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SITE-SPECIFIC MODIFICATION OF PROTEINS MEDIATED BY TRANSGLUTAMINASE

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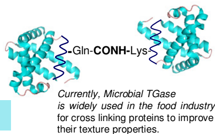
ABSTRACT

Transglutaminase (TGase) catalyzes an acyl transfer reaction between the γ-carboxamide group of glutamine (Gln) and the ε-amino group of lysine (Lys) residues to form a stable amide bond. The TGase reaction can be used for bioconjugation of an amino-derivative of poly-ethylene glycol (PEG) to protein drugs, leading to PEGylated proteins that display increased bioactivity and stability. The procedure was shown to lead to site-specific bioconjugation of few proteins, thus offering a valid alternative to the chemical methods of PEGylation in current use (1). Moreover, TGase can be used for site-specifically labeling of proteins with fluorescent groups at the level of Gln or Lys for diagnostic applications. The TGase attack is not entirely dependent on a specific consensus sequence around the Gln or Lys residues. However, a correlation between the TGase-mediated sites of PEGylation and the chain flexibility has been observed (2).
Our study was focused to elucidate the molecular features favoring the TGase mediated site-specific reactions on two extensively studied model proteins, *i.e.* apomyoglobin (apoMb) and lysozyme. Besides amino-PEG, we used dansyl-cadaverine as an acyl acceptor and N-carbobenzoxy-Gln-Gly-OH as acyl donor for the TGase reactions. The sites of protein modification were determined by fingerprinting and ESI mass spectrometry. Myoglobin in its holo form is not susceptible to TGase reactions due to its rigid conformation, but the apo form was conjugated with PEG and fluorescent labels at the level of helix F (chain segment 82–99). The NMR study on apoMb had earlier demonstrated increased flexibility of the helix F (3) and, moreover, several proteases cleave the 153-residue chain of apoMb at the level of helix F only (4). Therefore, the chain region attacked by both TGase and proteases is flexible or unfolded site(s). Lysozyme in its disulfide crosslinked native state is highly resistant to proteases and TGase attack. However, the more dynamic three-disulphide derivative of lysozyme, lacking the Cys6–Cys127 disulfide bridge, is susceptible to TGase-mediated reactions, as well as limited proteolysis. These results indicate that the sites of TGase reactions and the sites of limited proteolysis have a clear analogy for their presence in flexible/disordered regions of protein substrates.
Overall, our studies clearly demonstrate that TGase-mediated reactions occur only at disordered chain regions, as evidenced by the correlation between sites of the TGase reaction and sites of enhanced chain flexibility, this last deduced from the crystallographic B-factor.

Transglutaminases

Transglutaminase (TGase; protein-glutamine γ-glutamyl-transferase), EC (2.3.2.13) catalyzes an acyl transfer reaction between the γ-carboxiamide group of glutamine and the ε-amino group of lysine residues to form a stable amide bond.



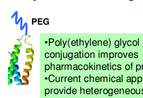
Currently, Microbial TGase is widely used in the food industry, for cross linking proteins to improve their texture properties.

The TGase enzymatic approach is highly selective towards specific Gln or Lys residues.

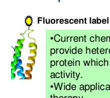
***Therefore*, primary amines or Gln donor peptides can be conjugated at the level of Gln or Lys respectively.**

TGase approach can be applied to obtain Site-specifically

PEGylated Protein drugs | **Labelled proteins**



•Poly(ethylene) glycol (PEG) conjugation improves pharmacokinetics of protein drugs.
•Current chemical approaches provide heterogeneous product .



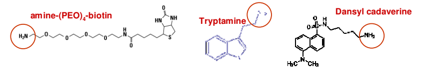
•Current chemical approaches provide heterogeneously labeled protein which affects the biological activity.
•Wide application in diagnosis and therapy

So far, no consensus sequence has been determined for the TGase selectivity

Our study was focused to elucidate the molecular mechanism of TGase selectivity on two extensively studied model proteins: apomyoglobin and lysozyme.

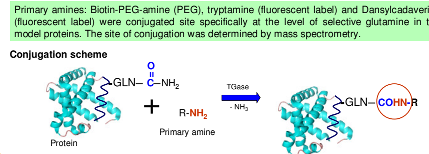
Experimental approach

Strategy A: Conjugation at reactive glutamine residues with primary amines



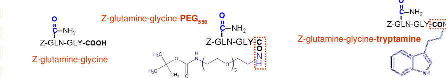
Primary amines: Biotin-PEG-amine (PEG), tryptamine (fluorescent label) and Dansylcadaverine (fluorescent label) were conjugated site specifically at the level of selective glutamine in the model proteins. The site of conjugation was determined by mass spectrometry.

Conjugation scheme



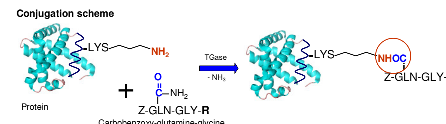
Commercially avaliable dipeptide, carbobenzoxy-GLN-GLY was modified by DCCI/HoBt method to obtain its derivatives containing the fluorescent label/ PEG at the carboxy terminus of GLY

Strategy B: Conjugation at reactive lysine residues



Carbobenzoxy-GLN-GLY and its derivatives containing PEG/ fluorescent label were conjugated site specifically at the level of selective lysine in the model proteins. The site of conjugation was determined by mass spectrometry.

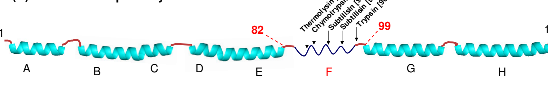
Conjugation scheme



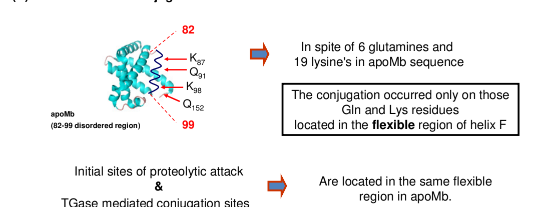
Apomyoglobin (apoMb)

ApoMb is a 153 a.a. highly helical monomeric protein, containing 8 helices (A-H). The holo form of apoMb is resistant to proteases, whereas, apoMb shows increased flexibility in the region of helix F as given by NMR measurements (3) and from the limited proteolysis experiments, which demonstrated that, helix F is more prone towards the proteolytic attack (4).

(A) Initial sites of proteolytic attack



(B) TGase mediated conjugation sites

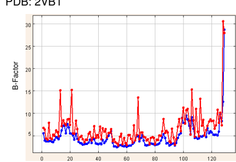


Hence, a strong correlation is seen between TGase and protease attack for the requirement of flexibility/disordered region.

Lysozyme

Hen egg white lysozyme consists of 129 a.a. with 4 intermolecular disulphide bridges. Native lysozyme is highly resistant for proteolysis and TGase mediated conjuagtion.

Crystallographic B-factor profile for Lysozyme
PDB: 2VB1



B-factor profile of native lysozyme shows a rigid conformation.

Therefore, We prepared a 3 disulphide derivative of lysozyme lacking Cys6-Cys127 disulphide bridge by the carboxymethylation (CM) of Cys6 and Cys127.

Overall 3D structure of the ^{CM6,127}lysozyme derivative was almost the same of native lysozyme (5).

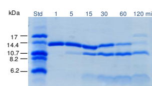
Plot of chemical-shift differences for lysozyme minus ^{CM6,127}-lysozyme (5).

Slight disorder was observed at the vicinity of the carboxymethylated Cys6 & Cys127

Our aim was to examine the possibility of protease and TGase attack on this slight disordered region of ^{CM6,127}-lysozyme

Limited Proteolysis of $^{CM\ 6,\ 127}$-lysozyme

Preliminary limited proteolysis experiments with endoproteinase Glu-C (V8 protease) on hen egg white $^{CM\ 6,\ 127}$-lysozyme



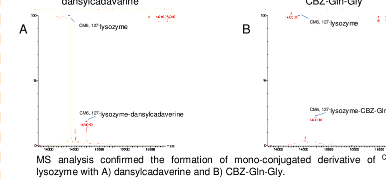
The initial V8 protease cleavage sites were located at the position Glu7-Leu8 and Glu35-Ser36.

Hence, these regions show a slight degree of flexibility which could favor TGase attack for site-specific conjugation.

Further experiments:
Limited proteolysis experiments on ^{CM6,127}-lysozyme with proteinase K, chymotrypsin and trypsin are currently in progess.

TGase mediated conjugation of $^{CM\ 6,\ 127}$-lysozyme

Preliminary TGase mediated conjugation experiments on the hen egg white $^{CM6,\ 127}$-lysozyme



MS analysis confirmed the formation of mono-conjugated derivative of ^{CM6,127}-lysozyme with A) dansylcadaverine and B) CBZ-Gln-Gly.

Inspite of 3 Gln and 6 Lys in $^{CM6,\ 127}$-lysozyme seq. → Only one Gln/Lys was conjugated

Further experiments:
Characterization of TGase conjugation site(s) are currently in progress.

Conclusions

- Gln and Lys residues located in the flexible/disordered regions were seen to be the prerequisite required for the TGase mediated site-specific PEGylation and fluorescent labeling.
- TGase mediated conjugation and limited proteolysis studies on apoMb and $^{CM\ 6,\ 127}$lysozyme clearly demonstrated the analogy between the TGase and proteases for their substrate selectivity.
- Chain flexibility as given by the crystallographic B-factor values can be used to estimate the TGase conjugation site(s).

Hence, the sites of PEGylation/ labeling in the target proteins could be predicted from the structure and dynamics as given by the X-ray or NMR data.

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