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The cysteine proteinase inhibitor chicken cystatin is a phosphoprotein

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Peptide maps obtained by reversed-phase HPLC of tryptic digests of isoelectric form 1 (p*I* = 6.5) and 2 (p*I* = 5.6) of chicken egg white cystatin revealed that the difference was located only in a single peptide (residues Ser-74–Lys-91). Ser-80 of cystatin 2 was subsequently identified as being modified by phosphorylation. Moreover, alkaline phosphatase treatment of a mixture of native cystatin forms 1 and 2 was shown by ion-exchange chromatography to cause the disappearance of isoelectric form 2 with a concomitant increase in form 1. Thus, the existence of two isoelectric forms of chicken cystatin is due to the phosphorylated form 2 and non-phosphorylated form 1.

Cystatin; Proteinase inhibitor; Protein phosphorylation; (Chicken egg white)

1. INTRODUCTION

Chicken cystatin is a reversible, tight-binding protein inhibitor of papain-like cysteine proteinases. It has been crystallized [1] and its three-dimensional structure elucidated by X-ray diffraction techniques [2]. Based on the crystal structure, a model for the interaction of chicken cystatin with papain has been proposed [2] and several properties of the model confirmed by biochemical data [3].

By ion-exchange chromatography chicken cystatin can be resolved into two immunologically identical forms with p*I* values of 6.5 and 5.6 [4], designated as forms A and B [5] or 1 and 2 [4], respectively. The structural differences responsible for the occurrence of two isoelectric forms are a matter of controversy. Initially, it was proposed that cystatin 1 is eight amino acid residues shorter than cystatin 2 [5], but the separation of either form into full-length and N-terminally truncated forms contradicted these results [3]. Moreover, both full-length molecules had identical inhibition constants for papain and no sequence differences were detected between their 18 N-terminal amino acids [3]. Thus, as all previously published data provided no explanation for the difference between the forms, it was implicit that the structures vary only to a small extent.

Here, we show that the structural difference between the two isoelectric forms of chicken cystatin is due to a phosphoserine residue in form 2. The location of the moiety in the polypeptide chain is identified and possible consequences of this modification are discussed.

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Abbreviation: PSer, *O*-phosphoserine

2. MATERIALS AND METHODS

2.1. *Materials*

Cystatin was purified from chicken egg white [5]. Forms 1 and 2 were resolved by ion-exchange chromatography (Pharmacia Mono Q FPLC column) and further separated into the

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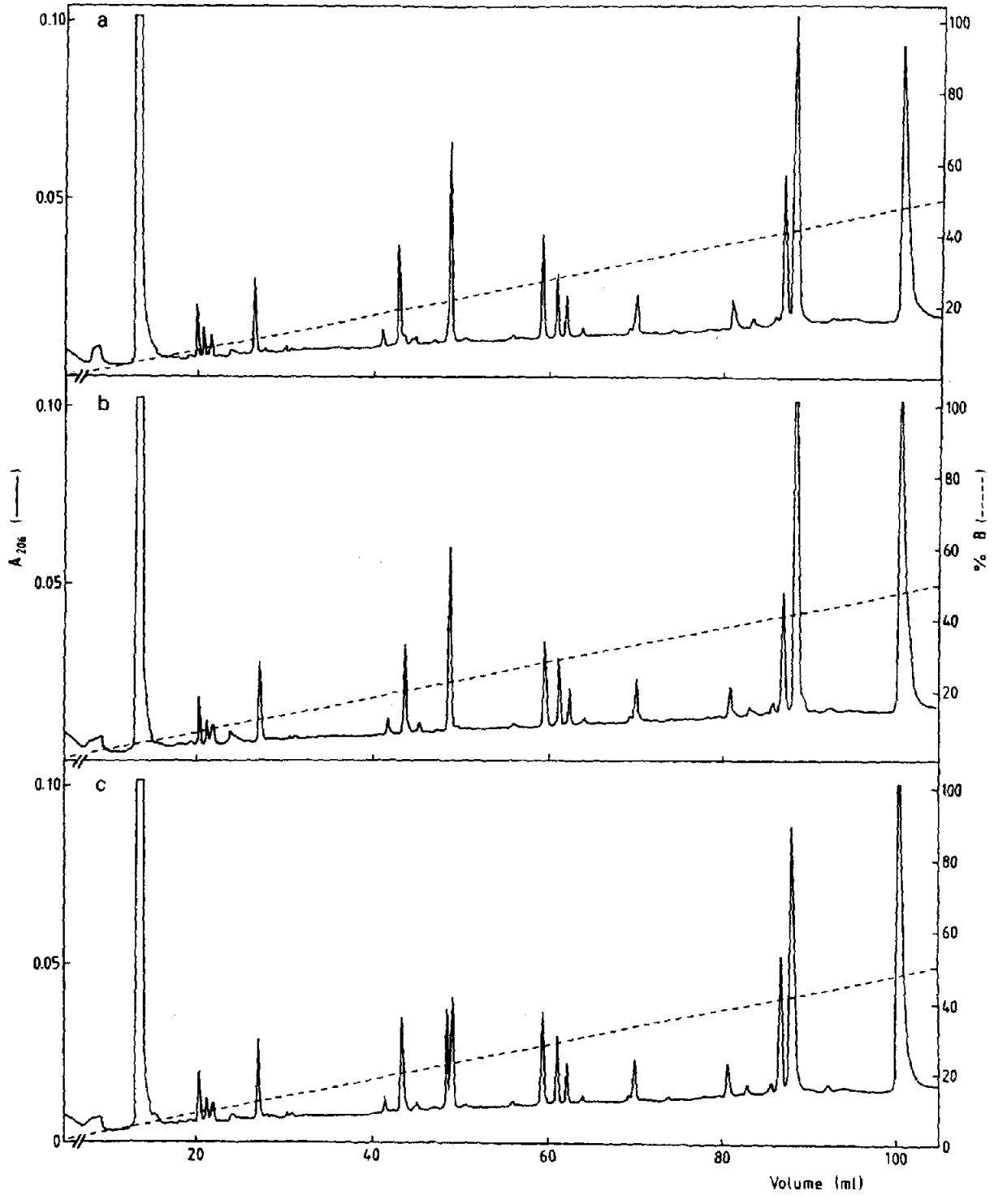

Fig.1. Reversed-phase HPLC separation of tryptic peptides of cystatin form 1 (a), form 2 (b) and of a mixture of equal amounts of forms 1 and 2 (c).

Table 1

Amino acid composition of peptides P-11 from cystatin 1 and 2 in comparison with that deduced from the corresponding sequence of cystatin 1 [5,10]

Amino acid	Composition found for peptide P-11 from		Composition from sequence
	Cystatin 1	Cystatin 2	
Asp/Asn	1.9	1.8	2
Ser	2.9	2.5	3
Glu/Gln	4.5	4.0	4
Gly	1.3	1.1	1
Ala	1.3	1.0	1
Met	0.7	0.8	1
Leu	1.1	1.1	1
Phe	0.8	0.9	1
Lys	1.0	1.3	1
His	0.9	0.8	1
Pro	+	+	1
Cys	+	+	1

(+) Amino acid present but not quantified; Cys was determined as *S*-β-(4-pyridylethyl)cysteine

full-length inhibitor and N-terminally truncated forms by hydrophobic-interaction chromatography [3]. Alkaline phosphatase from bovine intestine and TPCK-treated trypsin were from Sigma. Endoproteinases Glu-C and Asp-N were purchased from Boehringer.

2.2. *Peptide mapping*

N-terminally truncated cystatins 1 and 2 (peptide chain starting with Gly-9) were denatured, mercaptolysed and then alkylated with 4-vinylpyridine [6]. After digestion with trypsin [4], fragments were separated on a Macherey-Nagel ET 250/8/4 Nucleosil 300-10 C-18 HPLC column in an elution gradient from 0 (solvent A) to 80% acetonitrile (solvent B) in 0.1% TFA in 160 min.

2.3. *Endoproteolytic digestion*

Peptides were incubated at 37°C either for 2 h in 100 μl of 0.1 M NH_4HCO_3 (pH 7.8) with 5 μg endoproteinase Glu-C or for 16 h in 100 μl of 50 mM sodium phosphate buffer (pH 8.0) with 0.5 μg endoproteinase Asp-N and subsequently fractionated by HPLC.

2.4. *Alkaline phosphatase treatment*

Peptides were dissolved in 100 μl of 50 mM Tris-HCl, 1 mM $MgSO_4$, 0.1 mM $ZnSO_4$ (pH 9.0; P-buffer), incubated at 37°C for 1 h with 30 nkat (5.4 μg) alkaline phosphatase and subsequently separated by HPLC. 0.58 mg native chicken cystatin was incubated at 37°C for 3.5 h in 0.23 ml P-buffer with 30 nkat (5.4 μg) alkaline phosphatase and separated on the Mono Q column.

2.5. *Amino acid and sequence analyses*

Amino acid analyses were carried out on a Biotronic LC 600 amino acid analyzer after hydrolysis with 6 N HCl at 110°C for 24 h or 2 h. Peptides were sequenced by Edman degradation in a prototype spinning-cup sequenator [7,8]. Phosphoamino acid analyses were performed by electrophoresis on cellulose thin-layer plates at pH 3.4 [9]. Additionally, the presence of phosphoserine was proved by amino acid analysis.

3. RESULTS

Peptide maps obtained by reversed-phase HPLC of tryptic digests of isoelectric forms 1 and 2 of chicken cystatin gave elution patterns which revealed only a single difference (fig.1a,b).

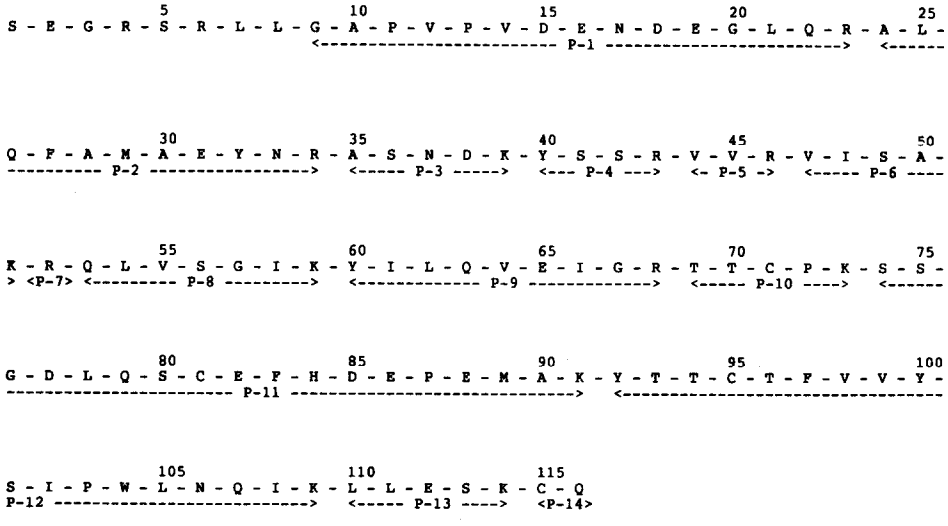

Fig.2. Amino acid sequence of chicken cystatin 1 [5,10]. Peptides produced by trypsin digestion are indicated and numbered.

Chromatography of a mixture of equal amounts of these peptides unequivocally confirmed this result (fig.1c). Only the peptide eluting at about 22% solvent B split into two separate peaks, other peptides of the map being superimposable. Amino acid compositions of the two differing peptides (table 1) corresponded to tryptic peptide P-11 (fig.2), were identical and in agreement with the previously published sequence of cystatin 1 [5,10]. Thus, rather than an amino acid substitution either deamidation or a charged substituent could explain the two different cystatin forms. Deamidation would have changed Gln-79 (the only amide in P-11) to Glu and consequently introduced an additional cleavage site for endoproteinase Glu-C, which specifically cleaves peptide bonds C-terminal to Glu residues [11]. Incubation of P-11 from cystatin 1 with endoproteinase Glu-C resulted in the formation of three fragments: S-S-G-D-L-Q-S-C-E, F-H-D-E-P-E and M-A-K. However, digestion of P-11 from cystatin 2 did not produce an additional fragment, thus constituting evidence against deamidation of Gln-79. Unexpectedly, only two fragments were obtained: S-S-G-D-L-Q-S-C-E-F-H-D-E-P-E and M-A-K.

Automatic sequence analysis of eight (form 1) and 15 (form 2) residues together with the amino acid compositions of tryptic and endoproteinase Glu-C digests gave S-S-G-D-L-Q-S-C-E-F-H-D-E-P-E-M-A-K as the complete sequence of P-11 from both cystatin forms (in agreement with the previously published sequence for residues 74–91 of cystatin 1; fig.2). The reason for endoproteinase Glu-C failing to cleave the C-E bond in P-11 from cystatin 2 whilst complete cleavage was observed in P-11 from cystatin 1 could not be explained.

Rechromatography of a mixture of equal amounts of isolated P-11 from the two cystatin forms clearly separated the peptides from each other (P-11 from cystatin 2 eluted first, indicating lower hydrophobicity). Treatment with alkaline phosphatase did not affect the retention time of P-11 from cystatin 1 but increased that of P-11 from cystatin 2 to such an extent that it coeluted with P-11 from cystatin 1 (fig.3b). Thus, a phosphoamino acid in P-11 from cystatin 2 is the difference between the two cystatin forms. Furthermore, the phosphorylated residue must be phosphoserine, since neither threonine nor tyrosine, whose phosphorylated derivatives have

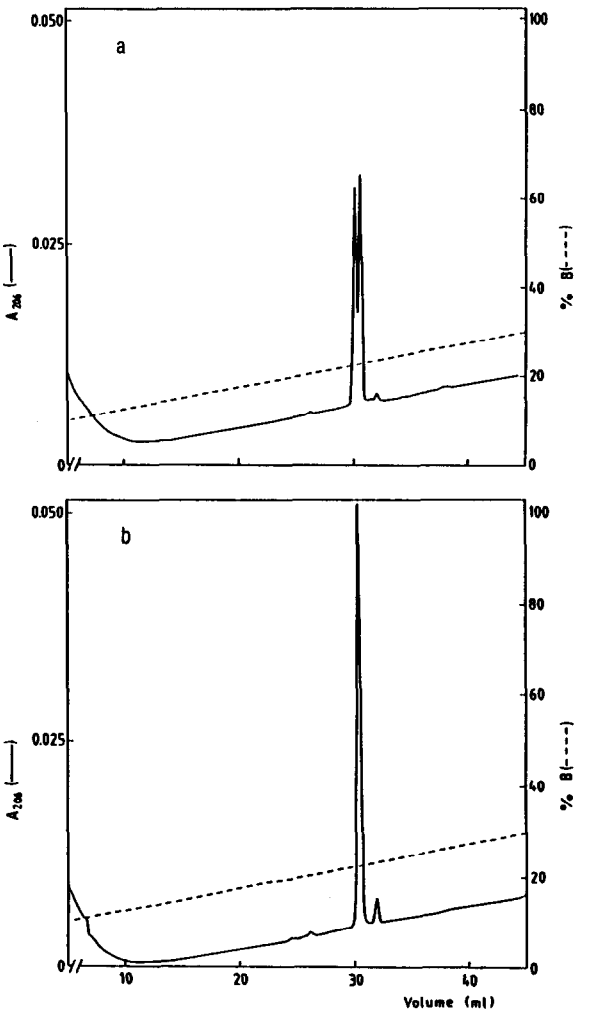

Fig.3. Reversed-phase HPLC separation of the untreated mixture of P-11 of cystatin form 1 and 2 (a) and of the alkaline phosphatase-treated mixture (b).

also been found in proteins [12], occurs in P-11.

To determine the exact location of the PSer-residue (position 74, 75 or 80) P-11 was treated with endoproteinase Asp-N, which specifically cleaves peptide bonds N-terminal to Asp residues [13]. Cleavage of P-11 from cystatin 1 gave the fragments S-S-G, D-E-P-E-M-A-K and D-L-Q-S-C-E-F-H. However, about 60% of the last fragment was unexpectedly cleaved further into D-L-Q-S-C and E-F-H (D-L-Q-S-C and D-E-P-E-M-A-K were not separated on the HPLC column). Cleavage of P-11 from cystatin 2 produced the fragments S-S-G, D-E-P-E-M-A-K and D-L-Q-S-

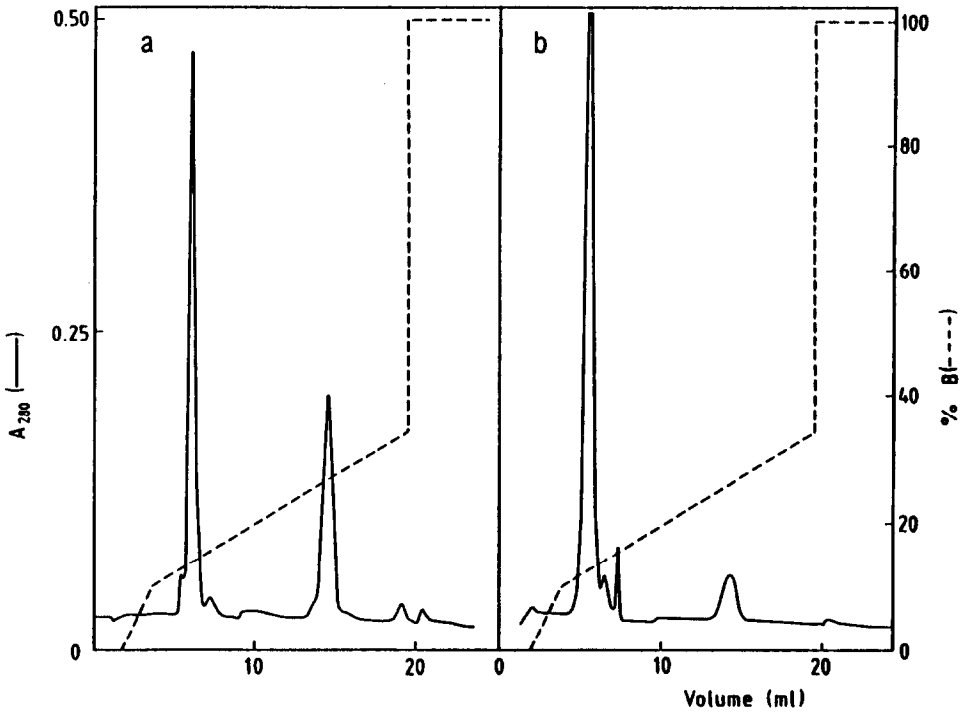

Fig.4. Ion-exchange FPLC separation of the untreated mixture of native cystatin forms 1 and 2 (a) and of the alkaline phosphatase-treated mixture (b).

C-E-F-H; no additional cleavage of the last fragment was observed. In both cases Ser-74 + Ser-75 were present in a different fragment from that of Ser-80.

For phosphoamino acid analysis both S-S-G fragments were hydrolyzed for 2 h – conditions which have been found to destroy PSer only to a minimal extent [14]. No PSer was detected when the hydrolysates were subjected to amino acid analysis. The possibility of modification of Ser-74 or Ser-75 of cystatin 2 by phosphorylation was thereby excluded.

After partial acid hydrolysis the Ser-80-containing P-11 fragments, obtained by digestion with endoproteinase Asp-N, were subjected to thin-layer electrophoresis. Upon spraying with ninhydrin, a spot with relative mobility similar to that of authentic PSer was visible only in the sample originating from cystatin 2. When the hydrolysates were subjected to amino acid analysis, again only the sample originating from cystatin 2 produced a peak at the position of authentic PSer. Thus, the two forms of chicken cystatin differ in residue 80, which is serine in form 1 and phosphoserine in form 2.

Ion-exchange chromatography of native chicken cystatin gave an average of about 63% form 1 and 37% form 2, eluting at 70 and 140 mM KCl, respectively (fig.4a). Treatment of native cystatin with alkaline phosphatase for 3.5 h prior to separation by ion-exchange chromatography caused the disappearance of form 2 with a concomitant increase in form 1 (fig.4b). After the 3.5 h incubation period only 10% of the material eluted at the position of cystatin 2, whereas 90% eluted as form 1. Thus, about 75% of cystatin 2 was transformed to cystatin 1 by alkaline phosphatase treatment, again indicating that a phosphorylated residue exists only in cystatin 2.

4. DISCUSSION

The present investigation has provided direct evidence for the occurrence of PSer at position 80 in form 2 (Ser in form 1) and an explanation for

the difference between the two isoelectric forms of chicken cystatin. The additional negative charge of the PSer moiety is responsible for the lowering of the isoelectric point from 6.5 (form 1) to 5.6 (form 2). Transformation of native cystatin 2 into cystatin 1 by phosphatase treatment confirmed that phosphorylation is the only difference between both forms.

According to the three-dimensional structure of cystatin 1 [2] the side chain of Ser-80 protrudes from the main body of the molecule (fig.5). Thus, the side chain is readily accessible to protein kinases as well as phosphatases, thereby explaining why the phosphomonoester linkage is rapidly hydrolyzed by alkaline phosphatase.

Attempts to crystallize cystatin 2 for X-ray structure analysis were unsuccessful. The failure to obtain crystals may be due to the additional negative charge introduced by the PSer residue, which causes a significant increase in solubility. Additionally, if the crystal packing of cystatin 2 were to be the same as that seen for cystatin 1, the negatively charged phosphoryl group of PSer-80 would be in close vicinity to the negatively charged carboxyl group of Glu-65 of a symmetry-related molecule. The repulsion between these adjacent groups most likely hinders the formation of a highly ordered structure and thus prevents crystal growth.

The amino acid sequence Ser-Cys-Glu at the phosphorylation site is in agreement with the consensus sequence PSer-X-Glu for phosphorylation sites of many other proteins [15–17]. This sequence (residues 80–82) is unique to chicken cystatin and has not been found in other members of family 1 and 2 of the cystatin superfamily [18]. However, it has been detected in a family 3 cystatin, human kininogen segment 3. Additionally, possible phosphorylation sites occur in human cystatins, e.g. S-V-E (residues 15–17) in cystatin C, and S-F-E (residues 96–98) in cystatins S and SN [18]. However, further investigations are required to determine whether modification by phosphorylation also occurs in other cystatins. The recent report of glycosylated and non-glycosylated isoforms of rat cystatin structurally related to human cystatin C [19] indicates that post-translational modifications of cystatins may be more frequent than previously expected and may play an important role in their biological activities.

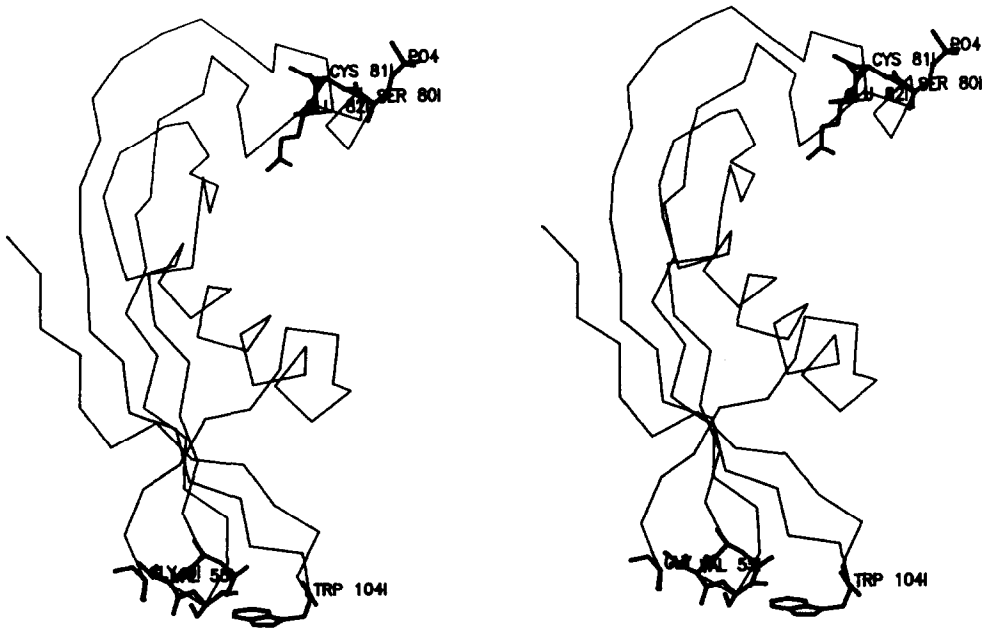

Fig.5. α-Carbon drawing of the structure of chicken cystatin. Residues Gly-9, Leu-54, Ser-56 and Trp-104 (which undergo major binding interactions with papain [2]) and residues around the phosphorylation site (PSer-80–Glu-82) are represented with side chains (thick connections).

The biological function(s) of the phosphorylation of cystatin 2 has remained unclear thus far. Nevertheless, regulation of the inhibitory activity seems quite unlikely as the two isoelectric forms have virtually identical inhibition constants for papain [3]. This is in complete agreement with the proposed model of the cystatin-papain complex [2] as the PSer residue lies opposite to the cystatin-papain binding area (fig.5) and is not in contact with the proteinase molecule. Consequently, binding of cystatin to papain is not influenced by the PSer residue. However, the PSer moiety may possibly be involved in the import of cystatin into the developing egg. Thus, it is known that dephosphorylation of riboflavin-binding protein (a phosphoglycoprotein necessary for the transport of riboflavin to the egg) greatly diminishes the uptake of this protein into oocytes [20]. Furthermore, since peptide P-11 from phosphorylated form 2 has been found to be less susceptible to endoproteolytic attack than P-11 from cystatin 1, the PSer moiety may protect the molecule against degradation.

Cystatin isolated from chicken egg white is a mixture of the non-phosphorylated and phosphorylated form. This raises the question as to whether both forms occur naturally or one is formed from the other during storage of eggs or the purification. It is known that PSer in peptides and proteins is rapidly destroyed by β-elimination of the phosphate residue when exposed to alkaline conditions [21,22]. Therefore, a certain amount of form 2 may be destroyed during the purification, which involves strongly alkaline conditions [5]. Additionally, cystatin 2 may be dephosphorylated in the egg, especially by the action of alkaline phosphatases, since the pH of chicken egg white is known to rise to 9.5 during storage [23]. Thus, it is quite uncertain as to whether the relative amounts of forms 1 and 2 found after the purification procedures reflect the in vivo situation. Furthermore, since chicken cystatin is not present exclusively in egg white but has also been detected in the serum and in muscle cells [4,24], variable amounts of the two isoelectric forms may be present in different tissues.

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