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Cysteines of chloroplast NADP-malate dehydrogenase form mixed disulfides

Oksana Ocheretina, Renate Scheibe*

Pflanzenphysiologie, Fachbereich Biologie/Chemie, Universität Osnabrück, D-49069 Osnabrück, Germany

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Abstract Chloroplast NADP-malate dehydrogenase (NADP-MDH) from pea and from spinach was N-terminally truncated by limited proteolysis with *Staphylococcus aureus* protease V8. The resulting monomeric enzymes lacking, respectively, the 37 and 38 N-terminal amino acids were inactive. Reduction and addition of low concentrations of guanidine-HCl (50–100 mM) resulted in a highly active enzyme of 850 units per mg protein. Equilibration of the truncated enzyme with various glutathione (GSH) redox buffers and assaying its activity in the presence of guanidine-HCl was used to establish the existence of protein–GSH mixed disulfides. This finding was further confirmed using incorporation of radioactively labelled thiol. The possible function of such cysteine modifications under oxidative stress and their regeneration by the thioredoxin system in the light is discussed.

Key words: NADP-malate dehydrogenase; Pea chloroplast; Limited proteolysis; *Staphylococcus aureus* protease V8; Mixed disulfide; Oxidative modification; Redox modification

1. Introduction

NADP-dependent malate dehydrogenase (NADP-MDH; EC 1.1.1.82) from chloroplasts undergoes a change in its redox state upon activation/inactivation [1]. Sequence data of NADP-MDH from pea [2], sorghum [3], maize [4], and ice plant [5] are available. All chloroplast NADP-MDH sequences contain 8 cysteine residues. The sequence is characterized by an N- and a C-terminal extension in addition to the conserved part which exhibits a high similarity to NAD-MDH [6]. Two cysteines involved in the redox change have been localized on the N-terminus [7,8]. However, there have been various reports suggesting the involvement of further cysteines in the redox change [9,10]. In particular, site-directed mutagenesis of recombinant NADP-MDH provided evidence that more than the two N-terminal cysteines are subjected to oxidation upon inactivation. Mutants of the sorghum enzyme with amino acid exchanges at one or both N-terminal cysteines were found to still require reduction for activation; the same was true for a mutant protein lacking the N-terminal extension [11]. This was confirmed also for the pea enzyme [2]. As a consequence, there have been attempts to obtain mutant proteins that are active without reductive treatment. A triple mutant of the sorghum enzyme was in fact active [12]. In contrast, for the pea enzyme two other triple cysteine mutants were found to be still partially dependent upon reduction; a quadruple mutant was completely active [2]. Since the exchange of only one cysteine forming a disulfide bridge should be sufficient to impair disulfide bridge formation, these results are not conclusive as far as the location of a second disulfide bridge is concerned.

In another approach we have produced N-terminally truncated pea NADP-MDH by limited proteolysis using aminopeptidase K [8]. Digestion of the complete N-terminal extension has been found to occur only with the DTT-reduced form. After removal of the protease and of DTT the product was still active, however, it appeared to be susceptible to oxidation by oxidized glutathione; yet the inactivation was immediately reversible upon addition of low concentrations of β-mercaptoethanol [13].

Here we present a similar approach but using instead limited proteolysis of the oxidized enzyme by an endoprotease, and provide evidence for the presence of a cysteine residue forming a disulfide bridge with a low molecular weight thiol.

2. Experimental

2.1. Preparation of N-terminally truncated NADP-MDH

Spinach and pea chloroplast NADP-MDH was purified from leaves as described [14]. Purification of spinach NADP-MDH often required an additional step using a Mono Q column (Pharmacia, Freiburg) and a gradient of 0–500 mM NaCl in 20 mM Tris-HCl, pH 8.0, to elute the enzyme. Incubation of the purified enzyme with *Staphylococcus aureus* proteinase V8 was performed for 3.5 h at 37°C at a ratio (w/w) of NADP-MDH:protease of 25:1 in 0.1 M potassium phosphate buffer, pH 7.8, containing 2 mM EDTA. Subsequent separation and retrieval of the degradation product was achieved by gel-filtration on a Superdex 200 column (Pharmacia, Freiburg) in 50 mM Tris-HCl, pH 8.0. Analysis of the product under denaturing conditions and N-terminal sequencing was carried out as described [13].

2.2. Enzyme activation and assay of activity

All media for incubation and assay were degassed and flushed with nitrogen. Incubations were carried out at 25°C under nitrogen atmosphere. Activity was determined in an assay mixture containing 0.1 M Tris-HCl, pH 8.0, 1 mM EDTA, 0.2 mM NADPH, and 1 mM oxaloacetate. Where indicated, guanidine (gdn)-HCl at the respective concentration was included.

2.3. Incorporation of ^{14}C-labelled cysteine residues upon oxidation of reduced truncated NADP-MDH

[^{14}C]Cystine (spec. act. 302.2 mCi/mmol; New England Nuclear, Dreieich, Germany) was used directly in a 2.4-fold excess over the total cysteines (8 per subunit) present in the intact NADP-MDH. The enzyme was incubated in 0.1 M Tris-HCl, pH 8.0, for 1 h at room temperature. Then equivalents of 1.5 μg protein were subjected to SDS-PAGE either in the absence or in the presence of β-mercaptoethanol. After staining the gel was dried and exposed to an X-ray film (Kodak X-OMAT, XAR-5) for 36 h at −80°C.

*Correspondong author. Fax: (49) (541) 969 2870.

Abbreviations: NADP-MDH, NADP-malate dehydrogenase; DTT, dithiothreitol; gdn-HCl, guanidine-HCl; GSH, reduced glutathione; GSSG, oxidized glutathione.

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2.4. Redox equilibration of purified truncated NADP-MDH with GSH/GSSG-redox buffer

The truncated NADP-MDH (V8-MDH) was incubated in the presence of 0.1 M Tris-HCl, pH 8.0, 0.1 M gdn-HCl with GSH (reduced glutathione) and GSSG (oxidized glutathione), dissolved in buffer and adjusted to pH 8.0 with 1 M KOH. The GSH concentration was either 25 mM or 50 mM, and GSSG was added to obtain the required GSH/GSSG ratios (R). Equilibrium was reached after 3 h under these conditions and the activity was found to be stable up to at least 2 h later. Measurements of activity in the presence of 50 mM gdn-HCl were performed after 3.5 h of incubation.

3. Results and discussion

3.1. Preparation and characterization of truncated NADP-MDH

Purified oxidized NADP-MDH from pea and from spinach was subjected to limited proteolysis by incubation with the endoprotease as described in section 2. After removal of the protease by gel-filtration, determination of the new N-termini of the enzymes revealed the cleavage sites to be the glutamic acid residues close to the conserved 'core' part of the sequence that is common to all malate dehydrogenases. The new N-terminus of the pea enzyme was TKSW..., and that of the spinach enzyme TRSW..., indicating that cleavage had occurred at similar positions. As a result of this treatment the enzymes had lost the complete N-terminal extra sequence [2]. Analysis of the truncated enzyme by SDS-PAGE revealed the homogeneous nature of the proteolysis product (Fig. 1). The truncated enzymes derived from both pea and spinach NADP-MDH possessed a residual activity of only 5–20 units per mg protein. Reduction with DTT or with reduced thioredoxin yielded enzyme of a specific activity of about 250 units per mg protein (data not shown).

3.2. Activation of V8-truncated NADP-MDH by gdn-HCl

Native oxidized NADP-MDH does not exhibit any significant activity, but the addition of moderate concentrations of gdn-HCl to the assay medium results in about 10% of the full activity of the reduced enzyme [15]. Similarly, the activity of the reduced NADP-MDH can be stimulated by low concentrations of gdn-HCl [16]. The same has been found for the recombinant pea enzyme [2]. Since limited proteolysis of oxidized NADP-MDH resulted in a form lacking the complete N-terminus but still exhibiting only marginal activity, we attempted the activation of this form by the addition of gdn-HCl. As with the intact form, the truncated oxidized form is also somewhat stimulated by the presence of gdn-HCl in the assay mixture (Fig. 2B). Reduction of the truncated form results in an increase in activity to 250 units per mg protein, considerably lower than for the intact enzyme. The addition of 50–100 mM gdn-HCl to the assay, however, is accompanied by a dramatic increase in activity, resulting in comparable maximal activities of about 850 units per mg protein for both the intact and the truncated reduced enzymes (Fig. 2B).

These results indicate that in addition to the mere reduction of the enzyme cysteines, some conformational change is also required to obtain full activity. This might be due to the presence of the C-terminal extension, since proteolytic removal of C-terminal amino acids had been shown to result in some activation of the enzyme [17]. Also from structure predictions for the maize enzyme it had been suggested that the C-terminus could act as an 'internal inhibitor' blocking the active site [18].

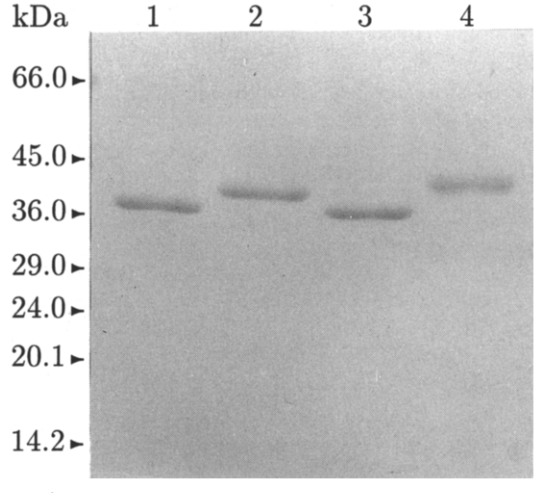


Fig. 1. Limited proteolysis by V8-protease of purified pea (lanes 1, 2) and spinach (lanes 3, 4) NADP-MDH. Either the intact protein (lanes 2, 4) or the proteolyzed enzyme after removal of the protease by gel-filtration (lanes 1, 3) were subjected to SDS-PAGE. The positions of the molecular mass standards (Sigma; Dalton Mark VII L) are indicated by arrows.

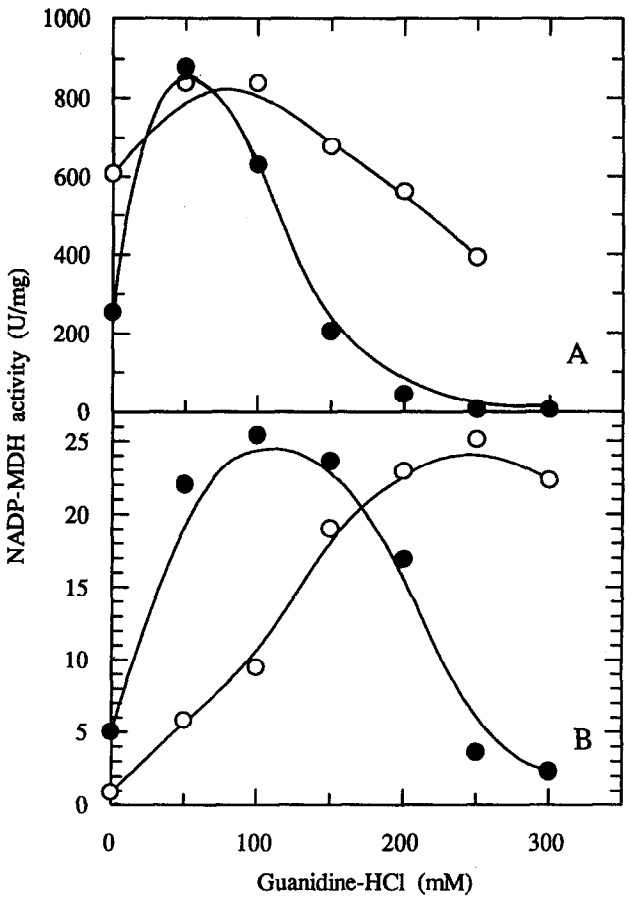


Fig. 2. Stimulation of NADP-MDH activity by gdn-HCl. The reduced (A) and the oxidized (B) enzymes were assayed in the presence of the indicated gdn-HCl concentrations under standard conditions. The open circles represent the intact spinach enzyme, the filled circles are for the truncated enzyme (V8-MDH).

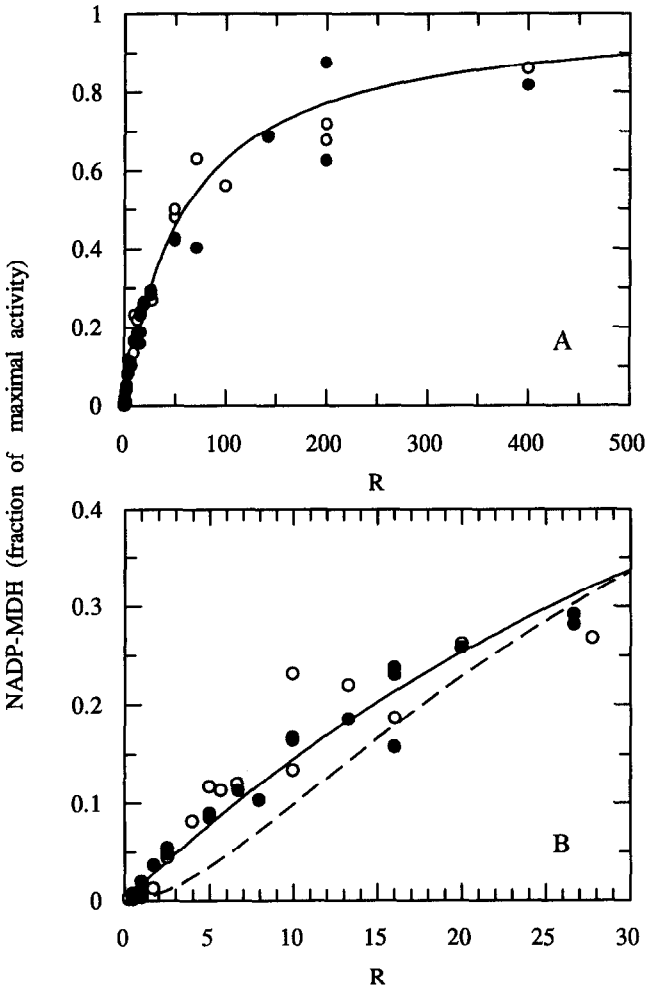
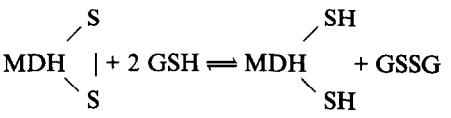


Fig. 3. Redox equilibration of V8-MDH with GSH/GSSG-redox buffers in the presence of 0.1 M gdn-HCl. The total GSH concentration was either 25 mM (○) or 50 mM (●). In B the lower range is shown with an expanded scale and the theoretical curves for the case of one mixed disulfide (solid line) and of two mixed disulfides (broken line) are shown.

3.3. *Redox equilibria between truncated NADP-MDH and GSH/GSSG-redox buffers*

Reduction of the N-terminal disulfide bridge can only be achieved with a strong reductant such as DTT, but not with similar concentrations of monothiols [19]. In contrast, reduction of a reoxidized truncated NADP-MDH had been achieved with 40 mM β-mercaptoethanol [13]. This suggests that the oxidized cysteines of the truncated V8-form could also be reduced by monothiols.

In order to determine how many further (non N-terminal) cysteines are oxidized and in which form (disulfide bridge or mixed disulfides with low molecular weight thiols), redox equilibration with GSH/GSSG redox buffers was used as suggested by Gilbert [20]. Since apart from reduction a conformational change is also required to obtain the fully active enzyme (see section 3.2), gdn-HCl was included in the assay of the truncated enzyme that had been equilibrated with various GSH/GSSG redox buffers. The reduction of a disulfide bridge requires two molecules of GSH, while its oxidation needs only one molecule of GSSG:



(1)

Thus, at equilibrium, the distribution of reduced and oxidized enzyme forms will depend on the fraction of $[GSH]^2/[GSSG]$, as a simple analysis of the equation of equilibrium concentrations shows. This fraction can be rewritten as $R \cdot [GSH]$, with $R = [GSH]/[GSSG]$. The fraction of reduced protein of the total MDH protein at equilibrium is described by the following equation:

$$\frac{[MDH_{active}]}{[MDH_{total}]} = \frac{R \cdot [GSH]}{K_{ox} + R \cdot [GSH]} \quad (1.1)$$

On the other hand, the reduction of a mixed disulfide, as its formation, requires only one molecule of glutathione:

$$\text{MDH-SH} + \text{GSSG} \rightleftharpoons \text{MDH-SSG} + \text{GSH} \quad (2)$$

The analysis of the equilibrium equation shows that the distribution of reduced and oxidized enzyme forms in this case is dependent only on R, and not on [GSH], when R is fixed:

$$\frac{[MDH_{active}]}{[MDH_{total}]} = \frac{R}{K_{ox} + R} \quad (2.1)$$

Our measurements of the relative activities of truncated NADP-MDH (V8-MDH) in 25 and 50 mM GSH buffers at equilibrium show them coinciding (Fig. 3), thus being independent of [GSH]. This excludes the possibility of the existence of a disulfide brigde.

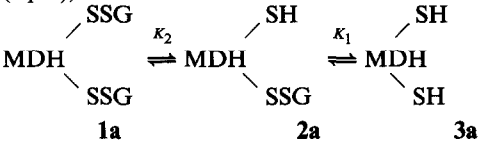
To further analyze the data we tried to approximate them using different assumptions about the number of the mixed disulfides and about the scheme of their reduction. We restrained ourselves with one- and two-disulfide models, because the rate of the reduction is defined by the hardest reducible disulfides, and one would not expect to have too many of them. On the other hand, if the number of mixed disulfides is more than two, then the qualitative character of the corresponding curves is very similar to that for two mixed disulfides.

We used three models: (i) with one mixed disulfide being reduced (eq. 3),

$$\text{MDH-SSG} \overset{K}{\rightleftharpoons} \text{MDH-SH} + \text{GSH} \quad (3)$$

$$\frac{[MDH_{active}]}{[MDH_{total}]} = \frac{R}{R + K} \quad (3.1)$$

where R is the ratio of GSH to GSSG and K is the equilibrium constant; (ii) with two sequentially reduced mixed disulfides (eq. 4),



(4)

$$\frac{[MDH_{active}]}{[MDH_{total}]} = \frac{R^2 + A \cdot R \cdot K_1}{R^2 + R \cdot K_1 + K_1 \cdot K_2} \quad (4.1)$$

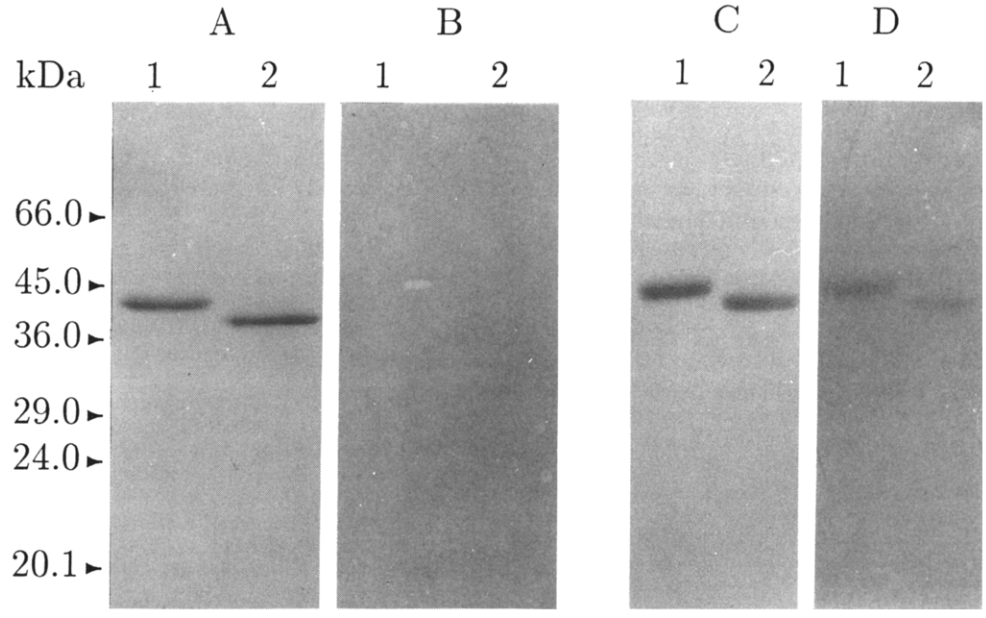
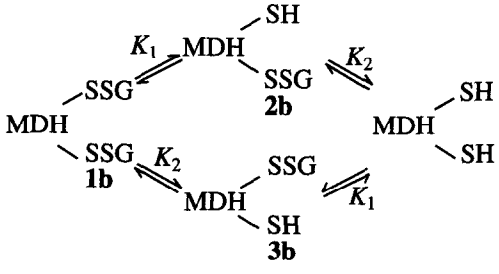


Fig. 4. S-Thiolation of NADP-MDH with ^{14}C-cystine. Intact (lanes 1) and truncated (lanes 2) NADP-MDH was incubated with [^{14}C]cystine as described in section 2. Aliquots were subjected to SDS-PAGE either with (A, B) or without (C, D) β-mercaptoethanol in the sample buffer. A and C represent the Coomassie-stained gels; B and D are autoradiograms of the gels.

where $A \leq 1$ is the relative activity of the intermediate form **2a**; and (iii) with two independently reduced mixed disulfides (eq. 5).



(5)

$$\frac{[MDH_{active}]}{[MDH_{total}]} = \frac{R^2 + A_1 \cdot R \cdot K_1 + A_2 \cdot R \cdot K_2}{R^2 + R \cdot K_1 + R \cdot K_2 + K_1 \cdot K_2} \quad (5.1)$$

where A_1 and A_2 are the relative activities of the intermediate forms **2b** and **3b**, respectively. The forms **1a** and **1b** are assumed to be inactive.

To fit the data, we used the software packet Grafit 3.00. In all cases the best fitting coefficients A (A_1, A_2), describing the activity of intermediate stages were very close to 0. The approximation using eq. 3 gives $K = 59 \pm 3$. The coefficient K_2 in eq. 4 happened to be very close to 0, so that this scheme reduced effectively to eq 3.1. The approximation using eq. 5 gave much higher errors than in eq. 3 (see Fig. 3B, broken line). This makes apparent that eq. 3 describes the reduction process best. Thus there is only one mixed disulfide. The above described observations are reminiscent of the redox properties of rabbit muscle phosphofructokinase, where in a similar way the formation of two protein–GSH mixed disulfides upon oxidation was proven [21].

The approach is based on the activity measurements, and in principle can give information only about those cysteines, the modification of which causes a change of activity. Thus we do not exclude the possibility of the formation of mixed disulfides at 5 further cysteines or even of disulfide bridges. However, it seems that their reduction is either very quick or is not connected with the activity regulation altogether.

In the case of chloroplast NADP-MDH, such modification of cysteine(s) apart from the N-terminal disulfide bridge could only be detected after removal of this part of the sequence, since the redox potential of the N-terminal cysteines appears to be rather negative, only DTT or reduced thioredoxin being capable of reducing and thereby activating the intact enzyme [19]. Interestingly, the observation of reductive formation of thiol groups without concomitant activation upon incubation with β-mercaptoethanol has led to the proposal of additional (pre-regulatory) disulfide bonds [9,10].

3.4. Incorporation of [^{14}C]cysteine

Finally an attempt was made to incorporate a small molecular weight thiol into intact as well as into V8-digested NADP-MDH. Using ^{14}C-labelled cysteine it was found that thiolation of SH-groups must have occurred in both cases (Fig. 4). Treatment with β-mercaptoethanol before gel-electrophoresis removed the label completely, indicating that a mixed disulfide was present.

3.5. Conclusions

The previous findings, of more than one regulatory disulfide bridge per subunit of NADP-MDH that appeared to be rather variable, can now be explained by the existence of a variable number of mixed disulfides depending on the 'history' of the enzyme. In addition, the fact that purified intact NADP-MDH exhibiting one band upon denaturing gel electrophoresis and a uniform N-terminus upon sequencing renders a very diffuse band upon isoelectric focusing (data not shown), underlines the possible presence of a variable number of cysteines modified by

S-thiolation. It is not yet possible, however, to deduce the actual number and position of the cysteines modified in vivo. Possibly, this is also dependent upon the redox state of the stroma or the degree of oxidative stress. Such cysteine modification could occur when the GSH/GSSG ratio drops below a certain value and would thus prevent more severe damage. Similar protective mechanisms are already known to be active in rat hepatocytes [22,23] and in human endothelial cells [24] during oxidative stress. In chloroplasts, S-thiolated cysteines will be easily restored in the light, when thiol compounds with negative redox potentials, such as reduced thioredoxins, are formed.

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