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Biochemistry

Sequence determination of the thiolester site of the fourth component of human complement

(primary structure/active site/reactive acyl group/covalent bond formation/sequence homology)

RICHARD A. HARRISON, MATTHEW L. THOMAS, AND BRIAN F. TACK*

Department of Pediatrics, Harvard Medical School and Department of Medicine, Children's Hospital Medical Center, Boston, Massachusetts 02115

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ABSTRACT **The fourth component of complement (C4) is inactivated by treatment with methylamine. This property is shared with the third component (C3) and with α_2-macroglobulin. In each instance, the reaction with methylamine is stoichiometric, covalent, and accompanied by the appearance of a thiol group. These data are consistent with the presence of an internal thiolester bond. Incubation of C4 with [^{14}C] methylamine in the presence of activated thiol-Sepharose resulted in immobilization of the protein via its active-site thiol. Analysis of bound C4 indicated incorporation of 1.12 mol of [^{14}C]methylamine per mol of protein. Digestion of the immobilized protein with porcine elastase resulted in the release of C4 β- and γ-chains and lower molecular weight fragments. The ^{14}C label, however, was retained on the Sepharose beads. Subsequent release of bound material with L-cysteine indicated that the radiolabel was associated with two polypeptides of M_r 25,000 [C4d(ela25)]. The released material was dialyzed and the active-site thiol was radioalkylated with iodo[2-^{3}H]acetic acid. C4d(ela25) was further purified by chromatography on Sephadex G-100 and, after reduction and alkylation, on Sepharose CL-6B in 0.2% NaDodSO$_4$. The C4d(ela25) pool, containing 0.83 mol of [^{14}C]methylamine per mol of iodo[2-^{3}H]acetic acid, was subjected to automated sequence analysis. *S*-carboxy-[^{3}H]methylcysteine was released at step 21 and γ-glutamyl-[^{14}C]methylamide was released at step 24. The recovery of radiolabel at positions 21 and 24 confirmed the originally calculated ^{14}C/^{3}H incorporation ratio and further indicated that the radiolabels were present at single sites in the C4 molecule. Comparison of the derived primary structure for the thiolester site in C4 with those for the corresponding regions in C3 and α_2-macroglobulin has shown sequence identity. Further comparisons among these three proteins have indicated additional homologies on both the NH_2- and COOH-terminal sides of the thiolester site.**

In the classical pathway of complement (C) activation, the fourth component, C4, is cleaved by $\overline{C1s}$, a subcomponent of activated C1, into two fragments, C4a and C4b. The larger of these, C4b, has the transient ability to bind to surfaces and functions as a component of the classical pathway C3 convertase, $\overline{C4b2a}$. These properties are analogous to those of C3, which, on activation, is split into the two fragments C3a and C3b. C3b also acquires a transient ability to bind to surfaces and functions as a component of the alternative pathway C3 convertase, $\overline{C3bBb}$ (1–3). The interaction between C3b and cell surface components is now known to be, in part, covalent (4). Studies of the chemical reactivity of this bond suggested that it was an oxygen ester, and that the acyl group donor was contained in C3b. These observations led to the hypothesis that covalent bond formation occurred via a transesterification reaction (5).

Both C4 and C3 have long been known to be inactivated by treatment with nitrogen nucleophiles such as ammonia or hydrazine (6–8) and by chaotropic salts such as KCNS (9). Recently, treatment of C3 with the nucleophilic reagent methylamine, or with the chaotrope KBr, was shown to result in the loss of potential for covalent bond formation and the appearance of a single thiol group (10–13). This thiol is also released on activation of C3 to C3b (13). These data, and the observed stoichiometric relationship between uptake of methylamine into a glutamyl residue and titration of a thiol (10), led to the hypothesis that an internal thiolester exists in C3 and that covalent bond formation involves transfer of the acyl group from the thiol to a hydroxyl contained on the acceptor molecule (10, 11, 14). These studies of C3 have now been extended to C4. Treatment with nitrogen nucleophiles such as methylamine again results in the titration of a single reactive group, the release of a thiol (which is also contained in C4b), and the loss of covalent bond-forming potential (14–19). Recently, in addition to ester bonds between these molecules and binding surfaces, amide (hydroxylamine-resistant) bonds have also been proposed (20, 21).

On incubation with denaturants, both C3 and C4 undergo internal autolytic cleavage. This reaction is accompanied by the release of a single thiol, and prior release of the thiol with nitrogen nucleophiles results in loss of autolytic potential (15, 16, 22–24). Thus, the autolytic cleavage is dependent on the integrity of the internal thiolester. Nucleophile inactivation, autolytic cleavage on denaturation, and the ability to form covalent bonds (with proteases) have also been described for α_2-macroglobulin (α_2M) (25–31). We and others have shown that nucleophile inactivation again correlates with the appearance of a thiol group (10, 32–34). Thus, it appears that C3, C4, and α_2M share a common reaction mechanism. Recent structural work has shown the close proximity of the reactive acyl and thiol groups in both C3 (10) and α_2M (35) and homology between the residues surrounding them. We therefore undertook sequence determination around these groups in C4, in order that comparative analysis might permit some insight into requirements for this unique site. The results of this study are presented here and confirm those recently reported elsewhere (36).

MATERIALS AND METHODS

Chromatographic media were purchased from Pharmacia. Before use, activated thiol-Sepharose (ATS) was treated with iodoacetamide (10). [^{14}C]Methylamine (Amersham) was used at a specific activity of 0.25 mCi/mmol (1 Ci = 3.7×10^{10} becquerels) and iodo[2-^{3}H]acetic acid (New England Nuclear) had a

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Abbreviations: C, complement; C4, C1, etc., fourth, first, etc., component of C; ATS, activated thiol-Sepharose; α_2M, α_2-macroglobulin.

* Present address: Dept. of Molecular Immunology, Scripps Clinic and Research Foundation, La Jolla, CA 92037.

specific activity of 5.54 mCi/mmol. $NaDodSO_4$/polyacrylamide gel electrophoresis was carried out in a discontinuous buffer system (37). Gels were stained with Coomassie brilliant blue R250 and impregnated with 2,5-diphenyloxazole before autoradiography (38). Automated Edman degradation was carried out in a Beckman 890C instrument modified with a cold trap accessory and fitted with a P-6 autoconvertor (Sequemat, Watertown, MA). A 0.1 M Quadrol program was used (39) and two coupling cycles were run before the initial cleavage reaction. All sequence analysis reagents and solvents were from Pierce. Methanolic HCl [AcCl (Sequemat)/MeOH (1:7)] was used as the conversion reagent. Phenylthiohydantoin derivatives were identified by high-performance liquid chromatography using a Zorbax ODS column (Du Pont) equilibrated in 0.01 M sodium acetate, pH 5.5/20% acetonitrile and developed with an acetonitrile gradient modified from that described previously (40). Acetonitrile and dichloroethane were purchased from Burdick and Jackson (Muskegon, MI).

C4 was isolated from fresh human acid citrate/dextrose plasma as described (41) with the following exceptions: (*i*) it was precipitated from the 5% polyethylene glycol 4000 supernatant by the addition of solid glycol to 15% (wt/vol); (*ii*) the precipitate was dissolved in 0.03 M K/Na phosphate, pH 7.6/0.12 M NaCl/0.03 M EDTA/0.03 M benzamidine at 0°C and treated with 5 mM diisopropyl fluorophosphate; (*iii*) after overnight incubation, the fluorophosphate-treated pool was adjusted to a specific conductance of 4.0 mS (measured at 0°C) and applied to DEAE-Sephacel equilibrated with 0.01 M K/Na phosphate, pH 7.6/0.04 M NaCl/0.01 M EDTA/0.01 M benzamidine. The column was developed with a linear salt gradient (0.04–0.24 M NaCl). C4-containing fractions were identified by hemolytic assay (42), pooled, and concentrated by using a Pellicon ultrafiltration system (Millipore). This pool was then treated with ATS in 0.01 M K/Na phosphate, pH 7.6/0.2 M NaCl/0.1 M EDTA/0.01 M benzamidine at 37°C for 1 hr to remove any molecules containing free thiol groups. Unbound (native) C4 was removed by filtration. C4 concentrations were determined spectrophotometrically ($E^{1\%}_{280\ nm}$ = 8.3) (43). Elastase (Worthington; twice crystallized) was further purified by chromatography on CM-Sephadex and DEAE-Sephadex A-50 (44).

RESULTS

Enzymic Digestion of C4 Conjugated to ATS. The preadsorbed C4 pool was incubated with ATS (225 A_{280} units; 9.4 A_{280} units/ml of ATS; final vol 49 ml) in the presence of 25 mM [^{14}C]methylamine for 4 hr at 37°C. Unbound material (96 A_{280} units) was removed by filtration and discarded. Treatment of a small aliquot of the ATS–C4 conjugate with 0.01 M L-cysteine followed by $NaDodSO_4$/polyacrylamide gel electrophoresis of the released protein showed the three-polypeptide chain structure of C4 to have been preserved. The released C4 had incorporated 1.12 mol of [^{14}C]methylamine per mol of protein.

Initial attempts to isolate the [^{14}C]methylamine-containing peptide after digestion of the ATS–C4 conjugate with trypsin in 4 M urea (10) were unsuccessful. Accordingly, the polypeptides produced by digestion with elastase were investigated. The ATS–C4 conjugate was washed with 0.1 M Tris·HCl, pH 8.0/0.01 M EDTA and suspended in 25 ml of this buffer, and 200-μl aliquots of the gel suspension were then incubated for 1 hr at 37°C with elastase (Fig. 1). At all elastase concentrations used, the released material contained undigested β- and γ-chains, as well as some low molecular weight material (Fig. 1 *Left*, tracks 1–4). However, essentially total digestion of the C4 α-chain, with concomitant release of β- and γ-chains, was seen only at protein/enzyme ratios (mol/mol) of 6.25:1 and 3.12:1. In these samples, a characteristic doublet with an apparent M_r of 25,000 was observed (tracks 8 and 9). Fluorographic analysis showed all of the [^{14}C]methylamine to be incorporated into the doublet (Fig. 1 *Right*), which has been designated C4d(ela25).

Preparation of C4d(ela25). The preliminary experiments indicated that high enzyme/protein ratios were required for complete cleavage of the C4 α-chain. Conditions for large-scale digestion were selected with the intent (*i*) to maximize release of C4 β- and γ-chains and (*ii*) to maximize production of the lower molecular weight component of the doublet retained on the ATS. Thus, 24 ml of ATS–C4 conjugate was digested with elastase at a protein/enzyme ratio (mol/mol) of 6.25:1 for 6 hr at 37°C. A second portion of elastase equal to the first was then added, and digestion was continued for 10 hr at 37°C. Enzymatically released material was removed by filtration and the

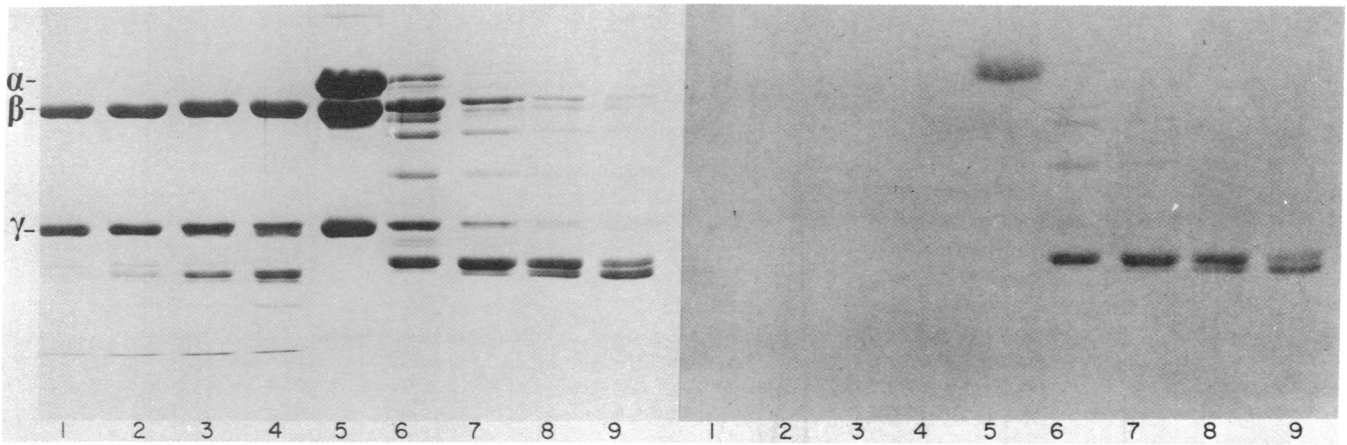

FIG. 1. Elastase digestion of [^{14}C]methylamine-inactivated C4 bound to ATS. After digestion, aliquots were centrifuged to pellet the gel and 270 μl of each supernatant was added to 30 μl of 0.1 M Tris·HCl, pH 8.0/0.1 M EDTA/10% $NaDodSO_4$/0.1 M dithiothreitol. The pellets were washed with two 0.5-ml aliquots of digestion buffer and then with two 0.5-ml aliquots of buffer/1% $NaDodSO_4$. They were then suspended in 100 μl of 0.1 M Tris·HCl, pH 8.0/0.1 M EDTA/1% $NaDodSO_4$/0.01 M dithiothreitol, and the degree of digestion was assessed by $NaDodSO_4$/polyacrylamide gel electrophoresis. (*Left*) $NaDodSO_4$/7.5% polyacrylamide gel electrophoresis of protein released by enzymatic digestion (tracks 1–4); of protein released, subsequent to digestion, by incubation of the gel with 0.1 M dithiothreitol (track 5); and of material released by incubation with 0.1 M dithiothreitol after enzymatic digestion (tracks 6–9). (*Right*) Autoradiographic analysis of the same gel. The positions of the α-, β-, and γ-chains of C4 are indicated. Protein/enzyme ratios (mol/mol): tracks 1 and 6, 31.2:1; tracks 2 and 7, 12.5:1; tracks 3 and 8, 6.25:1; tracks 4 and 9, 3.12:1.

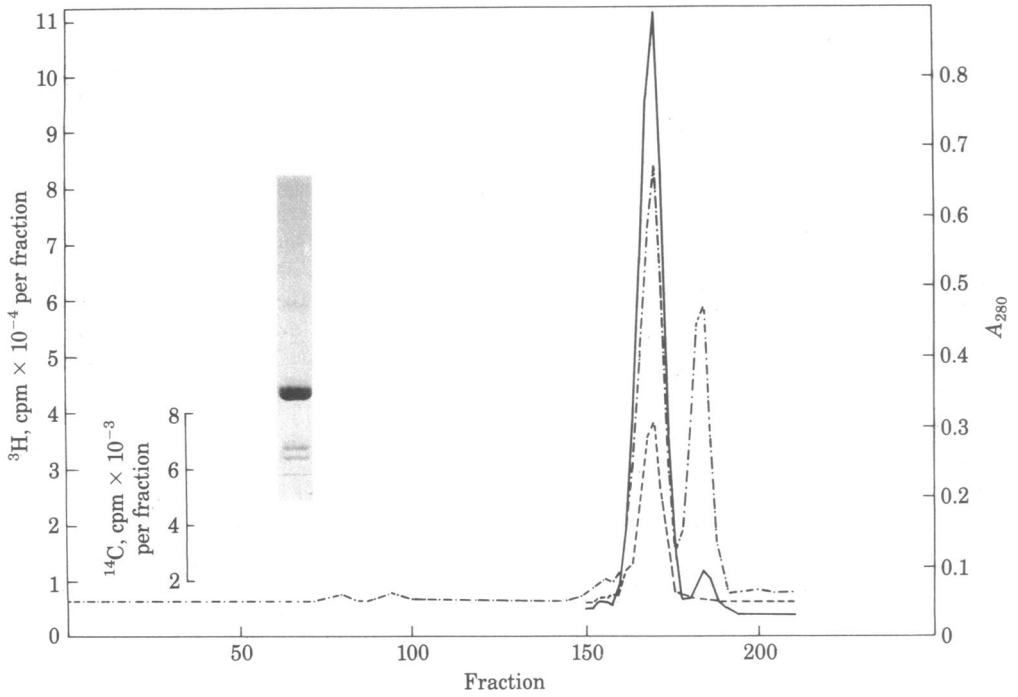

FIG. 2. Chromatography of C4d(ela25) on Sepharose CL-6B. The column (210 × 1.5 cm) was equilibrated with 0.01 M Tris·HCl, pH 8.0/1 mM EDTA/0.2% NaDodSO$_4$. (*Inset*) NaDodSO$_4$/polyacrylamide gel electrophoresis of the C4d(ela25) pool loaded onto the column. Curves: ——, ^{3}H; ----, ^{14}C; –·–, A_{280}.

gel was washed to ensure total removal of protease. The gel slurry was then suspended in 0.1 M Tris·HCl, pH 8.0/0.1 M EDTA/0.01 M L-cysteine and left at 4°C for 12 hr to equilibrate, and the released protein was collected by filtration. After lyophilization, the released material was redissolved in distilled water, dialyzed against 0.1 M Tris·HCl, pH 8.5/0.01 M EDTA/1 mM L-cysteine, and carboxymethylated by the addition of 60 μmol of iodo[2-^{3}H]acetic acid. Unreacted iodo[2-^{3}H]acetate was removed by reaction with excess L-cysteine and dialysis against 0.02 M K/Na phosphate, pH 7.0/1.0 M NaCl. Since

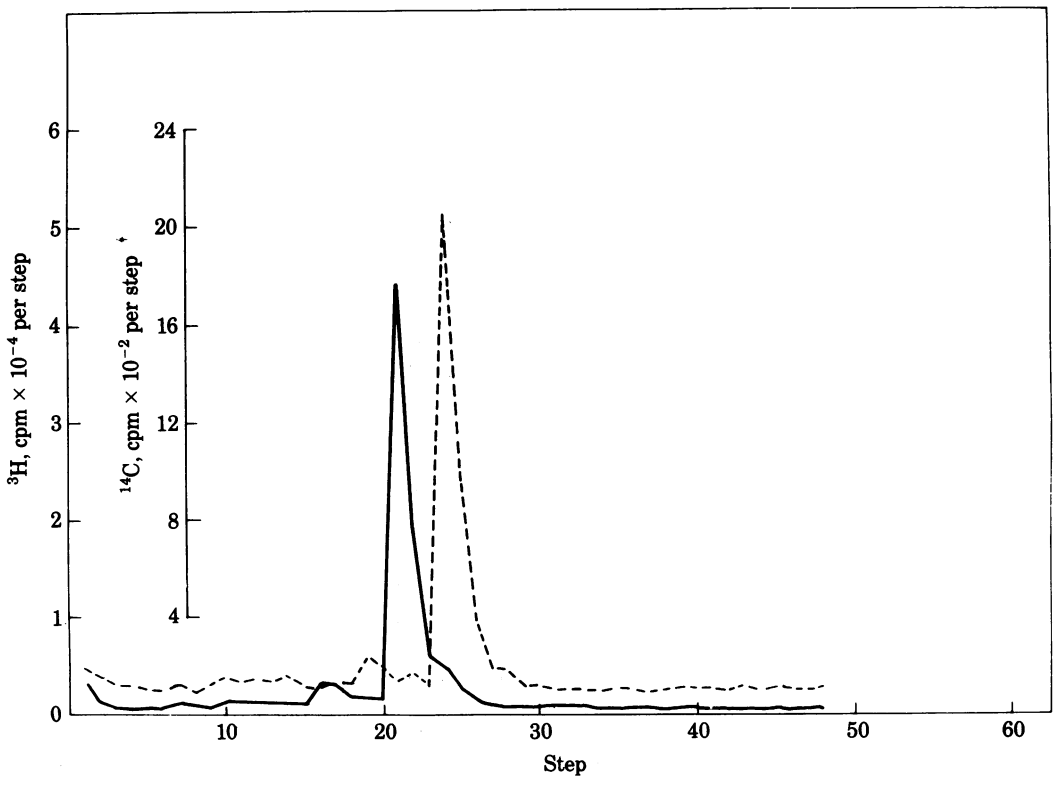

FIG. 3. ^{3}H (——) and ^{14}C (----) radioactivity released on Edman degradation of C4d(ela25).

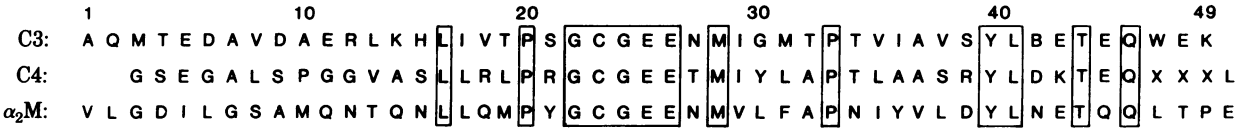

FIG. 4. NH_2-terminal amino acid sequence of C4d(ela25) and comparison with sequences around the thiolester sites in C3 (45) and α_2M (35). Alignment is based on the residues containing the cysteinyl and glutamyl groups involved in the internal thiolester. Numbering is for the elastase-derived C3d fragment. Thus, the cysteinyl residue at position 21 of the C4d(ela25) sequence aligns at position 23 of the C3d sequence, and the reactive glutamyl residue at position 24 aligns at position 26 of the C3d sequence. Positions of identity in all three proteins are boxed. A, Ala; B, Asx; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; X, unassigned; Y, Tyr.

preliminary experiments had shown the presence of appreciable amounts of higher molecular weight material, including some β- and γ-chains, the dialyzed protein was concentrated to 4 ml and applied to a Sephadex G-100 superfine column (110 × 3 cm) equilibrated in dialysis buffer. Although there was some separation of the characteristic C4d(ela25) doublet from other proteins, particularly a lower molecular weight polypeptide having an iodoacetic acid-reactive group, there was still a considerable amount of higher molecular weight polypeptide associated with the major C4d(ela25) peak. Fractions containing the C4d(ela25) polypeptide were therefore pooled, dialyzed against distilled water (three times, 500 ml each), and lyophilized. The lyophilized protein was redissolved in 1 ml of 0.1 M Tris·HCl, pH 8.5/9.0 M urea, reduced by the addition of 2 μmol of dithiothreitol, and carboxamidomethylated by the addition of 10 μmol of iodoacetamide. Alkylation was terminated by the addition of 500 μmol of 2-mercaptoethanol, and excess reactants were removed by dialysis against 0.1 M Tris·HCl, pH 8.0/1 mM EDTA/0.2% $NaDodSO_4$. The dialyzed protein was then applied to a Sepharose CL-6B column. The elution profile from this column is shown in Fig. 2. Fractions 160–175, containing essentially all of the [^{14}C]methylamine label and the bulk of the iodo[2-^{3}H]acetate label, were pooled, dialyzed against distilled water, and lyophilized. The overall yield, based on recovery of [^{14}C]methylamine, was 22%. This represented 2.4% of the A_{280} units contained in the C4 pool and 4.5% of the A_{280} units specifically bound to ATS. Assuming similar extinction coefficients for C4 and C4d(ela25), these represent yields of 17.8% and 33.3%. The stoichiometry of incorporation of radiolabel into C4d(ela25) was 0.83 mol of [^{14}C]methylamine per mol of iodo[2-^{3}H]acetate.

NH_2-Terminal Sequence Analysis of C4d(ela25). In a typical sequencer run, 46 nmol of C4d(ela25) in distilled water was applied to the cup. The initial recovery was 22 nmol (48%), and the repetitive yield, calculated from the recovery of proline at steps 8, 18, and 31, was 95%. The profiles of ^{14}C and ^{3}H radioactivities released at each cycle are shown in Fig. 3. A single peak of ^{3}H was released at step 21 and one of ^{14}C was released at step 24. A single sequence was followed for 50 cycles (Fig. 4). High-performance liquid chromatography indicated that *S*-carboxymethylcysteine was released at position 21. The actual recovery, calculated from the ^{3}H released and the specific activity of iodo[2-^{3}H]acetic acid, was 7.6 nmol. The theoretical recovery, calculated from a repetitive yield of 95% and recovery at the initial step of 22 nmol, was 7.9 nmol. The ^{14}C radioactivity was associated with a novel peak eluting between threonine and glycine. This position corresponded to that occupied by the ^{14}C-labeled residue in C3 and was identified, after back hydrolysis, as glutamic acid (10). The recovery at this position, calculated from the ^{14}C released and the specific activity of [^{14}C]methylamine, was 5.8 nmol. In addition, 1.2 nmol of unmodified glutamic acid was released at this position. The theoretical recovery for this residue was 6.8 nmol. These figures confirm the stoichiometry of uptake [^{14}C label recovered at position 24/^{3}H label released at position 21 (mol/mol), 0.76:1; after correction for loss through three sequencer cycles, this becomes 0.88:1] and further indicate that the respective radiolabels are contained within single sites in the C4 molecule.

DISCUSSION

Our primary objective was to obtain an extended sequence around the reactive acyl group in C4 such that comparison with the corresponding regions in C3 and α_2M could be made. For this reason, an approach analogous to that used previously for C3 (10) was made. Proteolytic digestion of the protein immobilized, via the thiol group released from the internal thiolester, on ATS has several attractive features. In particular, high concentrations of protease can be used, enhancing the prospects of obtaining a "limit" peptide. This is possible as the protease can subsequently be removed from the bound peptide by repeated washing of the gel. The approach also permits selection of a protease and digestion conditions such that cleavage close to the thiol can be obtained, enabling direct sequence determination in the region of interest. In this instance, the approach was only partially successful as a limit peptide was not obtained. However, autoradiographic analysis showed that, at high enzyme concentrations, the [^{14}C]methylamine label contained in the C4 α-chain was converted quantitively to two polypeptides with molecular weights of ≈25,000, C4d(ela25). NH_2-terminal sequence analysis of C4d(ela25) showed a single sequence and, thus, the heterogeneity can be deduced to lie at the COOH-terminal end of the molecule and to be inconsequential in this study.

The sequence around the reactive acyl group represents the third such structure determined; in Fig. 4, it is compared with the analogous sequences determined for C3 (10, 45) and α_2M (35). Striking homology is apparent. Within the region containing residues 3–46, there is 30% identity between the three proteins. If the region containing residues 16–46 only is considered, the homology increases to 42% and, if comparison is limited to C3 and C4, the values increase to 44% and 56%. (For C3 and α_2M, the values are 35% and 50%, and for C4 and α_2M, they are 36% and 48%.) This homology suggests considerable constraint on this region of each protein. The pentapeptide Gly-Cys-Gly-Glu-Glu (positions 22–26) is of particular note because it is present in each protein and contains the reactive acyl group (glutamate-26) that is specifically labeled with [^{14}C]methylamine and the thiol group (cysteine-23) that becomes reactive after methylamine incorporation. We have previously proposed that, in C3, an internal thiolester bond is formed between these groups (10). A space-filling model of this sequence has been made and indicates the steric feasibility of such a bond (45). The existence of the same pentapeptide sequence in all three proteins further strengthens this hypothesis. A second striking homology is that the pentapeptide is flanked by two prolyl resi-

dues (positions 20 and 33). These would impose steric constraints on this region and could well serve to stabilize an internal thiolester bond. Stabilization could also be afforded by the conserved hydrophobic nature of the regions comprising residues 16–20 and 28–41. These regions, flanking the proposed thiolester site, contain a further four invariant residues (leucine-16 and -41, methionine-28, and tyrosine-40), as well as conservative replacements at positions 17, 29–31, and 35–37. The region comprising residues 42–46 is probably best considered separately because the asparagine residue at position 42 in α_2M has been shown to be a site of carbohydrate attachment (35) while, in C4, only aspartic acid was detected and, in C3, the yields at this position were low and did not permit unambiguous assignment. Thus, the conserved residues threonine-44 and glutamate-46 may well relate to a potential for carbohydrate attachment (46).

Clearly, the thiolester site comprises a highly conserved region of the three proteins. However, differences in sequence are also noteworthy, and these may well contribute to specificity in the binding reaction. The NH_2-terminal regions (up to position 16) are of particular interest as there is no overall homology and only limited homology between C3 and C4 (glutamate-5 and alanine-7 and conservative replacements at positions 4, 8, and 13). In C3, this region contains seven charged groups and probably lies on the surface of the molecule, whereas in C4 and α_2M, single charged residues only are found, suggesting the regions to be at least partially interior. In addition, there is a strong potential for a β-turn (47, 48) around the proline at position 10 in C4 but no such potential in the coincident regions in C3 and α_2M. C4 also has differences to C3 and α_2M in the highly conserved region 16–46, notably the inclusion of four basic residues (arginine-18, -21, and -39 and lysine-43). The residues at positions 18, 21, and 39 are of particular note as these are the only positions in this region in which there is neither partial nor conservative homology between the three proteins.

Thus, with the determination of the sequence around the reactive acyl group in C4, it has been possible to identify structural features common to three proteins that form covalent bonds with a variety of unrelated molecules. Some may well be absolute requirements for a common reaction mechanism. Other features are variable and may provide, in part, the basis for specificity of binding. Although further identification of these will require either definition of the spatial arrangement of the residues around the proposed thiolester or identification and sequence analysis of other proteins sharing this reaction mechanism, knowledge of these sequences provides a framework for an understanding of the site.

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