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Molecular Modeling and Experimental Approaches toward Designing a Minimalist Protein Having Fc-binding Activity of *Staphylococcal* Protein A

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Protein A (PA), a cell wall constituent of *Staphylococcus aureus,* has got the unique property of binding with the Fc fragment of IgG from various species. The sequence data indicate five highly homologous Fc-binding regions in protein A. Computer sequence analysis provided the tryptic and chymotryptic fragments of IgG-binding domains of protein A. Molecular modeling in conjunction with molecular mechanical calculation has been used to search for the smallest possible proteolytic fragments of PA, still retaining Fc-binding activity. A 20-residue peptide (typtic fragment) and a 16-residue peptide (chymotryptic fragment) have been indicated, by molecular modeling studies, to possess IgG-binding affinity comparable to that of the B domain of Protein A. Binding of a 20-residue peptide has been substantiated experimentally by immunoprecipitation, capillary electrophoresis, and circular dichroism spectroscopic analyses.

Key Words: **Protein A; peptides; Fc-binding; interaction energy.**

Protein A (PA) is covalently bound to the peptidoglycan moiety of the cell wall of *Staphylococcus aureus,* Cowan I, (molecular weight of 42kD, 395 residues) and has the unique property to bind the Fc portion of IgG (Immunoglobulin G) from various species which has initiated its use as a tool in immunochemical and cell surface structural studies (1). The amino acid sequence of PA is composed of five homologous IgG binding domains (from the N-terminus: E,D,A,B,C—in this order) followed by the C-terminal cell-wall binding region X, approximately 150 residues long, which differs to a great extent from the other IgG binding active regions (2,3). A comparison of sequence data of different Fc-binding regions indicate not only a mutual homology but also internal homologies within the regions (4).

The X-ray crystal structure of domain B in complex with the constant domain (FC1) of IgG subclass 1 has been solved at a resolution of 2.8 A (2,5–6). It has been shown that B domain makes two contacts with Fc fragment in the crystal. In the Fc fragment, residues located at the interface between CH2 and CH3 domains contribute to the first contact. whereas the other contact is formed by residues only from the CH3 domain. Electron density for the Fc bound B domain was observed for the segment from F 6 to E 48; no information, however, was available for the A 2–K 5 and A 48–K 59 segments. Two antiparallel helical regions, Q10–L 18 and E 26–D 37, are the predominant elements of the secondary structure of the Fc bound B-domain. A medium resolution structure of domain B has been determined by NMR spectroscopy (7) which reveals an antiparallel three helix (Q10–H 19, E 25–D 37, and S 42–A 55) bundle motif. NMR and CD studies (8) show that *Staphylococcal* Protein A maintains its three helical bundle structure in the complex with human IgG (Fc) which is not shown in crystal structure.

Protein A has been shown to possess diverse array of biological properties (9–14). It was shown that some synthetic peptides containing sequences encompassed by Staphylococcal PA could induce immunomodulation and cytotoxcity (15).

In the present study, our aim was to find out a peptide fragment from Protein A molecule still retaining strong Fc binding activity. The IgG-binding property of PA was used to remove blocking factors from Cancer patients (16–17). The interaction between *Staphylococcal* Protein A and the Fc portion of human IgG has been studied in detail by various other laboratories (18–21). The specific interaction of protein-A with the Fc region of IgG has been exploited in many fields, with roles in Ig purification (22), biotechnology (23) and clinical therapy (24). The minimization of IgG binding domains of protein-A could have important

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applications. The smaller functional versions are more synthetically accessible. Since Protein A is a bacterial protein, and when inoculated into the mammalian host it would be subjected to proteolytic degradation, we raised the question whether or not its proteolytic products would retain the IgG-binding property.

Force field calculations were done to get minimized energy conformation for all molecular species studied, thus evaluating the stability of as well as interaction between peptides and Fc region of IgG. From the crystal structure it was revealed that the helix Gln(Q) 10-Leu(L) 18 has better contact with Fc (IgG) than the other part of the B-domain. This portion is common in A and C domains also. An octapeptide sequence (QNAFYEIL) from this helix part was designed earlier showing IgG binding property (10). Increasing two amino acids from the N-terminal end of the octapeptide ('deca' peptide) indicated somewhat better binding property than the octapeptide (12). Building on these sequences a 20-residue tryptic fragment (from N-terminus) was identified, alongwith a 16 amino acid peptide (chymotryptic fragment) which appeared to possess greater Fc binding property when compared to both octa and deca peptides. Immunoprecipitation data, capillary electrophoresis and circular dichroism spectroscopic analysis substantiated the IgG binding affinity of a 20-residue peptide.

MATERIALS AND METHODS

Freeze dried protein A (5 mg/vial) was procured from Pharmacia Fine Chemicals, Sweden. A 20-amino-acid-long synthetic peptide was synthesized from Genemed Synthesis Inc., USA. The purity was checked by HPLC. Human IgG (5.3 mg/ml) was procured from Sigma, purity was checked by HPLC. All the other chemicals used in the study were of analytical grade purity.

Molecular modeling. 'Peptide Map', 'Peptide Sort' and 'Peptide Structure' programs available in Wisconsin sequence analysis package (Genetics Computer Group, ver.8) were used to generate the peptide fragments following trypsin and chymotrypsin (this two proteolytic enzymes are used in this software) cleavage of different IgG-binding domains of protein-A sequence (3) and to predict their secondary structure.

Insight II (Biosym, USA), a comprehensive graphic molecular modeling program was used in conjunction with the molecular mechanics/dynamics program, DISCOVER (ver. 2.9.7, 95) to generate the peptide structure and get the minimized energy conformations. The amino acids linked together were assigned with a particular secondary structure *viz.*, α helix, or β sheet. These initial structures were then subjected for energy minimization study. Initially steepest descents, followed by conjugate gradient minimization, were used (25). Typically 1000 steps of steepest descent followed by 2000 steps of conjugate gradient minimization were performed after which the incremental decrease in energy was <0.01 kJ/mol and the r.m.s. derivative ~ 0.01kJ/mol/A. The conformation of peptide (α helix or β sheet) that gave the lowest energy was used as the initial conformation of peptide for docking. To evaluate the interaction energies of many orientations of peptides relative to IgG (Fc region), while searching for the orientation that results in the lowest interaction energy, Docking type calculations were used (26). This includes single point interaction energy calculation varying the mutual orientations of Fc and the peptide fragments. The orientation of peptide and IgG (Fc region) complex, which gave a low initial interaction energy, was subjected for energy minimization using force field methods. Non-bonding interaction energy, in terms of Van der Waal interaction (Evdw) and electrostatic interaction (Eel), was evaluated using CVFF (27) force field, where total nonbonding energy (Etot) was calculated as the sum of Evdw and Eel.

Calculated energy difference for different minimized energy conformation (α helix or β sheet) formed from extended peptide sequences (ΔEp) and calculated energy of the complex formation for the formal reaction process of peptide-IgG binding (ΔEs) can be obtained. Since all these peptides are of different length, energy per residue (ΔEp/res) was calculated to show comparative stability of the peptides. The B-domain of Protein A as a complex with Fc was also minimized. In this minimization only the contact segments of the Fc structure were relaxed, together with B-domain.

Protein concentration. Protein and peptide solutions were made by dilution of a known weight of salt free freeze-dried protein (or peptide) in Milli-Q water. Concentrations were determined by the Bradford dye binding procedure (28) with BSA as the standard.

Immunoprecipitation and capillary electrophoresis analysis. Protein A and normal human IgG (1:4) was incubated at 37°C for immunoprecipitation purposes. Similarly, the 20 amino acid synthetic peptide (EQQNAFYEILHLPNLNEEQR) was reacted with normal human IgG (20:1) and was incubated at 37°C for immunoprecipitation (BSA was used as a negative control). The dose of Protein A and peptide was selected from the dose response study (where precipitation occurs). The precipitate was dissolved in 0.2 ml phosphate buffered saline (pH 7.2) and used for Capillary electrophoresis (Beckman). Pure Protein A (5 mg/ml), normal human IgG (5 mg/ml), 20 amino acid synthetic peptide (.1mg/ml) were used directly as controls in Capillary electrophoresis. A Beckman P/ACE system 5010, equipped with an IBM PC (window-based control including system Gold software) was used in this study. neutral capillary (Beckman), with an inner diameter of 50 μm and total length 37 cm (UV detector at 30 cm), and as electrolyte a 20 mM phosphate buffer, pH 2.5 was employed. Samples were injected by 85 psi pressure.

Circular dichroism (CD) spectra. Far UV-CD spectra were collected in a J-600 spectropolarimeter (JASCO, Japan) using wave lengths of 250-200 nm at room temp. Curves were signal-averaged from ten scans and presented with similarly signal-averaged buffer baseline subtracted. The optical path length was 1 mm. Data points were given in millidegrees and converted to mean residue ellipticity at 222 nm.

Sample preparation for CD analysis. Samples for CD analysis were prepared in a 20 mM phosphate buffer at pH 7.2. Concentrations used were for IgG .06 mg/ml, for protein A .05mg/ml and for a 20-residue peptide .03 mg/ml, for protein A–IgG complex .01 mg/ml and for 20 aa–IgG complex .01 mg/ml.

RESULTS

Molecular Modeling

Using 'Peptide Structure' software (GCG Package) three α helices: K(8)-L(20); L(23)-D(38) and L(45)-A(57) were predicted for B-domain by Chou-Fasman method (29) and two helices, K(8)-L(18) and Q(41)-F(64), were predicted by Osguthorpe, Robson and Garnier method (30). Chau-Fasman method is more relevant if we compare these results with the conformations depicted by NMR (7) for B-domain. 'Peptide structure' prediction for other four IgG-binding domains were computed and compared with the B-domain conformations. All other four domains show

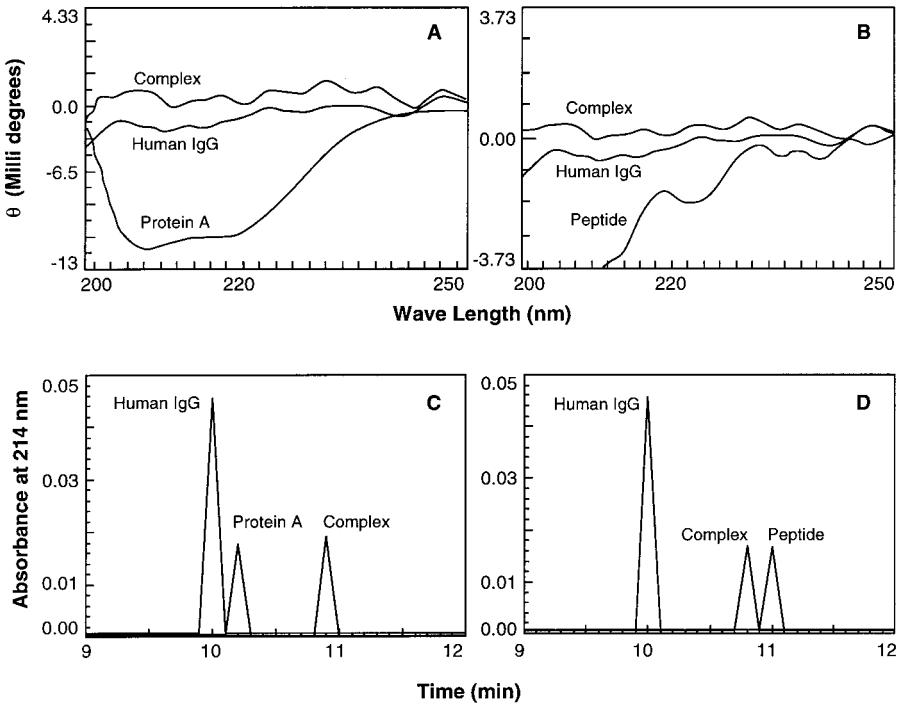

FIG. 1. Superimposition of far-UV CD spectra for protein/peptide and protein complexes with human IgG; (**A**) Protein A, human IgG, and complex of (1:4) Protein A and human IgG. (**B**) 20-residue peptide, human IgG, and complex of (20:1) 20 aa peptide and human IgG. θ is given in millidegrees, and separation by neutral capillary electrophoresis. (**C**) Protein A with normal human γ-globulin (1:4) complex precipitate dissolved in 5 ml PBS superimposed with IgG and Protein A. (**D**) 20 aa synthetic peptide with normal human γ-globulin (20:1) complex precipitate dissolved in 5 ml PBS superimposed with IgG and 20 aa synthetic peptide.

three α helical conformations in almost homologous regions of sequences.

Active regions (IgG binding) of B-domain of Protein A (4) are: ADNKFNKEQQNAFYEILHLPNLNEEQR (N-terminal halves of the active region) and NGFIQSLKDDPSQSANLLAEAKKLNDAQAPK (C-terminal halves of the active region).

Ideally, trypsin cuts at amino acid Arg (R) and Lys (K) positions and chymotrypsin cuts at Phe (F) and Tyr (Y) positions. Theoretical 'Peptide Map' prediction on trypsin degradation gives a 20 amino acid (EQQNAFYEILHLPNLNEEQR) from N-terminal active part and 8 amino acid (NGFIQSLK) and 14 amino acid (DDPSQSANLLAEAK) peptides from C-terminal active part. Chymotrypsin digestion gives 16 amino acid (EILHLPNLNEEQRNGF) and 8 amino acid (NKEQQNAF) fragments from N-terminal active part and a 33 amino acid (IQSLKDDPSQSANLLAEAKKLNDAQAPKADNKF) peptide froms C-terminal active part. The 20 residue fragment from the B-domain could not be isolated form its tryptic digest earlier (4), whereas it was isolated from the A domain. The differences in sequence were in two amino acids (N and M instead of H and L at the position of 11th and 12th residues) and this fragment also showed Fc-binding affinity theoretically, comparable to that of the B-domain interaction. We have considered in our study, the 20 aa synthetic peptide residues of the B-domain fragment, as this peptide sequence showed better interaction.

B-domain interaction with Fc was taken as the basis of our study and all energy values were compared with B-domain interaction because the crystal structure (minimum energy conformation) of this as a complex with IgG(Fc) is available.

The minimization calculations for each peptide (proteolytic fragments) from B domain for their different conformations (right handed α helix, β strand) were calculated and It is evident from thermodynamic stability point of view (ΔEp), that α-helix is the most stable conformation. This inference is unanimously attested to for all the peptides considered. Comparison of the average potential energy per residue (amino acid) data (ΔEp/res) were calculated to study comparative stability of the peptides, since the peptides are of different length. Figure 1B shows far UV CD spectra for 20 residues tryptic fragment of B-domain of protein-A which shows some % of α helicity (Characteristic molar ellipticity amplitude at 222 nm was $\sim 4 \times 10^3$ in degrees $\times$ cm^2 $\times$ dmol^1) and this fragment, inside B domain, has got mostly α helical conformation. Alpha helicity was predicted by Chau-Fasman (29) method also. For further investigation on interaction between peptides and IgG (Fc), α-helical conformation was taken into consideration for all peptides considered.

TABLE I

Interaction Energy Values (van der Waal, Evdw; Electrostatic, Eel; Sum of These Two, Etot) and Thermodynamic Stability Index (ΔEs) for Minimized Energy Conformation of Complexes of B-domain (X-ray Crystal Structure) and Proteolytic Peptide Fragments from B-domain with Fc Region of IgG

Complex with Fc (IgG)	Evdw	Eel	Etot	ΔEs	No. of H-bonds
B-domain	−131.7	−39.9	−171.6	−56.9	10
Tryptic fragments					
a. 20 aa	−83.1	−36.3	−119.4	−62.2	10
b. 14 aa	−65.4	−29.6	−85.1	−38.2	6
c. 8 aa	−36.6	−17.7	−54.3	−12.6	3
Chymotryptic fragments					
a. 16 aa	−71.6	−25.1	−96.7	−54.3	5
b. 33 aa	−54.4	−14.7	−69.1	−30.4	2
c. 8 aa	−48.9	−11.6	−60.5	−19.2	1
Previously studied peptides					
a. octa[a]	−55.5	−14.4	−69.9	−36.3	3
b. deca[b]	−49.5	−28.8	−73.4	−24.5	5

Note. Energy values in kcal/mol.
[a] Reference 10.
[b] Reference 12.

Table I presents calculated energy values—Van der Waals (Evdw), electrostatic (Eel) and total nonbonding energy (Etot)—as well as the thermodynamic stabilization energy (ΔEs) for each peptide from B-domain interacting with IgG (Fc region).

Twenty amino acid tryptic fragment formed from the B-domain (EQQNAFYEILHLPNLEEQR) could also be formed from D, A, C domains:

EQQNAFYEILNMPNLNEEQR (A domain)-2 aa difference
DQQSAFYEILNMPNLNEAQR (D domain)-5 aa difference
EQQNAFYEILHLPNLTEEQR (C domain)-1 aa difference

and 16 residues (chymotryptic) fragment with B-domain like sequence (EILHLPNLNEEQRNGF) could be formed from all other IgG binding domains A, C, D, and E.

EILNMPNLNEEQRNGF (A domain)-2 aa difference
EILNMPNLNEAQRNGF (D domain)-3 aa difference
EILHLPNLTEEQRNGF (C domain)-1 aa difference
QVLNMPNLNADQRNGF (E domain)-6 aa difference

The comparison is based on B-domain fragments and an amino acid is underlined when different from that.

Interaction energies (Evdw, Eel and Etot and ΔEs) for all 20 aa (tryptic) and 16 aa (chymotryptic) fragments from different domains were compared with similar fragments of B-domain and furnished in Table II.

Table III gives information regarding hydrogen bonds formed (between polar hydrogen and electronegative atom O/N) by 20 aa peptide (sequence used for experimental purposes) with Fc region of IgG.

CD Spectra

Far UV CD Spectra of Protein A and 20 aa peptide were collected together with spectra for IgG (human) alone as well as the complex precipitated out for the mixture of Protein A/peptide with IgG at the molar ratio described above. CD spectra are shown in Fig. 1A, 1B.

Immunoprecipitation and Capillary Electrophoresis

Twenty aa peptide was synthesized and their binding ability to IgG was studied using normal human IgG.

In capillary electrophoresis the standard PA, 20 amino acid synthetic peptide, and human IgG gave a single peak at 10.2, 11 and 10 minutes. Whereas the complex of PA with IgG, 20 amino acid synthetic peptide with IgG gave single peak at 10.9, 10.8 minutes (Fig. 1C, 1D).

DISCUSSION

Molecular Modeling

Crystallographic evidence suggested that B-domain of PA is composed of two antiparallel α helices when bound to Fc, with a disordered C-terminal region. On the other hand, NMR investigations demonstrate that unbound B-domain of PA consists of three α helices. First two helices contain the amino acids involved in the binding interaction and are essentially the same as in the crystal structure of the complex. The third helix (C-terminal region) does not participate in the binding interaction. Crystal structure was taken as the initial conformation since it should be one of the stable conformations.

To search for a peptide fragment, we have considered tryptic and chymotryptic fragments of PA, since it is logical to assume that PA would be subjected to enzymatic (proteolytic) degradation *in vivo.* The small pep-

TABLE II

Interaction Energy Values (van der Waal, Evdw; Electrostatic, Eel; Sum of These Two, Etot) and Thermodynamic Stability Index (ΔEs) for Minimized Energy Conformation of Complexes of 20 aa (Tryptic Digest) and 16 aa (Chymotryptic Digest) Residue Peptides from Other IgG-Binding Domains of Protein A with Fc Region of IgG

Complex	Evdw	Eel	Etot	ΔEs	No. of H-bond
Tryptic fragment					
20 aa (domain-B)	−83.1	−36.3	−119.4	−62.2	10
20 aa (domain-A)	−70.3	−33.5	−103.8	−60.5	8
20 aa (domain-C)	−52.9	−24.5	−77.4	−37.1	5
20 aa (domain-D)	−62.2	−19.8	−82.0	−38.4	5
Chymotryptic fragment					
16 aa (domain-B)	−71.6	−25.1	−96.7	−54.3	5
16 aa (domain-A)	−56.7	−18.7	−75.4	−34.7	4
16 aa (domain-C)	−62.4	−24.9	−87.3	−40.2	5
16 aa (domain-D)	−79.8	−16.5	−96.3	−51.8	4
16 aa (domain-E)	−50.2	−10.7	−60.8	−16.6	2

Note. Energy values in kcal/mol.

tides derived from degraded PA molecule were thought to be responsible for inducing its various biological properties *in vivo* (12) but no direct evidence was given. Discussion of energy aspects in Table I focuses chiefly on the stability of the complex with IgG (Fc) for different peptides. All these indices gave a possible measure of the level of formation of the complex with IgG (Fc) for each peptide in comparison with the whole B-domain of Protein-A. Comparison of the data predicted that, 20 aa residue peptide as a tryptic fragment and 16 aa residue fragment as a chymotryptic fragment gives high interactions with IgG (Fc) and the energy values are comparable to B-domain interactions. Though other fragments also appeared to show interaction to some extent, we have concentrated on the 20 aa and 16 aa fragments since they showed comparatively higher interactions among all those fragments. Sequences of both these fragments (tryptic and chymotryptic) are in the same stretch of the active N-terminus. Partial trypic fragments of PA, have been isolated and partly sequenced (4,31). Fragments of the tryptic digest of intact B-domain were assigned (based on Edman degradation) as BT1 (ADNK), BT2 (FNK) from the N-terminal part and BT4 (NGFIQSLK), BT5 (DDPSQSANLLAEAK) from C-terminal part. Only BT3 which is the 20 aa fragment of N-terminus could not be identified for some unknown reason. In fact, tryptic fragment of 20-residues were separated and identified from A domain (assigned by AT3), 14 residues from A and B domains, and 8-residues from A, B and C domains respectively (4). Octa peptide (QNAFYEIL) and deca peptide (EQQNAFYEIL) sequences mentioned earlier have been taken into account to compare the IgG-binding affinity with proteolytic peptide fragments.

It is directly evident from the comparative study of Tables I and II that interactions for B-domain fragments (both 20 and 16) with IgG is stronger than the interactions of similar fragments from the other domains. A comparison was made, based on the interaction energy between the peptides and IgG (Fc). Assignment of atoms of Fc (as D) and B domain (as C) was made according to the crystal structure data (2). Ex-

TABLE III

Donor and Acceptor Atom Pairs and Bond Distances (in Å) for Hydrogen Bonds Formed for Fc (IgG) Complexes with B-domain, 20 aa peptide (B-domain Tryptic Fragment, Used for Experimental Purpose) and 16 aa peptide (B-domain Chymotryptic Fragment)

Complex	Donor . . . acceptor	Bond length (Å)
Fc (IgG)–B-domain	(C129)GlnNE . . . LeuO(D251)	2.47
	(D254)SerN . . . AsnOD(C125)	2.22
	(C125)AsnND . . . SerOG(D254)	2.88
	(D254)SerOG . . . GlnOE(C128)	1.93
	(C154)LysNZ . . . HisND1(D310)	2.19
	(D312)AsnND . . . AsnOD(C147)	1.99
	(C146)ArgNE . . . AspOD(D315)	2.02
	(D315)AspND . . . ArgNE(C146)	2.06
	(D317)LysNZ . . . GluOE(C143)	2.18
	(C130)AsnND . . . AsnOD(D434)	1.89
Fc (IgG)–20 aa	(D317)LysNZ . . . AsnO(4)	2.09
	(4)AsnND . . . Val(D279)	2.12
	(D311)GlnNE . . . GluOE(8)	2.29
	(D317)LysNZ . . . GluOE(8)	1.67
	(8)GluOE . . . AsnOD(D312)	1.95
	(11)HisND . . . GluOE(D430)	1.97
	(D430)GluOE . . . HisND(11)	2.91
	(11)HisNE . . . GlyO(D341)	1.99
	(18)GluOE . . . LeuO(D343)	1.72
	(19)GlnNE . . . HisO(D433)	1.97
Fc(IgG)–16 aa	(1)GluNE . . . ValO(D279)	1.64
	(D315)AspND . . . AsnO(4)	1.87
	(4)AsnND . . . AspOD(D280)	2.16
	(11)GluNE . . . GlyO(341)	1.70

amination of all these data led to the prediction that the interaction energies in case of 20 aa peptide as a tryptic fragment and 16 aa peptide as a chymotryptic fragment are much higher than that of the other peptides (octa, deca etc.), and also all these indices are comparable with the total B-domain interaction indices. Since, among all fragments, 20 residues tryptic fragment from B-domain shows the strongest interactions (10 hydrogen bonds, Table III) with Fc of IgG, we have considered this peptide sequence for experimental verification for IgG binding.

Analysis of CD Spectra

The α helical content of proteins with limited amounts of other secondary structural elements, can accurately be estimated by the molar ellipticity amplitude at 222 nm (32). This is a characteristic wave length for α helices, whereas other secondary structural elements such as β-sheet, turn or random coil display only small CD signals at this wave length (33). Therefore, the application of CD spectroscopy to follow possible changes in a helicity is particularly suitable in the complex. Since Fc (IgG) is a β-sheet protein (6) with a small molar ellipticity ($\theta \times 10^{-3}$ in degrees $\times$ cm^2 $\times$ dmol^{-1} = 2.4). CD signal ($\theta \times 10^{-3}$ in degrees $\times$ cm^2 $\times$ dmol^{-1}) at 222 nm for both the complexes (protein A and 20aa with IgG) were very little (3.5 and 2.8 respectively) as compared with the protein A/20aa alone (23.2 and 3.9 respectively). That may be due to the fact that the helical part were buried inside IgG and lone pairs on amide nitrogen were involved in hydrogen bond formation (not available for nπ* transition). The spectra for protein-A showed minima at about 208 and 222 nm, indicating a helical conformation ($\theta \times 10^{-3}$ in degrees $\times$ cm^2 $\times$ dmol^{-1} = 23.2, Fig. 1A, B). The CD spectrum of protein A (34) indicates that it is composed 50% right handed helical structure and 10–20% β-structure and is consistent with the proposed extended shape.

Immunoprecipitation and Capillary Electrophoresis

These results (Fig. 1C, D) establish that PA as well as the synthetic peptide (20 aa) binds to the IgG. As a result of such binding there is a shift in retention time of each peak when separated by capillary electrophoresis. When voltage is applied separation occurs due to the combined actions of electrophoretic migration and electroosmotic flow.

Upon considering the results of this study, it can be predicted that the 20 aa residue peptide may act as the appropriate sequence still retaining the Fc-binding property. Since many immuno-active cells (*e.g.* macrophages) have got Fc-receptor on their cell surface, Fc binding ability of these peptides of PA may have some role for inducing various biological signals as was previously observed due to Protein-A. Cytotoxcity and immunomodulation were shown by some peptides (15) which are part of this 20 residue peptide. Our aim was to dissect out a peptide fragment of this protein which still retains IgG biding capacity. Thus, a peptide fragment which can act as a substitute for whole PA molecule, at least with respect to its IgG binding property, has been identified. Further studies with respect to their biological properties are in progress.

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