

Lactococcus lactis Thioredoxin Reductase Is Sensitive to Light Inactivation

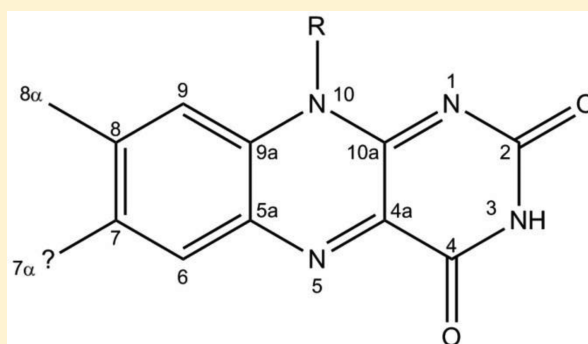
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S Supporting Information

ABSTRACT: Thioredoxin, involved in numerous redox pathways, is maintained in the dithiol state by the nicotinamide adenine dinucleotide phosphate-dependent flavoprotein thioredoxin reductase (TrxR). Here, TrxR from *Lactococcus lactis* is compared with the well-characterized TrxR from *Escherichia coli*. The two enzymes belong to the same class of low-molecular weight thioredoxin reductases and display similar k_{cat} values ($\sim 25 \text{ s}^{-1}$) with their cognate thioredoxin. Remarkably, however, the *L. lactis* enzyme is inactivated by visible light and furthermore reduces molecular oxygen 10 times faster than *E. coli* TrxR. The rate of light inactivation under standardized conditions ($\lambda_{\text{max}} = 460 \text{ nm}$ and 4°C) was reduced at lowered oxygen concentrations and in the presence of iodide. Inactivation was accompanied by a distinct spectral shift of the flavin adenine dinucleotide (FAD) that remained firmly bound. High-resolution mass spectrometric analysis of heat-extracted FAD from light-damaged TrxR revealed a mass increment of 13.979 Da, relative to that of unmodified FAD, corresponding to the addition of one oxygen atom and the loss of two hydrogen atoms. Tandem mass spectrometry confined the increase in mass of the isoalloxazine ring, and the extracted modified cofactor reacted with dinitrophenyl hydrazine, indicating the presence of an aldehyde. We hypothesize that a methyl group of FAD is oxidized to a formyl group. The significance of this not previously reported oxidation and the exceptionally high rate of oxygen reduction are discussed in relation to other flavin modifications and the possible occurrence of enzymes with similar properties.



Thioredoxin (Trx) is a ubiquitous 12 kDa dithiol protein regulating the cellular redox environment and providing reducing equivalents to enzymes like ribonucleotide reductase, peroxiredoxin, and methionine sulfoxide reductase, which employ redox-active cysteine residues in catalysis. After oxidation to the disulfide, Trx is recycled to the dithiol form by nicotinamide adenine dinucleotide phosphate (NADPH)-dependent thioredoxin reductase (TrxR, also termed NTR). TrxR and Trx together constitute the Trx system.¹

All known TrxRs are homodimers and carry one molecule of FAD per monomer but are divided into two types with distinctly different structures and molecular mechanisms. The high-molecular weight (HMW) TrxRs from, e.g., mammals, dipteran insects, and protozoan parasites and the low-molecular weight (LMW) TrxRs from bacteria, fungi, and plants have subunits of ~ 55 and $\sim 35 \text{ kDa}$, respectively. Both types of TrxR, like other flavoenzymes, can produce hydrogen peroxide upon reaction with molecular oxygen (O_2), a hazardous side reaction probably generally suppressed during evolution, except in cases of dedicated enzymes like macrophage NADPH oxidase that has a role in combating infections.²

HMW TrxR is similar in sequence to glutathione reductase (GR) and displays a similar mechanism of transfer of reducing equivalents that involves interfaces between monomers and domains.³ The hydride from NADPH is delivered to the *re* face of FAD and leaves the opposite *si* face to reduce the pair of redox-active cysteines within a conserved motif (CVNVGC), assisted by a histidine residue from the other subunit. A distinguishing feature between HMW TrxR and GR is the C-terminal extension of a flexible segment (of ~ 15 residues) having an additional redox center that shuttles reducing equivalents from the internal redox-active cysteines near the FAD to Trx.⁴ In HMW TrxRs from mammals, this additional redox center is a selenosulfide pair, but organisms such as *Drosophila melanogaster* instead have a second redox-active disulfide that intriguingly works with a similar efficiency.⁵ Several structures of HMW TrxRs are available as well as complexes with Trx.^{6,7}

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LMW TrxRs by contrast undergo a large conformational change allowing the transfer of electrons to Trx.⁸ The two FAD domains are central for LMW TrxR dimer formation and keep their relative positions, while the NADPH domains rotate, with respect to the FAD domains, by 66° to switch between the flavin-oxidizing (FO) and flavin-reducing (FR) conformations in the catalytic cycle. In the FO conformation, the FAD and the redox-active disulfide are buried and positioned for internal electron transfer (from the *re* face of FAD), which excludes both thioredoxin and NADPH from productive interaction. In the more open FR conformation, the *re* face of FAD is exposed for the delivery of hydride from NADPH and the active site cysteines are exposed for interaction with Trx. Reducing equivalents thus enter and leave FAD at the same face. A nearby aspartic acid residue (CA^T/√CD) assists the thiol–disulfide exchange with Trx.^{9,10} The differences in mechanisms between the HMW and LMW types of enzymes make bacterial TrxR a promising target for new antimicrobial drugs.^{11–13}

There is substantial structural information about LMW TrxRs from several eubacterial species, including a crystal structure of TrxR from *Helicobacter pylori* in reduced form at an excellent resolution of 1.45 Å.¹⁴ In addition, TrxR structures were determined from archaea, yeast, plants, and a unicellular eukaryote. The FR conformation, however, has so far only been observed for *Escherichia coli* TrxR (EcTrxR), in a structure of the complex with Trx, stabilized by an intermolecular disulfide bond formed using single-active site cysteine mutants.^{8,15,16} Stopped-flow analysis of EcTrxR with NADPH conducted under anaerobic conditions at 1 °C demonstrated that the reductive half-reaction is very fast.¹⁷ Although the oxidative half-reaction is slower, it is the rearrangement between the FO and FR conformations that appears to limit turnover.⁸

Lactococcus lactis is a model organism for lactic acid bacteria and industrially important as a starter culture in the dairy industry. Like many Gram-positive bacteria within the genera *Bacillus*, *Staphylococcus*, and *Streptococcus*, *L. lactis* lacks a glutathione biosynthesis system. *L. lactis* furthermore has no bacillithiol found in *Bacillus* species, *Staphylococcus aureus*, or *Deinococcus radiodurans*.¹⁸ Remarkably, the knockout mutant of *trxB* (encodes TrxR) in *L. lactis* is viable with no special requirements for the growth medium,¹⁹ while the corresponding gene appears to be more or less essential in *Bacillus subtilis* and *S. aureus*.^{20,21} LiTrxA is an efficient reductant for three different redoxin substrates: LiTrxA, similar to the well-characterized Trx1 from *E. coli* (EcTrx1), with a WCGPC active site motif; LiTrxD having a WCGDC motif; and LiNrdH.²² The latter is a glutaredoxin-like protein providing electrons to ribonucleotide reductase class Ib.^{23,24} LiTrxA appears to act as a general disulfide reductase, whereas LiTrxD seems to have more specialized functions, possibly including arsenate detoxification.²⁵ In this study, comparison of the *L. lactis* and *E. coli* Trx systems showed that the activity of LiTrxR is unstable *in vitro*. Ambient light in the laboratory is sufficient to destroy the activity, which motivated the closer investigation of the nature of light inactivation. Native LiTrxR, protected from light, and a light-inactivated form both show a high rate of turnover with molecular oxygen (O₂) as the electron acceptor.

■ EXPERIMENTAL PROCEDURES

Protein Purification. The genetic constructs, growth conditions, and purification of recombinant His-tagged TrxRs and Trxs from *E. coli* and barley have been described previously.^{26–28} LiTrxA and LiTrxR were prepared similarly

also using the pET15a expression vector.²² Cultures producing EcTrxR and LiTrxR were incubated either overnight at 20 °C or for 6 h at 30 °C following addition of IPTG (0.1 mM). The cell pellet from a 1 L culture was resuspended in 20 mL of Bugbuster protein extraction reagent (Novagen) containing Benzonase nuclease (Merck) and supplemented with FAD (0.2 mM). Without the FAD supplement, the occupancy of FAD in the preparations of EcTrxR and LiTrxR was 10–40% as judged from the comparison of the protein concentration and absorbance (456 nm). The extract was shaken slowly (30 min at room temperature). After centrifugation at 4 °C, the supernatant was filtered (pore size of 0.45 μm) and applied to a 5 mL HisTrap column (GE Healthcare) equilibrated with loading buffer [20 mM potassium phosphate (pH 7.4), 500 mM NaCl, and 10 mM imidazole]. The column was washed with 2 column volumes of loading buffer with 50 mM imidazole. Finally, the TrxRs were eluted by a linear gradient of 50 to 200 mM imidazole in loading buffer. Yellow fractions were pooled, dialyzed [6–8 molecular weight cutoff (MWCO), Spectra/Por 1] against 0.1 M potassium phosphate (pH 7.4) and 1 mM EDTA, and concentrated to at least 5 mg/mL in a volume of ≤5 mL (Ultra-15 10 kDa MWCO, Amicon). Protein samples were stored at –80 °C in aliquots. The yields of protein were higher for the TrxRs (>50 mg/L culture) than for the Trxs.

Spectrophotometric Analysis of Enzyme and Cofactor. Absorption spectra of oxidized TrxRs (300–600 nm) were routinely recorded (Ultrospec 2100 *pro*, Amersham Biosciences). The ϵ_{456} of FAD in EcTrxR determined to be 11300 M^{–1} cm^{–1} and equal to that of free FAD at 450 nm²⁹ was used for quantification of FAD in LiTrxR both before (456 nm) and after light inactivation (445 nm) as well as for isolated light-damaged cofactor (445 nm). FAD occupancy was assessed by comparison to the protein concentration determined by the aid of amino acid analysis after hydrolysis for 24 h in 6 M HCl.³⁰ Fluorescence emission spectra of 5.0 μM LiTrxR or EcTrxR were recorded (PerkinElmer luminescence spectrometer LS55). Excitation and emission wavelengths (10 nm slit widths) were 450 and 470–700 nm, respectively.

Measurement of TrxR Activity. LiTrxR, EcTrxR, and HvNTR2 were assayed in a 1 mL format at 2.0 μM LiTrxA, EcTrx1, and barley HvTrx1, respectively, close to the experimentally determined K_M values of 3.5, 2.2, and 1.2 μM, respectively.^{22,31} The assay mixture contained 0.2 mM NADPH with 0.2 mM DTNB as the final electron acceptor in 0.1 M potassium phosphate (pH 7.5), 2 mM EDTA, and 0.1 mg/mL BSA, and reactions were started by the addition of TrxR to a final concentration of 20 nM. The formation of TNB anion was measured at 412 nm (ϵ_{412} = 13600 M^{–1} cm^{–1}).

Activity of LiTrxR and EcTrxR was also measured as described above but in the absence of Trx and DTNB, forcing the enzymes to use O₂ as an electron acceptor, termed the NADPH/O₂ assay. The oxidation of NADPH was recorded at 340 nm (ϵ_{340} = 6220 M^{–1} cm^{–1}), and the concentration of TrxR was varied between 50 and 200 nM to obtain a turnover number. The two TrxRs were also compared with respect to the use of 40 μM dichlorophenolindophenol (DCPIP) as an unspecific electron acceptor monitoring the disappearance of absorbance at 600 nm (ϵ_{600} = 20000 M^{–1} cm^{–1}) at a TrxR concentration of 100 nM.

Conditions for Light Inactivation. The standard buffer during light incubations contained 0.1 M potassium phosphate (pH 7.5), 2 mM EDTA, 0.1 mg/mL BSA, and 2.0 μM TrxR in

an Eppendorf tube kept 20 cm below a 1225 Lumen (21 W) lamp in the cold room (4 °C). According to the manufacturer Leuci (Relco group), the color temperature is 6500 K corresponding to a λ_{max} of 460 nm. Samples (10 μL) were withdrawn at intervals during the inactivation and assayed after dilution to 1 mL in standard buffer (to obtain a final TrxR concentration of 20 nM). The LITrxR concentration was varied (2.0–40 μM) in separate experiments with intermediate dilutions to achieve a concentration of 20 nM in the assay. Overnight exposure for preparative-scale formation of light-damaged enzyme was performed at 50–200 μM LITrxR. To study the influence of pH on the light inactivation process, samples contained the standard components (2.0 μM LITrxR, 2 mM EDTA, and 0.1 mg/mL BSA), but the major buffering component of the standard buffer [0.1 M potassium phosphate (pH 7.5)] was replaced with potassium acetate, adjusted by acetic acid at pH 4.0 and 5.0, a mix of monobasic and dibasic potassium phosphate at pH 6.0, 7.0, and 8.0, and bicine adjusted with NaOH at pH 9.0.

The influence of dissolved molecular oxygen (O_2) on light inactivation was probed using an O_2 scavenging system in the standard buffer consisting of 10 mM glucose, glucose oxidase from *Aspergillus niger* (0.1 mg/mL), and bovine catalase (0.2 mg/mL). Glucose was omitted from the control sample. The influence of iodide ions was tested by including potassium iodide (0.1 M KI) in the standard buffer. As a reference, potassium chloride (0.1 M KCl) was included in the standard buffer to account for the increased ionic strength.

Reconstitution of Light-Treated LITrxR. To LITrxR (20 μM in 1 mL) exposed to light treatment for 3 h under standard conditions as described above (and a light-protected control) was added a large excess of FAD (8 mM in 1.5 mL). Subsequently, guanidinium chloride (GdmCl) was gradually added to a final concentration of 2 or 3 M followed by incubation for 21 h with gentle shaking at 4 °C. The samples were then diluted in 0.1 M potassium phosphate (pH 7.5), 2 mM EDTA, and 0.1 mg/mL BSA in several steps to reach GdmCl concentrations of 1, 0.5, and 0.1 M followed by incubations for 18, 1.5, and 3 h, respectively, at 4 °C. The samples were then concentrated to 1 mL by ultrafiltration (Ultra-15 10 kDa MWCO, Amicon), loaded on a PD-10 column, and further concentrated (Ultra-0.5 10 kDa MWCO, Amicon) to a final volume of 0.5 mL. The TrxR activity of the samples before and after reconstitution was analyzed as described above and compared to controls incubated without GdmCl.

Heat Extraction of the Light-Damaged Cofactor. A LITrxR sample (190 μM) was exposed for 16 h under standard conditions for light inactivation (see above) and inspected for the resulting spectrum to show the typical appearance of light damage. The buffer was exchanged by loading the sample (0.8 mL) on a PD10 column equilibrated in 62 mM ammonium acetate (pH 6.6). Protein from the recovered fractions was incubated at 75 °C over 20 min, resulting in a large precipitate. After centrifugation, the supernatant was loaded on an ultrafiltration device (Ultra-0.5, Ultracel-10 Membrane, Amicon) and the permeate was retained.

Mass Spectrometric Analysis of FADs. Undamaged and light-damaged FAD was analyzed by high-resolution mass spectrometry (HRMS) on a maXis G3 quadrupole time-of-flight (qTOF) mass spectrometer (Bruker Daltonics), equipped with an electrospray ionization (ESI) source and connected to an Ultimate 3000 ultra-high-performance liquid chromatog-

raphy (UHPLC) system with a UV/vis diode array detector (Dionex) and a Kinetex 2.6 μm pentafluorophenyl (PFP), 100 mm \times 2.1 mm column (Phenomenex, Torrance, CA) maintained at 40 °C. A linear 20 min, 0 to 100% gradient from 20 mM aqueous formic acid to 20 mM formic acid in acetonitrile at a rate of 0.4 mL/min was used, followed by isocratic elution for 3 min. HRMS was performed in both negative (ESI^-) and positive (ESI^+) ion mode with a data acquisition range of 10 scans per second at m/z 100–1000. Sodium formate was automatically infused before each run and provided a mass accuracy of <1.5 ppm. MS/MS was performed at 20 and 40 eV for both ESI^+ and ESI^- .

Ion-pair UHPLC–MS/MS analysis was conducted using an Agilent 1290 binary UHPLC system coupled with an Agilent 6460 triple quadrupole instrument equipped with an ESI source and operated in negative mode. Nitrogen was used as the collision gas at 30 eV. Separation was performed on an Agilent Poroshell phenyl column using a 10 mM tributylamine, 10 mM acetic acid (pH 5.5), and 90% methanol gradient system as described in detail by Magdenoska et al.³²

Reactions with Dinitrophenylhydrazine (DNPH). Light-inactivated, heat-extracted 40 μM FAD was incubated at room temperature with 80 μM DNPH (total volume of 200 μL) in 10 mM phosphoric acid to give pH values of 3.0–3.5. Extracted FAD from untreated LITrxR and EcTrxR was used as a reference. Cinnamaldehyde (40 μM) was used as a positive control. Blanks containing the compounds described above but no DNPH were incubated in parallel. After 3 days in the dark at room temperature, precipitates in samples were visually inspected.

Analysis of Ligand Binding in Three-Dimensional Structures of TrxR. The interactions between FAD and polypeptides in TrxRs were examined by the tool PDBsum (<http://www.ebi.ac.uk/thornton-srv/databases/pdbsum>), Ligand Explorer (<http://www.rcsb.org/pdb/home/home.do>), and Pymol.

RESULTS

LITrxR Is Light-Sensitive. The thoroughly studied *E. coli* Trx system (EcTrxR/EcTrx1) was used as a point of reference in the characterization of *L. lactis* TrxR. The *E. coli* system was subjected to steady state kinetics, and the saturation curve with EcTrx1 as the substrate for EcTrxR generated a k_{cat} of 25 s^{-1} and a K_{M} of 2.5 μM , in agreement with the parameters reported in the literature.^{17,33} The *L. lactis* system (LITrxR/LITrxA), however, showed an approximately 10-fold lower k_{cat} . It was noticed that the activity of fresh preparations was higher but decayed considerably upon storage for a few hours on ice. After various principal issues such as the integrity of the DNA clone and the solubility and stability of the enzyme had been interrogated, it became evident that LITrxR is light-sensitive. The problem of inactivation was solved by avoiding daylight exposure and storing LITrxR in brown tubes. Light-protected LITrxR with LITrxA as a substrate thus yielded a k_{cat} of $\sim 26 \text{ s}^{-1}$, essentially identical to that of the corresponding *E. coli* system.

The susceptibilities of LITrxR, EcTrxR, and barley TrxR (HvNTR2) to light inactivation were compared. A compact fluorescent lamp ($\lambda_{\text{max}} = 460 \text{ nm}$, i.e., blue light) situated in the cold room was used as the standard condition for light inactivation, and after incubation for 4 h, EcTrxR and HvNTR2 retained >70% activity using their cognate Trx acceptors (EcTrx1 and HvTrxh1) while LITrxR retained <4% activity (Figure 1). In the dark, all three enzymes retained >90%

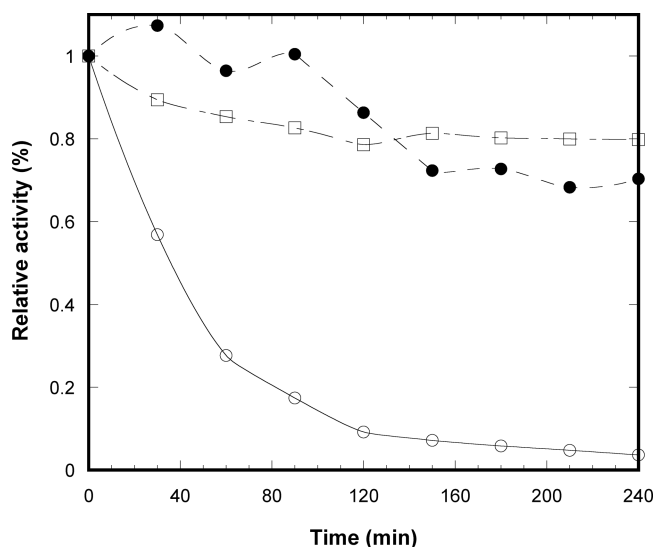


Figure 1. Inactivation of TrxR from *L. lactis*, *E. coli*, and *Hordeum vulgare* (HvNTR2) at pH 7.5. Samples at 2.0 μ M LITrxR (○), EcTrxR (●), and HvNTR2 (□) were exposed to light under standard conditions (20 cm below a 21 W lamp with a maximal intensity at 460 nm). Samples were withdrawn over 4 h and directly assayed together with their cognate Trx. The activities at different time points relative to the initial activity before light exposure are shown.

activity. Inactivation curves of LITrxR repeated on four separate occasions indicated a half-life of ~ 35 min (Figure S1 of the Supporting Information), and a very low level of residual activity (0.2%) was determined after 16 h. Inactivation of 2–40 μ M LITrxR (Figure S2 of the Supporting Information) showed that only the highest concentrations (20 and 40 μ M) provided significant protection.

Light-Damaged LITrxR Shows a Distinctly Changed Flavin Spectrum. Spectrophotometric analysis revealed that light inactivation is accompanied by major spectral changes in LITrxR (Figure 2). The spectra of a 40 μ M sample of LITrxR displayed large changes in three regions after illumination for 3 h. The main peak (1), giving rise to the yellow color, was blue-shifted from ~ 455 to ~ 445 nm but retained a slight shoulder toward higher wavelengths (465–475 nm in undamaged LITrxR). The magnitude of the second peak (2), around 380 nm, decreased. Finally, a large increase at 300–330 nm (3) not extending to the maximal absorbance at 260–270 nm (Figure S3 of the Supporting Information) where the adenosine moiety contributes. Additional spectra from the experiment showed a reinforcement of the described spectral features and a negligible degree of light scattering (Figure S4 of the Supporting Information). The presence of isosbestic points, e.g., at 400 nm, suggests that light damage involves direct conversion to a photoproduct without observable intermediates. The maintained maximal absorbance at 445 nm indicates a very low level of photodestruction of the isoalloxazine ring system (Figure S4 of the Supporting Information). Consistent with the spectral characteristics of oxidized flavin, diffuse daylight from a window was very harmful, while standard “warm-white” bulbs (typical λ_{max} values of >1000 nm) shifted the spectrum only slowly. EcTrxR and free FAD used as reference substances for these initial spectral observations were insensitive to light (data not shown).

The fluorescence emission of the flavin bound to LITrxR was 3-fold higher than that of EcTrxR before inactivation (Figure

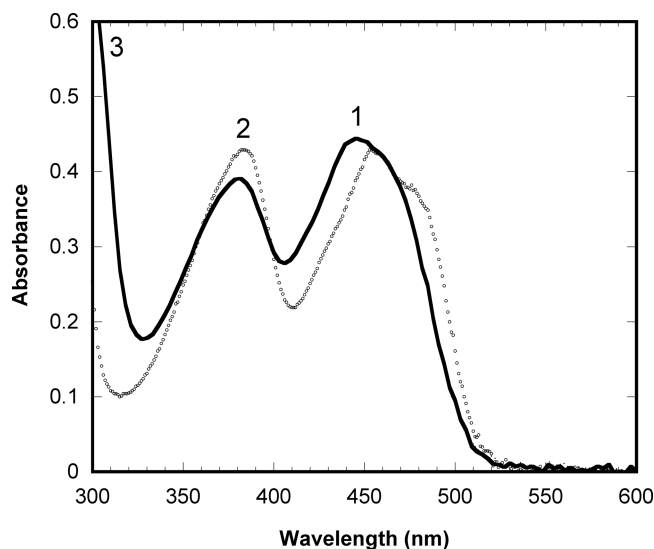


Figure 2. Spectral shift of LITrxR upon light treatment. The spectrum between 300 and 600 nm of LITrxR (40 μ M) is shown before (small empty circles) and after 3 h (solid line) of exposure under the 21 W lamp. Three major changes (1–3) are indicated: a blue shift of the main peak (1), a suppression of the second peak (2), and an increase in absorbance below 330 nm (3). Spectra of 10 μ M LITrxR to display the region at 250–300 nm are shown in Figure S3 of the Supporting Information. Additional spectra of time points from this experiment are shown in Figure S4 of the Supporting Information.

S5 of the Supporting Information). This difference in quantum yield between the two enzymes suggests that the isoalloxazine ring is less rigidly fixed in LITrxR.³⁴ After light exposure of LITrxR, the emission intensity decreased to a level slightly lower than that of EcTrxR, and the maximal emission showed a small shift from 515 to 510 nm. These properties of EcTrxR were not affected by light treatment (Figure S5 of the Supporting Information).

Analysis of LITrxR by reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis revealed a band of apparent cross-linked dimer that increased in intensity after light inactivation. More than 95% of light-inactivated LITrxR, however, appeared in the monomeric form (data not shown). Inactivated LITrxR was subjected to trypsin digestion, but subsequent analysis by matrix-assisted laser desorption ionization time-of-flight MS did not reveal any differences in the peptide profile compared to that of a sample not exposed to light.

Addition of a large excess of exogenous FAD to light-damaged LITrxR in the presence of 2–3 M GdmCl (see Experimental Procedures for details) resulted in increased activity from approximately 7 to 40% relative to the level prior to inactivation. No increase in activity was observed after reconstitution of light-protected samples.

Taken together, these observations indicate that light inactivation is accompanied by structural changes in the tightly bound flavin cofactor, but modification on the protein level cannot be excluded.

The Rate of Light Inactivation Is Affected by pH, Oxygen Concentration, and the Presence of Iodide Ions. LITrxR was subjected to light inactivation in buffers with a range of different pH values. At pH 4.0 and 9.0, activity declined already prior to light exposure [time zero (Figure 3)], suggesting that the enzyme is destabilized. The lowest pH

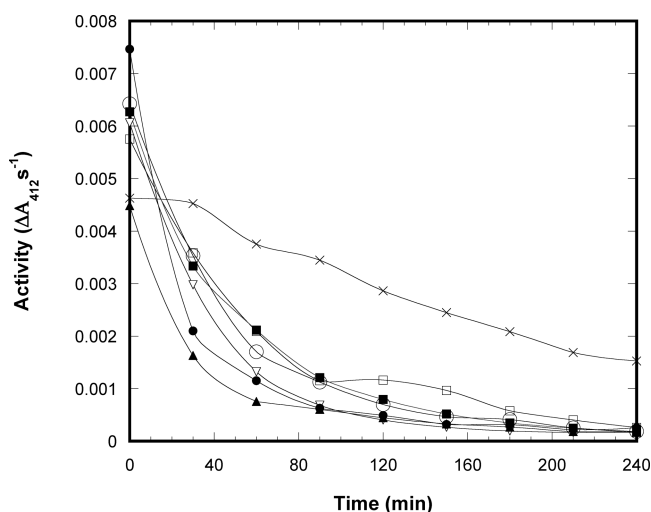


Figure 3. Dependence of pH on light inactivation. LITrxR was diluted to $2.0 \mu\text{M}$ in buffers in the pH range from 4.0 to 9.0, and samples were removed over a 4 h light inactivation period for activity measurements with LITrxA as the substrate and DTNB as the final electron acceptor. Symbols used for the pH buffers: (x) 4.0, (□) 5.0, (■) 6.0, (▽) 7.0, (○) 7.5 (standard buffer), (●) 8.0, and (▲) 9.0. Absolute activity values, in $\Delta A_{412} \text{ s}^{-1}$, are shown.

(4.0), however, conferred a strong protective effect against light inactivation possibly because of non-native conformations of LITrxR in the vicinity of the flavin. At pH 9.0, inactivation is clearly faster than at the standard pH of 7.5. A mechanism of breakdown initiated by hydroxylation similar to what is observed for riboflavin analogues at alkaline pH may take place if a hydroxide ion can be stabilized in the vicinity of the flavin.³⁵ However, the moderate increase in the rate of inactivation above pH 7.5 does not suggest that hydrolysis is involved in the light inactivation process.

The O_2 concentration in the inactivation buffer was lowered by addition of glucose, glucose oxidase, and catalase. The scavenging system was validated by a >10-fold decreased activity of LITrxR in the NADPH/ O_2 assay, likely explained by a correspondingly decreased amount of dissolved O_2 . The scavenging system strongly slowed the inactivation (Figure 4), suggesting the involvement of O_2 . Next, addition of potassium iodide, a quencher of photoexcited triplet state flavins, was investigated. Indeed, iodide has a protective effect at least as strong as that caused by a lowered O_2 content (Figure 5). To account for the additional ionic strength, potassium chloride was included in the reference sample.

The influence of LITrxA and LITrdH ($1 \mu\text{M}$), superoxide dismutase (0.02 and 0.1 mg/mL), FAD and riboflavin ($20 \mu\text{M}$), DTT (2 mM), and tryptophan (1 mM) as additives was tested separately using a 2 h illumination. Only riboflavin and FAD offered minor protection, albeit less than the O_2 -scavenging system. No effect on inactivation was obtained by removing 2 mM EDTA (often employed at a higher concentration in anaerobic photoreduction)³⁶ from LITrxR by gel filtration. These various findings suggested the involvement of molecular oxygen (O_2) and photoexcited FAD in the process of inactivation.

LITrxR Reduces Molecular Oxygen (O_2) Faster Than EcTrxR. EcTrxR is the most well-characterized LMW TrxR, and its sequence is 37% identical to that of LITrxR. These two TrxRs displayed similar k_{cat} values when they were assayed with their cognate Trx, and DTNB as the final electron acceptor.

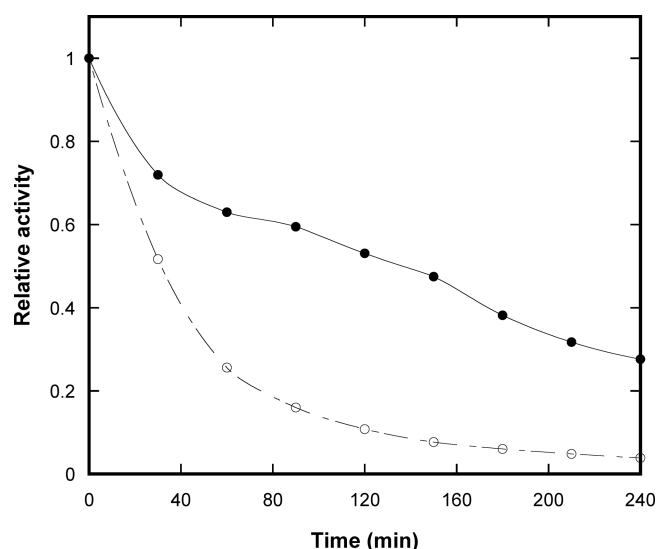


Figure 4. Influence of O_2 on light inactivation. LITrxR was diluted to $2.0 \mu\text{M}$ in standard buffer supplemented with glucose oxidase and catalase (0.05 and 0.2 mg/mL, respectively). Glucose (10 mM) was added to activate the enzymatic system for removal of O_2 and H_2O_2 (●) and a control without glucose (○). Samples were removed over a 4 h light inactivation period for activity measurements with LITrxA as the substrate and DTNB as the final electron acceptor. The activities relative to the initial activity before light exposure are shown.

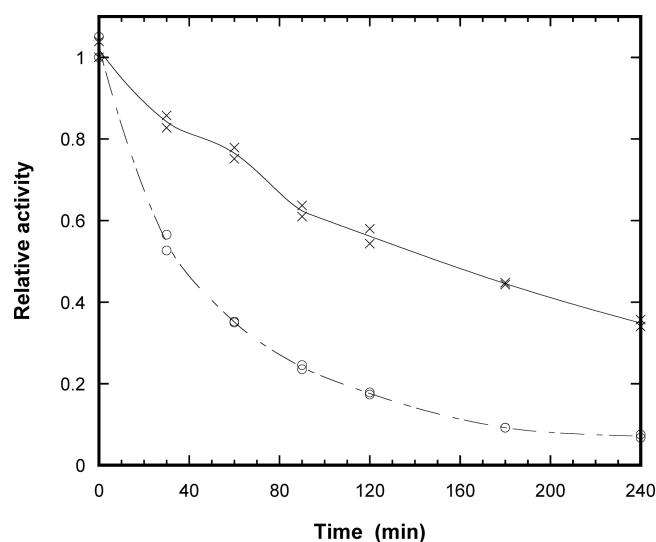


Figure 5. Influence of iodide ions on light inactivation. LITrxR was diluted to $2.0 \mu\text{M}$ in standard buffer supplemented with 0.1 M KI (x) or in the control with 0.1 M KCl (○). Samples were removed over a 4 h light inactivation period for activity measurements with LITrxA as the substrate and DTNB as the final electron acceptor. The activities relative to the initial activity before light exposure are shown.

EcTrxR displayed a turnover of 1.7 s^{-1} for reduction of the classical redox dye DCPIP, twice that of LITrxR (data not shown). These results suggest that the two reduced TrxRs have somewhat different surface properties.

When assayed without an added electron acceptor, TrxR reduces O_2 to H_2O_2 and LITrxR showed a turnover of 0.33 s^{-1} in the NADPH/ O_2 assay, 10-fold faster than that of EcTrxR (Figure 5). Assuming the commonly accepted O_2 concentration of $200 \mu\text{M}$ in aqueous solutions, EcTrxR reduces O_2 at a rate of $160 \text{ M}^{-1} \text{ s}^{-1}$, i.e., slightly more slowly than free reduced

flavin ($250 \text{ M}^{-1} \text{ s}^{-1}$),³⁷ while the corresponding 10-fold higher rate for LTrxR is $1600 \text{ M}^{-1} \text{ s}^{-1}$. In comparison, flavin-dependent oxidases dedicated to the use of O_2 typically reduce with much higher rate constants in the range of 10^5 – $10^6 \text{ M}^{-1} \text{ s}^{-1}$.^{38,39} Light-inactivated LTrxR with <2% residual activity in the DTNB assay retained the high reduction rate of O_2 as seen for the noninactivated enzyme. In fact, a slightly higher turnover of 0.4 s^{-1} was recorded, demonstrating that NADPH is able to reduce the light-inactivated enzyme. However, in the NADPH/ O_2 assay, the reductive half-reaction is conceivably at least 2 orders of magnitude faster than the oxidative.¹⁷ A correspondingly large decrease in the reductive half-reaction can thus pass undetected in the assay.

Mass Spectrometric Analysis of the Light-Damaged FAD Suggests Aldehyde Formation. Flavins from both light-inactivated and untreated material were extracted by heat, with a recovery of >50%, suggesting that a majority of the cofactors are not covalently bound to the enzyme. Analysis of the extract from light-inactivated LTrxR by UHPLC–ESI⁺–HRMS revealed a major ion at m/z 800.1433 with an elution profile similar to that of the nonmodified flavin (m/z 786.1642) on a PFP column (Figure S6 of the Supporting Information). The mass difference (m/z 13.979) unambiguously corresponds to the addition of one oxygen atom and the loss of two hydrogen atoms, consistent with conversion of a methyl group to an aldehyde. Furthermore, a minor ion at m/z 816.1385 indicated further oxidation of the aldehyde to a carboxylate group or, alternatively, but less likely, hydroxylation of a second methyl group (Figure S6 of the Supporting Information). The native and presumed aldehyde-modified forms of FAD were readily baseline separated by ion-pair chromatography, and the shorter retention time of the modified FAD is consistent with a higher polarity of the oxidized species. ESI⁺ MS/MS spectra of the presumed FAD aldehyde and the nonmodified flavin were highly similar and only displayed fragment ions originating from only the ribityl and phosphoadenosyl groups (data not shown). ESI[−] MS/MS of the nonmodified flavin on the other hand showed a low-intensity ion at m/z 241.0729 originating from the isoalloxazine ring system that was shifted up by m/z 13.979 in the aldehyde form, suggesting that the site of modification is localized within this part of the FAD molecule (Figure S7 of the Supporting Information).

To consolidate the results of the mass spectrometric analysis, we probed the inactivated LTrxR with DNPH that reacts with ketone and aldehyde groups to form insoluble derivatives. Brick-red precipitates appeared with FAD extracted from light-inactivated LTrxR and cinnamaldehyde (the positive control), while precipitate was not observed with FAD extracted from EcTrxR or untreated LTrxR. Although the precipitate was similar to the positive control, stoichiometric oxidation to an aldehyde cannot be concluded.

The two methyl groups defined as positions 7 α and 8 α on the isoalloxazine ring system are the possible sites of aldehyde formation. Oxidation of position 8 α was previously observed in a flavoenzyme.⁴⁰ In the R268K mutant of lactate oxidase from *Aerococcus viridans*, FMN was almost completely transformed into 8-formyl-FMN upon aging. However, the positional identification relied on the spectral properties being similar to those of the synthesized analogue 8-formyl tetraacetylriboflavin.⁴¹ Spectra of oxidized forms formylated at position 8 α are different from the spectrum of the light-damaged FAD in LTrxR (see Discussion). Edmondson⁴¹ showed that 8-formylriboflavin forms a blue 8-formylflavin hydroquinone

cation upon reduction by TiCl_3 in 6 M HCl, and the reaction does not require removal of O_2 . Incubating the extracted light-damaged cofactor from LTrxR under these conditions (a 5-fold excess of TiCl_3) did not increase absorbance above 550 nm, indicating that 8-formyl-FAD is not a major constituent of the light-damaged FAD in LTrxR.

DISCUSSION

Here we report on the surprising inactivation of LTrxR by blue light under aerobic conditions, a phenomenon not reported for any other TrxR. In fact, light inactivation under these conditions is unusual or at least slow for other flavoenzymes. On the basis of the mass spectrometric analysis and a positive reaction with DNPH, our results suggest generation of an aldehyde derived from a methyl group. Mass spectrometric fragmentation pinpointed the modification to be localized in the isoalloxazine ring, which has two methyl groups. Iodide seems to exert a protective effect by scavenging photoexcited FAD. It appears likely that triplet state flavin formed upon photoexcitation reacts with O_2 , generating a reactive oxygen species ($^1\text{O}_2$ or O_2^-), which in turn attacks and oxidizes the methyl group to a formyl group. A modification of the methyl group at position 7 α has, to the best of our knowledge, never been reported in a flavoenzyme, but it has been observed at the level of riboflavin. Yagi and co-workers thus isolated both 7 α - and 8 α -hydroxyriboflavin from urine and showed that liver microsomes catalyze this NADPH-dependent hydroxylation.^{42,43}

Modifications of position 8 α are implicated in covalent attachment of flavins to some enzymes, e.g., succinate dehydrogenase and monoamine oxidase.⁴⁴ The influence of these linkages on activity and redox potential has been studied using synthetic flavin analogues.⁴⁵ 8-Formyl-FAD, 8-formyl-tetraacetylriboflavin, and 8-formyl-FMN extracted from the aged R286K mutant of L-lactate oxidase show nearly identical spectral properties (in the range of 300–800 nm).^{40,41,46} Like that of light-damaged LTrxR, the spectrum of oxidized flavin with an 8-formyl substitution displays an elevated absorbance at 300 nm (Figure 2). Otherwise, the spectrum is very different; the main peak is red-shifted (460 nm) in contrast to the blue-shift in light-damaged LTrxR, and the second peak around 380 nm is elevated. These differences suggest that the aldehyde instead is formed at position 7 α , which is a testable hypothesis, and crystallization trials with the light-inactivated enzyme have been initiated. The strong negative effect on activity may be caused by changes in redox potential and/or positioning of redox-active flavin atoms (e.g., N5) hindering electron transfer to the dithiol motif. Experimental and theoretical work on 7,8-substituted flavin analogues predicts increases in redox potentials of at least 50 mV.⁴⁷ The light-damaged enzyme is, however, still redox-active with O_2 .

Is there any biological significance of the deviating properties of LTrxR? A naïve idea is simply that *L. lactis*, which thrives at low levels of O_2 , uses light inactivation as a sensor of light and O_2 with immediate effects on DNA replication via ribonucleotide reductase. There are several established examples of photoexcited flavoproteins that function as photoreceptors of blue light,⁴⁸ for example, YtvA from *B. subtilis*.⁴⁹ It could also be suggested that inactivation of TrxR results in an oxidized thiol redox environment that activates redox-sensitive transcription factors such as Spx.⁵⁰ In general, blue light at wavelengths of ≤ 470 nm has an antibacterial effect. Optimal cell killing of *S. aureus* was found to occur at 405 nm, and the mechanism has

been proposed to involve photoexcitation of porphyrins, reaction with O_2 , and subsequent formation of reactive oxygen species.^{51,52}

LlTrxR showed a rate of O_2 reduction 10-fold higher than that of EcTrxR (Figure 6). Similar results were obtained with

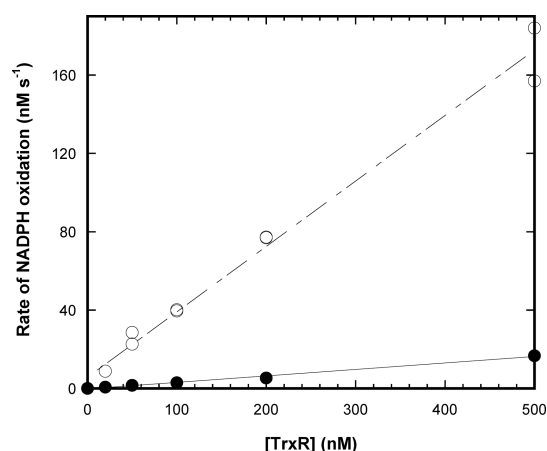


Figure 6. Oxidation of NADPH via TrxR using O_2 as an electron acceptor. LlTrxR (○) and EcTrxR (●) were incubated in the presence of NADPH (0.2 mM). A background rate of $0.00000762 \Delta A_{340} s^{-1}$, conceivably NADPH destruction, was subtracted from all rates, and the activity recorded ($\Delta A_{340} s^{-1}$) was converted to nanomolar NADPH oxidized per second.

the flavoenzyme dihydroorotate dehydrogenase from *E. coli* and *L. lactis*, i.e., a 7-fold higher reduction rate for the *L. lactis* enzyme.⁵³ During fermentative growth, *L. lactis* utilizes NADH oxidases to reduce O_2 to restore the NAD^+ pool. These flavoenzymes display different mechanisms and release either H_2O or H_2O_2 as main product of catalysis.⁵⁴ Similar to many other lactic acid bacteria, *L. lactis* lacks catalase and relies on peroxiredoxins and NADH peroxidases to degrade H_2O_2 . Strains of *Streptococcus pneumoniae* were shown to both tolerate and excrete H_2O_2 , which has been suggested to be a strategy for outcompeting other microorganisms, possibly utilized widely among lactic acid bacteria.^{55,56} *L. lactis* contains a low number of enzymes with iron–sulfur clusters, generally sensitive to H_2O_2 and O_2^- .

Might other LMW TrxRs have the features shown here for LlTrxR, i.e., sensitivity to light and a high rate of O_2 reduction? Both processes are oxidative; however, only the O_2 reduction in the NADPH/ O_2 assay is without doubt dependent on the fully reduced form, obtained by hydride transfer, while the comparatively slower light inactivation may involve a one-electron-reduced species of FAD. Furthermore, the site of oxidative attack is probably different; we identified carbon 7 α as a probable site for light inactivation, and the C4a carbon is likely to be employed for O_2 reduction. Both oxidases and hydroxylases, dedicated to the use of O_2 , employ C4a-hydroperoxide intermediates in catalysis.² Dehydrogenase flavoenzymes in contrast seem to have evolved toward low reactivity with O_2 , and reduction can be considered as an inappropriate side reaction. A113 on the *re* side of the C4a locus was identified as a gatekeeper for O_2 reduction in L-galactono- γ -lactone dehydrogenase, and the corresponding A113G mutation, creating more free space above the locus, converted the enzyme to a catalytically competent oxidase.⁵⁷ A change in the opposite direction, toward less reduction of O_2 ,

was achieved in lactate monooxygenase by a mutation that diminished the amount of free space above the C4a locus.⁵⁸ TrxR structures in the FO conformation are defined by having the active site disulfide within 3.0–4.0 Å of C4a and N5, thus preventing access of O_2 . Exposure of the reactive locus, however, probably takes place during the switch between conformations and in the FR conformation without bound NADPH or $NADP^+$. The structure of HvNTR2 shows a conformation between the FR and FO conformations, with the active site disulfide closer to carbon 7 α .⁵⁹ Like EcTrxR, HvNTR2 shows a low rate of O_2 reduction, observed as a background rate in measurements of insulin reduction by the barley Trxs.⁶⁰ Apart from the accessibility of O_2 , stabilization of the activated oxygen species in the reaction intermediate greatly affects the rate, e.g., by a lysine residue, identified to be primarily responsible for stabilization of the transient negative charge on FAD C(4a)-hydroperoxide in sarcosine oxidase.^{2,61}

At present, LMW TrxR structures in the FO conformation from 14 species are available in the Protein Data Bank (PDB). There are several interactions with FAD, most of which concern the ribityl group, the two phosphate groups, and the adenosine moiety. With respect to polar interactions with the chemically active isoalloxazine ring, only two direct hydrogen bonds recur in all 14 structures. They involve O2 as the acceptor and N3 as the donor, respectively. The corresponding residues involved are the main chain NH group of a nonpolar residue (A295_{EcTrxR}) and a completely conserved Asn residue (N51_{EcTrxR}). These two hydrogen bonds, also seen in the FR conformation of EcTrxR, appear to be central to LMW TrxRs, certainly serving both to keep the ring system in position and to control its reactivity. Other residues surrounding the isoalloxazine ring show less conservation, as outlined below.

The enzyme from *S. aureus* (SaTrxR) is the closest homologue of LlTrxR with a determined structure (PDB entry 4GCM). The sequence identity is 53% (with no need for introducing gaps) in a pairwise alignment (Figure S8 of the Supporting Information). Five residues in SaTrxR (Q42, N45, T46, Y133, and D138) have atoms within 6.0 Å of the 7 α -methyl carbon of FAD, and only one of them is completely conserved, D138, the active site acid/base corresponding to D139_{EcTrxR}. Q42_{SaTrxR} (Q42_{EcTrxR}) is strongly conserved, although its substitution with alanine in *Mycobacterium tuberculosis* TrxR maintains a conserved hydrogen bond to a phosphate through its main chain NH group.⁶² N45_{SaTrxR} and Y133_{SaTrxR} occupy less conserved positions, while T46_{SaTrxR} is conserved with substitutions only with serine. These residues in SaTrxR are conserved in the TrxRs from *L. lactis*, *Streptococcus mutans*, and *Bacillus anthracis* (BaTrxR), which group together in the alignment (Figure S8 of the Supporting Information). BaTrxR has been thoroughly investigated, including spectral characterization, but no unexpected properties were reported.⁶³

In comparison to EcTrxR, the 7 α -methyl carbon is buried less tightly in the SaTrxR structure; e.g., the distance to the phenolic oxygen atom of Y118_{EcTrxR} is 3.8 Å, while the closest atom (NZ) of the corresponding residue in SaTrxR, K117, is 7.6 Å from the 7 α -methyl carbon. Moreover, Ala134_{EcTrxR} (CB) is positioned within 4.0 Å, while the corresponding residue Y133_{SaTrxR} shows a closest distance of 5.4 Å (ring carbon CD). This substitution is notable in a segment that is highly conserved in the context of the active site cysteines and acid/base aspartate residue (SYCAVCDG). Furthermore, these residues are displaced during the transition to the FR conformation. Subtle differences related to the conformational

change can make LTrxR differently reactive with O₂, and the structure of SaTrxR is not a sufficiently good model for making predictions about the mechanisms. However, we find it likely that TrxRs from more closely related genera, e.g., *Streptococcus* (with ~70% sequence identity), display properties like those of LTrxR.

■ ASSOCIATED CONTENT

■ Supporting Information

Eight figures as indicated in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

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■ ABBREVIATIONS

DCPIP, dichlorophenolindophenol; DNPH, 2,4-dinitrophenylhydrazine; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); EcTrx1, *E. coli* Trx1; EcTrxR, *E. coli* TrxR; ESI, electrospray ionization; FO, flavin-oxidizing; FR, flavin-reducing; GR, glutathione reductase; HMW, high-molecular weight; HRMS, high-resolution mass spectrometry; HvNTR2, thioredoxin reductase from barley; LlnrdH, *L. lactis* NrdH; LTrxA, *L. lactis* TrxA; LTrxD, *L. lactis* TrxD; LTrxR, *L. lactis* TrxR; LMW, low-molecular weight; MS, mass spectrometry; MS/MS, tandem MS; qTOF, quadrupole time-of-flight; TNB, 2-nitro-5-thiobenzoate; Trx, thioredoxin; TrxR, NADPH-dependent thioredoxin reductase; UHPLC, ultra-high-performance liquid chromatography.

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