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Substitution of a single amino acid (aspartic acid for histidine) converts the functional activity of human complement C4B to C4A

(thioester/immune complexes/C4 polymorphism)

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ABSTRACT **The C4B isotype of the fourth component of human complement (C4) displays 3- to 4-fold greater hemolytic activity than does its other isotype C4A. This correlates with differences in their covalent binding efficiencies to erythrocytes coated with antibody and complement C1. C4A binds to a greater extent when C1 is on IgG immune aggregates. The differences in covalent binding properties correlate only with amino acid changes between residues 1101 and 1106 (pro-C4 numbering)—namely, Pro-1101, Cys-1102, Leu-1105, and Asp-1106 in C4A and Leu-1101, Ser-1102, Ile-1105, and His-1106 in C4B, which are located in the C4d region of the α chain. To more precisely identify the residues that are important for the functional differences, C4A–C4B hybrid proteins were constructed by using recombinant DNA techniques. Comparison of these by hemolytic assay and binding to IgG aggregates showed that the single substitution of aspartic acid for histidine at position 1106 largely accounted for the change in functional activity and nature of the chemical bond formed (ester vs. amide). Surprisingly, substitution of a neutral residue, alanine, for histidine at position 1106 resulted in an increase in binding to immune aggregates without subsequent reduction in the hemolytic activity. This result strongly suggests that position 1106 is not "catalytic" as previously proposed but interacts sterically/electrostatically with potential acceptor sites and serves to "select" binding sites on potential acceptor molecules.**

An important function of the classical pathway of complement is clearance of immune complexes (1). Activation of the fourth component of human complement (C4) and its covalent attachment to the target substance via a thioester transacylation mechanism (2) both inhibits formation of insoluble complexes (3, 4) and facilitates their uptake via C4b/C3b receptors on blood cells (5, 6). Human C4 is very polymorphic (7) and is the product of two coexpressed loci—i.e., *C4A* and *C4B*—located within the class III region of *HLA* (8). It has long been recognized that the isotypic forms of C4 displayed different hemolytic activities (9, 10). More recent studies (11, 12) have addressed the basis of this functional difference and have shown that the two isotypes of C4 differ dramatically in their efficiency of covalent binding to protein or carbohydrate, despite nearly identical structures (13). C4A preferentially forms amide bonds with protein, while C4B binds more efficiently to hydroxyl groups of carbohydrate and to some extent protein (11, 12). Complete deficiency of C4 in humans, albeit rare, invariably correlates with severe immune complex disease and is often fatal (14), whereas partial deficiency in one of the isotypes (i.e., C4A or C4B) is also associated with an increased susceptibility to immune complex disease (15–18). Whether the C4 deficiency is causative or simply serves as a marker for a linked disease susceptibility gene is not known. Nevertheless, C4 polymorphism in general, and coexpression of the C4A and C4B isotypes in particular, may ensure interaction with a wide range of surfaces, similar to what has been proposed for class I and II major histocompatibility molecules (19).

C4 protein is synthesized as a single polypeptide of 1706 amino acids (13), which is processed (20) into three subunits (α, 95 kDa; β, 75 kDa; and γ, 30 kDa) (21) before secretion. Comparison of the derived amino acid sequence of the C4A and C4B isotypes has shown that 10 of 12 differences were clustered in the C4d domain of the α subunit about 100 residues c-terminal from the covalent binding site (internal thioester) (13, 22). Of these 10, differences only at positions 1101, 1102, 1105, and 1106 (in C4A, Pro-1101, Cys-1102, Leu-1105, and Asp-1106; in C4B, Leu-1101, Ser-1102, Ile-1105, and His-1106) segregated with the functional differences (23). In this study, site-specific mutagenesis has been used to construct hybrid C4B–C4A proteins to gain insight into the chemical basis for the observed functional difference between the isotypes. We found that substitution of a single residue—i.e., aspartic acid for His-1106—converted C4B to C4A with respect to its binding activity, and we propose that the isotypic residues "select" specific sites on the immune complex for covalent binding through steric and electrostatic interactions.

MATERIALS AND METHODS

Construction of Plasmids. Hybrid plasmids were constructed using the gapped plasmid oligonucleotide-directed mutagenesis technique (24). After transfer of the repaired full-coding sequence for C4B (13, 22) into the pBluescript vector, respective hybrids were constructed using the following antisense oligonucleotides (5′–3′) OL1, CACTGGACAGGGGTCCTG; OL2, GCTCCTGTCTAACACTGG; OL4, CACTGGAGAGGGGTCCTG; OL5, TATCACTGGACAGAGGTC; OL6, CATGCTCCTGTCTATCAC; OL9, CATGCTCCTAGCTATCACTGG; OL10, GCTCCTATGTGCCACTGG. OL4-6 was constructed by mutating hybrid OL4 with OL6 oligonucleotide. The wild-type and hybrid C4 coding sequence was confirmed by dideoxy-sequencing (25) both before and after transferring the inserts into either expression vector pMT-2 (26) or pSV. Mutants OL9 and OL10 were made directly in expression vector pSVC4, which contains in addition to C4B coding sequence a simian virus 40

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Abbreviation: SV40, simian virus 40.

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(SV40) promoter and a polyadenylylation signal on a pBluescribe M13$^+$ background.

Expression of Recombinant C4. For transfection of COS-1 cells (1×10^6 cells per plate) the DEAE-dextran technique (27) was used. After 48 hr, cells were pulse-labeled for 1 hr in methionine-free medium [Dulbecco's modified Eagle's medium, which includes 10% (vol/vol) fetal calf serum, 2 mM L-glutamine, and 50 units of penicillin/streptomycin per ml] supplemented with [^{35}S]methionine (0.5 mCi/ml; 1 Ci = 37 GBq) and were chased overnight with an equal volume of complete medium. Supernatants (72 hr) were harvested and stored at 4°C. For electrophoretic typing, C4 was immunoprecipitated (rabbit anti-human C4; Cappel Laboratories) from 50-μl aliquots of [^{35}S]methionine-labeled supernatant and analyzed on a NaDodSO$_4$/8% low-bisacrylamide polyacrylamide gel (reducing) as described (28). Gels were then treated with EN^{3}HANCE (DuPont), dried, and autoradiographed at −80°C for 1 to several days. Supernatant from [^{35}S]methionine-labeled COS-1 cells transfected with vector only was negative in the functional assays.

Functional Assays. Serial dilutions of 100-μl aliquots of supernatants from COS-1 cells transfected with the respective hybrid or mutant (as described above) were assayed for activity in a C4 hemolytic assay as described (29). Comparisons were made on the basis of Z units/ng of immunochemically determined recombinant C4 antigen, where $Z = -\ln(1 - \text{fractional lysis})$ and is physically equal to the number of hemolytically effective molecules per erythrocyte. Relative thioester activity was assessed from susceptibility to C1s subunit cleavage (11) and was determined by treating 50-μl aliquots of supernatant with 5 μg of C1s (Cytotech, San Diego, CA). The C4 was immunoprecipitated, analyzed on NaDodSO$_4$/polyacrylamide gels (reducing), and autoradiographed (as described above). Binding of [^{35}S]methionine-labeled recombinant C4 to C1-bearing human erythrocyte membranes was performed essentially as described (30) with 2.5×10^8 EAC1 (activated C1 bound to antibody-coated sheep erythrocytes) and 0.8 ml of [^{35}S]methionine-labeled supernatant. Determination of ester vs. amide bond was based on the sensitivity of ester but not amide bonds to 1 M hydroxylamine. Following a first dimension of nonreducing NaDodSO$_4$/5% PAGE, the adduct lanes were excised and treated with 1 M hydroxylamine/0.1 M NaHCO$_3$, pH 9, at 37°C for 3 hr. Subsequently, the treated lane was fractionated during a second dimension of NaDodSO$_4$/PAGE (5%, nonreducing). The assay used for direct binding to immune aggregates was modified from Ishizaka *et al.* (31). Briefly, a 10-μl aliquot of a 2% suspension of heat-aggregated human IgG (IgG1 myeloma protein, gift from P. Schur, Brigham Women's Hospital, Boston) was first washed in 10 mM EDTA-GVB^{++} (GVB^{++} = gelatin at 1 g/liter in veronal-buffered saline (VBS; 4 mM veronal/0.15 M NaCl) and then in C1 buffer (10% sucrose in VBS) prior to fixing human C1 [100 μl of 1:10 dilution of the euglobulin fraction (29) of 10 mM EDTA containing plasma diluted in C1 buffer] and incubated at 37°C for 30 min. After two washes in GVB^{++}, C1-fixed and non-C1-treated control IgG aggregates were suspended in [^{35}S]methionine-labeled supernatant, and the total volume was adjusted to 0.6 ml with GVB^{++} and incubated at 37°C for 60 min. The reaction was stopped with 20 mM EDTA, and aggregates were washed in immune complex buffer (25 mM Tris·HCl, pH 7.5/150 mM NaCl/0.5% Nonidet P-40/1 mM EDTA) as described (32). To test for ester vs. amide bond formation, half of each sample was treated with an equal volume of 2 M hydroxylamine/0.2 M Tris chloride, pH 9.5, for 3 hr at 37°C prior to fractionation on NaDodSO$_4$/polyacrylamide gels (reducing). Gels were treated and autoradiographed as described above, and the relative intensity of adduct bands was determined by scanning with a laser densitometer.

RESULTS AND DISCUSSION

The general approach used in this study was to construct hybrid C4B/A proteins using a full-length plasmid coding for C4B as the template. In addition to the six hybrids (see Table 1) two mutant proteins were constructed in which a neutral residue (alanine) was substituted either for the charged residue at 1106 (i.e., OL9) or isoleucine at 1105 (i.e., OL10). After transfection of COS-1 cells with the respective cDNAs contained in either of the two expression vectors shown in Fig. 1, ^{35}S-labeled recombinant proteins were compared for functional differences by using both a hemolytic assay and an assay that measures C1-dependent binding to immune aggregates. Based on previous work (11, 12) showing a correlation between increased binding efficiency and hemolytic activity of C4B compared with C4A, the hemolytic assay was used as a measure of binding of the recombinant C4 proteins to the

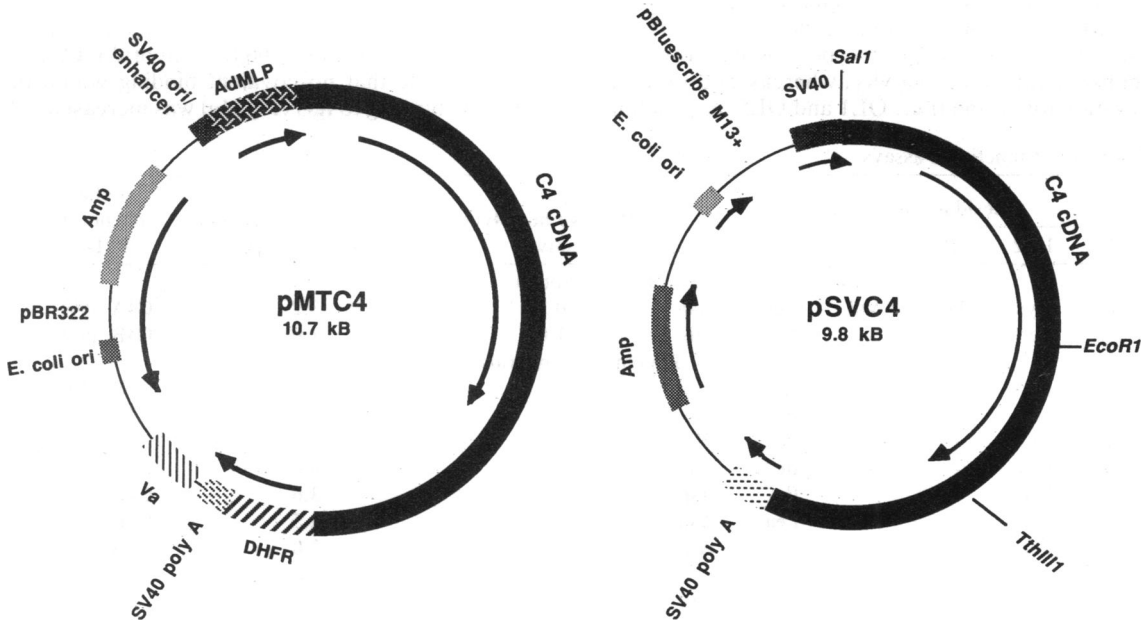

FIG. 1. Plasmid vectors used for construction and expression of recombinant C4 protein. Amp, ampicillin gene; AdMLP, adenovirus major late promoter; ori, origin of translation; DHFR, dihydrofolate reductase gene.

carbohydrate-rich surface of the sheep erythrocyte. The second assay was designed to detect the increased binding efficiency of C4A to immune aggregates. Direct covalent binding of the α subunit, which contains the internal thioester, to IgG heavy chain forms a major adduct of about 180 kDa (band 1) and a minor adduct of about 150 kDa (band 2). This pattern was demonstrated after $NaDodSO_4$/PAGE (reducing) and autoradiography as reported (33–35). A third assay was used to determine the nature of the covalent bond (ester vs. amide) formed between the C4 hybrids and the transacylation acceptor molecule and was based on the susceptibility of ester bonds but not amide bonds to scission by hydroxylamine. In addition, the "electrophoretic isotype" of the C4 hybrids was determined based on the differential migration of C4A and C4B α subunits in $NaDodSO_4$/PAGE.

Table 1 summarizes the results of all of the functional assays. Figs. 2, 3, and 4 show results from functional assays of mutants and hybrids considered critical to the study. For comparison, C4B was given a value of 1.0 in both the hemolytic and immune complex binding assays. Relative activity of the recombinant proteins was adjusted for differences in concentration (determined by immunoassay) and integrity of the thioester (determined by susceptibility to cleavage by C1s and analysis by $NaDodSO_4$/PAGE). In parallel transfections of the hybrid C4 cDNAs in COS-1 cells, the proportion of C1s-cleavable α-chain material was constant. As a further internal standard, C4A and C4B purified from plasma were subjected to the same series of functional assays. The results obtained with plasma proteins were in agreement with those published previously (11, 12, 34).

Typing of the recombinant hybrids on reducing $NaDodSO_4$/polyacrylamide gels showed that three hybrids—i.e., OL1, OL4, and OL4-6—all of which had proline for leucine at position 1101, converted the C4B electrophoretic phenotype to that of C4A (Fig. 2). Although substitutions of positions 1105 and 1106 were without effect in this assay, an intermediate shift of the α subunit was observed when cystine was substituted for serine at position 1102 in hybrid OL5 (Fig. 2).

The hemolytic activities of the various hybrid molecules are compared in Fig. 3*a* and their C1-dependent binding activities to IgG aggregates are compared in Fig. 4*a*. Relative to the wild-type C4B recombinant molecule, the activities of the hybrids fall into three groups. Changing leucine to proline at 1101 (i.e., OL4) was without effect in both assays. Thus, the switch in "electrophoretic phenotype" did not correlate with any change in functional activity, whereas, substitution in C4A of cystine for serine (i.e., OL1 and OL5) at position

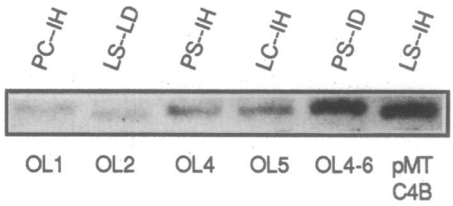

FIG. 2. Autoradiograph of $NaDodSO_4$/polyacrylamide gel showing that substitution of proline for leucine at position 1101 converts C4B to the C4A electrophoretic phenotype. Labels above each lane indicate the coding sequence in single-letter code at positions 1101 and 1102 and at 1105 and 1106 (pro-C4 numbering), and labels below each lane indicate the respective recombinant C4 analyzed.

1102 resulted in a 30–40% decrease in both hemolytic activity and IgG binding. The most dramatic effect on functional activity was observed when aspartic acid was substituted for histidine at position 1106. In each of three different hybrids—i.e., OL2, OL4-6, and OL6—hemolytic activity was reduced by a factor of about 2.5–3 (Fig. 3*a*), whereas C1-dependent binding to IgG aggregates was increased about 3-fold (Fig. 4*a* and Table 1). Furthermore, adducts formed between Asp-1106-containing hybrids and either erythrocyte surface molecules or immunoglobulin chains were resistant to cleavage with hydroxylamine (Figs. 3*c* and 4*b*; Table 1). Thus, replacement of His-1106 by aspartic acid resulted not only in a change from C4B to C4A hemolytic activities and target binding preferences but also in a conversion from ester to amide linkages. This agrees with the results of Dodds and Law (36), who compared the functional activity of human C3 and murine C4, which have histidine at the homologous position. In their assay of direct binding to glycine and glycerol, they found that both human C3 and murine C4 were more similar to human C4B than C4A. Interestingly, hybrid OL1, which is homologous to murine C4 at the isotypic residues (1101, 1102, 1105, and 1106) had a 30–40% decrease in activity in both assays. This is in contrast to their findings that the murine C4 was at least as active as human C4B in binding glycerol.

It has been proposed (12, 36, 37) that the charged group at position 1106 participates in the transacylation reaction as a general base and enhances the nucleophilicity of the acceptor amino or hydroxyl group by abstracting a proton from the nucleophile. To test this hypothesis, alanine was substituted for histidine at position 1106 (i.e., hybrid OL9). The prediction would be that no covalent binding would occur. Surprisingly, binding to IgG (Fig. 4*a*) was increased >2-fold with

Table 1. Summary of functional assays

Protein	Residue* at positions						Relative hemolytic activity	Relative C1-dependent binding to IgG	α-chain phenotype (kDa)	Covalent linkage
	1101	1102	1103	1104	1105	1106				
Plasma proteins										
C4A	Pro	Cys	Pro	Val	Leu	Asp	0.25	3.2	C4A (99)	Amide
C4B	Leu	Ser	---	---	Ile	His	1.0	1.0	C4B (97)	Ester
Recombinant hybrids										
C4B(w)	Leu	Ser	---	---	Ile	His	1.0	1.0	C4B (103)	Ester
OL4	**Pro**	Ser	---	---	Ile	His	1.0	1.0	C4A (105)	Ester
OL5	Leu	**Cys**	---	---	Ile	His	0.7	0.7	C4A (104?)	Ester
OL1	**Pro**	**Cys**	---	---	Ile	His	0.7	0.6	C4A (105)	Ester
OL6	Leu	Ser	---	---	Ile	**Asp**	0.4	3.6	C4B (103)	Amide
OL2	Leu	Ser	---	---	**Leu**	**Asp**	0.4	2.5	C4B (103)	Amide
OL4-6	**Pro**	Ser	---	---	Ile	**Asp**	0.4	2.8	C4A (105)	Amide
Alanine mutants										
OL9	Leu	Ser	---	---	Ile	**Ala**	0.8	2.4	ND	Amide
OL-10	Leu	Ser	---	---	**Ala**	His	0.4	0.5	ND	Ester

ND, not determined.
*Boldface residues signify replacement residues.

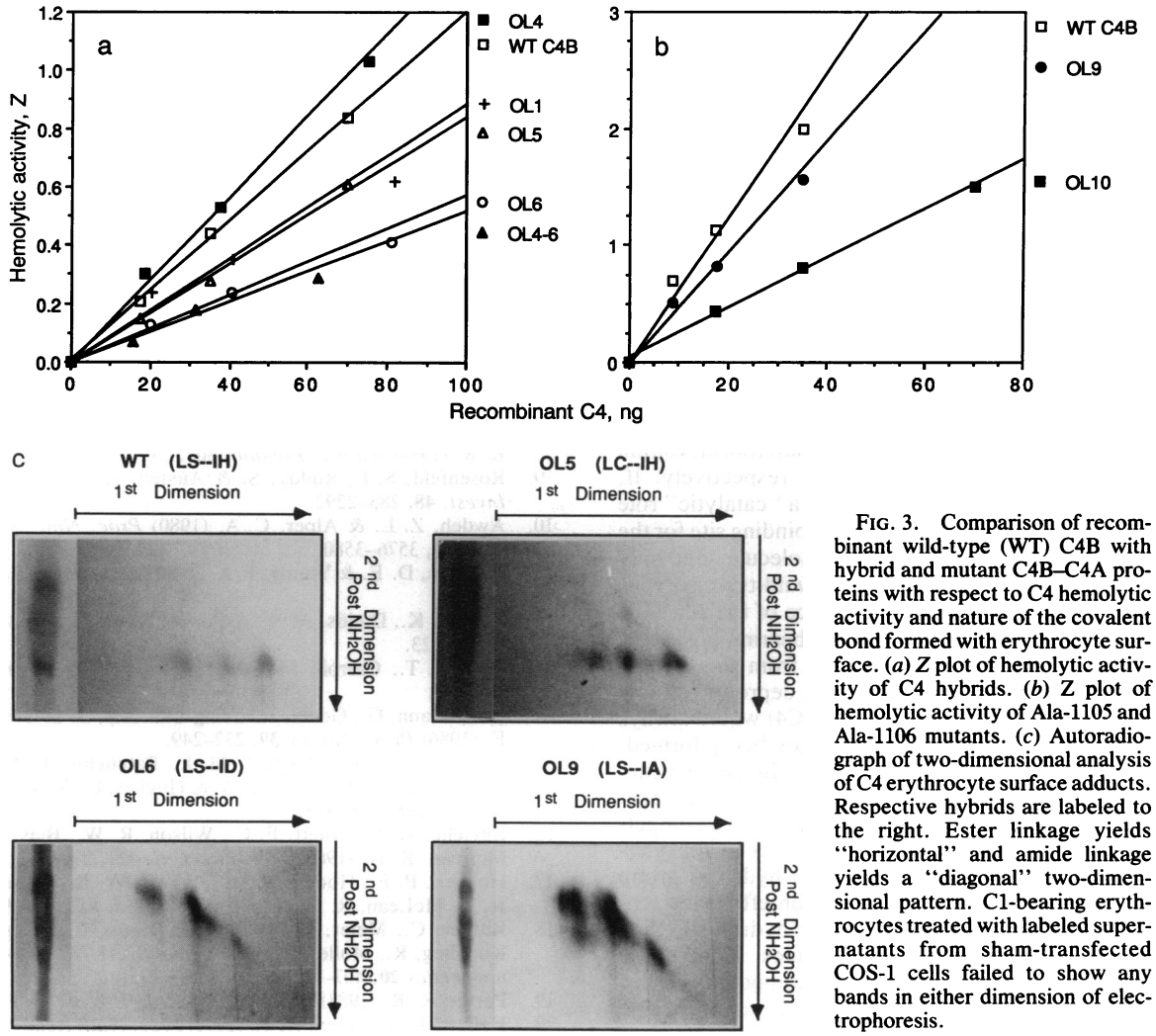

FIG. 3. Comparison of recombinant wild-type (WT) C4B with hybrid and mutant C4B–C4A proteins with respect to C4 hemolytic activity and nature of the covalent bond formed with erythrocyte surface. (*a*) *Z* plot of hemolytic activity of C4 hybrids. (*b*) Z plot of hemolytic activity of Ala-1105 and Ala-1106 mutants. (*c*) Autoradiograph of two-dimensional analysis of C4 erythrocyte surface adducts. Respective hybrids are labeled to the right. Ester linkage yields "horizontal" and amide linkage yields a "diagonal" two-dimensional pattern. C1-bearing erythrocytes treated with labeled supernatants from sham-transfected COS-1 cells failed to show any bands in either dimension of electrophoresis.

relatively little loss in hemolytic activity (Fig. 3*b*). Therefore, it seems unlikely that the charged group at position 1106 plays an essential catalytic role in the transacylation reaction. The hydroxylamine release experiments (Figs. 3*c* and 4*b*) showed that the presence in position 1106 of either aspartic acid or alanine resulted in amide bond formation, whereas recombinant C4 molecules having histidine at position 1106 form ester linkages. Although the results do not totally exclude a role for histidine in ester bond formation, a more probable mechanism would have the side chain of 1106 interacting electrostatically and/or sterically with potential acceptor molecules, either favoring access to the thioester—e.g., through ion pair

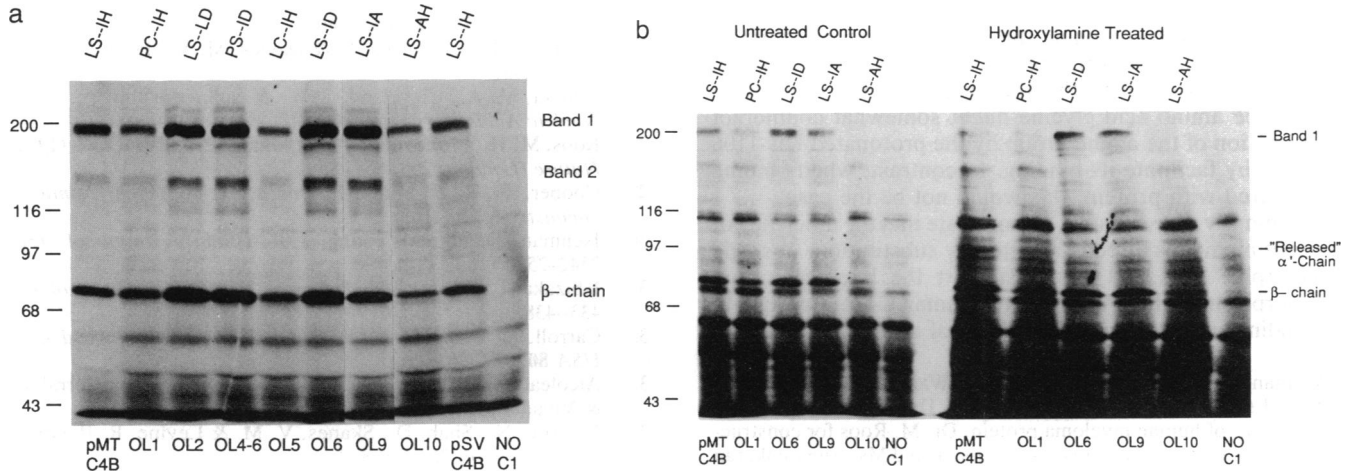

FIG. 4. Comparison of recombinant wild-type (WT) C4B with hybrid and mutant C4B–C4A proteins by C1-dependent binding to aggregated IgG. (*a*) Autoradiograph of $NaDodSO_4$/6% polyacrylamide gel (reducing) of recombinant [^{35}S]methionine-labeled C4 bound to aggregated IgG. (*b*) Same as in *a* except that samples were adjusted for relative C4 binding activity and half of each sample was treated with 1 M hydroxylamine before loading the gel. Lanes are labeled as in Fig. 2. Amino acids are in single-letter code.

formation between aspartic acid and an incoming amino-group nucleophile—or preventing it—e.g., through mutual charge repulsion of the partially protonated histidine and an incoming amino group. Further evidence that the isotypic residues form a binding site during the transacylation reaction comes from an examination of the importance of the long hydrophobic side chain of isoleucine at position 1105. Its replacement by alanine (i.e., OL10) reduced activity at least 50% in both binding assays (Figs. 3*b* and 4*a*) but did not change the nature of the covalent bond.

In speculating about possible mechanisms mediating the covalent binding reaction of C4, there is an apparent paradox in that any "catalytic" group capable of making a hydroxyl group an "acceptable" nucleophile for transacylation also would do so with an amino group, which is intrinsically more nucleophilic. However, this is not compatible with the nearly exclusive hydroxyl group versus amino group transacylation preferences displayed by C4B and C4A, respectively. If, however, the isotypic residues do not have a "catalytic" role but instead function to form a "substrate" binding site for the incoming nucleophile-bearing acceptor molecule, not only could the nucleophilic preferences of the wild-type isotypes be reconciled but also the amide preference of the Ala-1106 mutant. Specifically, if acceptor species bearing positively charged amino groups were not repelled from the binding site, then the free base amine (formed upon deprotonation by an as-yet-unidentified general base within C4) would always result in a predominance of amide linkages being formed. Only when amino groups are excluded from the binding site, as by His-1106 bearing at least a partial positive charge, would hydroxyl-bearing acceptor molecules have a competitive advantage in the transacylation reaction. The considerably more difficult task of deprotonating a hydroxyl group would result in a much lower transacylation efficiency (covalent binding rate of C4 relative to water hydrolysis) than with amino group-bearing acceptor molecules. Indeed, this efficiency difference is apparent in model compound studies using glycine and glycerol (36). The amino group of glycine has been shown to react with the exposed thioester of activated C4A at a rate that is ≈1000-fold greater than the reaction of glycerol hydroxyl groups with similarly activated C4B, when a similar rate of water hydrolysis is assumed. In these model systems, the relative reaction rate of glycine with C4B is also 8-fold greater than that of glycerol with C4B. Since the latter pair of reaction rates are within 1 order of magnitude of each other, it might be expected that a mixture of ester and amide linkages between target acceptor and C4B would be found. The fact that a strong preference for ester linkages is observed when the C4B residue histidine is present at position 1106 may reflect a peculiarity of the model system. One possibility is that the adjacent carboxylate group on the free amino acid glycine might somewhat counteract the repulsion of the amino group by the protonated His-1106 and thereby facilitate its binding. In contrast, when binding is measured with protein, this would not be the case.

In summary, these results demonstrate that the efficiency of covalent binding is directly affected by substitutions at single polymorphic sites and would support the hypothesis that polymorphism in the C4d domain of human C4 is important in the binding and subsequent clearance of immune complexes.

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