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Molecular anatomy of RNA polymerase using protein-conjugated metal probes with nuclease and protease activities

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Iron (*S*)-1-(*p*-bromoacetamidobenzyl)ethylenediaminetetraacetate (FeBABE) with the sequence-non-specific cleavage activity of nucleic acids and proteins was conjugated to protein Cys residues, and used for mapping the contact sites of both the α-subunit carboxy-terminal domain of *Escherichia coli* RNA polymerase on promoter UP elements and the σ^{70} and σ^{38} subunits on the respective promoters. The same chemical nuclease was also used as a chemical protease for mapping the subunit–subunit contact sites within the RNA polymerase. By using 2-iminothiolane as a linker, FeBABE could be conjugated to protein Lys residues and successfully used for mapping the contact surfaces of some *E. coli* transcription factors on the RNA polymerase holoenzyme.

Most of the biological reactions involved in transfer of genetic information such as replication, transcription and translation are carried out by macromolecular complexes consisting of a large number of proteins and nucleic acids. Regulation of these processes is mediated by rearrangements of protein–DNA, protein–RNA and protein–protein interactions. For instance, the transcription apparatus is composed of the DNA-dependent RNA polymerase, which consists of 4–5 (in the case of prokaryotes) to 12–18 subunits (in the case of eukaryotes), and hundreds of accessory transcription factors. Identification of the protein–DNA, protein–RNA and protein–protein contact networks within the transcription apparatus is crucial for understanding the molecular events carried out in transcription and its regulation. In order to meet the increasing demand for technical innovation for the molecular anatomy of these multi-component assemblies, a variety of new physical, chemical and genetic methods have been developed recently.

Akira Ishihama is Head of the Department of Molecular Genetics, National Institute of Genetics, Mishima, Japan. He obtained his PhD at Nagoya University in 1967, and subsequently worked with Jerard Hurwitz at the Albert Einstein College of Medicine, New York, on the structure–function relationship of bacterial RNA polymerase. He then returned to the Kyoto University Institute for Virus Research in 1970, and worked with the reconstitution of RNA polymerase. In Kyoto, he also began studying the molecular anatomy of viral RNA polymerases. In 1984, he moved to the National Institute of Genetics, and has contributed to the development of the novel research concerned with the molecular interaction between RNA polymerase and transcription factors. In Mishima, he also began studying the molecular anatomy of RNA polymerase II from the fission yeast. His current research interests include the functional modulation of RNA polymerases through protein–DNA, protein–RNA and protein–protein interactions.

Use of metals for cleavage of nucleic acids and proteins

Transition metals are known to catalyze a number of oxidative chemical reactions with deleterious effects in biological systems.[1–5] Iron promotes the formation of reactive radicals such as hydroxyl radicals, which indiscriminately damage all cellular components, even though iron is an indispensable element and the most abundant metal for living organisms. The high reactivities of iron have been put into practical use to explore the properties of macromolecular complexes.[6–11]

Initially free Fe^{2+} or FeEDTA was used to study the conformation of nucleic acids and their complexes.[12–18] The potential value of such reagents for protein mapping was first recognized by using untethered free FeEDTA, and a number of different approaches have been developed to use the metal complexes to map the overall surface of proteins.[19–21] Such methods are often called 'protein footprinting'. The broad specificity of hydroxyl radicals for attack at the backbones of nucleic acids and proteins permitted probing of nearly all the nucleotides and amino acids. FeEDTA complex offers the most general measure of surface accessibility, because its distribution around the target molecule is insensitive to charge, compared with free Fe^{2+}.[4]

The inception and development of methods for site-specific cleavage of nucleic acids and proteins with FeEDTA have led to development of artificial reagents with nucleolytic and proteolytic activities. Various attempts have been made to conjugate FeEDTA to proteins for monitoring of proximity mapping. For this purpose, bifunctional chelating agents were considered as linkers which possess both a strong metal-binding moiety and a group that binds to a biological molecule.[22] Applications of Cys-linked FeEDTA complexes have exploited oxidative chemistry to cut DNA[23] or RNA.[24,25] Most of these procedures are based on the high reactivity of H_2O_2 or O_2 and iron, which depends on the nature of the iron chelate involved.[4,26] For oxidative scission of nucleic acids, the generation of electrophilic species such as hydroxyl radicals is important.[14–16]

Likewise the artificial non-enzyme proteases have been developed in many laboratories.[27–29] It is essential that the protein cutting is only due to the tethered reagent and not to unchelated metal coordinated directly to sites on the protein. Protein-conjugated small metal chelates were shown to cleave polypeptide chains at sites determined by proximity to the chelates, apparently independent of the amino acid residues involved.[10,11,30] The net reaction involves hydrolysis, but not oxidation, of the peptide backbone. The peptide cleavage results from generation of a powerful nucleophile that selectively attacks peptide carbon rather than hydroxyl radicals.[31] The hydrolysis of protein backbones appears to be dominated by nucleophilic iron-peroxo complexes.[31] In the presence of H_2O_2

or O_2 and ascorbate, FeEDTA forms an intermediate oxygen-activated complex that leads to nucleophilic attack by oxygen on the carbonyl carbon of the peptide bond. The reactivities of nucleophiles, $HO–O^-$ and $CH_3O–O^-$, toward carboxylic esters are 100 times higher than hydroxide.

Development of chemical nucleases and proteases

A sulfhydryl-specific EDTA derivative, EDTA-2-aminoethyl 2-pyridyl disulfide (EPD) was developed to tether FeEDTA to protein Cys residues[32] and used for mapping the resolvase-binding site on DNA[23] and the binding sites of some RNA-binding proteins.[33] The newly developed method allowed the identification of regions or surfaces of proteins that are in close proximity to DNA or RNA, and of the portions of DNA or RNA that are closest to each EDTA-derivatized Cys, because the DNA cleavages are highly localized and their efficiency drops rapidly as a function of the distance between the Fe^{2+}EDTA complex and the DNA targets. EPD-Fe has also been applied for analysis of the protein–protein contact network within the non-native equilibrium protein folding intermediate.[32] Cys-linked FeEDTA chelates have since been used to study non-native conformation of Staphylococcal nuclease during folding of the protein.[14,32]

To avoid the need to add iron after conjugation of such metal-chelating reagents as EPD to target molecules, an iron-bound probe, iron (*S*)-1-(*p*-bromoacetamidobenzyl)ethylenediaminetetraacetate (FeBABE) was developed (Fig. 1),[29,34] which can

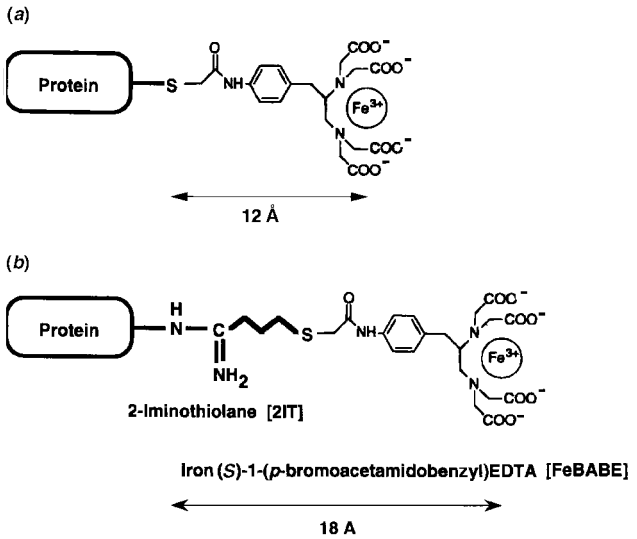

Fig. 1 Chemical probes with nuclease and protease activities. (*a*) Iron (*S*)-1-(*p*-bromoacetamidobenzyl)EDTA [FeBABE] can be conjugated to protein Cys residues.[29] (*b*) FeBABE in combination with 2-iminothiolane (2IT) can be conjugated to protein Lys residues.[53]

be handled in a manner analogous to many other protein-labeling reagents, such as fluorescent probes or cross-linkers. FeBABE can be tethered to proteins preferentially at Cys residues. Protein–FeBABE conjugates are formed under mild reaction conditions and are stable for long periods in the absence of activating reagents. Activation of the chelate iron is accomplished by a brief exposure, for instance for about 10 s, to H_2O_2 in the presence of ascorbate. This generates a highly reactive hydroxyl radical which induces cleavage of the backbones of DNA and RNA. In the presence of H_2O_2 and ascorbate, FeBABE forms an intermediate oxygen-activated complex which leads to nucleophilic attack by oxygen on the carbonyl carbon of the protein peptide bond and causes peptide hydrolysis.[31]

The chemistry of FeBABE was characterized by using proteins of known sequence or structure.[10,11,31] Since direct contact is required between FeBABE and the carbonyl carbon for protein cleavage to take place, the reaction is limited to peptide bonds that are within about 12 Å of the site of attachment of FeBABE (see Fig. 1). Because of this distance limitation and the required orientation for nucleophilic attack on the peptide carbon, it is expected that the number of cleaved sites will be relatively small. Using the human carbonic anhydrase as a model substrate, the efficiency of cleavage was estimated by measuring the cleaved fragments, as determined by the N-terminal sequences, to be 50–70%, indicating most of the cleavage was the hydrolysis of a peptide bond.[31] The high yield of products provides sufficient cleaved materials for sequence analysis. FeBABE is a powerful probe of protein structure, usable with relative ease in any system where Cys-linked reagents are applicable. The cleavage reaction is fast, highly selective, and proceeds in high yield under physiologial conditions. It forms hydrolytic products that are readily characterized by standard N-terminal sequencing of the resolved peptides. The results provide direct information on the proximity of the tethered probe to particular peptide bonds regardless of amino acid residue type, easily exceeding the scope and resolution of chemical cross-linking reagents.

Mapping of RNA polymerase contact sites on promoter DNA

FeBABE has been successfully used for the mapping of protein-bound sequences on DNA and RNA. In particular, a systematic search has been carried out in two cases: the RNA polymerase-contact sites on promoter DNA[35–39] and ribosomal protein-binding sites on rRNA.[40–44] The contact sites of proteins on DNA or RNA can be easily identified from the terminal sequences of fragments generated after cleavage with Fe-BABE.

The RNA polymerase core enzyme of *Escherichia coli* is composed of two α-subunits and one each of the β- and β′-subunits. The carboxy (C)-terminal domain (CTD; amino acid residues 234–329) of the RNA polymerase α-subunit plays a key role in molecular communications with class-I transcription factors and upstream (UP) elements of promoter DNA.[45,46] In order to identify possible differences in the functional roles of the two α-subunits, we established an *in vitro* reconstitution system of hybrid RNA polymerases containing two distinct α-subunit derivatives in a defined orientation.[35] In order to identify the binding sites of two αCTDs on the UP element DNA, a mutant α-subunit without a Cys residue was prepared, from which a number of α-derivatives with a single Cys residue at various positions along the CTD were constructed. After conjugation of FeBABE to the single-Cys mutant α-subunits, each was used, together with untethered wild-type α, for reconstitution of hybrid RNA polymerases containing one intact and one FeBABE-tethered mutant α-subunit. The reconstituted RNA polymerases were used for formation of promoter DNA complexes with or without a UP element, and the complexes were subjected to hydroxyl radical-based DNA cleavage mediated by FeBABE for identification of the contact DNA sequence of each αCTD. In the case of the P1 promoter of *E. coli* ribosomal RNA gene (*rrnB*) promoter, the two α-subunits were found to bind in tandem to two helix turns of the UP element, the β- and β′-associated α-subunits being bound to the promoter proximal and distal regions, respectively (Fig. 2).

The RNA polymerase σ-subunit is responsible for sequence recognition of DNA promoters, and in general bacteria contain multiple molecular species of the σ-subunit, each recognizing a specific set of promoter sequences. For instance, *E. coli* contains seven different σ-subunits.[47] The σ family proteins carry four regions of the conserved sequence. The contact-dependent DNA cleavage method was then applied for mapping spacial relations between the evolutionary conserved regions of various σ-subunits and each DNA strand along the respective promoter DNA. The library of single Cys mutants has been

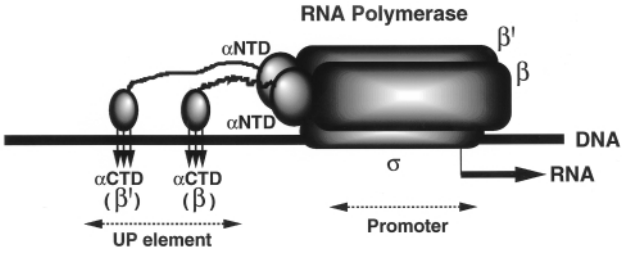

Fig. 2 Identification of the contact sites of RNA polymerase α-subunit on promoter UP element. FeBABE was conjugated at a single Cys residue (position 269) on the UP element contact surface of the α-subunit C-terminal domain (CTD). The reconstituted RNA polymerase holoenzyme containing the FeBABE-tethered α-subunits was used for mapping of the αCTD contact sites on the UP element of the *E. coli* ribosomal RNA (*rrnB*) gene, after sequencing the DNA cleavage sites by hydroxyl radical generated from the protein-tethered FeBABE upon addition of H_2O_2 and ascorbate.[35] The cleavage sites are shown by arrows.

constructed for σ^{70} (the σ major subunit for transcription of growth-related genes) and σ^{38} (the σ-subunit for stationary-phase genes). Results of the DNA cleavage mapping of open complexes using FeBABE-conjugated σ-subunits indicated that each conserved region of both σ^{70} and σ^{38} makes contact with different regions along the promoter DNA (Fig. 3).[37–39] The

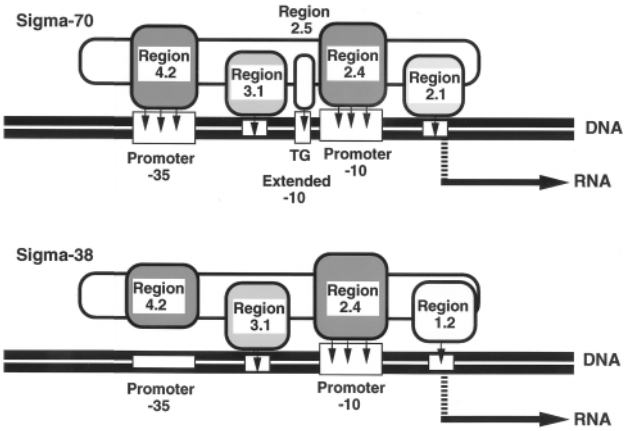

Fig. 3 Identification of the contact sites of RNA polymerase σ-subunits on promoters. FeBABE was conjugated at a single Cys residue located at various positions of single-Cys mutant σ^{70} and σ^{38} subunits of the *E. coli* RNA polymerase. The reconstituted RNA polymerase holoenzymes containing the FeBABE-tethered σ-subunits were used for mapping of the σ-subunit contact sites on the respective promoters, after sequencing the DNA cleavage sites by hydroxyl radical generated from the protein-tethered FeBABE upon addition of H_2O_2 and ascorbate.[37–39] The cleavage sites are shown by arrows. The σ family proteins contain four conserved regions. The regions 2, 3 and 4 are composed of 4, 2 and 2 subregions, respectively. In the case of the major σ^{70} subunit, the region 2.4 is involved in promoter -10 recognition while the region 4.2 recognizes the promoter -35 sequence.

results agreed well with the prediction from mutant studies of essential roles for two hexanucleotide sequences located at promoter -35 and -10. In addition, the determination of direct contact sites by the novel method revealed hitherto unidentified contacts involving sequences other than the -35 and -10 sequences. The comparison of promoter cleavage mapping by σ-conjugated FeBABE also revealed different modes of promoter DNA interaction between different σ-subunits.

Mapping of subunit-subunit contact sites within RNA polymerase

Proximity relationships between the two α-subunits of *E. coli* RNA polymerase were studied using a set of single Cys mutant α-subunits, each harboring a single Cys at various positions along the entire α polypeptide (Fig. 4). FeBABE conjugated to each of these Cys residues introduced both intramolecular and intermolecular cutting of the α-subunit in the presence of

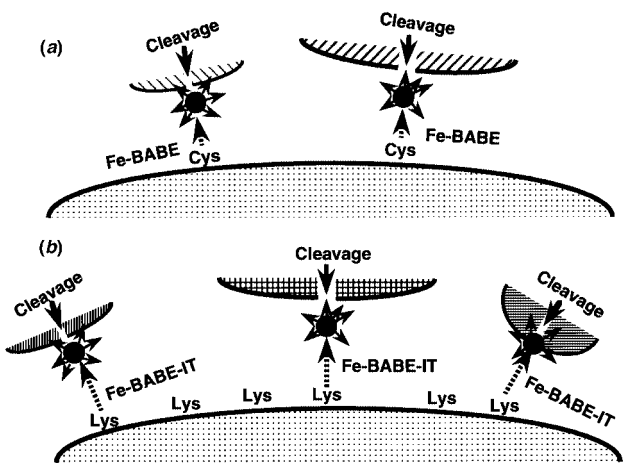

Fig. 4 Contact-dependent cleavage of proteins by the protein-conjugated FeBABE. (*a*) FeBABE can be conjugated to specific Cys residues of proteins (see Fig. 1). The polypeptide backbone of proteins, which are located near the protein-tethered FeBABE, is cleaved in the presence of ascorbate and H_2O_2. The protein–protein contact sites can be identified by tethering FeBABE at specific sites of the proteins using single Cys mutants. (*b*) FeBABE can also be conjugated to Lys residues using 2-iminothiolane (2-IT) as a linker (see Fig. 1). Lys is often involved in protein functions and exposed on protein surfaces. For mapping of protein–protein contact networks, mixtures of FeBABE-tethered proteins, each carrying a single FeBABE conjugation on average at one Lys residue, can be used for the peptide cleavage reaction after addition of H_2O_2 and ascorbate.

ascorbate and H_2O_2.[48] The intramolecular cutting was observed only within an individual N- or C-terminal domain, in agreement with the structural organization of α-subunits.[45,46] From the intermolecular cleavage mapping, the N-terminal assembly domains of two α-subunits were found to be assembled in an antiparallel fashion (Fig. 5).

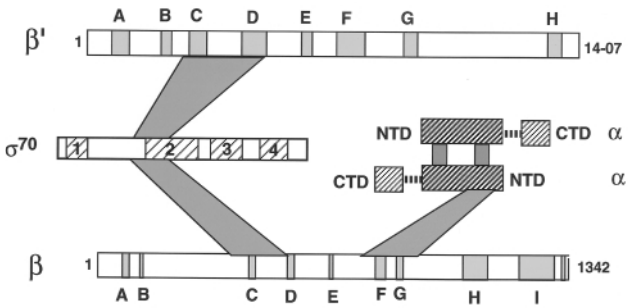

Fig. 5 Subunit–subunit contact sites within the RNA polymerase. FeBABE was conjugated at various positions of the α- or σ-subunits using collections of the respective single Cys mutant derivatives. The contact surfaces of these two small subunits on the two large subunits, β and β′, of the core enzyme were determined after identification of the contact-dependent cleavage sites by FeBABE in the presence of H_2O_2 and ascorbate.[48,49]

The RNA polymerase core enzyme acquires the promoter recognition activity by binding one of the σ-subunits. To characterize the proximity between the core enzyme and various σ-subunits, we analyzed the protein-cutting patterns produced by a set of covalently tethered FeEDTA probes positioned in or near all the four conserved regions. The protein cutting was identified at specific regions of both of the two large subunits, β and β′.[49,50] The σ-contact sites on the core enzyme are essentially the same between σ^{70} (or σ^{D}), the major σ-subunit for recognition of growth-related genes, and σ^{38} (σ^{S}) for recognition of the stationary phase-specific genes (Fig. 5). As proposed by Ghaim *et al.*,[51] Cys residues on the RNA polymerase fall into three categories: a small number of Cys residues are unreactive toward Fe-BABE, some are alkylated but produce no cleavage products upon treatment with ascorbate and peroxide, and some produce intra- and intersubunit cleavages.

The functional specificity of RNA polymerase holoenzymes (core σ-complexes) is further modulated by interaction with one or two of a total of 100–150 transcription factors.[47,52] The contact sites of these factors on RNA polymerase holoenzymes can also be monitored by using FeBABE tethered to each factor protein. For a large scale survey of the holoenzyme–transcription factor contact sites, a bifunctional linker probe, 2-iminothiolane (2-IT), was used for conjugation of FeBABE at Lys residues (see Fig. 1).[53] Transcription factors were conjugated with FeBABE at Lys residues, and the cutting sites of core enzyme subunits were successfully determined after mixing FeBABE-tethered transcription factors and holoenzyme followed by treatment with ascorbate and H_2O_2. The contact sites of σ^{70} and σ^{38} subunits on the β and β′ subunits determined using the newly developed method of a combination of 2IT and FeBABE were essentially the same as by direct conjugation of FeBABE to single Cys mutant σ-subnits. In addition to the σ family proteins, the contact sites have been determined for transcription elongation factors, NusA and GreA, and the RNA polymerase chaperon, owega.[51]

Protein-conjugated chemical probes with nucleolytic or proteolytic activities will be widely used for determining the molecular anatomy of multi-component complexes, in particular those involved in replication, transcription and translation.

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