{1/2} r^{3/2}(Å)$$

while diffusion control will prevail as long as

$$k_f\,(M^{-1}\,s^{-1}) \ge 7000[\nu(V/s)]^{1/2} r^{3/2}(Å)$$

For $r = 4$ Å, at a scan rate of 10 000 V/s, diffusion control prevails as long as k_f is larger than 6×10^6 M^{-1} s^{-1}.

(18) (a) Delahay, P. *Double Layer and Electrode Kinetics*; Interscience: New York, 1965. (b) See also ref 18c where the kinetic law was derived for a Temkin isotherm. (c) Delahay, P.; Mohilner, D. M. *J. Am. Chem. Soc.* **1962**, *84*, 10763.

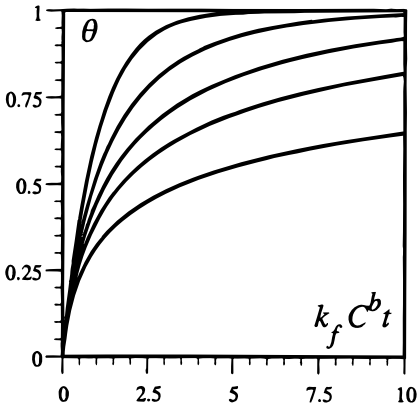

Figure 4. Kinetics of irreversible adsorption for Frumkin-isotherm interactions between adsorbates. From left to right $\alpha a = 0, -1, -2, -3, -5$.

Scheme 1

$$P \rightleftharpoons Q + e^-$$

$$FAD + G \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} FADG$$

$$FADG \xrightarrow{k_2} FADH^- + GL$$

$$FADH^- + 2Q \xrightarrow{k_3} FAD + 2P$$

and FAD are the active reduced form and oxidized form of the flavin adenine dinucleotide enzyme prosthetic group at pH 7.4, respectively. The resulting current, i, is the sum of the diffusion current of P, i_d, and of the catalytic current, i_{cat}, reflecting the enzymatic reaction that regenerates Q.[9] The diffusion current, i_d, can be measured independently by means of experiments carried out in the absence of glucose and subtracted from the total current to give i_{cat}. At potentials positive to 0.3 V the catalytic current reaches a plateau value where i_{cat} is given by eq 11.

$$i_{cat} = \frac{2FSk_3\Gamma_E C_P}{1 + k_3 C_P\left(\frac{1}{k_2} + \frac{1}{k_{red} C_G}\right)} \qquad (11)$$

where C_P is the ferrocene concentration, C_G the glucose concentration, F the faraday, S the electrode surface area, Γ_E the surface concentration of immobilized enzyme, and $k_{red} = k_1k_2/(k_{-1} + k_2)$.

Most of our experiments were carried out as follows. After deposition of the antigen, the electrode is thoroughly rinsed and then immersed and rotated in a PBS buffer (see Experimental Section) containing the glucose oxidase labeled antibody. The amount of adsorbed antibody is monitored at any time by measuring the catalytic activity of the attached enzyme. For this purpose the recognition reaction is quenched by removing the electrode from the antibody solution and rinsing it, the rate of the washout being very small as demonstrated further on. The electrode is then transferred into an antibody-free PBS solution containing 0.1 mM ferrocene methanol and 0.1 M glucose. If ferrocene and glucose are added initially to the antibody solution, the progress of the recognition reaction can also be monitored in situ, the current due to the catalytic activity of the enzyme in solution being negligible compared to that due to the enzyme immobilized at the electrode surface.[9] The proportionality coefficient between Γ_E and i_{cat} ($(0.30 \pm 0.01) \times 10^{-12}$ mol cm^{-2} μA^{-1}) is determined as described in the Experimental Section.

To ensure that the amount of immobilized enzyme thus determined is identical to the amount of immobilized antigen−antibody complex, the homemade glucose oxidase antigoat conjugate is purified so as to obtain antibody molecules labeled with only one, catalytically active, glucose oxidase moiety (see Experimental Section). The superficial concentration of antigen−antibody complex is thus given by Γ_E.

2. Kinetics of the Recognition Reaction. A first series of experiments was carried out with a glassy carbon disk electrode saturated with the whole IgG antigen. It has been shown earlier that adsorption of an IgG used as antigen onto a glassy carbon surface and consecutive recognition by a glucose oxidase conjugated antibody produce a quite stable, well-defined, immobilized monolayer of fully active enzyme corresponding to a monolayer of IgG antigen.[9] That the antigen deposit indeed consists of a single monolayer was confirmed by the fact that it could be quantitatively duplicated to form a multi-monolayer construction by successive recognition of the first enzyme layer by the appropriate anti-glucose oxidase antibody being in turn recognized by the conjugate. Even when this step-by-step procedure was repeated 10 times, the same amount of enzyme (i.e., of conjugate) was immobilized in each monolayer.[9b,19] Thus the adsorbed IgG layer does fulfill the condition of being actually an antigen molecular monolayer within which the interference of antibody diffusion can be excluded. The kinetics of binding with glucose oxidase labeled antibody may be followed as depicted in the preceding section. The results obtained at several concentrations of glucose oxidase labeled antibody and several rotation rates are displayed in Figure 5. The saturation surface concentration, reached after letting the system equilibrate overnight, was found to be $(2.2 \pm 0.2) \times 10^{-12}$ mol/cm^2.

In another experiment, we found that direct adsorption of the antibody glucose oxidase conjugate on a glassy carbon electrode free of antibody gives a saturation surface concentration of 1.1×10^{-12} mol/cm^2, i.e., half the preceding value. This observation points to the conclusion that each deposited antigen is recognized by two antibody glucose oxidase conjugates on average. This is the reason that the surface coverage values reported in Figure 5 have been normalized toward a saturation value of 2.

The role of diffusion on the adsorption kinetics is clearly visible in Figure 5a. The coverage−time curve first depends on the rotation rate, and this dependence ceases when the rotation rate is made larger than 550 rpm. The data gathered at higher rotations rates therefore represent the activation-controlled dynamics of the recognition reaction.

The binding of the antibodies is an irreversible process, as demonstrated by the observation that no loss could be detected after a two-day exposure of a covered electrode to a pure buffer solution (the electrode was rotated at 2200 rpm, to ensure that diffusion plays a negligible role). All the binding reactions investigated in this work are irreversible, in line with the remarkable stability of the immunological construction of mono- and multilayers of antigen−glucose oxidase labeled antibodies.[19] The irreversible recognition of immobilized haptens by monoclonal or polyclonal antibodies or by Fab fragments[20] has also been reported in several instances.[10a,21,22]

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We will come back to the analysis of the recognition dynamics after the description of complementary experiments.

The recognition of an antigen by two antibodies was confirmed by experiments in which the antigen on the surface was diluted by coadsorption of bovine serum albumin (BSA). BSA is a convenient diluting agent, for its presence prevents effectively the nonspecific adsorption of the glucose oxidase antibody conjugate onto the electrode surface. The antigen/BSA ratio in the solution used for coadsorption was adjusted so as to finally obtain an equilibrium surface concentration of immobilized antigen−antibody complexes of one tenth of the value obtained in the absence of BSA. The recognition kinetics were then followed over several hours, the electrode being rotated in the same PBS-buffered solution containing the antibody at 2200 rpm so as to avoid the interference of diffusion. The results are shown in Figure 6. The θ vs time curve cannot be fitted with a single exponential but can be simulated by a double-exponential expression in the framework of the following mechanism:

$$\text{A} + \text{B} \xrightarrow{k_1} \text{AB}_1$$

$$\text{AB}_1 + \text{B} \xrightarrow{k_2} \text{AB}_2$$

thus implying a double recognition of the antibody by the immobilized antigen. The variation of the apparent θ with time, revealed by the catalytic current, is thus expected to follow eq 12.

$$\theta = \theta_1 + 2\theta_2 = 2[1 - \exp(-k_1 C^b t)] + \frac{k_1}{k_1 - k_2}[\exp(-k_1 C^b t) - \exp(-k_2 C^b t)] \quad (12)$$

The best fit (Figure 6) is obtained for $k_1 = (8.8 \pm 1.5) \times 10^4$ and $k_2 = (1.1 \pm 0.1) \times 10^4$ M^{-1} s^{-1}. There are enough binding sites on the immobilized antigen to make possible the recognition of one antigen by more than two antibodies. However, these further recognition processes are likely to be severely hindered by the bulkiness of the antibodies. For immobilized receptor/solution target molecule systems as different as hapten/monoclonal antibody Fab fragments,[22] monoclonal antibody/soluble human T-cell receptor CD4,[23] and T-cell receptor/MHC class I peptide,[24] the recognition kinetics have previously been shown to deviate markedly from pseudo-first order. In these monoclonal systems, the deviation has been ascribed to a reactive heterogeneity caused by the more or less accessible orientation that the epitopes may take after adsorption.[23,25] In our case, this effect is statistically canceled by the polyclonal character of both the antibody and the antigen. This is the reason we rather ascribe the observed double-exponential behavior to a double recognition process.

Other complementary experiments were performed immobilizing onto the electrode a Fab fragment[20] of the antigen rather than the whole antigen itself. The Fab fragment has a much smaller size (molar mass: ca. 50 000) than the whole antigen (molar mass: ca. 150 000). We thus expect recognition to be limited to one antibody per Fab fragment ($\theta = 1$). Figure 7 shows the results obtained with an electrode saturated with Fab fragments and also those obtained upon dilution of the Fab fragments by bovine serum albumin. In the former case (Figure 7b), the saturation surface concentration of bound antibody was

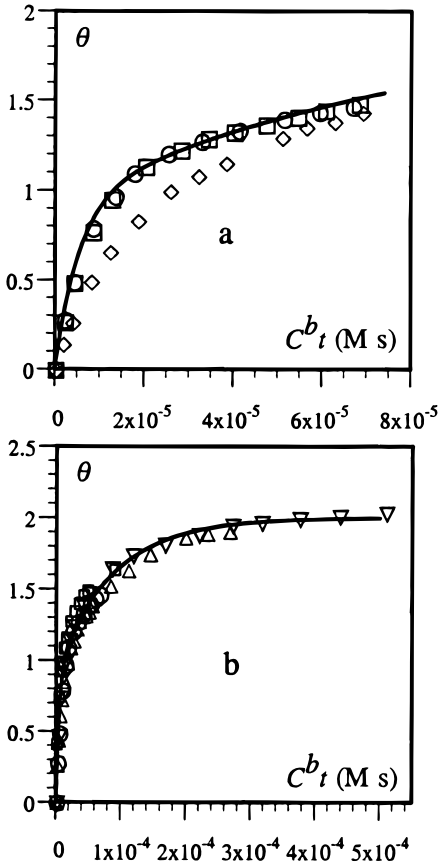

Figure 5. Binding of polyclonal antibody glucose oxidase conjugates to a saturated monolayer of whole antigen deposited on the surface of a GC rotating disk electrode. Variation of the coverage with time. (a) Effect of the rotation rate (□ 2200; ○ 550; ◇ = 140 rpm) at an antibody concentration C^b = 7 nM. (b) Rotation rate 2200 rpm; antibody concentration 7 (○), 14.2 (□), 27.7 (△), 54.3 (▽) nM. In a and b, the solid curve represents a double-exponential fitting of the data points with a saturation value of θ equal to 2 (see text). The concentrations are based on a molar mass of 300 000.

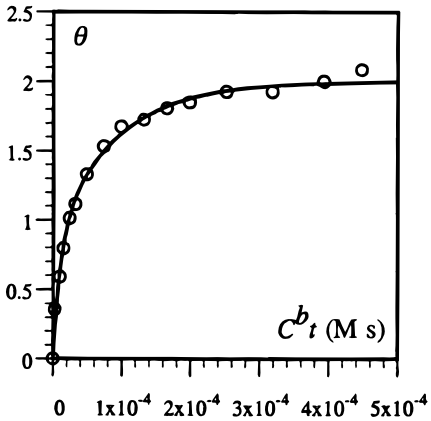

Figure 6. Binding of polyclonal antibody glucose oxidase conjugate to a BSA diluted monolayer of whole antigen deposited on the surface of a GC rotating disk electrode. Variation of the coverage with time. Rotation rate 2200 rpm; antibody concentration C^b = 27.7 nM. The solid curve represents a double-exponential fitting of the data points with a saturation value of θ equal to 2 (see text).

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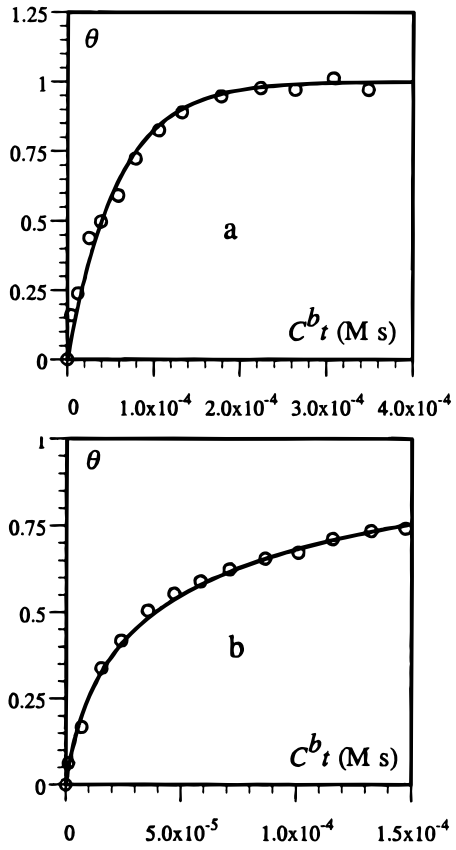

Figure 7. Binding of polyclonal antibody glucose oxidase conjugate to a monolayer of a Fab fragment of the antigen deposited on the surface of a GC rotating disk electrode. Variation of the coverage with time: (a) after dilution with BSA; (b) with no dilution. Rotation rate 2200 rpm; antibody concentration, $C^b = 22.3$ nM. In a, the solid curve represents a single-exponential fitting of the data points (see text). In b, it represents a simulation taking lateral interaction into account (see text)

found to be $(1.0 \pm 0.1) \times 10^{-12}$ mol/cm^2. In the latter experiment (Figure 7a), the amount of BSA was adjusted so as to obtain a surface concentration of bound antibody of 0.14×10^{-12} mol/cm^2. In both cases the rotation rate was 2200 rpm in order to avoid the interference of diffusion. The data points in Figure 7a can be fitted by a single-exponential equation in agreement with the expectation that one Fab fragment cannot bind with more than one antibody. The binding rate constant thus found is $(1.7 \pm 0.2) \times 10^4$ M^{-1} s^{-1}.

When the electrode is covered with undiluted Fab fragments, the data points can no longer be fitted with a single-exponential expression. Lateral interactions between the antibody glucose oxidase conjugates now have to be taken into account. The kinetic model proposed earlier, where the interactions are based on a Frumkin isotherm, allows a satisfactory simulation of the experimental data as shown in Figure 7b. The binding rate constant thus found is $(4.5 \pm 1.0) \times 10^4$ M^{-1} s^{-1} with a value of the parameter αa equal to 3 ± 0.3. If we assume that α is close to 0.5, $a = 6$, i.e., a repulsion free energy at full coverage, $\Delta G_i = 0.36$ eV. The fact that the binding rate constant is somewhat higher than in the BSA-diluted experiment may be rationalized by lateral interactions of the antibody with the BSA molecule, which is bulkier than the Fab fragment.

We may now come back to the recognition of the whole immobilized IgG by the antibody glucose oxidase conjugate as represented in Figure 5b. It is seen that the experimental points are satisfactorily fitted with a double-exponential curve according to eq 12. It is thus found that $k_1 = (1.8 \pm 0.2) \times 10^5$ and $k_2 = (1.2 \pm 0.15) \times 10^4$ M^{-1} s^{-1}. The second rate constant may be somewhat underestimated by the neglect of lateral interactions. It is however of little use to attempt introducing Frumkin-type interactions in the simulation because the resulting modification of the θ−time curve would hardly be distinguished from the effect of the second binding rate within experimental uncertainty.

Experimental Section

Chemicals. The goat IgG (whole molecule, affinipure grade) used as the sacrificial antigen was from Jackson Laboratories. The rabbit antigoat IgG (whole molecule) used for conjugation and the Fab fragment of goat antibody were from Biosys (Compiègne, France). Glucose oxidase from *A. Niger* was from Boehringer Manheim (grade I). The desalting column was a 10 mL Kwick-step column from Pierce. Between each use, the column was equilibrated with PBS buffer. The IMAC affinity column was a 1 mL Hitrap column from Pharmacia. Sephacryl S-300 HR from Pharmacia was the exclusion chromatography phase used to separate labeled from unlabeled antibodies. Centricon-30 was from Amicon. All other chemicals were purchased from Aldrich. They were used as received. The stock solutions of glucose were allowed to mutarotate overnight before use. The PBS buffer was made of 0.01 M KH_2PO_4 and 0.15 M NaCl, and the pH was adjusted to 7.4 with a 1 M NaOH solution. A 0.1% sodium azide solution was added when the buffer was used for protein storage.

Preparation of the 1/1 Rabbit Antigoat IgG−Glucose Oxidase Conjugate. The conjugation procedure was adapted from the literature.[26] Briefly, lyophilized glucose oxidase (19 mg) was dissolved in 1.2 mL of 10 mM PBS buffer. Periodate, 100 mL of a 8mM solution of $NaIO_4$ in water, was then added. After a 20 min incubation in the dark, periodate was removed on a desalting column equilibrated with a 0.2 M pH 9.6 carbonate buffer. The fractions containing the enzyme were pooled together. The rabbit antigoat IgG was then added: the activated enzyme and IgG concentrations were ca. 5 mg/mL and 1 mg/mL, respectively. After a 2 h incubation in the dark at room temperature, the mixture was allowed to react for 30 min with cyanoborohydride (30 μL of a 5 M solution in 1 M NaOH). To avoid the formation of polymers, the remaining aldehyde groups were blocked by reaction with ethanolamine (100 μL of a 1 M solution adjusted at pH 9.6 with concentrated HCl) for 30 min. Cyanoborohydridre (30 μL) was added again, and after a final 30 min incubation, the solution was passed twice through a desalting column equilibrated with PBS buffer. Then glucose oxidase labeled or unlabeled antibodies were separated from the excess of glucose oxidase molecules, not attached to an antibody, by nickel chelate affinity chromatography. The 1 mL IMAC affinity column was loaded with Ni^{2+} ions and equilibrated with PBS buffer. The reaction mixture was injected in the IMAC column and eluted with PBS buffer (10 mL). Under those conditions, all the antibody derivatives were specifically retained on the IMAC column, whereas the glucose oxidase molecules not attached to an antibody were not. The antibody derivatives were recovered by elution with ca. 3 mL of a buffer made of 0.1 M pH 5 acetate buffer and 0.5 M NaCl. Absorbance measurement at 280 nm was used to monitor the elution. The thus recovered mixture contained only labeled and not labeled glucose oxidase antibodies. To isolate the antibodies conjugated to only one glucose oxidase moiety, the solution was further purified by exclusion chromatography. The 55 cm long, 1.25 cm diameter column was filled with Sephacryl S-300 HR (100 mL) at a flow rate of 0.2 mL/min. Absorbance monitoring of the elution was performed at 280 and 454 nm, the latter wavelength being used to assay the glucose oxidase labeled species. At a peak retention volume of 35 mL we collected species absorbing at both wavelengths. A second peak, of similar height, appeared at 40 mL corresponding to species absorbing only at 280 nm. We therefore assigned the first peak to the presence of glucose oxidase labeled antibodies and the second to antibodies having not been labeled. The glucose oxidase/antibody ratio of the

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collected fractions under the first peak was deduced from the comparison of the absorbances measured at both wavelengths. The absorbance coefficients $\epsilon_{280,a}$ and $\epsilon_{280,GO}$ of the antibody and glucose oxidase are identical at 280 nm ($\epsilon_{280,a} = \epsilon_{280,GO} = 1.35$ (mg/mL)$^{-1}$ cm^{-1}), and at 454 nm, $\epsilon_{454,GO}$ is 0.135 (mg/mL)$^{-1}$ cm^{-1}. That gave a glucose oxidase/antibody ratio of 1, confirming that we actually obtained a 1/1 stoichiometry for the glucose oxidase−antibody conjugate. The conjugate was concentrated up to 0.35 mg/mL by ultracentrifugation through a Centricon 30 filter, sodium azide (0.1%) was added, and the conjugate was stored at 4 °C.

Adsorption of the Antigen Monolayer. The 3 mm diameter glassy carbon disk electrodes were prepared as described previously.[27] They were successively polished with sand paper and diamond pastes of 3 and 1 μm particle size. The electrodes were ultrasonicated in ethanol between each polishing step. Adsorption of goat IgG was obtained by wetting the electrode surface with a drop of a 1 mg/mL IgG solution in PBS buffer. The electrode was then thoroughly rinsed, washed with the buffer, dipped into a 0.1 mg/mL solution of gelatin in PBS for 10 min, and rinsed again. The gelatin treatment was used to prevent the occurrence of nonspecific binding. The electrode was then adapted to a EDI Tacussel rotating device and immediately used for the recognition experiments.

Cyclic Voltammetry. The instrumental setup was the same as previously described.[28] Unless otherwise specified, the temperature in all experiments was 25 °C. All solutions were purged from dioxygen before each voltammetric run.

Determination of the Proportionality Coefficient between i_{cat} and the Surface Concentration of Immobilized Antigen−Conjugate Complexes. The stationary electrode covered with a saturated monolayer of antigen−conjugate complexes was tested in the presence of phosphate buffer (pH 8.0, 0.1 M ionic strength), 0.4 mM ferrocene methanol, and 0.5 M glucose. The kinetic characteristics of glucose oxidase under such conditions were previously determined,[9a] and the enzyme surface concentration was thus determined by means of cyclic voltammetry as described in ref 9a. The same electrode was then immersed in the test solution used in the experiments reported in the present work (ferrocene 0.1 mM, glucose 0.1 M, PBS buffer). The catalytic current was measured at 0.32 V/SCE as described in the text. The proportionality coefficient between the enzyme surface concentration and the catalytic current was found to be $(0.30 \pm 0.01) \times 10^{-12}$ mol cm^{-2} μA^{-1}.

Conclusions

The rotating disk method allows a facile diagnosis of the rate control in the dynamics of adsorption or recognition by immobilized receptor molecules. Activation control is reached when, upon increasing the rotation rate, the time response ceases to depend on this factor. Diffusion control is characterized by a time response whose slope is proportional to the square root of the rotation rate. In the first case, the mechanism and kinetics of the adsorption (recognition) process are easily derived from the coverage time curves. Information about the intrinsic adsorption kinetics may still be obtained even in the presence of partial control by mass transfer, albeit with less accuracy. The possibility of reaching a situation where the activation-controlled process can be characterized is favored by an increasing size of the adsorbing molecule. An approximate estimate of the maximal accessible adsorption rate as a function of the equivalent radius of the molecule may be obtained from eq 6.

The effect of lateral interactions between adsorbed molecules on the dynamics of adsorption may be accounted for by a kinetic law based on Frumkin isotherm thermodynamics (eqs 9 and 10). Application to the recognition of the immobilized Fab fragments by the antibody glucose oxidase conjugate gave satisfactory results, assuming that a 1-to-1 recognition process is operating. The validity of this assumption was demonstrated by experiments in which the Fab fragments on the electrode were diluted by bovine serum albumin.

Double recognition of the antibody glucose oxidase conjugate by the immobilized whole IgG is clearly apparent both in experiments where the antigen was diluted and in experiments where it was not. The first attachment rate constants, $(1-2) \times 10^5$ M^{-1} s^{-1}, are among the largest reported in the literature for similar systems. For example, rate constants of 3×10^5 M^{-1} s^{-1}, [29] or 10^4 to 10^5 M^{-1} s^{-1},[27] have been found for the bindings of monoclonal antibodies with the corresponding antigens and 10^4 M^{-1} s^{-1} for the binding of a Fab fragment of an anti-paraquat monoclonal antibody with a paraquat analogue covalently attached to a surface.[22] For polyclonal species similar to those investigated here, rate constants of 10^3 M^{-1} s^{-1},[4] and of $1.5\ 10^5$ M^{-1} s^{-1},[30] have been reported, pointing presumably to an uncorrected diffusion control of the binding rate in the first case.

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