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Covalent Binding Efficiency of the Third and Fourth Complement Proteins in Relation to pH, Nucleophilicity, and Availability of Hydroxyl Groups†

Sai-Kit A. Law, Tana M. Minich, and R. P. Levine*

ABSTRACT: The binding of [^{3}H]glycerol and [^{3}H]putrescine to C3 was studied in a fluid-phase system using trypsin as the C3 convertase. The binding of glycerol showed little variation in the pH range between 6.0 and 10.0. The binding of putrescine (pK_a = 9.0) is rather ineffective below pH 7.5 but becomes more efficient as the pH of the reaction mixture increases. These results agree with the contention that the final step of the binding reaction is the transfer of the acyl group of the exposed thio ester of C3 to a nucleophile since the nucleophilicity of hydroxyl groups is rather independent of pH whereas only the unprotonated form of amino groups is nucleophilic. The inefficient reaction of amino groups with the exposed thio ester of C3 is also supported by the study of the inhibitory activity of serine and its two derivatives, *N*-acetylserine and *O*-methylserine, to the binding of [^{3}H]glycerol to C3. *N*-Acetylserine showed an inhibitory activity equivalent to that of serine, whereas O-methylated serine showed only minimal activity. It can be concluded, therefore, that serine reacts with the thio ester of C3 by its hydroxyl group but not by its α-amino group. The ability of the alcohol group of various alkanes to inhibit the binding of [^{3}H]glycerol to C3 was also studied. The primary alcohols inhibit the binding reaction with an efficiency that is similar to glycerol, and there are no significant differences in the binding efficiencies of methanol, ethanol, 1-propanol, and 1-butanol. Hence, the length of the alkyl chain does not appear to affect the reactivity of the hydroxyl group with the exposed thio ester. On the other hand, secondary and tertiary alcohols are less effective in their inhibition of the binding reaction, indicating that the thio ester, though exposed, is not freely accessible to bulkier molecules. The binding of [^{3}H]glycerol to C4 shows a pH dependence that is similar to C3. However, the binding efficiency of [^{3}H]putrescine to C4 is significantly higher in the pH range 5.0–10.0. These results suggest that there are some differences between the binding sites of C3 and C4, though the differences are not defined at present. The elevated activity of the exposed thio ester of C4 to amino groups may be of physiological significance, since the binding of C4b to IgG of immune complexes by amide bonds may be important in the efficient activation of the classical pathway of complement.

The covalent binding reaction of the third complement protein C3[1] is an integral part of its functional activity (Law et al., 1980b). It allows the protein to bind to all cell surfaces capable of activating the classical and/or the alternative pathways of complement [for review, see Law (1983b)], and it is currently understood as an acyl transfer reaction (Law et al., 1979; Sim et al., 1981) in which an internal thio ester (Janatova et al., 1980; Pangburn & Müller-Eberhard, 1980; Tack et al., 1980; Law et al., 1980b) between a cysteinyl residue and a glutamyl residue in C3 (Tack et al., 1980) becomes available for reaction upon the proteolytic activation of C3 to C3a and C3b* (C3b* is defined as the active state of the C3b molecule). The thio ester can react with water or other molecules that contain either amino or hydroxyl groups in the medium. If, however, C3 is activated sufficiently close to a cell surface, C3b* can react with surface-bound hydroxyl or amino groups to covalently link C3b to the cell surfaces via ester or amide bonds (Müller-Eberhard et al., 1966; Law & Levine, 1977; Law et al., 1979).

We have established a fluid-phase system to study the covalent bonding reaction of C3b* (Law et al., 1981). Radioactive small molecules (S) are incorporated specifically at the binding site of C3b with trypsin as the C3 convertase (Hostetter et al., 1982). The fluid-phase system has the advantage of being comprised solely of defined molecular components in a medium where kinetic parameters are defined. Uncertainties arising from the heterogeneity of cell-surface molecules and the molecular diffusional properties at cell surfaces are eliminated. Using this system, we have shown that the basic scheme of the binding reaction is

† From the James S. McDonnell Department of Genetics, Washington University School of Medicine, St. Louis, Missouri 63110 (T.M.M. and R.P.L.), and the MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, Oxford, U.K. (S.-K.A.L.). *Received October 19, 1983.* This research was supported by National Institutes of Health Grant AI16543. S.-K.A.L. is currently a Lister Institute Research Fellow.

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; SBTI, soybean trypsin inhibitor; $NaDodSO_4$, sodium dodecyl sulfate; PPO, 2,5-diphenyloxazole; Me_2POPOP, 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene; C3 and C4, third and fourth complement proteins; C3b* and C4b*, the active states of C3 and C4 induced by proteases; C3* and C4*, the active states of C3 and C4 induced by nonproteolytic reagents; S, substrate molecule in the binding reaction; I, inhibitor of the binding reaction; BE, binding efficiency; IgG, immunoglobulin G. The nomenclature of the complement proteins is that recommended by the World Health Organization (1968).

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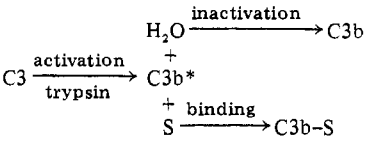

where S is the substrate molecule, C3b–S the covalently linked complex of C3b and S, and C3b* the active form of C3b. Furthermore, by showing that the binding reaction can also be initiated by chaotropes and denaturants, we concluded that the key feature in the activation mechansim is the physical exposure of the internal thio ester. Different reagents, including proteases, activate the binding reaction by inducing the necessary conformational change of the protein (Law, 1983a). Binding, therefore, is the reaction between the exposed thio ester with the substrate molecules.

In this paper, we report the studies of the binding of C3 to various alcohols and amines. Where the substrate molecules were not available in radioactive form, their effect on a standard binding reaction (the binding of [^{3}H]glycerol to C3 under specific conditions) was used as an indirect measurement of their binding activity with C3b* (see Appendix). The results of these experiments suggest that the nucleophilic nature of the substrate molecules is responsible for the binding reaction and that the binding efficiencies of different molecules may be governed by the conformation and environment around the thio ester site of C3b*.

To date, two other proteins have been documented to contain the same internal thio ester. They are the fourth complement protein C4 (Law et al., 1980a,b; Campbell et al., 1980, 1981; Gorski & Howard, 1980; Janatova & Tack, 1981; Harrison et al., 1981) and the serum protease inhibitor α_2-macroglobulin (Swenson & Howard, 1980; Salvesen et al., 1981). They also possess the ability to bind covalently to other molecules upon activation (Campbell et al., 1980, 1981; Law et al., 1980a; Law, 1983b; Salvesen & Barrett, 1980; Salvesen et al., 1981). In this paper, studies on the binding reaction of C4 similar to that of C3 are also reported.

Materials and Methods

Proteins and Reagents. C3 was purified from either pooled fresh human plasma or pooled outdated human plasma according to Hammer et al. (1980). C4 was purified from pooled outdated plasma by a modified procedure of Bolotin et al. (1977). Briefly, the euglobulin at pH 7.5 was fractionated on a DEAE-Sephacel column. The C4-containing fractions were further purified on a QAE-Sephadex column followed by a DEAE-Bio-Gel A column. The purified proteins were stored at 4 °C in the presence of 0.01% sodium azide (Law et al., 1983a). $\overline{C1s}$ was a gift from L. P. Chung of the MRC Immunochemistry Unit, University of Oxford, U.K.

N-Acetylserine and *O*-methylserine were obtained from Vega Biochemicals, Tucson, AZ. Trypsin, soybean trypsin inhibitor (SBTI), putrescine dihydrochloride, methylamine hydrochloride, and serine were from Sigma Chemical Co., St. Louis, MO. [2-^{3}H]Glycerol (200 mCi/mmol) and [^{14}C]-methylamine hydrochloride (44 mCi/mmol) were products of New England Nuclear, Boston, MA. [2-^{3}H]Glycine (15 Ci/mmol), [1,4(N)-^{3}H]putrescine dihydrochloride (19 Ci/mmol), and iodo[2-^{3}H]acetic acid (106 mCi/mmol) were from the Radiochemical Centre, Amersham, Bucks, U.K. NCS tissue solubilizer was from Amersham Corp., Arlington Heights, IL.

Binding of [^{3}H]Glycerol and [^{3}H]Putrescine to C3 and C4 at Different pH Values. [^{3}H]Glycerol was mixed with glycerol to give a specific radioactivity of 50 mCi/mmol. The volume of the solution was reduced in a Speed Vac concentrator (Savant Instruments, Hicksville, NY) such that the glycerol concentration was about 10 times that in the final reaction mixture. Buffers containing 200 mM acetate, 200 mM phosphate, and 200 mM borate, sodium salts, at different pH values ranging from 5.0 to 10.5 were prepared.

In a representative experiment, 7.5 μL of concentrated [^{3}H]glycerol, 37.5 μL of buffer of the appropriate pH value, and 15 μL of trypsin (0.067 mg/mL, diluted from a 1 mg/mL stock in 1 mM HCl with water) were added to each reaction tube. Finally, 15 μL of C3 (6.9 mg/mL in 25 mM potassium phosphate, 5 mM EDTA, 100 mM NaCl, pH 7.5) was added. The reaction mixture was incubated at 37 °C for 5 min, at which time the reaction was stopped by the addition of 75 μL of a solution of 1 M glycerol containing SBTI at 0.027 mg/mL. Trichloroacetic acid was added to the mixture to a final concentration of 10% to precipitate the proteins, and the precipitates were washed once with 5% trichloroacetic acid followed twice with acetone. They were then solubilized by incubation in 200 μL of 2.5% $NaDodSO_4$ at 80 °C for 2 h. The protein concentration of the samples and the radioactivity in association with the proteins were determined. For controls, preformed C3b was added in place of C3, and trypsin was omitted from the reaction mixture. The identical procedure was followed in order to determine the binding of [^{3}H]putrescine to C3.

For the binding of small molecules to C4, $\overline{C1s}$ was used as the convertase. The incubation time was 30 min. No protease inhibitor was added to stop the reaction since C4 is a specific substrate of $\overline{C1s}$ whereas C4b, the end product of the reaction, is not.

In the case where guanidine hydrochloride was used as the activating reagent, 8 M guanidine hydrochloride was added to the reaction mixture to a final concentration of 1 M. The incubation was carried out for 60 min. C3 or C4 inactivated in guanidine hydrochloride was used in control experients. In some experiments, the removal of unbound radioactivity from proteins was achieved by dialysis.

Since the pH of the reaction mixtures was not determined directly because of their small volumes, the pH of identical proportional mixtures of component buffers, less proteins and radioactive materials, was measured. These mixtures were estimated to approximate the pH of the acetate/phosphate/borate buffer to within 0.1 pH unit.

Since C3 and C4 lose activity upon storage, the activity of the proteins was determined for each experiment. The [^{14}C]methylamine binding capacity of the proteins was determined according to Law et al. (1980b). Alternatively, the specific appearance of the free sulfhydryl group in the presence of methylamine was quantitated with iodo[^{3}H]acetic acid (Janatova et al., 1980).

Inhibitory Effects of Nonradioactive Molecules on a Standard Binding Reaction. The binding of small molecules not readily available in radioactive form was studied by measuring their inhibitory effects on a standard binding reaction. The inhibiting small molecule was added to a solution of [^{3}H]glycerol (8.3 mM, 50 mCi/mmol) and C3 (about 5 mg/mL) and incubated in the presence of trypsin (0.8% w/w to C3) at pH 7.5, 37 °C, for 5 min.

$NaDodSO_4$–Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out in the Laemmli system (Laemmli, 1970) under reducing conditions using slab gels of 7% polyacrylamide. The gels were stained and destained according to Fairbanks et al. (1971).

Assays. Protein concentrations were determined by the method of Lowry et al. (1951). To determine the radioactivity

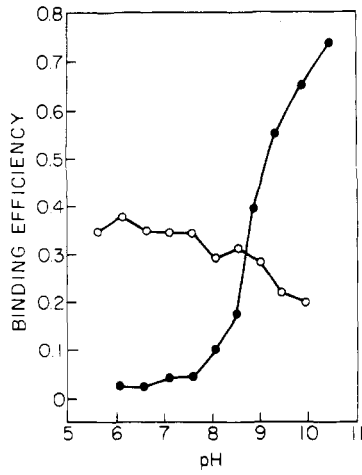

FIGURE 1: Binding of [^{3}H]glycerol (○) and [^{3}H]putrescine (●) to C3b* as a function of pH. The concentration of glycerol was 24 mM and that of putrescine was 14 mM. Trypsin was used as the convertase to activate the C3 binding reaction.

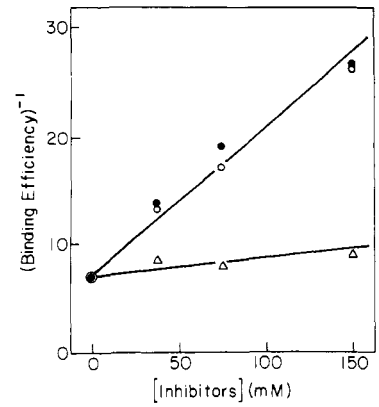

FIGURE 2: Inhibition of [^{3}H]glycerol binding to C3b* by serine (○), *N*-acetylserine (●), and *O*-methylserine (△). Different concentrations of serine, *N*-acetylserine, and *O*-methylserine were used to inhibit the binding of [^{3}H]glycerol to C3 in the presence of trypsin. The concentration of glycerol in these experiments was 8.3 mM. The pH of the reaction mixtures was 7.5.

in association with proteins, the polypeptides of the proteins were first separated by $NaDodSO_4$–polyacrylamide gel electrophoresis. The stained bands were cut out and solubilized in 1.5 mL of NCS tissue solubilizer (9:1 v/v, with respect to water) at 56 °C for 2 h. A 15-mL portion of toluene base scintillation fluid, containing PPO (5 g/L) and Me_2POPOP (0.3 g/L), was added. The counting efficiency for ^{3}H is 33%, and that for ^{14}C is 82%.

Results and Discussion

Binding of [^{3}H]Glycerol and [^{3}H]Putrescine to C3b as a Function of pH.* The direct binding of [^{3}H]glycerol (24 mM, 50 mCi/mmol) and [^{3}H]putrescine (14 mM, 25 mCi/mmol) to C3b* was measured at different pH values. As the results in Figure 1 show, the binding to glycerol at pH 6 is about twice that at pH 10. Thus, in agreement with Sim et al. (1981), who studied binding of ^{125}I-C3b to Sepharose–trypsin, the binding of glycerol to C3b* is more efficient at lower pH. On the other hand, the pH dependence of putrescine binding to C3b* is quite different, since the binding efficiency increases with increasing pH.

These results support the suggestion that binding occurs by a nucleophilic attack on the exposed thio ester in C3b* by hydroxyl or amino groups. The nucleophilicity of hydroxyl groups is rather insensitive to pH; hence, glycerol, which binds to C3b* by virtue of its hydroxyl groups, shows little variation across the range of pH studied. The slight drop in binding efficiency at higher pH can be accounted for by the increasing presence of the hydroxide ion, a strong nucleophile. On the other hand, only the unprotonated form of the amino group is nucleophilic, and accordingly, the binding efficiency of putrescine varies at different pH values as shown in Figure 1. The curve approximates the deprotonation of the first amino group of putrescine ($pK_a \simeq 9.0$).

Binding of Serine to C3b.* In a previous report (Law et al., 1981), the binding of [^{3}H]serine to C3 was studied. The C3b–serine complex is likely to be amide linked since treatment of the complex with hydroxylamine at alkaline pH does not lead to the dissociation of [^{3}H]serine from the protein. One can conclude that serine reacts with C3b* via its α-amino group. It is also possible, however, that serine reacts initially with C3b* via its hydroxyl group to form an ester-linked intermediate that subsequently undergoes an intramolecular O → N shift to yield the final amide-linked complex. Since it was unclear from these experiments whether serine reacts with C3b* via its hydroxyl or α-amino group, we investigated the inhibitory effect of serine and its two derivatives, *N*-acetylserine and *O*-methylserine, on the binding of [^{3}H]glycerol to C3b*. The results are shown in Figure 2. Whereas serine and *N*-acetylserine show a similar inhibitory effect on the binding of glycerol to C3b*, the inhibitory activity of *O*-methylserine is minimal. Because the methylation of the hydroxyl group abolishes the inhibitory activity of serine and the acetylation of the α-amino group does not, we conclude that at neutral pH the reaction of serine with C3b* is predominantly by way of its hydroxyl group and that an intramolecular rearrangement yields the final hydroxylamine-resistant C3b–serine complex.

The results of this experiment, and those described in the previous section, are consistent with earlier observations that the binding of ^{125}I-C3b to cell-surface molecules is mostly by way of ester (hydroxylamine-sensitive) bonds (Law & Levine, 1977). It must be stressed that direct reaction of amino groups with the exposed thio ester of C3b* can occur, though not efficiently, at neutral pH in a fluid-phase system. The binding of C3b to immune aggregates by way of an amide (hydroxylamine-resistant) linkage has been described (Gadd & Reid, 1980).

Effect of Alcohols on the Binding of [^{3}H]Glycerol to C3b.* It has been reported that the thio ester in native C3 is not freely accessible to nucleophiles. Only those of minimal size are at all effective in inactivating C3; a few examples are methylamine (Law et al., 1980), hydroxylamine (Budzko & Müller-Eberhard, 1969), and hydrazine (Müller-Eberhard, 1961). It is remarkable that ethylamine, though it differs from methylamine by only a methyl group, is only $^1/_{40}$th as efficient as methylamine in inactivating C3 (Pangburn & Müller-Eberhard, 1980).

The thio ester of C3b* is more accessible as evidenced by the binding of a larger variety of molecules to the binding site (Law et al., 1981). Nonetheless, molecules of very similar sizes and molecular structures bind with different efficiencies to C3b* (Law et al., 1981). We therefore studied the inhibitory effects of the alcohols of simple alkanes on the binding of [^{3}H]glycerol to C3b* as a simple model system in order to gain insight into the fine structure of the binding site of C3b*. The results are shown in Table I. The k_i values (see Appendix) expressed as a ratio to the k' value of glycerol, for the primary alcohols, are similar, indicating that the length of the alkyl chains attached to the hydroxyl group does not affect its reaction with the thio ester. However, the secondary and tertiary

Table I: Inhibition of [^{3}H]Glycerol Binding to C3b* by Alcohols[a]

inhibitors (0.1 M)	BE	$k_i[I]/k'$[glycerol]	k_i/k'
none	0.113	0	
glycerol	0.047	12.7	1[b]
methanol	0.044	14.1	1.12
ethanol	0.043	14.4	1.13
1-propanol	0.041	15.8	1.25
2-propanol	0.080	3.6	0.28
1-butanol	0.042	15.2	1.20
2-butanol	0.086	2.8	0.22
2-methyl-2-propanol	0.117	−0.3	−0.02

[a] Various alcohols, at a final concentration of 0.1 M, where added to the standard binding reaction: the binding of [^{3}H]glycerol (8.3 mM) to C3 in the presence of trypsin (0.8% w/w to C3), pH 7.5, 37 °C. BE and k' are defined for glycerol. Calculations are done according to the equations in the Appendix. [b] By definition.

alcohols are significantly less effective in inhibiting the binding reaction. It is therefore concluded that the protein structure must pose some restriction on the reactivity of the "exposed" thio ester.

Part of the protein structure must pose restriction on the accessibility of the thio ester of C3b* to bulkier molecules. The description of the nature of the restriction awaits further studies on the three-dimensional structure around the binding site (Levine et al., 1983).

Binding Reaction of C4. The description of C4 as a thio ester containing protein (Law et al., 1980b; Janatova & Tack, 1981; Harrison et al., 1981; Campbell et al., 1981) and the binding of C4b to surfaces by a covalent bond (Law et al., 1980a; Campbell et al., 1980) have been well documented. The binding reaction, perhaps for the reason that it has been assumed to be similar to that of C3, has not been well characterized, however, and there are fine distinctions between the binding properties of C3 and C4. The release of C4b from membrane polypeptides by alkaline hydroxylamine is not as complete as that for C3b under identical conditions, suggesting that there might be some amide-linked (hydroxylamine-resistant) complexes (Law et al., 1980a). Campbell et al. (1981) reported the efficient incorporation of [^{14}C]putrescine into C4 in the presence of $\overline{C1s}$ at neutral pH. It was speculated that, in contrast to C3, the activated form of C4, C4b*, may have preference for amino groups over hydroxyl groups as its binding substrate.

Recently, it has been reported that the binding efficiency of [^{3}H]glycerol to C4b* is similar to that of C3b* (Law, 1983b; also see Figure 3A). The preference of C4b* for amino groups does not appear to be the consequence of its diminished reactivity with hydroxyl groups, but rather of its enhanced reactivity with amino groups.

The binding of [^{3}H]glycerol and [^{3}H]putrescine to C4 was studied by using $\overline{C1s}$ to activate C4. The binding of the radioactive molecules to C3 was carried out in parallel for purposes of comparison with trypsin as the C3 convertase. The results are shown in Figure 3. Whereas the binding of glycerol (Figure 3A) as a function of pH is similar for the two proteins, that of putrescine (Figure 3B) is quite different. The binding of putrescine to C4 is more efficient across the pH range studied.

In another experiment, [^{3}H]putrescine was bound to C3 and C4 in the presence of 1 M guanidine hydrochloride, which was used to activate the binding reaction. In this way, the binding of putrescine to C3 and C4 can be measured under identical conditions. Moreover, we were able to extend the pH range in the experiment to cover from 5.0 to 10.0. This was not

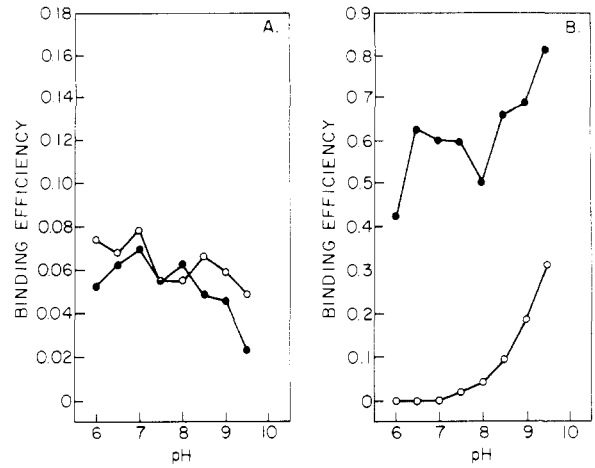

FIGURE 3: Binding of [^{3}H]glycerol (A) and [^{3}H]putrescine (B) to C3 (○) and C4 (●) as a function of pH. The concentration of glycerol was 10 mM and that of putrescine was 16 mM. Trypsin and $\overline{C1s}$ were used as the convertases for C3 and C4, respectively.

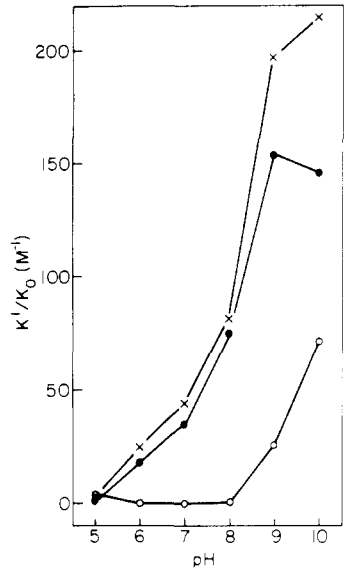

FIGURE 4: Binding of [^{3}H]putrescine to C3 (○) and C4 (●) as a function of pH using guanidine hydrochloride as the activating reagent. A mixture of C3 or C4, each at a final concentration of about 1 mg/mL, was incubated with [^{3}H]putrescine (3.6 mM) at different pHs in the presence of guanidine hydrochloride (1 M). The binding of [^{3}H]putrescine to C3 and C4 was determined by measuring the specific incorporation of radioactivity to the C3α and C4α polypeptides, respectively, after the polypeptides were first separated by NaDodSO$_4$–polyacrylamide gel electrophoresis. The k'/k_0 values were obtained by using the equations in the Appendix. The k'/k_0 values for the binding of [^{3}H]glycine (5 mM) to C4 (×) at different pHs are also shown.

possible in the previous experiment because of the diminished activities of both trypsin and $\overline{C1s}$ at the extremes of the pH range. The results obtained are similar to those using proteases to activate the reaction. Shown in Figure 4 are the reaction rates of putrescine, expressed in k'/k_0 values (see Appendix), with C3* and C4* as a function of pH.

Gorski et al. (1982) reported the elevated reactivity of diamines, putrescine and 1,3-diaminopropane, in comparison with monoamines, with the binding site of C4b*. They conjectured that the second amino group of the diamines plays a catalytic role in the binding reaction. However, they failed to observe that the first pK_a of the diamines is about 9.0 whereas the pK_a of the monoamines is about 10.5. In order to clarify this point, we studied the binding of [^{3}H]glycine, which does not possess a second amino group, to C4 using

guanidine hydrochloride as the activating reagent. The results, also shown in Figure 4, are similar to those of [^{3}H]putrescine binding. Since the pK_a values of both glycine and putrescine are similar (9.6 and 9.0, respectively), and because their reaction rates with C4* are also similar, we must conclude that the second amino group of putrescine does not play any part in the binding reaction as suggested (Gorski et al., 1982).

Covalent Binding Sites of C3 and C4. We have used the fluid-phase system to study the binding reaction of C3 and C4. The reaction can be described in two phases. The activation phase is the exposure of the internal thio ester. This can be accomplished by the proteolytic cleavage of the C3a and C4a portion from the respective molecules or by reagents that can induce the equivalent conformational change (Law, 1983a,b). The binding phase is defined as the reaction between substrate molecules with the exposed thio ester.

In our experiments with C3, it is apparent that binding is the transfer of the acyl group of the thio ester to nucleophilic groups in the medium. Three points should be noted: (1) The reaction only proceeds if the thio ester is exposed "correctly". Extensive denaturation of the protein results in the loss of binding activity (Law, 1983a).[2] (2) The thio ester is not equally accessible to all molecules. Different molecules have different binding efficiencies with the protein (Law et al., 1981). The alcohols of the simple alkanes show differences in their reactivity, depending upon the bulk of the alkyl groups (Table I). (3) The rate of the binding reaction, k' as defined here, is extremely fast. It is estimated that the reaction is at least a few orders of magnitude faster than the free reaction between thio esters and nucleophiles in a chemical system (Fedor & Bruice, 1965; Sim et al., 1981). For these reasons, we conjecture that the binding reaction may be catalyzed in some fashion and that there may be catalytic groups in the binding site. [For more details of the argument, see Law (1983b).]

This conjecture is supported by experiments on the other two known thio ester containing proteins, C4 (this study) and α_2-macroglobulin (Salvesen et al., 1981). If the binding reaction is only a chemical reaction made possible by the exposure of the thio ester in these proteins, one would expect to find the reactivity and selectivity of the binding reactions of the three proteins to be similar since the chemical structure of their thio esters is identical (Tack et al., 1980; Swenson & Howard, 1980; Harrison et al., 1981; Campbell et al., 1981). Our experiments show that C3 reacts preferentially with hydroxyl groups over amino groups at neutral pH, whereas the converse is true for C4. In this regard, α_2-macroglobulin is more C4-like than C3-like since Salvesen et al. (1981) have shown that lysine and glycine can be incorporated to α_2-macroglobulin quite efficiently at neutral pH. However, α_2-macroglobulin differs from the two complement proteins in another respect. Whereas the half-life of the activated state of α_2-macroglobulin is of the order of 100 s (Salvesen et al., 1981), that of C3 and C4 is likely to be in the millisecond range (Götze & Müller-Eberhard, 1970; Sim et al., 1981). It is apparent, therefore, that the covalent binding reactions for these three proteins are similar yet different and that the simplest postulate to account for the differences would be the presence of different catalytic groups in the three proteins. The identification of the hypothetical catalytic groups may require more extensive studies of the binding properties of these proteins as well as the three-dimensional structures of their respective binding sites.

[2] More accurately, C3 undergoes the autolytic cleavage reaction upon extensive denaturation. For reviews on the autolytic cleavage reaction of C3 and other thio ester containing proteins, see Erickson & Khan (1983) and Sim & Sim (1983).

Acknowledgments

We thank R. B. Sim, S. G. Davies, and R. Finn for valuable discussions.

Appendix

The binding reaction of C3 is described by the reaction scheme

$$\begin{array}{l} \qquad\qquad\qquad\quad H_2O \xrightarrow[\text{(inactivation)}]{k_0} C3b \\ \qquad\qquad\qquad\quad + \\ C3 \xrightarrow[\text{(activation)}]{k} C3b^* \\ \qquad\qquad\qquad\quad + \\ \qquad\qquad\qquad\quad S \xrightarrow[\text{(binding)}]{k'} C3b\text{–}S \end{array}$$

where k (first order), k_0 (first order), and k' (second order) are the reaction rates of the different steps. In the presence of excess substrate molecules, i.e., when [S] $\gg$ [C3], the binding efficiency (BE), defined as

$$BE = C3b\text{–}S/(C3b + C3b\text{–}S)$$

can be approximated, in terms of the reaction rates and the concentration of S, by the relationship

$$BE = k'[S]/(k_0 + k'[S])$$

By inverting both sides, the equation $(BE)^{-1} = 1 + (k_0/k')[S]^{-1}$ is obtained. This has been experimentally demonstrated by measuring the BE of glycerol to C3 at different concentrations of glycerol. The double reciprocal plot of BE vs. [glycerol] gave a straight line intersecting the y axis at 1 (Law et al., 1981).

When nonradioactive molecules are present in the reaction mixture as inhibitors (I), an additional reaction, C3b* + I $\rightarrow$ C3b–I, proceeding at a rate denoted by k_i (second order) has to be included in the scheme shown above. The binding efficiency of S in the presence of I, BE(I), can be expressed similarly:

$$[BE(I)]^{-1} = 1 + (k_0/k')[S]^{-1} + (k_2/k')[S]^{-1}[I]$$

or

$$[BE(I)]^{-1} = [BE(0)]^{-1} + (k_i/k')[S]^{-1}[I]$$

where BE(0) is the binding efficiency of S in the absence of I. If [S] is fixed in a given experiment where [I] varies, the plot of $[BE(I)]^{-1}$ vs. [I] should yield a straight line intersecting the y axis at $[BE(0)]^{-1}$.

Registry No. C3, 80295-41-6; C3b, 80295-43-8; C4, 80295-48-3; glycerol, 56-81-5; putrescine, 110-60-1; L-serine, 56-45-1; *N*-acetylserine, 16354-58-8; *O*-methylserine, 32620-11-4; methanol, 67-56-1; ethanol, 64-17-5; 1-propanol, 71-23-8; 2-propanol, 67-63-0; 1-butanol, 71-36-3; 2-butanol, 78-92-2; 2-methyl-2-propanol, 75-65-0.

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Evidence for Membrane-Associated Calpain I in Human Erythrocytes. Detection by an Immunoelectrophoretic Blotting Method Using Monospecific Antibody†

Michiyo Hatanaka, Nagahisa Yoshimura, Toshio Murakami,‡ Reiji Kannagi, and Takashi Murachi*

ABSTRACT: Low and high Ca^{2+}-requiring forms of Ca^{2+}-dependent cysteine proteinase are known as calpain I and calpain II, respectively. We have obtained, for the first time, monospecific antibodies for calpain I and for calpain II. Using these antibodies and an electrophoretic blotting method, we have found that a small, but reproducible, amount of calpain I was associated with human erythrocyte membranes while the bulk of the protease was contained in the cytosol. Most of membrane-associated calpain I was extractable with 1% Triton X-100, but not with 0.1% detergent. In the presence of 0.1 mM Ca^{2+} and 5 mM cysteine, membrane-associated calpain I degraded the membrane protein band 4.1 preferentially and band 3 protein only slowly. The Ca^{2+}-induced autodigestion of the membrane preparation was inhibited by leupeptin but not by a cytosolic calpain inhibitor, calpastatin, added to the incubation medium. No calpain II was detected in either erythrocyte cytosol or membranes when anti-calpain II antibody was used under the same conditions as those for the detection of calpain I.

The Ca^{2+}-dependent cysteine proteinase, collectively called calpain[1] [EC 3.4.22.17], has been found mainly in the cytosolic fraction of various mammalian and avian cells (Guroff, 1964; Huston & Krebs, 1968; Dayton et al., 1976; Phillips & Jakâbovâ, 1977; Murachi et al., 1981b; Murakami et al., 1981; Nelson & Traub, 1982). Although physiological functions of such widely distributed calpains are still obscure, recent works have shown that this enzyme degrades native cytoskeletal proteins including high molecular weight microtubule-associated proteins (Sandoval & Weber, 1978), actin-binding proteins (Davies et al., 1978; Truglia & Stracher, 1981), and

† From the Department of Clinical Science, Kyoto University Faculty of Medicine, Sakyo-ku, Kyoto 606, Japan. *Received November 16, 1983.* This work was supported in part by Grants-in-Aid for Scientific and Cancer Research, Ministry of Education, Science and Culture, Japan.
‡ Present address: Department of Home Economics, Kyoto Bunkyo Junior College, Uji, Kyoto 611, Japan.

[1] Recommended name is given by the Nomenclature Committee of the International Union of Biochemistry (1981). Calpain I denotes a low Ca^{2+}-requiring form and calpain II a high Ca^{2+}-requiring form. The 80K and 30K proteins represent subunits of calpain having molecular masses of 70 000–85 000 and 25 000–30 000 daltons, respectively.

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