JBS Methylation

Reductive Methylation of Lysine Residues to Alter Crystallization Properties of Proteins

JENA BIOSCIENCE

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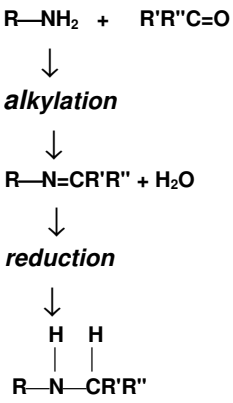
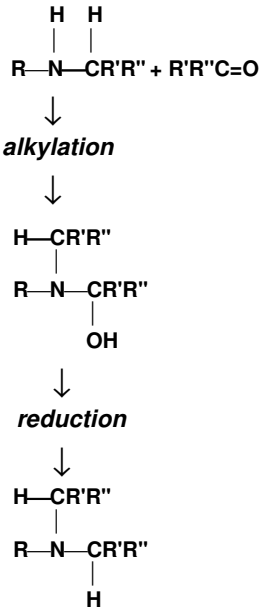
1. Introduction

Once sufficient amounts of a protein of interest have been obtained, crystal growth is obviously the first step in any X-ray diffraction analysis. At present, unfortunately, it is often the most troublesome and frustrating part of the structural studies as well. The ultimate target of crystallization experiments is the growth of crystals which diffract to a resolution of ≤ 3 Å. However, there are numerous examples where this quality could not be achieved with the native protein, and, hence, the X-ray data could not be evaluated for structural investigations.
One straightforward and easy to utilize way to tremendously improve crystal quality is the reductive alkylation of free amino groups; i.e. the lysine residues and the N-terminus, of the protein by a simple two-step reaction:

Step 1:



Step 2:



(R is a lysine or a N-terminal amino acid residue, R' and R'' are hydrogen or alkyl groups)

It was demonstrated that for hen egg white lysozyme the resolution of the X-ray analysis was improved from 2.4 Å to 1.8 Å by reductive methylation [2]. The magnitude of structural changes observed between the modified and the native lysozyme was similar to that seen when an identical protein structure is solved in two different crystalline lattices. Consequently, the methylation of lysine residues results in very little structural perturbations, however, can produce enormous effects on the crystallization properties of a protein. The described technique was absolutely critical for obtaining X-ray quality crystals of the myosin subfragment 1 [3] and has been successfully applied to several other proteins as well [4 - 7].
Thus, this method has proven to be a valuable tool for the crystallization of proteins that have so far resisted forming ordered crystals yielding the desired X-ray diffraction resolution for structural research.
Different protocols for the reductive alkylation of lysine residues in proteins have been reviewed [1]. Among other possibilities the reductive methylation, employing formaldehyde as alkylating reagent and the dimethylamine-borane complex (ABC) as reducing agent, shows the best selectivity and reactivity:

- High reactivity ensuring a complete degree of modification
- Mild effects on the biochemical properties of the protein
- Homogeneous population of protein

The JBScreen Methylation utilizes these most successful reaction conditions in user-friendly manners, thus, providing a convenient tool to substantially improve the quality of protein crystals to sufficiently ordered lattices for high resolution X-ray diffraction analysis.

2. Contents of the Kit

Each **JBScreen Methylation** kit contains all the necessary reagents for six methylation experiments:

(1) **Stock A**: pure ABC to yield a 1 molar solution in step (2) (6 vials)
(2) **Solution B**: 1 ml of 1 M Formaldehyde in purified water
(3) **Solution C**: 1 ml of 1 M ammonium sulfate in purified water
(4) **Solution D**: 1 ml of 50 mM DTT in purified water

The kit is stable at ambient temperature, however, should be kept at 4°C for long-term storage.

3. Protocol

All manipulations should be carried out at strictly 4°C:

*(1) Prepare the reagents immediately before use and keep them on ice. Set up **solution A** by adding 100 µl of purified water (reagent grade) to 1 vial of stock A (ABC), provided in the kit, with gentle mixing at 4°C.*
(2) Transfer the protein to a buffer at pH = 7.5 that does not contain any free amino groups and/or alcohols, such as 50 mM HEPES or phosphate. If necessary, additional salts can be included to stabilize the protein. Note that buffers that contain amino groups, such as amino acids, ammonium salts, TRIS, CAPS, CHAPSO etc. must be strictly avoided. The protein concentration should be in the typical range of 5 - 10 mg/ml whereas the higher concentration is for proteins with a low number of lysine residues (typically below 10) and the lower one is for proteins with higher number of lysine residues, respectively.
*(3) Add 20 µl of **solution A** (ABC) to 1 ml protein solution with gentle mixing.*
*(4) Immediately add 40 µl of **solution B** (formaldehyde), again with gentle mixing and leave the solution at 4°C for two hours.*
(5) Repeat steps (3) and (4) and incubate the reaction mixture at 4°C for additional two hours.
*(6) Add a final aliquot of 10 µl **solution A** (ABC) per ml of protein (initial volume) and leave the reaction mixture overnight at 4°C (The total reaction time is approximately 24 hours).*
*(7) Although little formaldehyde remains after 24 hours, the reaction should be quenched by adding 100 µl of **solution C** (ammonium sulphate) with gentle mixing and leave it for one addition hour.*
(8) The protein should be separated from the reaction mixture by extensive dialysis, size exclusion chromatography, microfiltration, or precipitation.
*(9) Subsequently add 50 mM DTT(provided with **solution D)** up to a total concentration of 1-5 mM to reverse any modifications of cysteine (S-bridges) or methionine residues.*

It is important to dialyze the modified protein extensively before the crystallisation set-up.

Please, note that methylation may alter the structure of the protein [2] in a way that the crystallization conditions successful for the native protein do not yield crystals of the methylated variant.

To confirm the success of methylation MS analysis can provide sufficient qualitative data as shown in the graphics below (Figure 1).

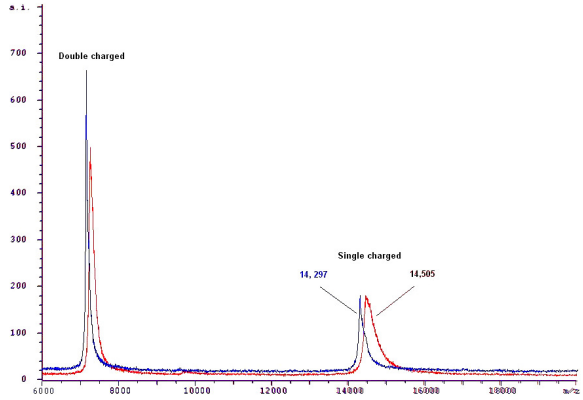


Figure 1: MALDI-TOF Spectra of native (blue) and methylated (red) hen egg-white Lysozyme: The mass difference of 208 hints a complete methylation of the six lysine residues and the N-terminal amino acid.

A complete amino acid analysis should be run when the modification is to be analysed quantitatively.

To re-optimize the crystallization strategy, please, refer to the other products of the JBScreen family.

4. References

[1] Rayment (1997) Reductive Alkylation of Lysine Residues to Alter Crystallization Properties of Proteins. *Methods in Enzymology* **276 (12)**:171.

[2] Rypniewski *et al.* (1993) Structural consequences of reductive methylation of lysine residues in hen egg white lysozyme: an X-ray analysis at 1.8-A resolution. *Biochemistry* **21**:9851.

[3] Rayment *et al.* (1993) Three-dimensional structure of myosin subfragment-1: A molecular motor. *Science* **261**:50.

[4] Kobayashi *et al.* (1999) Crystallization and improvement of crystal quality for X-ray diffraction of maltooligosyl trehalose synthase by reductive methylation of lysine residues. *Acta Crystallogr. D* **55**:931.

[5] Kurinov *et al.* (2000) X-ray crystallographic analysis of pokeweed antiviral protein-II after reductive methylation of lysine residues. *Biochem. Biophys. Res. Comm.* **275**:549.

[6] Kurinov *et al.* (2003) High resolution X-ray structure of potent anti-HIV pokeweed antiviral protein-III. *Biochem. Pharmacol.* **65**:1709.

[7] Kobayashi *et al.* (2003) Refined structure and functional implications of trehalose synthase from *Sulfolobus acidocaldarius*. *J. Applied Glycoscience* **50**:1.