Absence of Large-scale Conformational Change upon Limited Proteolysis of Ovalbumin, the Prototypic Serpin*

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1H and 31P NMR spectroscopies have been used to examine the effects of limited proteolysis with subtilisin Carlsberg on the global conformation of ovalbumin and on the local environment of phosphoserine 344, a residue two positions removed from the site of proteolysis. Such limited proteolysis has been shown to result in excision of a hexapeptide from the region of the protein that, in other serine protease inhibitors (serpins), contains the reactive center. Based on the structure of the related serpin α1-antitrypsin, it has been predicted that phosphoserine 344 should undergo a large change in environment upon proteolysis of ovalbumin (Löbermann, H., Tokuoka, R., Deisenhofer, J., and Huber, R. (1984) J. Mol. Biol. 177, 531–550). Proteolysis of ovalbumin produces a small upfield shift (0.15 ppm) of the 31P resonance of phosphoserine 344. In addition, the pKᵢ of phosphoserine 344 is raised by 0.1 pH unit. At pH 8.5, phosphoserine 344 in cleaved ovalbumin (plakalbumin) is as accessible to hydrolysis by Escherichia coli alkaline phosphatase as it is in native ovalbumin. 1H NMR shows that dephosphorylation of serine 344 has an imperceptible effect on the protein’s conformation. Similarly, little effect on conformation is seen by 1H NMR upon proteolysis of ovalbumin. These findings suggest that ovalbumin does not undergo a marked conformational change analogous to that inferred for the related members of the serpin superfamily, α1-antitrypsin and antithrombin III, nor do the residues close to the site of proteolysis appear to change environment from that of an exposed loop to a buried strand of β-sheet. These findings are not consistent with the hypothesis of Carrell and Owen (1985) Nature 317, 730–732) for the role of the exposed loop in serpins of directly facilitating conformational change upon cleavage of the loop. Instead, it is proposed that cleavage of the exposed loop alters the solvent accessibility of residues formerly covered by the loop and that this provides the thermodynamic impetus for conformational change, perhaps by disruption of a salt bridge crucial to the integrity of the native structure.

There are now more than 10 published sequences of proteins belonging to the serine protease inhibitor (serpin) family (1, 2). Inclusion of a protein in the serpin family is based on sequence homology of the C-terminal region of approximately 350 residues, which is believed to arise from a common ancestral gene (3). The N-terminal region of serpins shows great variation in length, composition, and degree of glycosylation and confers auxiliary properties on the protein such as the heparin binding sites in antithrombin III and heparin cofactor II (3–5).

All members of the family are thought to possess an exposed loop of polypeptide that is particularly susceptible to proteolysis and that contains a dipeptide that is the site of cleavage by the target protease (where known). Based on observations of a great increase in stability for the serpins α1-antitrypsin and antithrombin III upon cleavage at or near the active center dipeptide, it has been proposed that native serpins exist in a strained conformation that undergoes a large conformational change to a relaxed form upon limited proteolysis (6). The x-ray structure of the cleaved form of α1-antitrypsin supports such a large conformational change, since the two residues that constitute the active center dipeptide in the native protein are located 67 Å apart in the cleaved species (7). For antithrombin III, 1H NMR data on native and cleaved species support a large scale conformational change upon proteolysis (8).

Our interest in ovalbumin stems from the original sequence comparison that led to formulation of the notion of the serpin family. This comparison was between ovalbumin, α1-antitrypsin, and antithrombin III (9, 10). In keeping with the altered properties of the cleaved versus native forms of antithrombin III and α1-antitrypsin, it was observed many years ago that reaction of ovalbumin with subtilisin results in a very limited proteolysis and a change in properties such that the form in which the protein crystallizes changes from needles to large plates (11), hence the name plakalbumin for the cleaved form. Based upon the x-ray crystal structure of cleaved α1-antitrypsin and the sequence homology with ovalbumin, it has been predicted that the phosphorylated residue Ser-344 in ovalbumin should undergo a marked change in environment upon conversion of ovalbumin to plakalbumin (7). 31P NMR spectra of plakalbumin are reported here that permit comparison of the properties of phosphoserine-344 in native and cleaved ovalbumin to be made. It is found that this residue remains quite mobile and accessible to bacterial alkaline phosphatase in plakalbumin. The phosphate pKᵢ is raised only slightly and indicates a surface hydrophilic environment. The effects of proteolysis on the 1H NMR spectra of two forms of ovalbumin are also examined; normal bis-phosphorylated ovalbumin and ovalbumin dephosphorylated at Ser-344. In both cases there are only very slight changes in the spectra of the protein upon proteolysis, and the changes are nearly identical for the two proteins. These data suggest that the negatively charged phosphate group on Ser-344 does not have a significant effect on the conformational changes occurring upon proteolysis of ovalbumin and that the change in conformation is very limited and does not involve burial of...
residue 344. These conclusions suggest that the large conformational change inferred for antithrombin III and α,-antitrypsin upon cleavage does not occur for ovalbumin and that, therefore, cleavage of the exposed loop per se is not sufficient for conformational rearrangement, in contradiction of the hypothesis of Carrell and Owen (6).

MATERIALS AND METHODS
Plakalbumin was prepared from hen ovalbumin (Sigma, grade VI) by reaction with subtilisin Carlsberg at pH 8.5 in 50 mM Tris-HCl for 2 h using a protease/protein ratio of 1:8000. Protease was removed by several cycles of dilution and re-concentration in an Amicon ultrafiltration cell fitted with a PM-30 membrane. The first dilution-reconcentration step was achieved within 10 min and removed most of the subtilisin. After several cycles the residual amount of subtilisin was <0.1% of that used originally. This ensured that subsequent non-specific cleavage of plakalbumin by trace amounts of protease could be ignored.

Ovalbumin dephosphorylated at residue 344 was prepared by reaction of ovalbumin with bacterial alkaline phosphatase. 2 mM ovalbumin in 20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 1 mM ZnCl₂ was reacted with 20 units of bacterial alkaline phosphatase for 5 h at room temperature. The ³¹P NMR spectrum was monitored for selective removal of the Ser-344 phosphate group and showed complete removal after 5 h with only minimal hydrolysis of the phosphate group on Ser-68. The protein was fractionated on a G-100 column (1 X 90 cm) to remove alkaline phosphatase and the ovalbumin fractions pooled and dialyzed against 10 mM ammonium bicarbonate prior to freeze drying. Ovalbumin concentrations were determined spectro-photometrically using A₂₈₀ = 5.0 (12).

Subtilisin Carlsberg, Escherichia coli alkaline phosphatase type III, and 95.8% D₂O were purchased from Sigma.

³¹P NMR spectra were recorded at 81 MHz on a Bruker AC200 wide bore spectrometer. 2-ml samples in 10-mm tubes were employed. The buffer used was 50 mM Tris-HCl, 1 mM EDTA, 20% (v/v) D₂O for the pH titration experiments. Chemical shifts are given relative to an external standard of 85% phosphoric acid taken as 0 ppm. Proton decoupling using a low power WALTZ pulse sequence was employed. Typical acquisition parameters included a 30° pulse angle and a repetition rate of 0.75 s⁻¹. These conditions should minimize differences in peak intensities attributable to even large differences in T₁ values. A line broadening of 2 Hz was routinely used to improve the signal-to-noise ratio. pH measurements were made using a Radiometer 26 pH meter and a 3-mm Ingold combination electrode. pH values are given as direct meter readings uncorrected for deuterium isotope effects.

¹H NMR spectra were recorded on a narrow bore Bruker AM400 spectrometer equipped with a 5-mm ¹H probe. A sweep width of 6024 Hz, block size of 16 K points, and pulse angle of 60° were employed. Residual water intensity was diminished using a low power presaturation pulse of 0.5-s duration. Chemical shifts are given relative to an external standard of dimethyl silapentane sulfonic acid at 0 ppm.

RESULTS

³¹P NMR Spectra of Plakalbumin—The ³¹P NMR signals from the two phosphoserine residues in hen ovalbumin have been previously assigned (13). Resonance 1 (Fig. 1a) arises from phosphoserine 68 and resonance 2 from phosphoserine 344. To determine the effect of conversion of ovalbumin into plakalbumin on these two phosphonoanesters, the ³¹P NMR spectrum of a sample of ovalbumin at pH 6.3 was recorded as a function of time after addition of the protease subtilisin. The pH was chosen to be close to the pKₐ since the ³¹P chemical shift changes at its maximum rate at the pKₐ and even small perturbations should be detectable. These ³¹P NMR spectra are shown in Fig. 1. The resonance from phosphoserine 68 is insensitive to the cleavage, whereas phosphoserine 344 resonates at 3.03 ppm in ovalbumin and 2.82 ppm in plakalbumin. After 1h, resonances from both cleaved and uncleaved species are present (Fig. 1b), representing approximately 50% conversion to plakalbumin. After a further hour of reaction, the conversion is complete and no further change occurs (Fig. 1d).

To assess the origin of the upfield shift for phosphoserine 344 upon proteolysis of ovalbumin, a pH titration was performed on plakalbumin and the pKₐ for ionization from phosphomonoanion to dianion determined from the pH dependence of the ³¹P resonance chemical shift. Chemical shifts as a function of pH are shown in Fig. 2 for both phosphoserine residues. The pKₐ values and Hill coefficients (n) determined for residues 68 and 344 are pKₐ = 6.00 and 6.15 and n = 0.96 and 0.91, respectively. These values compare with pKₐ values of 6.00 and 6.04 for native ovalbumin (13). In addition the alkaline extremum of the phosphoserine 344 resonance is upfield shifted by 0.15 ppm relative to that found in ovalbumin (13). Two resonances were clearly seen at all pH values examined. This observation and the linear fit to a Henderson-Hasselbach plot obtained for the resonance chemical shifts make undetected cross-over of the resonances extremely unlikely.

In native ovalbumin it has been shown that the 2 phospho-
serine residues have different accessibilities to E. coli alkaline phosphatase such that phosphoserine 344 can be completely hydrolyzed whereas phosphoserine 68 is only 10% changed after reaction with 20 units of enzyme for 20 h at 37°C. To test whether phosphoserine 344 in plakalbumin had become less accessible to alkaline phosphatase, the time course of reaction with phosphatase was monitored by 31P NMR (data not shown, but qualitatively identical to those of Vogel and Bridger (13) obtained for ovalbumin). Over the time period examined (12 h), no hydrolysis of phosphoserine 68 was detected, whereas phosphoserine 344 was more than 70% hydrolyzed. This reaction was performed with only 5 units of phosphatase and at a lower temperature than employed by Vogel and Bridger. It thus seems that phosphoserine 344 is still readily accessible in cleaved ovalbumin.

Effect of Removal of Ser-344 Phosphate—α1-Antitrypsin differs from ovalbumin in containing no sites of phosphorylation. Whereas proteolysis of α1-antitrypsin results in burial in β-sheet of residues preceding and following the site of proteolysis, it is possible that a comparable burial in ovalbumin is precluded by unfavorable energetics associated with removing a doubly negatively charged phosphate group from an aqueous environment into the hydrophobic interior of the protein. The 'H NMR spectra of plakalbumin and plakalbumin dephosphorylated at Ser-344 were thus compared (Fig. 3). These spectra are almost identical and give very little intensity in a difference spectrum (Fig. 3c). The phosphate group on Ser-344 thus has almost no effect on the conformation of plakalbumin.

Effect of Proteolysis on 'H Spectrum of Ovalbumin—Cleavage of ovalbumin by subtilisin can be conveniently followed by 'H NMR in situ. The low level of protease used adds insignificantly to the observed ovalbumin resonances. Reaction is complete within 2 h under the conditions employed. Fig. 4 shows the initial and final spectra and the difference between them. The majority of the changes occur in the aliphatic region between 3.8 and 4.7 ppm and between 0.8 and 2.2 ppm and involve changes in chemical shift and sharpening of resonances upon proteolysis. The resonances at 1.54 and 1.93 ppm are doublets characteristic of methyl groups of terminal and internal alanine residues (14, 15), while the prominent negative peak at 0.93 ppm is at the position expected for valine, isoleucine, or leucine methyl resonances (15). The peak at 2.15 ppm is at an appropriate position for the CH group of valine. The group of perturbed resonances between 3.8 and 4.7 ppm are from α-CH protons. All of these prominent, sharp resonances result from the hexapeptide that is excised from ovalbumin by subtilisin in converting it to plakalbumin (16). This peptide has the sequence Ala-Gly-Val-Asp-Ala-Ala. The sharp resonances can be removed by dialysis. In contrast to these prominent resonances attributable to a free peptide, perturbations of other resonances due to the protein are very limited. Changes of environment of approximately 12 aromatic resonances by less than 0.1 ppm occur and two upfield-shifted methyl resonances at −0.17 and +0.23 ppm move downfield by 0.05 ppm. Almost no other changes are seen. The conformations of the two proteins thus seem to be almost identical, especially when the paucity of changes in the region of unexchanged core amide protons (8 to 11 ppm), upfield-shifted aromatic resonances (6.0 to 6.8 ppm), and upfield-shifted methyl resonances (0.7 to −0.4 ppm) is noted. These three regions are expected to be the most sensitive indicators of the tertiary structure of the protein since they either depend on the integrity and stability of the internal hydrogen bonding (for amide protons) or the proximity of nearby perturbing side chains.

DISCUSSION

The environment of phosphoserine 68 in plakalbumin is indistinguishable by 31P NMR from that in native ovalbumin. The phosphomonoester has the same pKα and the same chemical shift titration range. Phosphoserine 344 does sense the cleavage by subtilisin 2 residues away, but only to the extent of a slight change in chemical shift and increase of 0.1 pH unit in pKα. This phosphate group is still clearly in an aqueous environment, as judged by its pKα and ability to change ionization states and must be well exposed, since it retains its susceptibility to hydrolysis by alkaline phosphatase found for native ovalbumin.

If ovalbumin and α1-antitrypsin have homologous structures and undergo analogous conformational changes upon proteolysis at the active center bond, phosphoserine 344 in plakalbumin should become part of a six-strand β-sheet and...
unphosphorylated at this position appear to be identical (Fig. 4).

Proteolytic cleavage of ovalbumin results in exposure of the X-Ser dipeptide, which might be expected to result in a much greater perturbation in pK\textsubscript{a} and/or chemical shift for phosphoserine 344 than the small changes seen. An example of the change in \( ^{31}P \) chemical shift of phosphoserine between free solution and an interior protein environment is provided by apoalkaline phosphatase, where the phosphodiester resonance differs by 2.5 ppm between the two environments (17). Also it seems unlikely that such a relocation of the phosphate would leave accessibility to alkaline phosphatase unchanged from that found in the native protein. In ovalbumin it thus seems that the predicted burial of residue 344 and adjacent amino acids in an extensive \( \beta \)-sheet does not occur. This is not a result of the phosphorylation of this residue in ovalbumin that cleavage at the "reactive center" does not result in burial of the remainder of the loop. It should also be noted that the \( \beta \)-x-ray structure determination of the cleaved form of ovalbumin was performed on a truncated form lacking the N-terminal 11 residues. The observed incorporation of the previously exposed loop into the \( \beta \)-sheet of the modified protein may therefore be peculiar to this truncated form and may not even arise in the more physiologically relevant neutrophil elastase-cleaved \( \alpha_1 \)-antitrypsin.

A third, less direct, trigger mechanism involves a change in solvent accessibility of part of the serpin molecule as a result of loop cleavage. In the same way that cleavage of the "bait region" in human \( \alpha_2 \)-macroglobulin facilitates thiol ester hydrolysis and consequent conformational rearrangement (20), cleavage of the exposed loop of serpins may result in disruption of salt bridge(s) essential to the integrity of the "strained" conformation. Intriguingly, different proteases react with \( \alpha_1 \)-macroglobulin at slightly different sites within the bait region yet still result in the same thiol ester hydrolysis and conformational change. This pattern also seems to hold for serpins. A distinction between ovalbumin and other serpins may thus arise, either from failure to disrupt the crucial interactions upon cleaving the exposed loop or else from the absence of such interactions from the native structure. If ovalbumin's role is truly not that of a protease inhibitor, the evolutionary pressure to conserve residues crucial to operation of the protease-induced conformational change, and perhaps consequent regulatory processes, may be absent, thus permitting loss of those residues necessary for the conformational change while still retaining the exposed loop as an unneeded appendage.

The present data are consistent with this indirect trigger mechanism, although obviously the details of the interactions involved in disrupting the native structure of stabilizing the modified structure have not been identified.

In summary, ovalbumin undergoes very little change in conformation upon limited proteolysis in the region proposed to constitute an exposed loop containing the active center bond in serpins. As a result the \( ^1H \) NMR spectrum of ovalbumin is little changed by proteolysis, and the properties of phosphoserine 344 (2 residues removed from the site of proteolysis) are not consistent with the large change in environment proposed from the structure of the cleaved form of \( \alpha_1 \)-antitrypsin. These findings, together with earlier results on
antithrombin III are not consistent with the hypothesis of Carrell and Owen (6) for the mechanism of serpin conformational change. It is proposed here that the mechanism of conformational rearrangement observed upon limited proteolysis of serpins involves a change in stability brought about by change in solvent accessibility upon cleavage of the reactive center loop.

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REFERENCES