Crystallization and Preliminary Diffraction Data of a Major Pollen Allergen

CRYSTAL GROWTH SEPARATES A LOW MOLECULAR WEIGHT FORM WITH ELEVATED BIOLOGICAL ACTIVITY*

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Group V major allergen Phl p 5b of timothy grass pollen induces allergic rhinitis and bronchial asthma in 90% of grass pollen-allergic patients. In addition to its allergenicity ribonuclease activity has recently been attributed to this 29-kDa protein.

The allergen was expressed in Escherichia coli and subsequently purified. Spontaneous conversion of these preparations to a mixture of various forms with molecular sizes between 10 and 29 kDa was consistently observed. Surprisingly, crystals could be grown from this heterogeneous preparation. Single crystals, redissolved and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblot, yielded one distinct low molecular weight protein, which was identified by amino acid sequencing as the C-terminal 13-kDa portion of the allergen. Histamine release assays with single crystal solutions using basophils of an allergen patient demonstrated allergenicity comparable with that of the holallergen. By contrast, RNase activity of the crystallized C-terminal form was 23 times higher than that of the full-length parent allergen. Crystals were used to collect preliminary diffraction data; the space group was determined to I41 22 with cell dimensions of a = 87.7 Å, b = 87.7 Å, and c = 59.6 Å.

We conclude that preferential crystal growth of the 13-kDa form is indicative of a compact conformation of this particular C-terminal portion of the allergen. Thus, we show here that protein crystallization is not only a prerequisite for structural analyses, but it also can provide a unique separation technique to localize the functional domain of a major allergen.

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Grass pollen allergens induce allergic disease in up to 30% of northern European and North American populations (1). Sensitization to these molecules can lead to allergic rhinitis, bronchial asthma, and atopic dermatitis. Several groups of grass pollen allergens have been defined according to their biochemical properties (2). Group I and group V allergens are especially important proteins because more than 90% of grass pollen-allergic patients produce IgE antibodies recognizing these molecules (3). IgE antibodies play a key role in initiating the allergic response (4, 5). Hence, the structure of allergens and their ability to bind with IgE have been thought to reflect a fundamental difference between allergens and antigens. IgE-binding regions and epitopes have been identified on a number of allergens (6–9). It transpires that recognition of allergens by specific IgE antibodies and binding with high avidity is dependent on stable conformational conditions and probably involves continuous epitopes. Thus, knowledge of the three-dimensional structure of allergens would make a considerable contribution to the understanding of their functionally relevant molecular interaction with IgE antibodies. Crystal structures of pollen proteins, however, have not been determined so far.

Here we report the crystallization of a spontaneously emergent short form of recombinant Phl p 5b, an allergenic protein that has been reported to possess ribonuclease activity (11). Crystal growth preferentially separated this truncated version of the allergen. Its biological activity is investigated and compared with that of the full-length parent protein.

EXPERIMENTAL PROCEDURES

Purification of Recombinant Phl p 5b—The mature recombinant protein Phl p 5b was constructed by polymerase chain reaction techniques as described elsewhere (11). Purification was performed on a Ni²⁺-nitrotriacetate-resin column according to the instruction of the manufacturer (Qiagen), followed by gel filtration on a Superdex™ 75 HR 10/30 column (Pharmacia Biotech Inc., Sweden).

Immunoblotting and Monoclonal Antibodies (mAbs)—12% SDS-polyacrylamide gel electrophoresis/immunoblotting was performed as described earlier (6). For N-terminal sequencing of the protein redissolved from crystals we employed electroblotting onto ProBlott membrane (Applied Biosystems, Weiterstadt, Germany) using 10 mM CAPS, pH 11.0, as blotting buffer (12). mAb Bo1 is specific for group V grass pollen allergens (13) and was used at a concentration of 0.1 mg/ml for detection of Phl p 5b.

Crystalization and X-ray Investigation—Crystals were grown at a controlled temperature of 20 °C by the sitting drop vapor diffusion technique as described in Hampel et al. (14). Crystals grew within various time spans (2–2 weeks) with ammonium sulfate or sodium/potassium phosphate, pH 7–8, as precipitants. Two crystals were used to collect low resolution diffraction data on a conventional anode generator (Rigaku RU 200) with CuKα radiation and a graphite monochromator. 35 images were collected at room temperature using a MAR 300-mm image plate detector. The exposure time was set to 1 h per image and the rotation range to 3°. The crystals of about 0.15 × 0.15 × 0.25 mm³ in size were mounted for data collection together with some mother liquor in glass capillaries. The images were processed using the program DENZO (15).

Amino Acid Sequence Analysis—For N-terminal sequencing we used Coomassie Blue-stained electrobotted crystalline protein cut from ProBlott membrane or dissolved crystals from the x-ray capillaries. Microsequencing was performed by Edman degradation using the 473A protein sequencer with an on-line phenylhydantoin-derivative analyzer.
Crystallization of Pollen Allergen

RESULTS

Recombinant Phl p 5b was expressed in the 6xhis-tag vector pQE and purified via Ni$^{2+}$-chelate column followed by gel filtration. The purified protein presented as a single band at 29 kDa in immunoblots and was detected using a monoclonal antibody against group V grass pollen allergens (Fig. 1A). Upon storage at 4 °C this protein converted spontaneously to a mixture of peptides with molecular sizes between 10 and 29 kDa (Fig. 1B), all of which appeared to be recognized by mAb Bo1 (not shown) indicating that its epitope remained preserved in all products of limited proteolysis. In spite of the observed heterogeneity, the preparation was used for a preliminary crystallization experiment. Cubic crystals of about 0.1–0.4 mm in diameter were obtained (Fig. 1C). In order to characterize the crystallized protein a single crystal was selected, washed with high salt buffer, and dissolved, and finally the solution was separated on SDS-polyacrylamide gel electrophoresis/immunoblot (Fig. 1D). Two bands were recognized by mAb Bo1, a minor band at 13 kDa and a prominent band at 26 kDa. This result shows that only selected components of the initial protein solution built up the crystal. This finding was confirmed with other crystals.

In order to conduct further characterization of these components the two bands at 13 and 26 kDa were blotted and cut from the membrane. Both peptides as well as the protein from a dissolved single crystal were N-terminally sequenced. All three sequences were found to be identical. They represented Lys$_{138}$–Ile$_{146}$ of the published sequence of Phl p 5b (11) as shown in Fig. 2. Compared with the full-length sequence, a C-terminal peptide starting at Lys$_{138}$ was predicted to have a maximum size of 13,378 Da. This size was in accordance with the lower band shown in Fig. 1D. The presence of the 26-kDa band was possibly indicative of an unusual dimer formation (stable under reducing and denaturing conditions). Taken together, these results indicate that only one distinct peptide was separated by crystallization out of the multicomponent mixture.

In order to evaluate the biological relevance of the crystallized 13-kDa component we analyzed solutions prepared from single crystals. In a histamine release assay the ability of the C-terminal component to induce mediator release from human basophils of an allergic patient was tested and compared with that of the full-length 29-kDa allergen. This assay is regarded to be a relevant indicator for allergenicity. Fig. 3 shows the histamine release in relation to the concentration of the two molecules. Bovine serum albumin served as a negative control, and lack of mediator release was indicative of the specificity of the reaction. The 13-kDa protein from a dissolved crystal was biologically as active as the full-length recombinant allergen as documented by similar molar concentrations causing 50% of maximum histamine release. Thus, conversion of the Phl p 5b allergen to the C-terminal short form is not accompanied by a loss of allergenicity.

Phl p 5b is known to have ribonuclease activity. Therefore, we questioned whether this function is also associated with the 13-kDa short form of the allergen. In an RNase activity assay the 29-kDa protein and the 13-kDa peptide from a redissolved crystal were compared. Fig. 4 shows that the short form exhibited a much higher nucleolytic activity than the full-length Phl p 5b molecule; molar concentrations of both proteins at 50% of maximum rate of RNA hydrolysis differed by a factor of 23. This indicates that dissociation from the N-terminal portion of the protein results in a marked increase in RNase specific activity.

Since the major recognized functions of Phl p 5b reside in the crystallized 13-kDa protein it will be of great interest to determine its three-dimensional structure. So far, two crystals have been used to collect low resolution diffraction data up to 4.6 Å on a rotating anode generator equipped with an image plate scanner. From processing 35 images the space group was evaluated to $I4_1$22 with cell dimensions of $a = 87.7$ Å, $b = 87.7$ Å, and $c = 59.6$ Å. Assuming a molecular mass of 13 kDa the packing parameter $V_m$ was calculated to be $2.2 Å^3$/dalton; it indicates the presence of 2 molecules in the asymmetric unit and a solvent content of 44%. 5,581 reflections were merged to a final reduced data set containing 568 reflections and a completeness of 98%. The $R_{merge}$ defined as $R(I) = \Sigma[I - 1/N(I)] / \Sigma[I]$ is 7.7%. The data collection to higher resolution using synchrotron radiation is in progress.

DISCUSSION

Preliminary structural data on a few allergens based on modeling studies have been published (18, 19); NMR solution structures have been reported for two allergens from ragweed (20, 21), but this is the first report concerning the crystallization of a grass pollen allergen. Such studies are possible due to the availability of recombinant allergens in sufficient amounts, and our experience leads us to suggest that crystallographic techniques may make a substantial contribution to allergy research.

This paper describes the crystallization of one component from a mixture of various molecular forms of the grass pollen allergen Phl p 5b. Crystallization was achieved in spite of the heterogeneous composition of the protein preparation; and (rather unusual in protein crystallization) it served as a purification technique by separating from a multicomponent mixture the short form of the allergen and led to its identification as the essential functional domain. The fact that this component in preference to any others formed crystals suitable for X-ray analysis may indicate a particular three-dimensional
structure with reduced molecular flexibility. Facilitation of crystal growth by shortening a macromolecule has frequently been observed (22, 23). In our case, however, the crystallized component was generated in a spontaneous event. Since the initial 29-kDa allergen had been purified to homogeneity, an autoproteolytic process seems to be likely, although conversion by a protease co-purified from the bacterial culture cannot be completely excluded. In any case, however, it is remarkable that the crystallized 13-kDa component retained all recognized biological functions of the parent protein. The occurrence of a band with an apparent molecular mass of 26 kDa on SDS gels (instead of 13 kDa as calculated from the sequenced N terminus) is obviously the consequence of dimer formation. Such aggregates, which remain stable under the conditions of denaturing and reducing electrophoresis, have been described also for other proteins (24). Dimer formation is also supported by the crystallographic analysis; the packing parameters reflect the presence of two molecules in the asymmetric unit.

The observation of a marked increase in RNase specific activity associated with the short form compared with the parent protein suggests that the generation of the 13-kDa product may reflect a process of physiological activation. Interestingly, in this reaction the pollen protein is trimmed down to a molecular size, which is typical for a number of other RNases analyzed from different sources (25).

The biological role of RNases in grass pollen is not fully understood. It is known that group V allergens are exported from the pollen once they are released from the inflorescence (26). Novel data presented on pollen RNases in different plant species indicate that they may function as defense proteins against viral infection since a number of pollen RNases can degrade double-stranded RNA (27). The relatively low RNase specific activity of Phl p 5b placed a question mark over biological significance, but the increase in activity caused by cleavage of the N-terminal portion suggests that the protein may play a significant role in the pollen biology. The principle of gaining biological activity by cleavage of molecules is a general phenomenon observed in a number of different biological systems; processing by limited proteolysis to the active form may also play a role for the function of the allergen Phl p 5b.

Pollen proteins like Phl p 5b were originally identified as a result of their ability to induce allergic responses in man (28). It has previously been demonstrated that the C terminus of this group of allergens is preferentially recognized by IgE antibodies of most grass pollen-allergic patients (9, 10, 26). This is in accordance with the histamine-releasing activity that is wholly retained in the crystallized C-terminal 13-kDa component. The stability of the C terminus may contribute to its preferential presentation and recognition by the immune system. The structural stability of the 13-kDa domain is also reflected by the ease with which it crystallized. Solving the

FIG. 2. Alignment of the published sequence of the full-length 29-kDa allergen (11) and of the N terminus determined for the crystallized short form. N-terminal sequences were identical for the excised 13- and 26-kDa bands as well as for the protein redissolved from the crystal.

FIG. 3. Comparison of allergenicity associated with the 29- and 13-kDa forms of Phl p 5b. Different concentrations of recombinant Phl p 5b (circles), of protein from a redissolved crystal (squares), and of bovine serum albumin (BSA) as negative control (triangles) were assayed for induction of histamine release from human basophils.

FIG. 4. Comparison of specific RNase activity associated with the 29- and 13-kDa forms of Phl p 5b. 1 μg of RNA was incubated at 56 °C with different concentrations of full-length parent protein (circles) and of protein redissolved from crystals (squares). After 30 min RNA was precipitated, and the extinction of degraded RNA was measured in the supernatant at 260 nm.
crystal structure of this clinically relevant domain may possibly pave the way for the rational design of drugs blocking the interaction between IgE antibodies and allergen. The preliminary diffraction data presented in this paper are an important step to the structure analysis, which is now in progress. After identification of the 13-kDa peptide as the enzymatically active and allergenic portion of the parent molecule we also intend to directly produce this domain in an expression system and to test it for crystallizability from homogeneous solutions.

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