The principal site of glycation of human complement Factor B

Marilyn A. NIEMANN,*† Ajit S. BHOWN* and Edward J. MILLER†

*Department of Medicine, Division of Clinical Immunology and Rheumatology, and †Department of Biochemistry, University of Alabama at Birmingham, University Station, Birmingham, AL 35294, U.S.A.

Accumulating amino acid sequence data have made it increasingly evident that many essential complement proteins have potentially modifiable lysine residues in putative critical functional regions. Evidence is now presented that glucose is covalently attached to lysine-266 of purified human complement Factor B as a result of glycation. Purified B was treated with NaB³H₄, which reduces such bound glucose to a mixture of radiolabelled hexitols. Amino acid analysis revealed the expected radiolabelled hexitol-lysine epimers. In addition, fluorography of dried gels resolving the major high-molecular-mass h.p.l.c.-fractionated CNBr-cleavage peptides of NaB³H₄-reduced B indicated that this radioactivity was specifically associated with the 15 kDa fragment derived from the *N*-terminal region of fragment Bb. Amino acid sequence analysis suggested that the *C*-terminal lysine (residue 266 of B) of the *N*-terminal Lys-Lys doublet of this peptide is preferentially modified. If such glycation can subsequently be shown to occur *in vivo*, then perhaps this modification might also be found to affect the functional activity of B and offer a potential explanation for some of the immunopathological complications of diseases exposing key plasma proteins, such as this active-site-containing proteinase of the multimeric alternative-complement-pathway C3/C5 convertases, to long-term high concentrations of glucose, such as the decreased resistance to infection and impaired chemotaxis and phagocytosis characteristic of diabetes.

INTRODUCTION

Significant progress has been made in recent years towards elucidating the complete primary structure of the key enzymically active complement components Factor D [1], Factor B [2] and C2 [3] as well as the pivotal modulatory proteins C3 [4] and C4 [5]. Investigations correlating this emerging physical structure with functional activity resulting in the elaboration of biologically active peptides (C3a, C5a) as well as the membrane-attack complex (C5b–9) now appear feasible. Indeed, such studies seem to be the next logical step in expanding our knowledge of the sequence of events involved in initiating and regulating the activation of the complement cascade.

One relatively unexplored potential area for structural regulation of functional activity is glycosylation. A wide variety of proteins, for example, owe many of their properties to the covalent attachment of carbohydrates at specific residues in the polypeptide chain [6]. Much emphasis, moreover, has recently been placed on the potential biological importance of glycation of proteins and the implications of this post-translational modification in certain disease states, such as diabetes, as well as during aging. Such modification involves the formation of a keto-amine bond between glucose and free amino groups, such as the α -*N*-terminal amino acid of the ϵ -amino group of lysine, on the protein.

Accumulating primary amino acid sequence data have made it increasingly evident that these essential complement proteins have such potentially modifiable lysine residues in critical functional regions. B, for example, contains 53 lysine residues. Of these, 47 are in the larger enzymically active *C*-terminal Bb subunit, whereas only six are in the smaller *N*-terminal Ba subunit [2]. Two of these lysine residues, one from each subunit, flank the D-cleavage site. Modification of either of these amino acid residues thus might be envisaged to interfere with the normal activation of this protein. A role for lysine residues forming stabilizing ionic interactions with C3b is also suggested by the

fact that modification of this amino acid in the regulatory protein H, which inhibits alternative-complement-pathway C3 convertase activity by competing with B binding to C3b, markedly inhibits its activity [7]. The purpose of the present study therefore is to establish if such potentially biologically significant non-enzymically modified lysine derivatives do indeed occur in human complement protein B and, if so, to determine if this modification is, as appears to be the case in other proteins studied, site-specific, offering a unique opportunity eventually to relate a specific amino acid residue to the overall function of this enzyme.

EXPERIMENTAL

Materials

Poly(L-lysine), poly(L-glutamylalanyltyrosine), bovine pancreatic RNAase A and CNBr were from Sigma Chemical Co. All solvents and reagents for amino acid analysis and sequencing were from Beckman. All h.p.l.c. solvents (OmniSolve) were from EM Science. P-2 and all SDS/PAGE reagents were from Bio-Rad Laboratories. Aquasol scintillation fluid and Enlightening were purchased from New England Nuclear. NaB³H₄ and ACS scintillation fluid were purchased from Amersham International. X-Omat-AR film as well as GBX developer and fixer were from Kodak.

Protein purification

Complement components C2 [8], B [9], C3 [10] and D [11] were purified to homogeneity as assessed by SDS/PAGE [12] followed by silver [13] and/or Coomassie Blue R-250 staining as previously described [14]. B was cleaved into Ba and Bb and these fragments were purified as previously described [15]. C3b was generated by trypsin cleavage of C3 according to the method of Tack *et al.* [16]. Gels containing ³H-labelled peptides were treated with Enlightening for approx. 30 min before being dried and subjected to fluorography with X-Omat AR film.

† To whom correspondence should be addressed, at: Department of Biochemistry, University of Alabama at Birmingham, SDB Box 13, Birmingham, AL 35294, U.S.A.

Glycation

Poly(L-lysine) (40 kDa) was glycated according to the method of Yue *et al.* [17] as follows. A 12–13 mg portion of this homopolymer was incubated in 0.05 M-sodium phosphate buffer, pH 7.0, containing 25 mM-glucose at a protein concentration of 10 mg/ml for 10 days at 37 °C. This mixture was initially made 1 mM by the addition of an appropriate volume of a 10% (w/v) stock solution of NaN_3 , then sterilely filtered into a sterile tube to prevent microbial contamination. After incubation the free glucose was removed by dialysis against 5 litres of the above sodium phosphate buffer (four changes in 24 h) at 4 °C. RNAase A was glycated according to the method of Eble *et al.* [18] as follows. A 10 mg portion of RNAase A was dissolved in 500 μl of 0.2 M-sodium phosphate buffer, pH 7.4, containing 1 M-glucose and 0.02% NaN_3 . This mixture was then incubated at 44 °C for 2½ days. After incubation the remaining unincorporated glucose was removed by exhaustive dialysis against four changes of a 4000-fold excess of 0.05 M-sodium phosphate buffer, pH 7.0, at 4 °C.

NaB^3H_4 reduction

NaB^3H_4 reduction was performed according to the method of Yue *et al.* [17]. Briefly, 0.5–1 mg of the proteins glycated *in vitro* and 0.16–1 mg of purified B, C2, D or C3b in 0.05 M-sodium phosphate buffer, pH 7.0, were reduced by adding 37.5 μl (1.8 mCi) of NaB^3H_4 dissolved in the same buffer. This constituted an equimolar to 107-fold molar excess of NaB^3H_4 to lysine residues per protein. The reaction was allowed to proceed at room temperature for 40 min. The mixture was then rapidly desalted on a P-2 column washed with 30% (v/v) acetic acid. The appropriate fractions were immediately pooled, frozen and freeze-dried.

Amino acid analysis

After reduction, each protein was hydrolysed in 6 M-HCl at 110 °C for 24 h and analysed on a Beckman model 119C amino acid analyser [19]. The amino acids and their derivatives were quantified either by the automated ninhydrin reaction or by collecting the column effluent and assaying 1 min (1.6 ml) fractions for radioactivity.

Protein sequencing

Edman degradations were performed in a Beckman model 890M sequencer [20]. Less than 2 nmol of each peptide was used for sequencing. Repetitive yields were generally between 96 and 99%. Amino acid phenylthiohydantoin derivatives were identified by h.p.l.c. as described by Bhowan & Bennett [20]. After identification of the indicated number of *N*-terminal amino acids, the remaining residues of each peptide were resuspended in 1 ml of methanol, added to 10 ml of ACS and counted for ^3H radioactivity.

Chemical and enzymic cleavages

Cleavage of B by CNBr was performed in 70% (v/v) formic acid at room temperature for 4 h as reported previously [21]. Digestion of the 15 kDa CNBr-cleavage peptide of B by *Staphylococcus aureus* V8 proteinase was performed under conditions reported to favour cleavage at Glu–Xaa over Asp–Xaa peptide bonds as reported previously [1].

H.p.l.c.

H.p.l.c. was performed at room temperature by using a Waters Associates chromatographic system. Separation of the CNBr-

cleavage peptides derived from B was performed by size-exclusion chromatography using four I-125 (0.78 cm internal diam. $\times$ 30 cm) columns (Waters Associates) in tandem as reported previously [21]. The solvent was 20% (v/v) acetic acid/15% (v/v) propan-1-ol. The flow rate was 0.5 ml/min. For the reverse-phase purification of the 15 kDa CNBr-cleavage peptide as well as the *S. aureus*-V8-proteinase-digest peptides of this fragment, a C_{18} $\mu\text{Bondapak}$ (0.39 cm internal diam. $\times$ 30 cm) column (Waters Associates) was used. chromatography was performed at a flow rate of 1.5 ml/min with 0.1% (v/v) trifluoroacetic acid for 10 min followed by a linear gradient to 60% (v/v) acetonitrile over a 60 min interval as reported previously [1].

RESULTS

Detection of hexitol-lysine derivatives by amino acid analysis

Poly(L-lysine) (Fig. 1a) and RNAase A (Fig. 1b) were used as positive controls to identify the appropriate hexitol-lysine derivative peaks upon amino acid analysis. They were pre-incubated with glucose before NaB^3H_4 reduction and then subjected to amino acid analysis. Fig. 1 demonstrates two main peaks of radioactivity eluted just after the position of phenylalanine (between 215 and 220 min) and just before the position of hydroxylysine (between 225 and 230 min). The position and relative height of these peaks correspond to those previously shown to represent the hexitol-lysine derivatives of hydrolysed glycated lysine residues [18].

The heteropolymer poly(L-glutamylalanyltyrosine) (1:1:1) was used as a negative control. It was similarly treated with NaB^3H_4 and analysed. However, since it was not glycated and only its α -*N*-terminal amino acid could be potentially modified, it incorporated only a minimal amount of the NaB^3H_4 radioactivity, and none of this label was subsequently detected at the hexitol-lysine elution times upon amino acid analysis (results not shown).

The purified human complement proteins B, C2, D and C3b were subsequently treated with NaB^3H_4 and subjected to amino acid analysis (Fig. 2). Each of these analyses demonstrated radioactive peaks eluted in positions characteristic of the glycated poly(L-lysine) and RNAase A positive controls, confirming the presence of such modified lysine residues in these proteins. In addition, various amounts of other unidentified radiolabelled peaks were also detected. Such non-specific labelling has previously been reported [18]. These additional peaks may represent unidentified ^3H -labelled non-protein low-molecular-mass contaminant(s), since they appear to be greatly diminished when the protein is dialysed after P-2 chromatography. Alternatively, they may be radiolabelled glycated amino acids, such as the α -*N*-terminal alanine of C2 [3], threonine of B [9], serine of C3b [4] or isoleucine of D [1]. Interestingly, Bb (Fig. 3b) and RNAase A (Fig. 1b), which have essentially none of these artificial radioactive peaks, both contain lysine at their *N*-terminus. Although these amino acid chromatograms indicate that not all the NaB^3H_4 radioactivity incorporated into each of these proteins is associated exclusively with glycated lysine residues, all of these proteins nevertheless contain detectable amounts of such modified residues.

Since human complement protein B (Fig. 2a) appeared to contain the least amount of these unidentified radiolabelled peaks, it was selected for further analysis (Fig. 3). Most of the specific hexitol-lysine labelling appeared to be associated with the Bb region of the protein (Fig. 3b). The Ba peptide, on the other hand, contained less hexitol-lysine labelling, and relatively more unidentified label was eluted after the hexitol-lysine peaks (Fig. 3a).

Fig. 1. Portions of the amino acid analysis chromatograms of NaB^3H_4 -reduced glycated (a) poly(L-lysine) (500 μg) and (b) RNAase A (1 mg)

Poly(L-lysine) and RNAase A were preincubated with glucose before reduction in order to maximize hexitol-lysine labelling. Poly(L-lysine) and RNAase A were incubated with an equimolar and 5.5-fold molar excess of NaB^3H_4 respectively to lysine residues. The larger radioactive peak eluted just after phenylalanine (between 215 and 220 min) and the smaller radioactive peak eluted just before hydroxylysine (between 225 and 230 min) are characteristic of hexitol-lysine and its time-dependent rearrangement [18] or dehydration [42] product. Arrows indicate elution positions of known amino acids in this region of the chromatogram. The two hexitol-lysine peaks contain at least five data points each. Additional experimental details are given in the text.

Fig. 2. Portions of the amino acid analysis chromatogram of NaB^3H_4 -reduced B (1 mg) (a), C2 (500 μg) (b), D (160 μg) (c) and C3b (500 μg) (d)

These complement proteins were incubated with a 6-, 16-, 107- and 12-fold molar excess of NaB^3H_4 respectively to lysine residues. The radioactive peaks eluted just after phenylalanine and before hydroxylysine indicate the presence of glycated lysine residues in these proteins. Additional details are given in Fig. 1 legend.

Site of glucose attachment to B

Isolation and identification of the NaB^3H_4 -radiolabelled peptide.

In order to determine if specific lysine residues are preferentially glycated, purified human complement protein B was reduced with NaB^3H_4 followed by CNBr cleavage at methionine residues

and fractionation of the resultant peptides by gel-permeation h.p.l.c. [21] as illustrated in Fig. 4. The peptides in each h.p.l.c. peak were resolved by SDS/PAGE and the stained gel is shown in Fig. 4(a), Fig. 4(b) showing the corresponding fluorogram. The total radioactivity recovered from each peak is indicated

Fig. 3. Portions of the amino acid analysis chromatograms of NaB^3H_4 -reduced B cleavage fragments Ba (300 μg) (a) and Bb (500 μg) (b)

These peptides were incubated with a 60- and 9-fold molar excess of NaB^3H_4 respectively to lysine residues. The radioactive peaks eluted just after phenylalanine and before hydroxylysine indicate the presence of glycated lysine residues in these peptides. Additional details are given in Fig. 1 legend.

Fig. 4. Gel-permeation h.p.l.c. elution profile of the CNBr-cleavage peptides of B

A 5 mg portion of B was reduced with 20 mCi of NaB^3H_4 , then cleared with CNBr and fractionated by using four I-125 gel-permeation columns arranged in tandem. The columns were developed with 20% (v/v) acetic acid/15% (v/v) propan-1-ol at a flow rate of 0.5 ml/min. Peaks were collected as indicated. The total ^3H radioactivity associated with each peak is indicated below the appropriate fraction on the chromatogram. Insets show the same 10–20% non-reducing SDS/polyacrylamide gel. Inset (a) silver-stained gel. Inset (b) fluorogram of the dried gel (after 1 month exposure). Lane numbers of insets correspond to chromatogram peak numbers. The positions and molecular masses of marker proteins are indicated to the left of inset (a). Peaks eluted beyond 65 min are too small to be detected by gel electrophoresis. Only the peak 4 peptide, however, contained any lysine residues.

below the appropriate h.p.l.c. fraction on the chromatogram. Peak 1 is enriched in a 35 kDa peptide, which we have previously shown to be composed of a large 28 kDa peptide disulphide-linked to a smaller 5 kDa peptide [21]. Both of these peptides are derived from the C-terminus of the Bb domain. Peak 2 is enriched in a 28 kDa peptide, which we have previously shown to be derived from the N-terminal Ba region of B [21,22]. This fraction also contains 20 kDa and 15 kDa peptides, which we have previously determined to have identical N-terminal sequences beginning at residue 11 of the Bb subunit [22]. The latter

smaller peptide is a more completely digested fragment of the former larger one, which contains an uncleaved methionine about 30 residues from its C-terminus. Finally, peak 3 is enriched in an 8.5 kDa peptide, which we have previously determined to be derived from the Bb region of the intact protein [21]. It also contains a small amount of the 15 kDa peptide from the previous peak. Peaks eluted beyond 65 min are too small to be detected by SDS/PAGE. One additional fragment of interest eluted in this region of the chromatogram, however, is found in peak 4 and is composed of the Ba–Bb overlap peptide containing the

				Asn	Ile	Tyr	Leu	Val	250 Leu
Asp	Gly	Ser	Asp	Ser	Ile	Gly	Ala	Ser	260 Asn(CHO)-
Phe	Thr	Gly	Ala	Lys	Lys	Cys	Leu	Val	270 Asn
Leu	Ile	Glu	Lys	Val	Ala	Ser	Tyr	Gly	280 Val
Lys	Pro	Arg	Tyr	Gly	Leu	Val	Thr	Tyr	290 Ala
Thr	Tyr	Pro	Lys	Ile	Trp	Val	Lys	Val	300 Ser
Glu	Ala	Asp	Ser	Ser	Asn	Ala	Asp	Trp	310 Val
Thr	Lys	Gln	Leu	Asn	Glu	Ile	Asn	Tyr	320 Glu
Asp	His	Lys	Leu	Lys	Ser	Gly	Thr	Asn	330 Thr
Lys	Lys	Ala	Leu	Gln	Ala	Val	Tyr	Ser	340 Met

Fig. 5. Complete amino acid sequence of the 15 kDa B CNBr-cleavage peptide as reported by Gagnon [2]

Residue numbering represents the position of each amino acid within the intact B sequence. The amino acid residues sequenced to establish the identity of this purified peptide are underlined. Lysine residues are enclosed in boxes. Arrows (↑) indicate points of *S. aureus* V8 proteinase cleavage.

Fig. 6. ³H radioactivity associated with the N-terminal amino acids of the 15 kDa B CNBr-cleavage peptide

Residue number represents distance from the N-terminus. After 10% of the first ten amino acid residues were identified by h.p.l.c. to establish the identity of this peptide, the remaining 90% normalized to 100% (----) as well as 100% (—) of the next 50 residues were counted for radioactivity. In light of the small amount of protein sequenced and the expected low extent of glycation, monitoring of the phenylthiohydantoin derivatives by h.p.l.c. was stopped after the first ten steps to conserve the radioactivity recovered at each of the succeeding steps. Lysine residues (K) are enclosed in circles. Lys-22 of this Figure corresponds to Lys-266 of Fig. 5.

D-cleavage sequence Lys-Arg-Lys. These four peaks account for more than 95% of the total B sequence and all of the reported lysine residues. The fluorogram of the dried SDS/PAGE gel resolving these major CNBr-cleavage peptides thus clearly in-

dicates that most of the incorporated NaB³H₄ radioactivity is associated with a single 15 kDa peptide present in peaks 2 and 3 and previously shown to be derived from the N-terminal region of the Bb subunit. In addition, although peak 1 contains

Fig. 7. Reverse-phase h.p.l.c. elution profile of the *S. aureus*-V8-proteinase-digest peptides fractionated from the 15 kDa B CNBr-cleavage fragment

Approx. 100 μ g of this purified NaB^3H_4 -reduced CNBr-cleavage peptide was digested and subjected to reverse-phase h.p.l.c. The C_{18} μ Bondapak column was developed at a flow rate of 1.5 ml/min for 10 min in the A solvent (0.1% trifluoroacetic acid) and then eluted with the illustrated 60 min linear gradient to 60% (v/v) acetonitrile (—). Five peptides, namely Ile-317–Glu-320 corresponding to peak 1, Asp-321–Met-340 corresponding to peak 2, Ala-302–Glu-316 corresponding to peak 3, Lys-274–Glu-301 corresponding to peak 4 and Asn-245–Glu-273 corresponding to peak 5, were isolated and sequenced. The total ^3H radioactivity recovered in each peak is indicated below the appropriate h.p.l.c. fraction on the chromatogram.

substantial radioactivity, none of this label appears to be associated with any known CNBr-cleavage peptide of B. Perhaps the radioactivity associated with this peak is found in high-molecular-mass aggregates unable to penetrate the stacking gel and/or is distributed among the six incompletely cleaved higher-molecular-mass peptide bands (> 35 kDa) as well as the faint 15 kDa peptide band in amounts too low to be individually detected under the fluorimetric development conditions used.

Identification of the preferred NaB^3H_4 -radiolabelled lysine residue. The unlabelled 15 kDa CNBr-cleavage peptide present in peak 2 of Fig. 4 was further purified by reverse-phase h.p.l.c. The purity and identity of this peptide was confirmed by sequence analysis through ten amino acid residues at its *N*-terminus. As shown in Fig. 5, the complete sequence of this peptide indicates that it contains 11 lysine residues (enclosed in boxes) including two Lys-Lys doublets at positions 265–266 and 331–332 [2].

The position of the glycosylated lysine residue(s) in this 15 kDa fragment was localized by reducing the purified peptide with NaB^3H_4 followed by *N*-terminal amino acid sequence analysis. The peptide rather than intact B was reduced to maximize labelling. The first ten amino acid derivatives were identified by h.p.l.c. to establish unequivocally the identity of this peptide. The remaining radioactivity associated with these initial residues as well as the next 50 residues was measured. As shown in Fig. 6, Lys-22 (corresponding to Lys-266 in Fig. 5) appeared to be preferentially labelled. In addition, Lys-21, Lys-30 and Lys-37 were labelled to a lesser extent as well as Ser-11 and Asn-16. Upon further *S. aureus* V8 proteinase digestion of this CNBr-cleavage peptide, however, none of these lysine residues was found to incorporate NaB^3H_4 radioactivity significantly above background levels. Serine has previously been reported to be one of the amino acids other than the ketoamine adduct labelled by

this treatment [17,23–25], and Asn-16 is thought to be a potential site of *N*-linked glycosylation. A major peak of radioactivity is also associated with the *N*-terminal asparagine. Since NaB^3H_4 cannot chemically react with asparagine, the high radioactivity associated with this first residue may be due to glycation of its α -amino group or, more likely, non-protein-associated radioactivity washed out of the peptide during the first few sequencing cycles.

In order to determine if any other lysine residues in this peptide were similarly preferentially glycosylated, the labelled 15 kDa fragment was subsequently cleaved with *S. aureus* V8 proteinase at glutamic acid residues. The five expected *S. aureus*-V8-digest peptides accounting for the entire CNBr-cleavage fragment were isolated by reverse-phase h.p.l.c. (Fig. 7) and sequenced. The first three amino acid residues were identified by h.p.l.c. to establish the identity of each peptide. The residual radioactivity associated with these initial derivatives as well as all the remaining residues was measured. Although the total recovered radioactivity suggested that peak 2 containing amino acid residues Asp-321 to Met-340 (Fig. 5), including the second Lys-Lys doublet of the 15 kDa fragment, might also be glycosylated, upon amino acid sequence analysis of each of these *S. aureus*-proteinase-digest peptides, no additional lysine residues, including the now *N*-terminal Lys-275 (Lys-30 in Fig. 5) as well as the eighth Lys-282 (Lys-37 in Fig. 6) residues, other than Lys-266 appeared to be preferentially labelled.

Interestingly, Asp-303, but no other aspartic acid residue, contained substantial radioactivity (approximately 1800 c.p.m.). Such NaB^3H_4 labelling of acidic residues has been used previously to establish and confirm indirectly the internal thioester bond in C3 between Cys-9 and Glu-12 of the C3d trypsin-digest fragment [26]. Perhaps such a thioester bond is also formed between Asp-303 and the heretofore presumed free Cys-267 of B. Substantiation of such a novel putative thioester bond in B, however, would require further chemical characterization.

DISCUSSION

The data presented in this paper establish that glucose is covalently attached to human complement Factor B. Detection of the appropriate radiolabelled hexitol-lysine derivatives upon amino acid analysis of this NaB^3H_4 -reduced complement protein (Fig. 2a), moreover, substantiates the fact that the attachment of this monosaccharide is to lysine residue(s) presumably via glycation. In addition, this glucose appears to be associated with a specific region of the protein since the Bb subunit appears to be labelled to a greater extent than the Ba fragment (Fig. 3). Furthermore, fluorography of dried SDS/PAGE gels resolving the major CNBr-cleavage peptides of B (Fig. 4) indicates that this radioactivity is specifically associated with the 15 kDa fragment derived from the *N*-terminal region of Bb, whereas no Ba peptides are similarly labelled. Finally amino acid sequence analysis of this Bb CNBr-cleavage fragment (Fig. 6) indicates that Lys-266 of the intact protein, the *C*-terminal lysine residue of its *N*-terminal Lys-Lys doublet, is preferentially modified.

Interestingly, approximately half of the glycation of human serum albumin occurs at Lys-525, which is also the second or *C*-terminal lysine residue of a Lys-Lys dipeptide sequence [27]. Likewise, the two most prevalent sites of ϵ -amino glycation of human haemoglobin, β -Lys-66 and α -Lys-61, represent the second lysine residues of Lys-Lys dipeptide sequences [28]. B has six such Lys-Lys doublets, all of which are located in the Bb region of the molecule. The NaB^3H_4 -labelled 15 kDa CNBr-cleavage peptide derived from B has two such doublets, one near its *N*-terminus (residues 265–266) and the other near its *C*-terminus (residues 331–332). *S. aureus* V8 proteinase digestion

of the 15 kDa Bb CNBr-cleavage fragment indicated that the peptide containing the second C-terminal Lys-Lys doublet contained about half as much radioactivity as the N-terminal Lys-Lys doublet peptide. Upon amino acid sequence analysis, however, no significant radioactivity was recovered in association with any lysine residue other than Lys-266.

Although not known to be involved in the enzymic activity of B, this N-terminal region of the active site containing Bb subunit has been proposed to be involved in the binding of the C3b protein cofactor [29] required for expression of Bb proteolytic activity. Glycation of this region of the B molecule, therefore, might interfere with its ability to form an effective bimolecular C3 convertase complex similar to that reported to account for the decrease *in vitro* in thrombin-inhibiting activity of glycated antithrombin III [30,31]. Of course, glycation of C3b in its complementary Bb-binding region could similarly have a synergistic inhibitory effect on alternative-pathway convertase formation.

The physiological significance of such glycation of complement proteins such as B, however, still remains to be determined. Such modification, for example, may occur either *in vivo* or *in vitro* during storage of the plasma in 5% (w/v) glucose before purification of this complement component, which was usually purified from plasma stored refrigerated for not more than 2 weeks. A slight increase in the extent of glycation of haemoglobin, plasma proteins and erythrocyte membrane has been reported during such storage [32], although serum samples stored at -20°C for up to 3 months have been reported to show no changes in their content of glycated albumin [33]. In addition, the extent of glycation of a protein *in vivo* appears to be a function not only of its accessibility to serum glucose, but also of its half-life. Thus it might initially seem that such modification would be less likely to occur *in vivo* to an appreciable extent on complement proteins such as B because of their relatively short half-lives [34]. Glycation, however, begins in all cases with glucose attachment to protein amino groups via nucleophilic addition with formation of a Schiff base. This labile Schiff base rapidly reaches an equilibrium level *in vivo* reflecting ambient glucose concentration. An increased concentration of these reversible glucose-protein Schiff-base compounds *in vivo* could then significantly alter a certain constantly changing percentage of susceptible plasma proteins such as B [25]. This mechanism has previously been proposed to account for the short-term inhibition *in vitro* of alkaline phosphatase activity by glucose [35].

Previous investigations have demonstrated increased concentrations of complement proteins, including B, C3 and C4, in diabetics [36–38]. Our preliminary experiments (M. A. Niemann & J. E. Volanakis, unpublished work), in agreement with these earlier studies, using a tube assay measuring the lysis of rabbit erythrocytes in the presence of heat-inactivated normal human serum, also found that serum B concentrations of 15 diabetics were elevated almost 2-fold over those of four normal controls (1257.1 versus 607.9 CH_{50} units/ml). Such an increase would be consistent with an inhibition of D haemolytic efficiency in diabetic plasma, but whether these elevated B concentrations are a result of diabetes or an acute-phase response to secondary complications was not determined. More recent studies report decreased concentrations of Clq, C3 and C4 as well as no change in B and C2 concentrations in diabetic patients as measured by radioimmunoassay with monospecific antiserum [39]. Another study using the same method suggests that C3 and C4 concentrations are initially lower at disease onset, then become elevated as the disease progresses [40]. Clearly more experiments are needed to clarify the role of these complement proteins in the pathogenesis of this disease. If glycation can subsequently be shown to occur *in vivo*, however, then perhaps this modification

might also be found to affect the functional activity of B and offer a potential explanation for some of the immunopathological complications of diseases exposing key plasma proteins, such as this active-site-containing proteinase of the multimeric alternative-complement-pathway C3 and C5 convertases, to long-term high concentrations of glucose, such as the decreased resistance to infection and impaired chemotaxis and phagocytosis characteristic of diabetes [41].

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