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Differential Accessibility of the Carbohydrate Moieties of $\bar{C}1s$–$\bar{C}1r$–$\bar{C}1r$–$\bar{C}1s$, the Catalytic Subunit of Human $\bar{C}1$†

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ABSTRACT: The catalytic subunit of human $\bar{C}1$, $\bar{C}1s$–$\bar{C}1r$–$\bar{C}1r$–$\bar{C}1s$, is a Ca^{2+}-dependent tetrameric association of two serine proteases, $\bar{C}1r$ and $\bar{C}1s$, which are glycoproteins containing asparagine-linked carbohydrates. With a view to investigate the accessibility and the possible functional role of these carbohydrates, the isolated proteases and their Ca^{2+}-dependent complexes were submitted to deglycosylation by peptide:*N*-glycosidase F, an endoglycosidase that specifically hydrolyzes all classes of N-linked glycans. Treatment of isolated $\bar{C}1r$ and $\bar{C}1s$ led to the removal of the carbohydrate moieties attached to their N-terminal α region, whereas those located in the C-terminal γ-B catalytic domains were resistant to hydrolysis. Formation of the Ca^{2+}-dependent $\bar{C}1s$–$\bar{C}1s$ dimer and $\bar{C}1s$–$\bar{C}1r$–$\bar{C}1r$–$\bar{C}1s$ tetramer induced specific protection of the single carbohydrate attached to the α region of $\bar{C}1s$ and of one of the two carbohydrates located in the corresponding region of $\bar{C}1r$. Sequence studies indicated that the carbohydrates protected upon homologous ($\bar{C}1s$–$\bar{C}1s$) or heterologous ($\bar{C}1r$–$\bar{C}1s$) interactions are attached to asparagine residues 159 of $\bar{C}1s$ and 204 of $\bar{C}1r$, at the C-terminal end of the EGF-like domain of both proteases. These data bring further evidence that Ca^{2+}-dependent interactions between $\bar{C}1r$ and $\bar{C}1s$ are mediated by their N-terminal α regions and strongly suggest that, inside these regions, the EGF-like domains play an essential role in these interactions.

The first component of the classical complement pathway is a macromolecular protease assembled from C1q, the recognition unit, and from C1s–C1r–C1r–C1s, the catalytic unit, a Ca^{2+}-dependent tetrameric association of two serine proteases, C1r and C1s, that are sequentially activated upon C1 activation [reviewed by Cooper (1985), Arlaud et al. (1987a), and Schumaker et al. (1987)]. In both cases, activation occurs through cleavage of a single Arg–Ile bond (Arlaud et al., 1985; Spycher et al., 1986), converting the single-chain proenzymes (C1r, C1s) into active proteases ($\bar{C}1r$, $\bar{C}1s$)[1] comprising two disulfide-linked chains. Complete sequences of the N-terminal A chain and of the C-terminal B chain of each protein have been determined, indicating that the B chains are homologous to the catalytic chains of other mammalian serine proteases, whereas the A chains are subdivided into five structural units, referred to as I–V and including one EGF-like segment and two pairs of internal repeats (Arlaud & Gagnon, 1983; Journet & Tosi, 1986; Leytus et al., 1986; Arlaud et al., 1987b; Mackinnon et al., 1987; Tosi et al., 1987). Each monomeric protease is organized in at least two independent domains: a catalytic domain, which includes the B chain, associated to γ, the C-terminal portion of the A chain, and an interaction region α, derived from the N-terminal half of the A chain (Villiers et al., 1985; Weiss et al., 1986; Busby & Ingham, 1987). The former is considered a true globular domain (Arlaud et al., 1986), whereas the latter is more likely divided into a series of small subdomains responsible for specific interactions (Arlaud et al., 1987a; Tosi et al., 1987). These

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography; iPr_2P-F, diisopropyl phosphorofluoridate; PNGase F, peptide:*N*-glycosidase F; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tos-Phe-CH_2Cl, 1-chloro-4-phenyl-3-(L-tosylamino)butan-2-one; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EGF, epidermal growth factor; the nomenclature of complement components is that recommended by the World Health Organization; activated components are indicated by a superscript bar, e.g., $\bar{C}1r$.

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include calcium binding, formation of the Ca^{2+}-dependent $C\bar{1}s$–$C\bar{1}s$ dimer and $C\bar{1}s$–$C\bar{1}r$–$C\bar{1}r$–$C\bar{1}s$ tetramer, and probably interactions between the tetramer and C1q upon assembly of the C1 complex.

$C\bar{1}r$ and $C\bar{1}s$ are glycoproteins containing Asn-linked carbohydrates: four glycosylation sites are present on $C\bar{1}r$, two in the α region and two in the B chain (Arlaud & Gagnon, 1983; Arlaud et al., 1987b), whereas $C\bar{1}s$ contains only two carbohydrates, one being located in the α region and the other in γ (Spycher et al., 1986; Mackinnon et al., 1987; Tosi et al., 1987). Thus, in contrast with the remarkable homology that exists between the two proteases with respect to their amino acid sequence and domain structure, they widely differ from each other by their glycosylation patterns. The objective of the present study, based on the use of PNGase F, an endoglycosidase that specifically hydrolyzes all classes of Asn-linked glycans, was to investigate the accessibility and the possible functional involvement of these carbohydrate moieties, particularly in Ca^{2+}-dependent interactions between the two proteins. It is shown that, in each isolated protein, the carbohydrate moieties located in the α regions are accessible to the endoglycosidase, in contrast with those located in the γ-B catalytic domains. Formation of the Ca^{2+}-dependent $C\bar{1}s$–$C\bar{1}s$ dimer and $C\bar{1}s$–$C\bar{1}r$–$C\bar{1}r$–$C\bar{1}s$ tetramer induces in each protein specific protection of a carbohydrate located at equivalent position in the α regions.

Experimental Procedures

Materials

Flavobacterium meningosepticum (ATCC 33958) was obtained from the American Type Culture Collection and cultured for production of PNGase F as described by Tarentino et al. (1985). Trypsin (treated with Tos-Phe-CH_2Cl) was obtained from Worthington Biochemical Corp., and purified PNGase F was from Genzyme Inc. TSK HW-55(S) was obtained from Merck. Iodo[2-^{3}H]acetic acid (106 Ci/mol) was from the Radiochemical Centre, Amersham. CNBr was obtained from Aldrich Chemical Co.

$C\bar{1}r$ and $C\bar{1}s$ were purified from human plasma as described previously (Arlaud et al., 1979), and their concentrations were estimated by using respective values of A(280 nm, 1%) = 11.5 and 13.7, as determined by Sim et al. (1977) or calculated by the method of Edelhoch (1967) from the number of Trp, Tyr, and disulfides (Tosi et al., 1987). The $(\gamma\text{-B})_2$ catalytic domains of $C\bar{1}r$ were obtained from autolytic cleavage of the native protease and purified by high-pressure gel permeation (Arlaud et al., 1986). Quantitative estimation of the $(\gamma\text{-B})_2$ fragment was based on a value of A(280 nm, 1%) = 15.0, calculated by the method of Edelhoch (1967).

PNGase F was purified from cultures of *F. meningosepticum* by a modification of the method described by Tarentino et al. (1985). After step II [gel filtration on TSK HW-55(S)], fractions containing PNGase F were identified by SDS–PAGE analysis and from their ability to produce partial deglycosylation of isolated $C\bar{1}r$ and $C\bar{1}s$. Active fractions were pooled, concentrated by ultrafiltration (PM-10 membrane, Amicon Corp.) to 2.0 mL, and dialyzed against 0.1 M Na_2HPO_4/1 mM EDTA (pH 7.1). Final purification of PNGase F was realized by high-pressure hydrophobic interaction chromatography on a TSK-phenyl 5PW column (7.5 × 75 mm) (LKB). The dialyzed material was loaded onto the column equilibrated in 0.1 M Na_2HPO_4/1 mM EDTA/1.5 M $(NH_4)_2SO_4$ (pH 7.1), and elution was realized by decreasing the concentration of $(NH_4)_2SO_4$ from 1.5 M to 0 in 30 min (flow rate = 1 mL/min). Three major peaks were detected from absorbance at 230 nm, and PNGase F was contained in the second peak. SDS–PAGE analysis indicated that PNGase F (M_r 34 000) contained traces of a minor contaminant (M_r 44 000) already described by Tarentino et al. (1985) but no detectable amount of endo-β-N-acetylglucosaminidase F (Endo F). The final preparation obtained from a 5-L culture was dialyzed against 20 mM Tris-HCl/100 mM NaCl/1 mM EDTA (pH 7.1) and concentrated by ultrafiltration to 6.0 mL. Concentration of purified PNGase F was estimated spectrophotometrically by the method of Warburg and Christian (1941).

Methods

Deglycosylation of $C\bar{1}r$ and $C\bar{1}s$ by PNGase F. $C\bar{1}r$, $C\bar{1}s$, the $(\gamma\text{-B})_2$ catalytic domains of $C\bar{1}r$, and the Ca^{2+}-dependent complexes $C\bar{1}s$–$C\bar{1}s$ and $C\bar{1}s$–$C\bar{1}r$–$C\bar{1}r$–$C\bar{1}s$ were incubated with 3–12% (w/w) PNGase F at 25, 30, or 37 °C and for varying periods as indicated in the text. Unless otherwise stated, all incubations were performed in 145 mM NaCl/1 mM iPr_2P-F/15 mM triethanolamine hydrochloride (pH 7.4) containing 2 mM EDTA or 2.5 mM $CaCl_2$ as indicated. Protein deglycosylation was monitored by SDS–PAGE analysis of the samples after reduction and alkylation, using 8.75% or 12.5% polyacrylamide gels as described by Laemmli (1970). Protein staining was performed with Coomassie blue, and carbohydrate-containing bands were identified by specific staining as described by Kapitany and Zebrowski (1973). Bovine serum albumin (M_r 67 000), ovalbumin (M_r 45 000), glyceraldehyde-phosphate dehydrogenase (M_r 36 000), carbonic anhydrase (M_r 29 000), bovine trypsinogen (M_r 24 000), soybean trypsin inhibitor (M_r 20 100), and lactalbumin (M_r 14 200) were used as molecular weight markers.

Native and deglycosylated proteins were analyzed by high-pressure gel permeation on a TSK-G3000 SW column (7.5 mm × 600 mm) (LKB) equilibrated in the same buffer as used for deglycosylation and run at 1.0 mL/min. The esterolytic activity of native and deglycosylated $C\bar{1}r$ and $C\bar{1}s$ was determined from the rate of hydrolysis of the synthetic substrate N-α-acetylglycyl-L-lysine methyl ester by using a spectrophotometric assay based on the measurement of methanol released upon hydrolysis (Herzberg & Rogerson, 1985).

Location of the Single Carbohydrate Removed upon Treatment of Isolated $C\bar{1}s$ with PNGase F. Monoglycosylated $C\bar{1}s$ was prepared by incubation of native $C\bar{1}s$ (5.0 mg) in 145 mM NaCl/1 mM iPr_2P-F/2 mM EDTA/15 mM triethanolamine hydrochloride (pH 7.4) for 2 h at 30 °C in the presence of 6% (w/w) PNGase F, and the protein was dialyzed at 4 °C against 0.5% (v/v) acetic acid and freeze-dried. Reduction of monoglycosylated $C\bar{1}s$, alkylation by iodo[2-^{3}H]acetic acid, and separation of A and B chains were performed as described previously for $C\bar{1}r$ (Arlaud et al., 1982). The reduced and S-^{3}H-carboxymethylated monoglycosylated $C\bar{1}s$ A chain (3.4 mg) was dissolved in 70% (v/v) formic acid (2.0 mL) and cleaved with CNBr (100 mg) for 24 h at 4 °C in the dark. The CNBr digest was fractionated by gel filtration on a Sephadex G-75 superfine column (2.5 cm × 120 cm) equilibrated in 6 M urea/50 mM NH_4HCO_3 (pH 8.0) as described by Spycher et al. (1986) for CNBr-cleavage peptides obtained from intact $C\bar{1}s$ A chain. Five pools were collected, and peptides CB2 and CB4, which contain the two glycosylation sites of $C\bar{1}s$, were found in pools 3 and 5, respectively. CB4 was further purified by reversed-phase HPLC using solvent system 2, whereas CB2 was used without further purification.

Location of the Single Carbohydrate Removed from $C\bar{1}r$

upon Partial Deglycosylation of C$\bar{1}$s–C$\bar{1}$r–C$\bar{1}$r–C$\bar{1}$s by PNGase F. A mixture containing 21.0 mg of both C$\bar{1}$r and C$\bar{1}$s was prepared in 145 mM NaCl/15 mM triethanolamine hydrochloride (pH 7.4), and $CaCl_2$ was added to 10 mM. The resulting C$\bar{1}$s–C$\bar{1}$r–C$\bar{1}$r–C$\bar{1}$s tetramer (0.67 mg/mL) was submitted to partial deglycosylation by incubation for 1 h at 25 °C in the presence of 6% (w/w) PNGase F and cooled on ice. The tetramer was then dissociated by addition of 10 mM EDTA and, after dialysis at 0 °C against 5 mM EDTA/20 mM Na_2HPO_4 (pH 7.4), C$\bar{1}$r and C$\bar{1}$s were applied to a DEAE-cellulose column (Whatman DE 52) equilibrated in the same buffer and separated as described by Arlaud et al. (1979). C$\bar{1}$r eluted from DEAE-cellulose was dialyzed against 0.5% (v/v) acetic acid, freeze-dried, reduced, and alkylated by iodo[2-^{3}H]acetic acid, and the A and B chains were separated by high-pressure gel permeation as described previously (Arlaud et al., 1982). The reduced and S-^{3}H-carboxymethylated monoglycosylated C$\bar{1}$r A chain (4.5 mg) was submitted to CNBr cleavage in 70% (v/v) formic acid as described above for C$\bar{1}$s A chain, and the digest was fractionated by gel filtration on a Bio-Gel P-100 column (2.5 cm × 110 cm) equilibrated in 9% (v/v) formic acid. Five pools were collected. Peptide CN1a was contained in pool 1, whereas pool 5 contained peptides CN2a and CN4b, which were separated by reversed-phase HPLC with solvent system 2. CN1a was submitted to full tryptic digestion as described previously for C$\bar{1}$r B chain (Arlaud & Gagnon, 1983), and peptide CN1aT3 was purified by fractionation of the digest by reversed-phase HPLC with solvent system 1.

Peptide Separation by Reversed-Phase HPLC. Separations were performed on a Nova-PAK C_{18} column (Waters Associates) with two solvent systems as described previously (Arlaud & Gagnon, 1983). Briefly, system 1 consisted of 0.1% NH_4HCO_3 and acetonitrile, and system 2 consisted of 0.1% trifluoroacetic acid and acetonitrile/methanol/propan-2-ol, 1:1:1 v/v/v). Peptides were detected from absorbance at 215 nm.

Amino Acid Analysis and N-Terminal Sequence Determination. Samples were hydrolyzed for 24 h under reduced pressure at 110 °C in constant-boiling HCl containing 0.1% (v/v) 2-mercaptoethanol and 1% (w/v) phenol. Analyses were performed either on acid hydrolysates with a Beckman 7300 amino acid analyzer using ninhydrin for detection or by reversed-phase HPLC of amino acid phenylthiocarbamyl derivatives with a Pico-Tag amino acid analysis system (Waters Associates) using the standard procedure recommended by the manufacturer. The latter method was also used for identification of phenylthiocarbamylglucosamine, which was resolved from phenylthiocarbamylserine by lowering the pH of the starting buffer from 6.4 to 5.3 as recommended by Waters Associates. Automated Edman degradation was performed in an Applied Biosystems Model 470 A gas-phase protein sequencer, and identification of amino acid phenylthiohydantoin derivatives was performed according to the protocol recommended by Applied Biosystems on a Model 120 A HPLC system.

Peptide Nomenclature. Peptides obtained from CNBr cleavage of C$\bar{1}$s and C$\bar{1}$r A chains are designated according to the nomenclature used in the original articles reporting their purification (Spycher et al., 1986; Gagnon & Arlaud, 1985; Arlaud et al., 1987b).

Results

Deglycosylation of Isolated C$\bar{1}$r. C$\bar{1}$r was incubated for 2 h at 30 °C in the presence of 6% (w/w) PNGase F, and deglycosylation was monitored by SDS–PAGE analysis of reduced samples using 12.5% polyacrylamide gels (Figure 1). Under these conditions, the intact A chain (M_r 54000), which carries two carbohydrate moieties, was rapidly converted to its monoglycosylated form (M_r 51000) and then more slowly to its aglycosylated form (M_r 48000). Specific staining of carbohydrate (not shown) indicated that the intermediate species was still glycosylated, whereas the final species was not, thus demonstrating that deglycosylation was responsible for the observed increases in electrophoretic mobility. The B chain (M_r 37000) was much less susceptible to deglycosylation, as only small amounts of the monoglycosylated form (M_r 34000), which in this system comigrated with PNGase F, were obtained after 2 h. Increasing the temperature to 37 °C had no significant effect on the deglycosylation of the B chain, whereas deglycosylation of the A chain was facilitated at this temperature and reduced at 20 °C.

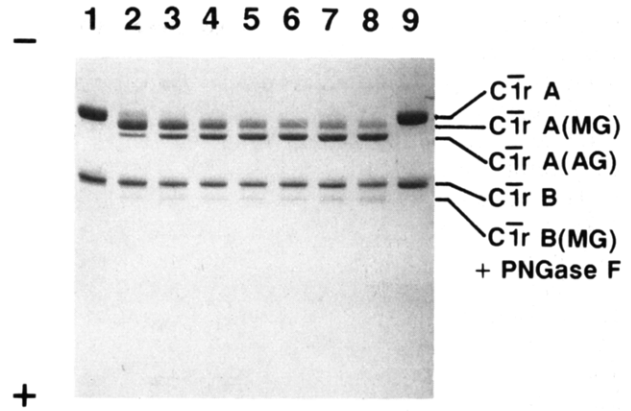

FIGURE 1: SDS–PAGE analysis of the deglycosylation of isolated C$\bar{1}$r by PNGase F. C$\bar{1}$r (0.5 mg/mL) in 145 mM NaCl/1 mM iPr_2P-F/15 mM triethanolamine hydrochloride (pH 7.4) was incubated with 6% (w/w) PNGase F for increasing periods at 30 °C. Reduced and alkylated samples were analyzed with 12.5% polyacrylamide gels as described under Methods. (Lane 1) Untreated C$\bar{1}$r; (lanes 2–8) C$\bar{1}$r incubated with PNGase F for 10, 20, 30, 45, 60, 90, and 120 min, respectively; (lane 9) control C$\bar{1}$r incubated for 120 min without PNGase F. (A) A chain; (B) B chain; (MG) monoglycosylated species; (AG) aglycosylated species.

Native C$\bar{1}$r submitted to high-pressure gel permeation eluted as a major peak corresponding to the dimer, with a small amount of aggregated material in the void volume. After removal of both carbohydrate moieties from the A chain, the major peak almost completely disappeared without significant increase of the material eluted in the void volume, indicating that most of the protein was lost, probably bound to the column. Complete deglycosylation of the A chain of C$\bar{1}$r had not effect on the esterolytic activity of this protease.

The accessibility of the carbohydrate moieties located on the B chain was further investigated by deglycosylation studies performed on the isolated $(\gamma\text{-B})_2$ domains. Incubation of these domains with up to 12% (w/w) PNGase F only led to partial removal of a single carbohydrate moiety from the B chain. Incubation for 3 h at 37 °C produced less than 50% of the monoglycosylated species, whereas no detectable amount of the aglycosylated species could be obtained. Partial deglycosylation of the B chain had no significant effect on the esterolytic activity of the $(\gamma\text{-B})_2$ domains.

Deglycosylation of Isolated C$\bar{1}$s. Incubation of C$\bar{1}$s with 3% (w/w) PNGase F at 30 °C in the presence of EDTA led to deglycosylation of the A chain (M_r 57000), which was progressively converted to a species of M_r 54000 that, as judged from specific staining, still contained carbohydrate and therefore corresponded to a monoglycosylated form. The second carbohydrate of the A chain was entirely resistant to PNGase F, as no detectable amount of the aglycosylated form

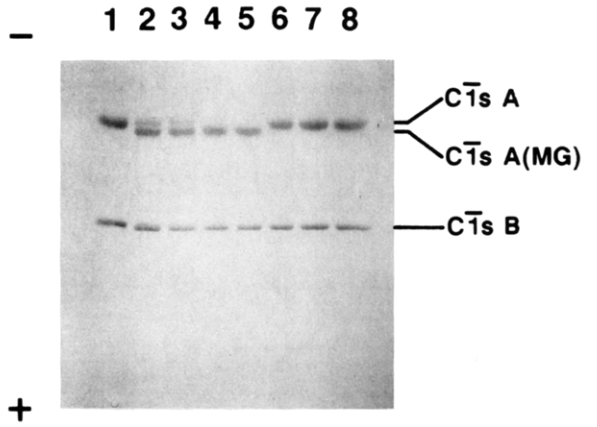

FIGURE 2: SDS–PAGE analysis of the deglycosylation of isolated C1s by PNGase F. C1s (1.0 mg/mL) in 145 mM NaCl/1 mM iPr_2P-F/15 mM triethanolamine hydrochloride (pH 7.4) was incubated with 3% (w/w) PNGase F for varying periods at 30 °C in the presence of EDTA or $CaCl_2$. (Lane 1) Untreated C1s; (lanes 2–5) C1s incubated with PNGase F for 15, 30, 60, and 120 min, respectively, in the presence of 2 mM EDTA; (lane 6) control C1s incubated for 120 min in the presence of 2 mM EDTA without PNGase F; (lanes 7 and 8) C1s incubated for 120 min with PNGase in the presence of 2.5 and 10 mM $CaCl_2$, respectively. Other experimental conditions are as described in the legend to Figure 1.

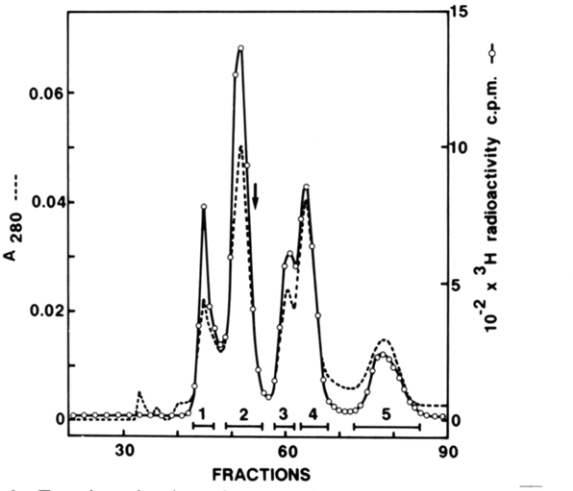

FIGURE 3: Fractionation by gel permeation on Sephadex G-75(SF) of CNBr-cleavage peptides from reduced and S-^{3}H-carboxymethylated, monoglycosylated C1s A chain. Radioactivity was measured on 60-μL aliquots, and pools were made as indicated by bars. The arrow indicates the approximate position of peptide CB2 in the fractionation obtained by Spycher et al. (1986).

Peptide		
	379	391
CB4	E N G G G G E Y H C A G_G S W	
	154 159	
CB2	K N C G V D C S G	
(CB3)	P C P K E D T P N	
	278	
	96	108
CN2a	L L T F H T D F S N E E D G T I	
	203	
CN1aT3	C_Y S I R	

FIGURE 4: N-Terminal sequences of selected glycopeptides obtained from partially deglycosylated C1r and C1s A chains. Residues are numbered according to their position in the sequence of C1s (CB4, CB2, CB3) or C1r (CN2a, CN1aT3).

could be obtained, even on prolonged incubation in the presence of increasing amounts of PNGase F.

When treatment with PNGase F was performed in the presence of calcium ions, deglycosylation of the A chain was completely prevented (Figure 2, lane 7), indicating that formation of the Ca^{2+}-dependent C1s–C1s dimer prevented accessibility of the only carbohydrate that is susceptible to hydrolysis by PNGase F in monomeric C1s.

Native C1s was eluted by high-pressure gel permeation in EDTA as a major peak corresponding to the monomer and, in contrast with C1r, partial deglycosylation only slightly increased the elution volume of the protein, without significant loss of material on the column. Using the same technique in the presence of calcium, monoglycosylated C1s was found to retain partially its capacity to dimerize and to form C1s–C1r–C1r–C1s tetramers when mixed with equal amounts of C1r. As already observed with C1r, partial deglycosylation of C1s had no effect on its esterolytic activity.

Location of the Single Carbohydrate Removed upon Treatment of Monomeric C1s by PNGase F. With a view to identify which one of the two carbohydrates of C1s is removed upon treatment with PNGase F, monoglycosylated C1s was reduced and S-^{3}H-carboxymethylated, and the monoglycosylated A chain, separated from the B chain by high-pressure gel permeation, was submitted to CNBr cleavage (see Methods). Previous studies (Spycher et al., 1986; Mackinnon et al., 1987; Tosi et al., 1987) have shown that the two carbohydrates of C1s are located on the A chain and contained in the CNBr-cleavage peptides CB2 and CB4. CNBr-cleavage peptides were fractionated by gel filtration on Sephadex G-75 (SF) in the same conditions as originally used by Spycher et al. (1986), and five pools were collected (Figure 3). Amino acid analysis indicated that peptides CB2 and CB4 were contained in pools 3 and 5, respectively. Compared to the separation obtained by Spycher et al. (1986), therefore, the elution position of CB4 was not modified, whereas the apparent M_r of CB2 was significantly lower (Figure 3). Peptide CB4 (residues 379–422), further purified by reversed-phase HPLC, eluted as a single peak, and its N-terminal sequence was determined for 16 cycles (Figure 4). No residue was identified at cycle 13, indicating that this position (391 in C1s A chain) was still occupied by an Asn-linked carbohydrate, in agreement with the identification of glucosamine in acid hydrolysates of CB4.

Attempts to further purify peptide CB2 by reversed-phase HPLC were unsuccessful, and N-terminal sequence analysis was performed directly on the material contained in pool 3 from Sephadex G-75. As shown in Figure 4, two sequences were obtained, a major sequence (approximately 90%) corresponding to peptide CB2 and a minor sequence corresponding to peptide CB3, the major component of pool 4. Aspartic acid was unambiguously identified at position 6 of CB2, clearly indicating that the Asn-linked carbohydrate normally found at this position (159 in C1s A chain) had been removed upon treatment with PNGase F (Plummer et al., 1984), in agreement with the absence of detectable amounts of glucosamine in acid hydrolysates of pool 3.

Deglycosylation of the Ca^{2+}-Dependent C1s–C1r–C1r–C1s Tetramer. The C1s–C1r–C1r–C1s tetramer, assembled from equal amounts of C1r and C1s in the presence of calcium, was treated with PNGase F at 30 °C, and deglycosylation of both proteins was monitored by SDS–PAGE analysis of reduced samples using 8.75% polyacrylamide gels, which allowed resolution of the native and various deglycosylated forms of the A chains of both proteins. Incubation of the tetramer for 1 h in the presence of 2.5 mM $CaCl_2$ and 6% (w/w) PNGase F resulted in the removal of a single carbohydrate from C1r A chain, whereas no deglycosylation of C1s A chain was

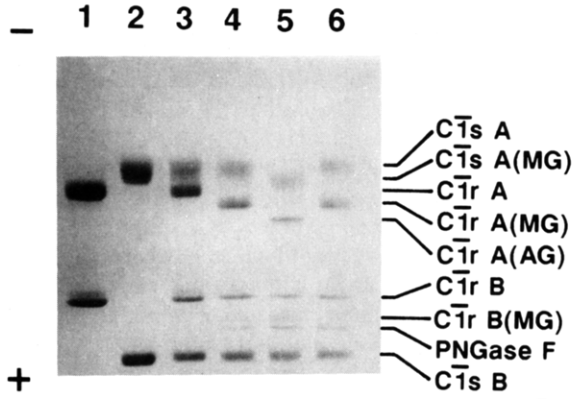

FIGURE 5: SDS–PAGE analysis of the deglycosylation of C1r and C1s upon treatment of the Ca^{2+}-dependent C1s–C1r–C1r–C1s tetramer by PNGase F. (Lanes 1–3) Untreated C1r, C1s, and C1s–C1r–C1r–C1s; (lane 4) C1s–C1r–C1r–C1s incubated for 1 h at 30 °C with 6% (w/w) PNGase F in the presence of 2.5 mM $CaCl_2$; (lane 5) C1s–C1r–C1r–C1s incubated as in lane 4 with further addition of 6% (w/w) PNGase F after 30 min; (lane 6) C1s–C1r–C1r–C1s incubated as in lane 5 in the presence of 10 mM $CaCl_2$. Electrophoresis was performed on 8.75% polyacrylamide gels.

observed (Figure 5, lane 4). In the same conditions, further addition of 6% (w/w) PNGase F after 30 min led to the complete deglycosylation of C1r A chain and to the removal of a single carbohydrate moiety from C1s A chain (Figure 5, lane 5). Increasing the concentration of $CaCl_2$ to 10 mM markedly reduced the deglycosylation process, as a single carbohydrate of C1r A chain was removed under these conditions, even when a second addition of PNGase F was performed after 30 min (Figure 5, lane 6). Similar results were obtained when incubations were performed at 25 or 37 °C.

The intact C1s–C1r–C1r–C1s tetramer submitted to high-pressure gel permeation eluted as a single peak, and the elution profile was not modified after removal of a single carbohydrate from C1r A chain. In contrast, treatment of C1s–C1r–C1r–C1s with PNGase F under conditions leading to complete deglycosylation of C1r A chain and to partial deglycosylation of C1s A chain resulted in the dissociation of the tetramer. In agreement with the results obtained with individual proteins, C1r was not recovered from the column, whereas C1s eluted as a peak corresponding to the dimer.

Location of the Single Carbohydrate Removed from C1r A Chain upon Partial Deglycosylation of C1s–C1r–C1r–C1s by PNGase F. The Ca^{2+}-dependent C1s–C1r–C1r–C1s tetramer, assembled from 21.0 mg of both C1r and C1s in the presence of 10 mM $CaCl_2$, was treated with PNGase F under conditions (see Methods) where a single carbohydrate of C1r A chain is susceptible to hydrolysis. After dissociation of the tetramer in EDTA, C1r was separated from C1s by ion-exchange chromatography and then reduced and alkylated by iodo[2-^{3}H]acetic acid. The reduced and S-^{3}H-carboxymethylated, monoglycosylated C1r A chain, separated from the B chain by high-pressure gel permeation, was cleaved with CNBr, and the digest was fractionated by gel filtration, as shown in Figure 6. CN1a (residues 113–351), the large peptide containing one of the two glycosylation sites of the A chain (Arlaud et al., 1987b), was found in pool 1, whereas pools 2 and 4 contained peptides CN1b (residues 1–89) and CN3 (residues 352–376), respectively. Analysis of pool 3 by reversed-phase HPLC with solvent system 1 showed that it only contained peptide CN2b (residues 397–446), whereas the same pool obtained from CNBr cleavage of the intact A chain also contained CN2a (residues 96–112), the other glycopeptide of the A chain. Pool 5 contained both peptides CN4b (residues 377–396) and CN2a, which were separated by reversed-phase

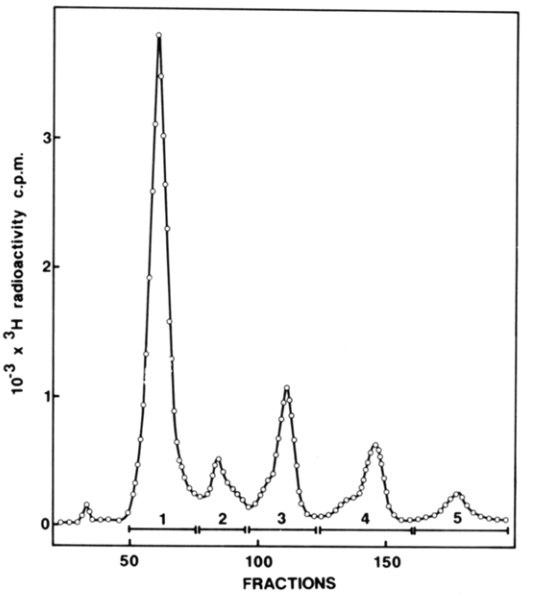

FIGURE 6: Fractionation by gel permeation on Bio-Gel P-100 of CNBr-cleavage peptides from reduced and S-^{3}H-carboxymethylated, monoglycosylated C1r A chain isolated from the PNGase-F-treated C1s–C1r–C1r–C1s tetramer. Radioactivity was measured on 50-μL aliquots, and pools were made as indicated by bars.

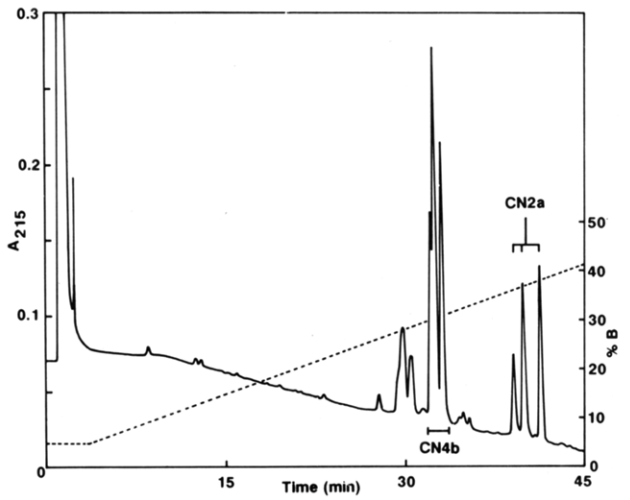

FIGURE 7: Separation of CNBr-cleavage peptides CN4b and CN2a by reversed-phase HPLC. Pool 5 from Bio-Gel P-100 (see Figure 6) was fractionated on a Nova-PAK C_{18} column using solvent system 2 consisting of 0.1% trifluoroacetic acid and acetonitrile/methanol/propan-2-ol, 1:1:1 (eluent B).

HPLC using solvent system 2 (Figure 7). CN2a was resolved into three peaks of identical amino acid composition. The complete sequence of each form was determined, with the exception of the C-terminal homoserine residues that could not be identified. The sequences obtained were identical (Figure 4), suggesting that the apparent heterogeneity of CN2a (which was also observed with the intact glycopeptide) was due mostly to this particular residue. Aspartic acid was identified at cycle 13 (Figure 4), indicating that the Asn-linked carbohydrate found at this position (108 in C1r) had been removed by PNGase F, in agreement with the absence of detectable amounts of glucosamine in acid hydrolysates of CN2a.

With a view to verify that the other carbohydrate moiety, linked to the asparagine residue at position 204 of C1r A chain, was still present, peptide CN1a (residues 113–351) was submitted to full tryptic cleavage, and peptide CN1aT3 (residues 203–208), resulting from cleavage of arginyl bonds at positions

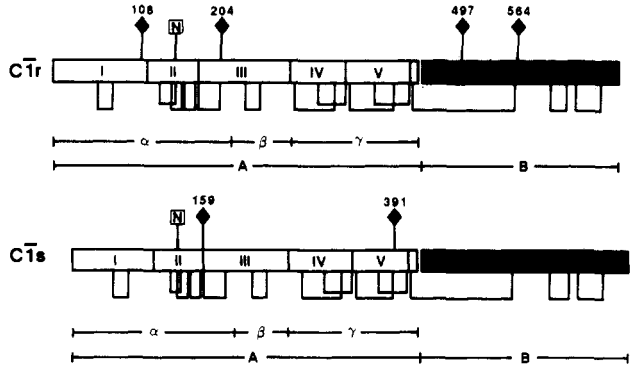

FIGURE 8: Schematic representation of the linear structures of C1r and C1s. The positions of the β-hydroxyasparagine residues (N) and Asn-linked carbohydrates (◆) are indicated (Arlaud et al., 1987b,c; Arlaud & Gagnon, 1983; Spycher et al., 1986; Mackinnon et al., 1987; Tosi et al., 1987). Disulfide bridge patterns are based on published data (Arlaud & Gagnon, 1985; Gagnon & Arlaud, 1985) and on homology with serine proteases and other proteins (Leytus et al., 1986; Arlaud et al., 1987a; Mackinnon et al., 1987).

202 and 208, was purified by fractionation of the whole digest by reversed-phase HPLC using solvent system 1. CN1aT3 was contained in a single peak, and its complete sequence was obtained, with the exception of the residue at position 204 (Figure 4). This position was therefore still occupied by the Asn-linked carbohydrate, a conclusion that was also supported by the identification of glucosamine in acid hydrolysates of CN1aT3 and which confirmed that the single carbohydrate moiety removed by PNGase F is that attached to the Asn residue at position 108.

DISCUSSION

Preliminary experiments performed with commercially available PNGase F led to significant but restricted deglycosylation of isolated C1r and C1s. Further experiments conducted with high amounts (3–12% w/w) of PNGase F, purified from cultures of *F. meningosepticum*, gave identical deglycosylation patterns and allowed us to obtain more extensive deglycosylation and to identify in each protein those carbohydrate moieties that are susceptible to hydrolysis.

Treatment of isolated C1r with purified PNGase F indicated that the two carbohydrates of the A chain, which are both attached to the N-terminal α region via Asn residues at positions 108 and 204 (Figure 8), were removed by the enzyme, although one was cleaved more rapidly than the other. In contrast, the B chain, which also contains two carbohydrates, attached to Asn residues at positions 497 and 564 (Figure 8), was much less susceptible to deglycosylation. One of these carbohydrates proved entirely resistant to the enzyme, whereas the other was only removed to a limited extent, even when PNGase F treatment was performed on the isolated $(\gamma\text{-B})_2$ domains.

C1s contains only two carbohydrate moieties, which are both located on the A chain (Figure 8). Again, treatment of the monomer indicated that these are not equally susceptible to PNGase F: the carbohydrate attached to Asn residue at position 159 in the N-terminal α region was removed by PNGase F, whereas that located in the C-terminal γ region (Asn residue 391) was resistant to the enzyme. Thus, although C1r and C1s differ from each other by the number and location of their carbohydrate moieties, both proteins behave in a comparable manner with respect to PNGase F treatment: all carbohydrates located in the N-terminal α region are susceptible to enzymatic hydrolysis, in contrast to those located in the C-terminal γ-B catalytic domains, which are entirely or partially resistant to the enzyme. PNGase F is known to hydrolyze all classes of Asn-linked glycans, provided that both the α-amino and carboxyl groups of the asparagine residue are in peptide linkage (Tarentino et al., 1985). In addition, although the detailed structure of C1r and C1s carbohydrates has not yet been determined, previous analyses have shown that they have comparable compositions (Sim et al., 1977). Furthermore, both carbohydrates of C1r B chain, of which one is partially removed by PNGase F, whereas the other is not, have almost identical compositions with respect to *N*-acetylglucosamine, mannose, galactose, and sialic acid (Arlaud & Gagnon, 1983) and should therefore have comparable structures. Hence, the most likely hypothesis is that the observed lack of cleavage by PNGase F of the carbohydrate moieties located in the catalytic domains of C1r and C1s is a consequence of a lack of accessibility, as was already observed for other native proteins (Tarentino et al., 1985). In the case of C1r, a possible explanation could arise from the dimeric structure of the protein, considering that the carbohydrates of the B chain could be partly involved in monomer–monomer interactions, which occur through the γ-B catalytic domains (Villiers et al., 1985; Weiss et al., 1986; Arlaud et al., 1986). This hypothesis, however, is not valid for C1s, which does not associate through its catalytic domains. The most likely hypothesis is therefore that the observed lack of accessibility of the carbohydrates located in the catalytic domains arises mostly from steric hindrances inherent in the globular nature of these domains, which could prevent access of the susceptible asparagine–*N*-acetylglucosamine bonds to the active site of PNGase F. In this respect, it is noteworthy that these domains are also particularly resistant to extrinsic proteolytic cleavage (Villiers et al., 1985; Arlaud et al., 1986).

From a functional point of view, the removal of the carbohydrates located in the α region of C1r and C1s had no consequence on the esterolytic activity of either protease. However, this does not necessarily imply that the physiological enzymatic functions of C1r and C1s are unaffected by deglycosylation. In the case of C1s, this treatment did not apparently modify the overall stability of the protein, which also partially retained its ability to dimerize or to interact with C1r in the presence of calcium. In contrast, the removal of the two carbohydrates of the α region of C1r deeply modified the physicochemical properties of the molecule, which acquired a strong tendency to bind to the TSK G3000 SW matrix used for high-pressure gel permeation. This tendency, which could result from the exposure of hydrophobic amino acids, did not make possible further studies on the ability of deglycosylated C1r to associated with C1s in the presence of calcium.

Formation of the Ca^{2+}-dependent C1s–C1s dimer completely prevented the hydrolysis by PNGase F of the carbohydrate linked to the asparagine residue at position 159, thereby rendering the protein totally insensitive to deglycosylation. Treatment of the Ca^{2+}-dependent C1s–C1r–C1r–C1s tetramer with PNGase F showed that formation of the tetramer also resulted in protection of the same carbohydrate moiety and of the carbohydrate linked to the asparagine residue at position 204 of C1r, whereas the accessibility of the carbohydrate linked to the asparagine residue at position 108 of C1r was not modified. Interestingly, this protective effect was reinforced by increasing calcium concentration to 10 mM, even in the presence of high amounts of PNGase F, probably due to an increased stability of the tetramer in these conditions. Thus, two carbohydrates, linked to asparagine residues at positions 204 of C1r and 159 of C1s, are specifically protected upon homologous (C1s–C1s) or heterologous (C1r–C1s)

Ca^{2+}-dependent interactions. These asparagine residues are both located in the homologous region III of each protein (Figure 8), more precisely on each side of half-cystine residues that likely form the N-terminal disulfide bridge of these regions (positions 176–203 in C$\bar{1}$r and 160–187 in C$\bar{1}$s) (Leytus et al., 1986; Arlaud et al., 1987a; Mackinnon et al., 1987). Thus, although these carbohydrates do not appear to be located at homologous positions from direct alignment of the sequences of C$\bar{1}$r and C$\bar{1}$s (Figure 8), they are likely located at homologous positions in the three-dimensional structures of both proteins and hence can be considered as "functionally homologous".

Another interesting feature of these carbohydrates is their location at close proximity of the C-terminal end of homologous regions II of C$\bar{1}$r and C$\bar{1}$s (Figure 8). Both regions show extensive homology with epidermal growth factor, including six half-cystine residues distributed in the characteristic pattern of EGF-like domains. In addition, region II of C$\bar{1}$r contains at position 150 an *erythro*-β-hydroxyasparagine residue resulting from posttranslational hydroxylation of an asparagine (Arlaud et al., 1987c; Leytus et al., 1986; Journet & Tosi, 1986), and the same modified amino acid is probably found at position 134 of C$\bar{1}$s (Przysiecki et al., 1987; Tosi et al., 1987; Mackinnon et al., 1987). Similar cases of EGF-like sequences containing a β-hydroxyaspartic acid or β-hydroxyasparagine residue have been reported in other proteins, including protein C (Foster et al., 1985), protein S (Stenflo et al., 1987), blood coagulation factors IX and X (Anson et al., 1984; McMullen et al., 1983), and protein Z (Hojrup et al., 1985). Although the precise functional role of these sequences has not yet been fully established, their involvement in calcium binding has been proposed in the cases of factor IX (Morita et al., 1984) and factor X (Sugo et al., 1984) and indirectly shown in the case of protein C (Öhlin & Stenflo, 1987). This has led us to suggest that EGF-like regions II of C$\bar{1}$r and C$\bar{1}$s contain a calcium-binding site involved in C$\bar{1}$r–C$\bar{1}$s and C$\bar{1}$s–C$\bar{1}$s interactions (Arlaud et al., 1987a). This hypothesis is strongly supported by the data discussed above and particularly by (i) the observed protection of the carbohydrates linked to asparagine residues 204 of C$\bar{1}$r and 159 of C$\bar{1}$s upon formation of the Ca^{2+}-dependent C$\bar{1}$s–C$\bar{1}$s dimer and C$\bar{1}$s–C$\bar{1}$r–C$\bar{1}$r–C$\bar{1}$s tetramer, (ii) the location of these carbohydrate moieties in the vicinity of EGF-like regions II, and (iii) the fact that increasing the concentration of calcium ions increases this protection.

It cannot be established whether the carbohydrate moieties of regions III are directly involved in C$\bar{1}$r–C$\bar{1}$s and C$\bar{1}$s–C$\bar{1}$s interactions or simply hidden upon these interactions due to a major involvement of the neighboring regions II. The observed dissociation of the C$\bar{1}$s–C$\bar{1}$r–C$\bar{1}$r–C$\bar{1}$s tetramer upon removal of these carbohydrates favors the first hypothesis, although this effect could likely be the consequence of a local denaturation resulting from the removal of these carbohydrates, particularly in C$\bar{1}$r, which appears to be deeply modified upon deglycosylation. Moreover, the fact that C$\bar{1}$s lacking the carbohydrate linked to asparagine residue 159 partially retains its interaction capacity would favor the hypothesis that the carbohydrate moieties attached to regions III are not essential participants of calcium-dependent interactions.

It appears now clearly that the structures responsible for the Ca^{2+}-dependent assembly of C$\bar{1}$s–C$\bar{1}$r–C$\bar{1}$r–C$\bar{1}$s, the catalytic subunit of C$\bar{1}$, are all located in the N-terminal α region of each protein (Villiers et al., 1985; Weiss et al., 1986; Busby & Ingham, 1987). The data presented in this paper entirely support this scheme and strongly suggest that the binding of calcium by EGF-like regions II is an essential step in this process. However, further investigations will be needed to elucidate the precise mechanism of C$\bar{1}$r–C$\bar{1}$s interaction, particularly with respect to the possible involvement of other structures of regions α.

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Amino-Terminal Nucleotide-Binding Sequences of a *Lactobacillus* Deoxynucleoside Kinase Complex Isolated by Novel Affinity Chromatography†

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ABSTRACT: A highly efficient new affinity medium for deoxycytidine kinase, deoxycytidine 5′-tetraphosphate–Sepharose (dCp_4–Sepharose), has been constructed. A dCp_4–Sepharose column effects a one-step, 19 000-fold, purification to homogeneity of dCyd kinase from the ammonium sulfate fraction of *Lactobacillus acidophilus* R-26 extract, with 60% recovery. dCTP, a potent end-product inhibitor, is used as an eluent, and it also stabilizes the extremely labile purified enzyme. A noncompeting deoxyadenosine kinase activity accompanies the deoxycytidine kinase activity eluted. Native polyacrylamide gel electrophoresis shows a single protein band, which coincides with both deoxycytidine kinase and deoxyadenosine kinase activities at several gel concentrations. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis reveals a single polypeptide band of 26 000 daltons. Since the native enzyme is known to have an M_r of 50 000, it appears that the enzyme is composed of two subunits of similar size. Sequence analysis of the intact protein from the N-terminus reveals but a single amino acid species per residue up to the 17th residue; at the 18th, 21st, 26th, and 27th residue positions of the sequence, however, there appear to be two different amino acids in almost equal amounts. This may indicate that the enzyme is composed of two nonidentical subunits having the same amino acid sequence near the N-terminus. Residues 6–13 contain the highly conserved Gly-X-X-Gly-X-Gly-Lys sequence found at the active sites of kinases and other nucleotide-binding proteins.

Lactobacillus acidophilus R-26 has two unique paired deoxynucleoside kinases, namely, dCyd/dAdo kinase and dGuo/dAdo kinase (Deibel & Ives, 1977a). Kinetic studies have revealed that these two paired activities do not share a common active site in either case but exhibit positive allosteric interactions (Deibel et al., 1977; Chakravarty et al., 1984). The dGuo/dAdo enzyme was purified to homogeneity in very small amounts, with a combination of broadly specific affinity media, and shown to consist of a monomeric polypeptide. Selective chemical inactivation of its dGuo phosphorylation site concurrently eliminated the ability of dGuo, mediated by that site, to stimulate dAdo phosphorylation (Chakravarty et al., 1984).

In sharp contrast with the *Lactobacillus* enzyme, competition and mutation experiments with human cytosol dCyd kinase have revealed that its multiple deoxynucleoside specificities are associated with a common active site, which phosphorylates dCyd, dAdo, and dGuo with varying efficiencies (Verhoef et al., 1981; Hershfield et al., 1982; Bohman & Eriksson, 1988). A human mitochondrial deoxypyrimidine kinase isoenzyme also appears to phosphorylate both dCyd and dThd at the same active site (Lee & Cheng, 1977). Only one other bacterial dCyd kinase has been characterized; a dCyd/dAdo kinase isolated from *Bacillus subtilis* exhibited weak mutual inhibition by these substrates, while dCTP or dATP was each most effective in inhibiting the phosphorylation of its own cognate deoxynucleoside (Møllgard, 1980). This behavior suggests the possibility of separate but noninteracting sites on the *B. subtilis* kinase.

Studies on the structure–function relationship of the interesting multifunctional-type deoxynucleoside kinases of *L. acidophilus* have been impeded by the extremely small amounts of purified proteins attainable by conventional purification procedures (Chakravarty et al., 1984). We have developed several affinity media using deoxynucleosides linked to Sepharose through the 3′-hydroxyls or through various positions on the purine or pyrimidine bases. Although deoxynucleoside kinases from other sources were purified successfully on some of these media, none of them retained the bacterial kinases effectively (Ikeda et al., 1984). Greater success was attained with multisubstrate-type affinity media (dNp_4A–Sepharose) directed specifically toward the dCyd, dAdo, or dGuo sites (Ikeda & Ives, 1985). Copurification of two activities (dCyd/dAdo or dGuo/dAdo kinase) on these media, in conjunction with competition experiments, has provided further proof of the existence of two distinct active sites on each protein. However, even these highly specific media had relatively limited capacity for the *Lactobacillus* kinases. Recently, we have found that natural triphosphate end products (dNTP)[1] bind still more tightly (K_i = 0.4–3 μM)

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[1] Abbreviations: dNp_4A, deoxynucleoside 5′-adenosine 5‴-P^1,P^4-tetraphosphate; dNTP, deoxynucleoside triphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; dNp_3A, deoxynucleoside 5′-adenosine 5‴-P^1,P^3-triphosphate; dCp_4–Sepharose, deoxycytidine 5′-tetraphosphate–Sepharose.

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