Structure-based Mapping of DAF Active Site Residues That Accelerate the Decay of C3 Convertases*

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Focused complement activation on foreign targets depends on regulatory proteins that decay the bimolecular C3 convertases. Although this process is central to complement control, how the convertases engage and disassemble is not established. The second and third complement control protein (CCP) modules of the cell surface regulator, decay-accelerating factor (DAF, CD55), comprise the simplest structure mediating this activity. Positioning the functional effects of 31 substitution mutants of DAF CCP2 to -4 on partial structures was previously reported. In light of the high resolution crystal structure of the DAF four-CCP functional region, we now reexamine the effects of these and 40 additional mutations. Moreover, we map six monoclonal antibody epitopes and overlap their effects with those of the amino acid substitutions. The data indicate that the interaction of DAF with the convertases is mediated predominantly by two patches ∼13 Å apart, one centered around Arg69 and Arg300 on CCP2 and the other around Phe148 and Leu171 on CCP3. These patches on the same face of the adjacent modules bracket an intermodular linker of critical length (16 Å). Although the key DAF residues in these patches are present or there are conservative substitutions in all other C3 convertase regulators that mediate decay acceleration and/or provide factor I-cofactor activity, the linker region is highly conserved only in the former. Intra-CCP regions also differ. Linker region comparisons suggest that the active CCPs of the decay accelerators are extended, whereas those of the cofactors are tilted. Intra-CCP comparisons suggest that the two classes of regulators bind different regions on their respective ligands.

The C3 convertases of the classical and alternative pathways are the central enzymes of the complement cascade (1). These bimolecular complexes, C4b2a and C3bBb, produce anaphylatoxin C3a, locally amplify C3b deposition, and serve as sites for the assembly of the C5 convertases, C4b2a3b and C3bBb3b. These trimeric convertases, in turn, generate anaphylatoxin C5a and initiate the terminal pathway, leading to formation of lytic C5b-9 membrane attack complexes. The relative rates of assembly and disassembly of the C3 convertases lie at the heart of complement regulation, and their physiologic modulation ensures that complement acts in a proportionate and targeted fashion.

To focus complement activation on foreign targets, prevent nearby activation in the fluid phase, and simultaneously protect self tissues from complement-mediated injury, the C3 convertases are controlled by both serum- and cell-associated regulatory proteins (2, 3). Because the convertases assemble on C4b and C3b fragments that condense indiscriminately with free hydroxyl and amino groups on foreign targets and these same acceptor groups are present on all biological membranes, their formation on self cells at the single enzyme level cannot be avoided. To prevent further propagation of the cascade, which would induce self cell injury, self cells possess decay-accelerating factor (DAF or CD55). By virtue of its glycosylphosphatidylinositol anchor, which allows it to move rapidly in the plane of the membrane, this regulator immediately decays the single C3 convertase enzymes wherever they form on self cell surfaces (4).

DAF is ∼70 kDa in size (4). Its functional region, consisting of four ∼60-amino acid-long repeating units, termed complement control protein (CCP) modules (also known as short consensus repeats or sushi domains), is suspended above the cell surface on a heavily O-glycosylated, Ser- and Thr-rich stalk (5–7). Studies with antibody-sensitized sheep erythrocytes (E4A) bearing defined complement components have shown that the DAF decay-accelerating activity (DAA) against the alternative pathway (AP) convertase, C3bBb, lies in its CCP2, -3, and -4, whereas its activity against the classical pathway (CP) enzyme, C4b2a, resides entirely within its CCP2 and -3 (8).

Although an NMR-derived solution structure of CCP2 and -3 (9) and a crystal structure of CCP3 and -4 (10) were reported earlier, an x-ray crystal structure of the full DAF CCP1 to -4 regulatory region has become available more recently (11). In the present study, we have employed this full-length structure to map the effects of the 31 previously reported principally alanine substitution mutants and to guide the design of 40 additional mutants. The new mutations include four insertions/substitutions of residues in the linkers between CCP2 and -3 and CCP3 and -4 (12). In conjunction with this analysis, we

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* This work was supported by National Institutes of Health Grants R01 AI23598 (to M. E. M.) and R01 AI05143 (to D. E. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: CCP, complement control protein; DAA, decay-accelerating activity; AP, alternative pathway; CP, classical pathway; MCP, membrane cofactor protein; mAb, monoclonal antibody; CF, cofactor.
Refined Mapping of DAF Active Site

TABLE 1
Sense primers (5′–3′) used in site-directed mutagenesis

| Mutation | Primer |
|----------|--------|
| R69C     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| R69W     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| R70A     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| S74A     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| K76A     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| Y79A     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| Y94A     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| E94A     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| R96L     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| P97G     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| G98S     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| R100C    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| K108A    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| E102Q    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| K116A    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| K126C    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| E122A    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| K126E    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| N131A    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| K126C    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| N131G    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| R69W     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| R69W/L70P| GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| R96L     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| P97G     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| G98S     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| R100C    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| K108A    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| E102Q    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| K116A    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| K126C    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| N131A    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| K126C    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| N131G    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| R69W     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| R69W/L70P| GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| R96L     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| P97G     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| G98S     | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| R100C    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| K108A    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| E102Q    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| K116A    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| K126C    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| N131A    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| K126C    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |
| N131G    | GCCTGAGAGGTGCCAACATGCCTAAATTCTGCATCCCTC |

* N131G was obtained.

localized the epitopes of six mAbs and considered, in light of the three-dimensional structural information, the effects of their binding on DAF function. Since other regulators that mediate decay acceleration of the C3 convertases (i.e. C3b receptor (CR1 or CD35), factor H, and C4-binding protein (C4bp)) are more complicated in that 1) their decay-accelerating regions are larger (three or four CCP modules) and 2) they also mediate a second regulatory function (i.e. cofactor activity for factor I cleavage of C3b (factor H), C4b (C4bp), or both (CR1)), the information derived from the simpler DAF CCP2 and -3 structure, through sequence and structure comparisons between other decay accelerators and factor H cofactors, sheds light on differences between the two classes of regulators.

**EXPERIMENTAL PROCEDURES**

Preparation of the cDNA Encoding the Native DAF CCP1 to -4 and Substitution Mutants—The DAF cDNA derivative HuDAF-6H, the native DAF parental control, encoding CCP1 through CCP4 with a C-terminal His$_6$ (6H) tag was prepared as described in Ref. 12 using human DAF 13:2 cDNA in pBT-KS$^+$ (13). For generation of the substitution mutants, the QuikChange (II) site-directed mutagenesis kit (Stratagene) was used in conjunction with HuDAF-6H. Sense primers are listed in Table 1. All mutations were confirmed by sequencing. Additionally, CCP1 through CCP4 was sequenced in full for all of the following mutants that adversely affected function: R69C, R69W, R96/L70P, R96L, P97G, G98S, R100C, K126C, K126E, K127GS128 (glycine inserted between Lys$^{127}$ and Ser$^{128}$), T151A, F169C, D207A, E205A, and D207A.

Recombinant DAF Protein Expression by Transient Transfection—Samples of 7.5–15 μg of cDNA, preincubated for 20–30 min at 20 °C with 54 μl of Lipofectamine (Invitrogen) in 1.8 ml of Opti-MEM I (Invitrogen), were mixed with 7.2 ml of Opti-MEM I and added to COS1 cells grown to near confluence in Falcon 3084 flasks (BD Biosciences). After 5 h at 37 °C, 30 ml of Dulbecco’s modified Eagle’s medium (10% fetal bovine serum, with or without 1% glutaMAX I (Invitrogen) and 1% penicillin/streptomycin) was added, and the cells were incubated overnight. The following day, the cells were washed twice with phosphate-buffered saline (Dulbecco’s), and 30 ml of Opti-MEM I was added. The Opti-MEM I supernatant containing the recombinant protein was harvested 2–3 days later.

Supernatants were concentrated and buffer-exchanged into phosphate-buffered saline using a Millipore (Bedford, MA) Ultrafree-15 centrifugal filter device, Biomax 5-kDa molecular mass cut-off. A two-site immunoradiometric assay (14) and densitometry (UN-SCAN-IT Gel Digitizing Software, version 5.1 for Windows (Silk Scientific, Orem, UT) and Dell AIO printer/scanners A940 and A920) were used to quantitate DAF protein concentrations. The two-site immunoradiometric
assay used CCP1-specific mAb IA10 at 5 μg/ml as capture mAb and
and 125I-labeled CCP4-specific mAb 2H6 for detection. As a second method for quantitating mutants, all of which contained a C-terminal His6 tag, a horseradish peroxidase-conjugated anti-His6 (C-terminal) mAb (mouse, clone 3D5, IgG2b; Invitrogen) was used in conjunction with densitometry.  

Monoclonal Antibodies—To map the epitopes of the mAbs to DAF, supernatants of the native DAF (wild type or N61Q substitution) CCP1 to -4 control, substitution mutants, and vector-only control were run on 10% SDS-polyacrylamide gels, and proteins were transferred to polyvinylidene difluoride membranes. These were done in duplicate to allow blotting with IA10, a mAb that binds CCP1 of DAF, as antibody control, and with the mAb under investigation. The mAbs tested included 8A7 (14), 2H6 (14), BRIC216 (BIOSOURCE International, Camarillo, CA), 1C6 (Wako Chemicals, Richmond, VA), 2D2-2 (15), 2D2-3 (15), and 1H4 (16). Table 2 lists substitution mutants tested with each antibody. Lanes were compared qualitatively to determine the binding site.  

Hemolytic Assay Buffers—Isotonic Veronal-buffered saline (DGVB2+) contained 72.7 mM NaCl, 1.56 mM barbital (Fisher), 0.91 mM sodium barbital (Fisher), 1 mM MgCl2, 0.15 mM CaCl2, and 2.5% (w/v) dextrose (pH 7.3–7.4) to which 0.1% gelatin was added. Isoionic Veronal buffer (GVB2+) consisted of 145 mM NaCl, 3.12 mM barbital, 1.82 mM sodium barbital, 1 mM MgCl2, and 0.15 mM CaCl2 (pH 7.3–7.4) to which 0.1% gelatin was added. Metal-chelating Veronal buffer (GVB-E) substituted 10 mM EDTA for MgCl2 and CaCl2 of GVB2+.  

DAA Assays—Alternative pathway C3 convertase DAA was determined by enzyme-linked immunosorbent assay (17). Microwell plates were coated overnight at 4 °C with C3b (1 determined by enzyme-linked immunosorbent assay (17). The plates were blocked with 1% (w/v) bovine serum albumin and 0.1% (v/v) Tween 20 and incubated at 37 °C for 2 h with factor B (400 ng/ml), factor D (25 ng/ml; Advanced Research Technologies), 2 mM NiCl2, 25 mM NaCl in 10 mM phosphate buffer, pH 7.4, supplemented with 4% (w/v) bovine serum albumin and 0.1% (v/v) Tween 20. After extensive washing of the wells, plate-bound C3Bb (N2+) complexes were incubated for 15 min at 37 °C with increasing concentrations of mutant or control DAF proteins in 25 mM NaCl-supplemented phosphate buffer or with buffer alone. Remaining plate-bound C3Bb complexes were detected with polyclonal goat anti-factor B antibody followed by peroxidase-conjugated rabbit anti-goat antibody (17). In each experiment, dose-response curves for mutant and control DAF protein were generated by regression analysis, and mutant activity was calculated from the curves as percentage activity of the wild-type protein.  

Classical pathway C3 convertase DAA was determined using a hemolytic C4b2a decay assay (4). In this assay, hemolysin-sensitized sheep erythrocytes (E48A; 1 × 107 in 100 μl of DGVB2+) were incubated with ~30 site-forming units of human C1 (Advanced Research Technologies) (15 min, 30 °C). The cells were pelleted, resuspended in 100 μl of DGVB2+, and incubated for 20 min at 30 °C with ~15 site-forming units of human C4 (Quidel, San Diego, CA). The cells were again pelleted and resuspended in 100 μl of DGVB2+ and then treated for 5 min at 30 °C with human C2 (Advanced Research Technologies) predetermined to yield ~1 C4b2a site/cell after subsequent decay for 15 min at 30 °C. The parental DAF, mutated DAF, or DGVB2+ control was added to the cells during this decay step, after which guinea pig serum (C3-9) (Colorado Serum, Denver, CO) in GVB-E was added for 1 h at 37 °C to develop lysis. After pelleting of unlysed cells, the A412 value of the supernatant was measured, and residual C4b2a sites were calculated.  

Calculations—In each assay, a dose-response curve of percentage activity versus concentration was established for mutants and controls, and the concentration of DAF protein required for 50% activity was determined. The concentrations obtained for mutants were compared with the concentrations of their corresponding controls, and percentage activity was calculated, with the native DAF control assigned a score of 100%. For classical pathway C3 convertase assays, either two or three independent experiments were performed. For alternative pathway C3 convertase assays, between two and five assays were performed.  

Sequence Alignments—Sequences of DAF CCP2 to -4 (P08174), CR1 CCP1 to -3 and CCP15 to -17 (P19729), factor H CCP1 to -3 (CAA68704), C4bpa CCP1 to -3 (AAA36507), membrane cofactor protein (MCP) CCP1 to -4 (P15529), Kaposica CCP1 to -4 (AAAC57082), VCP CCP1 to -4 (P68638), and SPICE CCP1 to -4 (NP_042056) were initially aligned using ClustalW (version 1.8) (18). Subsequently, CCP4 of factor H and CCP4 of C4bpa were added, and the sequence alignment was subjected to further manual editing on the basis of conservation of residues.  

PSI-BLAST and Position-specific Substitution Matrix—To test whether the residues in the linker region are conserved in regulators that have decay acceleration activity, a PSI-BLAST (19) alignment using Cys111–Ile140 as the seed sequence was performed using blastp. The PSI-BLAST alignment of the reference sequence from the NCBI Protein Data Bank yielded
an alignment of DAF homologues from a variety of species. The position-specific substitution matrix from this alignment was used in a PSI-BLAST search of the nonredundant data base. The convergence of this PSI-BLAST search yielded all of the known proteins that have DAA in the classical pathway. A parallel PSI-BLAST with the homologous sequence from MCP yielded an alignment limited to MCP analogs. The cut-off score (in bits) for DAF and MCP (see Tables 4 and 5) is 50. The DAF sequences in Table 4 are based on iteration 6, whereas the MCP sequences in Table 5 are based on iteration 4. Examination of the position-specific substitution matrix identifies those residues that are strongly conserved (log odds value $>6$) in the DAF and MCP alignments and is the basis for Fig. 4, C and D.

RESULTS

Mapping of the DAF Active Site by Mutational Analyses—The positions of our 31 previously reported and 40 new DAF mutants (a total of 71 substitutions in 60 of 190 amino acids in the functionally relevant CCP2 to -4 of DAF) on the crystal structure of the four CCP modules are shown in two views (differing by 180°) in Fig. 1, A–D. The activity of each mutant relative to that of unmodified recombinant DAF CCP1 to -4 with respect to accelerating decay of the CP and AP pathway convertases is summarized in Table 3. For mapping the DAF interface with the C3 convertases, solvent-exposed side chains were distinguished from buried residues (Leu$^{70}$, Gly$^{98}$, Phe$^{123}$, and Phe$^{154}$). The functional effects of substitutions of these exposed residues are represented schematically by the following color code.

To grade function, we designated substitutions resulting in retention of $\geq$4% of native function as having no significant effect (green), 17–33% as having a limited effect (yellow), 5–16% as having a considerable effect (orange), and $\leq$4% as having an essentially obliterative effect (red). Fig. 1, A and B, shows the effects of the substitutions on DAF CP and AP activity on one face of the CCP2 to -4 modules, and C and D show effects of the substitutions on the opposite face of the two modules. As seen in Fig. 1, A and B, solvent-exposed Arg$^{59}$, Arg$^{86}$, and Leu$^{171}$ in CCP2 and Leu$^{171}$ in CCP3 are critical for DAF function in both pathways. Consistent with Phe$^{148}$ being buried in the full-length CCP1 to -4 crystal structure, its alanine substitution totally abolished DAF function. Lying on the same face of the crystal structure, these two sets of residues, each of which is

![Figure 1. Positions of mutations are shown on the x-ray crystal structure of DAF (Protein Data Bank code 1OK3). Red, $\leq$4% DAA of wild-type DAF; orange, $\leq$16%; yellow, $\leq$33%; green, $>33%$. CP activity is shown in A (front) and C (back), whereas AP activity is shown in B (front) and D (back).](image-url)
linked immunosorbent assays. Note that substitution mutants were made in a human DAF CCP1 to -4 module with N61Q (no C-terminal tag) (HuDAF(N61Q)) or AsnH + C-terminal His$_6$ tag (HuDAF-6H).

Table 3—continued

| Mutant | Alternative pathway | Classical pathway |
|--------|---------------------|-------------------|
| K211A-6H | 44 ± 33 | 85 ± 3 |
| E228A | 69 ± 9 | 57 ± 8 |
| H229A-6H | 169 ± 142 | 123 ± 36 |
| Y232A-6H | 67 ± 39 | 87 ± 29 |
| D258A-6H | 50 ± 14 | 71 ± 6 |
| E239A-6H | 61 ± 25 | 56 ± 17 |
| Other | | |
| RhDAF-6H | 28 ± 12 | 92 ± 32 |

almost contiguous in the two adjacent CCPs, are separated by 13 Å from each other. In contrast, on the opposite face, none of the tested residues were found to be important for DAF function except for portions of Arg$^{100}$, Lys$^{126}$, and Lys$^{127}$, which wrap around the CCP2 and -3 interface. Consequently on the face shown in Fig. 1, A and B, the two closely spaced clusters of 1) externally oriented, 2) functionally critical, and 3) separated but juxtaposed residues on the two adjacent CCPs provide full function in the classical pathway and contain all of the residues where function is not fully lost in the alternative pathway.

Alternative mutations of the above residues identified as being critical were subsequently made in which we 1) substituted residues occupying equivalent positions in other regulators with decay acceleration activity or 2) altered either charge or hydrophobicity. The results of these substitutions are summarized in Table 3. With regard to the functional patch on CCP2 (Arg$^{69}$ and Arg$^{96}$), although the substitution R69A abolished DAF CP and AP activity, R69W (as in CR1 CCP1) had little effect. In contrast, R69C, like R69A, abolished DAF CP and AP activity. Similarly, although alanine substitution of Arg$^{96}$ (conserved in both CR1 CCP1 and factor H CCP1) totally abolished DAF CP and AP activity, the substitution R96L (present in C4bp CCP1) retained partial activity, more in the AP than in the CP. In contrast to these findings involving the functionally important Arg$^{69}$, Arg$^{96}$ patch on CCP2, the substitutions K76A and E94A, 16 Å from Arg$^{96}$, and the substitutions R100C, E102Q, K108A, K116A, and E122A on the opposite face had little effect. With respect to residues in a patch on CCP3 previously reported to affect DAF and AP activity (i.e. Phe$^{149}$, Leu$^{171}$, and F169C), similarly to F169A, D181A abolished DAF CP activity. Concerning the buried residue Phe$^{149}$, although the substitution F148A completely abolished DAF CP and AP activity, F148Y had no effect, implicating the phenyl ring as the important element in maintaining structure.

To help resolve the differences in interpretations based on previous partial structures with respect to the flexibility of the CCP2 and -3 linker, we made new substitutions or insertions in this site. Elongation of the CCP2 and -3 linker by insertion of a G between Lys$^{177}$ and Ser$^{184}$ totally abolished DAF CP and AP activity. The new substitutions K126C and K126E nearly abolished DAF AP activity, similar to the substitution K126A, whereas they had lesser effects on DAF CP activity. At the CCP3 and -4 junction (Arg$^{187}$, Glu$^{188}$, Ile$^{189}$, and Tyr$^{190}$), the addition of a Gly between Arg$^{187}$ and Glu$^{188}$ greatly reduced DAF AP activity while not affecting DAF CP activity, arguing that only CCP2 and -3 are important in DAF CP function and that for CP function, fixation of the CCP2 and -3 spatial relationship by CCP4 precisely in its native orientation is not essential.
FIGURE 2. A–F, epitopes on DAF recognized by anti-DAF mAbs 1H4, 1C6, 2D2-3, 8A7, BRIC216, and 2H6 mapped on the DAF x-ray crystal structure. Highlighted are those where Western blots with mAbs were negative. G, overlap of epitopes mapped by mAb with loss of DAA function.
Refined Mapping of DAF Active Site

Mapping of the DAF Active Site by Epitope Analysis and Functional Effects of mAbs—The epitopes of six anti-DAF mAbs on the crystal structure (as mapped by the inability of each to bind the mutant proteins) are shown in Fig. 2, A–F. The amino acids comprising the epitopes of those mAbs that impair DAF function are combined in Fig. 2, G. As seen in Fig. 2, A, the epitope of 1H4 (which completely inhibits both DAF CP and AP activity) includes structurally relevant Phe148, Phe169, Leu171, and Asp181; that of 1C6 includes Thr151 (Fig. 2, B); that of 2D2-3 includes Lys126, Thr151, and Phe169 (Fig. 2, C), and that of 8A7 includes Arg69, Phe148, and Leu171 (Fig. 2, D). The substitution F169C was poorly recognized by BRIC216 (Fig. 2, E), which has been reported to have inhibitory effects on DAF CP activity (15) (Fig. 2, F). Thus, as found by alanine substitution analysis on CCP3, the epitope mapping reinforces the proposition that L171 is a contact site for DAF CP activity, and the three residues Phe169, Leu171, and Asp181 are contact sites for DAF AP activity.

Correlation of the DAF Active Site Residues with Corresponding Residues in Other C3 Convertase Regulators That Mediate Decay Acceleration—The sequences of CCP modules in other C3 convertase regulators that decay-accelerate the bimolecular enzymes are aligned with that of DAF CCP2 to -4 in Fig. 3, A and B. Focusing on the functionally critical residues identified above (seen in the orientation where the major negative effects on function grouped together), Arg96–Arg100 in CCP2 in conjunction with Lys125–Lys127 in the CCP2 and -3 linker and buried Phe148 in CCP3 are conserved in CCP1 and -2 of CR1 (DAA), factor H (DAA + CF), Kaposica, and C4bp (DAF + CF). Whereas Arg96–Arg100 and Phe148 are also highly conserved in C3 convertase regulators, which lack substantial DAA, but control C3 convertases principally by providing cofactor activity for factor I cleavage (i.e. MCP (membrane cofactor protein), CR1 CCP15 to -17, VCP (vaccinia virus complement control protein), and SPICE (smallpox inhibitor of complement enzymes)), Lys125–Lys127 in the CCP2 and -3 linker and buried Phe148 in CCP3 are conserved in CCP1 and -2 of CR1 (DAA), factor H (DAA + CF), Kaposica, and C4bp (DAF + CF). Whereas Arg96–Arg100 and Phe148 are also highly conserved in C3 convertase regulators, which lack substantial DAA, but control C3 convertases principally by providing cofactor activity for factor I cleavage, the crystal structure of DAF CCP1 to -4 shows an elongated arrangement of CCP2 and -3 (Fig. 4, A and B) (20). Armed with the above information concerning the region containing the residues in the DAF active site that are critical for decay acceleration, we performed a PSI-BLAST alignment using Cys113–Ile142 encompassing the CCP2 and -3 linker and its preceding and succeeding strands as the seed sequence in the NCBI protein database. As shown in Table 4, this analysis yielded DAF homologs from a variety of species as well as other proteins. Among known proteins having DAA are CR1 (site 1; CCP1 to -3) (21), Kaposica (22), mouse Crry (23), and the recently reported but only partially character-
ized rat CSMD1 (24). The only missing decay accelerator is factor H (+ factor H-related proteins). Interestingly, this analysis identified several additional sequences of uncharacterized function. A parallel PSI-BLAST search for homologous sequences with MCP, which lacks DAA but has cofactor function, did not identify any DAF orthologs other than CR1-related sequences (in particular CR1 site 2), CR1 having been shown to possess cofactor activity but also to retain DAA, and yielded an alignment primarily limited to MCP homologs (Table 5).

Examination of the position-specific substitution matrix identified those residues that are strongly conserved (log odds value > 6) in the DAF alignment and those strongly conserved in the MCP alignment. Those conserved in the DAF alignment are colored blue on the DAF CCP2 and -3 backbone structure (Fig. 4C), and those conserved in the MCP alignment are colored yellow on the MCP CCP1 and -2 backbone structure (Fig. 4D). The two representations show that conservation associated with decay acceleration is highest around the linker region, whereas that associated with cofactor function extends into the opposing modular arms.

DISCUSSION

Our characterization in this study of surface patches on DAF that mediate its DAA of the C3 convertases utilized positioning of the effects of substitution mutants that diminish its CP and AP activities, in conjunction with localization of the epitopes of mAbs reported to diminish its function (16) on the crystal structure of CCP1 to ~4 (11). Armed with this information on DAF, we then analyzed the extent of conservation of residues in DAF and in other C3 convertase regulators determined by mutagenesis to be important, either for cofactor activity or for DAA (12, 18, 21, 22, 25–41). Whereas we found an absence of strict conservation of residues which distinguish the two functions, by focusing alignment on the sequence flanking the CCP2 and -3 junction where DAF function resides, we identified differences between those regulators that 1) mediate DAF and 2) mediate cofactor activity.

The mutational analyses, together with the epitope mapping, revealed two functionally important patches on the tandem CCP2 and CCP3 surfaces of DAF that spatially are positioned 13 Å apart on the same face of the molecule but on two adjacent modules as shown in Fig. 1. These patches are centered around Arg69 and Arg96 of CCP2 and Phe148 and Leu171 of CCP3. The alternative pathway functional surface on CCP3 appears larger than the classical pathway surface, since mutation of the nearby residues Phe169 and Asp181 diminishes AP function more than CP function. The functional importance of these patches is corroborated by the mAb footprint data, since all five inhibitory mAbs include residues from the hydrophobic patch on CCP3, and the footprint of one of these mAbs, 8A7, which spans CCP2 and -3, also includes Arg69.

Although mutations that have a minimal effect on function are sometimes viewed as less informative, they are important in that they rule out uninvolved residues and thereby focus attention on the relevant region(s) of contact. The mutations near the CCP1 and -2 junction of CCP2 (K76A, Y79A, Y84A, K108A, and K116A), on the opposite face of CCP3 from the 148/171 patch (N131G), and near the CCP3 and -4 junction in CCP3 (E185S, R136S) caused no deficits in DAF function in either CP or AP. Newly generated mutations in CCP4, including E205A, D207A, H208A, Y211A, K221A, H229A, and Y232A (Table 3, Figs. 1 and 2F), likewise failed to affect DAF activity in either

**Refined Mapping of DAF Active Site**

**FIGURE 3—continued**
Consequently, in not finding any new functionally critical regions, we more precisely honed in on the DAF active site. Although two amino acid residues not located in either of the above patches were previously identified (12) as being important for AP function, one is localized in the linker between CCP2 and -3, and the other is in CCP4. Specifically, mutation of the two positively charged linker residues Lys125 and Lys126 decreased AP DAA to 32 and 9%, and mutations of Arg206, Arg212, and Asn220 decreased AP DAA to 25, 25, 32%. The epitope mapping with mAb 2H6 supports the previously hypothesized contact region in CCP4. Interpretation of the modest functional deficits introduced by mutation, however, is necessarily ambivalent.

The new substitutions (P97G, G98S, and D181A) near the two patches on the tandem CCP2 and CCP3 surfaces previously identified as constituting the active sites of DAF (11, 12) are suggestive of pathway differences in that P97G and G98S, both in the linker region close to the interface between CCP2 and -3, decreased DAF CP function to 16 and 4% while having a lesser effect on its AP function (72 and 43%). Since the Pro97 lies within the loop composed of the D and E strands of CCP2 (DE loop) that is close to CCP3 and is substantially exposed, it is a potential hydrophobic contact point. Gly98 is a buried residue in the CCP2 and -3 linker region, so that no CP/AP comparison can be made. On the other hand, the finding that mutating Asp181, which lies downstream of the AP-only critical Phe169 on CCP3, negatively affected DAF AP function only, slightly extends the region on CCP3 associated with DAF AP function, since Phe169 is contiguous with the Phe148/Leu171 patch.

Bringing the collective mutagenesis and epitope mapping data together with the crystal structure of DAF provides a number of new insights. Different substitutions at key residues (Arg69, Arg96, Lys126, and Phe169), by virtue of having similar deleterious effects as the alanine substitution, verify that these residues are important in function. An example is that the exposed Lys126 substitutions K126C and K126E have the same effect as K126A, indicating that K126 is functionally important with respect to charge. The importance of the rigidity and relative orientation of successive CCPs was evaluated by insertion of a glycine residue...
into the linker sequence. Glycine residues enhance the flexibility of the linker region by virtue of the conformational freedom introduced into the backbone. Additionally, insertion of the residue will necessarily extend the sequence distance between the modules and, in the absence of strong intermodular contacts, serve to randomize the relative orientations. Insertion of a glycine into the CCP2 and -3 linker region between Lys127 and Ser128 rendered DAF unable to decay-accelerate either C3 convertase. Conversely, insertion of a glycine in the CCP3 and -4 linker interval between Arg187 and Glu188 left the ability of DAF to accelerate decay of C4b2a unaffected, implying that the relative orientation of CCP3 and CCP4 is unimportant for CP function. However, consistent with the mutational analysis, this orientation would affect the alignment of Arg212 and Arg206 in CCP4 relative to CCP3, and the decrease in activity for the AP was therefore consistent.

The functional orientation of the two identified patches on CCP2 and CCP3 must be determined to define the interface of DAF with the C3 convertases. As shown in Fig. 1, these two patches lie on opposite sides of a concavity defined by the tilt between modules. Any significant change in either the skew or twist angles would rotate these two patches away from each other and interpose the bulk of one of the CCPs. This observation supports the proposition that the orientation determined crystallographically approximates the functional orientation.

The orientations of the two CCPs are determined in large measure by two considerations: first, favorable (or unfavorable) intermodular contacts can constrain the relative orientation; second, the linker sequence may adopt preferred conformations based on interactions within the linker and with each of the CCPs. There are few intermodular contacts detected either in the crystal structure or by analysis of nuclear Overhauser effects in the NMR spectra of DAF CCP2-CCP3 (9). The absence of intermodular contacts suggests that key determinants of the orientation of CCP2 and CCP3 would be present in the linker sequence and nearby loops.

To investigate whether there is a difference in conserved sequence in the linker region for complement control proteins that have DAA and CF activity, PSI-BLAST analyses were performed starting with human DAF, which possesses only DAA function, and MCP, which possesses only CF function. The results permitted a position-specific substitution matrix to be generated, which was capable of identifying proteins containing tandem CCPs that have DAA. Other attempts at aligning CCP units have focused on alignments of sequences within modules. The success of the PSI-BLAST alignment, based on the 30 res-
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idues that comprise the linker and the two adjacent strands and β turns that define the strands in DAF versus that in MCP, suggests that the linker region has a significant role in supporting the DAA of these proteins. Stronger conservation of the linker region and of the immediate residues in CCP3 adjacent to the linker supports the proposal that these residues help constrain the relative orientations of CCP2 and CCP3 needed for its DAA activity. The same CCP domains of C4bp, factor H, and CR1 can exhibit both DAA and CF activity. The proposition that distinct functional orientations are required for CF activity and DAA requires that the linker region between these CCPs be flexible enough to permit the orientations required for both DAA and CF activity to be sampled.

The PSI BLAST analyses, in conjunction with the mutagenesis insertion data, argue that in regulators that possess DAA, an open arrangement (i.e., small tilt angle) between the two CCP modules exists. The crystal structure and mapping of the epitope of mAb 8A7 both imply that this orientation is accessed in solution. This open arrangement would allow one CCP to interact with C3b or C4b and the other CCP to interact with Bb or C2a. In contrast, the greater tilt angle (less open structure) found in MCP versus versus the open arrangement would allow one CCP to

Acknowledgments—We thank Joann Mould (Drexel University) for the DAF mAbs 2D2-2 and 2D2-3 and Douglas Lublin (Washington University) for the DAF mAb 1H4.

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