– a similar approach has been adopted for the study of insulin and its semi-synthetic analogues the crystal structure of some of these was solved, in parallel with computer-aided modelling of the effect of the synthetic modifications

– the field of computer-aided drug design is heavily dependent on the availability of both suitable computers and suites of programmes, some of which are expensive The possibilities of collaborations offered by the programme were thus very useful and allowed progress in the studies of the delivery of drugs with the help of polymer carriers and the modelling of drugs acting on excitable membranes

– further developments using Patterson methods in small and macromolecular crystallography were pursued

– finally, several groups among the participating laboratories found common research themes that have allowed significant progress For example, several groups are interested in the understanding of the molecular mechanisms underlying the process of sporulation

While the European Comission-sponsored programme comes to an end, the interest in the field of structural biology will remain Many of the collaborations can and will continue to provide opportunities for interesting results as well as means for the training of scientists and students in the different techniques involved

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Structural Studies of HIV-1 Protease-inhibiting Antibodies

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Key words: HIV-1 protease, monoclonal antibodies, three-dimensional structure

The protease of Human Immunodeficiency Virus type 1 (HIV-1) is essential for the maturation of infectious viral particles (Katz and Kalka, 1994) It cleaves the Gag and Gag/Pol polyprotein precursors into the structural proteins and enzymes of HIV (including the protease itself) as well as some subsidiary polypeptides The enzyme is a homodimer belonging to the aspartate protease family in which the hydrophobic active site covered by two identical flap regions contributed by each of the respective monomers (Wlodawer *et al* 1989) The flap regions must undergo substantial conformational changes in order that the polyprotein substrate be bound at the active site of the enzyme (Fitzgerald and Springer 1991) The dynamic behaviour of the protease is therefore an essential element in understanding the function of the enzyme and its role in the viral life cycle We have studied the effect of monoclonal antibodies raised against HIV-1 protease to probe conformational changes that might be important for enzyme activity To this end,

we have selected those monoclonal antobodies which induce a significant reduction in proteolysis with a view to analyse the three-dimensional structure of complexes formed between the protease and the Fab fragments of inhibiting antibodies by crystallographic methods From these structures we hope to relate eventual conformational constraints or changes imposed by the bound antibody on the protease to dynamic behaviour that is essential for proteolytic activity In addition, the structures of such antibody-protease complexes might conceivably serve as a basis for the conception of inhibitors to the viral enzyme Such compounds would form a novel set of inhibitors since the active site of the protease is not assessable by antibodies

We have obtained two murine monoclonal antibodies fulfilling the criterion for HIV-1 protease inhibition One of these, F11 2 32, was shown by epitope mapping studies to bind to a region of the protease contained between residues 36 to 46 From kinetic studies, the inhibition constant was determined to be 35 nM The second monoclonal antibody, 1696, proved to be an even more effective protease inhibitor, having an inhibition constant of 1 nM The epitope recognised by antibody 1696 was shown by peptide mapping to include the first seven amino-terminal residues, a result corroborated by its ability to efficiently bind the HIV-2 protease, which carries only three conservative amino acid differences with respect to the HIV-1 enzyme in this region The HIV-2 protease, by contrast, does not cross-react with F11 2 32

Our attempts to crystallise F11 2 32 as an Fab complex with HIV-1 protease have been unsuccessful so far since this species has proved to be unstable and difficult to isolate We have succeeded, however, in crystallising the Fab as a complex with the peptide fragment 36-46 from HIV-1 protease and have solved its structure at 2 2 Å resolution using molecular replacement to obtain an initial model for refinement (Space group $P2_12_12_1$, $a = 82\ 2$Å, $b = 96\ 3$A, $c = 105\ 8$Å) We have also crystallised the uncomplexed Fab from F11 2 32 (space group $P2_1$, $a = 67\ 6$Å, $b = 94\ 7$Å, $c = 70\ 3$Å, $b = 105\ 8°$) and refined the structure at 2 6Å resolution This has allowed us to obtain the conformation of the bound peptide fragment from the protease, providing a opportunity to compare its conformation with the equivalent region in the native enzyme (Lescar *et al* 1996, 1997)

The structure of the complex shows ten of the eleven amino acids to be ordered and in direct contact with the antibody Three buried water molecules are located at the antibody-antigen interface, acting as bridges between the peptide and the Fab fragment and perfecting the complementarity of the binding surfaces Comparison of the Fab structures in the complexed and unbound states revealed a small change in the relative orientation of the V_H and V_L domains as well as differences in conformation of certain of the complementarity-determining regions (CDR) These changes allow residues forming the antigen-binding site to move closer to the antigen than would have been otherwise possible had the antibody preserved the same conformation as observed in the uncomplexed state The induced fit between the antibody and antigen has therefore led to a more intimate contact and thus tighter binding between the two components

The conformation adopted by the peptide when bound to the Fab corresponds to that of a b-hairpin strand Its structure is therefore more closed than that of the corresponding segment of the native protease structure Although segments 36-39 and 42-44 of the bound peptide have an extended b-strand conformation as in the native enzyme, the main-chain conformation of the segment 40-41 is completely different, and it is impossible to superimpose the entire peptide onto the equivalent region of the protease structure Nonetheless, the antibody F11 2 32 does bind the protease since the enzyme activity is greatly inhibited in its presence Thus, if the peptide complex accurately describes the interaction between the antibody and the protease, then the latter must be induced into

a conformation that is significantly different from the native form, at least in the region 36-46 Since this segment forms the amino-terminal base of the functionally important flap region, such a distortion could account for the observed inhibition of proteolytic activity in the presence of the antibody Given the intimate contacts between ten of the eleven peptide amino acids and the antibody, we would further conclude that F11 2 32 was probably induced by a non-native form of the protease Since HIV protease has a limited stability, we cannot exclude the possibility that the immunisation procedures we used to obtain the hybridoma cell line producing F11 2 32 (e g , using Freund's adjuvant) might have led to the presentation of the protein antigen in a form different from its native state Indeed, this could explain our lack of success in crystallising the complex formed between F11 2 32 and the protease itself

Although we have not yet succeeded in crystallising the complex formed between the HIV protease and the second protease-inhibiting antibody, 1696, inhibition of proteolytic activity in this case is probably due to dissociation of the dimeric enzyme The association of the protease subunits to the active dimeric enzyme is governed by the formation of a four-stranded b-pleated sheet by the amino- and carboxy-terminal regions of each monomer Since the antibody 1696 binds to the amino-terminal region of the protease, it conceivably decreases the stability of the dimeric species, favouring its dissociation to the monomer Several inhibitors directed to this region of the protease have been designed (Zhang *et al* 1991) but none have in inhibition constant approaching the nanomolar range that we have observed for 1696

We have, nonetheless, succeeded in crystallising the Fab fragment of 1696 (space group $P2_12_12_1$, $a = 46\ 6$Å, $b = 58\ 8$Å $c = 143\ 0$Å) as well as a single-chain Fv fragment expressed in *E coli* (space group $P2_12_12_1$, $a = 126\ 9$Å, $b = 61\ 2$Å, $c = 57\ 3$Å), each in the uncomplexed form The Fv construction consists of the heavy chain and light chain variable domains of 1696 and represents the smallest immunoglobulin fragment carrying the intact antigen-binding site The two variable domains are linked by a flexible peptide linker of 20 amino acids which join the carboxy-terminus of the V_L domain to the amino-terminus of the V_H domain to give the recombinant antigen-binding molecule a higher stability The structure of the Fab has been refined at 3 0 Å resolution while that of the Fv fragment has been refined at 1 7 Å resolution (measured at the ESRF, Grenoble) Differences in conformation are evident in the third CDR of the V_H domain, but this can be attributed to differences in intermolecular contacts in the two crystal forms Attempts are currently underway to crystallise the complex with HIV-2 protease which is more stable than the HIV-1 enzyme

This study has therefore provided two antibodies which might serve as a basis for the conception novel protease-inhibiting compounds These could be in the form of peptide mimetics (Saragovi *et al* 1991) based on the structure of those CDR that are most intimately in contact with the antigen For example, in the case of F11 2 32, the first CDR of V_L and the third CDR of V_H, which form the most important contacts with the peptide antigen, could be used to this end The use of F11 2 32 or 1696 as intra-cellular antibodies (Chen *et al* 1994) offers an alternative means of exploiting these protease-inhibiting antibodies when this potentially interesting therapeutic approach becomes feasible in practice

Acknowledgements. This work is supported by the Grant Agency of the Czech Republic, the Prague Biochemical fund, the Pasteur Institute, Centre National de la Recheche Scientifique and the European Commission

References

Chen S , Khouri Y , Bagley J , Marasco W A (1994) Combined intra- and extracellular immunization against human immunodeficiency virus type 1 infection with a human anti-gp120 antibody Proc Natl Acad Sci USA **91**, 5932-5936

Fitzgerald P M D , Springer J P (1991) Structure and function of retroviral proteases Annu Rev Biophys Biophys Chem **20**, 299-320

Katz R A , Skalka A M (1994) The retroviral enzymes Annu Rev Biochem **63**, 133-173

Lescar J , Stouracova R , Riottot M-M, Chitarra V , Brynda J , Fabry M , Horejsi M , Sedlacek J , Bentley G A (1996) Preliminary crystallographic studies of an anti-HIV-1 protease antibody which inhibits enzyme activity Protein Science **5**, 966-968

Lescar J , Stouracova R , Riottot M -M , Chitarra V , Brynda J , Fabry M , Horejsi M , Sedlacek J , Bentley G A (1997) Three-dimensional structure of an Fab-peptide complex structural basis of HIV-1 protease inhibition by a monoclonal antibody J Mol Biol **267**, 1207-1222

Sarngovi H U , Fitzpatrick D , Raktabutr A , Nakanishi H , Kahn M , Greene M I (1991) Design and synthesis of a mimetic from an antibody complementarity determining region Science **253**, 792-795

Wlodawer A , Miller M , Jakolski M , Sathyanarayana B K , Baldwin E , Weber I T , Selk L , Clawson L M , Schneider J , Kent S B H (1989) Conserved folding in retroviral proteases crystal structure of a synthetic HIV-1 protease Science **245**, 616-621

Zhang Z -Y , Poorman R A , Maggiora L L , Heinrikson R L , Kézdy F J (1991) Dissociative inhibition of dimeric enzymes J Biol Chem **226**, 15591-15594

Anti-HIV Proteinase Monoclonal Antibody F11.2.32 that Inhibits Enzyme Activity

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Key words: HIV-1 protease, epitopes, crystal structure

Introduction

The hybridoma that produce inhibitory monoclonal antibody (mAb) termed F11 2 32 originate from mice immunized with recombinant proteinase of HIV-1 This mAb belongs to IgG1 isotype, and its binding and inhibitory properties are also preserved in the corresponding Fab fragment HIV-1 protease is a homodimeric enzyme belonging to the family of aspartyl proteinases The monomer comprises 99 amino acid residues containing a triplet AspThrGly which is located near the dimer interface Thus, in the functional homodimer the two amino acid triplets are adjacent to each other, forming a pepsine-like catalytic site at the bottom of a hydrophobic cavity

The catalytic site is covered by two flap regions, one contributed by each subunit, which undergo substantial movement during binding the substrate