A Two-Site Model for Reaction Kinetics in the Immunoradiometric Assay (IRMA) of Skeletal Alkaline Phosphatase (sALP)

García Gómez, J. and Moreno Frígols, J.L.¹,²
¹Radioisotope Service. Valencia Hospital Clínico Universitario.
²Dpt. of Physical Chemistry. Faculty of Pharmacy. Valencia.

Dos sitios de modelos de cinética de reacción en la prueba inmunoradiométrica (IRMA), de Fosfatasa Alcalina Ósea (sALP)

Dos llocs de models de cinètica de reacció en la prova inmuno-radiomètrica (IRMA), de Fosfatasa Alcalina Òssia (sALP)

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RESUMEN
Se ha elaborado un modelo matemático para el ajuste de la cinética de la reacción de asociación antígeno-anticuerpo con el fin de distinguir los procesos con uno o varios sitios de unión en las reacciones implicadas en la determinación analítica de la Fosfatasa Alcalina Ósea (sALP) por medio del análisis inmunoradiométrico (IRMA). El modelo biexponencial dio el mejor ajuste y la más precisa estimación de los parámetros. El análisis de los datos cinéticos explica satisfactoriamente la influencia de las concentraciones de antígeno y anticuerpo admitiendo que el enlace entre ellos tiene lugar a través de dos tipos diferentes de sitios de unión. Esta conclusión no había podido alcanzarse en base al análisis de los datos de equilibrio.

Palabras clave: Cinética. IRMA. sALP. Sitios de unión. Viscosidad. Fuerza iónica.

Key words: Kinetics. IRMA. sALP. Binding sites. Viscosity. Ionic strength.

RESUM
S’ha elaborat un model matemàtic per a l’ajustament de la cinètica de la reacció d’associació antígen-anticòs per tal de distingir els processos amb un o diversos llocs d’unió en les reaccions implicades en la determinació analítica de la Fosfatasa Alcalina Òssia (sALP) mitjançant l’anàlisi inmunoradiomètric (IRMA). El model biexponencial va donar el millor ajustament i la més precisa estimació dels paràmetres. L’anàlisi de les dades cinètiques explica satisfactoriament la influència de les concentracions d’antígen i anticòs admetent que l’enllaç entre ells té lloc a través de dos tipus diferents de llocs d’unió. Aquesta conclusió no havia pogut aconseguir-se en base a l’anàlisi de les dades d’equilibri.

Mots clau: Cinètica. IRMA. sALP. Llocs d’unió. Viscositat. Força iònica.

A mathematical model was chosen to fit the antigen-antibody association kinetics with a view to distinguishing processes with either one or several binding sites in the reactions involved in the analytical determination of Skeletal Alkaline Phosphatase (sALP) by means of immunoradiometric immunoassay (IRMA). The biexponential model gave the best fit and the most precise estimation of its parameters. The analysis of the kinetic data conducted satisfactorily explains the influence of the antigen and antibody concentrations, by admitting that the antigen-antibody binding takes place through two different binding site types. This conclusion would have not been reached on the basic of equilibrium data analysis.

* Correspondence address:
Dr. José Luis Moreno Frígols,
Dpto. Química Física, Facultad de Farmacia,
Avda. Vicent Andrés Estellés s/n, 46100 Burjassot (Valencia) SPAIN,
Tel.: +34 96 3543289, Fax: +34 96 3544892,
e-mail: Jose.L.Moreno@uv.es
SYMBOLS

P = Antibody bound to the bead; Q = Skeletal alkaline phosphatase (sALP); M = $^{125}$I-labelled anti-skeletal alkaline phosphatase tracer antibody; PQ = Immunocomplex made of the antibody bound to the tube with the sALP; PO, PM, PQ = Sandwich-type radioactive immunocomplex; |P|, |Q|, |M|, |PO|, |PM| = Mol / L concentrations; P0, M0, Q0 = Initial concentrations in arbitrary units; Z = cpm activity measured in each tube after reaction; (Z = Zsp + Z0). A sub-index is added in the tables indicating the experiment number; Z0 = Activity specifically bound to the tube wall, directly proportional to the radioactive immunocomplex concentration; Zs = Value of Z obtained at t = 0, corresponds to unspecific binding; Z∞ = Value of Z obtained at t = infinity; Zs = Value of Zs at equilibrium (Zs = Z − Z0); t = Time, in minutes; v0 = Initial rate k = Rate constant; K = Equilibrium constant, r = Correlation coefficient; SS= addition of residual squares; N= number of points; P= number of parameters.

1. INTRODUCTION

The Tandem-ROstase assay is a solid-phase, two-site immunoanalytical assay. Samples containing sALP are reacted with plastic beads (solid phase) coated with a monoclonal antibody directed towards a site on the sALP molecule and, simultaneously, with a radio-labelled monoclonal antibody directed against a different antigenic site on the same sALP molecule. Following the formation on the solid-phase/sALP/labelled antibody sandwich, the beads are washed to remove any unbound labelled antibody. The radioactivity bound to the solid phase is measured in a gamma counter. The amount of radioactivity measured is directly proportional to the concentration of sALP present in the sample. The Tandem-ROstase Immunoanalytical assay is a suitable in vitro device for the quantitative measurement of skeletal alkaline phosphatase (sALP), an indicator of osteoblastic activity, in human serum. The device is intended to be used as an aid in the management of post-menopausal osteoporosis and Paget’s disease.

Kinetics and equilibrium in antigen-antibody reactions are determining factors in the sensitiveness and accuracy of immunoanalytical techniques (1-3). In previous research, different characteristics have been studied in relation with the antigen-antibody reactions used in analytical techniques that employ radioactivity as a measurable magnitude.

Equilibrium data analysis is used to a great extent in determining the capacity of a substance to bind to one or several receptor populations. Nonetheless, as pointed out by Weber (4), detecting two binding sites through such an assay requires the ligand to have very different affinity for the two binding sites.

In their analysis of a hypothetical two-receptor site model, Tomlinson and Hnatowich (5) argued that apparent competitive inhibition can be produced in different ways depending on the specificity of the sites and the interactions between them. Ideally, the validation of such a model would include: a) an independent study of the binding of the ligand and the inhibitor in each other’s absence, b) saturation and displacement experiments in a ligand and inhibitor concentration range as broad as possible, c) statistical fitting and analysis.

From a kinetic point of view, Giraudo et al (6) characterised the antibody population involved in the binding of testosterone to its anti-serum. To that end, the authors followed up the reaction between tritium-labelled testosterone and a rabbit serum with pH = 7.4, constant ionic strength, a temperature range from 2 to 37°C, and concentrations of a similar order to those used in the radioimmunoassay. The dissociation process was followed up as from the addition of an excess of unlabelled testosterone. The results point to the existence of two binding site types in the antibody.

Drawn from the kinetic data, equilibrium constants were very similar for both binding site types and in line with those obtained through Scatchard representation. Voss and Mummet (7) revised relationships between anti-metatype antibody reactivity and the ligand-induced conformational state of monoclonal antibodies on the fluorosecin hapten as a small molecule model system. One characteristic result of the interaction of anti-metatype antibodies with liganded antibodies is a significant delay in the dissociation rate (k2) of the ligand bound within the primary immune complex. Incorporation of principles inherent in the anti-metatype concept and their application to assay development were summarized.

Our research is aimed at applying a previously described kinetic model (8) to the reaction between sALP and its specific antibodies. Such a model should be able to account for the influence of the concentration of the reagents for both the global reaction and its stages. The ultimate goal is to distinguish between single-site and two-site binding models by analysing kinetic data, as proposed by Motulsky and Mahan (9) and later by Karlsson and Nei (10). These authors noticed that the distinction between single-site binding and two-site binding models was in many cases impossible through equilibrium analysis, while at the same time it was indeed feasible on the basis of kinetic experiments. The latter authors proposed a method which was applied to the study of the binding of tetrade Noscapine (antitussive) to guinea pig brain homogenate which can have a general application for single and double site binding model receptor populations with ligand excess. This would allow for the discrimination between binding models and the study of binding parameters by using kinetic data only.

2. MATERIAL AND METHODS

2.1. Reagents

Anti-Skeletal Alkaline Phosphatase Tracer Antibody: Solution of mouse monoclonal IgG labelled with $^{125}$I in a bovine/mouse/horse protein matrix. Anti-Skeletal Alkaline Phosphatase Coated Beads: Mouse monoclonal IgG coated on plastic beads in a buffer containing 0.1% sodium azide as a preservative.

Skeletal Alkaline Phosphatase standard solutions. All the reagents used were included in the Tandem, Ostase, immunoanalytical assay kit manufactured by Hybritech.

2.2. Instruments

LKB Gammamaster Automatic Gamma Counter. Brookfield DV-II digital viscometer. Brookfield Digital Viscometer DV-II. Viscosity measurements were performed at 60 rpm with a UL ADAPTER at room temperature. Beads washing systems.

2.3. Experimental Procedure

Reaction kinetics were studied by placing the reagents in the plastic tubes and letting them react at different times and at 48 hours, this being considered infinite time. Each bead is washed to remove any unbound labelled antibody. Any radioactivity present in the remaining bound labelled antibody is then measured using a gamma counter.
All experiments were conducted at 5°C, since sALP changes above 9°C. 15 experiments were performed, arranged as follows:

Experiments 1-9
Study of the influence of sALP (Q) and tracer (M) concentrations upon the global reaction. P and 100 μl of Q and 100 μl of M from different concentrations were left to react.

Experiments 10-12
Study of the influence of the concentrations of the previously mentioned factors upon the first process stage, i.e. the binding of Q to the antibody bound to the bead (P). Q-coated beads were incubated at different times; later on and once washed, M was added and left to react for 24 hours.

Experiments 13-15
Study of the influence of the same factors upon the second process stage, namely the binding of M to the PQ immunocomplex. Beads and Q were left to react for 24 hours, and once washed M was added and left to react at different times.

2.4. Data Analysis
The Statistica programme (Copyright© StatSoft, Inc.; 1993) was used with specific non-linear regression equations. As the statistical criterion\textsuperscript{(16, 17)} that allows a choice from different equations, SS and Corrected Akaike’s Information Criterion (AIC\textsubscript{c}) was used, expressed as

\[ AIC_c = N \cdot \ln \left( \frac{SS}{N} \right) + \frac{2P + 2P(P + 1)}{N - P - 1} \]

where N is the number of points, SS the addition of residual squares, and P the number of parameters in the equation. The fitting with the lowest AIC\textsubscript{c} must be chosen. In order to distinguish equations from monoexponential and biexponential models, AIC\textsubscript{c} and ANOVA (F test) were used.

2.5. Determination Of Initial Rate
Z values obtained depending on time were fitted to the equation in all cases:

\[ Z = a + b \cdot t + c \cdot t^2 \]

Since Z was initially assumed to be proportional to the immunocomplex concentration, the following could be written:

\[ v = \frac{dZ}{dt} = \frac{d[PM]}{dt} = b + 2c \cdot t \]

Based on the above, coefficient b could be identified to the process initial rate, provided the immunocomplex activity in cpm is accepted as a concentration measurement in arbitrary units. Conversion into mol·l\textsuperscript{–1} would demand the knowledge of constant \( \alpha \).

3. RESULTS AND DISCUSSION
Influence of M and Q concentration. Global reaction and stages.
This is the global process:

\[ P + Q + M \rightarrow PQM \]

It can be broken down as follows:

\[ k_1 \]

\[ k_2 \]

P + Q ⇄ PQ (quick) and PQ + M ⇄ PQM (slow)

The second stage is slower, as can be seen in Table II; the initial rates of this stage are quite close to those in the global process at equal concentrations (Table I).

The rate equation for this process is:

\[ Z = \frac{a \cdot M_0 \cdot Q_0}{(M_0 + b) \cdot (Q_0 + c)} \cdot (1 - \exp(-d \cdot M_0 - e \cdot Q_0 + f \cdot t)) + g \]  

(Eq. 01)

### TABLE I

<table>
<thead>
<tr>
<th>M₀ (μl)</th>
<th>Q₀ (μl)</th>
<th>t(min)</th>
<th>0</th>
<th>15</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>300</th>
<th>∞</th>
<th>v₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>100</td>
<td></td>
<td>165.6</td>
<td>549.9</td>
<td>1237.0</td>
<td>1565.0</td>
<td>1658.3</td>
<td>2872.4</td>
<td>7697.3</td>
<td>9.90</td>
</tr>
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<td>80</td>
<td>100</td>
<td></td>
<td>376.5</td>
<td>906.5</td>
<td>1423.8</td>
<td>2132.6</td>
<td>2790.4</td>
<td>4120.3</td>
<td>11479.6</td>
<td>14.31</td>
</tr>
<tr>
<td>60</td>
<td>100</td>
<td></td>
<td>409.6</td>
<td>1290.5</td>
<td>1958.8</td>
<td>3075.2</td>
<td>3450.1</td>
<td>6452.4</td>
<td>13334.0</td>
<td>15.50</td>
</tr>
<tr>
<td>40</td>
<td>100</td>
<td></td>
<td>396.9</td>
<td>1690.7</td>
<td>2203.0</td>
<td>3914.0</td>
<td>5085.0</td>
<td>5960.6</td>
<td>14604.2</td>
<td>33.16</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
<td></td>
<td>668.0</td>
<td>1693.0</td>
<td>2366.6</td>
<td>4368.3</td>
<td>5561.4</td>
<td>7673.3</td>
<td>12962.9</td>
<td>31.56</td>
</tr>
<tr>
<td>100</td>
<td>14.9</td>
<td></td>
<td>479.9</td>
<td>748.7</td>
<td>679.6</td>
<td>1339.7</td>
<td>1679.8</td>
<td>1494.0</td>
<td>3329.0</td>
<td>9.57</td>
</tr>
<tr>
<td>100</td>
<td>31.3</td>
<td></td>
<td>706.7</td>
<td>1017.6</td>
<td>1122.3</td>
<td>2055.9</td>
<td>2184.9</td>
<td>2216.9</td>
<td>5032.5</td>
<td>13.09</td>
</tr>
<tr>
<td>100</td>
<td>44.8</td>
<td></td>
<td>856.0</td>
<td>1147.0</td>
<td>1373.5</td>
<td>2246.6</td>
<td>2776.6</td>
<td>3200.6</td>
<td>7132.7</td>
<td>13.73</td>
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<tr>
<td>100</td>
<td>60.3</td>
<td></td>
<td>499.1</td>
<td>1104.5</td>
<td>1515.2</td>
<td>2870.1</td>
<td>3379.0</td>
<td>4285.5</td>
<td>8959.0</td>
<td>21.08</td>
</tr>
</tbody>
</table>
The detailed deduction and validation of this equation can be seen in previous papers\(^7\),\(^8\).
Which can be written as follows:
\[
Z = \frac{{a_1 \cdot M_0 \cdot Q_0}}{\left( {M_0 + b_1} \right) \left( {Q_0 + c_1} \right)} \cdot \left[ {1 - \exp \left( { - t \cdot d_1 \cdot (M_0 + e_1 \cdot Q_0 + f_1)} \right)} \right] + g_1
\]  
(Mono-exponential equation)
And for two binding sites, we have:
\[
Z = \frac{{a_2 \cdot M_0 \cdot Q_0}}{\left( {M_0 + b_2} \right) \left( {Q_0 + c_2} \right)} \cdot \left[ {1 - \exp \left( { - t \cdot d_2 \cdot (M_0 + e_2 \cdot Q_0 + f_2)} \right)} \right] + \frac{{g_2 \cdot M_0 \cdot Q_0}}{\left( {M_0 + h_2} \right) \left( {Q_0 + j_2} \right)} \cdot \left[ {1 - \exp \left( { - t \cdot k_2 \cdot (M_0 + m_2)} \right)} \right] + m_2
\]  
(Bi-exponential equation)
The results obtained in the global reaction for different M and Q concentrations were studied in experiments 1-9, whose results and correlation equations are shown in Table I. Globally, the values fit with the equation:
\[
Z = \frac{{a_1 \cdot M_0 \cdot Q_0}}{\left( {M_0 + b_1} \right) \left( {Q_0 + c_1} \right)} \cdot \left[ {1 - \exp \left( { - t \cdot d_1 \cdot (M_0 + e_1 \cdot Q_0 + f_1)} \right)} \right] + g_1
\]  
Its parameters, coefficient of correlation (r), sum of squares of residuals (ss) and AICc are:
\[
a_1 = 172618 \hspace{1em} b_1 = 16.98 \hspace{1em} c_1 = 1007 \hspace{1em} d_1 = 0.223 \times 10^{-4} \hspace{1em} e_1 = 0.51 \hspace{1em} f_1 = -37.70 \hspace{1em} g_1 = 758 \hspace{1em} r = 0.994 \hspace{1em} ss = 8.15 \times 10^6 \hspace{1em} AICc = 760.20
\]
Or with:
\[
Z = \frac{{a_2 \cdot M_0 \cdot Q_0}}{\left( {M_0 + b_2} \right) \left( {Q_0 + c_2} \right)} \cdot \left[ {1 - \exp \left( { - t \cdot d_2 \cdot (M_0 + e_2 \cdot Q_0 + f_2)} \right)} \right] + \frac{{g_2 \cdot M_0 \cdot Q_0}}{\left( {M_0 + h_2} \right) \left( {Q_0 + j_2} \right)} \cdot \left[ {1 - \exp \left( { - t \cdot k_2 \cdot (M_0 + m_2)} \right)} \right] + m_2
\]  
(Bi-exponential equation)
The results obtained in the global reaction for different M and Q concentrations were studied in experiments 10-15, whose results are shown in Table II.

Comparison of Mono and Bi-Exponential Models
For the comparison of mono- and bi-exponential models, AICc and ANOVA (F test) were used\(^{15,16}\).
Calculations were completed with the online calculator GraphPad Quickcals\(^5\).
Model 1 corresponds to the monoexponential model, and model 2 corresponds to the biexponential one.
Note that the F test assumes Model 2 to be a simpler case than Model 1.
Model 2 (biexponential) has a lower AICc than Model 1 (monoeXponential) and is more likely to be the correct model for all cases. In the global reaction, Model 2 is 620.2 times more likely to be correct than Model 1.
Since the P value is below the traditional significance level of 5% (p < 0.005), we can conclude that the data fit significantly better to Model 2 (Biexponential) than to Model 1 (Monoexponential).

4. CONCLUSION
The analysis of the kinetic data satisfactorily explains the influence of the antigen and antibody concentrations. The bi-exponential model fits better than the mono-exponential one as far as the obtained results are concerned, this justifying the influence of Q and M concentrations. Therefore, the conclusion could be drawn that experimental results are better explained by admitting that two different processes occur—corresponding to the two-site binding reactions—in the interaction between sALP and its antibody immobilised on a spherical surface.

### TABLE II

Z and v₀ values as a function of time for various concentrations of M and Q (Stages).

<table>
<thead>
<tr>
<th>Stage</th>
<th>t(min)</th>
<th>0</th>
<th>15</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>300</th>
<th>∞</th>
<th>V₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M₀ = 100, Q₀ = 100</td>
<td>902.0</td>
<td>1631.0</td>
<td>1958.8</td>
<td>2455.8</td>
<td>3494.0</td>
<td>3673.6</td>
<td>3983.8</td>
<td>16.24</td>
<td></td>
</tr>
<tr>
<td>M₀ = 40, Q₀ = 100</td>
<td>601.9</td>
<td>783.1</td>
<td>1265.3</td>
<td>2004.0</td>
<td>2549.5</td>
<td>2335.2</td>
<td>2710.6</td>
<td>16.99</td>
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</tr>
<tr>
<td>M₀ = 100, Q₀ = 44.8</td>
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<td>943.3</td>
<td>1272.9</td>
<td>1409.4</td>
<td>1759.4</td>
<td>2038.5</td>
<td>2797.5</td>
<td>5.37</td>
<td></td>
</tr>
<tr>
<td>Stage 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M₀ = 100, Q₀ = 100</td>
<td>735.5</td>
<td>1625.3</td>
<td>1592.8</td>
<td>1831.1</td>
<td>2640.5</td>
<td>4133.4</td>
<td>6094.5</td>
<td>5.69</td>
<td></td>
</tr>
<tr>
<td>M₀ = 40, Q₀ = 100</td>
<td>414.5</td>
<td>790.9</td>
<td>1178.5</td>
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<td>4091.2</td>
<td>9.76</td>
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</tr>
<tr>
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<td>1948.4</td>
<td>2034.5</td>
<td>2637.5</td>
<td>5.70</td>
<td></td>
</tr>
</tbody>
</table>

Stage 2 is the slowest and so limits the process rate.
BIBLIOGRAPHY


