

Circular dichroism analysis of allergens

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Abstract

Recombinant allergens have gained a lot of importance lately for the diagnosis of allergic diseases and for specific immunotherapy. To characterize recombinant allergens and potential hypo-allergenic derivatives thereof circular dichroism (CD) spectroscopy is used widely. It is a convenient, fast method to assess the structural integrity of the recombinant proteins, compare them with the allergens isolated from natural sources, and to determine the effects of mutations on the structural properties. In this paper, we will describe the techniques and the most useful applications of CD spectroscopy to the field of allergy research.
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1. Introduction

Cloning and expression of allergens, which are responsible for the sensitization of human individuals and the eliciting of allergic diseases, has enabled new approaches for allergy diagnosis and therapy [1–6]. The concepts of CRD (component resolved diagnosis) and CRIT (component resolved immunotherapy) have raised hope that in the near future it will be possible to cure allergic diseases with an immunotherapy using synthetic or recombinant hypo-allergenic allergen derivatives [7,8]. To characterize the recombinant allergens and derivatives designated for clinical purposes, fast and reliable methods are needed.

Circular dichroism (CD) is an optical phenomenon resulting from the interaction of polarized light with chromophores that are either inherently chiral or placed in an asymmetric environment. In such a case, the sample exhibits different absorption coefficients for left- and right-circularly polarized light. This difference in absorption, which also transforms linear polarized light into elliptically polarized light, can be measured in a wavelength dependent manner yielding the CD spectrum. (For a detailed description and theoretical background of the phenomenon see [9,10].)

In proteins, the major optically active groups are the amide bonds of the polypeptide backbone and the aromatic side chains [11]. Whereas the CD signal of the former can be monitored in the so-called far UV-region (i.e., at wavelengths below 260 nm), the signal of the latter chromophores is observed in the near UV-region (260–310 nm). The far UV-CD signal arises from the optical transitions of the amide bonds, which depend on the orientation of the peptide planes in the well-ordered secondary structure elements. Exploiting this phenomenon the overall secondary structure of proteins in solution can be determined, disclosing the main conformational motives such as α -helices, β -sheets, β -turns, and random coil (for reviews see [11,12]). In the near UV-region, aromatic side chains and cystines (disulfide bridges) exhibit absorption. These CD contributions are very sensitive to changes in the environment of the chromophore and are therefore well suited to follow changes in the secondary and tertiary structure (such as domain movements and folding studies) as well as the binding of ligands and protein–protein interactions. Because the occurrence of aromatic side chains and disulfides is generally low compared to the total number of amino acids, the CD signal in the near UV-region is at least an order of magnitude lower than the far UV-CD signal.

In addition to secondary structure determination, CD spectroscopy can be used to follow protein folding and

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unfolding to investigate the thermal and chemical stability of proteins [13]. Furthermore, protein–ligand interactions can be studied, provided that a conformational change occurs upon binding. Thus, binding affinities and kinetics can be determined [14]. With respect to allergen research, CD spectroscopy has proven to be very useful, because it allows for a fast determination of secondary structure contents of recombinant allergens and thus their structural integrity. If the native allergens isolated from the natural sources are available for comparison, the proper fold of the heterologously expressed proteins can be assessed. Mutants, which are designed to characterize the IgE epitopes of the allergen, or to yield hypo-allergenic derivatives, can be characterized with respect to the native allergen.

High resolution methods such as X-ray crystallography and NMR-spectroscopy exist to determine the three-dimensional structure of allergens. However, in contrast to CD spectroscopy, these methods are time consuming and require a comparably large amount of protein sample. It turns out that CD spectroscopy is not only a fast and reliable method to determine the secondary structure contents of recombinant allergens and potential hypo-allergenic derivatives thereof, but there is also a remarkable correlation between structural integrity and allergenicity of the recombinant proteins. We will present CD spectroscopy as a method complementary to immunological and biochemical approaches of allergen characterization.

2. Secondary structure determination of recombinant allergens

As CD spectroscopy allows for fast and convenient assessment of the structure content of proteins in solution, this method can yield valuable information about newly identified, cloned, and over-expressed allergens. Having the purified recombinant allergens in hand, the overall fold and the secondary structure content can be quickly determined in a qualitative way. From the shape of the far UV-CD spectrum it can be inferred whether the structure is mainly α -helical, β -sheet or of a mixed folding type, or whether it is folded at all (for a review see [14]). If the allergen isolated from natural sources is available in a pure form, the CD spectra of the native and the recombinant proteins can be compared directly, enabling the determination of the degree to which the recombinant protein adopts a “native-like” fold.

The structural integrity of the recombinant allergen is a very important indicator, because it has been shown that IgE epitopes are mostly conformational epitopes [4]. Thus, the degree of folding correlates with the IgE-binding capacity and allergenicity of the protein. An example of a direct comparison between native and recombinant allergens is shown in Fig. 1: the far UV-CD

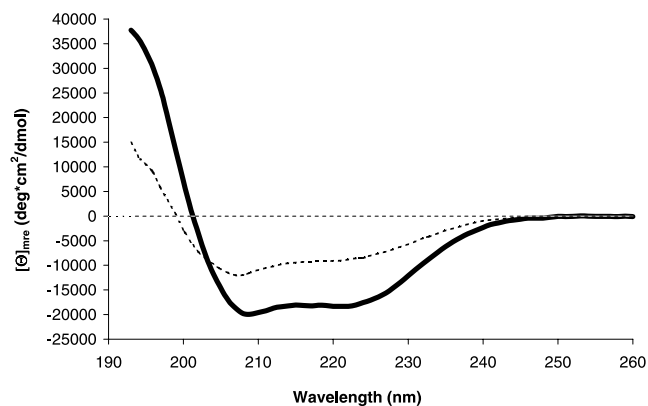


Fig. 1. Room temperature far UV-CD spectra of native and recombinant cat albumin. The native protein (continuous line) shows a mainly α -helical secondary structure, whereas the recombinantly prepared protein (dotted line) shows a significantly reduced fold.

spectra of native and a recombinantly produced cat albumin are superposed (R. Reininger et al., manuscript in preparation). The native protein which was isolated from the natural source shows a mainly α -helical conformation (58% α -helix content as calculated with the secondary structure estimation program J-700 (Jasco) according to Yang [10]). In contrast, the recombinant allergen is only partially folded showing about 20% α -helix content.

Natural allergens often turn out to be difficult to isolate or purify, making the direct comparison with the recombinantly produced proteins impossible. In these cases, the CD spectra of recombinant allergens yield an estimate of the secondary structure contents. The degree of “native-like” fold can be evaluated in a semi-quantitative manner using secondary structure prediction algorithms based on the protein sequences. Such algorithms work very reliably if analogous proteins with known three-dimensional structures exist, and still have a reliability of about 70% when no structural information is available [15–17].

A typical example for a recombinant allergen, without availability of the corresponding native protein, is the timothy grass pollen allergen Phl p 7 [18]. Due to the fact that the protein occurs only in very low amounts in the pollen (less than 1% of the total pollen protein content), the isolation and purification of sufficient quantities of the native allergen turned out to be impossible (K. Westritschnig and R. Valenta, unpublished data). However, CD spectroscopic investigation revealed that the recombinantly produced Phl p 7 allergen represents a well-folded α -helical protein [18]. This result proved to be in good agreement with sequence-based secondary structure predictions (e.g., with the program PsiPred [19]), where five α -helices were predicted (63% α -helix content) (P. Verdino and W. Keller, unpublished data). The solution of the three-dimensional structure [20] corroborated the predictions and revealed that Phl p 7 in

fact comprises five α -helices and two very short β -strands (overall: 69% α -helix content, 2.6% β -sheet content).

3. Characterization of hypo-allergenic mutants

The key step in triggering type I allergic responses is the recognition of conformational epitopes on the allergen surfaces by IgE antibodies [21]. To generate recombinant allergen derivatives which are hypo-allergenic and can therefore be used in immunotherapy approaches, several routes can be chosen [22]: one approach is the modification of the recombinant allergens by mutation of key residues, which are important for the proper three-dimensional fold [6]. These modified proteins exhibit disrupted IgE epitopes, resulting in a diminished or even totally abolished IgE-binding capacity. Another approach is based on the observation that immediate allergic reactions are triggered by the cross-linking of IgE bound to high affinity receptors on mast cells or basophils and the following release of histamine and leukotrienes [23,24]. For this cross-linking process, the responsible allergen has to carry at least two IgE epitopes. Changes in the structural context of the IgE epitopes (for instance, a fragmentation of the recombinant allergen) prevent this cross-linking reaction and the subsequent release of biological mediators from the effector cells.

The effect of modifications (e.g., point mutations, insertions, deletions, fragmentation, and oligomerization) yielding potential hypo-allergenic derivatives can be conveniently investigated by CD spectroscopy. Point mutations of residues playing a key role for structural integrity affect the overall structure content (and thus conformational IgE epitopes), which can be easily monitored via CD spectroscopy. The same is valid for the fragmentation of allergens, which affects the structural integrity and therefore IgE-binding. But even if the IgE epitopes are conserved, epitopes placed on different domains will be separated. Both types of changes are illustrated by mutational studies of the pollen allergen Phl p 7 (K. Westritschnig et al., manuscript in preparation), which has been shown to belong to a new family of 2 EF-hand calcium binding proteins [20]. Based on the observation that calcium depletion leads to a largely reduced IgE-binding capacity, mutants which are deficient in calcium binding were generated. One of these mutants showed a significant decrease in α -helicity corresponding to a diminished IgE-binding activity (Fig. 2). A protein fragment, comprising the N-terminal domain, proved to be essentially unfolded and its IgE-binding capacity was completely abolished (K. Westritschnig et al., manuscript in preparation) [20]. With this example the strong correlation between the overall fold as determined by CD spectroscopy and the IgE-binding capacity of the allergen is demonstrated.

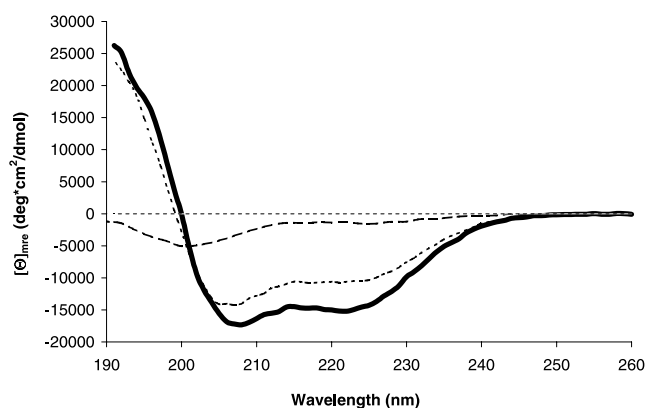


Fig. 2. Far UV-CD spectra at 20 °C of the timothy grass pollen allergen, Phl p 7, and mutant forms thereof. The recombinant calcium-binding protein shows a mainly α -helical secondary structure (continuous line). The α -helix content of a calcium-deficient point mutant is significantly reduced (dotted line). An N-terminal Phl p 7 fragment adopts random coil secondary structure (dashed line).

4. Allergen stability and refolding capacity

Solubility, stability, size, and the compactness of the overall fold are likely to be relevant for allergenicity. These features influence the allergen transport over mucosal barriers and its susceptibility to proteases [25]. CD spectroscopy is a valuable tool to gather information on some of these features of allergens. Measuring the changes in the CD spectrum with increasing temperature (or upon titration with denaturing agents) yields the thermodynamic parameters of the protein (un)folding. On one hand, one can record CD spectra at various temperatures and analyze them with respect to their secondary structure contents (Fig. 3A). On the other hand, one can record the temperature dependency of the CD signal at a significant wavelength (i.e., a wavelength at which pronounced changes between secondary structure elements upon thermal treatment can be monitored) (Fig. 3B). If the unfolding of the allergen occurs as a two-state transition, meaning that there is only a folded and an unfolded state without any intermediates, the unfolding mechanism can be described by a simple mathematical model [11]. Such a case is characterized by a sigmoidal unfolding curve with horizontal lower and upper branches. The point of inflection yields the transition temperature (T_m) and the steepness of the curve yields the folding enthalpy, ΔH_v . (For a detailed description of the evaluation of unfolding curves see [26].)

Differences in the thermodynamic stability and refolding capacity are indicators for the structural stability of proteins. CD spectroscopy can thus be exploited to evaluate conditions that selectively either stabilize or destabilize protein integrity. This is of particular interest for allergens and mutant forms thereof. Figs. 3A and B show the unfolding curves of the pollen allergen Phl p 7

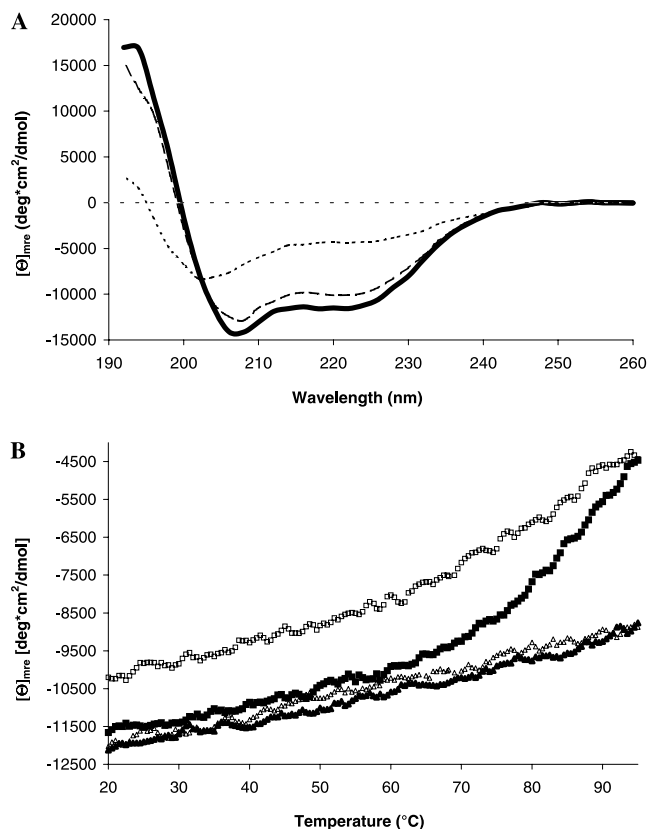


Fig. 3. Heat denaturation of the timothy grass pollen allergen, Phl p 7. (A) The far UV-CD spectra of the calcium-depleted protein are shown at 20 °C (continuous line), at 95 °C (dotted line), and after re-folding to room temperature (dashed line). The calcium-depleted protein adopts an α -helical secondary structure at room temperature. Upon heat treatment in the presence of EDTA, the protein unfolds and refolds only partially. (B) Heat denaturation of calcium-bound and calcium-depleted Phl p 7 monitored at a wavelength of 220 nm. The calcium-bound protein is rather unaffected (up-scan: black triangles) and refolds to its initial secondary structure (down-scan: white triangles). In the presence of EDTA, Phl p 7 shows a sigmoidal unfolding curve (up-scan: black squares) and does not refold completely (down-scan: white squares). Reprinted with permission from [18]. Copyright by the Federation of American Society for Experimental Biology.

[18], which turns out to be remarkably heat stable: even at 95 °C the protein remains nearly unchanged and regains its initial secondary structure upon cooling to room temperature. In contrast, the calcium depleted protein (in the presence of chelating reagents) has a well-defined transition at T_m 75 °C. The refolding capacity of the calcium depleted protein is only about 80%, whereas addition of calcium during the refolding process yields essentially 100% folded protein.

5. Comparison of homologous proteins cross-reactivity

The comparison of homologous proteins is another example demonstrating how CD spectroscopy may be used to characterize allergens. This is a particular in-

teresting aspect for the evaluation of cross-reactivity between different allergens. Cross-reactivity is largely determined by structural aspects, hence, cross-reactive allergens are proposed to share structural features such as homology in their three-dimensional fold. But not all proteins with similar folds are necessarily cross-reactive [25]. CD spectroscopy allows us to evaluate putative cross-reactivity: if two potentially cross-reactive proteins adopt completely different folds, cross-reactivity can be excluded. This permits us to verify the possibility of cross-reactivity within different proteins that were assigned to the same allergen groups. On the other hand, the secondary structures of the homologous protein from different sources (e.g., parvalbumin from carp, cod or pike) can be investigated, yielding information on potential cross-reactivity. A well-documented example is the cross-reactive two EF-hand pollen allergen family [27]. The CD spectra of proteins belonging to this family turn out to be nearly identical under native conditions, thermal denaturation as well as upon calcium-depletion [18,28–31].

Primary structure is used to determine sequence homology between proteins and to infer fold homologies. The fact that high sequence homology is not necessarily a good indicator for structural and immunological similarity of two proteins is demonstrated by the comparison of Bet v 1 and the highly homologous T1 protein. Bet v 1 is the major birch pollen allergen and belongs to a family of pollen and fruit allergens exhibiting high cross-reactivity between each other [32,33]. The cytokinin-inducible periwinkle protein T1 shows a high sequence homology with proteins from the Bet v 1 allergen family and with intracellular pathogenesis-related plant proteins [34]. The sequence identity with Bet v 1 has been found to be 40%, the similarity amounts to approximately 74%. In spite of this high sequence homology, the periwinkle protein T1 shows no cross-reactivity whatsoever with the Bet v 1 protein. This discriminative behavior in terms of allergenicity can be monitored via CD spectroscopy (Fig. 4A): the Bet v 1 CD spectrum shows mainly β -sheet features (24% α -helix, 58% β -sheet, as determined by a fitting procedure according to [10]). These values are in very good agreement with previous measurements [35] and with the three-dimensional structure [36]. The room temperature CD spectrum of the T1 protein looks somewhat similar, although it shows a higher α -helix and random coil content (Fig. 4A). When a heat scan is applied to both proteins, significant differences show up for the thermal stability and refolding capacity (Fig. 4B): Bet v 1, when heated, has a well-defined unfolding transition at about 68 °C and shows a mainly random coil CD spectrum at 95 °C. After slowly cooling to 20 °C, the protein regains about 70% of its initial fold [49]. In contrast, the T1 protein appears to have a higher heat stability than Bet v 1. The unfolding transition of T1 protein lies above 90 °C

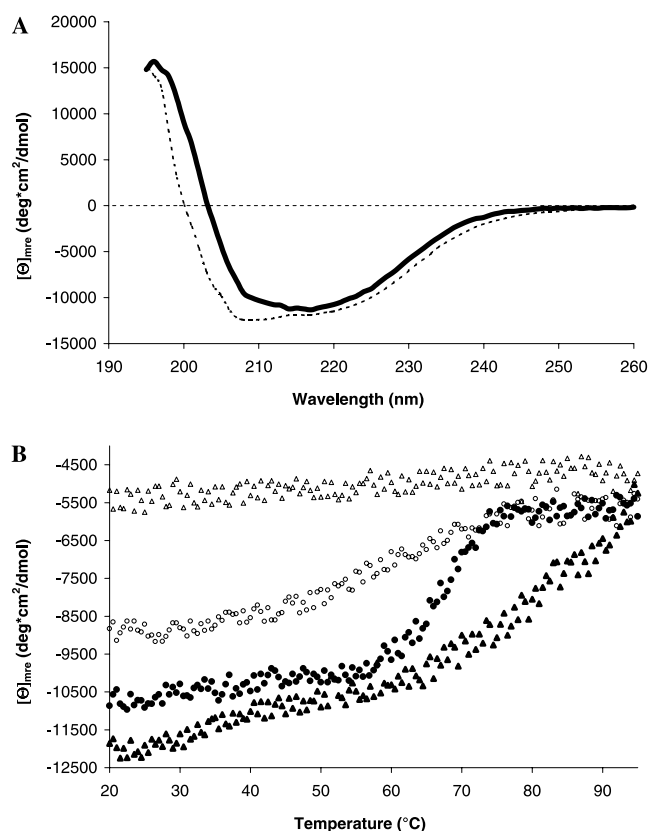


Fig. 4. Far UV-CD spectra of Bet v 1 (solid line) and T1 (dotted line). (A) At room temperature the spectrum of Bet v 1 reflects its β -sheet features (continuous line), whereas T1 shows a higher α -helical and random coil content (dotted line). (B) Upon heat denaturation monitored at 212 nm, Bet v 1 shows a discrete sigmoidal unfolding curve (black circles) and refolds partially (white circles). In contrast, T1 appears to have a much higher transition point above 90 °C (black triangles) and stays trapped in an (intermediate) β -sheet-like conformation (white triangles).

and at 95 °C the CD spectrum shows mainly β -sheet features. Upon cooling, the T1 protein does not regain its initial fold, but it appears to be trapped in the (intermediate) β -sheet-like conformation. Therefore, one can propose that conformational differences between the two proteins are responsible for the lack of cross-reactivity [49].

6. Allergen–antibody and IgE–receptor interactions

Frequently, the formation of complexes (protein–protein or protein–ligand complexes) involves conformational changes of the binding partners. This so-called “induced fit mechanism” has been described for protein–DNA interactions [37–39] and for interactions of proteins with peptides or protein targets [14,40,41]. Provided that such a conformational change occurs upon binding of an allergen to its specific antibody, the binding reaction can be characterized by far UV-CD

spectroscopy. Even if there is no change in overall conformation occurring, as it is frequently the case for binding of a small ligand to the much larger antibody, a signal may be detected in the near UV-CD-region, which

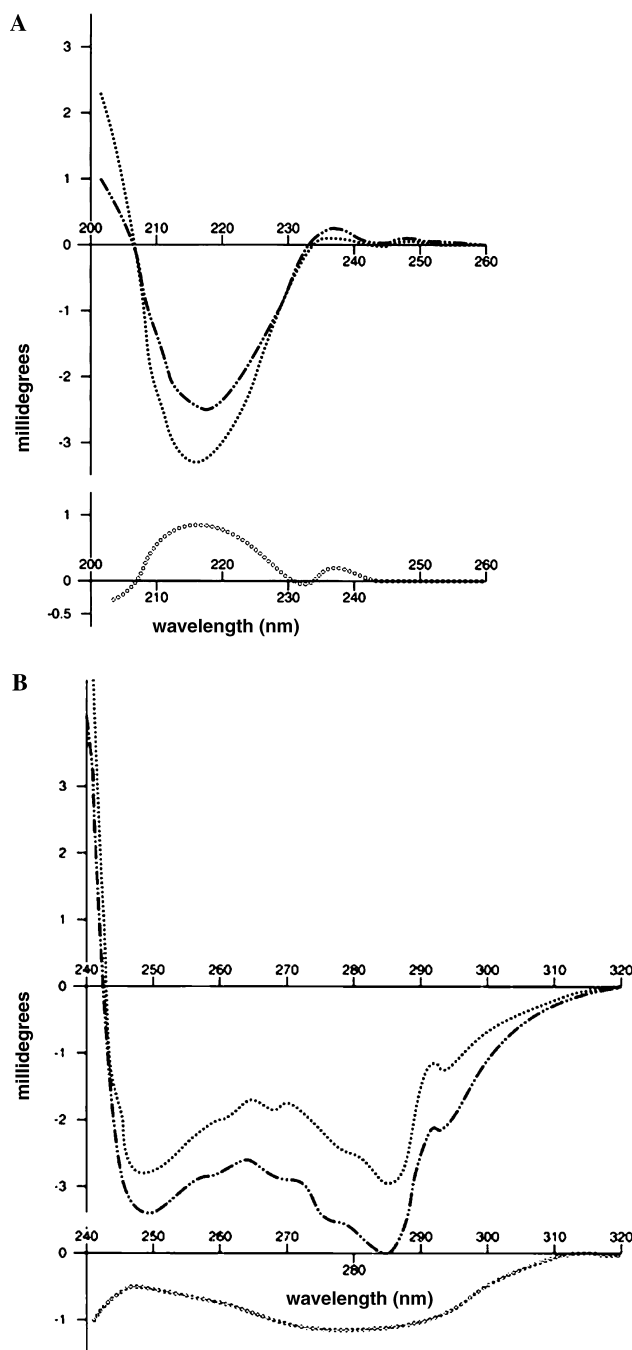


Fig. 5. CD spectra of a chimeric human IgE and the soluble domain Fc ϵ RI. The sum of the individual component spectra (dotted lines) and complex spectra (dash-dotted lines) is shown for (A) the far UV-region and (B) for the near UV-region. The differential spectra (white circles) obtained by subtraction of the spectra before and after binding are shown in the lower panels. Reprinted with permission from [44]. Copyright by the American Society of Biochemistry and Molecular Biology.

is sensitive to changes in the local environment of aromatic side chains. This principle is demonstrated, e.g., by the comparative CD studies of a highly specific anti-fluorescein antibody, the derived F_{ab} , and a specific single chain antibody [42]: the binding of fluorescein to all three proteins led to a large and comparable increase of the CD signal in the near UV-region between 280 and 310 nm.

Another allergy related field where CD spectroscopy has been used extensively is the structural characterization of IgE antibodies, the IgE receptors, and their interactions. The isolated C ϵ 3 domain of IgE and its interaction with the high affinity receptor Fc ϵ RI was investigated using a combination of plasmon resonance, fluorescence, and CD spectroscopy [43]. It was found that the isolated C ϵ 3 domain alone is unfolded, but still binds to the extra-cellular domain of Fc ϵ RI (without a detectable change in conformation).

In another study, the interaction of a chimeric human IgE with soluble domain of Fc ϵ RI was investigated [44]. The complex exhibited a significantly reduced CD signal in the far UV-region (around 217 nm) as compared to the sum of spectra of the individual compounds (Fig. 5A). This led to the conclusion that a major conformational rearrangement within IgE occurs upon binding. In addition, it was shown that the binding caused a pronounced increase of the near UV-CD signal with negative peaks at 286 and 293 nm (Fig. 5B), suggesting a major involvement of aromatic chromophores in the binding. Interestingly, the binding of a specific murine antibody to the soluble Fc ϵ RI domain exhibits no significant changes in the far UV-CD spectrum, but still a strong increase in the near UV-region, demonstrating that the large conformational change is specific for the IgE–Fc ϵ RI interaction.

7. Conclusions

CD spectroscopy is a useful method in the field of allergy research, because it allows for the fast acquisition of data on allergens, which are complementary to the information obtained from standard immunological methods:

- (i) Quick estimation of the secondary structure content of allergens.
- (ii) Assessment of a “native-like” fold of the recombinant allergens, which is especially important when recombinant proteins have been purified from inclusion bodies and therefore have to be refolded *in vitro*. In the case of post-translational modifications of the native allergens, changes in the modification pattern due to the expression procedure can lead to significant conformational changes on the recombinant allergen, which can also be assessed by CD spectroscopy.
- (iii) Effects of mutations on secondary structure.
- (iv) Evaluation of protein stability and conditions favoring a “native-like” fold.
- (v) Comparison of homologous proteins (determination of putative cross-reactivity).
- (vi) Studies of allergen–antibody and antibody–receptor interactions.

In future, recombinant allergens and hypo-allergenic derivatives will play a major role in the diagnosis of allergic diseases and for the development of immunotherapies and vaccines. CD spectroscopy allows for a fast and complementary characterization of potential hypo-allergenic derivatives and will therefore represent a standard method for the pre-clinical evaluation of these allergen derivatives.

Appendix A. Equipment

Modern laboratory-based CD spectrophotometers use a xenon lamp as light source and provide a scan range from 165 to 900 nm, extending the far UV-range considerably. To avoid absorption effects from oxygen in the air and damage to the optics by ozone, the sample compartment and the optics have to be purged with a constant nitrogen flow. To extend the range to either longer or shorter wavelengths, special equipment and experimental setups have to be used: extension to the IR-region led to the development of the method of vibrational CD (VCD), which yields complementary information to conventional CD spectroscopy in the UV/Vis-range [45]. At the short wavelength side of conventional CD spectroscopy, an extension of the scan range into the vacuum UV-region (VUV) has been enabled by the use of synchrotron radiation (SRC) [46]. This technique reveals additional bands for proteins and peptides, improving the accuracy of secondary structure determination [47].

UV-CD spectrophotometers consist of a light source, the optical system, the sample compartment, and the detector. Most commercial CD-instruments use the modulation method [48] to determine the CD signal of a sample. In these instruments, the optical system uses a double monochromator setup to select the wavelength and to produce linearly polarized light. The polarized light beam is then focussed and guided through a photoelectric modulator, producing left- and right-circularly polarized light. After leaving the optical unit, the light beam is led through the sample chamber where the probe is placed in a cuvette (for various cuvette formats see Appendix B). Most CD spectrophotometers feature a thermostating device for the sample cells, either a computer-controlled water bath or a thermoelectric device, allowing to perform temperature scans or to maintain a constant temperature during the measurements. Stop-flow kinetics or titration systems can be

attached optionally. The transmitted light leaves the sample compartment and enters the detector, a photomultiplier tube, which is electronically coupled with the modulator. Hardware control, data acquisition, and analysis are performed by standard personal computers.

Appendix B. Practical considerations

This section shall give a short overview over the practical aspects of CD spectroscopy of allergens. In particular, we briefly want to discuss sample preparation, selection of cuvettes, setting measurement parameters for standard and heat denaturation experiments, and arranging data for normalization and fitting procedures.

Many allergen preparations are supplied as proteins solved in distilled water without any buffer system. On one hand, this is very convenient for measuring purposes as water does not interfere with the CD signal (due to increased absorption effects) down to approximately 180 nm (in a 1 mm light pathlength cell). On the other hand, keeping the proteins in an unbuffered, no-salt solution bears the danger of inducing conformational changes, partial denaturation, and aggregation. Therefore, it is necessary to select a solvent system, which promotes the solubility and structural integrity of the protein to be examined, while minimizing the signal degrading effects (e.g., absorption in the scan range). Well-suited buffer systems are potassium-phosphate, sodium-phosphate, and Tris-HCl in a concentration range between 10 and 100 mM. If addition of salts is necessary to maintain a certain ionic strength, phosphate, sulfate, and fluoride salts are preferable over chloride salts, because the chloride ion strongly absorbs below 195 nm.

Typical allergen concentrations range between 0.1 and 1 mg/ml, depending on their absorption behavior and availability. In general, the signal to noise ratio can be increased by working with high protein concentrations and short light pathlength cells (0.1 or 0.05 mm), decreasing solvent absorption significantly and permitting to scan down to lower wavelengths. As a rule of thumb the absorption should be less than 1.0 OD-units over the whole scan region to yield a reliable CD signal. Oxygen also absorbs strongly below 200 nm. Therefore, it is necessary to purge the sample chamber thoroughly (e.g. with nitrogen) before accurate measurements in the far UV-region can be performed. Samples and buffer should be filtered through a 0.45 μ m filter or intensively centrifuged to remove dust or aggregated protein particles.

For the measurements of protein CD spectra in most cases cylindrical fused quartz cuvettes with light pathlengths between 0.05 mm and 1.0 cm are employed. These cuvettes are also available in water-jacket editions for thermostating. Sample volumes typically range be-

tween 0.1 and 0.5 ml, depending on the cell type and size. Cuvettes are either supplied by the manufacturers of the CD spectrophotometers or by companies such as Hellma GmbH & Co. KG.

CD spectrophotometers are manufactured by companies such as Jasco, Olis, and Aviv Instruments. Experimental parameters for the various scan modes (e.g., wavelength scan, temperature scan, and step scan) have to be adjusted to optimize the signal-to-noise ratio. Typical wavelength scan settings for a far UV protein scan are: (i) scan range of 260 to 180/190 nm (data to be truncated before saturating the photo-multiplier detector); (ii) step resolution of 0.2 nm; (iii) scan speed of 10–50 nm/min for normal to high resolution measurements and 100–500 nm/min for preliminary scans and/or sensitive samples; (iv) response time adjusted to the scan speed to optimize the signal-to-noise ratio (only relevant in continuous scan mode); and (v) band (slit) width of 1 nm for standard measurements (can be increased to improve the signal-to-noise ratio, but above 2 nm bears the risk of stray light effects).

Heat scans can be conveniently conducted using cylindrical water-jacket quartz cuvettes connected to a water thermostating device in a temperature range of 15–95 °C. The protein samples should be always degassed before use to avoid the formation of bubbles upon heating. Depending on the thermostating device and cuvettes used, a temperature lag may occur which can be assessed and corrected for using a thermocouple temperature sensor inserted directly into the cuvette.

The raw CD spectra (in units of mdeg) are baseline corrected by subtracting the corresponding buffer spectrum. These baseline corrected spectra are then normalized for pathlength and protein concentration, yielding molar ellipticity $[\theta]$ in units of $\text{deg cm}^2 \text{dmol}^{-1}$. This normalization is applied to ligand spectra and to protein spectra in the near UV-region, which depend mainly on the chromophores of aromatic side chains and to a lesser extent on disulfide bridges. Protein spectra in the far UV-region, which are generated by the electronic transitions within the amide groups, are additionally normalized for the number of peptide bonds ($n - 1$ where n is the number of amino acids of the protein), yielding the mean residue ellipticity $[\theta]_{\text{mre}}$. This normalization renders CD spectra of different molecular weight proteins comparable and improves the accuracy of the secondary structure determination. The resulting mean residue ellipticity spectra can then conveniently be fitted using one of the various secondary structure estimation programs available [12].

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