# Identification of a cryptic protein kinase CK2 phosphorylation site in human complement protease C1r, and its use to probe intramolecular interaction

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**Abstract Treatment of human $C\bar{1}r$ by CK2 resulted in the incorporation of [$^{32}$P]phosphate into the N-terminal α region of its non-catalytic A chain. Fragmentation of $^{32}$P-labelled $C\bar{1}r$ followed by N-terminal sequence and mass spectrometry analyses allowed identification of $Ser_{189}$ as the phosphorylation site. Accessibility of $Ser_{189}$ was low in intact C1r, due in part to the presence of one of the oligosaccharides borne by the α region, further reduced in the presence of calcium, and abolished when C1r was incorporated into the C1s-C1r-C1r-C1s tetramer or the C1 complex. In contrast, phosphorylation was enhanced in the isolated α fragment and insensitive to calcium. Taken together, these data provide support for the occurrence of a $Ca^{2+}$-dependent interaction between the α region and the remainder of the C1r molecule.**

*Key words:* Complement; C1r; Protein phosphorylation; Protein kinase CK2

## 1. Introduction

The first component of complement, C1, is a complex protease assembled from a non-enzymic protein C1q, and two modular serine proteases C1r and C1s, themselves associated in a $Ca^{2+}$-dependent tetramer C1s-C1r-C1r-C1s. Binding of C1 through C1q to various activators triggers self-activation of C1r into $C\bar{1}r$, which in turn converts C1s into $C\bar{1}s$, the protease responsible for enzymic activity of $C\bar{1}$ (for reviews, see [1-3]). Activation of both C1r and C1s occurs through cleavage of a single peptide bond generating active proteases comprising two disulfide-linked chains A and B. The former are mosaic-like polypeptides comprising five modules, whereas the latter are trypsin-like serine protease domains. Both $C\bar{1}r$ and $C\bar{1}s$ molecules can be split into two regions. The α regions, corresponding to the N-terminal half of the A chain, bind calcium, mediate the $Ca^{2+}$-dependent assembly of the C1s-C1r-C1r-C1s tetramer [4], and are very likely also involved in the C1q/C1s-C1r-C1r-C1s interaction [5,6]. The other regions, γ-B, comprising the C-terminal end of the A chain and the B domain, mediate catalytic activity.

Analysis of the amino acid sequences of C1r and C1s [7-9] reveals the presence of consensus phosphorylation motifs (**Ser/Thr**-Xaa-Xaa-Glu/Asp) for protein kinase CK2 (previously named casein kinase II) [10], all occurring at serine residues located at positions 104, 135, and 189 in C1r, and at positions 19, 100, 185, 287, and 427 in C1s. C1r also contains one potential phosphorylation site (Arg-Ser-Lys-$\mathbf{Ser}_{135}$) for the calcium/calmodulin-dependent kinase-II (CaMK-II) [10]. The objective of this study was to check whether these sites are effective substrates for these kinases. Our data show that CK2 phosphorylates $Ser_{189}$ of C1r, and provide information on the accessibility of this residue under various conditions, suggesting that the neighbouring segment (the C-terminal end of the α region) interacts with the catalytic region of the protease.

## 2. Materials and methods

### 2.1. Materials

Trypsin (sequencing grade) was from Boehringer Mannheim. PNGase-F was purified from cultures of *Flavobacterium meningosepticum* as described in [11]. Recombinant oligomeric CK2 from *Drosophila melanogaster* was overexpressed in Sf9 cells and purified as described by Filhol et al. [12]. CaMK-II was purified from rat cerebral cortex according to the method of Yamaguchi and Fujisawa [13]. Calmodulin was a kind gift from Dr J. Baudier (DBMS/BRCE, CEA-Grenoble, France). DFP, pNpGB and pentafluoropropionic acid were from Sigma. [γ-$^{32}$P]ATP (3000 Ci/mmol) was purchased from ICN Biomedicals.

### 2.2. Proteins

C1q, $C\bar{1}r$, $C\bar{1}s$, proenzyme C1r and C1s were isolated from human plasma as described previously [14,15]. The concentrations of purified C1q, $C\bar{1}r$ (C1r), $C\bar{1}s$ (C1s) were determined by using values of $A$ (1%, 1 cm) at 280 nm of 6.8, 12.4 and 14.5, and $M_r$ values of 459 300, 86 300 and 79 800, respectively [4,16,17]. The activated or proenzyme C1s-C1r-C1r-C1s tetramer was assembled from equimolar amounts of $C\bar{1}r$ (C1r) and $C\bar{1}s$ (C1s) in 145 mM NaCl/50 mM triethanolamine-HCl/0.25 mM $CaCl_2$ (pH 7.4). Proenzyme C1 was reconstituted by incubating equimolar amounts of C1q and the proenzyme tetramer for 10 min at 0°C in the above buffer. The C1rα fragment was prepared by limited proteolysis with trypsin as described previously [4] and its concentration was determined by using a value of $A$ (1%, 1 cm) at 280 nm of 7.2 and a $M_r$ value of 27 600.

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*Abbreviations:* Activated complement components are indicated by an overbar, e.g. $C\bar{1}r$; CaMK-II, calcium/calmodulin-dependent kinase II; CK2, protein kinase CK2; DFP, diisopropyl phosphorofluoridate; PNGase-F, peptide *N*-glycosidase F; pNpGB, *p*-nitrophenyl-*p'*-guanidinobenzoate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis


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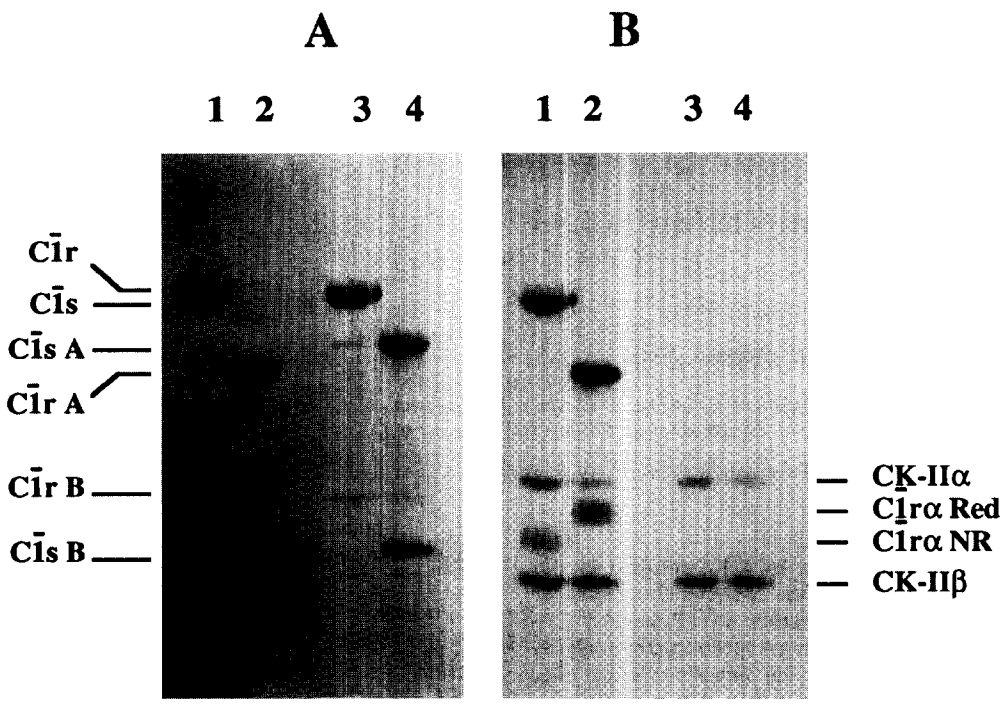


Fig. 1. Phosphorylation of C̄1r and C̄1s by CK2: analysis by SDS-PAGE and autoradiography. C̄1r and C̄1s were submitted to phosphorylation by CK2 in the presence of [$^{32}$P]ATP for 15 min at 30°C and then analyzed by SDS-PAGE followed by Coomassie Blue staining (A) and autoradiography (B). Lanes: 1, C̄1r, non-reduced; 2, C̄1r, reduced; 3, C̄1s, non-reduced; 4, C̄1s, reduced. C̄1r A, B and C̄1s A, B, chains A and B of C̄1r and C̄1s. C̄1rα, fragment α resulting from autolytic cleavage of C̄1r. Red, reduced, NR, non-reduced. CK-IIα, CK-IIβ, phosphorylated α and β subunits of CK2.

*2.3. Deglycosylation of C̄1r with PNGase-F*

C̄1r (0.43 mg/ml) in 100 mM NaCl/50 mM triethanolamine-HCl/0.5 mM pNpGB (pH 7.4) was incubated with 10% (w/w) PNGase-F at 30°C for varying periods. Deglycosylation was monitored by SDS-PAGE analysis of the reduced samples, as described by Laemmli [18].

*2.4. Phosphorylation assays*

C̄1r (1.5 μM) was incubated for various periods at 25°C, 30°C, or 37°C in 40 μl of 150 mM NaCl/50 mM triethanolamine-HCl/15 mM $MgCl_2$ (pH 7.4) in the presence of 0.1 μM CK2 and 100 μM ATP containing [γ-$^{32}$P]ATP (500 cpm/pmol). Phosphorylation by CaMK-II was assayed as above, in the presence of 5 mM $MgCl_2$, 3 μM calmodulin and 0.5 mM $CaCl_2$. The reaction was stopped in both cases by addition of an equal volume of gel electrophoresis sample buffer, and analyzed by SDS-PAGE, followed by Coomassie Blue staining and autoradiography. The extent of phosphorylation was quantitated by excising the bands corresponding to phosphorylated proteins from the dried gel and counting the associated $^{32}$P radioactivity. The protein content of each band was estimated by gel scanning. The stoichiometry of phosphate incorporation was calculated with reference to the specific activity of ATP.

*2.5. Location of the phosphorylated residue*

C̄1r (40 nmol) in 100 mM NaCl/50 mM triethanolamine-HCl/0.5 mM pNpGB (pH 7.4) was incubated with 10% (w/w) PNGase-F for 2 h at 30°C, then phosphorylated with CK2 for 30 min at 30°C as described above, using a specific activity for [$^{32}$P]ATP of 1000 cpm/pmol. The reaction mixture was dialyzed against 0.5% acetic acid to remove free ATP, freeze-dried, dissolved in 6 M urea/0.2 M formic acid, and then fractionated by high-pressure gel permeation on a 4.5×600 mm TSK G-3000 SW column (Tosohaas). Fractions were scanned for absorbance at 280 nm and $^{32}$P radioactivity. The fraction containing deglycosylated phosphorylated C̄1r was freeze-dried, dissolved in 70% formic acid, then cleaved with CNBr (CNBr/protein ratio=30/1 (w/w)) for 24 h at 4°C. The CNBr digest was reduced with dithiothreitol and alkylated by iodoacetic acid, dissolved in 0.1 M $NH_4HCO_3$/1 mM $CaCl_2$ (pH 7.8) and incubated twice with 2% (w/w) trypsin for 2 h at 37°C. After addition of 6 M guanidine-HCl to the digest, the tryptic peptides were fractionated by reverse-phase HPLC on a 4.6 mm×25 cm Vydac C18 column equilibrated with a mixture of 0.1% trifluoroacetic acid/acetonitrile (95:5, v/v). Elution was carried out with a linear gradient to give a final ratio of 40:60 (solvent system 1). The material contained in the single radioactive peak was further fractionated on the same column equilibrated with a mixture of 0.07% (v/v) pentafluoropropionic acid and acetonitrile in the ratio 95:5, and elution was carried out using the same gradient as above (solvent system 2).

*2.6. N-terminal sequence analysis and identification of the C1r phosphorylation site*

Automated Edman degradation was performed by using an Applied Biosystems model 477A gas-phase protein sequencer with on-line analysis of the phenylthiohydantoin-amino acid derivatives, as described previously [4]. For determination of the radiolabelled phosphoserine residue, peptide 170–195 was covalently immobilized on a Sequelon arylamine disk (Millipore) prior to sequencing, by using carbodiimide activation as described by Garin et al. [19], and $^{32}$P radioactivity released at each cycle of Edman degradation was measured.

*2.7. Mass spectrometry analysis*

Analysis of peptide $His_{170}$–$Arg_{195}$ was carried out on a Perkin-Elmer Sciex API III+ triple-quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray (ionspray) source. The instrument was tuned and calibrated in the positive mode and polarities were inverted for operation in the negative mode (detection of anions). Analysis was performed at a declustering potential of −85V. The sample (approx. 2 pmol/μl) was infused into the source at a flow rate of 3 μl/min using a Harvard 22 syringe pump and a Valco C6W injector equipped with a 1 μl internal loop. The solvent used to promote deprotonation of the peptide contained 70% acetonitrile and 0.1% triethylamine in water. The mass spectrum was an average of four scans obtained with a dwell time of 2 ms per 0.5 *m/z* step in an *m/z* range from 400 to 1100. The reconstructed molecular mass profile was obtained by using a deconvolution algorithm (PE-Sciex).

## 3. Results

*3.1. Evidence for phosphorylation of C̄1r by CK2*

A first series of experiments provided no evidence for phos-

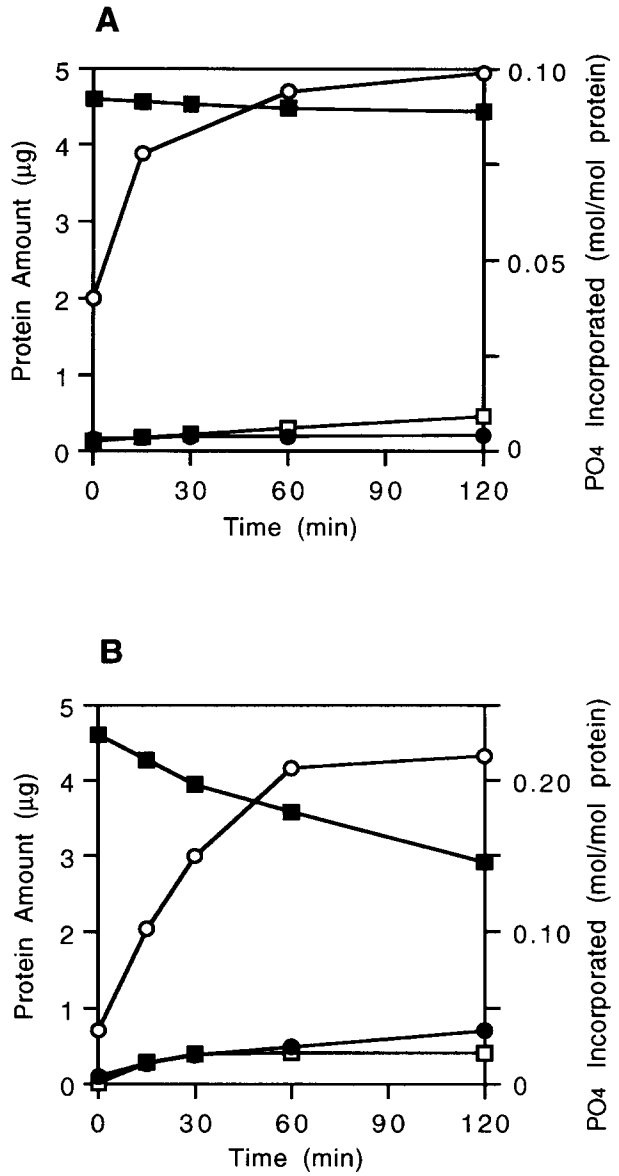


Fig. 2. Kinetics of phosphorylation of C1̄r and its autolytic α fragment by CK2. C1̄r was submitted to phosphorylation by CK2 in the presence of [$^{32}$P]ATP for various periods at either 25°C (A) or 37°C (B). The amounts of C1r (■) and its autolytic α fragment (●), and their respective phosphorylation stoichiometries (□,○) were estimated after SDS-PAGE analysis as described under section 2.

phorylation of purified human C1r and C1s by CaMK-II. This was expected in the case of C1̄s since this protein contains no potential phosphorylation site for this kinase. In contrast, C1̄r contains one consensus sequence ($Arg_{132}$-Ser-Lys-$Ser_{135}$), which was therefore not recognized by CaMK-II under the conditions used.

C1̄r and C1̄s were next submitted to phosphorylation by CK2 in the presence of $^{32}$P-labelled ATP, and then both proteins were analyzed by SDS-PAGE followed by autoradiography. As shown in Fig. 1, C1̄s was not phosphorylated, indicating that none of the five potential consensus sequences identified in this protein was recognized by CK2. In contrast, treatment of C1̄r under the same conditions led to the appearance of a radioactive species co-migrating with the unreduced protein (Fig. 1B, lane 1). Analysis under reducing conditions showed that labelling was associated with the N-terminal A chain (Fig. 1B, lane 2). Interestingly, incorporation of radioactivity was also detected at the level of fragment C1rα, resulting from autolytic cleavage of C1̄r [15], and migrating in between the phosphorylated α and β subunits of CK2 (Fig. 1B, lanes 1,2). This suggested that C1̄r phosphorylation occurred in the N-terminal α moiety of the A chain. Comparative estimation of the phosphorylation extent indicated that this was higher in fragment C1rα than in whole C1̄r.

### 3.2. *Accessibility of the CK2 phosphorylation site(s) of C1r under various conditions*

C1r was phosphorylated by CK2 under various conditions and the extent of phosphorylation was estimated in each case. In view of the likely location of the phosphorylation site(s) in the N-terminal α region of C1r, and considering that this region contains the $Ca^{2+}$-binding site of C1r [4], the effect of calcium ions was studied. Addition of 1 mM $CaCl_2$ into the reaction medium inhibited strongly, but not completely, incorporation of radioactivity into intact C1̄r. When C1̄r phosphorylation was performed in the presence of both calcium ions and purified C1̄s (i.e. within the $Ca^{2+}$-dependent C1̄s-C1̄r-C1̄r-C1̄s tetramer), a further decrease of radioactive labelling was observed. Complete inhibition of C1r phosphorylation was observed when reaction was performed on the proenzyme form of the C1s-C1r-C1r-C1s tetramer, or on the whole C1 complex, assembled from the proenzyme tetramer and C1q. It should be mentioned that no phosphorylation was detectable at the level of the C1q chains, although this protein contains 4 potential phosphorylation sites in its C-terminal globular regions, at residues $Thr_{116}$, $Ser_{145}$, $Ser_{208}$ (A chain), and $Thr_{169}$ (B chain). With respect to C1r, it is noteworthy that prior treatment with DFP, a reagent able to block the active-site serine residue of this protease [20], inhibited by about 60% subsequent phosphorylation by CK2, suggesting that this occurred at the level of (a) serine(s) residue(s).

### 3.3. *Accessibility of the phosphorylation site(s) in the C1rα fragments generated by autolytic cleavage or limited proteolysis with trypsin*

Phosphorylation of C1̄r by CK2 for 2 h at 25°C yielded low amounts of the autolytic fragment C1rα which, under these conditions, incorporated 0.1 mol of phosphate/mol of protein (Fig. 2A). Raising the temperature to 37°C resulted in an increased production of C1rα, and also increased its phosphorylation stoichiometry, which reached more than 0.2 mol of phosphate/mol of protein, i.e. about 10 times the value estimated for intact C1̄r (Fig. 2B). This strongly suggested that the accessibility of the phosphorylation site(s) was greater in the isolated C1rα fragment than in the intact protein. Also, in contrast with C1̄r, calcium ions had no significant inhibitory effect on the phosphorylation of C1rα.

Phosphorylation by CK2 was also performed on the C1rα fragment generated by limited proteolysis with trypsin. The purified tryptic fragment also incorporated radioactive phosphate as shown by SDS-PAGE analysis and autoradiography, but the phosphorylation stoichiometry (about 0.05 mol of phosphate/mol of protein) was consistently lower than that measured for the autolytic C1rα fragment. However, in view of the inhibitory effect exerted by DFP on the phosphorylation of intact C1̄r, this decreased phosphorylation was likely the consequence of the DFP treatment used after cleavage of

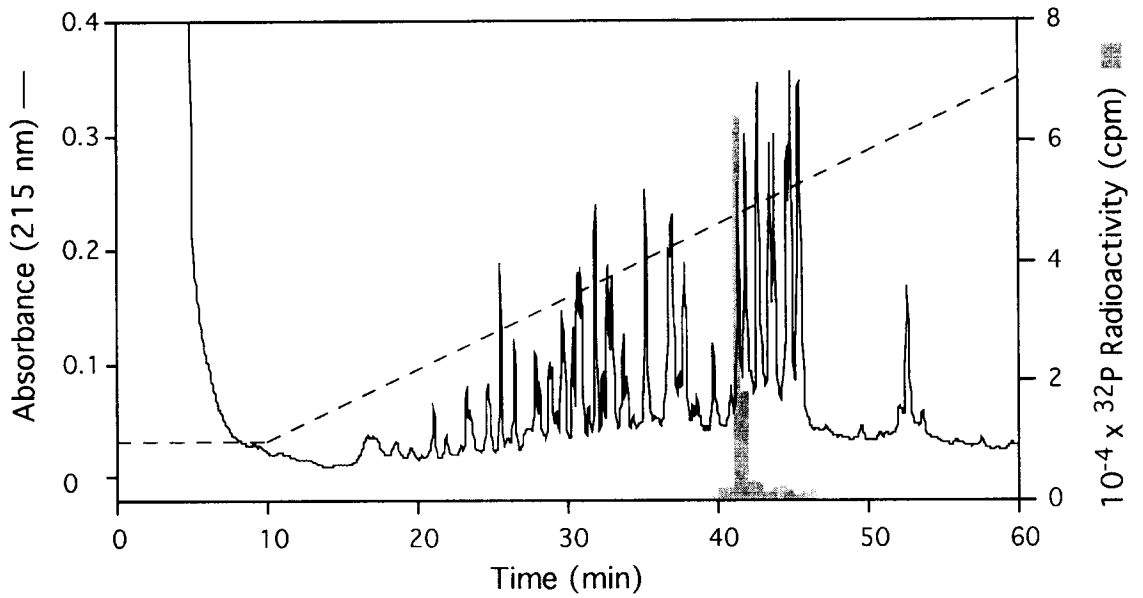


Fig. 3. Initial fractionation by reverse-phase HPLC of the tryptic peptides from $^{32}$P-labelled deglycosylated C$\bar{1}$r. C$\bar{1}$r was deglycosylated with PNGase-F, submitted to phosphorylation by CK2 in the presence of [$^{32}$P]ATP, cleaved with CNBr, then finally cleaved with trypsin after reduction and alkylation. The resulting tryptic peptides were fractionated by reverse-phase HPLC using solvent system 1 (see section 2). Fractions were scanned for absorbance at 215 nm and $^{32}$P radioactivity.

C$\bar{1}$r by trypsin [4]. Again, as observed for autolytic C1rα, calcium ions had no inhibitory effect on the phosphorylation of tryptic C1rα.

### 3.4. *Effect of enzymatic deglycosylation on C1r phosphorylation*

The occurrence of two N-linked oligosaccharides at positions 108 and 204 of C1r, in the N-terminal α region of the A chain [11], suggested that these could be partly responsible for the low accessibility of the phosphorylation site(s). In order to test this hypothesis, C$\bar{1}$r was treated with PNGase-F, then submitted to phosphorylation by CK2. As judged from SDS-PAGE analysis, PNGase-F treatment of C1r for 2 h at 30°C successively removed both carbohydrates, converting the native A chain ($M_r$ 54 000) into a transient monoglycosylated species ($M_r$ 51 000), then into the aglycosylated form ($M_r$ 48 000), in agreement with previous data [11]. Phosphorylation of deglycosylated C$\bar{1}$r by CK2 in the presence of $^{32}$P-labelled ATP led to the appearance of a major radioactive band corresponding to the phosphorylated form of the aglycosylated C$\bar{1}$r A chain, migrating slightly more slowly than its non-phosphorylated counterpart. Comparative estimation of the phosphorylation stoichiometry indicated that this was about 0.3 mol of phosphate/mol of protein for deglycosylated C$\bar{1}$r, compared to only 0.01–0.02 mol of phosphate/mol of protein in the case of native C$\bar{1}$r.

### 3.5. *Identification of the CK2 phosphorylation site of C1r*

In order to facilitate identification of the phosphorylation site, C$\bar{1}$r was first deglycosylated with PNGase-F, then phosphorylated by CK2 in the presence of $^{32}$P-labelled ATP, as described under section 2. SDS-PAGE analysis of the starting material indicated that approx. 25% of the aglycosylated C$\bar{1}$r species was phosphorylated, and the phosphorylation stoichiometry estimated on this species was close to unity (0.75 mol of phosphate/mol of protein). C$\bar{1}$r was separated from both CK2 and PNGase-F by high-pressure gel permeation under denaturing conditions, and the fraction containing $^{32}$P-labelled aglycosylated C$\bar{1}$r was submitted to CNBr cleavage. As judged from SDS-PAGE analysis (not shown), this treatment yielded a large radioactive fragment of $M_r$ approx. 30 000, due to the presence of the disulfide bridges. In order to generate smaller fragments, the CNBr-cleavage reaction mixture was therefore reduced and alkylated, then submitted to tryptic cleavage. Initial fractionation of the tryptic peptides by reverse-phase HPLC using solvent system 1 (Fig. 3) yielded several peaks, of which one was labelled with $^{32}$P. N-terminal sequence analysis of this fraction yielded three major sequences $Gly_{116}$-Phe-Leu-Ala-Tyr..., $His_{170}$-Ser-Cys-Gln-Ala..., and $Phe_{592}$-Val-Arg-Leu-Pro..., present in comparable amounts. Further purification by reverse-phase HPLC using solvent system 2 (not shown) allowed separation of the three peptides and showed that a single one, eluting in the first position, was labelled. As illustrated in Fig. 4, Edman degradation of this peptide allowed identification of residues $His_{170}$ to $Pro_{194}$, indicating that it likely resulted from cleavage of the arginyl bonds at positions 169 and 195. Solid-phase sequencing followed by measurement of the $^{32}$P radioactivity released at each degradation cycle showed unambiguously that phosphate was bound to $Ser_{189}$ (Fig. 4). The electrospray ionisation mass spectrum of the peptide is shown in Fig. 5. The reconstructed molecular mass (3107.3 ± 0.3) is consistent with the average mass calculated for the monophosphorylated form of peptide $His_{170}$–$Arg_{195}$ (3107.2). Taken together, these data clearly established that C1r phosphorylation by CK2 occurs at the level of $Ser_{189}$, within the sequence $Ser_{189}$-Ser-Leu-Glu, which is one of the three consensus sequences for CK2 in C1r.

## 4. Discussion

Our data indicate that none of the five phosphorylation motifs contained in human C1s is recognized by CK2. In contrast, we provide evidence that this kinase phosphorylates

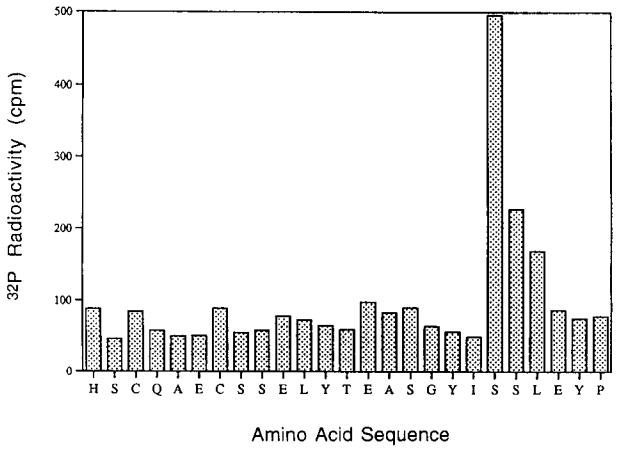


Fig. 4. Edman degradation of peptide 170–195. The amino acid sequence of the peptide was determined by gas-phase sequencing, and identification of the $^{32}P$-labelled residue was performed by Edman degradation of the peptide covalently linked to the support (see section 2).

C1r at the level of $Ser_{189}$, one of the three potential phosphorylation sites of this protein, located within its N-terminal α region. It should be mentioned that all of the motifs identified in C1r and C1s match the minimal consensus sequence (**Ser/Thr**-Xaa-Xaa-Glu/Asp) required for phosphorylation by CK2 [10], but that only three of them ($Ser_{19}$-Glu-Val-Glu and $Ser_{427}$-Asp-Ala-Asp in C1s, $Ser_{104}$-Asn-Glu-Glu in C1r) contain an acidic amino acid at position $i+1$ or $i+2$, i.e. a feature conferring optimal recognition by CK2 [10]. That these sites are not phosphorylated by CK2 strongly suggests that they are not accessible to the kinase, and therefore not exposed to the solvent.

As judged from the phosphorylation stoichiometry estimated in this study (0.01–0.02 mol of phosphate/mol of protein), the accessibility of $Ser_{189}$ is low in intact C1r. Based on the effect of enzymatic deglycosylation, it is clear that this low accessibility of $Ser_{189}$ is due, in large part, to the presence of one or both of the oligosaccharides attached to Asn residues 108 and 204, each contained in the α region. Indeed, kinetic analysis of the deglycosylation process followed by phosphorylation by CK2 (not shown) indicated that removal of a single oligosaccharide is sufficient to allow increased phosphorylation of $Ser_{189}$, suggesting that this residue lies in the vicinity of, or is hidden by one of the carbohydrate chains. In this respect, the fact that treatment of $C\bar{1}r$ with DFP inhibits subsequent phosphorylation of $Ser_{189}$ suggests that this residue has characteristics comparable to those of an active-site serine residue, and therefore probably lies in an hydrophobic environment.

Considering the low reactivity of $Ser_{189}$ in intact C1r, it appears unlikely that its phosphorylation plays a significant structural and/or functional role. However, comparison of the extent of phosphorylation of $Ser_{189}$ under various conditions provides information on the relative degree of exposure of the region surrounding this residue. Thus, our data show that binding of $Ca^{2+}$ to C1r, which is known to be mediated by the α region [4], strongly decreases the accessibility of $Ser_{189}$. Further, incorporation of C1r into the $Ca^{2+}$-dependent C1s-C1r-C1r-C1s tetramer, which again involves interaction of the α region of C1r with the corresponding region of C1s [4], leads to complete inhibition of the phosphorylation of $Ser_{189}$, which remains unaccessible to CK2 in the whole C1 complex. In contrast, removal of the α fragment from the remainder of the C1r molecule strongly increases reactivity of $Ser_{189}$ with respect to the kinase. In addition, contrary to what is observed in whole C1r, phosphorylation of $Ser_{189}$ in the isolated α fragment is not inhibited in the presence of $Ca^{2+}$ ions. Taken together, these data suggest that the low accessibility of $Ser_{189}$ in intact C1r compared to the isolated α fragment arises from the interaction of the neighbouring region (i.e. the C-terminal end of the α region) with the rest

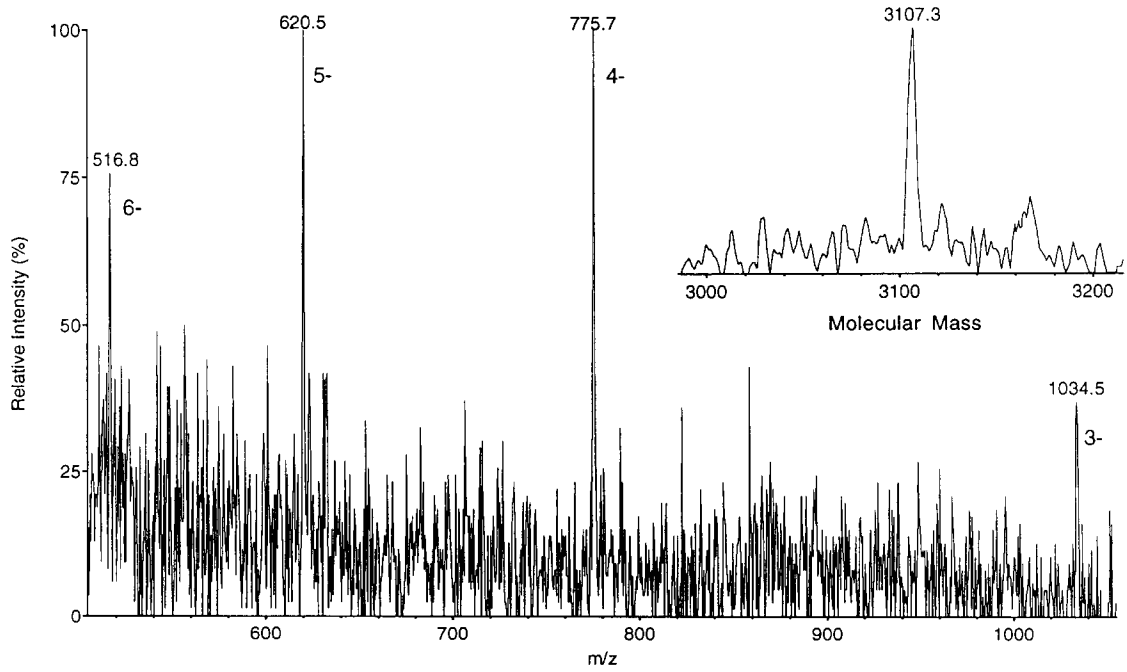


Fig. 5. Electrospray ionisation mass spectrum of peptide 170–195. The spectrum was obtained as described under section 2. The inset shows the reconstructed molecular mass profile derived from the series of negatively charged ions.

of the C1r molecule, and that this interaction is reinforced in the presence of $Ca^{2+}$ ions. Previous studies [6] have led to the conclusion that activation of the serine-proteinase domain of C1r is controlled by a $Ca^{2+}$-dependent intramolecular mechanism involving the $Ca^{2+}$-binding α region. The present data provide further support to this hypothesis and suggest that this control mechanism occurs through direct interaction between the $Ca^{2+}$-binding α region and the catalytic domain of C1r.

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