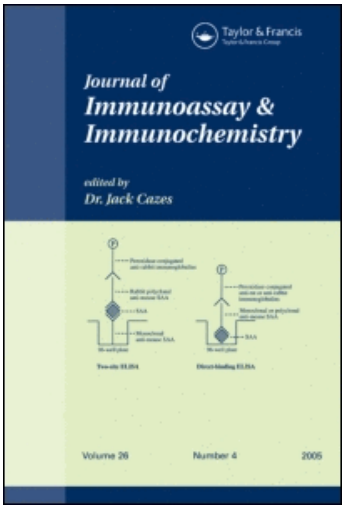

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Deciphering a Conformation-Specific Epitope of hCG-β Through Immunokinetics

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Abstract: Proteins and peptides are comprised of both sequence-specific and conformation-specific epitopes. Sequence-specific epitopes are delineated by a peptide approach and other robust methods like competition assays, gene expression assays, synthetic peptide library based assays, etc. Available methods for deciphering conformation-specific epitopes are cumbersome (X-ray crystallography, etc.), time-consuming, and require expensive equipment. Therefore, it is indispensable to develop a simple method for identification and mapping of conformation-specific epitopes. In the present investigation, the radiolabeled human chorionic gonadotropin-β (^{125}IhCGβ) was employed as a probe and nitrocellulose (NC) as a solid support to immobilize monoclonal antibody (MAb) $G_1G_{10}.1$. The NC-$G_1G_{10}.1$-^{125}IhCGβ complex (NC_{com}) was prepared and the dissociation of radiolabeled hCGβ was carried out in the presence of excess unlabeled ligate. From the experimental dissociation data under varying ionic strength, dissociation constants (k_{-1}), association constants (k_{+1}), and affinity constants (k_a) were calculated. The values obtained were utilized in exploring the amino acid residues constituting an epitopic region of hCGβ involved in interaction with the complementary paratope on MAb $G_1G_{10}.1$. Kinetic data of the present study supported our recently published findings [using single step-solid phase radioimmunoassay (SS-SPRIA)] that the core region of a conformation-specific epitope of hCGβ

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consists of Arg (94, 95) and Asp (99) while a Lys (104) and a His (106) are in proximity to the core epitopic region. Therefore, the results of the present investigation suggested that the dissociation kinetics coupled with SS-SPRIA unequivocally assists in deciphering amino acid residues constituting a conformation-specific epitope of hCGβ.

Keywords: Conformation-specific epitope, Dissociation kinetics, hCGβ, Immunokinetics, MAb G_1G_{10}.1, SS-SPRIA

INTRODUCTION

Epitope identification and mapping has relevance in the diagnosis and prognosis of disease, in immunointervention, in the design of drugs and generation of vaccines. The precise identification of conformation-specific epitopes allows mapping the regions of amino acid sequence located in the three-dimensional structure of the protein. Therefore, it is needless to say that the epitope mapping techniques are instrumental in the development of synthetic epitopes to be used in immunotherapeutics, immunodiagnostics, vaccines etc.[1–3]

All available methods of epitope mapping[4–14] are exclusively dependent on the principle of peptide synthesis and suitable for mapping the linear sequence-specific epitope. Their merits and limitations are briefly discussed in our recent report.[15] It is well known that sequence-specific epitopes form a very small component of the total antibody produced in the humoral system and the majority of antibodies are directed against the conformation-specific epitopes.[16–18] The majority of conformation-specific epitopes are deciphered by X-ray crystallography[16,19–22] in its native 3 D conformation but it is time-consuming, cumbersome, requires highly specialized and expensive equipment[7,23] and a large quantity of pure Ag and Ab for crystallization of each MAb-Ag complex. Considering the limitations of the existing methods for conformation-specific epitopes, the present investigation was designed utilizing dissociation kinetics.[24] Using this method, it is possible to find k_{-1}, k_{+1}, and k_a in a single experiment. In analyzing the MAb-Ag interaction, the k_{-1} is considered exclusively, as repeated experiments have shown that both the intra-assay and inter-assay precision of measurement of k_{-1} is high ($\pm 10\%$). In contrast, the k_{+1} and k_a are derived parameters.[24] The precision of measurement of these parameters in the experiments done in our laboratories were $\pm$ 35%. For this reason, interaction has been studied on the basis of experimentally determined k_{-1}. This approach has an advantage that the quantity of a material required is very small (in micrograms) and can be adopted in any laboratory and does not require expensive, sophisticated equipment.

The present study of Ag-MAb interaction has been assumed to follow:

1. A typical Arrhenius reaction mechanism (conventional reaction kinetics);
2. The structure of Ag, MAb and interacting region remained same in the salt concentrations used;
3. Any change in Ag-MAb interaction observed was due to an effect of modified amino acid residues present in the Ag epitopic region and varying salt concentrations;
4. No new regions of interactions were exposed due to the small change in ionic strength;
5. The values of rate constants (k_{+1} and k_{-1}) were determined by the Arrhenius activation energy of association and dissociation reactions, respectively. Using kinetics, we determined the rate constants for the interaction between chemically- or enzymatically-modified hCGβ epitopes with the paratope of an MAb $G_1G_{10}.1$, in buffer solutions containing varying salt concentration. We used these rate constants to assess which amino acids in an epitopic region of hCGβ were likely to be involved in the interaction with a paratope of MAb.

EXPERIMENTAL

Materials

Analytical grade chemicals were purchased from Sigma Chemical Co., St. Louis, MO., USA. The MAb $G_1G_{10}.1$ was developed and characterized in-house.[15] The chemically modified derivatives of hCGβ [viz., Trinitrobenzene sulphonate treated hCGβ (TNBS.hCGβ), Tetranitromethane modified hCGβ (TNM.hCGβ), Carbodiimide modified hCGβ (CDI. hCGβ), Acetylated hCGβ (Ac.hCGβ), Phenyl glyoxal treated hCGβ (PG.hCGβ), Diethylpyrocarbonate treated hCGβ (DEPC.hCGβ)] and enzymes digested derivatives [viz., Trypsin treated hCGβ (Tryp.hCGβ), Chymotrypsin treated hCGβ (Cht.hCGβ)] were prepared and characterized.[25,26] Carrier free sodium iodide (NaI^{125}) was purchased from Amersham, UK.

Buffers

The RIA buffer (50 mM phosphate buffer), 2.5 mM, 10 mM, 25 mM, and 100 mM phosphate buffers were made. All buffers mentioned contained 0.1% BSA and the pH was adjusted to 7.4.

Methods

Radioiodination of the Hormone

Iodination of hCGβ was carried out by the Iodogen method.[27]

Immobilization of MAb G_1G_{10}.1 on Nitrocellulose Discs

The NC discs (6 mm diameter) were punched out and coated with MAbs. The discs (60 no.) were incubated with G_1G_{10}.1 at an optimum 1:1,000 dilution in RIA buffer, overnight at room temperature (RT) on a rotator. They were subsequently blocked with 2 mL of 1% BSA overnight. After blocking, NC discs were washed with RIA buffer and stored at 4–8°C. The discs retained binding ability to ^{125}IhCGβ for 3–4 months.

Determination of Standard Curve

The MAb adsorbed NC discs (3) were incubated overnight with 100 μL of RIA buffer containing different doses of hCGβ (4 to 500 ng). After addition of 400 μL of RIA buffer, the supernatant was subjected to determine unbound hCGβ by RIA. A standard curve was plotted and the ED_{50} (effective 'or' efficient dose) was determined by choosing a point on the plot, which has shown half value of maximum displacement.

Determination of Ligate Binding Capacity of MAb Adsorbed Nitrocellulose Disc

In order to determine ligate (hCGβ) binding capacity of G_1G_{10}.1 immobilized NC disc, 3 discs were taken in each tube with 100 μL RIA buffer containing different quantities of hCGβ, as shown in Table-1. Following overnight incubation, 400 μL RIA buffer was added to each tube and the resulting supernatant was subjected to determination of unbound hCGβ by RIA. The unbound ligate in each tube was quantitated using a standard profile (as mentioned in the previous section 'Determination of standard curve').

An NC disc saturated with BSA, incubated under the same conditions, served as a control. The difference between experimental and control was used for calculation of the capacity of MAb coated NC disc to bind hCGβ. After determining unbound hCGβ in the supernatant of each tube by RIA, the total amount of bound hCGβ by 3 discs per tube was calculated by deducting unbound hCGβ from the respective dose of ligate (total hCGβ) added per tube. Subsequently, the ligate binding capacity per disc was calculated by dividing the total amount of bound hCGβ by 3.

Table 1. Determination of ligate binding capacities of NC discs immobilized with optimum density of ligand

Description of tubes*	Total amount of hCGβ added in each tube (ng)	Unbound hCGβ in the supernatant detd. by RIA (ng)	Total amount of ligate bound to 3 discs (ng)	Ligate binding capacity per disc (ng)	Average ligate binding capacity per disc (ng)
#1	10	1.9	8.1	2.70	–
#2	20	2.1	17.9	5.96	–
#3	40	3.2	36.8	12.26	–
#4	50	1.3	48.7	16.23	(17.41–0.43) = 16.98 or ~ 17 ng[a]
#5	100	48.3	51.7	17.23	
#6	200	144.9	55.1	18.36	
#7	500	446.6	53.4	17.80	
Control NC disc saturated with BSA	500	498.7 (500)[b]	1.3 (0)[b]	0.43 (0)[b]	0.43 (0)[b]

*Data presented are an average of three experiments, each with duplicates.

[a]Shows average ligate binding capacity per disc (in ng) was determined by using values from experimental tubes # 4 to 7, by deducting amount of ligate bound to each control NC disc saturated with BSA.

[b]Theoretical values are indicated in parentheses.

Preparation of Immobilized Complex

The MAb immobilized disc was taken in a plastic tube (3 mL capacity) and incubated overnight with 300 μL of ^{125}IhCGβ ($\sim 200 \times 10^3$ CPM) in RIA buffer. The disc was washed three times with 1.0 mL RIA buffer to remove all unbound ^{125}IhCGβ. The NC-MAb-^{125}IhCGβ complex (NC_{com}) was counted in a gamma counter to determine an extent of total binding. This NC_{com} was used in all dissociation experiments. The absence of hCGβ in the wash buffer ensured that no dissociation took place during washing.

Dissociation Studies with MAb G_1G_{10}.1 using Intact, Enzyme-treated and Chemically Modified Ligates Under Varying Ionic Strength and Data Analysis

The method used for the study of dissociation was similar as described elsewhere.[24] In brief, NC_{com} was placed in a small column made of glass and counted in an LKB multigamma counter. It was placed in a 5 mL glass test-tube containing 10 μg hCGβ in 1.0 mL RIA buffer, to start the dissociation. The column was taken out (at 5 mins. interval for initial 30 mins., at 10 mins. interval in the next 30 mins. followed by 1 hr interval till 24 hrs.) and the radioactivity of the solution in the tube (arising due to dissociation of ^{125}IhCGβ from NC_{com}) was measured to determine the extent of dissociation. The column was immediately placed back into the same glass tube to continue dissociation. When the column was taken out, a drop of buffer always remained at the bottom of column, allowing dissociation to continue even during the counting period. Similarly, a control dissociation of NC_{com} was performed by placing it in a 5 mL glass test-tube containing 10 μg BSA in 1.0 mL RIA buffer, to start the dissociation. In the case of dissociation experiments with intact ligate (hCGβ), enzyme-treated ligate (Tryp.hCGβ, Cht.hCGβ) and chemically modified ligate (TNBS.hCGβ, TNM.hCGβ, CDI.hCGβ, Ac.hCGβ, PG.hCGβ, and DEPC.hCGβ) all parameters remained same except that a 5 mL glass test-tube contained 10 μg of intact ligate (hCGβ) or enzyme-treated ligate (Tryp.hCGβ, Cht.hCGβ), or chemically modified ligate (TNBS.hCGβ, TNM.hCGβ, CDI.hCGβ, Ac.hCGβ, PG.hCGβ, and DEPC.hCGβ) in 1.0 mL RIA buffer, respectively, to start the dissociation.

In order to study the effect of varying ionic strength on the real-time kinetic constants of NC_{com} interaction with hCGβ, similar dissociation experiments were performed separately using 100 mM, 50 mM, 25 mM, 10 mM, and 2.5 mM phosphate buffers, pH 7.4 at 37°C.

It is important to note that the dissociation of NC_{com} was carried out in the presence of excess of unlabeled ligate, thereby making this reaction of competitive nature. Therefore, the dissociation becomes unidirectional but the reaction characteristics remain 'reversible'.

The mathematical treatment of data and their analysis were similar as reported earlier.[24]

Determination of Real-Time Kinetic Constants of the Interaction

The NC_{com} was subjected to quantitative dissociation in the presence of excess of unlabeled, intact ligate (hCGβ), enzyme-treated ligate (Tryp.hCGβ, Cht.hCGβ), and chemically modified ligate (TNBS.hCGβ, TNM.hCGβ, CDI.hCGβ, Ac.hCGβ, PG.hCGβ, and DEPC.hCGβ). The dissociation profile (Fig. 1) was analysed to obtain both k_{-1} and k_{+1}, as previously described.[24] In brief, k_{-1} was determined by the curve-fitting analysis, and k_a by the formula mentioned in Ref. [24]. The k_{+1} was obtained by equating it to $k_a \times k_{-1}$.

RESULTS

Standard Curve Analysis

Unbound hCGβ in the supernatant was determined by RIA. As the concentration of unbound hCGβ increased (with the increasing concentration of standard dose, 4–500 ng hCGβ), the competition with a constant dose of radiolabeled hCGβ for binding to the $G_1G_{10}.1$, also increased. Consequently, the assayed radioactivity gradually decreased with increasing concentration of unbound hCGβ. The concentration of

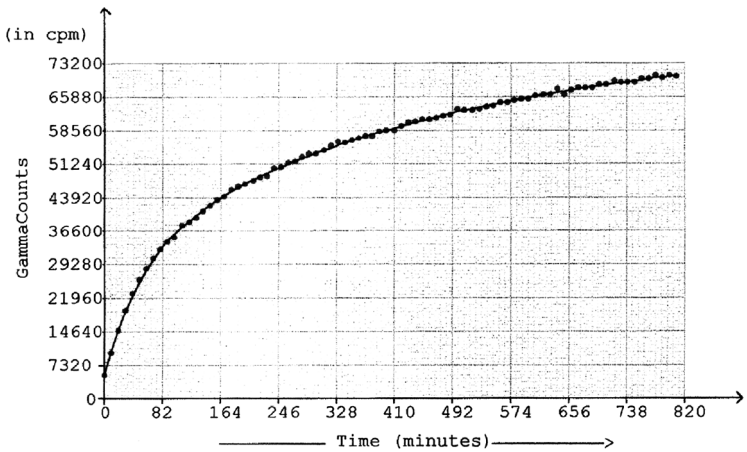

Figure 1. Representative dissociation profile of hCGβ-$G_1G_{10}.1$ complex. The profile shows experimental and theoretical data points run almost parallel indicating the accuracy of theoretically and experimentally obtained values.

hCGβ assayed in the supernatant reflected the standard performance of $G_1G_{10}.1$ coated NC discs (Fig. 2). The $G_1G_{10}.1$ coated NC discs showed displacement over 4 ng to 500 ng of hCGβ with ED_{50} at 28 ng. At 100 ng, maximum displacement occurred.

Ligate Binding Capacity of MAB $G_1G_{10}.1$ Adsorbed NC Disc

Irrespective of the added dose of ligate (hCGβ), the data of ligate binding capacity were found to be at equilibrium among experimental tubes 4 to 7, starting from 16.23, 17.23, 18.36, and 17.8, respectively. Therefore, only these tubes were considered for calculating the average ligate binding capacity per disc. The average hCGβ binding capacity of each NC disc-coated with $G_1G_{10}.1$ was determined to be approximately 17 ng.

Dissociation Studies with MAB $G_1G_{10}.1$ using Intact, Enzyme-Treated and Chemically Modified Ligates Under Varying Ionic Strength

(a) Using 100 and 10 mM Phosphate Buffers, pH 7.4

The kinetic constants of interaction of each ligate (intact, enzyme-treated, and chemically modified) with $G_1G_{10}.1$ was investigated using 100 and

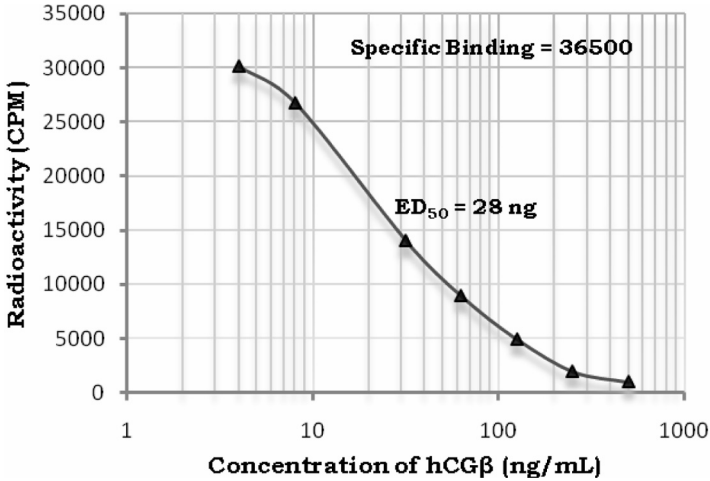

Figure 2. Standard curve analysis of NC discs immobilized with optimum dilution (1:1000) of ligand. The data on X-axis are in log scale and Y-axis in linear scale, plotted by MS Excel. The optimal dose of hCGβ (ED_{50}) showing half of the maximal displacement was found to be 28 ng. The specific binding was determined to be 36,500.

10 mM phosphate buffer, pH 7.4. Data presented in Tables 2 and 3 are the results of epitope-paratope interaction in 100 and 10 mM buffer, respectively. In both buffers, the k_+ and k_a were observed to be maximum (27.84 and 4.10 in 100 mM; 14.45 and 1.52 in 10 mM, respectively) with intact hCGβ-G_1G_{10}.1 (control), while phenyl glyoxal treated hCGβ-G_1G_{10}.1 exhibited nil k_{+1}, k_{-1} and k_a. Tables 2 and 3 also show percent decline in antibody binding ability, k_{+1} and k_a following modification of antigen. In both buffers, the PG.hCGβ-G_1G_{10}.1 has exhibited maximum loss (100%) of antibody binding ability, k_{+1} and k_a. The binding ability (7.14%), k_{+1} (18.35% in 100 mM and 14.88% in 10 mM) and k_a (23.17% in 100 mM and 19.63% in 10 mM) were least affected with chymotrypsin treated hCGβ-G_1G_{10}.1 system. From both tables, it is obvious that, as the percent binding abilities of modified derivatives of hCGβ with G_1G_{10}.1 has decreased, the values of k_a and k_{+1} also dropped.

(b) Effects of Various Ionic Strength on Kinetic Constants of Interaction

An effect of various ionic strengths on the kinetic constants of hCGβ-G_1G_{10}.1 interaction was studied in order to explore the nature of interactions (electrostatic or hydrophobic) occurring between ligand and ligate. Table 4 presents real-time kinetic constants of hCGβ-G_1G_{10}.1 interaction at differing ionic strengths, at pH 7.4. It was interesting to note that k_{+1}, k_{-1}, and k_a have exhibited gradually increased values with the increasing ionic strength. Their graphical presentation is shown in Fig. 3.

DISCUSSION

In an attempt to decipher a conformation-specific epitope of hCGβ, we employed MAb G_1G_{10}.1 as ligand and hCGβ as a ligate. It is well established that the Ag-Ab reaction principally involves electrostatic and hydrophobic interactions.[28] In the present study, the kinetic constants (reaction rates) of an intact hCGβ-G_1G_{10}.1 system have increased concomitantly with an increase of ionic strength. This indicated that a hydrophobic bonding is the principal bonding force between an hCGβ epitope and G_1G_{10}.1 paratope, as a hydrophobic bond is promoted by salt concentration.

In the present investigation, the dissociation experiments involving chemically- and enzymatically-modified derivatives of hCGβ-G_1G_{10}.1 complex under varying ionic strengths (100 and 10 mM phosphate buffer) showed that the affinity constants (k_a) and association constants (k_{+1}) have decreased consistently with the percent rise in binding ability loss.

Table 2. Real-time kinetic constants of hormone-MAb interaction in 100 mM phosphate buffer, pH 7.4 at 37°C

Ligate-ligand Complex	$k_{+1}(\times 10^{-6})$ $[M^{-1}min^{-1}]$	k_{-1} $[min^{-1}]$	k_a $(\times 10^{-8})$ $[M^{-1}]$	Percent decline in Ab binding ability as detd. by SS-SPRIA [Ref. # 15]	[1]Percent decline in k_{+1} $(\times 10^{-6})$	[2]Percent decline in k_a $(\times 10^{-8})$
hCGβ-G_1G_{10}.1 (Control)	27.84 ± 0.63	0.068 ± 0.002	4.10 ± 0.23	No loss	No loss	No loss
Tryp.hCGβ-G_1G_{10}.1	1.48 ± 0.27	0.075 ± 0.0005	0.20 ± 0.03	90.4	94.68	95.20
Cht.hCGβ-G_1G_{10}.1	22.74 ± 0.11	0.072 ± 0.0001	3.15 ± 0.10	7.14	18.35	23.17
TNBS.hCGβ-G_1G_{10}.1	5.86 ± 0.07	0.076 ± 0.0005	0.77 ± 0.15	76.12	78.96	81.20
TNM.hCGβ-G_1G_{10}.1	19.30 ± 0.33	0.068 ± 0	2.86 ± 0.47	10.71	30.58	30.37
CDI.hCGβ-G_1G_{10}.1	1.11 ± 0.01	0.072 ± 0.0003	0.15 ± 0.01	93.77	96.05	96.26
Ac.hCGβ-G_1G_{10}.1	14.87 ± 0.31	0.067 ± 0.002	2.22 ± 0.20	38.84	46.51	45.85
PG.hCGβ-G_1G_{10}.1	–	–	–	100 (Complete inactivation)	100 (Complete inactivation)	100 (Complete inactivation)
DEPC.hCGβ-G_1G_{10}.1	8.78 ± 0.04	0.091 ± 0.0002	0.96 ± 0.05	74.85	68.42	76.54

hCGβ, Human chorionic gonadotropin β-subunit; Tryp.hCGβ, Trypsin treated hCGβ; Cht.hCGβ, Chymotrypsin treated hCGβ; TNBS.hCGβ, Trinitrobenzene sulphonate treated hCGβ; TNM.hCGβ, Tetranitromethane modified hCGβ; CDI.hCGβ, Carbodiimide modified hCGβ; Ac.hCGβ, Acetylated hCGβ; PG.hCGβ, Phenyl glyoxal treated hCGβ; DEPC.hCGβ, Diethylpyrocarbonate treated hCGβ.

[1]Percent decline in $k_{+1} = \{[(k_{+1}$ baseline$) - (k_{+1}$ E−/C-M hCGβ-G_1G_{10}.1 complex$)]/(k_{+1}$ baseline$)\} \times 100$ where, E-/C-M refers to enzymatically- or chemically-modified derivative.

[2]It is arbitrarily taken that if the loss of affinity is $> 90\%$, the modified amino acid is at the core region of epitope, 25–90% proximal to the core region, and $< 25\%$ away from the core region of epitope.

*Data presented are an average of six dissociation experiments using a single NC_{com} in each dissociation reaction.

Table 3. Real-time kinetic constants of hormone-MAb interaction in 10 mM phosphate buffer, pH 7.4 at 37°C

Ligate-ligand Complex	k_{+1} ($\times 10^{-6}$) [M^{-1} min^{-1}]	k_{-1} [min^{-1}]	k_a($\times 10^{-8}$) [M^{-1}]	Percent decline in Ab binding ability as detd. by SS-SPRIA [Ref. #15]	[1]Percent decline in k_{+1} ($\times 10^{-6}$)	[2]Percent decline in k_a ($\times 10^{-8}$)
hCGβ-G_1G_{10}.1 (Control)	14.45 ± 0.45	0.096 ± 0.0001	1.52 ± 0.14	No loss	No loss	No loss
Tryp.hCGβ-G_1G_{10}.1	1.02 ± 0.05	0.089 ± 0.0005	0.12 ± 0.01	90.4	92.94	92.37
Cht.hCGβ-G_1G_{10}.1	12.28 ± 0.17	0.101 ± 0.0004	1.22 ± 0.13	7.14	14.88	19.63
TNBS.hCGβ-G_1G_{10}.1	4.12 ± 0.60	0.094 ± 0.02	0.44 ± 0.04	76.12	71.49	70.99
TNM.hCGβ-G_1G_{10}.1	10.70 ± 0.01	0.099 ± 0.0004	1.08 ± 0.13	10.71	25.95	28.95
CDI.hCGβ-G_1G_{10}.1	0.50 ± 0.38	0.069 ± 0.005	0.07 ± 0.11	93.77	96.56	95.24
Ac.hCGβ-G_1G_{10}.1	7.17 ± 0.06	0.1 ± 0.0007	0.72 ± 0.06	38.84	50.31	52.58
PG.hCGβ-G_1G_{10}.1	–	–	–	100 (Complete inactivation)	100 (Complete inactivation)	100 (Complete inactivation)
DEPC.hCGβ-G_1G_{10}.1	4.38 ± 0.05	0.088 ± 0.0001	0.50 ± 0.09	74.85	69.69	67.12

hCGβ, Human chorionic gonadotropin β-subunit; Tryp.hCGβ, Trypsin treated hCGβ; Cht.hCGβ, Chymotrypsin treated hCGβ; TNBS.hCGβ, Trinitrobenzene sulphonate treated hCGβ; TNM.hCGβ, Tetranitromethane modified hCGβ; CDI.hCGβ, Carbodiimide modified hCGβ; Ac.hCGβ, Acetylated hCGβ; PG.hCGβ, Phenyl glyoxal treated hCGβ; DEPC.hCGβ, Diethylpyrocarbonate treated hCGβ.

[1]Percent decline in $k_{+1} = \{[(k_{+1}$ baseline)-(k_{+1} E-/C-M hCGβ-G_1G_{10}.1 complex)]/(k_{+1} baseline)$\} \times 100$ where, E-/C-M refers to enzymatically- or chemically-modified derivative.

[2]It is arbitrarily taken that if the loss of affinity is > 90%, the modified amino acid is at the core region of epitope, 25–90% proximal to the core region, and < 25% away from the core region of epitope.

*Data presented are an average of six dissociation experiments using a single NC_{com} in each dissociation reaction.

Table 4. Effect of ionic strength on the real-time kinetic constants of hCGβ-G_1G_{10}.1 interaction in phosphate buffer, pH 7.4 at 37°C

Ionic strength (mM)	2.5 mM	10 mM	25 mM	50 mM	100 mM
$k_{+1}(\times 10^{-6})$ $[M^{-1}min^{-1}]$	4.29 ± 0.09	6.15 ± 0.14	10.83 ± 0.05	15.22 ± 0.47	21.95 ± 0.60
k_{-1} $[min^{-1}]$	0.030 ± 0.01	0.036 ± 0.01	0.038 ± 0.0005	0.044 ± 0.01	0.056 ± 0.02
$k_a(\times 10^{-8})$ $[M^{-1}]$	1.52 ± 0.02	1.71 ± 0.02	2.85 ± 0.01	3.46 ± 0.04	3.92 ± 0.02

*Data presented are an average of six dissociation experiments using a single NC_{com} in each dissociation reaction.

This clearly indicated that the structural integrity of an epitope is very crucial in its interaction with the complementary paratope.

Depending on the degree of k_a loss and the consequent drop in k_{+1} following each modification, the role of individual amino acid residues in the epitope integrity has been deduced. The higher the k_a and k_{+1} loss (in comparison with unmodified hCGβ, control), the greater the role of modified amino acid would be in maintaining the structural and conformational integrity of an epitope, and vice-versa. Based on these presumptions, the present study has shown maximum loss of k_a and k_{+1} on Arg

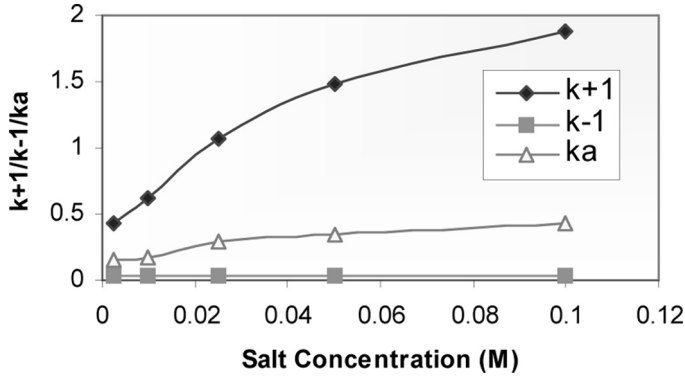

Figure 3. Effect of various ionic strength on the real time kinetic constants of hCGβ-G_1G_{10}.1 interaction using NC_{com}. As the ionic strengths of buffers have become more stringent, the values of k_{+1}, k_{-1} and k_a have increased. The rise in values of $k_{+1}$is most pronounced than k_a and k_{-1} values. The rise in k_{-1} values with increasing ionic strength (refer table 4) is so small that the plot is unable to reflect rising k_{-1} values and appears to run almost parallel to X-axis with increasing salt concentration. The units of k_{-1}, k_{+1} and k_a are min^{-1}, M^{-1} min^{-1} and M^{-1}, respectively.

modification using PG, followed by Asp, Glu modification by CDI; Lys and Arg modification by trypsin; Lys modification by TNBS; His modification by DEPC; Lys modification by acetylation; Tyr modification by TNM; and Phe, Tyr modification by Chymotrypsin. The maximum or approximately maximum loss of k_a and k_{+1} upon Arg and Asp modification in both buffers clearly pointed out that Arg (94th and 95th residues in the hCGβ sequence) and Asp (at 99th position in the hCGβ) were among the amino acid residues which constituted the core region of an hCGβ epitope. The moderate loss of k_a and k_{+1} following Lys and His modification indicated that Lys and His were not involved in the core epitopic region formation. Had they been constituents of the core region, their modification would have certainly led to maximum (or approximately maximum) loss of k_a and k_{+1}. Therefore, Lys (at 104th position) and His (at 106th position) were in proximity to the core epitopic region. The Tyr (at 37th position) became a distant amino acid in epitope integrity, as their modification did not cause appreciable loss in k_a and k_{+1}. Based on the variation in kinetic constants, the conclusion drawn about involvement of amino acids in the epitope integrity of hCGβ was in congruence with our recent report using SS-SPRIA.[15]

The present investigation was exclusively dependent upon the previously published method.[24] However, to the best of our knowledge, the interpretation of the kinetic data in structural terms is reported for the first time. From the present study, it can be concluded that dissociation kinetics seems to be a suitable method of studying ligand-ligate interactions. Moreover, dissociation kinetics coupled with SS-SPRIA[15] could be proved to be a valid analytical method for epitope analysis and/or deciphering conformation-specific epitope of a given antigen.

In a continuing quest of developing an effective immunocontraceptive (birth control or anti-fertility vaccine), several anti-hCG vaccines have entered clinical trials and have shown promising results[29–32] (these vaccines operate by bioneutralizing hCG as they prevent implantation of fertilized egg into the uterus and inhibit estrogen and progesterone synthesis which are vital for sustaining pregnancy). Moreover, reports of the feasibility of using anti-hCG vaccines for immunotherapy of certain cancers are also available.[29,33,34] The drawbacks of currently available vaccines are that they are either relatively weakly immunogenic or that they induce antibodies that cross-react with human luteinizing hormone (hLH).[34] Attempts are being made to improve the immunogenicity of vaccines and to further modify the sequence to eliminate the cross-reactivity by creating mutant epitope.[35,36] Recently, a heterodimer-specific discontinuous epitope, present only in hCG (distinct from the unique C-terminal peptide of hCGβ and absent in hLHβ or hLH), has been identified and seems to be an ideal candidate antigen for immunocontraception.[37] The application of dissociation kinetics in studying an

hCGβ epitope-paratope interaction and the results obtained not only strengthens our previous report[15] of epitope analysis, but also unequivocally helps in identification of a conformation-specific epitope of hCGβ more precisely. The epitope thus identified could pave a way in its native format or in mutated form for developing an effective immunocontraceptive or anti-hCG vaccines for cancer immunotherapy. Moreover, the amino acids identified in maintaining the structural integrity of the core epitopic region fall within the receptor-binding site [comprised of amino acids 94–114, as reported elsewhere[38,39]] of hCG. Therefore, this epitope could be a viable synthetic substitute for obtaining receptor blocking antisera *in vitro* or *in vivo* systems.

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