THE EQUILIBRIUM KINETICS OF THE ENZYMATIC LABELED ANTI-8OHdG ANTIBODY-IMMUNOSORBENT SYSTEM IN THE PRESENCE OF FREE ANTIGEN

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Abstract. The affinity constants, the rate constants and the optimal time to achieve a chemical equilibrium represent the essential immunogenic properties of the antibody-antigen systems required to define optimal conditions for ELISA (Enzyme Linked ImmunoSorbent Assay) analyses. Our paper evaluate the affinity constant of the 8OHdG (8-hydroxy-2-deoxyguanosine) antigen, covalently bounded on the glass tube surface, for alkaline phosphatase labeled anti-8OHdG antibody conjugate in the presence or absence of free 8OHdG antigen.

Key words: 8OHdG, anti-8OHdG antibody, ELISA.

INTRODUCTION

In modern immunobiotechnology, there is an increasing demand for efficient immunoassay analytical tools in the field of clinical analysis and biochemical studies linking bioanalytical techniques with microelectronics technology. In particular, there are immuno- or affinity-sensing techniques, which involve biospecific interactions such as antigen-antibody, ligand-receptor and protein-protein recognition reactions. The *in vitro* interaction between antigen and antibody is widely used for diagnostic purposes, for the detection and identification of either antigen or antibody by ELISA techniques. Additionally, development of gravimetric immunosensors (quartz crystal microbalance) also involves coupling an immune component on the surface of quartz crystal by physical adsorption or biocatalytical precipitation [1, 5, 10, 14].

The antigen-antibody interaction is characterized by relatively weak binding forces. Mainly, these consist of Van der Waals, electrostatic and hydrophobic forces and also imply a perfect match between an epitope (antigenic valence) and

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the antibody. The intrinsic association constant that characterizes the antibody binding with an epitope or a hapten is termed *affinity*.

As a DNA adduct, 8-hydroxy-2deoxyguanosine (8OHdG) is an important molecular marker of DNA degradation processes [7]. For this reason 8OHdG levels in human samples, such as leukocytes and urine, is considered as a marker of oxidative DNA damage and is used to evaluate the oxidative stress of individuals [6].

In most studies the 8OHdG levels have been measured by expensive and laborious HPLC-EC (high pressure liquid chromatography coupled with electrochemical detection) procedures [9]. In the last decade, there are some attempts to develop a cheaper and more popular ELISA immunoassay for 8OHdG [12, 13].

The main step in developing an immunoassay (either ELISA or gravimetric) technique concerns the binding of antibody on a solid surface (the immunosorbent phase) [2]. In our study we investigated the affinity of 8OHdG covalently bound to glass tube surface for anti-8OHdG antibody.

MATERIALS AND METHODS

Anti-8OHdG antiserum and alkaline phosphatase were achieved from Sigma Chemical Company. Conjugation of anti-8OHdG antibody to alkaline phosphatase was performed as described in [5]. Coated glass tubes with 8OHdG were made in two steps. First, the glass tubes surface is activated by incubation with a 5% α-aminopropyltriethoxysilane solution for 8 hours at 80 °C [14]. Then, the activated tubes were incubated with 2.5% glutaraldehyde in 0.1 M NaH_2PO_4 (pH = 7) for 1 hour at room temperature [11]. Coating with 8OHdG (100 ng/ml in distilled water) was done at 37 °C for 6 hours.

Into the glass tubes coated with 8OHdG was introduced 1 ml 8OHdG solution (0 – 100 ng/ml) in 0.05 M phosphatase buffer pH = 7.4 and 100 µl alkaline phosphatase-anti-8OHdG antibody conjugate in 0.05 M phosphatase buffer pH = 7.4. Tubes were incubated for 2 hours at the room temperature followed by storage for 24 hours at 4°C. They were washed with distilled water followed by addition of 0.5 ml 5 mM p-nitrophenyl phosphate, a substrate for alkaline phosphatase in 0.02 M Na_2CO_3 and 0.25 mM $MgCl_2$ buffer (pH = 9.8). The reaction was stopped by the addition of 50 µl 1M NaOH and the absorbance at 400 nm (the characteristic wavelength of p-nitrophenyl phosphate) was measured using a Carry 100 Bio UV-VIS spectrophotometer.

In order to calculate the affinity constant of 8OHdG antibody, the following algorithm describing the equilibrium kinetics of the antigen (Ag)-antibody (Ab) system was developed.

The systems formed by antigen 8OHdG coupled on a solid phase as immunosorbent or free in solution and the alkaline phosphatase anti-8OHdG antibody conjugate are typical examples in which bimolecular reversible reactions are produced:

$$Ag_b + Ab^* \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} Ag_b\text{-}Ab^* \tag{1}$$

$$Ag_f + Ab^* \underset{K'_{-1}}{\overset{K'_{+1}}{\rightleftharpoons}} Ag_f\text{-}Ab^* \tag{2}$$

where:

Ag_b is the 8OHdG antigen covalent bound on solid phase (immunosorbent)

Ag_f is the 8OHdG antigen free in solution;

Ab* represents anti-8OHdG antibody-alkaline phosphatase conjugate;

Ag_b-Ab* and Ag_f-Ab* are the immune complexes bound and free;

K_{+1}, K_{-1}, K'_{+1}, K'_{-1} are rate constants of the forward and respectively backward chemical reactions.

The following symbols are used:

a – the initial concentration of 8OHdG bound on the solid phase;

b – the initial concentration of 8OHdG from solution;

c – the initial concentration of the enzymatic labeled anti-8OHdG antibody;

x – the concentration of the immune complex bound on solid phase;

y – the concentration of the soluble immune complex.

The formation rates for both immune complexes are:

$$v_{f1} = \frac{dx}{dt} = K_{+1}[c-(x+y)][a-x] - K_{-1}x \tag{3}$$

for the immune complex bound on the solid phase and

$$v_{f2} = \frac{dy}{dt} = K'_{+1}[c-(x+y)][b-x] - K'_{-1}y \tag{4}$$

For the soluble immune complex.

After reaching the chemical equilibrium (theoretically, after infinite time) the rates become zero and consequently will result:

$$K_e = \frac{K_{+1}}{K_{-1}} = \frac{[x_e]}{[c-(x_e+y_e)][a-x_e]} \tag{5}$$

and

$$K'_e = \frac{K'_{+1}}{K'_{-1}} = \frac{[y_e]}{[c-(x_e+y_e)][b-y_e]} \tag{6}$$

where K_e, K'_e, $[x_e]$ and $[y_e]$ represent the equilibrium constants and the concentrations of the immune complex at the chemical equilibrium.

Assuming that affinities of the labeled and nonlabeled antigen against antibody are the same, $K = K_e = K'_e$, results:

$$\frac{[x_e]}{[a-x_e]} = \frac{[y_e]}{[b-y_e]} = K[c-(x_e+y_e)] \tag{7}$$

leading to

$$\frac{x_e}{y_e}=\frac{a}{b} \text{ and } y_e=\frac{b}{a}x_e. \tag{8}$$

Therefore, the final result is:

$$\frac{x_e}{a-x_e}=K\left[c-x_e\left(\frac{a+b}{a}\right)\right] \tag{9}$$

where a and c are constants and b is variable.

Considering the sample "*i*" ("*i*" counting the tube with b_i ng of 8OHdG in solution) the above relation results in:

$$\frac{x_i}{a-x_i}=K\left[c-x_i\left[\frac{a+b_i}{a}\right]\right] \tag{10}$$

By replacing $\frac{x_i}{a}=\Theta$, where $\Theta\in(0,1)$, Θ_i being the covering fraction, after rearrangement, the relation (10) becomes:

$$\frac{\Theta_i}{1-\Theta_i}+K\Theta_i(a+b_i)=Kc \tag{11}$$

$b_i = 0$ results in $\Theta_i=\Theta_{max}$ (the enzymatic marker reacts only with the immunosorbent). The immunosorbent sites will be occupied only by enzymatic antigen and *a* and Θ_{max} can be experimentally determined using enzymatic activities for the chosen immunosorbent.

In our study, the following values are considered: $\Theta_{max} = 0.27$, $a = 4.27\times10^{-9}$ M, $c = 3.45\times10^{-9}$ M (preliminary data not shown).

Considering the proportionality between Θ_i and A_i (A_i is the absorbance read at 400 nm) the ratio $\frac{\Theta_i}{\Theta_{max}}=\frac{A_i}{A_0}=R_i$ results in

$$\Theta_i=0{,}27\frac{A_i}{A_0} \tag{12}$$

Replacing (12) in (11) results in $\Theta_i=0{,}27R_i$, $R_i<1$ and

$$\frac{0{,}27R_i}{1-0{,}27R_i}+0{,}27KR_i(a+b_i)=Kc \tag{13}$$

The relation (13) was used for the experimental determination of the equilibrium constant.

RESULTS AND DISCUSSIONS

The goal of our study was to determine the equilibrium constants for the enzymatic labeled anti-8OHdG antibody-immunosorbent (8OHDG) system in the presence of free (8OHdG) antigen. Similar procedures are already used to

characterize the equilibrium constant of the antigen-antibody systems in solutions [4], but only in rare cases for the antibody-immunosorbent reaction [8]. In the ELISA experiment, 8OHdG is used as an antigen bound on the solid phase surface (glass tube) and as a free antigen. The competition between bound and free antigen against anti-8OHdG antibody labeled with alkaline phosphatase is carried out for 24 hours at 4 ºC in order to reach the chemical equilibrium.

The samples absorbances values corresponding to different 8OHdG concentrations are shown in Table 1 and Fig. 1. The equilibrium constants values were calculated using equation (13) for each value of the 8OHdG concentration in solution and the results are listed in Table 1. At higher concentrations, a lack of linearity is observed (Fig. 1), determining artefactual values for K [4] and for this reason these values are eliminated. The measurements of the affinity constants for the 8OHdG-anti-8OHdG antibody system in the above conditions lead to an equilibrium constant (affinity constant) of $\overline{K} = (1.31 \pm 0.27) \times 10^8$ l/mol.

Table 1

Optical absorbances at 400 nm for the 8OHdG-anti-8OHdG antibody system

Sample	$[b_{8OHdG}]$ (ng/ml)	Absorbance at 400 nm, A_i	$A_i/A_0 \times 100$ (%)	K_i (l/mol)
1	0	0.837	100.0	1.6×10^8
2	1	0.611	73.1	1.3×10^8
3	5	0.319	38.2	1.0×10^8
4	10	0.226	27.0	1.5×10^8
5	50	0.203	24.3	–
6	100	0.207	24.8	–

The application of ELISA procedure by using immunosorbent (8OHdG covalently coupled on glass surface) is a promising development for a fast and precise determination of the affinity constant of antigen-antibody complexes, parameter that characterizes the ELISA test sensitivity. The affinity constant determined in this way differs by the true constant defined for the antibody-antigen

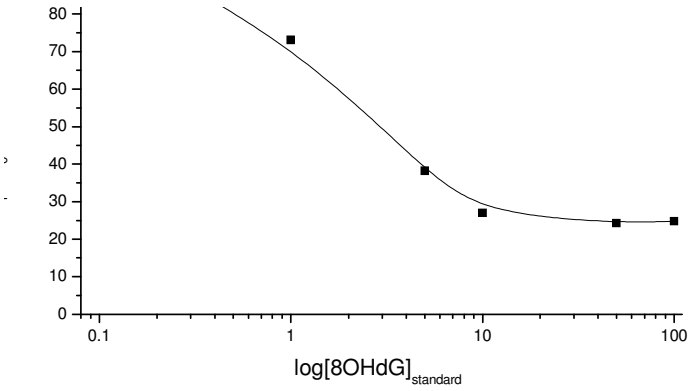

Fig. 1. Relative absorbance vs. 8OHdG concentration in ELISA test.

system in the same phase, for proteic antigens usually in solution [4]. The reason is given by the fact that in ELISA procedure the antigen and antibody are in different phases, immunosorbent and solution, respectively, and the structure of protein can be strongly modified by adsorption on the surface. This results in changing the interaction of the protein with the antibody [3]. In contrast, our analysis method for the antigen 8OHdG, which is a small molecule in comparison with proteins, proved to be a successful technique for characterizing the equilibrium kinetics in biphasic systems.

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