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Review

Structural and energetic aspects of protein-protein recognition*

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Specific recognition between proteins plays a crucial role in a great number of vital processes. In this review different types of protein-protein complexes are analyzed on the basis of their three-dimensional structures which became available in recent years. The complexes which are analyzed include: those resulting from different types of recognition between proteinase and protein inhibitor (canonical inhibitors of serine proteinases, hirudin, inhibitors of cysteine proteinases, carboxypeptidase inhibitor), barnase-barstar, human growth hormone-receptor and antibody-antigen. It seems obvious that specific and strong protein-protein recognition is achieved in many different ways. To further explore this question, the structural information was analyzed together with kinetic and thermodynamic data available for the respective complexes. It appears that the energy and rates of specific recognition of proteins are influenced by many different factors, including: area of interacting surfaces; complementarity of shapes, charges and hydrogen bonds; water structure at the interface; conformational changes; additivity and cooperativity of individual interactions, steric effects and various (conformational, hydration) entropy changes.

Specific protein-protein recognition is a key event in many biological processes. Nowadays, the problem what are the rules which determine specificity and energy of protein-protein associations is particularly attractive as the structural information about different complexes is rapidly growing [1–4]. This is particularly due to protein X-ray crystal-

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Abbreviations: BPTI, basic pancreatic trypsin inhibitor (Kunitz); CMTI I, *Cucurbita maxima* trypsin inhibitor I; CPI, carboxypeptidase A inhibitor from potato; hGH, human growth hormone; hGHbp, extracellular domain of human growth hormone receptor; OMTKY3, turkey ovomucoid third domain; ASA, solvent accessible surface area; PPE, porcine pancreatic elastase; SGPB, *Streptomyces gryceus* proteinase B; ΔG_{ass}, ΔH_{ass}, ΔS_{ass} and $\Delta C_{p,ass}$ denote free energy change, enthalpy change, entropy change and heat capacity change during protein-protein association reaction; K_a, association constant; k_{on}, second order association rate constant for the association of two proteins; k_{off}, first order dissociation rate constant of the protein-protein complex.

lography methods, since protein-protein complexes are generally too big for NMR solution studies. The protein-protein binding interfaces are generally quite large (500 to 1200 Å^2) and involve about 10–30 amino-acid side chains from each protein. It is generally believed that association energy is proportional to the surface area buried upon complexation [5].

The problem of protein-protein recognition appears to be, at least in principle, simpler than the protein folding/stability problem, since it is possible to obtain high quality structural information about the reactants (free proteins) and reaction product (complex). In the case of studies on protein folding, structural information is available only for the folded state, and that for the unfolded protein remains poorly understood. In recent years conformational analysis of interacting surfaces became increasingly popular. With increasing computer power, there is a substantial progress in prediction of protein binding sites by careful considerations of protein surface topography [6–12].

Recent structural data broaden significantly our knowledge about ways of complexation developed by nature. Further interest is due to potential pharmaceutical and biotechnological applications of many interacting proteins, including proteinases and their protein inhibitors, antibodies and hormones.

Unfortunately, structural data give little understanding of energetics of protein-protein associations. The individual contribution of contacting side chains to the binding energy and the relative contribution of hydrogen bonding, van der Waals interactions, hydration and entropic effects remain to be elucidated. Similarly, it is not clear what is the molecular meaning of ΔH_{ass}, $\Delta C_{p,ass}$ and ΔS_{ass}. Fortunately, with the advent of highly sensitive titration calorimetry some progress can be observed also in this area. Moreover, since the protein-protein interaction problem is in many ways analogous to the intensively studied protein stability/folding problem, parallel consideration of energetics of the two processes can facilitate progress in both areas [13].

In this review we analyze the representative structures of enzyme-inhibitor, antibody-antigen and hormone-receptor complexes, especially those for which the high resolution structures are available. It appears that, from the structural point of view, energy and specificity of interaction is achieved in many different ways. Special attention is paied to the systems for which structural information is well supplemented with thermodynamic and kinetic data.

PROTEINASE-PROTEIN INHIBITOR INTERACTION

Canonical or standard mechanism of inhibition of serine proteinases

Serine proteinases and their canonical protein inhibitors are the most intensively studied group of protein-protein complexes [14–17]. Protein inhibitors do not form a single large group but can be divided into about 18 different families [14, 18]. A large number of three dimensional structures is available for representatives of most of the inhibitor families in complexes with many different serine proteinases. Figure 1a shows the recognition of bovine β-trypsin by *Cucurbita maxima* trypsin inhibitor I (CMTI I). Similar recognition is a feature of all canonical inhibitors because despite having completely different scaffolds, they share a very similar conformation of the binding loop. The extended conformation of the loop is defined by its main chain torsional angles [16, 17]. The hydrophobic and convex proteinase binding loop of inhibitor which spans from position P3 to P3′ (notation of Schechter & Berger, [20]) is highly complementary to the concave active site of the enzyme. Rigid conformation of the loop is maintained *via* hydrogen bonds from the side chains of the distal part of the molecule to carbonyls of P2 and P1′ residues. Such spacers are commonly observed in different inhibitor families. Besides the P3-P3′ segment, also side chains from surrounding residues (P9 to P4′) and other parts of inhibitor make numerous van der Waals and/or hydrogen bonds with pro-

Figure 1a

Figure 1b

Figure 1c

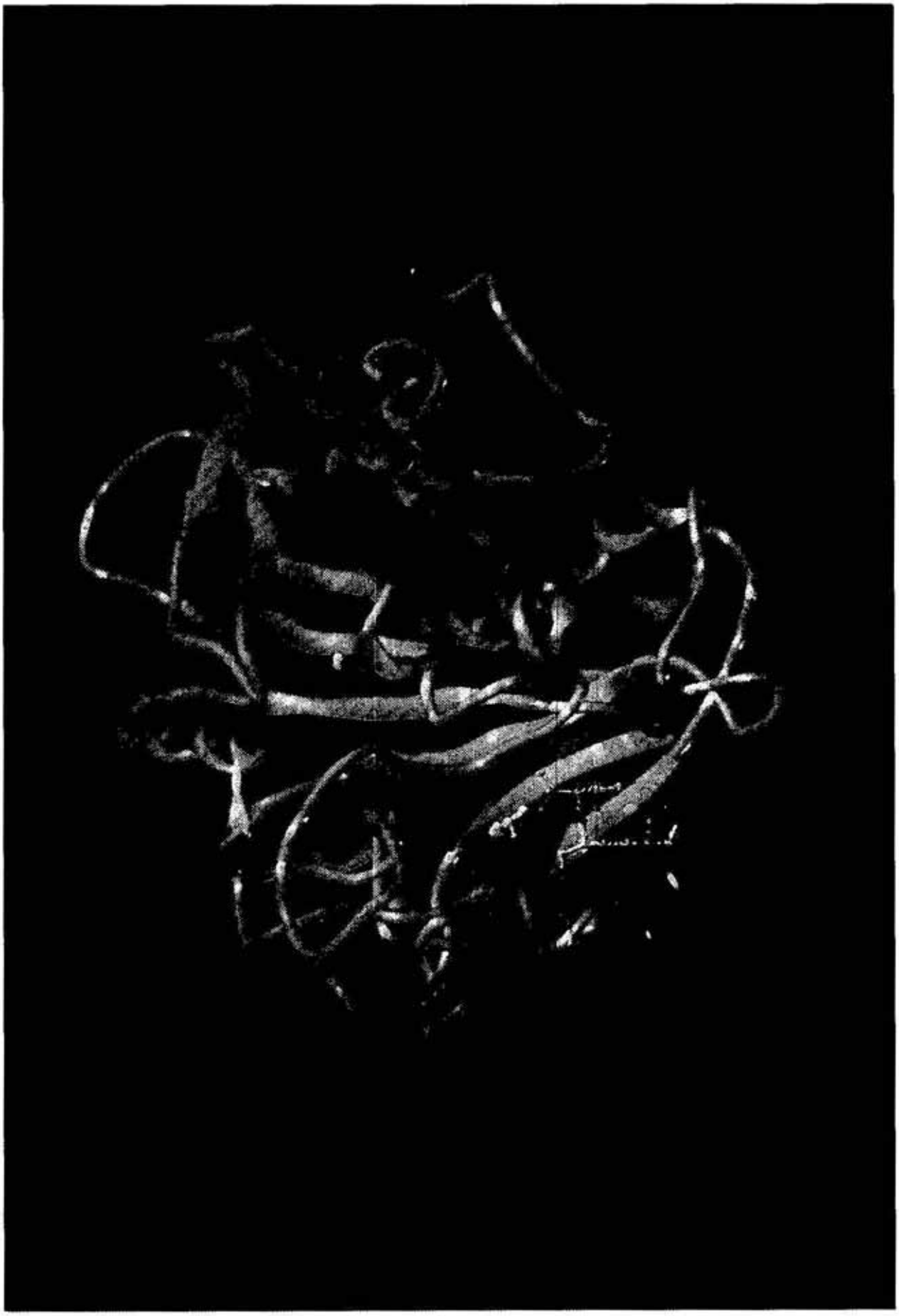

Figure 1d

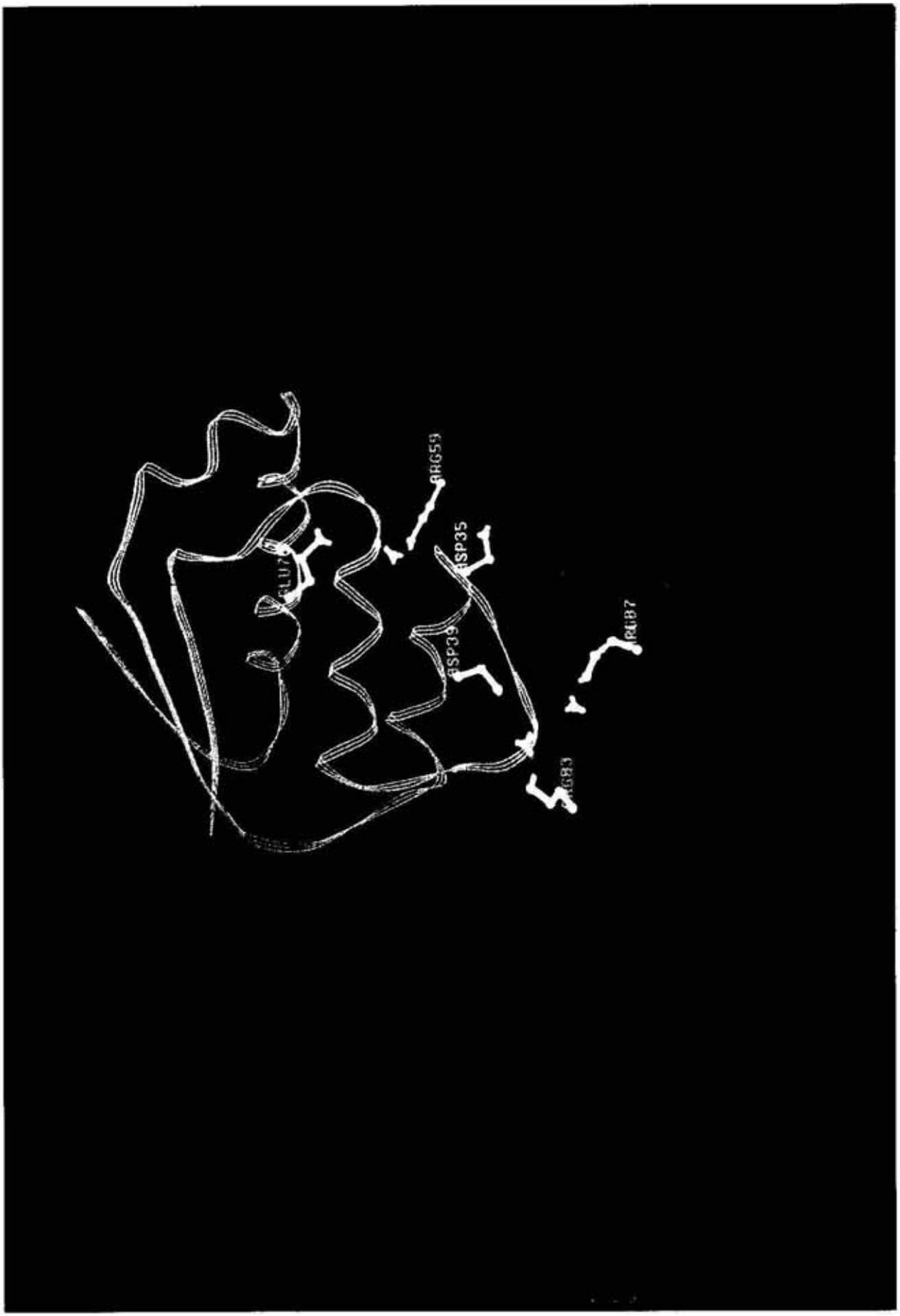

Figure 1e

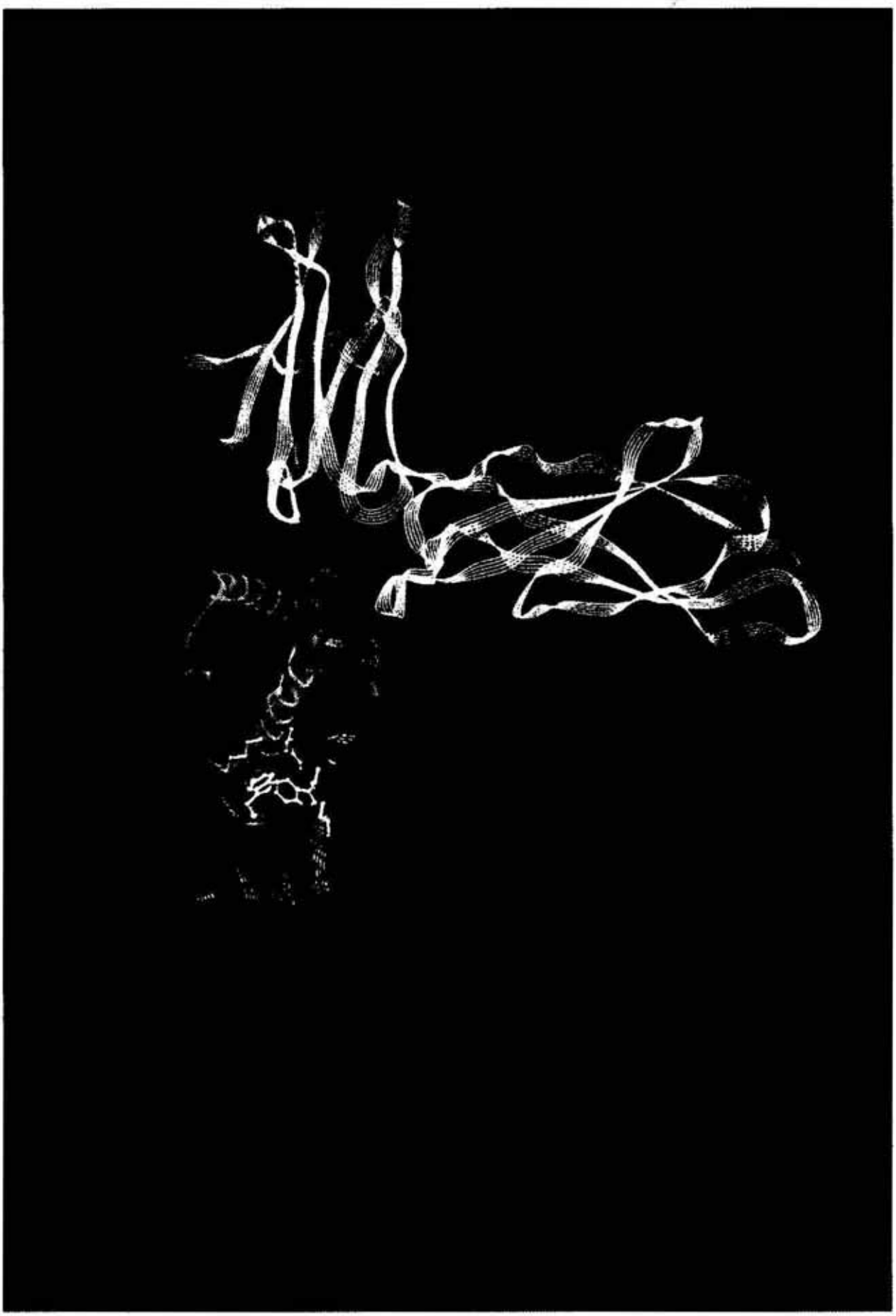

Figure 1f

Figure 1g

teinase. A similar number of contacting residues — about 10–12 on inhibitor side and 20–25 on enzyme side characterizes all complexes. Typical intermolecular contact area is of each protein about 600–900 $Å^2$. Hydrogen bonds and electrostatic interactions at the interface are well developed. Of particular importance is the short antiparallel β-sheet formed by main chain-main chain hydrogen bonds between P3 and P1 residues and the 214–216 segment of the enzyme. Other very important features are: short 2.7 Å contact between P1 carbonyl carbon and catalytic Ser195 O^{γ}, and two hydrogen bonds formed between carbonyl oxygen of P1 and Gly193–Ser195 amides (the oxyanion binding hole). All the above mentioned hydrogen bonds and shape complementarity of interacting areas ensure very similar recognition of different proteinases and inhibitors.

It appears that inhibitor binding resembles that of the ideal substrate. Indeed, inhibitors are substrates and their recognition involves short contacts with catalytic residues. Slow hydrolysis of the P1–P1′ peptide bond with high index of k_{cat}/K_m was observed [21, 22]. It should be stressed that, in this particular case, there is a distinct linkage between protein-protein recognition and a typical proteinase-catalyzed reaction.

The strength of the proteinase-inhibitor interaction is usually expressed in equilibrium association/dissociation constants. Measurable range of association constants (K_a) is from about 10^3 to 10^{12} M^{-1}. Canonical proteinase inhibitors bind to proteinases at a high rate; the second order association constant usually exceeds 10^6 M^{-1} s^{-1}, i.e. 3 orders of magnitude below that for the diffusion-controlled reaction. Similar values of association rate constants have been reported for many other types of protein-protein interactions [23]. Since K_a is equal to k_{on}/k_{off}, it appears that differences in thermodynamic stability of complexes result from differences in k_{off} values. In the most dramatic example of the complex between BPTI and bovine β-trypsin, $K_a = 1.6 \times 10^{13}$ M^{-1}, $k_{on} = 1.1 \times 10^6$ $M^{-1}s^{-1}$ and $k_{off} = 6.6 \times 10^{-8}$ s^{-1} at pH 8.0 [24]. Clearly, stability of the complex results from

Figure 1. Three-dimensional models of different protein-protein complexes determined by X-ray crystallography. Structural details are described in the text.

a. Structure of the complex between serine proteinase inhibitor CMTI I and bovine β-trypsin (PDB code 1PPE) at 2.0 Å resolution [19]. The main chain of the inhibitor is shown as red tube, proteinase main chain as green line ribbon. Side chain of the catalytic Ser195 and the recognition of Arg5 (P1 residue) of the inhibitor by side chain of Asp189 at the bottom of the S1 pocket are shown as ball-and-sticks models. **b**. Model of the recognition between hirudin inhibitor form leech and bovine thrombin, according to the crystal 2.8 Å resolution structure of the complex (PDB code 1HRT) [41]. The main chain of hirudin is shown as green tube. The N-terminal chain of thrombin (A chain) as orange tube and its catalytic domain (B chain) as magenta ribbon. Two areas of hirudin-thrombin recognition are represented by models of the important side chains. **c**. Papain-human stefin B interaction, according to the 2.4 Å model of the crystal structure (PDB code 1STF) [47]. Inhibitor's main chain is shown as cyan tube and three segments interacting with papain as green ribbon. Papain secondary structure is indicated as red helices, blue β-strands and irregular structure as yellow tubes. Catalytic Cys25 is also shown. **d**. Structure of the bovine carboxypeptidase A-CPI complex at 2.5 Å resolution (PDB code 4CPA) [49]. The enzyme's secondary structure is shown in green and irregular structure of the inhibitor in magenta. Three C-terminal residues of inhibitor are shown in orange and Gly39 (cut-off from the inhibitor) in yellow. **e**. The complex between barnase and its inhibitor barstar, according to the 2.0 Å structure of the complex (PDB code 1BRS) [52]. Barnase and barstar main chains are shown as violet and yellow ribbons, respectively. Charged residues which influence kinetics and thermodynamics of the interactions are indicated as ball-and-sticks models. **f**. Structure of the complex between hGH and its receptor (PDB code 3HHR) at 2.8 Å resolution [55]. Hormone is shown as green ribbon and two receptor molecules as magenta and yellow ribbons. Residues forming major interactions between hGH and the first molecule of hGHbp are shown in green and orange, respectively. **g**. The complex between F_{ab} fragment of monoclonal antibody HyHEL-5 and chicken egg-white lysozyme, according to 2.5 Å resolution crystal structure (PDB code 2HFL) [82]. Lysozyme main chain is shown in red, heavy and light chains of antibody in green and blue, respectively. Major side chains of interacting proteins are also shown.

extremely low dissociation rate constant, reflected in a half lifetime of 17 weeks.

Association energy, in general, strongly depends on pH. The single group which in deprotonated state facilitates the interaction is catalytic His57 of the proteinase. In trypsin, however, concerted three-proton transition with an apparent pK of about 4.0 is responsible for the huge, by 7 orders of magnitude drop in association constant when pH is lowered from 5.5 to 2.5 [21, 22].

Complementarity between interacting surfaces is very good — a gap index defined as the ratio of gap volume between proteinase and inhibitor molecules to interface area is particularly low for these complexes [3]. Water is predominantly excluded from the interface area, yet several water molecules which can be observed in the S1 binding pocket and at interface, possibly influence the association energy [25]. Generally, inhibitor-enzyme interaction is rigid in this sense that no conformational changes are observed upon complex formation. This is best seen from a comparison of free and complexed structures of inhibitor [26–28]. Moreover, thermal motions of the binding loop are significantly lowered upon complex formation. Although the conformational stabilization of the binding loop is entropically unfavourable, the numerous van der Waals interactions and hydrogen bonds well compensate for this effect. The interface is as well packed as protein interior. These factors are considered as leading to high association energy.

A large part of contacts is made just by P1 residue, which penetrates deeply to the S1 specificity binding pocket of the proteinase. Often the principal inhibitory activity stems from P1 residue (trypsin inhibitors have Lys or Arg; chymotrypsin inhibitors — Leu, Met or Tyr). However, even Gly introduced at P1 of turkey ovomucoid third domain gives an inhibitor with significant association constants against PPE ($K_a = 9 \times 10^8\ M^{-1}$) or subtilisin Carlsberg ($K_a = 6.4 \times 10^8\ M^{-1}$) [29]. In the case of BPTI-trypsin, interactions from Lys15 (P1) are of utmost importance: substitution of P1 Lys with Gly removes 70% of total association energy, leading to a huge, by 9 orders of magnitude decrease in association constant (unpublished results from our laboratory). In other complexes the relative importance of P1 residue can be somewhat lower, yet this residue is a principal determinant of inhibitor specificity and binding energy, as discussed below. Other contact residues usually do not influence the association energy so much. Non-contact residues only seldom influence the association with proteinase. Semiempirical free energy study of Krystek *et al.* [30] also shows the predominant role of P1 residue interactions. Free energy contributions from other contact residues are smaller at least by a half. An interesting general observation from that study is that the energetic contribution of inhibitor residues is larger than that donated by enzyme. In the case of subtilisin family of proteinases that theoretical study and experimental data indicate that both P1 and P4 side chains contribute significantly to association energy [30].

Single amino-acid substitutions at P1 cause enormous changes in association energy. The observed effects depend on many factors, including: size and hydrophobicity of side chain, its branching, presence of heteroatom or charge [29, 31]. Particularly deleterious are steric effects. For example, Pro is invariably an improper side chain for serine proteinases, β-branched side chains are bad for most of enzymes, and large side chains are bad when introduced to a small pocket. Also introduction of charged side chains to a hydrophobic pocket leads to dramatic effects on association constant. Hydrophobic pocket of SGPB binds the neutral form of His, Asp or Glu 10^2–10^5-fold stronger than the charged one [32]. The contrary will be, of course, true for binding pockets of e.g. trypsin, thrombin, Glu-specific proteinases, factors X_a and XII_a, which will recognize much more strongly charged forms of respective side chains.

It is important to stress that despite very severe steric conflicts in the S1 pocket, even very large side chains can be accommodated without distortions of the recognition motif. Recently determined X-ray structure of the SGPB-OMTKY3 complex (Leu18Trp mutation at P1 of OMTKY3) shows that such a large side chain is accommodated in the S1 pocket adopting an unfavourable χ_2 angle

but without pocket distortion [33]. Such steric effects usually lead to much lower association constants, compared to those for optimal side chains, and are not reflected in differences of interface areas.

In recent years several inhibitors containing a non-optimal P1 residue, yet inhibiting strongly the target enzyme, have been isolated from natural sources. An example is two-domain inhibitor rhodniin isolated from hematophagous assassin bug, potent ($K_a = 5 \times 10^{12}$ M^{-1}) inhibitor of thrombin [34]. The N-terminal domain of this inhibitor with His at P1 recognizes in canonical way the S1 pocket. The interaction is clearly strengthened by electrostatic attraction of the C-terminal domain with the fibrinogen recognition exosite. Another interesting example is ecotin from periplasm of *Escherichia coli*. The protein which acts as a 2×142 amino-acids dimer is highly effective against trypsin, chymotrypsin, elastase, kallikrein, factor X_a and urokinase [35]. Yet, it contains Met84 at P1, which is non-optimal, especially for highly specific above mentioned enzymes. In this case strong interactions at a secondary contact region, which results from ecotin dimerization, lead to a tetrameric complex in which one enzyme molecule is in contact through the active site with one ecotin molecule and at the same time with another ecotin through its secondary site. The inhibitor contact area at this secondary site is half that at the primary recognition site. It appears that these secondary interactions, combined with the observed structural adaptation of ecotin to different proteinases, supplement well those at the binding loop. In fact, specificity of ecotin is insensitive to mutations at P1 site [36] and analysis of P1 Met in different complexes shows its peculiar behaviour. Particularly, in the case of crab collagenase complex this side chain does not enter at all the S1 pocket [35].

The proteinase binding loop can be considered as a sequential epitope. Weak interactions which are formed by other residues (e.g. the C-terminus of CMTI I or Phe33–Arg39 segment of BPTI) often do not influence dramatically the energy of association. For example, recent alanine-scanning of contact residues of BPTI has shown that mutation of Lys15 (P1) to Ala produces the largest effect, about 10 kcal/mole, on ΔG_{ass} with trypsin [37]. Only three other contact residues: Gly12, Ile18 and Gly36 affected the interaction at the level of 4–4.5 kcal/mole. It should be stressed, however, that, particularly in the case of highly specific proteinases with extended binding sites (e.g. factor X_a), the substitution effects beyond P1 residue may also be pronounced. For example, factor XII_a is known to discriminate squash inhibitors which differ at P4′ position: Lys being 62-fold more effective than Glu [38].

Since many residues of inhibitor interact with the enzyme, the question arises: do these residues influence each other or the energetic effects are independent (additive). This problem was carefully studied over many years in M. Laskowski's, Jr., laboratory. The answer is that in an overwhelming number of the cases studied the effects are additive. This has been most often anticipated from double mutant thermodynamic cycles, which indicate that sum of effects on association energy of two single mutants are equal to that measured for a double mutant [39]. In those cases when lack of additivity was observed, it could often be explained by interaction of mutated residues (hydrogen bonding, van der Waals contacts) or structural changes accompanying introduced mutations. The additivity phenomenon appears to be general for protein-protein interactions and occurs also in other types of biological reactions, like folding, stability or substrate binding and catalysis [40]. Additivity significantly simplifies designing of strong and specific protein inhibitors.

Thrombin-hirudin interaction

Interaction between thrombin and hirudin (potent and selective 65 amino-acid residues inhibitor from leech) reveals other aspects of serine proteinase-protein inhibitor recognition. The hirudin-thrombin interaction is very strong ($K_a = 10^{13}$ M^{-1}) and specific due to binding at two different surfaces of the enzyme (Fig. 1b). The interface area is much larger (1800 $Å^2$) than in the case of canonical inhibitors, since three segments of hirudin interact with thrombin [41]. The amino-ter-

minal fragment binds at the active site region of thrombin and three N-terminal residues form a *parallel* β-sheet with the Ser214–Gly219 fragment of thrombin. As it was mentioned before in all serine proteinase-canonical inhibitor complexes this fragment interacts *via* a short *antiparallel* β-sheet. Moreover, in contrast to the canonical proteinase inhibitors, the hirudin-thrombin interactions are mediated through several water molecules and the residue Ser195 of thrombin is not blocked. There are no interactions within the S1 pocket, which in canonical inhibitors are extremely important in determining the strength and specificity of proteinase-inhibitor recognition. However, specific substitutions of these N-terminal residues (e.g. Val1 to Leu) markedly reduce the binding energy [42]. The carboxy-terminal tail of hirudin, which is flexible in solution [43] interacts with many residues in the (anionic) fibrinogen binding exosite and has a defined extended conformation in the complex. Recent thermodynamic analysis of this interaction indicates that, besides the inhibitor tail, also the three surface loops of thrombin (W60d, W148 and the fibrinogen exosite) undergo conformational transitions, which are coupled to inhibitor binding [44]. Most of the 17 residues forming the negatively charged tail participate in electrostatic (several salt bridges) or hydrophobic interactions in this region. According to kinetic data, initial recognition starts from association of negatively charged tail of hirudin and positively charged fibrinogen binding exosite of thrombin. This electrostatic component ensures very fast interaction with k_{on} values reaching 4×10^7 $M^{-1}s^{-1}$ [45]. Mutational analysis confirms this hypothesis: the association rate constant is effectively reduced by mutations that eliminate negative charges at the C-terminus of hirudin [42]. Also hydrophobic interactions of the C-terminal tail (particularly Pro60) with fibrinogen exosite are important in complex stabilization, as revealed by mutagenesis studies [42].

Protein inhibitors of cysteine proteinases

The cystatin family of protein inhibitors comprises proteins of about 11–14 kDa, which strongly inhibit cysteine proteinases: papain and cathepsins B, H, S and L [46]. Spatial structure is available for the cystatin B-papain complex [47]. The inhibitor is composed of a long α-helix and a five stranded β-sheet, which wraps around the helix. A papain binding hydrophobic wedge is formed by two β-hairpin loops and the N-terminal fragment. The wedge is highly complementary to the enzyme binding site (Fig. 1c). Of particular importance are hydrophobic interactions of the first hairpin loop, which contains the conservative sequence GlnXaaValXaaGly (often GlnLeuValSerGly). Mutational analysis, however, shows that this sequence is quite tolerant to substitutions, which do not lead to loss of association energy. The N-terminal end of inhibitor binds over the catalytic Cys25 and interacts with the subsite S2 similarly as observed in a productively bound substrate. The catalytic Cys25 residue is only sterically blocked and is not directly involved in the recognition mechanism. The shorter forms of papain starting at Gly9 or Ala10 bind about 10^4-fold weaker, compared to the full length form ($K_a = 2 \times 10^{11}$ M^{-1}). Comparative inhibition studies performed on chicken cystatin interaction with different cysteine proteinases revealed that N-terminal truncation comprising ten amino acids leads to differential effects on the rate constants of association and dissociation with different proteinases [48]. For papain and ficin truncation has no effect on the rate constant of association, but a huge effect (up to 2×10^6-fold increase) on the rate of dissociation. On the contrary, for cathepsin B truncation does not affect the rate of dissociation, but decreases 60-fold the association rate constant.

Carboxypeptidase A-potato carboxypetidase inhibitor complex

Currently there is only one available structure of an metalloproteinase-protein inhibitor complex [49]. Carboxypetidase A inhibitor (CPI) is a small protein of 39 amino-acid residues, which exhibits a compact and rigid structure (Fig. 1d). Its association constant for complex formation with carboxypeptidase is 2×10^8 M^{-1}. Interestingly, the global fold

of this protein, including relative orientation of disulfide bonds, is very similar to that of squash proteinase inhibitor [19].

The protein tail of CPI is quite flexible in solution. Its conformation becomes well-defined when bound in the active site groove of the enzyme. In the complex the C-terminal Gly residue is cut off and the three preceding residues favourably interact with the enzyme (particularly important are the interactions to Tyr248 and Arg71). Thus, the recognition resembles the enzyme-product stage and clearly indicates involvement of catalytic machinery of carboxypeptidase during complex formation.

BARNASE-BARSTAR COMPLEX

Bacterial ribonuclease barnase is a 110-residue protein which forms a tight complex ($K_a = 10^{14}\ M^{-1}$) with an 89-residue inhibitor barstar. Both proteins are produced in the same organism — *Bacillus amyloliquefaciens* and their association is of physiological importance. Structures are available both for free proteins, and for the complex [50–52]. The active site of the enzyme is blocked by a helical segment of barstar with catalytic His102 placed in the pocket on the surface of barstar (Fig. 1e). Other catalytic enzyme residues: Arg83, Arg87, and Lys27 together with His102 interact with barstar forming multiple ion pairs and hydrogen bonds. Of fourteen hydrogen bonds, six involve both charged donor and acceptor and four involve one charged partner. There is a clear electrostatic complementarity of interacting surfaces: negative Asp35 and Asp39 on the exposed helical segment of barstar interact favourably with a cluster of positive charges: Lys27, Arg83, and Arg87 from the barnase active site. Distinct similarity between barnase-barstar and barnase-nucleotide interaction can be observed. Although shape complementarity is high, the interface gaps contain together 35 water molecules. Six of them fill the region of poor complementarity (the guanine binding site), nine mediate hydrogen bonds and have low temperature factors, comparable to those of protein side chains at the interface.Thus, the barnase-barstar interface seems to be considerably more polar and filled with many more water molecules than other protein-protein interfaces. Barnase interacts with minor conformational adjustments. Larger, rigid body movements of four helices were observed in barstar structure.

The association rate constant for the barnase-barstar interaction is extremely high, about $10^8\ M^{-1}s^{-1}$ (in the case of some barnase mutants with replaced charged residues even up to $4.5 \times 10^9\ M^{-1}s^{-1}$, [53]) several hundred times faster than for other protein-protein complexes. Clearly, the electrostatic steering effect from favourably oriented charged side chains is responsible for the rate constant approaching the diffusion limit. Similarly as for the serine proteinase-protein inhibitor interaction, mutations of either Asp35 or Asp39 of barstar to alanine have no effect on the association rate constant, but have dramatic effects on the rate of dissociation increasing it by factors of 3800 and 900000, respectively [53].

Mutagenesis analysis of all possible interacting pairs at the barnase-barstar interface using the double-mutant cycle indicates strong interaction energy, up to 7 kcal/mol [54]. Interestingly, strong interactions occur between residues separated by 4.5 Å, which was not anticipated from the crystal structure analysis. In general, the interactions between charged and/or uncharged residues decrease with distance. Significant interactions could be detected for pairs of residues separated by even up to 7–8 Å. The effects of mutations on the kinetics of association are generally additive, with exceptions for charged residues separated up to 10 Å. Clearly, the transition state for association occurs before most interactions have been formed.

INTERACTION OF hGH WITH ITS RECEPTOR

The structural and functional data on the complex between human growth hormone (hGH) and soluble extracellular domain of its receptor (hGHbp) provide extremely interesting information how protein hormone ac-

tivates its cellular receptor. hGH is a small four helix bundle protein which binds two receptor molecules at two different contact sites (Fig. 1f). Initially, the studies were performed without support from structural data.

Virtually all contacts at the hormone-receptor interface are through side chains and do not involve main chain atoms [55]. Alanine-scanning mutagenesis of the hormone revealed that only with eight mutants clear effects of the mutation on kinetics of the complex formation at the recognition site 1 could be observed [56]. In each case the off-rate was increased (5 to 30-fold), while on-rates were only marginally affected. Thus, of the 31 side chains which become buried upon complex formation, eight account for about 85% of the association energy and can be called the functional epitope. Alanine-scanning mutagenesis of the receptor side showed a similar situation: 9 of the total 33 contacting side chains were responsible for total binding energy [57]. In particular, two Trp (104 and 169) side chains, which bury only about 220 $Å^2$ of total 1300 $Å^2$ ASA buried on the receptor side account for about 75% of the binding energy. When hormone and receptor sides are compared, they reveal that functional and unimportant regions on the two molecules fit each other. The important side chains of both tryptophans occupy the hydrophobic pocket formed by functionally important side chains on hormone surface. These centrally localized hydrophobic interactions are surrounded by less important 5 hydrogen bonds and salt bridges. The peripherial part of the interface appears to be not as tightly packed as the central part and contains more of water molecules.

The work on hGH and its receptor suggests that very small molecules can specifically bind protein, since only a few residues are important for function. In fact, using phage-display technology, successful minimization of the three different receptor-binding proteins has been very recently achieved [58–60]. In all three cases minimization of protein did not lead to a substantial decrease of the association constant.

In the case of the hGH-hGHbp interaction there is lack of correlation between the extent of side chain burial, number of contacts and functional importance of the side chain. For example, side chains of Phe25, Tyr42 and Gln46 bury 200 A^2 of ASA in a complex which accounts for 16% of total ASA of hGH buried [57]. Neither of these three side chains has any effect either on the association constant or individual on- and off-rate constants. Thermodynamic analysis of the three respective alanine mutants revealed that they influenced significantly the ΔH_{ass}, ΔS_{ass} and $\Delta C_{p,ass}$ parameters [58]. Changes in enthalpy and entropy were compensatory, however, leading to negligible changes in association free energy. Another interesting aspect of this analysis was lack of correlation between heat capacity change and buried hydrophobic area, and lack of $\Delta C_{p,ass}$ additivity for the triple mutant, compared to the sum of single mutants. Since prediction of thermodynamic binding parameters from structural data is based on the correlation between (polar and nonpolar) buried surface area and the magnitude of individual thermodynamic parameters [61–63], the observed effects suggest a complex behaviour of interacting proteins. Nevertheless, successful thermodynamic analyses of protein-ligand interactions based on such correlations have been recently reported [64, 65].

Two receptor binding sites of the hGH do not react randomly with the hGHbp molecules, they bind rather two receptors in a sequential manner [66, 67]. The receptor uses a virtually identical site to bind at either site of the hormone. While the structural epitope buries 1300 $Å^2$ of ASA at site 1 of hGH, less intensive interactions (ΔASA = = 850 $Å^2$) occur at site 2. An additional interface of 500 $Å^2$ is formed, however, between the first and second hGHbp molecule when the first receptor molecule is bound to the hormone. It appears that binding of the second receptor molecule to hormone is stronger when the first molecule is already bound, due to the additional interface area between the two receptor molecules.

ANTIBODY-ANTIGEN RECOGNITION

Specific recognition of an unlimited number of antigens by antibodies occurs *via* six CDRs

(complementarity determining regions): three from light and three from heavy chain of antibody (Fig. 1g). Although the conformations adopted by the main chain of these loops are limited in number, differences in sequence and length of each CDR allow for great variability of antigen combining sites. It has been suggested, however, that only about 10, the so called canonical combinations, out of the 300 possible are predominantly used to recognize antigens [68]. Together, about 12 to 20 amino-acid residues on the antibody side and 10 to 20 on the protein antigen side are in contact upon complex formation [69]. Changes in the buried surface area are generally larger for loops from the heavy chain (330 to 480 $Å^2$) than from the light chain (200 to 420 $Å^2$) [70, 71]. While in smaller antigens the antigen combining surfaces are concave, in protein antigens they are planar [69]. The antigen-antibody complexes are less complementary than enzyme-inhibitor complexes [3].

Particularly careful structural studies were performed on lysozyme interacting with four different monoclonal antibodies. Epitopes for these antibodies cover almost half of the total surface of the lysozyme — it appears that all surface area of lysozyme is antigenic. Presence of water at the interface appears to be a rather general feature of lysozyme-antibody complexes [71, 72]. However, the interpretation of water structure is difficult due to moderate resolution of available structures. In the hen egg lysozyme-F_vD1.3 complex at 1.8 Å resolution about 50 water molecules could be localized at the interface, some of them being completely buried in the interface [72, 73]. The role of these water molecules seems to mediate antigen-antibody interactions. In a recent study, single mutations of residues at the interface of lysozyme-F_vD1.3 [truncation of Tyr32 (light chain), Tyr50 (heavy chain) and Tyr101 (heavy chain) to Ala, Ser and Phe, respectively] led to alterations in solvent structure.

The role of water is nicely underlined in the structural study of Chacko *et al.* [74]. Single amino-acid replacement of Arg68 by Lys in lysozyme results in a 1000-fold lower binding affinity for monoclonal antibody HyHEL-5. Semiquantitative study of Novotny *et al.* [75] indicates that Arg68 together with Arg45 contribute most significantly to the free energy change accompanying complex formation. Structures of both Arg68 and Lys68 complexes reveal only local changes at the interface including introduction of a new water molecule which mediates the hydrogen bond between the lysine and residues of antibody. Thus, a small rearrangement of hydrogen bonds can produce large effects on affinity, despite virtually identical changes in accessible surface areas. Interestingly, a similar rearrangement of hydrogen bonds occurs for Arg and Lys variants of CMTI I or BPTI inhibitors interacting with trypsin [76]. In the case of inhibitor, however, both side chains are recognized with the same affinity [22, 76].

Another interesting aspect of different modes of antibody-protein recognition was addressed in the recent study of Mariuzza and coworkers [77]. They found that antibody D1.3 interacts with hen lysozyme and the anti-d1.3 antibody E5.2 using virtually the same set of combining residues and even most of the same atoms. However, when single alanine substitutions were introduced in the combining site of D1.3, different energetic patterns were observed. The energetics of D1.3-lysozyme interaction is dominated by only 3 out of 13 contact residues: Trp92 of the light chain and Asp100 and Tyr101 of the heavy chain (effects larger than 2.5 kcal/mol). These residues form a patch at the center of the interface. Surrounding interface residues contribute much less (below 1.5 kcal/mol) to affinity energy. In contrast, 11 of the 15 contacting residues contribute more than 1.5 kcal/mol to free energy of D1.3–E5.2 interaction. Thus, in this particular case, the association free energy results from many interactions over the entire contact region.

A study on human growth hormone interacting with 21 different monoclonal antibodies shows, however, that the situation observed in the D1.3-lysozyme complex is perhaps more common [78]. Single alanine mutations of the entire surface of hGH indicate that only 3–5 side chains could account for more than 80% of the binding affinity to any of the 21 different monoclonal antibodies used. Moreover, alanine mutations in groups

of 7 to 16 of the residues surrounding this functional epitope showed that even with 16 alanine mutations introduced, the effect on binding to antibody was less than 10-fold [79]. This contrasts with huge effects observed on the functional epitope, where mutation of any of the four charged residues (Arg8, Arg16, Asp112, or Asp116) with similar residues (e.g. Arg to Lys or Asp to Glu) produced effects comparable to alanine replacement. Thus, the thermodynamic mapping of the antibody-antigen interface reveals a situation similar to those for trypsin-inhibitor or hGH-receptor complexes. A decisive role is played by a few critical side chains placed in the center of the interface. This is in good agreement with earlier semitheoretical calculations for the HyHEL-5-lysozyme system [75]. In that complex both Arg45 and Arg68 of lysozyme, which are protruding to the solvent, play a decisive role in energetics of recognition. On the antibody side, the energetically most important residues are located at the bottom of the antigen binding cavity. The area which surrounds this functional epitope seems to be energetically passive.

Energetic aspects of antibody-protein antigen interactions have been recently studied by isothermal titration microcalorimetry. The reaction is generally enthalpy-driven with different contributions from entropy change [80, 81]. For example, two monoclonal antibodies E3 and E8 bind to cytochrome *c* with very different heat capacity changes for association: $\Delta C_{p,ass} = -165$ cal/mol deg for E8 and $\Delta C_{p,ass} = -350$ cal/mol deg for E3 [80]. Thus, for E3-cytochrome *c* there is a greater entropic gain from loss of solvent during complex formation than for E8-cytochrome *c* (the respective values of solvation entropy change are: 90 and 42 cal/mol deg). This effect is offset, however, by larger loss in configurational entropy for E3 than for E8 complexes (the respective values are: –77 and –34 cal/mol deg). Clearly, the larger cost of restrictions in the number of conformational degrees of freedom is paid for by the larger entropic effect due to solvent release.

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