SEMISYNTHETIC PEPTIDES AND PROTEINS

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I. INTRODUCTION

The chemical synthesis of large, biologically active polypeptides has proven a bittersweet experience for peptide and protein chemists. Willful synthesis of chains of amino acids is a tempting one, representing a potential playland in which we not only reproduce natural peptides but also, and perhaps more intriguingly, make analogue sequences with altered properties. Sequence modification through synthesis therein provides a flexible route toward delineating the code of rules whereby primary structure directs higher order properties of folded conformation and biological function. Ultimately, such information could help to evolve a broad-based understanding of how proteins work mechanistically and thus could lead to rational design of therapeutically or industrially useful polypeptides.

In the face of such promise, total synthesis of large polypeptides and proteins remains elusive. Successful methods certainly have evolved for small and even modestly sized peptides. Yet, problems of purity, yield, and sheer cost in time and materials make efforts for larger species much more chancy and impractical as a general rule. While syntheses have been reported for a few cases, such as ribonuclease,[1-3] these important achievements have not been routinely applied, especially for analogue synthesis.

Given the conflict between what is desirable and what is doable by total synthesis, semisynthesis has evolved as an alternative for making peptides and proteins. By this approach, small or modestly sized synthetic peptide fragments are prepared and then combined with fragments derived from the native polypeptide to create an intact species. The more routine synthesis of modest peptides thus is utilized, while the synthetic product allows one to make and study large polypeptide entities.

Formally, semisynthesis can be defined simply as the rebuilding of polypeptides from components at least one partner of which is synthetic. This is represented schematically in Figure 1. The essential operations here are (1) limited proteolysis of naturally occurring polypeptides to yield a workable set of fragments; (2) the chemical synthesis of at least one of these fragments; and (3) reconstitution of synthetic and native partners. Improvements in all three methodological areas have been steady, allowing the advance of semisynthesis into a flexible and, in many cases, preferred way to build large polypeptides. Semisynthesis thus has opened the way for exploration of polypeptides by manipulation of their chemical structures. The fruits of this endeavor, an understanding of how these macromolecules work and, therein, a basis for ultimate design of new structures, have begun to emerge.

In this section an effort is made to cite and discuss recent developments and achievements that have helped define and advance the use of semisynthesis. Review is

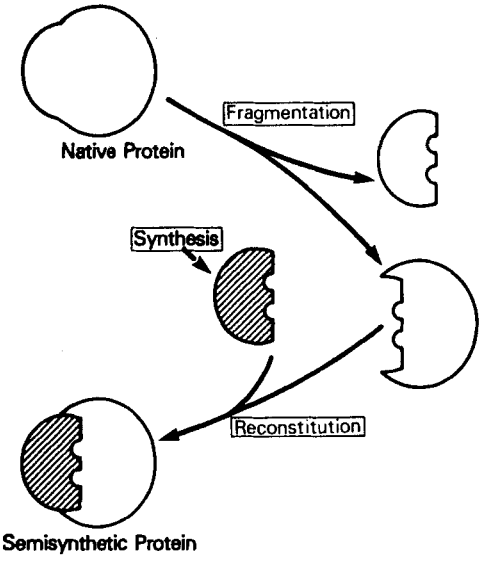

FIGURE 1. Scheme of the major experimental manipulations of semisynthesis. (With permission from Chaiken, I. M., *Semisynthetic Peptides and Proteins*, Offord, R. E. and DiBello, C., Eds., 1978, 349. Copyright by Academic Press Inc. (London) Ltd.)

made for methodological advances, synthetic successes, and application to the elucidation of properties of native polypeptides and proteins. Discussion also is offered of ways in which semisynthesis can profitably interface with related experimental efforts for producing polypeptides, such as biosynthesis through recombinant DNA methods.

Several important reports to which the interested reader should be directed have been published over the past few years on the subject of peptide and protein semisynthesis. A brief evaluation of the field appeared in 1975,[5] and an international conference held in Bressanone, Italy in 1977 provided the first published collection of research reports devoted exclusively to experimental progress in semisynthesis.[6] More recently, both a review[7] and a textbook[8] have provided excellent presentations of the developing chemical methods of semisynthesis. And the published proceedings of the sixth American Peptide Symposium[9] contain two general discussions and a useful set of communications of quite recent studies in the field.

II. METHODOLOGY AND APPLICATION

A. Polypeptide Fragmentation

A *sine qua non* of semisynthesis is production of polypeptide subfragments that are suitable for subsequent reconstitution to an intact, active polypeptide or protein species. (Figure 2 can be used to place this and other procedures in the context of overall semisynthesis manipulations.) Both enzymatic and chemical cleavages,[10-14] methodologies in many cases developed for protein sequencing, have been used profitably. Trypsin and, to a lesser extent, chymotrypsin have provided obvious primary tools for limited enzymatic endoproteolysis. Given the potential for stable and reversible protection of lysyl residues, as by methylsulfonylethoxycarbonyl,[15] trifluoroacetyl,[16] or acetimidyl[17] groups, trypsin has enjoyed a dominant role so far in limited proteolysis for purposes of

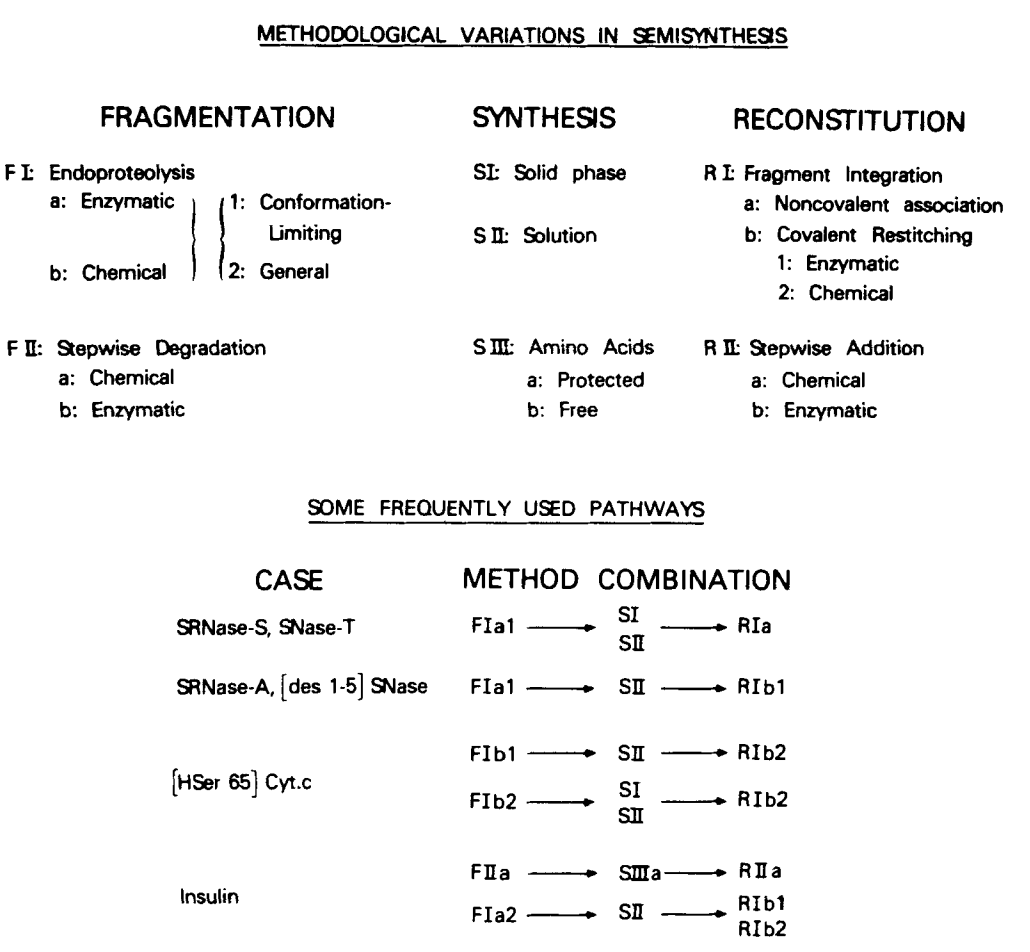

FIGURE 2. Chart of the types of methods that have been adopted for making semisynthetic polypeptides. Combinations of methods used in a few typical cases are given in the lower part of the figure. SRNase S and SRNase A denote semisynthetic ribonuclease-S and -A, respectively; SNase T and SNase, semisynthetic nuclease-T and -(6-149), respectively; and [HSer 65] cytochrome c, [homoserine 65] cytochrome c.

producing peptide candidates for synthesis. Clostripain and other arginine-favoring proteases[13,18] could become important alternatives to trypsin when too many trypsin-sensitive cleavage sites present themselves and strategic arginyl residues occur.

Whatever the protease used, a centrally useful adjunct for enzymatic fragmentation has been the restriction of peptide bond hydrolysis by the constraint of a compact, folded conformation which offers only one or a few of the potentially scissile bonds (based on amino acid sequence) for actual cleavage. This constraint has proven to be a frequent theme for many, if not most, of the large polypeptides and proteins which have been subjected to semisynthesis so far. Conformation-induced proteolytic resiliency can be observed, for example, in the trypsin cleavage of staphylococcal nuclease,[19] wherein only a few bonds — those carboxyl to residues Lys 5, Lys 48, and Lys 49 — are attacked when the protein is treated while in its native folded state (as stabilized by calcium ligands and deoxythymidine-3′, 5′-diphosphate). The nuclease case is striking given the fact that the protein conformation is stabilized only by noncovalent interactions, without disulfide cross-links. In this case, it typifies the potential for utilizing proteolytic resistance, a view promoted originally by the classical observation of conversion of bovine pancreatic

ribonuclease-A to ribonuclease-S by the normally fairly general protease, subtilisin.[20] Against the native, four disulfide-containing ribonuclease, the latter protease acts predominently on peptide bonds in the loop around residues 20 and 21. Besides the benefit of allowing isolation of restricted large fragments from proteolysis at a relatively few sites, the effects of conformation become strikingly useful for the reconstitution of synthetic and native fragments, since such limited proteolytic clips often occur in conformationally loose areas in the first place, and therefore produce only minimal conformational destabilization (see section below on reconstitution).

While chemical cleavage enjoys less benefit from conformational constraint, due to the chaotropic nature of the conditions often required, several such cleavage agents have been helpful due to their actions at sites that exist relatively infrequently in most natural polypeptides. Cyanogen bromide cleavage at methionyl residues[21] has allowed high yield fragmentation of cytochrome c[22-25] and pancreatic trypsin inhibitor,[26] to name only two examples of systems applicable or potentially applicable for semisynthesis. While this has remained the most widely used chemical reagent for semisynthesis effort so far, cleavages at tryptophanyl residues by 2-(2-nitrophenylsulfenyl)-3-methyl-3′-bromoindolenine (BNPS-skatole)[27] and iodosobenzoic acid[28,29] and at cysteinyl residues by 2-nitro-5-thiocyanobenzoate[30,31] have significant potential.

While it is generally true that fragment systems suitable for semisynthetic operations usually have been derived straightforwardly from limited degradation of native polypeptides, more-indirect procedures also could prove helpful. For example, Lin et al.[32] devised a semisynthetic ribonuclease carboxyl-terminal fragment complex based on combination of pepsin-produced inactive RNase-(1-120), or truncated variants, and the synthetically produced sequence (111-124). Their design succeeded because, while RNase-(1-120) could not be productively complemented by the pepsin-released C-terminal tetrapeptide (121-124),[33] sufficient stabilizing, noncovalent interactions could be produced by the extended carboxyl-terminal synthetic peptide to effect a suitable replacement for the disrupted sequence around the essential residue His 119. A similar fragment complex has been described[34] for RNase-(1-118) (obtained by pepsin and carboxypeptidases[32] and RNase-(105-124) (obtained from trypsin digestion of performic acid oxidized ribonuclease-A). Furthermore, combination of selective cleavage modes of the carboxyl-terminal ribonuclease complex with those of the ribonuclease-S system has formed the basis for a three-fragment noncovalent complex, consisting of RNase-S-(1-20), RNase-(21-118), and synthetic-(111-124).[32] More recently, Juillerat et al.[35] have devised another three-fragment complementing system, from cytochrome c, by combining fragments from different cleavage procedures. The functional system was arrived at by mixing the horse heart cytochrome c fragments (1-65, heme), (39-104), and (1-65, apo [heme depleted]) and then subjecting the resulting active complex to trypsin hydrolysis in order to remove redundant sequences. The final three-fragment system, which retained cytochrome activity, contained fragments (1-25, heme), (28-38), and (56-104). Thus, active complexes can be obtained by deriving active fragment mixtures with larger initial fragments in order to provide all necessary sequence information and then stripping away the unnecessary redundant stretches of polypeptide by proteolysis. The implications of such indirect approaches for defining systems for semisynthesis could well be instructive for future designs with other proteins.

B. Fragment Synthesis and Purification

Both solution and solid phase synthetic methods have been used in making fragments for semisynthetic peptides and proteins.[4-9] Each technique has well-discussed strong points and equally well-discussed weaknesses that make it more or less appealing to a particular investigator. Solution synthesis has carried with it the greater hope of

obtaining a chemically clean product, but at the expense of requiring considerable technical and empirical wisdom, especially in achieving peptide bond formation between increasingly large and insoluble protected fragments. The extensive use of small-fragment condensations in the recent successful synthesis of ribonuclease-A[3] may signal an important turning point for solution synthesis of large polypeptides. In the meanwhile, the solid phase approach, due to its intrinsic repetitive simplicity, has seemed a reasonable alternative for making significantly sized peptides. Wariness as to the lack of purity of final crude peptide products is essential, though, and methods must be developed which can allow separation of the product of desired sequence from closely related "deletion" variants or other contaminants.

Recent advances in solid phase synthesis methodology, especially in new solid phase matrix design, bode well for the optimistic use of this approach for preparing modestly sized peptides used in semisynthesis. Merrifield et al.[36] have described several resins which have performed well in trials so far. A striking example of the value of one of these, the phenylacetamidomethyl (or Pam) resin,[36,37] is the synthesis recently achieved[38] of the relatively large fragment of staphylococcal nuclease corresponding to the amino terminal third of the protein. Synthetic-(6-47) prepared using the earlier described chloromethylated polystyrene had been obtained previously in the crude form, after cleavage from resin and complete deprotection, with about 2 to 4% potential enzymatic activity (the activity exhibited upon addition to the complementary native fragment nuclease-T-(49,50-149)).[39,40] On the other hand, a preparation of synthetic-(6-49) and a Gly 48 analogue made using the Pam resin has yielded crude products of 35 to 50% potential activity.[38] The increased functional purity appears due mainly to the use of the new resin and presages the increased ease with which the correct product will be separable, in this case from the much less awesome set of contaminants. Other solid phase supports, described by Atherton and Sheppard[41] and Sparrow,[42] and insoluble active esters of amino acids such as that described by Fridkin and Patchornik,[43] also could be helpful in improving the performance of solid phase-based syntheses. However, as with many of the new techniques being introduced in chemical synthesis, sufficiently informative comparisons have not been made between the various technical alternatives to define any single best set of procedures.

The use of solid phase synthesis also has benefited from advances in chromatographic and electrophoretic peptide purification methods. Most noteworthy is high-performance liquid chromatography (HPLC). Even with the use of new resins, crude solid phase product (the total peptide grown on the insoluble support and then released in deblocked or partially deblocked form) is likely to contain several types of unwanted peptide contaminants with amino acid sequences closely related to one another and to the correct sequence. While functional purification of synthetic peptides has been a powerful device for fragments from such mixtures when these have specific binding properties,[44] this procedure does not guarantee chemical purity. Further, not all synthetic peptides can be handled in this way. Within the last few years, the use of reverse phase high-performance liquid chromatography has evolved for the separation of peptides.[45,46] This procedure is applicable for the high-resolution separations needed for solid phase synthesized products. As with other types of chromatography that have been of general benefit in peptide and protein chemistry, reverse phase HPLC effects separation via several different interactive characteristics of the polypeptides, including the presence and sequence-specific nature of hydrophobic centers as well as the possibility of ion pairing.

Successes in peptide mapping and purification have indicated that HPLC will be applicable for large peptides, such as insulin, and thus should be useful for large, solid phase-derived protein fragments prepared for semisynthesis. The HPLC approach could provide important benefits for separation of solution-derived synthetic products as well.

It is likely that preparation of fragments of native polypeptides also can be facilitated by this technique.

As is often asserted for synthesis of individual peptides, routes for making semisynthetic polypeptides, whether they are solid phase- or solution-based, must be developed according to the individual needs of the particular polypeptide system in question. Coupling and deprotection steps need to be tailored to accommodate such problems as purification and coupling with synthetic intermediates for solution procedures and constantly varying availability of terminal α-amino group (and related peptide solvation) in the solid phase route. However, even given the ability to make a particular sequence, flexibility also must be maintained in choosing a synthetic sequence that can be incorporated as part of a semisynthetic polypeptide or protein. Reconstitution of synthetic and native fragments usually can be achieved only in certain combinations. Synthetic expectations must be geared to these limitations.

C. Reconstitution to Intact Forms

The capacity to combine synthetic and native fragments to produce intact, functional polypeptides and proteins is at the very core of semisynthesis effort. Depending on the peptide system, this process has been achieved both covalently and noncovalently. The simplest option, when it presents itself, is through binding of fragments using the driving force of noncovalent interactions to hold the fragments together in a productive, conformationally intact form. Though intuitively simple-minded, noncovalent reconstitution has been a powerful option due to the discovery of several polypeptides and proteins in which limited peptide bond cleavage, usually enzymatic, can be effected without loss of biological function and further in which the produced fragment partners, after isolation, can regain a conformationally productive set of noncovalent interactions when mixed together, usually in aqueous solution. Two-fragment functionally active complexes have been produced successfully from bovine pancreatic ribonuclease A,[20,32,34] staphylococcal nuclease,[19,47] cytochrome c,[48,49] somatotropin,[50] and *bacillus amyloliquefaciens* ribonuclease,[51] to name a few examples. A limited number of stable three-fragment noncovalent systems also exist, prominantly for cytochrome c[35] and ribonuclease.[32]

While quite useful for the semisynthetic manipulation of polypeptide sequence by making analogues, noncovalent fragment complexes are clearly not going to be a universal occurrence. For a given protein, it is more likely that semisynthesis efforts must proceed by covalent reconstitution. And herein lies one of the major technical pitfalls in all semisynthesis work. For an otherwise undirected noncovalent attachment to occur, selective peptide bond formation usually can be achieved only if side chain and main chain reactive moieties to be preserved from peptide bond formation or side reactions are protected reversibly, leaving the appropriate α-carboxyl and α-amino groups free to react. Synthesis of suitably protected, modestly sized peptide fragments is not necessarily a problem, as discussed above. And, preparation of such fragments from native proteins can be accomplished, even when this requires significant chemical modification followed by high enough yield proteolysis. More critically, chemical coupling of large protected peptide fragments can be slow, if at all, and thus of low yield. Indeed, it is the very problem of coupling large protected peptides that has been a serious block in the total synthesis of large polypeptides. And it is this problem which has proven rate-limiting for many covalent semisynthesis objectives.[8]

In spite of the above, there have been several successes in the covalent reconstitution of large polypeptide and protein fragments. Functionally active cytochrome c has been produced successfully from three fragments, one synthetic and two from the native protein.[23,25] Notable covalent fragment stitching also has been achieved for myoglobin,[52,53] phospholipase A_2,[54] and insulin.[55,56]

A major improvement in prospects of achieving covalent semisynthesis has been made through the use of conformationally directed resynthesis. This was observed with cytochrome c, for which a covalent species could be reformed from the nonoverlapping cyanogen bromide fragments, [HSer 65] cytochrome-c-(1-65, heme) and cytochrome-c-(66-104), through reaction of the activated homoserine lactone in the amino terminal component, to yield [HSer 65]cytochrome-c.[57] Here, covalent restitching is directed by the steric proximity of homoserine lactone 65 to the α-amino group of cytochrome-c-(66-104), with this proximity produced by the initial folding of the two fragments into a conformationally cytochrome c-like noncovalent complex. A similar covalent reattachment has been described for pancreatic trypsin inhibitor, where two cyanogen bromide fragments, [HSer 52]PTI-(1-52) and PTI-(53-58), are held together by disulfide bonds.[26] While such conformation-driven covalent reattachment through an activated homoserine lactone is unlikely to be general, it does circumvent the problems of side chain protection and deprotection in covalent semisynthesis and thus is of striking usefulness for the limited cases where it occurs.

A significantly more general utilization of conformation-directed covalent restitching has been developed with proteases. Homandberg and Laskowski[58] have found that the noncovalent complex ribonuclease-S, consisting of ribonuclease-S-(1-20) and -(21-124), can be reconverted to the intact 124-residue ribonuclease-A by subtilisin catalysis in mixtures of aqueous buffer and organic cosolvents such as glycerol. Thus, the enzyme which acts in the first place to effect a limited proteolysis in fully aqueous solution to produce ribonuclease-S from ribonuclease-A can act to effect a limited peptide bond synthesis in organic cosolvent mixtures to remake ribonuclease-A. The restitching proceeds sufficiently due in large part to the enhancement derived from juxtaposing residues 20 and 21 by initial folding into the ribonuclease-S complex and the perturbation by the cosolvent of the free energy of peptide bond hydrolysis.

The above work, which evolved from an incisive series of studies on protease-catalyzed peptide bond synthesis in protease inhibitors,[59-61] has signaled the beginning of generalized use of enzymes in covalent semisynthesis. The approach has had a quite practical result in the semisynthesis of human insulin, by trypsin-catalysed restitching of desoctapeptide(B23-B30) insulin, obtained from porcine hormone, with an excess of synthetic octapeptide (B23-B30) made with the human sequence.[62] This manipulation, enhanced by mass action in addition to the thermodynamic effects of cosolvent, has allowed substitution of the Ala at position B30 in porcine insulin with the Thr specific for human hormone.

The successful use of enzymatic restitching for ribonuclease-S has led to several further experiments showing that, as a class, nonoverlapping, but sequence-contiguous noncovalent complexes produced by limited proteolysis have a good chance of being viable candidates for covalent semisynthesis by enzymatic restitching. The trypsin-produced staphylococcal nuclease-T can be reconverted to a covalent form using trypsin in 90% glycerol;[38,63] and promising results also have been reported for the clostripain-catalyzed restitching of cytochrome c fragments.[64] The nuclease-T case has revealed both the generality as well as some possible pitfalls in the use of enzymatic resynthesis. Here, attempts to reform an intact nuclease-like species from the native, nonoverlapping fragments nuclease-T-(6-48) and -(49-149) led to an inactive covalent form, [des Lys 49]nuclease-(6-149) due to the presence of two adjacent trypsin-susceptible bonds in the . . . Lys 48 — Lys 49 — Gly 50 . . . region.[63] The sequence-caused problem was overcome by using another set of nonoverlapping peptides, [Gly 48]synthetic-(6-49) and nuclease-T-(50-149), which provides only one trypsin-sensitive bond in the critical region, namely Lys 49 — Gly 50.[38]

A recent striking observation is that enzymatic resynthesis can be carried out efficiently between fragments even when these are not bound together noncovalently.

This so far most generalized use of enzymes involves the participation of kinetic traps. It has been found[65] that the ribonuclease-related peptide (1-15) can be produced from the noninteracting (1-10) and (11-15) pieces when these latter are incubated with clostripain in aqueous buffer in the presence of RNase-S-(21-124). Here, the (21-124) fragment acts as a kinetic trap for resynthesized (1-15), by binding to this latter sequence as it is produced to make a ribonuclease-S complex. The latter is relatively resistant to proteolysis under the conditions used, thus acting to protect the desired peptide from redegradation. While this type of resynthesis requires introduction of a new element, the kinetic trap, this latter in fact exists, or can be engineered, for many polypeptide systems. Any substance which binds the product of resynthesis, but not the reactants, can be considered a potentially usable trap. In systems where pieces of the particular proteins are unavailable as binding complements, antibodies specific for the restitched polypeptide could be elicited and used. The number of systems susceptible to enzymatic resynthesis using such traps, antibody or otherwise, could be considerable.

D. Stepwise Degradation and Elongation

In contrast to the more extensive introduction of synthetic sequences into proteins as afforded through peptide fragments, limited manipulation can be achieved by stepwise semisynthesis, through sequential degradation and buildup of polypeptides at their amino and carboxyl termini. Given the limited scope of such fragmentation, synthesis, and reconstitution these individual procedures are generally easier to achieve than they are in fragment-based semisynthesis. Such simplification makes this an attractive shortcut for introducing modifications aimed at studying the role of critical terminal regions, as by direct replacement or even extension. Additionally, introduction of nonperturbing isotopic labels or chromophoric probes can be accomplished readily by such a route.

For the most part, stepwise degradation and elongation have proceeded using chemical reagents in all stages. For fragmentation, the Edman degradation reaction[66] has been used most frequently since it allows sequential removal of residues with enough kinetic control to ensure high yields of polypeptide cores. While such a degradation usually requires side chain protection to avoid unwanted reactions at ϵ-amino groups of lysyl residues, the use of selective and reversible blocking has been achieved in several cases, including Boc (tert-butyloxycarbonyl), Msc (methylsulfonylethoxycarbonyl), and Tfa (trifluoroacetyl) groups in the exemplory case of insulin[56], or Msc, Acm (acetimidyl), and Boc groups for cytochrome c.[23-25] Given suitable protection of amino groups, buildup of intact or sequentially degraded polypeptide chains also can proceed chemically using activated amino acids or peptides.[24,25] In these cases, synthesis of the small building blocks required is a relatively routine matter compared to the more demanding synthesis of larger fragments. The major limitation may well be solubility of protected intermediates cores.

The alternative use of enzymes in stepwise semisynthesis, while possible in theory, has received only limited attention in practice. For fragmentation, use of exopeptidases is limited by difficulty in controlling removal of only a few selected residues. Nonetheless, the fortuitous occurrence of exopeptidase-resistant peptide bonds could allow use of enzymes in appropriate cases, therein obviating the need for side chain protection. For example, in semisynthetic manipulations of cytochrome c, the placement of Lys at residue 79 and Thr at 78 has allowed sequential enzymatic degradation of [HSer 80]cytochrome-c-(66-80) by removal first of HSer 80 with carboxypeptidase A and then of Lys 79 with carboxypeptidase B.[24] For elongation, the use of carboxypeptidase Y has been demonstrated as a potentially effective agent for building polypeptide chains at the carboxyl termini.[67] Even when degradation is achieved chemically, elongation of the polypeptide cores could proceed via enzyme-catalyzed stepwise resynthesis. For proteins

for which fully protected cores are insoluble, such enzymatic buildup could prove invaluable.

Internal enzyme-assisted stepwise semisynthesis, the replacement of individual residues into internal positions, has been of quite limited use but is represented by at least one cogent example. For the general class of protease inhibitors which act on proteases by binding to the latter and providing a particular peptide bond for proteolysis, residue deletion and reconstitution has been achieved around the internal bond hydrolysed in the inhibitor.[59-61] When possible, this type of internal sequence manipulation via enzymes can be quite clean and powerful, as evidenced by the variety of manipulations of the local sequences around the protease-cleaved bonds in the inhibitors.

III. SEMISYNTHESIS SYSTEMS DEVELOPED

By combining the basic processes of fragmentation, chemical synthesis, and reconstitution of purified natural and synthetically derived pieces, a variety of polypeptide and protein systems have been produced and studied. A representative list of these accomplishments is given in Table 1. In addition to the polypeptide and protein systems cited therein, a few other systems have been shown to be reasonable but as yet unfulfilled candidates for extended semisynthesis. In the cases of lysozyme,[86] carbonic anhydrase,[87] and myoglobin,[53,88] fragmentations have been developed for future incorporation into semisynthesis schemes. In addition, the limited cyanogen bromide-cleaved derivative of pancreatic trypsin inhibitor,[41] in which the cleaved internal bond can be reformed through the homoserene lactone, represents another potential tool. While the internal bond reformation in the inhibitor has been demonstrated only for protein fragments held together by disulfide linkage, the challenge clearly exists for fragment separation and incorporation of synthetic components. Also, a variety of closely related stepwise semisyntheses, as for the prominant case of insulin, have been omitted from Table 1 for simplicity. For example, Borrás and Offord,[89] in a pioneering effort, removed the amino-terminal Phe of the B-chain by Edman degradation and then added this residue back via a selectively protected amino acid derivative. Offord describes many other examples of stepwise processes in Reference 8. It seems reasonable to consider all of the above systems together with the cases of Table 1 in developing an overall impression of the current status of the semisynthesis field.

IV. STUDIES OF FOLDING AND BIOLOGICAL FUNCTION

From the point of view of using semisynthesis to study the relationship between sequence, conformation, and function, the approach has been most broadly applied so far to a relatively few large polypeptides. Notable examples are bovine pancreatic ribonuclease, staphylococcal nuclease, and cytochrome c. A central impetus in the use of these cases has been the availability of convenient fragment systems which include relatively small polypeptide components. By allowing routine preparation of sequence analogues, these few well-developed cases continue to offer a productive means to study both specific structure-function questions for these particular proteins as well as general characteristics of proteins as a class. Nonetheless, this limited set of protein cases cannot be expected to allow an infinitely broad range of issues to be addressed just by their study alone. However, many other systems, such as those derived from myoglobin, insulin, somatotropin, and immunoglobulin, are well developed enough to indicate promise. These recently developed cases have broadened the potential scope of the types of questions that can be addressed through semisynthesis.

Table 1
SEMISYNTHESIS SYSTEMS DEVELOPED

Category	Active semisynthetic peptide or protein[a]	Synthetic (or nonnative) component(s): Sequence[a]	Synthetic (or nonnative) component(s): Synthetic route[b]	Native component: Sequence[a]	Native component: Fragmentation route	Reconstitution route[c]	Ref.
Bovine pancreatic ribonuclease	RNase-S (1-20):(21-124)	(1-20)	Solution	(21-124)	Limited proteolysis (subtilisin)	Noncovalent association	68,69
		(1-20)	Solid phase	(21-124)	Limited proteolysis (subtilisin)	Noncovalent association	70
	[des 16-20]-RNase-S (1-15):(21-124)	(1-10) + (11-15)	Solid phase	(21-124)	Limited proteolysis (subtilisin)	Combination of covalent-enzymatic and noncovalent association	65
	RNase-"P_{rec}"[d] (1-115):(116-124)	(116-124)	Solid phase	(1-115)	Limited proteolysis (pepsin), then controlled CPase treatment	Noncovalent association	71
	RNase-A (1-124)	(1-20)	Solid phase	(21-124)	Limited proteolysis (subtilisin)	Covalent-enzymatic	64
	[Nle 13, HSer 20] analogue of RNase-A	[Nle 13 Hser-Lact 20] analogue of (1-20)	Solid phase	(21-124)	Limited proteolysis (subtilisin)	Covalent-chemical	72
Staphylococcal nuclease	Nuclease-T (6-47):(49,50-149)	(6-47)	Solid phase	(49,50-149)	Limited proteolysis (trypsin)	Noncovalent association	9,40
	Nuclease-"type II" (1-126):(99-149)	(99-149)	Solid phase	(1-126)	Limited proteolysis (trypsin) after amino protection	Noncovalent association	73
	[Gly 48]nuclease-(6-149)	[Gly 48] analogue of (6-49)	Solid phase	(50-149)	Limited proteolysis (trypsin)	Covalent-enzymatic	38
Porcine pancreatic phospholipase A_2	Protected [Phe 10]-phospholipase A_2	Protected [Phe 10] analogue of (8-10)	Solution	Protected [des 8-10] phospholipase A_2	Stepwise Edman degradation	Covalent-chemical	54

Cytochrome c	[HSer 65]cytochrome c (1-104)	(66-104)	Solid phase	[HSer Lac 65] (1-65, heme)	Chemical cleavage (cyanogen bromide)	Covalent-chemical	74
		Protected (66-80)	Solution	[HSer Lac 65] (1-65, heme) + protected (81-104)	Chemical cleavage	Covalent-chemical	23
		Protected (66-80)	Solid phase	[HSer Lac 65] (1-65, heme + protected (81-104)	Chemical cleavage (cyanogen bromide)	Covalent-chemical	75
		Protected 80	—	[HSer Lac 65] (1-65, heme) + protected (66-79) + protected (81-104)	Chemical cleavage (cyanogen bromide)	Covalent-chemical (after stepwise addition of 80 to (81-104))	24
	Cytochrome c-T (1-38,heme):(39-104)	Protected 39	—	Protected (1-38) + protected (40-104)	Limited proteolysis (trypsin)	Noncovalent association (after stepwise addition of 39 to (40-104))	76
	Cytochrome c — 3-fragment complex (1-25,heme):(28-38): (56-104)	(28-38)	Solid phase	(1-25,heme) + (56-104)	Limited proteolysis (trypsin)	Noncovalent association	77
Sperm whale myoglobin	Myoglobin (intact)	(1-5)+(6-13) + 14 (all protected)	Solid phase	Protected (15-153)	Chemical cleavage (BNPS-skatole)	Covalent-chemical	52
		Protected 1	—	Protected (2-153)	Edman degradation	Stepwise covalent coupling	78
Insulin	Various species of insulin	A chain (A_1-A_{21})	Solution	B chain (B1-B30)	Sulfitolysis	Reduction, air oxidation (to reform disulfides)	79
		B chain (B1-B30)	Solution	A chain (A1-A21)	Sulfitolysis	Reduction, air oxidation (to reform disulfides)	79
	Human insulin	Protected (B23-B30)(human sequence)	Solution	Protected [des B23-B30]porcine insulin	Proteolysis (trypsin)	Covalent-chemical Covalent-enzymatic	55,56 62

Table 1 (continued)
SEMISYNTHESIS SYSTEMS DEVELOPED

Category	Active semisynthetic peptide or protein[a]	Synthetic (or nonnative) component(s)		Native component		Reconstitution route[c]	Ref.
		Sequence[a]	Synthetic route[b]	Sequence[a]	Fragmentation route		
	[Lys B3]insulin	Protected [Lys B3] analogue of (B1-B3)	Solution	Protected [des B1-B3] insulin	Stepwise Edman degradation	Covalent-chemical	56
	[Ala A1]insulin	Protected Ala A1	—	Protected [des A1] insulin	Stepwise Edman degradation	Covalent-chemical	56
Somatotropin	[Nle 170, Ala 165, 182,189, Cys (Cam) 53] analogue of HGH[c] (1-134):(150-191)	[Nle 170, Ala 165,182,189] analogue of (150-191)	Solid phase	[Cys (Cam) 53] derivative of (1-134)	Limited proteolysis (*plasmin*)	Noncovalent association	80
Adrenocorticotropin	ACTH sequence (1-24)	Protected (1-4)	Solution	Protected (5-24)	Chemical cleavage (cyanogen bromide)	Covalent-chemical	81
Melanotropin	[Phe 9]analogue of αMSH (1-13)	Protected 9	—	Protected pieces (1-8) + (10-13)	Limited proteolysis (trypsin) followed by Edman degradation of (9-13)	Covalent-chemical (after stepwise (10-13))	82
	[Lys 10]analogue of human βMSH (1-22)	Protected (1-4)	Solution	Protected	Protected porcine βMSH (intact peptide contains Lys at position 10)	Covalent-chemical	83
Immunoglobulin	F_v fragment of MOPC 15	V_L (1-115)	Solid phase	V_H (1-120(~))	Limited proteolysis (pepsin)	Noncovalent association	
Clostridial Ferredoxin	[Leu 2]analogue of intact (1-55) sequence	Protected 1,2	—	[des 1-2] apoferredoxin	Stepwise Edman degradation	Stepwise addition followed by addition of iron and sulfide	85

Protease Inhibitors	[Lys 63 or Trp 63] soybean trypsin inhibitor	63 (free amino acid)	—	[des Arg 63] soybean trypsin inhibitor	Limited proteolysis (trypsin, CPase B)	Covalent-enzymatic	59,60
	[Arg 15 or Trp 15] trypsin-kallikrein inhibitor	5 (free amino acid)	—	[des Lys 15] trypsin-kallikrein inhibitor	Limited proteolytic (trypsin, CPase B)	Covalent-enzymatic	61

Note: This listing includes a representative set of peptides and proteins for which active semisynthetic species have been obtained successfully. Analogue variations are not included except when these represent primary semisynthesis successes for a given species. The reader is referred to the text and references for systems currently under development.

[a] Numbers denote residue positions in parent sequence. For noncovalent comlexes, a *colon* is used to denote noncovalent association between fragments of sequences defined by residue numbers and set in parentheses.

[b] The terms "solution" and "solid phase" refer generally to syntheses by solution-based (classical) and solid phase-based procedures, respectively, as defined in text.

[c] Distinction is made between methods of fragment reconstitution by mixing under noncovalently interacting conditions ("noncovalent association"), enzyme-catalyzed covalent restitching ("covalent-enzymic"), and covalent restitching either through specifically located activated homoserine lactones or through general coupling reactions using suitable side chain protection to direct appropriate bond formation ("covalent-chemical").

[d] The denotation RNase-"P_{rec}" has been adopted as a shorthand name for this review and refers to the nature of this species as a reconstituted, pepsin-treated ribonuclease.

[e] Cam stands for S-carboxamidomethyl.

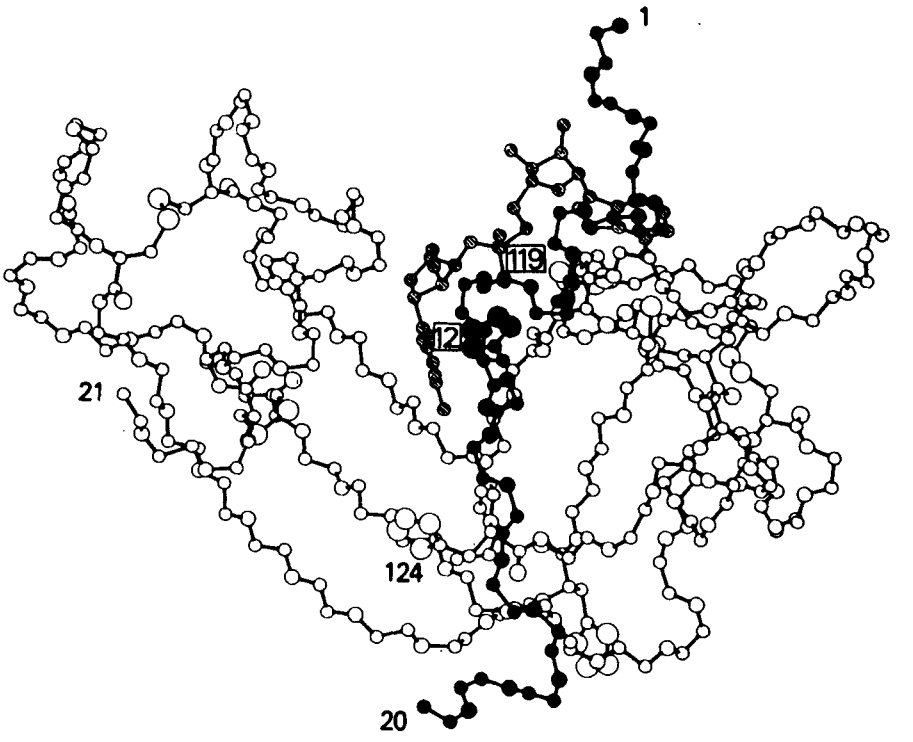

FIGURE 3. High resolution structure of ligand-interacting bovine pancreatic ribonuclease-S, as defined by X-ray crystallographic analysis. The figure distinguishes the fragments RNase-S-(1-20) (containing residues 1 through 20 of ribonuclease, ●); RNase-S-(21-124) (containing residues 21 through 124, ○); UpCA (a dinucleotide phosphonate analogue of uridylyl-3', 5'-adenosine which binds in the active-site region, ⊘); and the active site-involved His 12 and 119 side chains (located immediately to the right of the respective inserted boxed numbers in enlarged circles — filled for 12, open for 119). Enlarged circles also represent the 4 disulfides and 2 nonactive site His residues. (With permission from Chaiken, I. M., *Semisynthetic Peptides and Proteins,* Offord, R. E. and DiBello, C., Eds., 1978, 349. Copyright by Academic Press Inc. (London) Ltd.)

A. The Example of Ribonuclease-S

Bovine pancreatic ribonuclease-S (Figure 3) continues to enjoy popularity as a prototypic semisynthetic system. As one of the earliest elucidated noncovalent fragment complexes derived from a protein by limited proteolysis,[20] it has provided an elemental tool, via the S-peptide (RNase-S-[1-20]), for technological development of peptide synthesis per se. The solution-based synthesis efforts of Hofmann and his colleagues in the U.S.[90] and Scoffone and his colleagues in Italy[91] pioneered the approach of noncovalent incorporation of synthesized peptide analogues into a large protein entity. Noncovalent semisynthesis of ribonuclease-S has come to be the touchstone for studies aimed at answering questions about the mechanisms by which sequence dictates conformation and biological function.

In spite of its usefulness, the ribonuclease-S system sometimes has been viewed as an anomaly, representing a situation — a noncovalent complex — which is not likely to be available for many proteins. From the methodological point of view, then, the procedures used for ribonuclease-S are not extrapolable in themselves for the fundamental needs of typical semisyntheses, which most often must proceed through covalent fragment restitching in order to lead to an active protein species. However, as cited in Table 1, results of the last few years have shown that ribonuclease-S forms the

basis for several different types of covalent semisynthesis, such as conversion of semisynthetic ribonuclease-S to corresponding ribonuclease-A and restitching using kinetic traps. These encouraging results not only bode well for covalent semisynthesis of ribonuclease itself, but also should provide an impetus to try out such ideas as enzyme-assisted resynthesis and introduction of specifically activated amino acids residues on other fragments and fragment complexes.

1. Production and Conversion to Semisynthetic Ribonuclease-A

The preparation of purified ribonuclease-S (see Table 1 and Figure 4 for summaries) has been approached in solution synthesis by making a RNase-S-(1-20) species which, while not homogeneous after completion of bond synthesis, can be purified alone by classical techniques and then mixed with native RNase-S-(21-124).[68,69] For solid phase synthesis, the crude synthetic RNase-S-(1-20) species (with maximally about 50% potential activity, at least using chloromethylated polystyrene as the synthesis resin) also requires further purification. This latter, for a solid phase-derived product which probably contains contaminants more closely related to the correct sequence than does product of solution synthesis, has been achieved (Figure 5) by direct functional purification via complementation with RNase-S-(21-124) and isolation of reconstituted semisynthetic complexes.[44] If desired, purified synthetic-(1-20) or analogue can then be recovered from the isolated complex by gel filtration in acid.[92]

From the practical point of view, the end result for products of both solid phase and solution syntheses has been considerable success in generating semisynthetic ribonuclease-S of homogeneity sufficient to allow extensive protein biochemical characterization. The use of more recently defined solid phase synthesis resins and purification of synthetic products by HPLC and other high resolution procedures make the solid phase approach still more attractive, since the crude solid phase-derived peptide products are less likely to be chemically heterogeneous and the semisynthetic ribonuclease-S derived therefrom is less likely to contain closely related sequence variants which cannot be separated adequately during functional purification. Nonetheless, the choice of method for the total synthesis of the (1-20) fragment of ribonuclease-S probably will continue to be based on the methodological tendencies of the investigator and the nature of the questions being asked rather than on any overriding technical advantage.

Enzymatic restitching of subfragments of RNase-S-(1-20) offers yet an additional simplification for making analogues for semisynthetic ribonuclease-S. The approach has been suggested[65] by the successful covalent recoupling of synthetic fragments (1-10) and (11-15) (see Table 1 and earlier discussion). While the above has been carried out with synthetic subfragments, one could obtain either of these by proteolysis of the native RNase-S-(1-20) peptide using arginine-specific proteases. Thus, it can be expected that viable semisynthetic ribonuclease-S could be obtained by clostripain incubation of a mixture of native RNase-S-(21-124) with some combination of synthetic and native subfragments of RNase-S-(1-20) (such as synthetic [1-10] and native [11-20]). The resultant noncovalent complex could be isolated directly from the incubation mixture, as by ion exchange, affinity chromatography, or gel permeation HPLC. The need in this case to synthesize only a decapeptide makes the synthetic aspect of ribonuclease-S semisynthesis that much simpler by either solution or solid phase chemistry.

In those cases wherein semisynthetic alteration of sequence gives rise to characteristics that require study under conditions in which ribonuclease-S is insufficiently stable (as for study of activity at pH values below 4), conversion of the particular semisynthetic ribonuclease-S to the corresponding ribonuclease-A species can be a useful adjunct in an overall semisynthesis study. That this now is possible by enzymatic restitching in the presence of organic cosolvents has been cited earlier. Using the original observation for

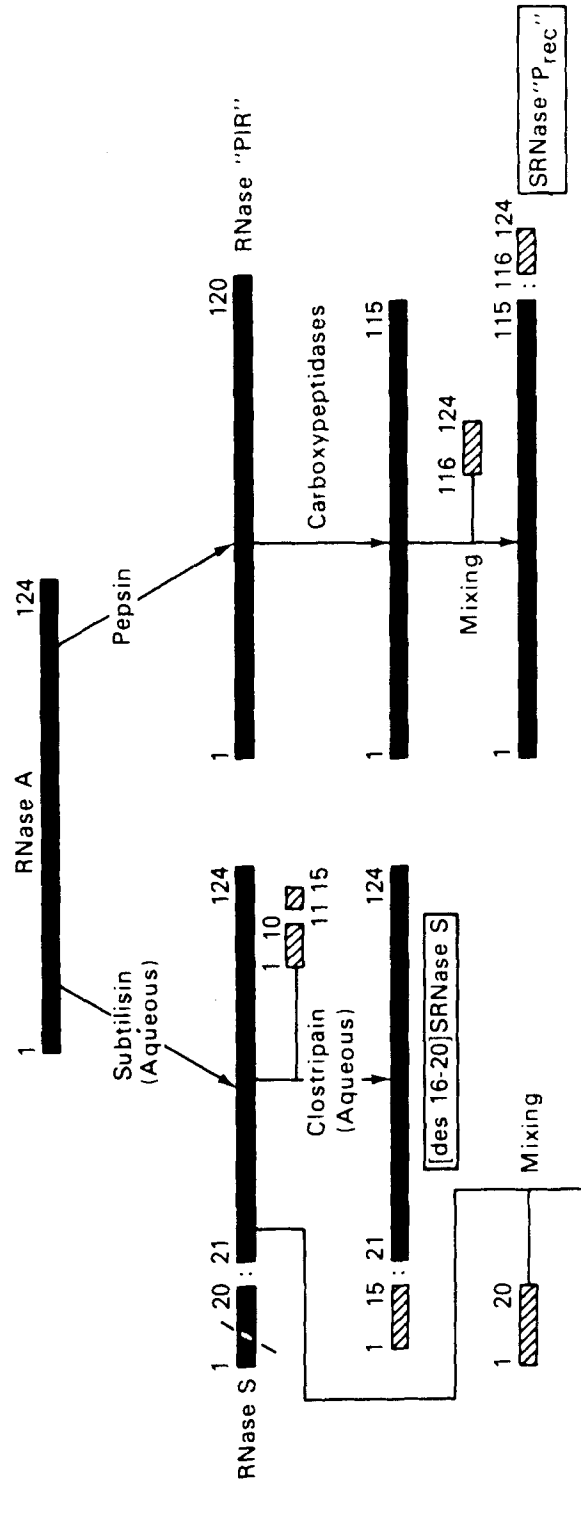

FIGURE 4. Scheme of types of semisynthetic derivatives produced from bovine pancreatic ribonuclease. Proteins or fragments of proteins are defined by amino- and carboxyl-terminal residue numbers, with native components shown as solid bars and synthetic components as hatched bars. Discard fragments, i.e., those not used for semisynthetic derivative production, have a diagonal dashed line drawn through them. The major semisynthetic products, denoted by an "S" preceding the protein type, are enclosed in boxes. Colons are used to indicate noncovalent interactions between fragments. The reader is referred to Table I and the text for further explanatory details.

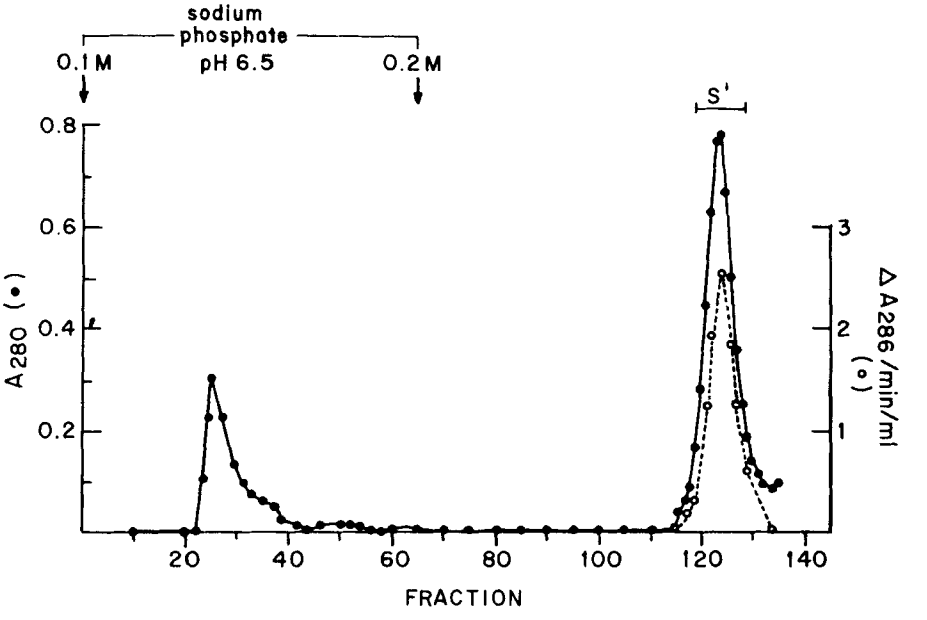

FIGURE 5. Production of semisynthetic ribonuclease-S by functional purification. Solid phase-derived synthetic-(1-15) (corresponding to residues 1 through 15 of ribonuclease) was mixed with native RNase-S-(21-124), and the resultant complex fractionated by sulfoethyl Sephadex® chromatography. Elution was followed by measuring protein content (A_{280}) and enzymatic activity (ΔA_{286}/min/mℓ) of fractions. The reconstituted semisynthetic complex, denoted S', was obtained as an approximately 100% active species (vs. native complex). (With permission from Chaiken, I. M., *Methods in Enzymology*, Vol. 37, Jakoby, W. B. and Wilchek, M., Eds., 1974, 631. Copyright by Academic Press Inc. (New York) Ltd.)

native ribonuclease-S as a basis, it has been possible to obtain semisynthetic ribonuclease-A (Figure 6) by mixing crude synthetic-(1-20) with native RNase-S-(21-124) in 90% glycerol containing subtilisin, and then isolating the intact A species by direct fractionation of the incubation mixture.[64] While this procedure requires the use of a synthesized S-peptide of full sequence (the [1-15]:[21-124] combination for example will not allow productive restitching), the preparation of such full-sequence peptides has not proven to be significantly more difficult than that of the shortened sequences.

2. Characterization of the Semisynthetic Species as a Protein

For all practical purposes, it is possible to treat (and interpret) the characterization of semisynthetic ribonuclease-S species, or derived semisynthetic A species, just as one would that of fully native complex or intact protein. This assertion is an important one from the point of view of the protein biochemist, who wishes to use synthesis to understand naturally occurring proteins; but its acceptance hinges (as, of course, it does for native proteins) on the extent of purity of the semisynthetic product. This condition has been, somewhat unfairly, a bone of contention more for solid phase-derived than for solution-derived product but surely should be established no matter what the synthetic route. That the assertion is valid for the semisynthetic ribonuclease-S system is increasingly clear. Here, native-like properties initially were confirmed for solution-derived normal-sequence product by full specific enzymatic activity and normal 1:1 noncovalent peptide-protein fragment association.[68] These same points can be demonstrated for solid phase-derived product.[70] In the latter case, substantiation of structural identity with native complex has been extended to the demonstration that both

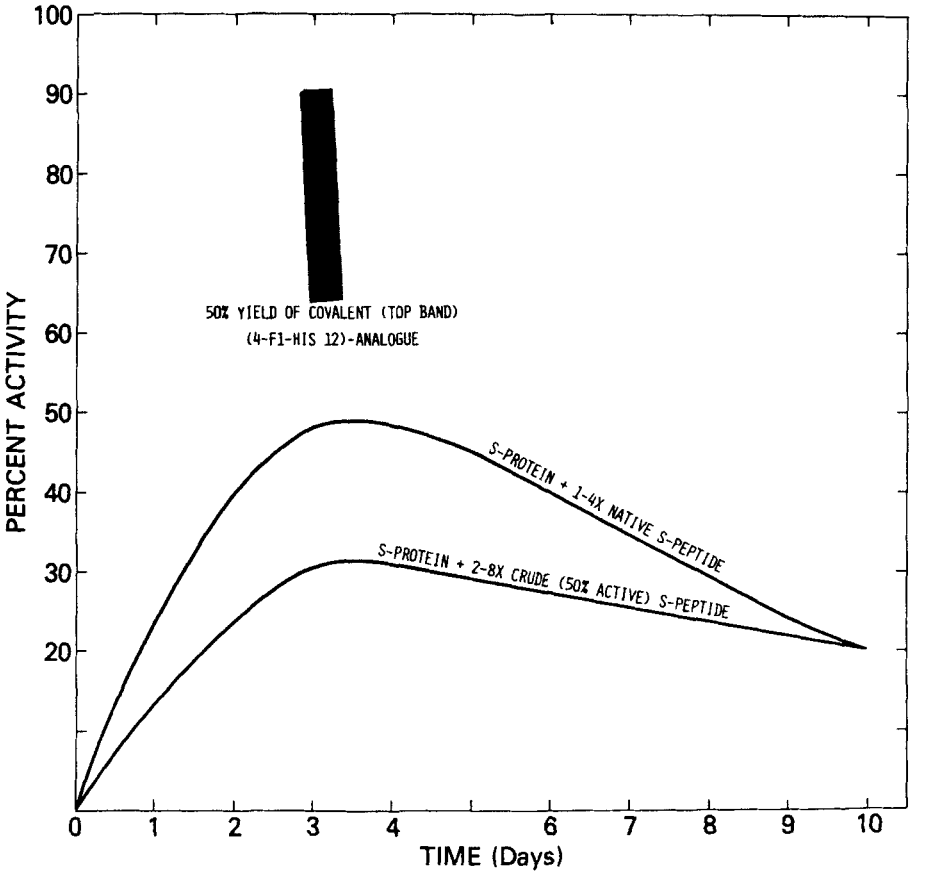

FIGURE 6. Conversion of native (RNase-S-(21-124) + RNase-S-(1-20), upper curve) and semisynthetic (RNase-S-(21-124) plus crude, solid phase-derived synthetic-(1-20), lower curve) ribonuclease-S complexes to covalent forms. The reaction mixture consisted of 90% glycerol, 10 m*M* 4-morpholine-ethanesulfonic acid (apparent pH 6.2), containing 1 mg/mℓ of complex (as defined) and 0.05 mg/mℓ subtilisin BPN'. Covalent forms were distinguished from noncovalent complexes by assay against cyclic cytidine-2':3'-monophosphate in 40% dioxane (to dissociate complex). Maximum yield of covalent form (ribonuclease-A and semisynthetic ribonuclease-A) occurred at about 3 days. The inset is a stained SDS polyacrylamide gel of a resynthesis mixture from [4-F-His 12] semisynthetic ribonuclease, with the top band representing the covalent semisynthetic ribonuclease-A analogue produced and the middle and lower bands representing the fragments of the reacted complex, namely RNase-S-(21-124) and [4-F-His 12] synthetic-(1-20), respectively. (Adapted from Homandberg, G. A., Komoriya, A., Juillerat, M., and Chaiken, I. M., *Peptides — Structure and Biological Function*, Gross, E. and Meienhofer, J., Eds., Pierce Chem. Co., Rockford, Ill., 1979, 597. With permission.)

semisynthetic and native ribonuclease-S behave virtually identically (see Figures 7 and 8) in crystallization and X-ray diffraction analysis.[70,93]

Given the high quality of semisynthetic ribonuclease-S, we are in a position to characterize this species, or semisynthetic ribonuclease-A derived therefrom, using all of the protein chemical techniques that are applicable to the native species, dependent only on the production of enough material. Indeed, in one or another case, an impressive range of protein chemical techniques now have been applied to the semisynthetic ribonuclease-S system. To cite only a few examples, functional properties have been studied by spectrophotometry, for catalytic activity measurements on a variety of

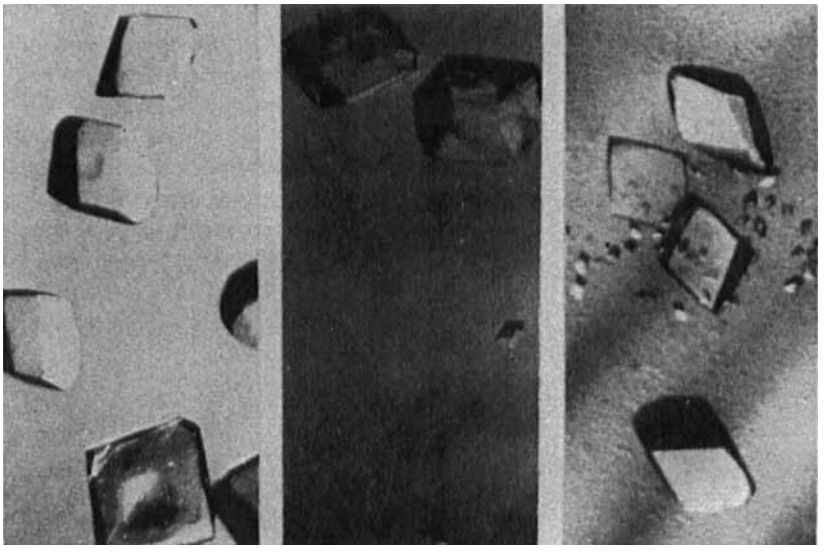

FIGURE 7. Large crystals (in the range of 0.4 to 0.7 mm maximum dimension) of normal-sequence semisynthetic ribonuclease-S (center), native ribonuclease-S (left), and [des 16-20] semisynthetic ribonuclease-S (right). (With permission from Chaiken, I. M., *Semisynthetic Peptides and Proteins,* Offord, R. E. and DiBello, C., Eds., 1978, 349. Copyright by Academic Press Inc. (London) Ltd.)

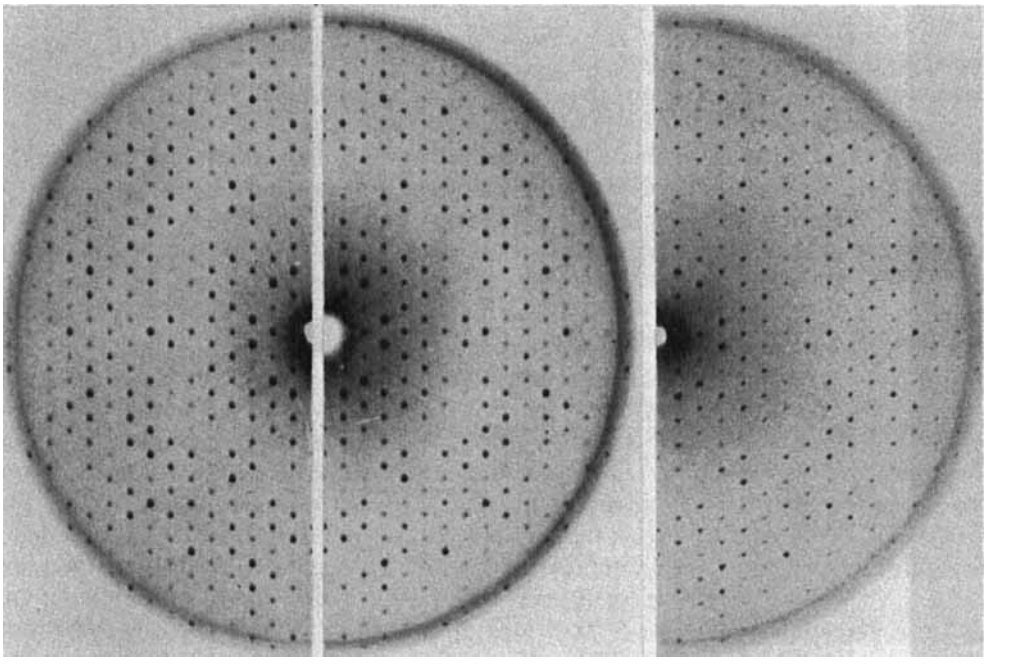

FIGURE 8. Half-precession (15°) diffraction photographs of hkiO nets of normal-sequence semisynthetic ribonuclease-S (center), native ribonuclease-S′ (left), and [des 16-20] semisynthetic ribonuclease-S (right). (With permission from Chaiken, I. M., *Semisynthetic Peptides and Proteins,* Offord, R. E. and DiBello, C., Eds., 1978, 349. Copyright by Academic Press Inc. (London) Ltd.)

substrates (such as RNA,[94,95] nucleotides,[96] and cyclic nucleotide monophosphates[97]), and by quantitative affinity chromatography, for binding of both substrates and competitive active site ligands.[98] Peptide-protein fragment interactions have been characterized by spectroscopic titration in the absence of substrate,[99] and by the response of activity to fragment titration in the presence of substrate.[95] The study of conformational features has ranged from general spectroscopic characterizations[96,99] and susceptability to proteolysis[96] to detailed study by nuclear magnetic resonance spectroscopy,[97] and X-ray diffraction of large crystals.[70,93] We have come to expect that when a semisynthetic ribonuclease-S species is made it can be characterized quite fully for functional properties of the active site region, conformational dynamics, and the detailed structure of the protein molecule as a whole. This is an expectation increasingly applicable to a variety of semisynthetic proteins.

3. Active Site Function

Semisynthesis studies of biological function benefit largely from the ability to effect selective and minimal modification of sequence. Residues which are suspected to have roles in catalysis based on prior evidence, from such studies as chemical modification and X-ray crystallography, can be tested for presumed roles. Where the residues of interest are accessible through replacement of a fragment containing them, modifications effected synthetically can be chosen willfully — in contrast to what is possible with more random methods such as chemical modification and biological mutation. These latter approaches also can lead to introduction of bulky new groups. In contrast, semisynthesis allows subtle modification to be effected with relative ease and can lead directly to information as to what structural or functional aspects of residues are essential for activity.

In retrospect, it would seem that semisynthetic sequence alteration directed at catalytically implicated residues, or those required for catalytically competent complexes, has been a major preoccupation in ribonuclease-S analogue work. The direct functon of His 12 in ribonuclease catalysis has been perhaps most exhaustively elucidated by the preparation of semisynthetic analogues; these have involved such replacements at position 12 as listed in Table 2. The message from all such studies is that when the chemical nature of the imidazolyl group is tampered with, especially by changing pK, this synthetic alteration alone results in suppression of catalytic activity. An exemplary case in the study of His 12 is that of the [4-fluoro-L-histidine 12]semisynthetic ribonuclease-S analogues. These derivatives of normal ribonuclease-S contain a solid phase-derived synthetic S-peptide with 4-F-His in place of the normal histidine at position 12. Studies of the full sequence analogue [4-F-His 12]semisynthetic ribonuclease-S, as well as the des 16-20 variant, have shown these species to be essentially inactive, exhibiting only about 0.2 to 0.4% of native ribonuclease-S activity against uridyl-3', 5'-adenosine and cyclic cytidine-2':3-monophosphate at pH 5 and 7.[96,98,101] At least a large percentage of this observed low activity appears due to contamination with native ribonuclease-A, based on measurement of Km and effects of dioxane.[101] In contrast to the loss of activity, the analogues were found to have a highly conserved overall conformation in solution, as indicated by such data as those in Figure 9. Further, it was shown directly by quantitative affinity chromatography (Figure 10) that both [4-F-His 12]- and [4-F-His 12, des 16-20]semisynthetic ribonuclease-S have high affinity for various active site nucleotide ligands and substrates.[98] Clearly, the fluorination of the imidazole, which lowers the imidazolium pK from about 6 to 2, does not obviate substrate binding. Moreover, in recent kinetic studies it has been observed that as the pH is lowered from 5 to 3.5, there is in fact some increase in the relative enzymatic activity of the analogues.[101] The results so far suggest that there indeed may be some intrinsic activity in the analogue complex

Table 2
LIST OF SOME SEMISYNTHETIC RIBONUCLEASE-S SEQUENCE VARIANTS USED TO EVALUATE THE FUNCTION OF RESIDUES IN THE S-PEPTIDE REGION

Normal residue	Semisynthetic variant	Properties of variant	Predicted major role(s) of normal residue(s) in ribonuclease-S	Ref.
His 12	[Pyrazole-3-Ala 12] (1-14)	0% activity; $K_d' = K_d$ of (1-14)[a]	Catalytic component; helps promote proper ribonuclease-S conformation	100
	[4-F-His 12] (1-15) (and 1-20)	0% activity; $K_d' = 2 \times K_d$ of (1-20); normal X-ray diffraction pattern; binds active site ligands, substrates		96,98,101
	[NorHis 12] (1-14)	100% activity at saturation vs. (1-14); $K_d' = 10^3 \times k_d$ of (1-14)		102
	[HomoHis 12] (1-14)	90% activity at saturation vs. (1-14) $K_d' = 10^2 \times K_d$ of (1-14)		103
	[Ser 12] (1-14)	0% activity; $K_d' = 7 \times K_d$ of (1-14)		104
	[Orn 10,Orn 12] (1-20)[b]	0% activity; no binding to RNase-S-(21-124)		105
Glu 9	[Leu 9] (1-15)	100% activity at saturation $K_d' = 3 \times K_d$ of (1-20)		92
	[Gly 9] (1-15)	High activity at saturation $K_d' = 20 \times K_d$ of (1-20)		92
Lys 1 Thr 3 Glu 9 Gln 11 Asp 14 Ser 15	[Ala 1,3,9,11,14,15]-(1-15) (model peptide)	~ 40% activity at saturation; $K_d' \simeq 10 \times K_d$ of (1-20); crystals of complex isomorphous with those of native SRNase-S	Much of S-peptide sequence provides α-helical backbone to orient a few critical residues for key contacts with S-protein and substrate	106

[a] K_d' denotes the dissociation constant of the analogue peptide to RNase-S-(21-124), and is defined by comparison to the dissociation constant for the normal-sequence peptide as shown.
[b] Orn denotes ornithine.

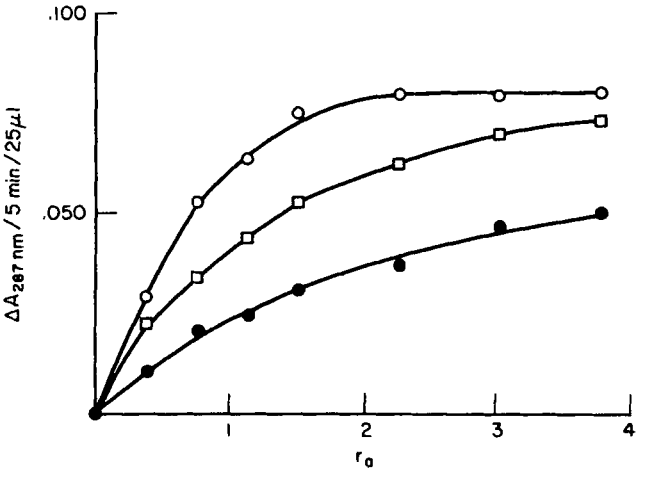

FIGURE 9. Assessment of noncovalent interaction of RNase-S-(21-124) to [4-F-His 12]-synthetic-(1-15) as indicated by titration of 5×10^{-4} mol of the former with RNase-S-(1-20) in the absence (○) or presence of fixed amounts of synthetic peptide (1.29 (□) or 5.16 (●) equivalents vs. the (21-124) fragments). Association of native fragments was followed by enzymatic activity, against cyclic cytidine-2':3'-monophosphate, measured as the initial rate of increase of absorbance at 287 nm as a function of the ratio (r_a) of (1-20) to (21-124). Association between synthetic peptide and (21-124) was measured by inhibition of native complex activity by the fixed amounts of synthetic component. By this analysis, the 4-F-His 12 peptide was shown to have an association constant with native (21-124) of $2.2 \times 10^7\ M^{-1}$ at pH 7.13, vs. $5.5 \times 10^7\ M^{-1}$ for native fragments (1-20) and (21-124). (From Dunn, B. M., DiBello, C., Kirk, K., Cohen, L. A., and Chaiken, I. M., *J. Biol. Chem.*, 249, 6295, 1974. With permission.)

which can be "switched on" by lowering the pH as the 4-F-His 12 is increasingly protonated. Taken together, the data for these derivatives provide compelling evidence consistant with the idea that His 12 acts as an obligatory catalytic component, probably dependent on its protonation at least transitorily, in the mechanism of action of native ribonuclease.

Interpretation of the 4-F-His 12 analogue data depends heavily on the demonstration that there is little perturbation of protein conformation, especially in the active site region, due to incorporation of the fluorine atom. Confirmation of the intactness of conformation has been obtained by X-ray diffraction analysis. The [4-F-His 12] variants of semisynthetic ribonuclease-S can be crystallized under conditions similar to those used for native[107] and normal sequence semisynthetic[70] ribonuclease-S species to yield similar "Y"-type crystals. Recently, a collaborative study at Duke University and the National Institutes of Health has shown that the conformations of the fully active [des 16-20]semisynthetic ribonuclease-S and the enzymatically deficient [4-F-His 12, des 16-20]semisynthetic ribonuclease-S are essentially indistinguishable at 2.6 Å resolution.[101,108] This absolute structural result confirms the assertion, inferred from spectroscopic characterization and comparison of affinities of nucleotide substrates and inhibitors, that the fluorination of histidine 12 effects little steric perturbation of the active site region.

Although the above conclusions have been made based on data for the noncovalent ribonuclease-S complex, extrapolation of conclusions from such semisynthesis studies to

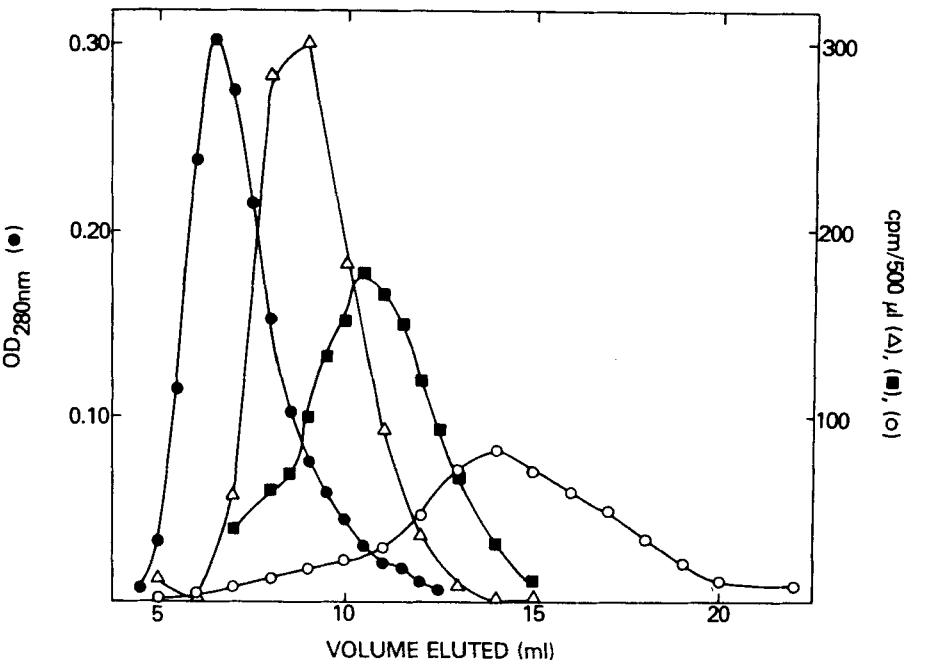

FIGURE 10. Quantitative affinity chromatography analysis of active-site ligand binding by [4-F-His 12, ^{3}H-Phe 8, des 16-20] semisynthetic ribonuclease-S by elution of semisynthetic ribonuclease-S analogue (0.1 mg) on the ribonuclease affinity matrix uridine-5′-(Sepharose-4-aminophenylphosphoryl)-2′(3′) phosphate with 0.4 *M* ammonium acetate, pH 5.2 (○) or with this buffer containing 2.2×10^{-3} *M* of the inhibitor 3′-uridine monophosphate (△) or 2.0×10^{-4} *M* of the ribonuclease substrate uridylyl-3′,5′-adenosine (■). The control (●) was bovine serum albumin (2.0 mg) eluted in buffer without added ligands. (From Taylor, H. C. and Chaiken, I. M., *J. Biol. Chem.*, 252, 6991, 1977. With permission.)

the intact parent, ribonuclease-A, has been common. Given the homology of conformational and functional properties of the A and S species,[109] such correlation normally is reasonable. Nonetheless, the case of the F-His 12 species does present difficulties in testing for possible intrinsic activity across a significant pH range due to the innate instability of the ribonuclease-S species at low pH. However, an opportunity to study the [4-F-His 12]semisynthetic ribonuclease-A derivative has been provided by the potential to enzymatically restitch the S-complex. Resynthesis of the 4-F-His 12 analogue complex into the intact covalent form has been shown, as indicated in Figure 6, to proceed as with normal sequence semisynthetic complex.[64] Study of the restitched analogue has allowed substantiation of at least low-level intrinsic activity in the 4-F-His 12 species.

4. Folding and Attainment of Active Conformation

Beyond the study of function, semisynthesis offers a specific way to modify amino acid residues which direct and stabilize the native secondary and tertiary structures, and thereby help to form an active conformation with an intact active site. Among numerous structure-stabilizing interactions present in a globular protein — including apolar interactions, hydrogen bonding and salt bridges — some play an essential role in directing and stabilizing tertiary structure. A key objective is to identify and test residues that provide such essential interactions.

In the ribonuclease-S system there is a wealth of data on conformation obtained from the pioneering semisynthetic studies of Hofmann, Scoffone, and colleagues,[90,91] as well as from more recent work.[4,101,110] A few examples are given in Table 2. Such studies have given extensive insight into the functions of several nonactive site residues of the native S-peptide sequence for the formation of a productive ribonuclease-S complex. For example, a study[92] on glutamic acid at position 9 has indicated that the tendency of this residue to support the integrity of α-helical conformation in this region is more important than the polarity of this side chain. This was suggested by the productive complex that results from an S-peptide with a single replacement of Glu 9 by leucine, a residue with strong side chain hydrophobicity (in contrast to Glu) but main chain α-helical propensity equally as high as that of Glu. The effect of residue replacement as well as data on sequence homology in the N-terminal region of ribonuclease have indicated[111-113] the importance of the hydrophobic residues, Met 13 and Phe 8, for stabilizing the ribonuclease-S complex. The one order of magnitude changes in the S-peptide:S-protein binding constant that was observed with changes at the sulfur atom of Met 13[114] or at the side chain of Phe 8[99] tends to support the apparent importance of these apolar interactions. Some recent empirical estimations of local interaction free energy changes associated with changes in side chain to side chain interactions for the ribonuclease-S complexation reaction revealed[115] that a major favorable free energy contribution came from the interactions involving Met 13 and Phe 8. Further, the analysis suggested that the role of the N-terminal α-helix, which dominates the backbone conformation of the S-peptide from residues 3 through 13 (Figure 11), is mainly to provide a structural frame on which the above two residue side chains can be properly oriented to optimize favorable interactions with nonpolar groups on S-protein. Taken with the proven importance of His 12, as well as the above-reviewed data, it was proposed that the key minimal features for S-peptide to produce a productive binding to S-protein are a stable α-helix which can orient Met 13 and Phe 8 for complex stabilization and His 12 for active site function.

The above postulate has been tested by designing a model peptide[106] employing the logic defined in Figure 12. This peptide, of 15 amino acids, retains the above essential structural (α-helix), complex stabilizing (Phe 8, Met 13) and active site (His 12) features and contains an α-helical matrix provided by poly-L-alanine. Besides incorporation of the above-mentioned Phe 8, His 12, and Met 13, the residues Glu 2 and Arg 10 were included to provide an ionic interaction which appears important for stabilizing the α-helix,[91] and Lys 7 was included for its contribution to the cationic environment of the active site.[116] This model sequence S-peptide, as prepared by solid phase synthesis, was found to be highly productive in generating an enzymatically active model semisynthetic ribonuclease-S.[106] From the enzymatic activation of S-protein by model S-peptide at various mole ratios of the two components the dissociation constant, K_d, was determined to be about 10^{-6} *M*. This value is only an order of magnitude larger than that of native S-peptide. When this increased K_d value for model peptide is compared with the same order of magnitude increase observed with peptide complexes with only a single residue replacement of native sequence (such as Glu 9 by Gly or Met 13 by methionine sulfone), the remarkable success of the model peptide is apparent. Further, model semisynthetic ribonuclease-S exhibited about 35% of the specific enzymatic activity at saturating peptide concentrations. The substantial activity shows that the model complex can generate the ribonuclease active site with a high degree of fidelity.

The absolute structural verification of the presumed ribonuclease-S-like conformation of the complex must rely on crystallographic analysis. As in the active site residue modification study, this is now possible. Crystals have been obtained of the model complex, once again in the native ribonuclease-S space group. Preliminary results indicate that the crystals are suitable for high resolution structure analysis and confirm

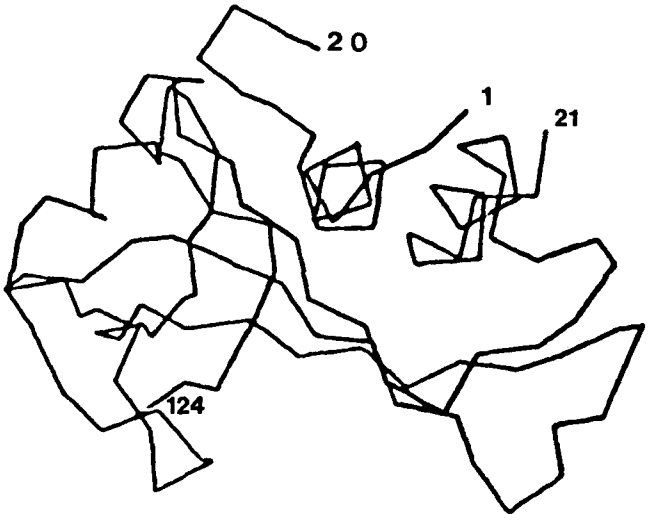

FIGURE 11. Backbone structure of bovine pancreatic ribonuclease-S, as in Figure 3 but oriented to emphasize the helical array in the RNase-S-(1-20) sequence. (Adapted from Komoriya, A., Krigbaum, W. R., and Chaiken, I. M., *Peptides 1978*, Siemion, I. Z. and Kupryszewski, G., Eds., Wroclaw University Press, Warsaw, 1979, 657. With permission.)

the interpretation that the model semisynthetic ribonuclease-S retains at least the overall conformation of the normal sequence complex.

5. Conformational Propensity vs. Chemical Detail

Semisynthesis studies with the ribonuclease-S system have allowed at least initial generalities to be deduced concerning the duality by which sequence provides information for conformation and function. Results such as those with the 4-F-His analogues show that, in certain cases, a definite set of chemical details is required to produce a functionally intact protein. In the ribonuclease case, only His at position 12 or a residue preserving similar side chain imidazole pK characteristics will provide enzymatic viability. However, the model peptide study demonstrates that for a large portion of the sequence, chemical detail can be perturbed dramatically as long as one preserves conformational integrity. A reasonable generalized interpretation of these semisynthesis results is that, at least in the ribonuclease S-peptide region, the information of amino acid sequence provides the overall propensity to fold to a packed conformation in which a relatively few groups in the sequence, with a particular set of chemical details, are required to express biological function. By this view, the precise detail of a large part of the sequence probably is in fact mutable, as long as the conformational propensity is preserved and the limited critical groups of the active site (or sites) are not disturbed. This same theme seems applicable to other protein cases also, as deduced from sequence variation studies cited later for staphylococcal nuclease and cytochrome c.

6. Insertion of Probes

In understanding the relationship between primary structure and higher order properties, an important issue is the nature of the microenvironments at specific loci and the way in which such microenvironments change upon conformational and functional

Logic of Peptide Design

Residues Included	Predicted Function
Phe-8 and Met-13	to provide stabilizing non-bonding peptide-protein interactions
His-12	to participate in enzymatic reaction
Ala residues	to provide rigid α-helix for proper spacing of Phe-8, His-12 and Met-13
Glu-2, Lys-7, Arg-10	to provide hydrophilic surface and other stabilizing interactions

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
Model Sequence:	H_2N-	Ala-	Glu-	Ala-	Ala-	Ala-	Ala-	Lys-	Phe-	Ala-	Arg-	Ala-	His-	Met-	Ala-	Ala-COOH
Native Sequence:	H_2N-	Lys-	Glu-	Thr-	Ala-	Ala-	Ala-	Lys-	Phe-	Glu-	Arg-	Gln-	His-	Met-	Asp-	Ser-COOH

FIGURE 12. Design of model S-peptide sequence. (From Komoriya, A., Krigbaum, W. R., and Chaiken, I. M., *Peptides 1978,* Siemion, I. Z. and Kupryszewski, G., Eds., Wroclaw University Press, Warsaw, 1979, 657. With permission.)

perturbation. Semisynthesis offers a straightforward way to incorporate reporter moieties into polypeptide sequences for characterizing such properties. Site-specific insertion of probes, such as stable isotopes of nonzero nuclear spin (for nuclear magnetic resonance spectroscopy) or probes with unique fluorescent characteristics, provides a means to obtain minimally perturbed protein analogues for study of particular conformational transitions and active site geometry. The synthetic introduction of NMR probes also can help in assigning multiple resonances for the native protein. In addition, radioisotopic residue incorporation through synthesis can provide species for following conformational transitions, as for example by presenting a handle to follow the stability of a particular sequence during in vivo processing[117] or the distribution of fragments between isolated and noncovalently complexed forms in fragment exchange analysis of kinetic and thermodynamic folding parameters.[118]

In the ribonuclease-S system, a particularly promising set of analogues has been made with ^{13}C enrichment introduced both in the α-helical region[97,106,119,120] as well as in the catalytically central His 12 residue.[120,121] Analogues enriched at residue positions 5,6, and 8 have allowed the conformational transition of the N-terminal α-helix to be followed between unfolded S-peptide and the folded ribonuclease-S complex. As shown in Table 3, several of the chemical shift changes, namely for C_o, C_α, and where applicable C_β, are assignable to the expected helix-coil transitions. Such transitions are not observed in systems, such as that involving the interaction of oxytocin with neurophysin,[124] in which helix formation is not expected. Thus, while the precise, first principles interpretation of chemical shift changes must be made with caution,[120] these results indicate the possibility of following helix folding and unfolding at particular chosen loci in proteins by use of semisynthetically enriched derivatives. The Phe 8-^{13}C and Ala 5-^{13}C species have been used to show that the temperature-dependent stability of the α-helical conformation of these residues is close to that of the overall complex.[106,120] Similar analyses for other semisynthetic analogues[120] have allowed the temperature stabilities to be measured for the microenvironment around several other loci, including His 12, Met 13, and Asp 14 in the S-peptide sequence. In all cases except for Met 13, these temperature stabilities are similar to that for the overall ribonuclease-S complex itself. For the Met 13 side chain methyl group a lower Tm is found,[120] indicating that this side chain moiety is less rigidly ordered in the complex. The Met 13 result suggests that some local conformational features may be perturbed without overall disruption of the ribonuclease-S complex. Nonetheless, the bulk of the results obtained so far from ^{13}C-enriched semisynthetic ribonuclease-S analogues would indicate that at least in a thermodynamic sense, the conformational stability of the S complex is closely linked to the stability of the S-peptide backbone α-helical structure.

For the His 12 case, incorporation of ^{13}C into the imidazolyl moiety of this residue in semisynthetic ribonuclease-S has allowed proton dissociation properties to be evaluated for this residue in the protein complex.[120,121] The potential to delineate the effects on pK of added active site ligands could provide a highly sensitive future approach to describe the modes by which the His 12 imidazolyl group participates in substrate interactions during catalysis.

B. Other Proteins — Other Questions

It is obvious that, even in the limit, ribonuclease-S semisynthesis will allow the study of only a limited set of enzymatic and general protein properties. To be sure, excellent semisynthesis opportunities also exist with the related and useful carboxyl-terminal noncovalent ribonuclease complex (between fragments [1-120] and [111-124] or truncated versions of these) as exploited by Merrifield, Moore, and their colleagues.[32,71] Several analogues have been produced based on this complex, such as those showing the important participation of several of the carboxyl-terminal residues, including Phe 120,

Table 3
CHEMICAL SHIFT CHANGES FOR C_o, C_α, and C_β ^{13}C NMR RESONANCES OCCURRING IN VARIOUS MACROMOLECULAR COIL-TO-HELIX TRANSITIONS[a]

			Atom	Direction of chemical shift change	Shift change magnitude (Δ ppm)	Ref.
Poly (γ-benzyl-L-glutamate)	Random (29% Tfa) ⟶	Helix (3% Tfa)	Glu C_o (backbone)	Downfield	2.0	122
			Glu C_α	Downfield	3.1	
			Glu C_β	Upfield	1.2	
Poly(N^δ-benzyloxycarbonyl-L-ornithine)	Random (23% Tfa) ⟶	Helix (2% Tfa)	Orn C_o (backbone)	Downfield	2.8	123
			Orn C_α	Downfield	3.2	
			Orn C_β	Upfield	0.8	
[Phe 8-$^{13}C^u$]SRNase-S[b]	Phe 8 random (synthetic-(1-15) peptide) ⟶	Phe 8 helix (SRNase-S)	Phe C_o	Downfield	1.3	97
			Phe C_α	Downfield	0.7	
			Phe C_β	Upfield	0.5	
[Gly 6-$^{13}C_{o,\alpha}$]SRNase-S	Gly 6 random (synthetic-(1-15) peptide) ⟶	Gly 6 helix (SRNase-S)	Gly C_o	Downfield	2.3	119
			Gly C_α	Downfield	0.7	
[Ala 5-$^{13}C_u$]SRNase-S	Ala 5 random (synthetic-(1-15) peptide) ⟶	Ala 5 helix (SRNase-S)	Ala C_o	Downfield	2.8	120
			Ala C_α	Downfield	2.9	
			Ala C_β	Upfield	0.2	

[a] Tfa: trifluoroacetic acid; Orn: ornithine.
[b] u denotes that residue is uniformly enriched in all carbon atoms.

in stabilizing the active ribonuclease conformation.[32,71,125,126] And the direct importance of His 119 in catalysis has been emphasized via study of the minimally perturbed semisynthetic complex of ribonuclease-(1-115) with [4-fluoro-His 119]-synthetic-(116-124).[101] However, ribonuclease-related issues, such as its particular type of active site, the role of helix propensity in folding, and the involvement of hydrophobic contact in peptide-protein interactions, represent only a few of the numerous phenomena that we wish to understand in proteins as a whole. In this regard, investigations akin to those with ribonuclease-S will be necessary for a variety of semisynthetic polypeptides.

A glance at the accomplishments so far (Table 1) reveals that the potential does exist to use semisynthetic polypeptides as routes to study a wide spectrum of protein-related phenomena. A few of these cases have reached a stage of development sufficient to have already allowed broad-based questions to be asked through partial synthesis.

1. Enzymes: Staphylococcal Nuclease, Phospholipase A_2, Carbonic Anhydrases, Trypsin

Staphylococcal nuclease is a 149-residue, disulfide-less polypeptide (Figure 13) for which several semisynthetic routes have been fully developed (see Figure 14 and Table 1). Two types of noncovalent fragment complexes exist,[19,47] providing the basis for semisynthetic manipulation of sequence by making modestly-sized peptides. This has been accomplished, for example, for the amino-terminal (6-48) fragment, which can be reassociated with native fragments (49-149) or (50-149) to produce a folded, enzymatically active species defined as semisynthetic nuclease-T.[39] Additionally, synthetic-(99-149) can be reassociated with native nuclease-(1-126) to produce another functional semisynthetic species, in this case of the nuclease "type II" complex.[73] Covalent semisynthesis of the intact nuclease molecule now is possible via enzymatic restitching of nuclease-T.[38,63] A productive covalent species has been produced by trypsin-catalysed condensation of [Gly 48]synthetic-(6-49) and nuclease-T-(50-149). While the resultant intact form is useful in itself as a parent, fully active (6-149) species, a straightforward further extrapolation of this work would be resynthesis using [Gly 5, 6, 48]synthetic-(1-49), which with the native (50-149) fragment should yield a full-sequence covalent semisynthetic parent nuclease. Further, the kinetic trap manipulation used for the synthesis of ribonuclease-(1-15) from (1-10) and (11-15)[65] could (at least in theory) be applied to the nuclease case. A (6-49) fragment could be made from nuclease-(6-36) (derived from native nuclease-T-(6-48) by cleavage at the single Arg at position 36 in the peptide) and [Gly 48]synthetic-(37-49), using nuclease-T-(50-149) as a kinetic trap for enzymatic resynthesis of the total fragment as catalyzed by clostripain. The resultant complex between [Gly 48]-semisynthetic-(6-49) and nuclease-T-(50-149) then could be enzymically condensed in glycerol, with trypsin, to produce another version of active semisynthetic nuclease. Thus, semisynthesis could be carried out in nuclease with actual synthesis limited, if desired, to the region of residues 37-49.

Staphylococcal nuclease-T semisynthesis has offered an opportunity to study a variety of protein properties. The nuclease type of active site, including the essential Ca^{++}-binding residues, has been the object of significant semisynthesis study.[128-130] Of note, analogues at positions Asp 19, 21, and 40, and Glu 43 have shown that even small steric alterations in the positions of the carboxyl groups lead to a serious loss of function (Figure 15). The participation of these residues in orienting Ca^{++} in a productive mode in the active site, as deduced by X-ray crystallographic analysis,[131] is substantiated. The results for Arg 35 (Figure 15) are consistent with the view[131] that this residue participates in active site nucleotide binding.

In addition, since the 10-36 sequence of nuclease is almost totally involved in β structure, including three strands of antiparallel β-pleated sheet (Figure 13), synthetic manipulation of residues in this region has been initiated in order to address questions

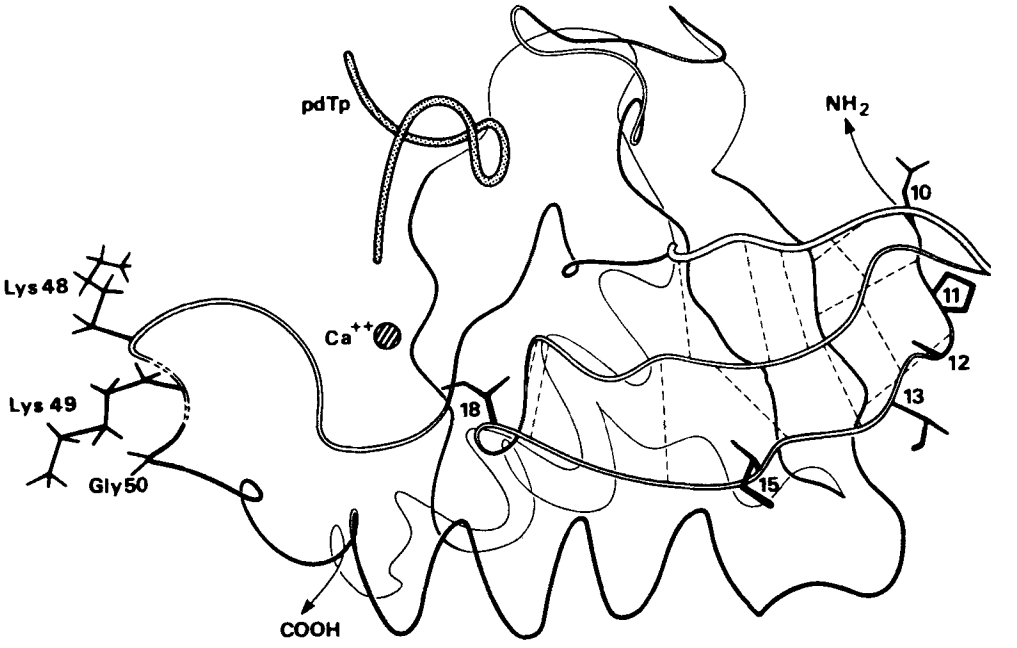

FIGURE 13. Schematic representation of staphylococcal nuclease structure as derived by X-ray crystallographic analysis. The backbone is indicated by the open line. Specific side chains are drawn in and numbered for reference, including Glu 10, Pro 11, Ala 12, Thr 13, Ile 15, Ile 18, Lys 48, Lys 49, and Gly 50. Main chain hydrogen bonds contributed by residues in the amino-terminal region are indicated by dashed lines. The backbone tracing is broken between residues 48, 49, and 50 to indicate sites of selective tryptic hydrolysis and resynthesis. The two active-site ligands Ca^{++} and deoxythymidine-3',5'-diphosphate (pdTp) are denoted. (Adapted from Chaiken, I. M., *J. Biol. Chem.*, 247, 1995, 1972. With permission.)

about formation of β-pleated sheet and β-bends and the role of these in folding and conformational stabilization. Information is available so far from two types of analogues. First, a study of truncated amino-terminal variants of this region[127,132] has indicated that as antiparallel pleated sheet-stabilizing hydrogen bonds are eliminated by serial amino-terminal deletion through residue 15, the ability to form a productive nuclease-T complex is progressively reduced and finally lost. Truncation of the total segment 6-13, leading to a 14-47 sequence and concomitant loss of several potential β-stabilizing hydrogen bonds, causes critical elimination of productive complex formation.[127] In addition, substitution of Ile residues 15 and 18 with the progressively less β-pleated sheet-frequent Leu and Gly residues, with resultant progressive decrease in stability of the analogue complexes, has shown that β-pleated sheet propensity probably is an overriding feature of the information provided by these residues.[4,127] These results, combined with the results with Ca^{++}- and nucleotide-liganding residues, again reflect the duality of use of sequence information, on the one hand providing specific chemical structure and on the other providing a propensity to form the conformation necessary to bring the few critically interacting groups to their proper places.

The nuclease case, by providing useful studies for α-helices in the carboxyl terminal region as well as for the above-mentioned properties in the amino terminal third of the molecule, forms a useful complement to the ribonuclease case. Together they allow not only different types of catalytic sites, but also a variety of ordered conformational elements to be explored from the synthetic point of view. Several other enzymes are subjects of ongoing semisynthesis study. These include carbonic anhydrases B and C,[87,133] trypsin,[134] and phospholipase A_2. For the last, analogue studies have advanced

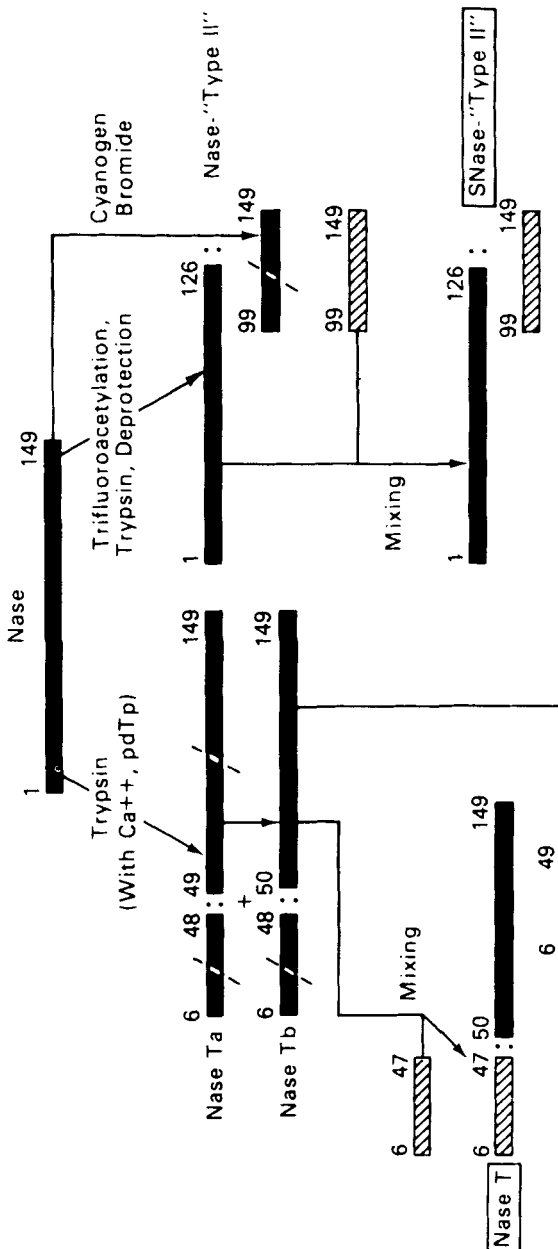

FIGURE 14. Scheme of types of semisynthetic derivatives produced from staphylococcal nuclease (Nase). See legend of Figure 4 for explanatory notes.

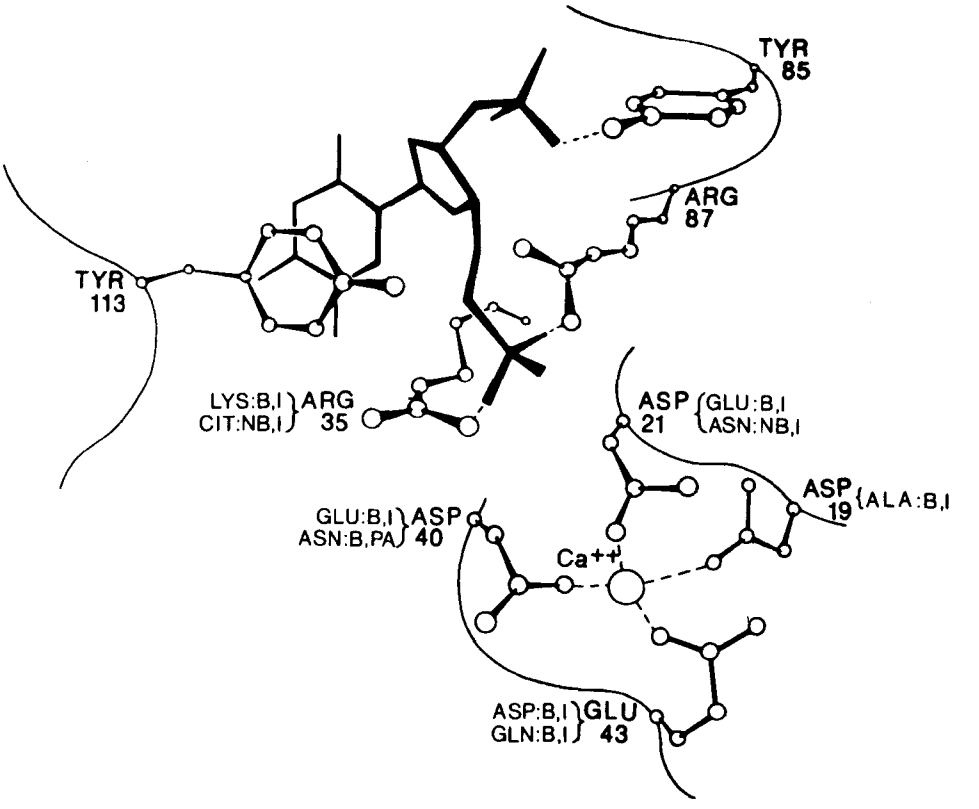

FIGURE 15. Correlation of results obtained for nuclease active-site analogues with a scheme of the deoxythymidine-3′,5′-diphosphate and Ca^{++} binding site region of nuclease. The symbols B and NB indicate, respectively, whether the synthetic-(6-47) analogue, with the residue substitution indicated, was or was not bound to nuclease-T-(49-149). The symbols I and PA indicate, respectively, whether the resulting complex or fragment mixture was enzymatically inactive or partially active. (Adapted from Chaiken, I. M. and Anfinsen, C. B., *J. Biol. Chem.*, 246, 2285, 1971. With permission.)

significantly,[54,135] using predominantly the stepwise semisynthesis approach and leading for example to a des 1-3 species to which different substituents were added. These studies have demonstrated the importance of residues in the amino-terminal portion of the native sequence for enzyme function through interactions with lipid-water interfaces.[136]

2. Heme Proteins: Cytochrome c, Myoglobin

For cytochrome c, as for staphylococcal nuclease, several cogent semisynthesis routes have been devised leading to both covalent[23,24,74,75] and noncovalent[76,77] reconstituted forms (see Figure 16 and Table 1). The fragment condensation of Corradin and Harbury[57] of the cyanogen bromide-derived heme fragment [HSer 65] (1-65, heme), with (66-104) through the carboxyl terminal-activated homoserine lactone (present in equilibrium with homoserine), has formed the basis for several types of covalent semisynthesis. The segment of residues (66-104) has been incorporated in the above condensation as a totally synthesized species.[74] Alternatively, to reduce the extent of synthesis necessary, a semisynthetic hybrid of (66-104) has been formed from subpieces.[23,24,75] In this latter case, [HSer 80](66-80) and (81-104) have been derived by cyanogen bromide fragmentation at Met 80. Then, sequences (66-79) and (80-104) have been prepared from these native pieces, or the (66-79) substituent has been obtained by

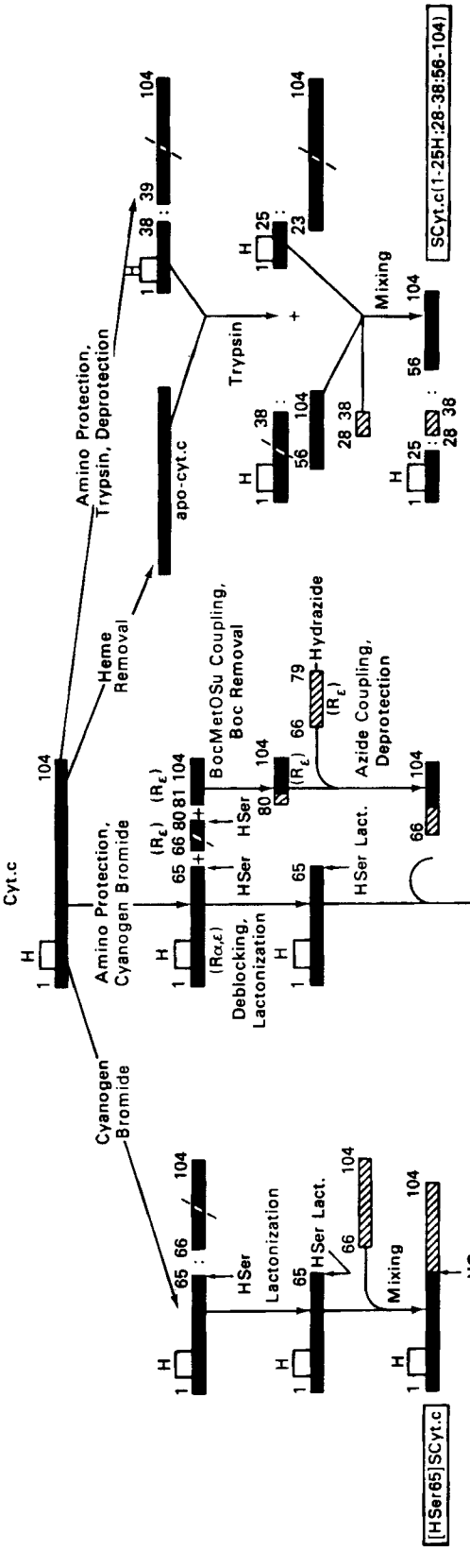

FIGURE 16. Scheme of types of semisynthetic derivatives from cytochrome c (cyt. c). H indicates heme; R, amino blocking group; OSu, succinimide ester; Boc, t-butyloxycarbonyl; apo-cyt c, cytochrome c with heme removed; HSer and HSer Lact, homoserine and homoserine lactone, respectively. See legend of Figure 4 for other explanatory notes.

total synthesis. Whatever the source, the subpieces then were combined to form a complete (66-104) using protected fragments to direct proper peptide bond formation.

Cytochrome c semisynthesis also is possible using the set of noncovalent complexes formed by proteolytic fragments.[35,48,49,76] Several systems have been derived, including those forming functional complexes involving fragment combinations (1-38, heme):(39-104) and (1-25, heme):(28-38):(56-104). In the latter, synthetic (28-38) has been incorporated into a semisynthetic three-fragment complex.[77] Also, both of the above systems contain relatively small heme fragments that seem attractive for synthesis. While such syntheses will require not only preparing the peptide sequences but also stereospecific covalent incorporation of the heme moiety, this latter perhaps will be attainable using enzymatic systems, as demonstrated for apocytochrome c.[137,138] Finally, in the above two-fragment case of (1-38, heme):(39-104), covalent semisynthesis can be achieved since it has been found that the components can be enzymically condensed by clostripain catalysis in 90% glycerol.[64]

Since both the 66-80 and 28-38 regions of cytochrome c contain a pronounced set of evolutionarily conserved residues in the protein, as indicated in Figure 17, the synthetic modification of these sequences made possible with the systems described above can be quite powerful for decoding the specific information preserved therein. For example, successful replacement of Met 80 (a heme ligand) by ethionine, but not by S-methylcysteine, has demonstrated a strict geometric constraint on the essential side chain sulfur atom in position 80.[140] When considered with the extensive evolutionary variation of much of the cytochrome sequence, this requirement at residue 80 can be considered a reflection of the view, discussed above for ribonuclease-S and nuclease-T, of needing particular elements of detail amongst an overall set of conformational propensity information.

Among other positions altered in the 66-80 sequence, Tyr 74 has been replaced by Leu with some alteration in stability but without loss of most functional properties.[141] Interestingly, while position 74 is almost always Tyr and invariably aromatic in eukaryotic cytochromes c, Leu can be found at position 74 in the cytochrome c from the bacterium *Paracoccus denitrificans*. Understanding the meaning of evolutionary conservation for such residues that can be replaced synthetically provides an interesting challenge for further semisynthetic study. More generally, the semisynthesis approach could prove useful in elucidating the functional and structural limitations which lie behind the patterns of evolutionary sequence variation, such as covarions, as deduced for cytochrome c.[139,142,143]

Only limited analogue studies have been conducted thus far with the proteolysis-derived noncovalent cytochrome c fragment complexes, with those carried out being via stepwise semisynthesis.[76] Nonetheless, given the fact that the crucial heme is placed in the amino-terminal region, further study offers an excellent opportunity to correlate sequence with such properties as structural requirements for the heme ligand at position 18 (tuna sequence numbering as in Figure 17) and the determinants for stereospecific covalent incorporation of heme.

Another heme protein, myoglobin, has been the object of significant effort to develop large-fragment noncovalent semisynthetic systems. However, these have been met by fragment solubility difficulties.[53] In the meanwhile, an elegant set of semisynthesis routes has been devised by Gurd and colleagues[52,78] for manipulation at the amino-terminal end of the molecule. These include not only derivatives designed for stepwise procedures,[78] but also a fragment system of (1-14) and (15-153) derived by BNPS-skatole cleavage at tryptophan 14.[52] While analogue studies have been limited so far, stepwise deletion of Val 1 and Glu 2 and replacement of Gly for Val 1 already have been used[144] to evaluate the contributions of electrostatic properties in the amino-terminal sequence to protein

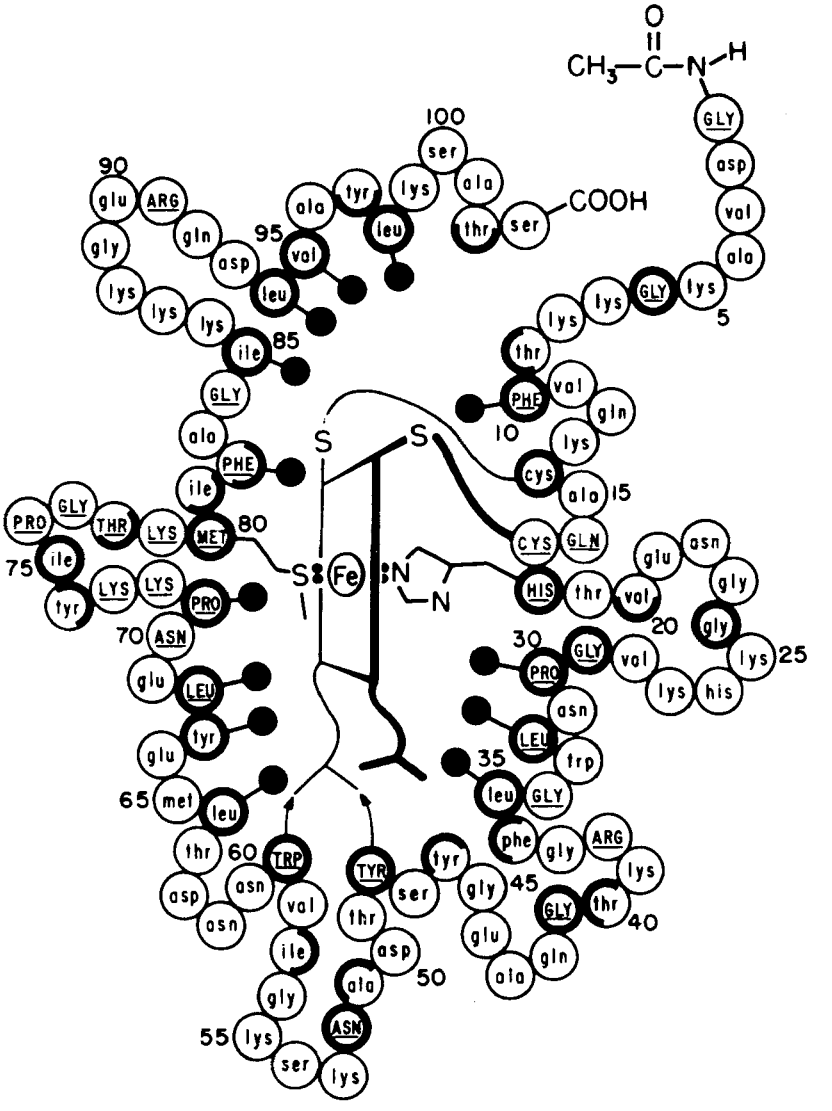

FIGURE 17. Chain-packing diagram of tuna reduced cytochrome c, showing amino acid residues evolutionarily invariant in all eukaryotic species examined (underlined capitals), buried side chains (heavy circles), external side chains (thin circles), side chains half-buried in the surface (half-heavy and half-thin circles), and side chains packed against the heme (black dots). (With permission from Dickerson, R. E. and Timkovich, R., *The Enzymes,* 3rd ed., Vol. 11, Boyer, P. D., Ed., 1975, 397. Copyright by Academic Press Inc. (New York) Ltd.)

conformation. The prospective use of this system to study such general issues as electrostatics, helix formation, noncovalent heme incorporation, and O_2 binding should provide an important stimulus to myoglobin semisynthesis. Furthermore, the above procedures devised for modification of the amino-terminal region could be applied directly to homologous polypeptides, such as the α chain of hemoglobin which has a single Trp residue at position 14.

3. Hormones: Insulin, Somatotropin, Adrenocorticotropin, α- and β-Melanotropins

While methods for preparing semisynthetic hormones have been worked out for several species, including somatotropin, adrenocorticotropic hormone, and α- and β-melanotropins (see Table 1), the most notable hormonal objective so far has been insulin. This last-mentioned case has been intensely developed, leading to several types of semisynthetic processes for both stepwise and fragment condensation manipulations (see Figure 18 and Table 1). Early studies led to successful total synthesis of one of the chains (A or B) and recombination with the second via intra- and interchain disulfide formation.[79] However, the inefficiency of chain recombination[145] due to the loss of sequence information from the originally biosynthesized proinsulin makes this route somewhat unattractive for studies of an extensive series of analogues. The more recently described use of reversible interchain linkages, between the α-amino group of the A chain and ε-amino group of Lys 29 of the B chain, has improved this efficiency by providing an effective mimic of the C-peptide of the "pro" form.[145] This type of linkage, or the C-peptide itself, could provide the basis for future cogent semisynthetic work.

In the meanwhile, most semisynthetic analogue studies have utilized other approaches. With the demonstration that the three amino groups of insulin — two α-amino groups (of the A and B chains) and the Lys B29 ε-amino — differ in reactivity,[146] general procedures have been devised to produce selective amino-terminal degradation and extension of the one or the other of the two chains. The scheme in Figure 18 shows a useful example of such reactions. In addition to the above, internal proteolytic fragmentation and reconstitution with synthetic fragments has been possible with insulin due to the presence of only two sites of trypsin digestion, at Arg 22 and Lys 29 of the B chain. This situation has led, for example, to preparation of desoctapeptide (B23-B30) insulin and readdition of synthetic sequences corresponding to residues 23-30 through either chemical fragment condensation[55,56] or enzymatic resynthesis.[62] Fragment-based semisynthesis routes also have become an objective for the biosynthetic precursors of insulin, namely proinsulin and preproinsulin.[147]

So far, analogue studies of insulin via semisynthesis have been limited largely to those deriving from stepwise procedures. The Phe B1 position has been found to be dispensible, providing the basis for probe insertion at this locus of the hormone.[89] Modifications at the amino terminus of the A chain have shown the involvement of this region in function. For example, Geiger and Obermeier[56] have observed that D-amino acids are more favorable substituents at residue A1 than the corresponding L forms. They have reasoned that these results may reflect the conformational constraints expected in this region (see Figure 19).

4. Other Promising Systems: Immunoglobulins, Protease Inhibitors, Ferredoxins

Of other proteins for which semisynthetic routes have been devised, those for the ferredoxins have been of the stepwise variety at the amino termini.[85] For soybean trypsin inhibitor and the related trypsin-kallikrein inhibitor, semisyntheses leading to replacement of internal residues have been developed.[59-61] These routes depend again on stepwise degradation and addition and are possible by virtue of internal peptide bonds in the inhibitors which are selectively hydrolyzed by the appropriate proteases (as a part of the mode of action of these as inhibitors). For bovine pancreatic trypsin inhibitor, a case which does not provide the same protease-mediated internal bond hydrolysis, cyanogen bromide cleavage has been studied, as cited above, as a way to provide a useful semisynthesis system for future analogue preparation.[72]

Immunoglobulin semisyntheses have been attempted by synthetic replacement of large polypeptide segments (as with the early insulin studies), based heavily on cases where a two-chain F_v fragment (the amino-terminal domain of the Fab fragment) has been

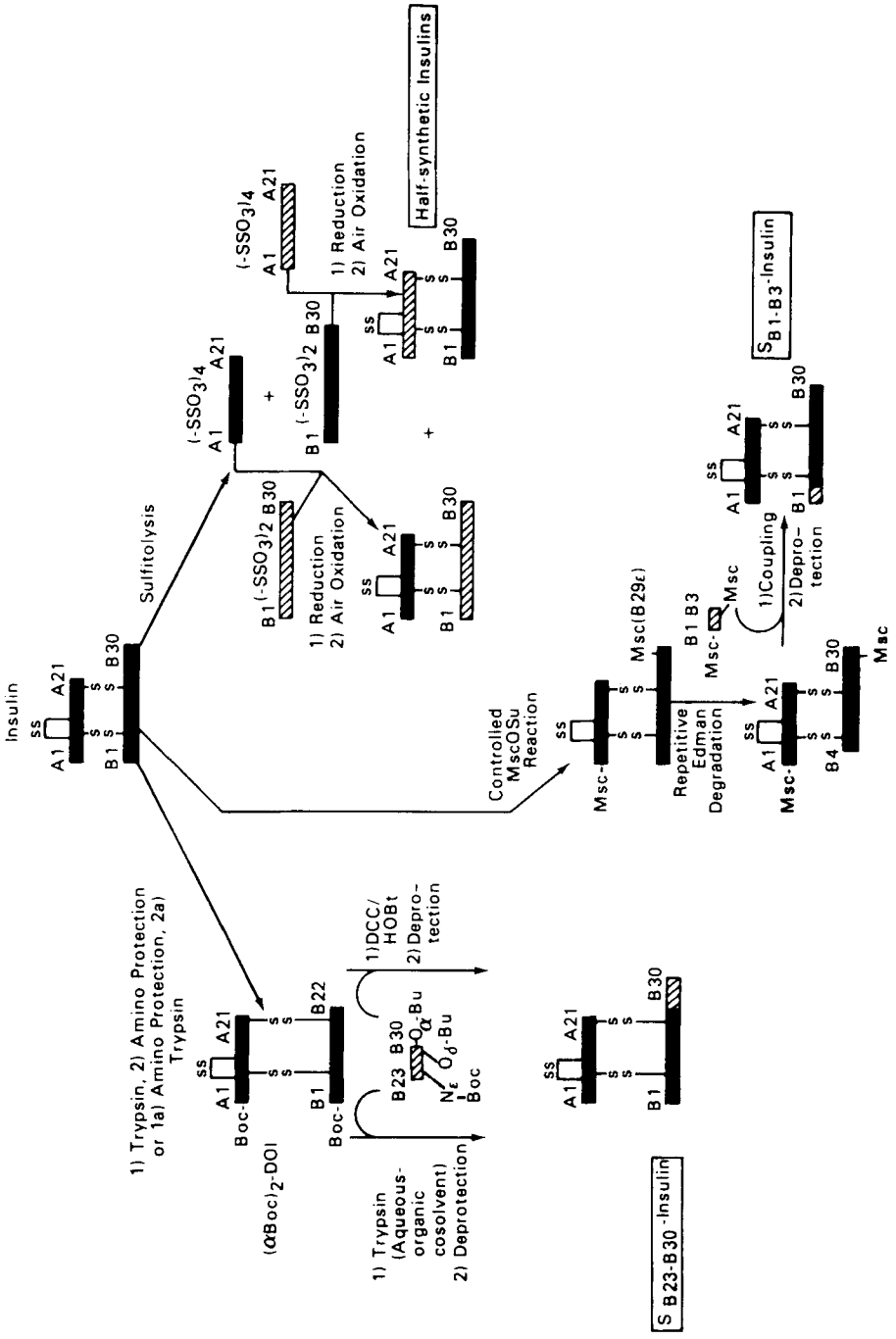

FIGURE 18. Scheme of types of semisynthetic derivatives obtained from insulin. DOI indicates desoctapeptide (B23-B30) insulin; α- or δ-OBu, butyl ester or ether, respectively, of Thr B30; DCC, dicyclohexylcarbodiimide; HOBt, hydroxybenzatriazole. The notation "$S_{x\text{-}y}$ insulin" is used to define the synthetic portion (x-y) in the semisynthetic species. See legend to Figure 4 for other explanatory notes.

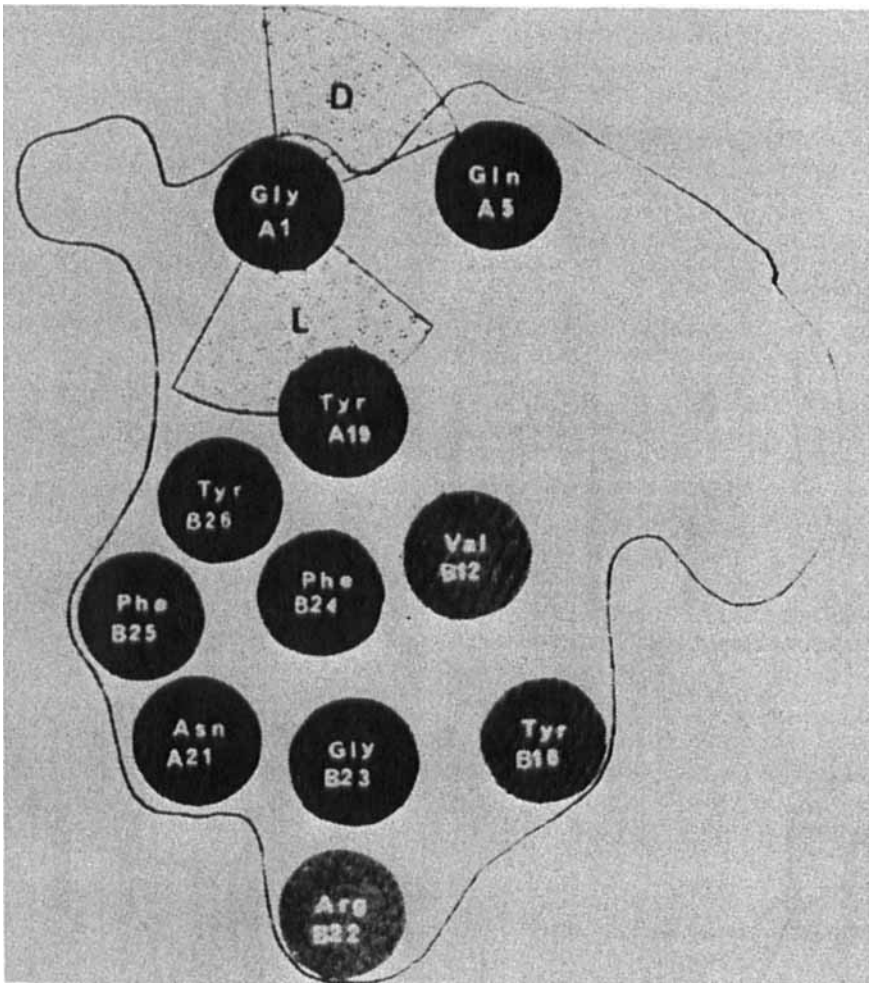

FIGURE 19. Possible arrangements of the side chains of D- and L-amino acids introduced into position A1 of insulin by semisynthesis. (From Geiger, R. and Obermeier, R., *Proinsulin, Insulin, and C-peptide,* Baba, S., Kaneko, T., and Yanaihara, N., Eds., Excerpta Medica, Amsterdam, 1979, 62. With permission.)

obtainable and shown to have intact antigen-binding activity.[36,84,148] Using such systems, work has proceeded on solid phase synthesis of either the heavy- or light-chain component (V_H or V_L, respectively). With reasonable synthetic products, the potential use of these to investigate the importance of various sequences in folding and antibody function should be an exciting chapter in semisynthesis studies.

C. Future Directions

Given a set of polypeptides such as the above, a reasonable attack can be made on a variety of protein-related properties and concepts using the systematic, controlled sequence modification afforded by semisynthesis. Semisynthesis routes either are available or are close to fruition for most of the cases cited. Hopefully, these routes will provide models to stimulate development of similar systems for a far greater variety of proteins for which dependable amino acid sequences and, if possible, three-dimensional

structures are known. Given the availability of such species to study the relationship between sequence and higher order properties, the usefulness of the approach will be limited by the questions we ask.

V. PRODUCING USEFUL POLYPEPTIDES

While the ability to employ semisynthesis to understand structure-function relationships seems at hand, the role of the method (or total synthesis, for that matter) to produce clinically or industrially useful proteins is less clear. Definitive semisynthesis methods have been devised to produce human insulin from porcine insulin through either chemical or enzymatic fragment condensation of the desoctapeptide (B23-B30) of human sequence.[55,56,62] Even more simply, this conversion has been effected through stepwise deletion of B30 Ala from porcine insulin and reconstitution with the human-specific B30 Thr.[149] In the ribonuclease-S case, it has been found that the semisynthetic complex produced by model S-peptide, with several of its residues converted to Ala, has a much lowered affinity for S-peptide-directed ribonuclease antibodies.[101] It is reasonable to expect that, in a general sense, semisynthesis could be a powerful method for massaging an amino acid sequence so as either to wash out undesirable properties (such as antigenicity), or to enhance desirable ones (such as a given enzymatic specificity or conformational stability). Thus, semisynthesis offers a way to define nonnative sequences which could be useful alternatives to the native sequence. In spite of this promise, it does not necessarily follow that such alternative sequences, once defined by semisynthesis, should be produced on a large scale by this same method, which would be a commercially expensive and unattractive route requiring large amounts of native polypeptide.

Recent progress in genetic engineering suggests an alternative. Several clinically important or potentially important proteins, such as somatotropin, insulin, and interferon, are being produced by cloning the genes for their sequences via recombinant DNA procedures.[150] This methodology may well be the method of choice, when the appropriate messenger RNA can be isolated, for producing large amounts of such proteins. But, the above clone-producing systems generally have used native DNA codes, so that only protein of native sequence would be produced. This would not take advantage of semisynthetically defined nonnative sequences which have more beneficial properties than those of the native sequence.

There have been, however, experimental alternatives that could allow recombinant DNA procedures to effect production of new sequences. In the case of insulin, DNA has been synthesized to code for each of the A and B chains and then incorporated into plasmids to produce A and B chain-synthesizing bacterial clones.[151] This result suggests the prospect that the cloning route could utilize nonnative DNA to produce polypeptides of nonnative sequence.[152,153] When such sequences are suggested by semisynthesis study, a DNA species could be synthesized to code for the appropriate amino acid sequence and then be incorporated to effect protein production. The ability to screen bacteria for the proteins they make can allow selection of the appropriate clone, thus avoiding problems with synthetic DNA heterogeneity. In addition to this "purification" aspect, cloning leads to amplification of the amount of DNA to produce greater amounts of protein. A shortcut to the above approach of total DNA synthesis could be the point mutation of native DNA sequences.[154] Using either option, an approach such as shown in Figure 20 perhaps could be invoked to allow genetic engineering to bear the fruits of semisynthesis. It remains for future development of chemical synthesis and recombinant DNA methods to indicate just how much sense this scheme makes at a practical level.

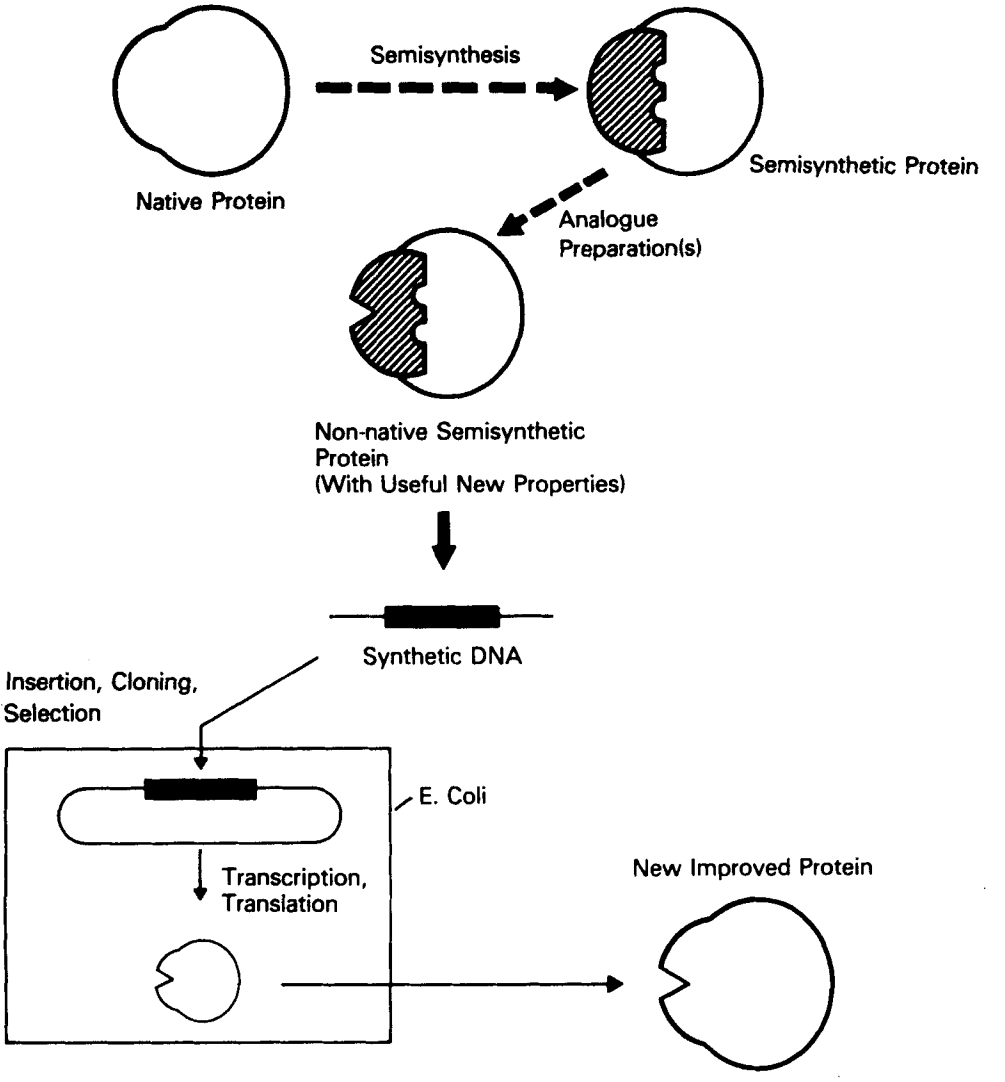

FIGURE 20. Scheme depicting manner in which information from analogue semisynthesis can be interfaced with recombinant DNA technology to produce nonnative polypeptide sequences with beneficial new properties. Here, development of a semisynthesis process is shown to lead to analogue preparations and ultimately to an analogue with desired properties. Based on this sequence a synthetic or partially synthetic DNA is made and incorporated into clones. Selected clones then can produce amplified amounts of the new polypeptide with improved properties.

VI. CONCLUDING COMMENTS

A central feature of semisynthesis is the interplay of techniques and ideas, from a variety of fields, that have helped shape and define its utility. The initial evolution of the approach has been fueled by the marriage of the tools and insights of peptide and protein chemistry and biochemistry. The result is a viable set of techniques which can allow important theoretical and practical issues to be addressed. Thus, by being able to make purified semisynthetic products suitable for detailed chemical, physical, and functional characterization, the effects of sequence engineering can now be described for several

protein systems. Therein, it is possible to arrive with precision at assertions for these cases about roles of particular amino acids in dictating conformation and function. To be sure, there is still much to achieve in the development and use of semisynthesis for important but as yet untried or only partially fulfilled peptides and proteins representing vehicles for studying new issues. And while such studies can lead to rational designs for analogue polypeptides with clinically or industrially useful new properties, the manner by which such specifically tailored nonnative species are ultimately produced is open to question. Perhaps another interdisciplinary marriage, that between sequence engineering through semisynthesis methods (for defining new sequences) and genetic engineering through recombinant DNA methods (for producing the analogues), will prove useful. Whatever the uncertainties, it seems fair to conclude at this juncture that opportunities are at hand for biochemists to use semisynthesis in the study of large peptides and proteins.

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