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The effect of residue 1106 on the thioester-mediated covalent binding reaction of human complement protein C4 and the monomeric rat α-macroglobulin α_1I3

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Abstract The histidine at position 1106 of the C4B isotype of human complement is involved in catalyzing the covalent binding of the thioester to glycerol and water. By replacing the histidine with other residues, it was found that tyrosine is also capable of mediating the reaction. We propose that they act as nucleophiles by first attacking the thioester, upon activation, to form acyl intermediates, which subsequently react with the hydroxyl groups of glycerol or water. The monomeric α-macroglobulin, α_1I3 of the rat, was also studied. Unlike α_2-macroglobulin, which is a tetramer, α_1I3 has binding properties similar to those of C4A.

Key words: Complement; α-Macroglobulin; Thioester; Covalent binding

1. Introduction

The covalent binding of the activated forms of the complement proteins C3 and C4 to immune complexes and pathogens is a key step in their elimination [1,2]. This reaction is made possible by the internal thioester in C3 and C4. Activation of these proteins involves the cleavage of a single peptide bond, splitting C3 into C3a and C3b, and C4 into C4a and C4b. It is thought that concomittant conformational changes take place in the major fragments C3b and C4b, resulting in the exposure of the thioester bond, which becomes reactive with nucleophiles. The most abundant nucleophiles in biological systems are the hydroxyl and amino groups found on carbohydrates and proteins, which react with the thioester to form ester and amide bonds. Although this description of the binding reaction is generally correct, it cannot account for the difference between C3 and C4 in their reaction with hydroxyl and amino groups. Whereas C3b reacts predominantly with hydroxyl groups at neutral pH, C4 reacts with both amino and hydroxyl groups (see later) [3]. $\alpha_2$macroglobulin (α_2M), another protein with an internal thioester, reacts only with amino groups with low efficiency [4].

The two isotypes of human C4, C4A and C4B, have been most useful in the investigation of this binding reaction. They differ by only four amino acids within the sequence of six residues from position 1101 to 1106, but have very different binding properties. C4A, with the residues PCPVLD in the 1101–1106 hexapeptide, reacts efficiently with amino groups but poorly with hydroxyl groups, whereas C4B, with the sequence LSPVIH, reacts moderately with both nucleophilic groups [5–8]. Using site-directed mutagenesis at the cDNA level followed by expression of the modified proteins, it was found that the key residue lies in position 1106. The His at this position is crucial in conferring C4B-like binding acitivity whereas an Asp, Asn or Ala at this position renders the protein C4A-like [9,10]. Based on these observations, it was argued that the His of C4B plays a role in catalyzing the reaction of the thioester with hydroxyl groups on water and other compounds; thus, its reaction with amino groups is apparently less effective. C4A, on the other hand, does not have a His at position 1106. The thioester, once exposed upon activation, is hydrolyzed more slowly and so reacts more efficiently with amino groups. This interpretation is supported by the kinetics of the binding reaction with respect to that of activation of C4A and C4B. As predicted, the half-life of the exposed thioester in C4A is substantially longer (~10 s at 22°C) than that of C4B (< 1 s and not measureable in the experiment) [10].

The importance of the His of C4B in catalyzing a reaction with hydroxyl groups may also be applicable in C3 since the binding properties of C3 and C4B are similar [4]. However, it is more difficult to reconcile the differences between C4A and α_2M since their reactivities with glycine differ by 100-fold [4] and replacement of the residues 1101–1106 of C4 with the corresponding ones of α_2M (SGSLLN) yielded a C4A-like molecule [10]. In this paper, we report our investigation on the role of the residue at position 1106 by replacing it with other basic/nucleophilic residues, namely cysteine (C4-H1106C), lysine (C4-H1106K), serine (C4-H1106S), arginine (C4-H1106R) and tyrosine (C4-H1106Y). In addition, we report our finding on α_1-inhibitor-3 (α_1I3), a monomeric α-macroglobulin of the rat, which, when activated with a non-binding protease, has binding properties similar to those of C4A.

2. Materials and methods

C4 variants, in pBluescript (KS-), were generated from a C4 cDNA

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Abbreviations: α_1I3, α_1-inhibitor 3; α_2M, $\alpha_2$macroglobulin; C1s, activated complement subcomponent of C1; EA, sheep erythrocytes coated with sensitizing antibody; EAC1, EA with activated C1 on the cell surface; HNE, human neutrophil elastase.

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clone coding for the B2 allotype as described [10]. The oligonucleotides used for introducing the various residues in position 1106 were:

G9C for cysteine (CCCTGCATGCTCCTACATATCACTGGAG);
G10K for lysine (CCCTGCATGCTCCTCTTTATCACTGGAG);
G11R for arginine (CCCTGCATGCTCCTACGATTCACTGGAG);
G12S for serine (CCCTGCATGCTCCTACTTATCACTGGAG) and
G13Y for tyrosine (CCCTGCATGCTCCTATATATCACTGGAG).

After sequence confirmation, the cDNA inserts were transferred to the pEE6.HCMV.GS plasmid (a gift from Celltech Ltd) and transfected into CHO-K1 cells by calcium phosphate precipitation using the reagents in the Mammalian Transfection kit (Strategene). Clones expressing the C4 variants were screened for C4 hemolytic acitivity (for C4-H1106R, C4-H1106S and C4-H1106Y) or C4 antigenic activity (for C4-H1106C and C4-H1106K). Postive clones were grown up and C4 was purified from the culture supernatant by ion exchange chromatography on Q-Sepharose followed by affinity chromatography on a LOO3 (monoclonal antibody against human C4) column. C4 was further purified by ion exchange on a mono-Q column using the same buffer and elution conditions as for the Q-Sepharose [10].

C4 hemolytic activity was measured using sensitized sheep erythrocytes (EA) and C4 deficient guinea pig serum [11]. C4b bound to sheep erythrocytes bearing activated C1 (EAC1) was detected with ^{125}I-labeled $F(ab')_2$ fragments of rabbit anti-human C4 [6]. [^{3}H]glycine and [^{3}H]glycerol (Amersham) binding to C4 [12], and the pH dependence of binding [3,6], were performed as described previously. Rat α_1I3 was prepared according to [13] and human neutrophil elastase (HNE) was a gift from J. Travis. Rat α_1I3 and HNE were incubated at 37°C in a molar ratio of 60 : 1 for 30 min in the presence of [^{3}H]glycine or [^{3}H]glycerol. The amount of glycine or glycerol covalently bound to the protein was determined by the same method used for C4.

C4 antigenic activity was determined by the inhibition of the binding of a rabbit anti-C4 antiserum to microtiter wells coated with human C4. 50 μl of C4 containing samples were incubated with an equal volume of rabbit anti-C4 antiserum (1:160,000 dilution in PBS containing 2% w/v bovine serum albumin) at 37°C for 60 min. The mixtures were then transferred to microtiter wells each precoated with 100 ng of human C4 and incubated for 45 min. After washing, 100 μl of sheep anti-rabbit IgG-alkaline phsophatase conjugate (1:1000 dilution; Sigma) was added and incubated for 60 min at 37°C. The wells were washed and 100 μl of p-nitrophenyl phosphate (1 mg/ml) was added. Color intensity at 405 nm was determined after 20–30 min at room temperature. The amount of C4 in the sample was determined by comparison to a curve obtained from controls with known amounts of C4.

3. Results

Stable CHO cell lines secreting C4-H1106C, C4-H1106K, C4-H1106R, C4-H1106S and C4-H1106Y were obtained. High levels of C4 antigenic activities were detected in the culture supernatant of the C4-H1106C cell lines but no C4 specific hemolytic activity was detected. C4 purified from the supernatant, by following antigenic activity, did not give the characteristic three-chain structure of C4 in SDS-PAGE analysis under reducing conditions (data not shown). It was concluded that the replacement of the His with a Cys at position 1106 did not yield a stable protein.

Hemolytically active C4 was purified from the cell lines expressing the other four C4 variants. Since the C4 proteins from the different cell lines were expected to have different binding and therefore different hemolytic activites, the amount of active C4 in each preparation was determined by [^{3}H]methylamine incorporation. Other activities were normalized to the amount of active C4 in the preparations.

The specific hemolytic activities of the four C4 variants, together with plasma C4A and C4B, are shown in Fig. 1. As expected, C4B showed ~3 times the specific hemolytic activity of C4A. The four C4 variants have lower hemolytic activities with C4-H1106K only ~1% that of C4B. The C4 proteins, at several concentrations, were incubated with EAC1. The amount of C4b bound to the erythrocytes was determined by the subsequent binding of ^{125}I-labeled anti-C4 $F(ab')_2$. Thus, the relative binding activities of the C4 variants to EAC1 correlates with their specific hemolytic activities.

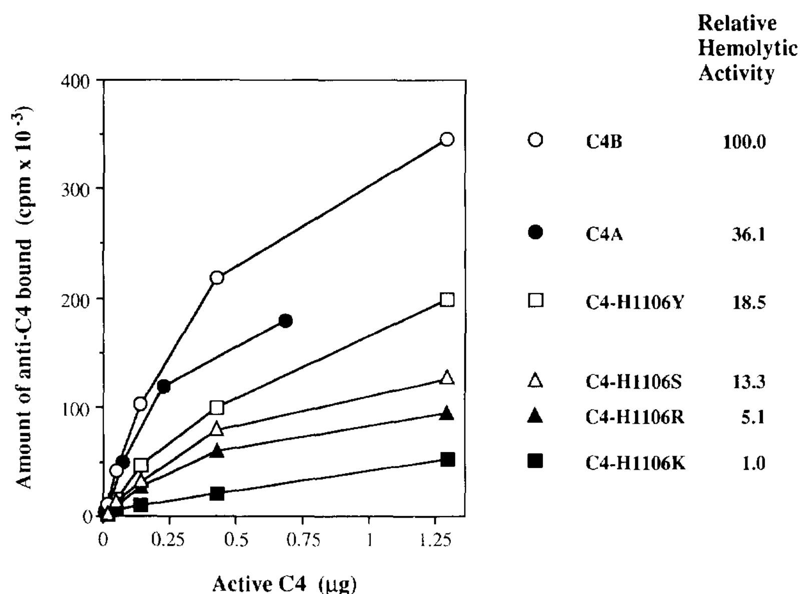

Fig. 1. The binding of C4 to EAC1 cells. Different amounts of C4A, C4B and their variants were incubated with sheep erythrocytes coated with antibody and C1. Activation of C4 results in the covalent binding of the activated C4b fragment on the cell surface. The amount of C4b bound was determined by ^{125}I-labeled $F(ab')_2$ fragment of rabbit anti-human C4. The hemolytic activities of the C4 variants are also shown relative to that of C4B, taking the value for C4B as 100.

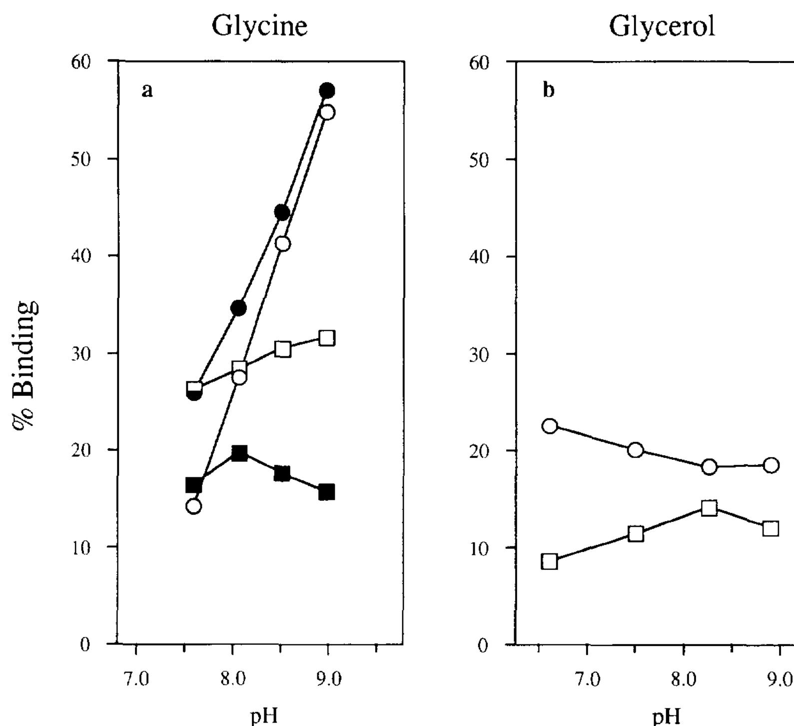

Fig. 2. Binding of [^{3}H]glycine (a) and [^{3}H]glycerol (b) to C4A, C4B and the two variants C4-H1106Y and C4-H1106K as a function of pH. The concentration of glycine used for C4A (●) was 12.5 μM, C4B (○) 2.5 mM, C4-H1106Y (□) 0.5 mM and C4-H1106K (■) 5 mM to give medium range binding values. The concentration of glycerol used was 15 mM for both C4B and C4-H1106Y.

We also examine the effect of the residue at position 1106 on the C4 binding to amino and hydroxyl groups using glycine and glycerol as representative compounds. C4 was incubated with [^{3}H]glycine or [^{3}H]glycerol in the presence of activated C1s, which splits C4 to yield C4a and C4b. The reactions were allowed to proceed to completion (15 min). Binding efficiency (BE) is defined as the fraction of C4b bound with either glycine or glycerol. Thus, BE = $k_2[G]/(k_0 + k_2[G])$, where k_2 is the second-order rate constant of binding to glycine or glycerol, [G] the concentratrion of the small molecules in the experiments and k_0 the first-order hydrolysis rate of the thioester. The k_2/k_0 values for the binding of the thioester proteins to glycine and glycerol, calculated as BE/[G](1 − BE), are shown in Table 1. The concentration of glycerol was 10 mM for all C4 variants. However, different amounts of glycine were used since the reactivity of the C4 variants to glycine span a large range. All four variants showed some level of binding to glycine but only C4-H1106Y showed significant binding to glycerol.

Table 1
Glycine and glycerol binding activity of C4, its variants and other thioester proteins

	Residues 1101–1106	k_2/k_0 (M^{-1})	
		Glycine[1]	Glycerol[2]
C4A	PCPVLD	16,300	0.6
C4B	LSPVIH	75	16.6
C4-H1106K	LSPVIK	65	0.3
C4-H1106R	LSPVIR	1,500	0.1
C4-H1106S	LSPVIS	5,500	0.7
C4-H1106Y	LSPVIY	850	6.5
C4-(α_2M)[3]	SGSLLN	15,300	1.0
α_2M[4]	SGSLLN	206	1.2
Rat α_1I3	SGSLLN	5,500	1.5

[1] The experiments were carried out at different concentrations of glycine to give non-saturating binding. The glycine concentrations were 0.1 mM for C4A, C4-H1106S, C4-(α_2M) and rat α_1I3, 1 mM for C4-H1106R and C4-H1106Y, and 2.5 mM for C4B, C4-H1106K and α_2M.
[2] All experiments were carried out at a glycerol concentration of 10 mM.
[3] C4 with the residues 1101–1106 substituted with those of α_2M, data from ref. [10].
[4] Human α_2M, data from [4]. Values are expressed as per mol of monomeric subunit of the α_2M tetramer.

The effect of pH on the binding activities of the C4 variants was also investigated (Fig. 2a). The binding of C4-H1106K and C4-H1106Y to glycine are relatively insensitive to pH. C4A, C4B, as well as C4-H1106R and C4-H1106S (not shown), on the other hand, bind more effectively at higher pH. Glycerol binding activities of C4B and C4-H1106Y were not significantly affected by pH (Fig. 2b).

The α_1I3 of the rat, a monomeric member of the α-macroglobulin family, was also studied for its binding activity to glycine and glycerol. Human neutrophil elastase (HNE) does not have any lysine residues and does not bind covalently to α_1I3 and, presumably, for this reason, its protease activity is not inhibited by α_1I3 [13]. It was therefore chosen to activate α_1I3 in these experiments. The binding results are included in Table 1. Unlike human α_2M, which is a tetramer, α_1I3 showed substantial binding to glycine. No significant binding to glycerol can be detected.

4. Discussion

We have constructed five C4 variants with the His at position 1106 substituted with Cys, Lys, Ser, Arg and Tyr. The background C4 sequence is that of C4B of the B2 allotype.

Although large quantities of C4 could be detected antigenically from the culture supernatant of C4-H1106C transfected cells, no C4 hemolytic acitivity could be detected. The introduction of a Cys residue at position 1106 is apparently sufficient to prevent proper synthesis of the protein, possibly by disrupting the normal course of disulphide bond formation. However, it should be noted that C4A has a cysteine at position 1102 and this does not affect the structure, and function, of the protein.

The other four constructs yielded active C4 proteins. The level of active C4 was quantitated by the covalent incorporation of [^{3}H]methylamine to the thioester site. Determinations of other properties of C4 are normalized to methylamine incorporation. All four variants have lower specific hemolytic activities when compared with C4B and C4A. The difference in specific hemolytic activity can be correlated with the ability of the proteins to bind EAC1 cells, which can in turn be correlated with the binding activities to glycine and glycerol. Since sheep erythrocytes have large amounts of carbohydrate on the cell surface in the form of glycoproteins and glycolipids, the binding of the C4 proteins to cells is primarily reflective of their capacity to react with hydroxyl groups.

We have previously shown that the difference in the binding activities between C4A and C4B is due to the His at position 1106 of C4B. We conjectured that the His plays a part in catalyzing the reaction of water and other hydroxyl groups with the internal thioester upon activation. In the absence of the His, hydrolysis of the thioester is not catalysed, thus, it is susceptible to the nucleophilic attack by the amino groups, such as that on a glycine molecule [10]. The mechanistic role of the His was not defined.

The substitution of H1106 with other basic/nucleophilic residues resulted in C4 variants with very different binding properties. All four variants show intermediate glycine binding activity with respect to C4A and C4B. In addition, C4-H1106Y also shows a significant level of glycerol binding. Thus, in any catalytic mechanism proposed for the reaction of hydroxyl groups with the 'exposed' thioester, His and Tyr, at position 1106, have to assume equivalent functional roles in the mechanism. Since the pK_a of the imidazole group of His is ~6.0 and that of the phenolic group of Tyr is ~10.0, it is difficult to propose a model involving these residues either both as acids or both as bases at neutral pH.

An alternative mechanism is that either group can act as a nucleophile, which necessarily predicts an intermediate. This hypothesis is possible although the phenolic group of Tyr would be less effective and may account for the difference in glycerol binding between C4B and C4-H1106Y. Furthermore, we can extend our hypothesis to explain the binding activity of C4-H1106K. The ε-amino group of Lys can attack the thioester to form a stable acyl-amide bond. Thus, C4-H1106K has very low specific hemolytic activity and glycine binding can only be detected at relatively high concentration (>2.5 mM) (Fig. 1, Table 1). Both the guanidinium group of Arg and the hydroxyl group of Ser are poor nucleophile at neutral pH and they do not promote the binding of C4-H1106R and C4-H1106S to hydroxyl groups. Indeed, this hypothesis finds support in the pH dependence of glycine binding to the C4 variants. Since the pK_a of the ε-amino group of Lys and the phenolyic group of Tyr are ~10.5 and ~10.0, respectively, they would be effective competitors with the α-amino group of glycine (pK_a ~9.5) over the pH range of 7.5–9.0. Thus, a relatively similar level of glycine binding to C4-H1106K and C4–1106Y is observed across this pH range. However, we are still left with the problem of how the reaction proceeds from the acyl intermediates. In particular, a base may still be required to catalyze the reaction between the acyl intermediates and oxygen nucleophiles. Furthermore, it seems unnecessarily complex to have one activated acyl group (the thioester) replaced by another (acyl-imidazole or acyl-phenol) in the reaction.

C4A and human α_2M do not have a His at position 1106 and they do not bind glycerol. However, their binding to glycine differs significantly (Table 1). In addition, when the residues 1101 to 1106 of C4 was replaced by those of α_2M, the resultant molecule has binding properties like C4A but not α_2M [10]. Unlike C4, which does not bind C1s during activation, α_2M is known to bind the activating proteases [13]. Thus, potiential competition between the activating proteases and ligand for covalent binding poses difficulties in the interpretation of binding data of α_2M. Further difficulties also arise from the fact that human α_2M is a tetramer [14,15] and negative cooperativity between the subunits has been demonstrated for their reaction with the activating proteases [15–17]. The rat α_1I3 is a monomeric α-macroglobulin and has the identical sequence of SGSLLN as α_2M in the region of interest. It is also not an inhibitor of HNE, nor does it bind the enzyme covalently. Thus, a system using α_1I3 and low levels of HNE affords a better comparison to C4 binding by eliminiating several confounding factors. Indeed, α_1I3 shows similar binding properties to C4A and C4-(α_2M) (Table 1).

In this paper, we have shown that the covalent binding reaction of monomeric α-macroglobulins are C4A-like. In addition, we have obtained results suggesting that the His residue at position 1106 of C4B may act as a nucleophile and that the reaction may proceed by first forming an acyl-imidazole intermediate. We are currently engaged in experiments to test whether this hypothesis is correct, by showing that such an intermediate exists and by identifying the base necessary to catalyze the subsequent reaction of the intermediate with substrate molecules.

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