Site-Directed Photocleavage for Mapping Protein Architecture

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Cleavage of the polypeptide backbone is a new approach to study proteins in solution, revealing proximity relationships between interacting sites in protein complexes.[1−3] Reagents such as the metal chelate iron (*S*)-1-(*p*-bromoacetamidobenzyl)ethylenediaminetetraacetate (FeBABE) can be attached to cysteine sulfhydryl groups;[4] or by using a 2-iminothiolane as a spacer, FeBABE can be tethered to the ϵ-amino groups of lysine residues, which are distributed over protein surfaces.[5] For FeBABE, the cleavage reaction is induced by adding an oxidizing agent such as H_2O_2 and a reducing agent such as ascorbate.

Photochemistry can also be used for protein cleavage. Site-specific photocleavage of hen egg lysozyme and bovine serum albumin by *N*-(1-phenylalanine)-4-(1-pyrene)butyramide (Py-Phe) has recently been reported.[6] When a mixture of protein, Py-Phe, and the electron acceptor $(NH_3)_6Co^{III}$ is irradiated, quenching of the excited state of Py-Phe by $(NH_3)_6Co^{III}$ leads to cleavage of the polypeptide backbone.

Nitroaromatic moieties are used for photolabile protecting or "caging" groups;[7−9] when irradiated, they transfer an oxygen atom from the nitro group to an acceptor, with concomitant bond cleavage. The photocleavage of nucleic acids with nitroaromatics has also been described.[10] We reasoned that photolysis of molecules containing nitroaromatic groups might lead to cleavage of polypeptide chains in a single step, avoiding not only the complications of a catalytic reaction but also the need for additional reagents.

Readily available reagents 2-bromo-4′-nitroacetophenone ($BrAcPhNO_2$) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent[11]) were attached to the cysteine residues of a set of engineered mutants of the *Escherichia coli* RNA polymerase σ^{70} protein, each of which contains a single cysteine at a unique position.[1] $BrAcPhNO_2$ and 2-bromoacetophenone (BrAcPh) conjugation reactions were performed as for FeBABE.[4] DTNB was conjugated by incubating each mutant σ^{70} protein (90 μM) and DTNB (180 μM) at room temperature for 15 min in 0.1 M phosphate buffer (pH 8.0) containing 1 mM EDTA. The absorbance of the 2-nitro-5-thiobenzoate (TNB) leaving group was quantitated at 412 nm to determine the conjugation yield. Excess DTNB and TNB were removed by gel filtration.

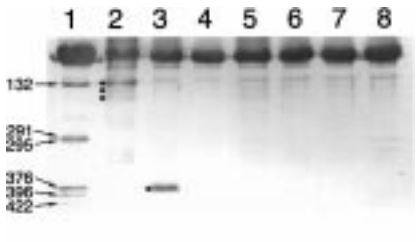

Figure 1. $BrAcPhNO_2$ conjugates of a set of engineered single-cysteine mutants of the *Escherichia coli* RNA polymerase σ^{70} protein were irradiated with UV, separated by 12% SDS-PAGE, blotted, and visualized with C-terminal specific σ antibody. The bands with "•" on the left side are cleavage fragments. Lane 1: MW markers prepared from σ subunit digestion with 2-nitro-5-thiocyanobenzoate (NTCB) (wild type σ, σ376Cys, σ396Cys, and σ422Cys);[1,15]lane 2: σ132Cys; lane 3: σ376Cys; lane 4: σ396Cys; lane 5: σ422Cys; lane 6: σ496Cys; lane 7: σ517Cys; and lane 8: σ581Cys.

The conjugation yield and biological activity of the conjugates are summarized in Table 1. To determine the effect of the conjugation of each probe, transcriptional activities relative to wild type σ^{70} were determined by a single-round transcription activity assay[12] with correction for the presence of unconjugated σ. Except for σ422Cys, the DTNB, $BrAcPhNO_2$, and BrAcPh conjugates show good retention of biological activity. The FeBABE conjugates show much lower activity. The gallium chelate GaBABE is similar in size to FeBABE, but does not exhibit its redox chemistry. Comparison of the two chelate conjugates suggests that the lower activity with FeBABE is due to oxidative side reactions, rather than to the presence of the probe.

$BrAcPhNO_2$, BrAcPh, and DTNB conjugated proteins were irradiated with either a UV lamp or an excimer laser. For UV lamp irradiation, the protein sample was placed in a Pyrex test tube and irradiated in a Rayonet photochemical reactor for 30 min (λ > 300 nm, 2.8 mW/cm^2 at 310 nm). For laser irradiation, the sample solution in a quartz cell was irradiated at 308 nm with 300 pulses from an excimer laser (Lambda Physik EMG 101) operated at 10 Hz. The laser has a pulse width of 20 ns and output energy of 40 mJ/cm^2. Purging with argon gas before irradiation led to reduced nonspecific cleavage.

Self-cleavage was surveyed with seven single-Cys σ mutants conjugated with $BrAcPhNO_2$. Only two mutants, with the probe at residue 132 or 376, lead to cleavage (Figure 1). No cleavage products were observed with the other five mutants, even with longer irradiation. Presumably, the probe at those Cys sites was not oriented in such a way as to produce cleavage. Cut sites similar to $BrAcPhNO_2$-σ, and only with the 132Cys and 376Cys proteins, occur with both DTNB and FeBABE conjugates. A significant amount of protein cross-linking also occurs under cleavage conditions with any of these reagents. Attempts to sequence a cleaved fragment of the 376Cys conjugate by Edman degradation show that the new N-terminus is blocked. While the mechanism of cleavage is not known in detail, hydroxylation of α-carbon would lead to a fragment with a blocked N-terminus.[13]

To identify the cleavage sites, the protein fragments were separated by SDS-PAGE and visualized by immunoblotting. The fragment sizes were assigned by comparing their R_f values with

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Table 1. The Conjugation Yield (%) and Transcriptional Activity (%, in parentheses) of Each Conjugate, Determined from at Least Two Experiments[a]

	σ132C	σ376C	σ396C	σ422C	σ496C	σ517C	σ581C
$BrAcPhNO_2$	80 (97)	68 (90)	65 (70)	64 (32)	95 (50)	74 (82)	73 (84)
DTNB	88 (100)	89 (90)	72 (60)	70 (22)	93 (66)	81 (62)	88 (91)
FeBABE	43 (43)	51 (40)	66 (31))	47 (10)	92 (39)	72 (26)	80 (42)
GaBABE	44 (86)	51 (84)	68 (56)	47 (14)	91 (64)	72 (79)	82 (82)
BrAcPh	82 (100)	71 (89)	65 (60)	65 (28)	95 (67)	72 (57)	75 (89)

[a] The estimated error for both conjugation yield and transcriptional activity is ±5%.

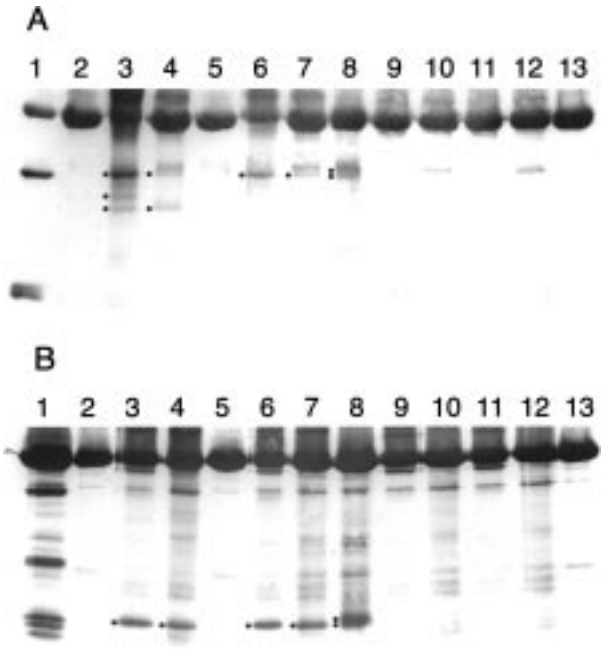

Figure 2. The cleavage reactions of (A) σ132Cys and (B) σ376Cys were separated by (A) 7% and (B) 10% SDS-PAGE, blotted, and stained with C-terminal specific σ antibody. Lane 1: σ MW marker; lane 2: $BrAcPhNO_2$-σ, unirradiated; lane 3: $BrAcPhNO_2$-σ, irradiated with UV lamp; lane 4: $BrAcPhNO_2$-σ, irradiated with laser; lane 5: DTNB-σ, unirradiated; lane 6: DTNB-σ, irradiated with UV lamp; lane 7: DTNB-σ, irradiated with laser; lane 8: FeBABE-σ, treated with ascorbate and H_2O_2;[4] lane 9: BrAcPh-σ, irradiated with UV lamp; lane 10: BrAcPh-σ, irradiated with laser; lane 11: unconjugated σ, irradiated with UV lamp; lane 12: unconjugated σ, irradiated with laser; and lane 13: unconjugated σ, treated with ascorbate and H_2O_2.

markers prepared by chemical cleavage of the σ^{70} protein. All cutting reagents attached to 132Cys cleave σ at similar locations, as do those at 376Cys (Figure 2). The same cleavage fragments are produced by either UV or laser irradiation; however, UV lamp irradiation lanes show lower backgrounds than laser irradiation lanes (Figure 2: lane 3 vs 4, 6 vs 7, 9 vs 10, and 11 vs 12). These cleavage fragments were not observed in control samples, BrAcPh conjugates, or unconjugated proteins (Figure 2, lanes 9−13), indicating the necessity of a nitroaromatic group.

The assigned cleavage sites (Table 2) may be compared to the crystal structure,[14] which shows that amino acid residues 132 and 376 are located on different α helices. When attached to 376Cys, $BrAcPhNO_2$ and DTNB cleave the polypeptide chain at sites separated by one turn of the helix, on the same side. The larger FeBABE cleaves one to two helix turns away. When attached to 132Cys, $BrAcPhNO_2$ and FeBABE also cleave at sites farther away in the primary sequence, but evidently nearby in the three-dimensional structure. Cleavage occurs with no obvious preference for amino acid type or degree of hydrophobicity of the neighboring environment. The approximate distances from Cys-sulfur to nitro oxygen of the attached $AcPhNO_2$ and TNB groups, determined from CPK models, are 9 and 8 Å. The distance from Cys-sulfur to iron of FeBABE is 12 Å. Using the crystal structure, these are in good agreement with the distances between the Cys-sulfur, where cleavage reagents were attached, and hydrogen on the α-carbon of each cleaved residue (Table 2), with one exception: cleavage at position 165 by $AcPhNO_2$ attached to 132Cys apparently requires some protein flexibility.

Table 2. The Cleavage Sites of σ132Cys and σ376Cys Conjugates, Assigned by SDS-PAGE with σ^{70} MW Markers[a]

	σ132C	σ376C
$BrAcPhNO_2$	130 (6.6 Å,1 helix turn) 165 (14.1 Å, in loop) 183 (not solved)	379 (7.9 Å, 1 helix turn)
DTNB	135 (8.3 Å,1 helix turn)	379 (7.9 Å,1 helix turn)
FeBABE	109 (not solved) 128 (4.5 Å, 1 helix turn)	368 (10.7 Å, 2 helix turns) 373 (4.4 Å, 1 helix turn)

[a] Distances between Cys-sulfur and hydrogen on α-carbon were determined from crystal structure. The averages of the results of 5−10 experiments are shown. The estimated accuracy is ±3 AA residues and the precision is ±1 AA residue.

Photocleavage is an excellent technical approach for studying complex biological systems. Nitroaromatic reagents cut cleanly, and they are not activated by thiols or oxygen. While a low degree of oxidative cleavage begins as soon as FeBABE is conjugated to a protein, photocleavage with a nitroaromatic does not occur without exposure to UV radiation; therefore, a DTNB- or $BrAcPhNO_2$-conjugated protein molecule is less subject to degradation. This should allow even lengthy preparation of elaborate macromolecular complexes, such as transcription complexes, prior to cleavage. FeBABE is a catalytic cleavage reagent, producing multiple reactive species. Photocleavage with a nitroaromatic is expected to be a single event, simplifying the interpretation of results. The blocked N-terminus produced by photocleavage with a nitroaromatic prevents Edman sequencing of the product; however, as the biological system becomes more complicated, Edman sequencing becomes less practical.

DTNB, which is used in biochemical assays for free sulfhydryl content in peptides and proteins, is a particularly interesting site-directed photocleavage reagent because of its simple conjugation chemistry, high conjugation yield, and exquisite selectivity for Cys residues. With DTNB, both the conjugation yield determination and the cleavage can be run from a single reaction. In addition, DTNB behaves as a "traceless" probe; when cleaved fragments are prepared for SDS-PAGE, the TNB group is removed from the protein by disulfide reduction. Analysis of unconjugated protein fragments provides for accurate assignment of cleavage sites. For situations that require the presence of a reducing reagent such as dithiothreitol or 2-mercaptoethanol, the stable thioether linkage formed by $BrAcPhNO_2$ may be used instead.

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