Perspectives
The last decade has witnessed a sort of renaissance in synthetic protein chemistry. The researches at the interface of chemistry and biology have lead to the unprecedented development of several innovative methods for construction of new and novel proteins. The ambit of solid phase peptide synthesis (SPPS) has been expanded to the synthesis of small proteins (Gutte & Merrifield, 1971; Li et al, 1983; Schneider & Kent, 1988). The chemistry of facile ligation, referred to as 'native chemical ligation', has been developed for condensation of large synthetic fragments to obtain moderate size proteins (Dawson et al, 1994). The methods based on the natural 'protein splicing' chemistry for in vitro joining of polypeptide segments have been realized. Further, clever manipulations of the facets of 'native chemical ligation' and 'protein splicing' have yielded a 'expressed protein ligation' strategy wherein a synthetic fragment can be ligated to the carboxy terminus of a recombinant protein (Muir et al, 1998). Contemporaneously, variants of enzyme subtilisin, 'subtiligase', have been engineered for efficient catalysis of peptide ligation in aqueous solution (Chang et al, 1994). The incessant efforts to expand the scope of ribosomal protein-synthesizing machinery for incorporation of nonstandard amino acids in proteins using innovative approaches in expression systems continue (Noren et al, 1989).

The resuscitation of a variety of chemical, enzymatic and genetic approaches for protein construction alluded to above has come about to provide a better handle of the protein assembly process amenable to precise introduction of nonstandard amino acids disposed with stereochemical elements of 'fixed secondary structure' or labeling of specific residues with spectroscopic probes. The use of only twenty proteinogenic amino acids is not sufficient to delineate the molecular codes of proteobiology (Balaram, 1992; Xu et al, 1999). For example, atomic level details are usually constrained by steric factors associated with replacement of one amino acid with another as true isosteric substitutions are not possible within the repertoire of twenty natural amino acids, and lack of useful amino acid side chains (except tryptophan) as spectroscopic reporter puts a limit on studies related to protein dynamics (Chaiken, 1981). Thus generation of altered covalent structure of proteins through incorporation of nonstandard amino acid residues is desirable for their precise structural and functional
characterizations. Although, as mentioned earlier, several elegant synthetic and biosynthetic procedures are currently available for incorporation of nonstandard amino acids in proteins of native or de novo design origin, there are inherent mechanistic, synthetic and practical problems associated with each of these methodologies restricting their utility to specific cases. It seems likely that a combinatorial strategy comprising of the established or novel chemical, enzymatic and genetic methods of peptide ligation would generate profound synergy in protein engineering studies as compared with the use of any given method in isolation.

The protease-catalyzed ligation of protein/peptide fragments is an elegant methodology that can act as a bridge between genetic and chemical approaches for assembly of new and novel proteins (Chaiken, 1981). This strategy is ideally suited for assembly of proteins as it provides facility for ligating a chemically manipulated protein 'module' to its complementary fragment in a regio- and stereo-specific fashion under mild reaction conditions. Unfortunately, protease-catalyzed peptide ligations usually occur under special situations in organic cosolvents and for those polypeptide fragments that are held together by non-covalent interactions as in 'fragment complementing' protein systems such as ribonuclease A (RNAse A) (Homandberg & Laskowski Jr, 1979). In situations where interaction or complexation between the reacting fragments is absent, product-specific 'molecular traps' are employed to drive the equilibrium of a protease-catalyzed reaction towards peptide ligation (Homandberg et al, 1982; Nyberg, 1988). Such traps facilitate the ligation reaction by specifically binding to and removing the product from the chemical equilibrium.

The proteolysis at a given site is determined by the flexibility of the peptide bond (Fontana et al, 1986). The peptide bonds in unstructured regions of the polypeptide chain are generally more susceptible to proteolysis than those that are contained within the rigid structured regions of the protein. It follows that "reverse proteolysis" should be facilitated through the intermediary of helices, sheets, coiled-coils or other higher order protease-resistant conformation of the product. The notion of using the stabilized, proteolysis-resistant structure (conformational trap) of the product that results from two less structured
fragments as a mechanism to drive protease-catalyzed peptide ligation forms the central theme of this thesis. As a prelude to the work described in the thesis, a brief review of the current status of the available synthetic and biosynthetic approaches that are employed in peptide ligation strategies, and their convergent evolution towards the objective of total synthesis of proteins is presented below.

**Review of literature**

**Chemical synthesis and peptide ligation**

The total chemical synthesis is a powerful approach for construction of proteins as every step of the assembly process is under complete control of the protein chemist. The unprecedented flexibility for systematic variation of protein covalent structure is currently unattainable by any other method. The synthesis of an all D-isomer of HIV-1 protease and its derivatives containing nonstandard amino acid are hallmark achievements of this methodology (Milton et al, 1992). The successful assembly and characterization of many other proteins viz., IL-3, insulin like growth factor, IL-8, RNAse A and phospholipase A2 (Clark-Lewis et al, 1986; Li et al, 1983; Clark-Lewis et al, 1991) suggest that solid phase peptide synthesis (SPPS) should be a method of choice for construction of proteins of about 100 residues or less in length. However, a fragment condensation approach using purified fragments is required to overcome the length limitations and other synthetic shortcomings that contribute to the micro-heterogeneity all too often present in the products generated by SPPS (Muir & Kent, 1993).

The need for segment condensation approaches for peptide ligation was realized much before the advent of SPPS. Nonetheless, the early segment condensation reactions were marred by maximal protection resulting in poor solubility, low coupling efficiency, and possibility of racemization because of over-activation of the carboxyl group. Subsequent developments focussed on the use of minimally protected peptide segments and improvements in the activation methods (Blake, 1981; Yamashiro & Li, 1988). Nonetheless, the problem of the poor coupling efficiency between large peptide segments persisted due to intrinsic difficulty in obtaining effective molar concentrations for high molecular weight molecules.
The intrinsic problems of the early fragment condensation strategies provided the motivation for development of facile ligation methodology. An astonishing observation of CNBr derived protein fragments presumably may have been the inspiration for these approaches. Dyckes et al, (1974) showed in pancreatic trypsin inhibitor that, under appropriate conditions, contiguous CNBr fragments would not only effectively refold to give non-covalent complexes, but also spontaneously reform the missing peptide bond. The thermodynamic and kinetic barriers to this occurrence were clearly overcome by the formation of a homoserine lactone (internal ester) generated by CNBr cleavage and the high local concentration of the reacting groups provided by non-covalent complex formation. This conformationally directed resynthesis of peptide bond proved to be an effective semisynthetic approach for introducing desired modifications into one or both the complementary fragments of the protein. Wallace (1993) used this ‘autocatalytic fragment ligation’ (AFR) system to insert replacements at almost 50% of the residues present in segment 1-65 of cytochrome C. However, this approach is unlikely to be general because methionine residues are not always ideally located for generating ‘fragment complementing’ systems. Nonetheless, AFR can be followed subsequent to placement of relevant methionine residues by site-directed mutagenesis (Wallace, 1995).

Alternative condensation methods exploited high and specific reactivity between complementary groups but these resulted in the introduction of hydrazone or thioether or thioester surrogate bonds in the polypeptide chain (Gaertner et al, 1992; Schnolzer & Kent, 1992). Although these methods produce facile coupling of peptide fragments, ether and ester bonds are generally not stable at either acidic or basic pH and, more importantly, they are stereochemically different from the peptide bond, which may lead to conformational distortions.

The ‘native chemical ligation’ technique that involves joining of synthetic fragments through a peptide bond by a precise reaction between two complementary reactive groups is currently the method of choice for fragment condensation. The chemo-enzymatic strategy of Liu & Tam (1994) consisted of three steps. In the first step, the participating α-carboxyl group was esterified
with a masked glycolaldehyde through trypsin-catalyzed reverse proteolysis reaction. In the second step, the amino group of the terminal cysteine, serine or threonine residue of the second peptide reacted with the unmasked aldehyde to form a thiazolidine or oxazolidine ring. The peptide bond formation then occurred through an intramolecular O- to N- acyl migration.

Dawson et al, (1994) introduced a remarkable refinement in the native chemical ligation method. Here high efficiency of ligation could be achieved between two peptide segments, one containing thioester at its α-carboxyl group and the other containing a cysteine residue at the amino terminus. The initial nucleophilic attack of the cysteine side chain to the thioester (thioesterification) is followed by a rapid intramolecular rearrangement (S→N acyl shift) reaction (because of five membered ring formation) leading to peptide bond formation. Native chemical ligation approach has been developed to facilitate the synthesis of moderate size proteins (Baca et al, 1995; Dawson et al, 1997; Hackeng et al, 1997; Muir et al, 1997). Because of its speed and efficiency, native chemical ligation has become the method of choice for the rapid preparation of small cysteine-rich proteins including the ‘open reading frames’ produced by the genome project. However, the method is not infallible and has its own limitations; besides the size limit of the protein, the preparation of thioester derivative of peptides is often a technically demanding process.

Interestingly the native chemical ligation bears remarkable resemblance with the naturally occurring protein splicing reactions (Holford & Muir, 1998). The first step in protein splicing involves an acyl shift (N→S or N→O) in which the N-extein unit is transferred to the side chain, SH or OH, of a cystein, threonine or serine residue located at the amino terminus of the intein. The N-extein unit is then transferred to a second conserved cys/thr/ser residue at the intein-extein boundary in a transesterification step. This is followed by excision of the intein as a succinimide derivative formed by a cyclization reaction involving a conserved asparagine. Finally a peptide bond is formed between the two exteins through an S→N or O→N acyl shift. This step is common to both, native chemical ligation and protein splicing reactions (Gimble, 1998; Holford & Muir, 1998).
The mechanistic similarities between the native chemical ligation and protein splicing have lead to a major breakthrough in segment ligation strategy referred to as ‘expressed protein ligation’ (Muir et al, 1998). Proteins expressed as in-frame amino terminal fusions to an engineered intein can be cleaved by thiol via an intramolecular transthioesterification reaction to generate a recombinant protein containing a α-thioester derivative to which a synthetic peptide (incorporated with desired modifications) with a N-terminal cysteine residue can be tethered through native chemical ligation (Cotton & Muir, 1999; Severinov & Muir, 1998).

The application of ‘expressed protein ligation’ to the structure and functional relationship of protein has been demonstrated in the construction of a novel Csk tyrosine kinase. The Csk tyrosine kinase catalyzes the tyrosine phosphorylation of Src family kinases at a specific position on the carboxy-terminal tail. The phosphorylation event triggers the intramolecular association of phosphotyrosine and SH2 domain resulting in repression of in vivo Src kinase activity. Although Csk is related to Src family, it itself lacks this tail and therefore its activity is not subject to regulation by phosphorylation. In order to assess, if the introduction of the phosphorylated carboxy terminal tail into Csk regulates its activity through structural changes as seen in other Src kinases, a phosphotyrosine tail was ligated to Csk by ‘expressed protein ligation’. Structural analysis of the semisynthetic tail-phosphorylated Csk construct showed that phosphorylation of Csk, like that of other Src proteins, induced a conformational change, suggesting the presence of an intramolecular interaction in phosphotyrosine-SH2 domain within the protein. Interestingly, the engineered protein is approximately five fold more active in the phosphorylated state, unlike Src, which is repressed by carboxy-terminal phosphorylation. (Muir et al, 1998). The expressed protein ligation has been proven to be invaluable in both synthesizing native polypeptides as well as modified proteins with desired properties useful for unambiguous elucidation of structure-function inter-relationships that are generally not achievable by other existing methodologies.
Although powerful, each of these currently available techniques for introduction of unnatural molecules into proteins, are associated with certain synthetic or practical limitations that have limited their widespread application. Notwithstanding the synthetic achievements, the lack of generality of each of these approaches to introduce modification at any position in any given protein, suggests that a combination of methods is likely to provide the greater flexibility and robustness in protein design and engineering.

**Enzymatic peptide ligation**

The use of enzymes for peptide ligation is an attractive alternative because enzymatic reactions are catalytic, regio- and stereo-selective, and racemization-free. Furthermore, the enzymatic reactions usually proceed with unprotected peptide fragments under mild conditions yielding homogeneous product. The reaction captures the maximum benefit of molecular recognition between substrate and enzyme. However, a close to universal peptide ligase is needed, which in principle would display high catalytic efficiency for all theoretically possible combinations of amino acids. Since the ribosomal peptidyltransferase is not suitable for practical use, and the multi-enzyme complexes involved in bacterial peptide synthesis do not seem to support general applications, only the proteases come into consideration at present. Although, designed catalytic antibodies and ribozymes have been conferred with peptide ligation propensity, their current status do not allow synthesis of larger fragments (Hirschmann et al, 1994; Zhang & Cech, 1997).

The use of proteases for peptide bond synthesis is based on the principle of microscopic reversibility predicted by van't Hoff more than a century ago (Van't Hoff, 1898). Protease-mediated synthesis of peptides can be accomplished by two distinct approaches that are mutually exclusive. The first one, involving the equilibrium controlled (thermodynamic) approach, is purely dependent upon the intrinsic property of both reactants and product(s) involved, and the experimental conditions that can be manipulated to shift the equilibrium in favor of peptide bond synthesis. The formation of acyl-enzyme complex and the competitive deacylation by water are two major rate-limiting steps in equilibrium controlled protease-catalyzed peptide bond synthesis that make this a slower
process. In contrast, in the second approach, namely the kinetically controlled peptide bond synthesis, the reaction is accelerated owing to the use of an activated C-terminal ester that rapidly acylate a serine or cysteine protease. This acyl-enzyme intermediate then undergoes acylation by an added nucleophile to yield a transient accumulation of the desired product.

**Kinetically controlled peptide synthesis**

Kinetically controlled syntheses are performed with both native enzymes as well as engineered enzymes, but only with those proteases that can form covalent acyl-enzyme intermediates; serine and cysteine proteases could be used. This method has been used extensively to synthesize di or tri peptides and also to synthesize many small bio-active peptides by segment condensation strategy (Schellenberger & Jakubke, 1991; Bongers et al, 1994; Mihara et al, 1993; Kullman, 1981). The use of free amino acids as nucleophiles has been demonstrated in chymotrypsin-catalyzed acyl-transfer reactions that occur in frozen solutions (Tougu et al, 1993).

The use of substrate mimetics that can function as artificial recognition sites for proteases has been explored for peptide ligations. Trypsin-catalyzed segment condensations have been studied using 4-guanidinophenyl esters of acylated peptide segments (Bordusa et al, 1997). This cationic leaving group mimics trypsin primary specificity and binds to the active site of trypsin in a way that Lys and Arg side chains bind. As a result, peptide coupling occurs irreversibly and independent of specificity. An anionic leaving group which satisfies the primary specificity of V8 protease also has been designed and reported to be useful in peptide ligations (Wehofsky & Bordusa, 1999).

Though it has been observed that serine proteases are most useful in reverse proteolysis under synthesis favoring conditions, because of their higher esterase activity, the hydrolysis of the acyl-enzyme intermediate is strongly favored over aminolysis. Modification of active site chemical environment, which would favor aminolysis over hydrolysis, has been useful in acyl transfer reactions to amine nucleophiles. Both, chymotrypsin as well as subtilisin, whose active site histidine residue were methylated, showed higher aminolysis /
hydrolysis ratio as compared with the respective native enzymes. Nakatsuka et al. (1987) showed that thiosubtilisin, a derivative of subtilisin in which the active site Ser221 was chemically converted to a Cys (S221→C), exhibited a thousand fold rate-enhancement in aminolysis relative to hydrolysis. The possible explanation for the higher aminolysis is the inherently greater kinetic liability of thioesters towards amines as opposed to water. Wu and Hilvert (1989) showed that selenosubtilisin had a 14,000 fold shift in preference for aminolysis over hydrolysis. However, it was found that catalytic efficiency by either thiol or selenosubtilisin for aminolysis of chemically activated esters were about $10^2$-$10^4$ fold below the esterase activity of wild-type subtilisin.

Further improvement in ligation activity of subtilisin derivative (Ser221→C) was achieved by engineering an additional mutation at Pro225. The double mutant (S221→C, P225→A) exhibited 10-fold higher peptide ligase activity and at least 100-fold lower amidase activity compared with the single mutant subtilisin (Ser221→C). The P225→Ala mutation enhanced the ligation activity as it alleviated the steric crowding at the active site resulting from S221→Cys substitution (Abrahmsen et al., 1991).

The use of this modified enzyme, subtiligase, to synthesize peptide bonds in a segment condensation strategy was demonstrated in a beautiful piece of work related to the total synthesis of ribonuclease A from six peptide fragments based on the substrate specificity of subtiligase (Jackson et al., 1994). The protein synthesis starts from the carboxy terminus of the protein and proceeds towards the amino terminus through five ligation steps. The ligation strategy involved coupling of an esterified (glycolate-phenylalanyl amide ester; glc-F-NH$_2$) and α-amino (isonicotinyl protection; iNOC) protected donor peptide to a fully deprotected acceptor peptide. The ester at the C-terminus efficiently acylates subtiligase to form a thiol-acyl enzyme intermediate (enzyme exhibits avid preference for esters containing the glc-F-NH$_2$ leaving group) which is attacked by amino group of the acceptor peptide to release the enzyme. The iNOC protection, which was introduced to prevent self-ligation of the donor peptide, was removed to generate the product as the acceptor peptide for the next ligation.
step. The subtiligase-mediated procedure for total synthesis of RNase A was utilized to introduce unnatural catalytic residues in the protein for unequivocal assignment of the roles of active site histidines, His12 and His119 (Jackson et al, 1994).

Equilibrium-controlled protease-catalyzed peptide bond formation

In comparison to equilibrium-controlled peptide synthesis, kinetically controlled synthesis proceeds relatively at a faster rate with lower enzyme concentration but the mechanistic imperatives limit the choice of enzymes only to serine and cysteine proteases. This kinetic approach also demands esterified substrates that limit the length of reactants. The necessity of a different type of activated esters for each protease, as the substrate specificity of the protease used must be satisfied, and the dependability of acyl transfers on the type of nucleophile limit the use of this approach.

The thermodynamic approach, although sluggish, is not limited by technical problems faced in kinetically driven synthesis. The use of unactivated peptides do not put a restriction on the size of reactants. The experimental conditions, that can shift the equilibrium towards peptide bond synthesis, are the major determinants of the success of a thermodynamically controlled protease-catalyzed splicing of peptide bond.

The first use of proteolytic enzymes to synthesize peptide bond was reported in the 1930s by Bergmann & Fraenkel-Conrat. The next twenty years witnessed considerable interest in this area with the notion that proteases may have a role in protein biosynthesis (Bergmann & Fraenkel-Conrat, 1938; Borsook, 1953). However, when it became apparent that proteins were synthesized in vivo on ribosomes, the interest in studying how to shift the peptide bond equilibria to peptide bond synthesis largely diminished. The work in this area was stimulated when porcine insulin was converted to human insulin by exchanging the C-terminal Ala$^{830}$ for Thr via a two-step enzymatic process (Morihara et al, 1979).

The proteolytic condensation of two native protein segments under physiological conditions is thermodynamically unfavorable. The enthalphic and
entropic barriers to peptide bond synthesis originate from ionization of both α-carboxyl and amino termini, and the lack of stereochemical proximity of these reacting groups. The enthalpic barrier to peptide bond synthesis can be lowered by the addition of an organic cosolvent which enhances the pKa of the carboxyl group while proximity of both reacting termini overcomes the large entropic barrier. The entropic barrier, a major hurdle in condensation of two fragments, can be compensated for when both the reactants are non-covalently interacting with each other. Other tactics as well can be applied to manipulate the protease-catalyzed peptide bond equilibria to favor synthesis over hydrolysis.

In accordance with the law of mass action, the product yields can be improved by using high initial concentration of one of the reactant peptide. However, this itself is not sufficient as the synthesized peptide bond remains susceptible to proteolysis. This can be overcome if the product is precipitated out. The equilibrium is shifted towards synthesis as a result of decreased concentration of the product. Morihara & Oka (1982) studied chymotrypsin-catalyzed synthesis of a series of dipeptides and found a direct correlation between product solubility and initial concentration of the starting material i.e., lower concentration of the starting peptide was needed as the product became less soluble. The thermolysin-catalyzed synthesis of aspartame is a classic example of the utility of ‘precipitation’ as a method for driving protease-mediated ligations; the aspartame precursor is obtained in nearly quantitative yields as it precipitates almost totally as an insoluble salt (Oyama & Kihara, 1984).

Martinek & Semenov (1981) used a biphasic system consisting of water and a water immiscible organic solvent, such as ethyl acetate or chloroform for chymotrypsin-catalyzed synthesis of dipeptides. Here the enzyme is in the aqueous phase while the reactants are dissolved in the organic phase. The reactants from the organic phase diffuse into the aqueous phase and undergo enzyme-catalyzed peptide synthesis: the synthetic product will diffuse back into the organic phase. This system is suitable for the formation of water insoluble products. However, in general, both precipitation and biphasic systems cannot be used for the synthesis of water-soluble products, such as short peptides.
containing basic amino acid residues or in the semisynthesis of higher molecular weight peptides and proteins.

Ingalls et al. (1975) reported an increased synthesis of esters by chymotrypsin and elastase respectively in 80% alcohol. Butler & Reithel (1977) obtained significant urease-catalyzed synthesis of urea in the presence of large concentrations of organic cosolvents. Although, the potential of organic cosolvents in shifting the equilibria to peptide bond synthesis was realized, the unequivocal role of organic cosolvents in peptide ligation reactions were established by Homandberg et al (1978) who suggested that the main cause for this effect was an increase in the pKa of the carboxyl group of the reactants or more appropriately, the decrease in the equilibrium constant for the transfer of a proton from the \( \alpha\)-COOH terminus to the \( \alpha\)-NH\(_2\) terminus of the reactants. Thus the enthalpic barrier to peptide bond synthesis, in general, may be overcome by incorporating organic solvents in the reaction milieu. The applicability of this approach was amply demonstrated in the synthesis of small peptide segments by proteases (Barbas, 1988; Chen & Wang, 1988).

Laskowski and coworkers were the first to apply the principle of shifting equilibria from proteolysis to synthesis by the use of organic cosolvents to the semisynthesis of proteins (Homandberg et al, 1978). They found that protease-catalyzed splicing of peptide bond in organic cosolvent was facilitated in protein/peptide systems which were held together by –S–S– bond or non-covalent interactions as both the enthalpic as well as entropic barrier to peptide bond synthesis would be overcome under this condition. The enzymatic resynthesis of proteolytically nicked proteinase inhibitors and RNAse A are representative examples of such splicing reactions (Mattis & Laskowski Jr, 1973; Jering & Tschesche, 1976; Homandberg & Laskowski Jr, 1979).

The classical conversion of non-covalent ‘fragment complementing’ S-peptide-S-protein complex of RNAse S to covalent RNAse A (native protein) by subtilisin in the presence of 90% glycerol solution was a hallmark in semisynthesis of proteins (Homandberg & Laskowski Jr, 1979). The successful restitching of ribonuclease S led to several further experiments showing that, sequence contiguous non-covalent complexes whose conformation was similar to
the native covalent form, could be genuine candidates for semisynthesis by enzymatic restitching in organic cosolvents. Indeed 'fragment complementing' systems of staphylococcal nuclease, and somotropin (Komoriya et al, 1980; Grafi & Li, 1981) have been successfully converted into their respective covalent forms. The most recent remarkable example has been the regeneration of native triosephosphate isomerase from four proteolytic fragments generated by subtilisin; subtilitic cleavage of triose phosphate isomerase into four fragments is reversed in 60% acetonitrile within 10 min yielding native triosephosphate isomerase. The concept of non-covalent reconstitution of two or more fragments to achieve the 'entropic activation' and high effective molarity of both the reacting termini has been at the core of the ligation of 'fragment complementing' protein systems. Although, conceptually simple, this approach is stymied by the availability of a non-covalent complex for any given protein.

Komoriya, et al, (1980) reported a striking observation that enzymatic resynthesis can be carried out efficiently between fragments even when these were not bound together non-covalently. They found that the N-terminal RNase A peptide (1-15) can be produced from the non-interacting (1-10) and (11-15) segments when the latter were incubated with clostripain in aqueous buffer in the presence of RNase S (21-124). The S-protein (21-124) fragment acted as a 'kinetic trap' for resynthesized peptide (1-15), by binding to it to produce a RNase S complex. The product, peptide (1-15) accumulated as the 10-11 peptide bond was rendered resistant to proteolysis in the complex. In systems where such product-specific binding proteins are unavailable, the use of product specific antibodies are suggested (Chaiken, 1981). In fact, enzymatic synthesis of substance P was achieved using product-directed antibodies as 'molecular traps' (Nyberg, 1988).

The introduction of a new element, which binds only the product, but not the reactants, and thereby offers protection to the nascent ligated product from to proteolysis, can be considered potentially a 'molecular trap'. An interesting variation of the product-directed 'molecular trap' concept is the 'conformational trap' proposed to explain the facile ligation of non-interacting complementary peptide segments of α-globin. It was observed that unlike the other protease-
catalyzed semisynthetic reactions, glycerol was not a suitable solvent for the α-globin semisynthetic reaction. Instead, the helix-inducing solvents, namely, 1-propanol, trifluoroethanol and 2-propanol were efficient organic cosolvents for this semisynthetic reaction (Acharya et al, 1985; Sahni et al, 1989). It was concluded that helix formation in the enzymatically ligated peptide (product) acted as a driving force for the protease-catalyzed ligation reaction (Roy et al, 1992).

The present investigation

The 'conformational trap' concept can be reconciled with the fact that proteolysis event is dictated by the stereochemistry and accessibility of the protein substrate (besides the activity and specificity of the protease) (Fontana, 1986; Wang & Kallenbach, 1998). Thus protease-catalyzed ligations would be facilitated in situations where the product can assume defined structures (helices, sheets, coiled-coils, helix bundles etc.) in which the stabilizing interactions can reduce conformational flexibility of the slicing/splicing site. The ability of proteases to produce selective and restrictive cleavage of proteins in the presence of organic cosolvents such as 1-propanol and TFE could be viewed as a manifestation of the enhanced conformational stability or decreased segmental mobility of the proteins in the presence of organic cosolvents (De laureto et al, 1995; Fontana et al, 1995; Fontana, 1986).

The feasibility of ‘conformational trap’ as a mechanism to drive protease-catalyzed splicing of totally unprotected and non-interacting peptide segments has been tested in the present thesis. The fragments spanning helical regions of proteins have been chosen as test cases with the assumption that intrinsic helix forming propensity of these sequences would be enhanced in the presence of favorable organic solvents used in the protease-catalyzed ligation reaction and act as a conformational trap. Staphylococcus aureus V8 protease (endoproteinase Glu-C) has been chosen for the study as it has been used extensively for both, hydrolysis as well as synthesis of peptide bond (Seetharam & Acharya, 1986; DeFilippis & Fontanna, 1990; Schuster et al, 1991; Roy et al, 1992).
The thesis is divided into three chapters. The first chapter presents studies of the helix-enhancement process through a comparative analysis of effects of fluoro- and alkyl alcohols on protein segments derived from RNAse A and thermolysin. The results of V8 protease-catalyzed ligation of peptide fragments in the presence of helix-promoting organic cosolvents is described in the second chapter. The follow-up studies of an accidental observation of V8 protease-catalyzed peptide ligation in neat aqueous solution is presented in the third chapter.