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Mini-review

Analysis of protein folding and function using backbone modified proteins

Xiaoye Yang, Min Wang, Michael C. Fitzgerald*

Department of Chemistry, Duke University, Durham, NC 27708, USA

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Abstract

With the recent development of chemical and biological methods to introduce backbone modifications into the polypeptide chains of proteins, there have been a growing number of site-directed mutagenesis experiments focused on understanding the role of the polypeptide backbone in protein folding and function. The substitution of a main chain amide bond with an ester bond is now a popular mutation to investigate the role of the polypeptide backbone in ligand, binding, enzyme catalysis, and protein folding. Here we review the results of studies on some 25 ester-bond containing analogues from nine different protein systems. The structural, thermodynamic, and functional consequences of introducing backbone amide- to ester-bond mutations into these protein systems are discussed.

Keywords: Analogues; Protein systems

1. Introduction

Protein mutagenesis experiments have provided a wealth of information about protein folding and function over the past several decades. Until recently, such experiments have been largely focused on understanding the role of individual amino

* Corresponding author. Fax: 1-919-660-1605.
E-mail address: mfitz@chem.duke.edu (M.C. Fitzgerald).

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acid side chain functionalities in such processes as ligand binding, enzyme catalysis, and protein folding. Only in the last decade have protein mutagenesis experiments been expanded to include investigations on the role of the polypeptide backbone in these processes. This expansion of site-directed mutagenesis experiments has been made possible due to the recent development of new experimental methods that enable the introduction of backbone modifications into proteins. The recombinant DNA methods that have been traditionally employed to introduce site-specific mutations into protein molecules do not permit the introduction of mutations into the polypeptide backbone of proteins.

Two different technologies (one relying on total chemical synthesis strategies [1,2b] and one relying on nonsense suppression techniques [3,4]) now facilitate the preparation of backbone modified protein analogues. The substitution of a main chain amide bond with an ester bond (see Fig. 1) has been the most common mutation introduced into the polypeptide backbone of proteins using the above technologies. Such ester-bond containing protein analogues are the focus of this review.

The ester bond is an especially useful mutation for probing the role of the polypeptide backbone in protein folding and function. On the one hand, the ester bond is a fairly conservative substitution for an amide bond as both types of bonds have similar conformational preferences (i.e., *trans* planar), have similar bond angles, and have similar bond lengths [5–7]. Thus, in polypeptide chains ester bonds and amide bonds have similar Ramachandran conformational energy plots [7]. On the other hand, the ester bond and the amide bond differ in their hydrogen bonding capabilities (see Fig. 1). The carbonyl group in an ester bond is a weaker hydrogen bond acceptor than the carbonyl group in an amide bond [8]. Also, in contrast to the

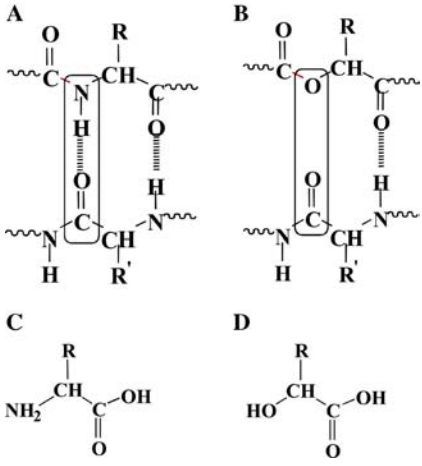

Fig. 1. Schematic representation of the backbone amide to ester-bond mutation in proteins. (A) Backbone amide bond in the polypeptide backbone of a protein. (B) Backbone ester-bond in the polypeptide backbone of a protein. (C) Naturally occurring amino acid residue. (D) α-Hydroxy acid. The R group represents the amino acid side chain.

amide bond the ester bond lacks a hydrogen bond donor (i.e., there is an O atom in place of the NH group).

The different hydrogen bonding characteristics of ester bonds and amide bonds make the amide- to ester-bond mutation in proteins an attractive means by which to probe the role of backbone hydrogen bonding interactions in protein folding and function. Hydrogen bonds involving backbone C=O and/or backbone NH groups constitute a large number of the native contacts in folded proteins, and the

Table 1
Chemically derived ester-bond containing protein analogues studied to date

Protein system	Ester-bond analogue[a]	Structural analysis	Protein folding $\Delta\Delta G_f$ (kcal/mol)	Functional analysis
Turkey ovomucoid third domain (OMTKY3)	OL18[b]	wt-X-ray[c]	—	ΔG_B = 1.5 kcal/mol[b,c]
Eglin c	VOL43[d]	—	—	ΔG_B = 0–2.9 kcal/mol[d]
	TOL44[d]	—	—	ΔG_B = −0.4 to −3.6 kcal/mol[d]
	OL45[d]	—	—	ΔG_B = 2.1–4.5 kcal/mol[d]
	OL47[d]	—	—	ΔG_B = 1.4–2.0 kcal/mol[d]
	OG70[e]	wt-NMR[e]	—	ΔG_B = 2.7 kcal/mol[e]
Metal-assembled helical bundle protein	OQ8[f]	wt-CD[f]	0.7[f]	wt electron transfer rate[f]
	OH9[g]	wt-CD[g]	1.1[g]	wt electron transfer rate[g]
	OQ8[g]	wt-CD[g]	0.6[g]	wt electron transfer rate[g]
	OL7[g]	wt-CD[g]	0.7[g]	wt electron transfer rate[g]
High-potential iron protein (HiPIP)	OA42[h]	wt-CD[h]		$\Delta\Delta E^0_{1/2}$ = 86 mV[h]
	OV57[h]	wt-CD[h]		$\Delta\Delta E^0_{1/2}$ = 126 mV[h]
P22 Arc repressor	POL8[i]	wt-CD[i]	2.5[i]	—
Chymotrypsin inhibitor (CI2)	[OV13,OA16, OV19,OA22][j]	—	2.93[j]	wt inhibition[j]
4-Oxalocrotonate tautomerase (4OT)	OI2[k]	wt-CD[k]	21.9[k]	wt catalysis[k]
	OI7[k]	wt-CD[k]	24.6[k]	wt catalysis[k]
	OL8[l]	wt-CD[l]	—	k_{cat} Reduced 20-fold[l]
	[OI2,ROA39][m]	wt-CD[m]	—	wt catalysis[m]
GCN4	OL6[n]	wt-CD[n]	0.7[n]	—
	OV10[n]	wt-CD[n]	3.2[n]	—
	OL13[n]	wt-CD[n]	3.09[n]	—
	OL20[n]	wt-CD[n]	2.37[n]	—
	OV24[n]	wt-CD[n]	3.56[n]	—
	OL27[n]	wt-CD[n]	3.17[n]	—
HIV-1 Protease	OI50[o]	—		k_{cat} Reduced 2-fold[o]

[a] The ester-bond analogues are denoted OXN, where O indicates the backbone replacement of a backbone NH group with an oxygen atom, X denotes the type of amino acid residue N-terminal to the ester-bond mutation (identified with its normal single letter code), and N denotes the position of the ester-bond mutation in the protein's polypeptide chain. XOXN signifies that the wild-type amino acid N-terminal to the ester-bond was mutated (the single letter code of the wild-type residue is listed first, then O, then the single letter code of the mutation). The [ ] notation signifies that the protein analogue contains multiple mutations.

[b] Ref. [11], [c]Ref. [12], [d]Ref. [13], [e]Ref. [14], [f]Ref. [15], [g]Ref. [16], [h]Ref. [17], [i]Ref. [18], [j]Ref. [19], [k]Ref. [5], [l]Ref. [20], [m]Ref. [21], [n]Ref. [22], [o]Ref. [23].

large majority of these contacts involve so-called backbone–backbone hydrogen bonding interactions [9,10]. Such backbone–backbone hydrogen bonding interactions are ubiquitous in α-helices, in β-sheets, and in protein–ligand interfaces. The recent ability to prepare ester-bond containing protein analogues by chemical and biological methods has allowed for the detailed experimental analysis of backbone–backbone hydrogen bonding interactions in protein folding and function.

Here we highlight the recent use of ester-bond containing protein analogues to elucidate the role of the polypeptide backbone in protein folding and function. The results of studies on some 25 different ester-bond containing analogues from nine different protein systems are reviewed, and the structural, thermodynamic, and functional consequences of introducing backbone amide- to ester-bond mutations into proteins are summarized (see Table 1). A brief discussion of the experimental methods used to generate ester-bond containing protein analogues is also presented below.

2. Preparation of ester-bond containing protein analogues

Ester-bond containing protein analogues have been prepared by both chemical and biological methods. The general strategy employed in both types of methods is similar, and it involves the site-specific incorporation of α-hydroxy acids into a protein's polypeptide chain (see Fig. 1). Chemical synthesis methods typically rely on stepwise solid phase peptide synthesis strategies to chemically couple the α-hydroxy acid into the growing polypeptide chain. Standard protocols for Boc-chemistry [24] have been used to assemble the growing polypeptide chain and special protocols [11] have been developed for coupling α-hydroxy acids to the growing polypeptide chain on the solid phase. Biological methods for incorporating ester bond into a protein's polypeptide chain rely on a nonsense suppression technique that was first reported by Schultz and co-workers [3]. The technique utilizes specialized in vitro [4,25] or in vivo [26] translation systems for the enzymatic coupling α-hydroxy acids to a growing polypeptide chain. These biological methods utilize specially charged tRNA molecules and exploit the translation machinery of the cell.

The relative ease with which large quantities of protein (i.e., 10's of milligrams of pure material) can be produced by chemical synthesis methods, has meant that the majority of ester-bond containing protein analogues prepared to date, have been prepared by total chemical synthesis strategies. All the ester-bond containing protein analogues highlighted in this review were prepared by total chemical synthesis methods. One notable limitation of total chemical synthesis methods is the length of pure polypeptide product that can be recovered in reasonably good yield after stepwise solid phase peptide synthesis. This length is somewhat dependent on the particular amino acid sequence but it is typically on the order of about 55 ± 10 amino acids.

One approach to extend the size range of proteins amenable to total chemical synthesis has been to develop chemical ligation strategies for the chemo-selective ligation of unprotected polypeptide chains prepared by solid phase peptide synthesis methods. Using a native chemical ligation strategy developed by Kent and co-work-

ers [2a] the total chemical synthesis of proteins in the 100 amino acid size range is routine, and there are several examples in the literature where proteins in the 100–200 amino acid size range have been prepared by total chemical synthesis using the native ligation strategy. (see reference [2b] for an overview of proteins prepared using the native chemical ligation strategy).

The absence of an inherent protein size limitation is an attractive feature of biological methods to prepare ester-bond containing protein analogues. In fact, the ester-bond containing analogues prepared to date using biological methods have been in protein systems with >150 amino acids [25–27]. However, one issue with using biological methods for the preparation of ester-bond containing analogues for mutational studies of protein folding and function is that the amount of material these methods yield can be relatively low. This limits the extent to which biologically prepared ester-bond containing protein analogues can be characterized by conventional biophysical techniques. For example, detailed structural data from high-resolution X-ray crystallographic and/or NMR methods are not feasible with the relatively small amounts of material that these biological methods currently yield.

3. Structural analysis of ester-bond containing proteins

Circular dichroism (CD) spectroscopy, nuclear magnetic resonance spectroscopy (NMR), and X-ray crystallography have all been utilized to analyze the three-dimensional structure of ester-bond containing protein analogues prepared by total chemical synthesis. The relative ease with which CD spectra can be acquired and analyzed makes CD spectroscopy an attractive technique for studying the structural properties of ester-bond containing protein analogues. In fact, a large majority of all the ester-bond containing protein analogues studied to date have had their three-dimensional structures analyzed exclusively by CD spectroscopy. Only in a few cases have the three-dimensional structures of ester-bond containing protein analogues been determined by high-resolution NMR spectroscopy and X-ray crystallographic methods.

Summarized in Table 1 are the structural results obtained on all of the chemically synthesized ester-bond containing protein analogues reported in the literature, to date. It is noteworthy that wild-type structures were detected for all the ester-bond containing protein analogues in Table 1 that were subjected to structural analyses. However, not included in Table 1 are unpublished results from our group on several additional ester-bond containing analogues of 4OT and the P22 Arc repressor that folded into nonnative-like three-dimensional structures as judged by far UV-CD spectroscopy. These 4OT analogues included several analogues (i.e., OA3, OI5) in which the ester-bond mutation specifically deleted backbone–backbone hydrogen bonding interactions in the middle of β-sheet regions of structure that defines the major subunit interface in this homohexameric enzyme system (see Fig. 2). A nonnative-like three-dimensional structure, as judged by far UV-CD spectroscopy, was also found for a P22 Arc repressor analogue in which the ester-bond mutation specifically deleted a backbone–backbone hydrogen bonding interaction at the end of the first α-

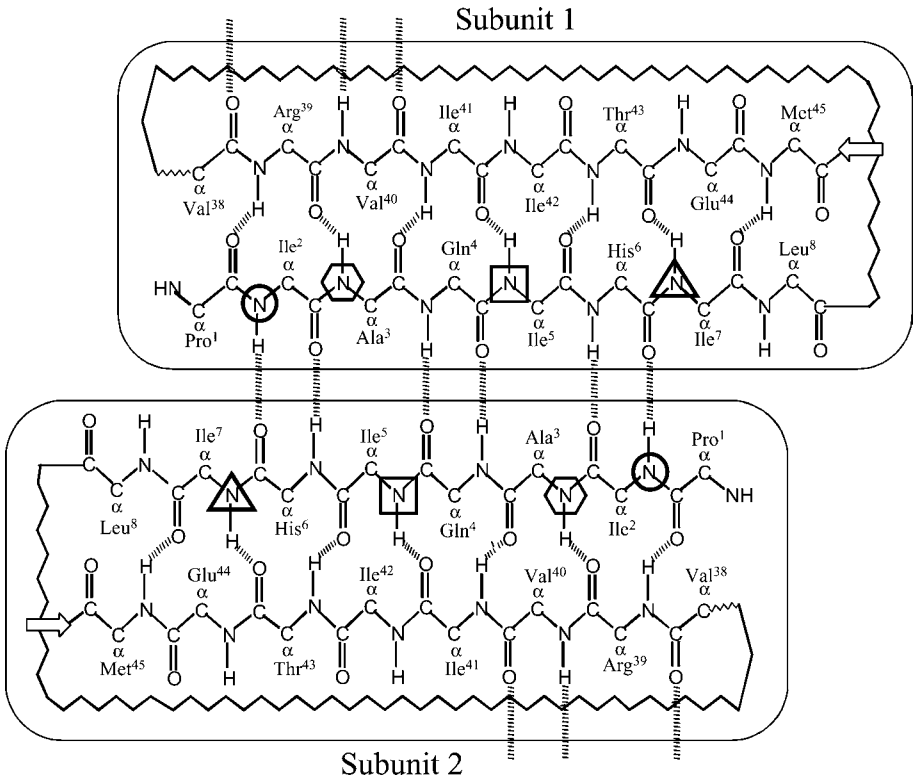

Fig. 2. Schematic representation of the β-sheet region of the major subunit interface in 4OT. The structure shown is repeated three times in the native three-dimensional structure of the 4OT's homohexameric structure. The site of the ester-bond mutations in the OI2, OA3, OI5, and OI7 analogues of 4OT are indicated with circles, hexagons, squares, and triangles (respectively).

helix in this protein's three-dimensional structure (see Fig. 3). Work is in progress to characterize the detailed structural properties of the nonnatively folded structures of these 4OT and P22 Arc repressor analogues.

Most of the ester-bond containing analogues in Table 1 were only subject to a CD analysis in order to ascertain whether or not they folded into a wild-type structure. Unfortunately, CD spectroscopy is only sensitive to gross structural changes that might result from the incorporation of an amide- to ester-bond mutation. Clearly, more sensitive structural probes are needed to assess more subtle structural perturbations that might introduced in a protein's structure when an amide- to ester-bond is incorporated into a protein's covalent structure. In some cases the functional properties of a particular analogue can provide useful information about the integrity of its three-dimensional structure. These functional assays can be related to the protein's biological function (see below). In other cases, the interaction of a protein with special nonbiological molecules can provide useful structural information. For example, in order to determine if the hydrogen bonds deleted in the GCN4 analogues

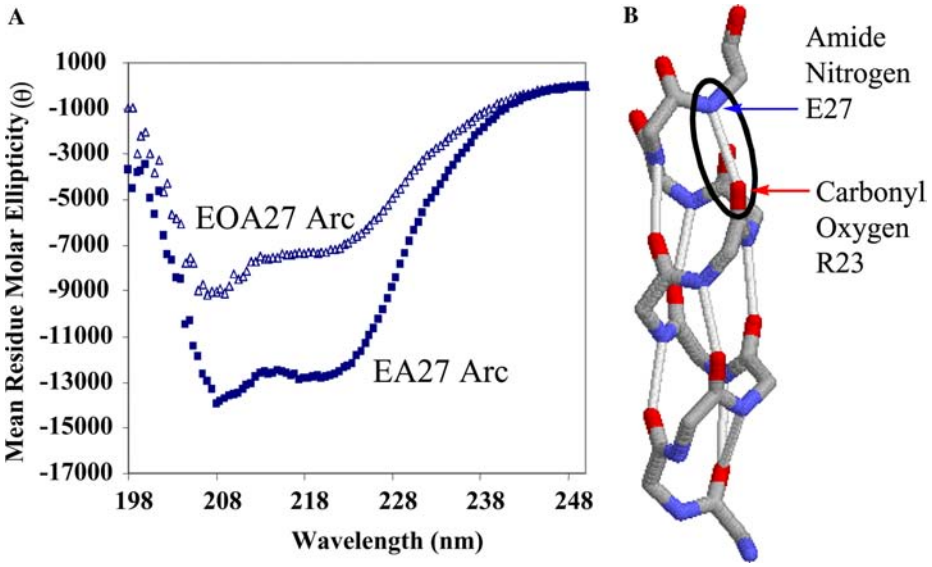

Fig. 3. (A) Far UV-CD analysis of the ester-bond containing analogue of P22 Arc repressor EOA27 and a wild-type control construct EA27. (B) Schematic representation of the hydrogen deleted in the EOA27 analogue of P22 Arc repressor analogue.

listed in Table 1 disrupted the protein's hydrophobic core, the ability of the ester-bond containing constructs to bind the hydrophobic dye, bis ANS, was compared to this same ability of the wild-type protein to bind this dye. The results were consistent with the ester-bond variants of GCN4 retaining a compact hydrophobic core similar to the compact hydrophobic core of the wild-type protein [22].

It is noteworthy that only two of the ester-bond containing analogues in Table 1 were subject to high-resolution structural analyses. In one case, two-dimensional homonuclear ^{1}H NMR experiments were used by Lu et al. [14] to analyze the three-dimensional structure of an ester-bond analogue of eglin c in which Gly-70 was replaced with α-hydroxyacetamide. The NMR analysis of this analogue revealed its secondary structure was essentially identical to that of the wild-type protein. However, the NMR experiments also revealed that the conformation of the ester-bond containing analogue's binding loop was altered from that of the wild-type protein. In general, NMR studies of ester-bond containing proteins have been limited to standard homonuclear ^{1}H NMR techniques, as heteronuclear NMR techniques require that the protein analogue under study be uniformly labeled with ^{13}C and ^{15}N nuclei. Unfortunately, the high cost of appropriately labeled amino acid starting materials prohibits such isotopic labeling in chemically synthesized proteins.

Currently, only one ester-bond protein analogue has been studied by X-ray crystallography. This study involved the high-resolution structural analysis of the enzyme–inhibitor complex formed between *Streptomyces griseus* Proteinase B (SGPB) and an ester-bond containing turkey ovomucoid third domain (OMTKY3) analogue

[12]. The X-ray diffraction data collected on a single crystal of this enzyme–inhibitor complex enabled the three-dimensional structure of the complex to be determined at 1.7 Å resolution. Significantly, the X-ray crystallographic data revealed that the structure and solvation of the ester-bond containing enzyme–inhibitor complex were essentially unchanged from the wild-type complex. Of particular interest was that the positions of the ester-bond oxygen and amide nitrogen atoms in the three-dimensional structures of the ester-bond analogue and wild-type construct, respectively, were within 0.23 Å of each other (i.e., the same within experimental error). This was despite the effective deletion of an intermolecular, backbone–backbone hydrogen bond in the complex with the ester-bond containing OMTKY3 analogue.

4. Protein folding properties of ester-bond containing proteins

Chemical denaturant-induced equilibrium unfolding experiments have been employed to evaluate protein folding free energies (i.e., ΔG_f values) of a number of chemically synthesized ester-bond containing protein analogues. To date, the ΔG_f values of 14 such ester-bond containing protein analogues in five different protein systems have been evaluated and compared to the ΔG_f values of their wild-type controls. The resulting $\Delta\Delta G_f$ for these ester-bond containing protein analogues are summarized in Table 1. In all cases the ester-bond mutation decreased the thermodynamic stability of each protein studied. These destabilizing effects were observed when the ester-bond mutation was incorporated into α-helical regions of protein secondary structure (e.g., the GCN4 analogues, the metal-assembled helical bundle protein analogues, and the CI2 analogue in Table 1) and when the mutation was incorporated into β-sheet regions of protein secondary structure (e.g., the P22 Arc repressor analogue and the 4OT analogues in Table 1).

The destabilizing effects reported for the ester-bond containing protein analogues studied to date range from 0.7 to 24.6 kcal/mol. The wide range of these values is in large part related to the number of putative hydrogen bonds that are effectively deleted from a protein's native three-dimensional structure upon introduction of the ester bond. For example, the single ester-bond mutations in 4OT effectively delete two backbone–backbone hydrogen bonds per monomer for a total of 12 hydrogen bond deletions in the enzyme's homohexameric structure. If the $\Delta\Delta G_f$ values in Table 1 are normalized to the number of hydrogen bonds deleted by the ester-bond mutation, they would range from approximately 0 to 2.0 kcal/mol per hydrogen bond deleted.

To a first approximation, the destabilizing effects of amide- to ester-bond mutations in proteins can be attributed to the strength of the deleted hydrogen bond(s). However, the exact thermodynamic contribution of hydrogen bonding interactions to protein stability is difficult to determine from $\Delta\Delta G_f$ values of ester-bond containing protein analogues. Several additional factors can contribute to the $\Delta\Delta G_f$ values measured for ester-bond containing protein analogues. In addition to the actual strength of the hydrogen bond, it is possible that these $\Delta\Delta G_f$ values include contributions from the electrostatic and van der Waals interaction between the two oxygen

atoms in the hydrogen bond deleted complex [27], from the differences in solvent exposure, as well from the effects on the denatured state of the mutant protein.

All the $\Delta\Delta G_f$ values determined for the ester-bond containing protein analogues in Table 1 indicate that the mutation is destabilizing. However, it is interesting to note that in the case of the TOL44 analogue of eglin c (see Table 1) the ester-bond substitution rendered the protein more stable to thermal denaturation (i.e., the temperature at which the protein unfolded increased by 7 °C) [13]. This apparent increase in stability was explained by the authors to result from an enthalpically more stable native structure that was stabilized enough to compensate for the entropic destabilization in the denatured state. The authors hypothesized that this could result from a significant local conformational adjustment in the binding loop upon ester incorporation [13].

It is noteworthy that all the folding properties of the ester-bond containing protein analogues analyzed to date have been thermodynamic properties. Currently, no kinetic analyses on ester-bond containing protein analogues have been performed to assess the relative rates of their folding and unfolding. Work is currently in progress in our laboratory to dissect the kinetic properties of the folding reactions of several ester-bond containing protein analogues of Protein L and the P22 Arc repressor. The acquisition of such kinetic data is expected to help uncover the role of the polypeptide backbone in defining the structure of transition states and partially folded intermediate states in protein folding reactions.

5. Functional properties of ester-bond containing proteins

The functional studies that have been performed on ester-bond containing protein analogues have had one of either two goals. In some cases functional analyses were used to confirm that the analogue of interest folded into a wild-type structure by demonstrating that it had wild-type function. In these cases the site of the ester-bond mutation is typically removed from the protein's active site. Functional analyses have also been used to assess the effect that amide- to ester-bond mutations have on the biological functions of proteins. In these types of studies, the site of the ester-bond mutation is typically near the active site, and the mutation typically deletes backbone hydrogen bonding interactions that were hypothesized to be important for the biological function of the protein under study.

The metal-assembled helical bundle protein, the 4-ester-bond containing CI2 analogue, and the first two 4OT analogues in Table 1 were all subject to a functional analysis in order to confirm that each of these analogues folded into three-dimensional structures similar to their respective wild-type proteins. In the case of the metal-assembled helical bundle protein, McLendon and co-workers [15,16] used the efficiency of an intra-protein electron transfer reaction to report on structural changes that might result from the incorporation ester-bond mutations into a metal-assembled helical bundle protein. The electron transfer rate of the ester-containing metal-assembled helical bundle proteins did not significantly change, suggesting a constant and invariant structure. In the case of the CI2 analogue it was noted that

the protein's function as a tight-binding inhibitor to subtilisin was not significantly changed with the introduction of four ester bonds into the protein's sole α-helix [19]. Three of the 4OT analogues in Table 1 (i.e., OI2, OI7, and the double mutant [OI2,ROA39]) also had catalytic properties that were similar to that of the wild-type enzyme [5,21]. This was despite the presence of hydrogen bond deletions at the end of the intra-subunit β-sheet region of the enzyme's structure (see Fig. 2A). The catalytic activity measurements on these three analogues served as a very sensitive structural probe, as the sites of the ester-bond mutation in each of these two 4OT analogues were very close to the active site of the 4OT enzyme which includes Pro-1 as the catalytic base.

One of the ester-bond containing 4OT analogues in Table 1, OL8, was specifically designed to quantify the catalytic contribution of a specific backbone hydrogen bond formed between the enzyme's polypeptide backbone and its substrate, 2-hydroxymuconate [20]. The K_M of this analogue was found to be unchanged from that of a wild-type control. However, the k_{cat} was reduced 20-fold relative to that of a wild-type control. Interestingly, this experimental result was consistent with theoretical calculations on this enzyme system which predicted the hydrogen bond deletion in (OL8)4OT should increase the energy barrier of the enzyme catalyzed reaction by 1.0 kcal/mol. This was the first study of backbone interactions and enzyme catalysis in which theory and experiment have been applied to the same protein system.

In another study on enzyme catalysis Kent and Baca [23] synthesized an ester-bond containing analogue of the human immunodeficiency virus protease (HIV-1PR) in order to determine whether the normal catalytic activity of HIV-1PR requires functional hydrogen bonding from one or from two flaps. The substrate specificity and enzyme kinetics were determined for both the ester-bond containing analogue of HIV-1PR and a wild-type control. The enzymatic properties of the ester-bond containing analogue were very similar to that of the wild-type control. These results supported the conclusion that the HIV-1PR uses only a single flap in catalysis.

In studies of protein–protein interactions Lu et al. [11,13] used ester-bond containing protein analogues to investigate the contributions of selected backbone hydrogen bonding interactions to the stability of serine protease–protein inhibitor complexes. Two serine proteinase inhibitors (OMTKY3 and eglin c) were investigated. In the OMTKY3 study, the inhibitor–enzyme association equilibrium constants were measured for the interaction of OMTKY3 ester-containing construct and wild-type OMTKY3 control with six serine proteinases. The backbone NH to O substitution in OMTKY3 weakened the binding of this inhibitor to the six serine proteinases by an average of 1.5 kcal/mol. The structural analysis of ester-containing OMTKY3 by X-ray crystallography has indicated that 1.5 kcal/mol decrease in binding free energy was largely due to the intermolecular hydrogen bond deleted between the inhibitor and protease [11,12]. Similarly, the binding properties of ester-bond containing eglin c analogues were evaluated, and it was found that the energetic contribution of intermolecular backbone hydrogen bonds deleted in these complexes ranged from 0 to 4.5 kcal/mol depending on the serine proteinase that was assayed for inhibition [13].

An investigation of the reduction potential the high-potential iron protein (HiPIP) when it was loaded with Fe_4S_4 also revealed that it was significantly reduced when selected amide groups in the proteins active site were replaced with ester bonds [17]. The ester-bond mutations in these analogues effectively deleted specific hydrogen bonds from backbone NH groups to the S in the Fe_4S_4 ligand. The absence of a hydrogen bond to the S in the ligand apparently reduced the charge density on the S which lowered the oxidation state of Fe in the ligand.

6. Conclusions

The ester-bond containing proteins prepared and characterized to date provide strong experimental evidence that the polypeptide backbone of proteins can play a role in ligand binding, enzyme catalysis, and protein folding. They have also provided some insight into the contribution of backbone–backbone hydrogen bonds to these processes. But clearly, there is additional insight to be gained by studying more backbone modified analogues from these and other protein systems. Important information that is especially lacking is data on the kinetic properties of the protein folding reactions of ester-bond containing analogues as well as data on the high-resolution structures of such backbone modified proteins.

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