DEST: A Novel Amidinating Protein Cross-linking Reagent

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Overview

- First reagent to cross-link amines at physiological pH without eliminating their basicity
- Use of this reagent is unlikely to perturb native protein structure
- Cross-links can be easily enriched by strong cation exchange

Introduction

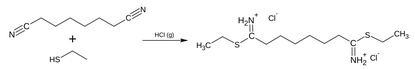
Advancing technologies in mass spectrometry, including high resolution and high mass accuracy, have led to a resurgent interest in chemical cross-linking as a method of studying protein structures and interactions, because it is now possible to identify peptide cross-links formed from proteolysis of derivatized protein samples with reasonably high confidence.[1, 2]

Nevertheless, analytical challenges hinder what can currently be accomplished. For example, the detection of cross-linked peptides in the proteolytic digests of derivatized proteins is often impaired by the combination of their low stoichiometric yield and the presence of other peptide species. Development of a simple and efficient technique for enriching cross-linked peptides from all components of proteolytic digests, including dead-end modified peptides, is therefore worth pursuing. Another challenge in cross-linking is ensuring that the reaction of a particular covalent probe does not have a deleterious effect on the stability of native protein structure. Amine-reactive succinimidyl esters are frequently used even though their labeling eliminates the native basicity of amines and thereby potentially disturbs native protein structure. Although amine-reactive imidates introduce basic amidine modifications, these reagents are effective only at alkaline pH, so their use may perturb native protein structure.

Each of these shortcomings is addressed by the use of a new protein cross-linking reagent, known as DEST (diethylsuberthioimidate).

Method

Diethyl suberthioimidate (DEST) was prepared from ethanethiol and suberonitrile via the Pinner synthesis (Scheme 1).

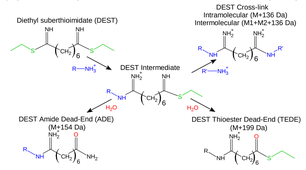


Scheme 1. Pinner Synthesis of Diethylsuberthioimidate (DEST).

Suberonitrile (9 mmol) in anhydrous dichloromethane (1:3 v/v) was added to ice-cold ethanethiol (90 mmol). The reaction mixture, under constant stirring, was sparged with hydrogen chloride gas for one hour, and subsequently kept at 0°C for an additional 16 hours. Anhydrous diethyl ether was then added to aid precipitation, and the mixture was stored at -20°C until a solid had formed. This solid was washed several times with anhydrous diethyl ether and stored in a vacuum desiccator at room temperature. Cytochrome c (5µM) was reacted with DEST or DMS at reagent to protein molar ratios of 20:1, 100:1, or 1000:1 in 20 mM sodium phosphate/150 mM sodium chloride (pH 7) . After being allowed to proceed at room temperature for 12 hours, reactions were quenched by adding 0.5 M Tris to a final concentration of 50 mM. Whole protein mass analysis was performed with a QTOF mass spectrometer. Analysis of tryptic peptides from cytochrome c modified by DEST at a 100:1 molar ratio was accomplished with an LTQ Orbitrap. DEST interpeptide cross-links were separated from other tryptic digest components using strong cation exchange (SCX) chromatography.

Results

Diethyl suberthioimidate (DEST) is an amine-reactive, water-soluble cross-linking reagent with an 11 Å 8-atom spacer arm. It is an analog to commercially available reagents such as DMS and BS3. As shown in scheme 2, DEST reacts with amines to form two types of products, cross-links (XL) and dead-ends (DE).



Scheme 2. Major Reaction Products of DEST Cross-linking.

Intramolecular Protein Cross-linking with DEST

Cytochrome c is an N-terminally acetylated small protein consisting of 104 amino acid residues and 19 modifiable primary amines (Lys residues). As such, it is an ideal substrate for probing the potentially complex array of cross-linking reaction products caused by modification with DEST. Whole protein MS of modified cytochrome c revealed that DEST, but not its imidate analog DMS, was capable of introducing intra-protein cross-links at pH 7 (Figure 1).

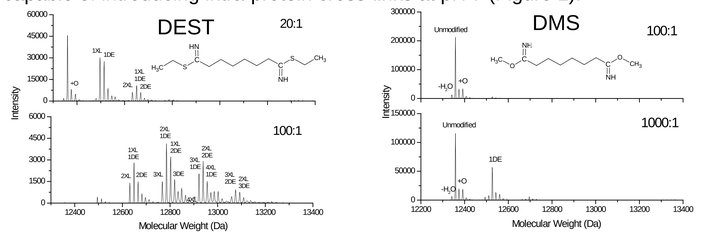


Figure 1. Deconvoluted ESI-QTOF Mass Spectra of Modified Cytochrome C.

Peptide Analysis of DEST Cross-linked Cytochrome C

Cytochrome c, intramolecularly cross-linked with DEST, was subjected to peptide analysis to test the true utility of our reagent.

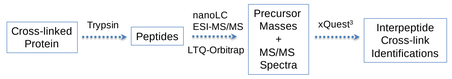


Table 1. LC-MS/MS Results.

cross-link	distance (C_α-C_α, Å)	xQuest hits
K5-K13	13	4
K7-K39	23	1
K22-K100	12	1
K27-K73	23	1
K27-K79	13	4
K27-K86	24	1
K39-K55	8	3
K39-K60	10	17
K39-K72	17	2
K53-K73	15	5
K53-K79	12	1
K55-K72	12	3
K55-K73	12	6
K60-K86	19	2
K72-K86	14	9
K73-K86	13	5
K87-K99	19	2
K88-K99	17	10

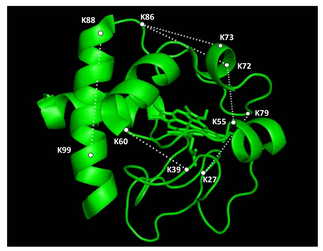
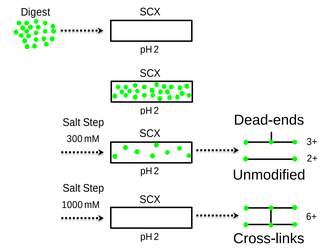


Figure 2. Crystal Structure of Cytochrome C.[4] Cross-links observed in all three LC-MS/MS replicates are indicated.

In total, 77 spectra for interpeptide cross-links were matched with an FDR <0.5%. This led to the identification of 18 unique cross-linked residue pairs, all of which were in complete agreement with the known crystalline structure of the protein and maximum cross-linkable distance between alpha carbons of lysine residues for an 11Å spacer arm reagent (24Å).

SCX Enrichment of DEST Cross-links



Interpeptide DEST cross-links were separated from other components of a tryptic digest using SCX chromatography. Since the basicity of amines is maintained upon amidination, interpeptide DEST cross-links contain six strongly basic residues. They therefore adsorb more strongly to SCX material than unmodified and dead-end modified peptides, which contain only 2 and 3 strongly basic residues, respectively.

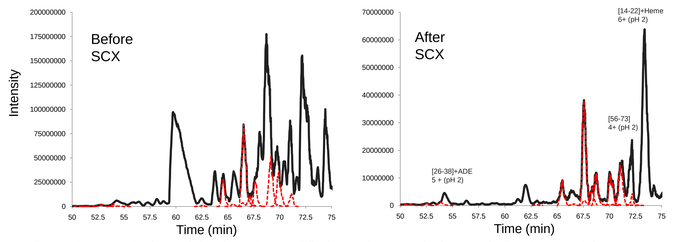


Figure 3. LC -MS of trypsinized, DEST-modified cytochrome c before and after SCX enrichment. Base peak chromatograms (black) are shown along with reconstructed selected ion chromatograms of interpeptide cross-links (red).

SCX enrichment of interpeptide DEST cross-links appears to be very effective. This pragmatic method does not require tagged reagents or additional reactions, is capable of separating cross-links from dead-ends, and therefore holds great promise for simplifying future bottom-up analyses.

Conclusion

We have introduced a novel bifunctional thioimidate cross-linking reagent (diethyl suberthioimidate, DEST) that modifies amines without sacrificing their basicity. Study of a model system by LC-MS demonstrated that DEST is effective under physiological conditions. This reagent is therefore a compelling alternative to imidate and succinimidyl ester reagents for structural studies, because it does not require alkaline pH for reactivity, does not perturb the electrostatic properties of a protein, and is thus less likely to lead to artifactual conclusions about native protein structure.

The reagent also has a particularly attractive analytical characteristic in that the interpeptide cross-links it forms can be readily detected by LC-MS/MS and easily separated from other components of tryptic digests using strong cation exchange chromatography. The use of this novel amidinating protein cross-linking reagent holds great promise for large-scale structural analysis of complex systems.

References

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