Replacement of Pro^{109} by His in TlpA, a thioredoxin-like protein from *Bradyrhizobium japonicum*, alters its redox properties but not its in vivo functions

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Abstract **TlpA, the membrane-anchored, thioredoxin-like protein from *Bradyrhizobium japonicum*, is essential for cytochrome aa_3 biogenesis. The periplasmic domain of TlpA was previously shown to have protein thiol:disulfide oxidoreductase activity and reducing properties similar to those of cytoplasmic thioredoxins. Here, we replaced the proline-109 in its active-site sequence $C^{107}V^{108}P^{109}C^{110}$ by a histidine residue. The resulting active-site motif (CVHC) resembles that of oxidizing thiol:disulfide oxidoreductases such as protein disulfide isomerase (PDI) and DsbA. Indeed, the TlpA variant P109H was by 66 mV more oxidizing than the wild-type protein. Nevertheless, the altered protein was even more efficient in catalyzing the reduction of insulin disulfides by dithiothreitol than the wild-type due to a faster recycling of its catalytically active, reduced form. Cells of *B. japonicum* expressing only the mutated *tlpA* gene had the same phenotypes as wild-type cells, suggesting that the change in the redox potential of TlpA was not critical for its in vivo function.**

Key words: *Bradyrhizobium japonicum*; Periplasm; Redox potential; Site-directed mutagenesis; Thioredoxin

1. Introduction

The TlpA protein of *Bradyrhizobium japonicum*, a Gram-negative soil bacterium able to live in root-nodule symbiosis with soybean, belongs to the family of protein thiol:disulfide oxidoreductases and has been shown to be essential for cytochrome aa_3 maturation [1]. The monomeric 23 kDa protein is anchored to the cytoplasmic membrane with its N-terminal transmembrane domain (residues 12–35), whereas the hydrophilic, thioredoxin-like domain (residues 36–221) faces the periplasm [1]. The latter domain was purified as a soluble protein ($TlpA_{sol}$) after cleavage of a MalE-$TlpA_{sol}$ fusion protein. Apart from its active-site disulfide bond which is common to all protein thiol:disulfide oxidoreductases, $TlpA_{sol}$ possesses a second, structural disulfide bond [2]. It was found that reduced glutathione (GSH) selectively reduces the active-site disulfide bond, which leads to a 10-fold increase of the intrinsic tryptophan fluorescence of $TlpA_{sol}$. The redox state-dependent fluorescence of $TlpA_{sol}$ provides a means to determine its redox properties. The $TlpA_{sol}$:glutathione equilibrium constant at pH 7.0 was found to be 1.9 M, corresponding to a standard redox potential of −0.213 V. This suggests a reducing function for TlpA in vivo, similar to cytoplasmic thioredoxins [3], which is consistent with the observation that $TlpA_{sol}$ is not a substrate of oxidizing periplasmic disulfide oxidoreductases such as DsbA [3].

TlpA, like thioredoxins, contains the amino acid sequence motif **CXPC** in its active site (Fig. 1). By contrast, bacterial DsbA proteins and eukaryotic PDIs contain the motif **CXHC**. These proteins have a higher redox potential which makes them ideal oxidants of protein thiols. A mutation of Pro^{34} to His in thioredoxin from *Escherichia coli* changed its redox potential to a more positive value [13] and also increased its disulfide isomerase activity [14]. Consistent with this finding, an exchange of His^{32} by Pro in *E. coli* DsbA decreased the redox potential of that protein [15]. In this work we mutated the active-site residue Pro^{109} of TlpA to His to possibly generate an oxidizing variant of TlpA. The mutated *tlpA* gene was introduced into the genome of a *B. japonicum* $tlpA^-$ strain to investigate the influence of TlpA's redox potential on several phenotypes associated with the function of TlpA in *B. japonicum*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

All bacterial strains used are listed in Table 1. *E. coli* strains DH5α and TG1 were used for cloning and propagation of phage M13, respectively. Expression of $TlpA_{sol}$ for protein purification was performed in strain BL21. Strain S17-1 served as donor to mobilize plasmids into *B. japonicum* recipients by conjugation. *E. coli* cells were usually grown aerobically in Luria Bertani (LB) medium at 37°C. For production of $TlpA_{sol}$, *E. coli* BL21 was grown at 26°C. *B. japonicum* cells were grown aerobically in peptone-salts-yeast extract (PSY) medium [20] at 28°C. Antibiotics were added at the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 100 μg/ml; tetracycline, 15 μg/ml for *E. coli* strains, 60 μg/ml for *B. japonicum* strains; chloramphenicol, 10 μg/ml.

2.2. Recombinant DNA work

Plasmid isolation, cloning, transformation, Southern blotting and DNA sequencing were performed according to standard protocols [22,23]. DNA probes for hybridization were labelled with the digoxigenin DNA labelling kit from Boehringer (Mannheim, Germany).

2.3. Site-directed mutagenesis

The exchange of Pro^{109} to His in TlpA was created by in vitro

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Abbreviations: DTT, dithiothreitol; GSH, reduced glutathione; GSSG, glutathione disulfide (oxidized); PDI, protein disulfide isomerase

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mutagenesis (version 2 of the system from Amersham, Buckinghamshire, UK). For this purpose, we used the phage M13mp18 derivative, M13mp18*tlpA-7*, carrying the *Bam*HI–*Sal*I fragment from *tlpA*, and the oligonucleotide primer P109H (5′-CCTTCCGACAGTGCACGCACCAGG-3′). The introduction of the correct mutation was confirmed by sequencing, and the mutated clone was named M13mp18*tlpP109H*.

2.4. Construction of plasmids and B. japonicum mutant strains

The plasmids used in this work are listed in Table 1. Plasmid pRJ3580 was created by isolating the *Bam*HI–*Hin*dIII fragment with the *tlpA* mutation from M13mp18*tlpP109H* RF-DNA and ligating it into plasmid pRJ3548 [2] from which the corresponding *Bam*HI–*Hin*dIII wild-type fragment had been removed.

For overproduction of the mutant protein, a 620-bp *Ban*I fragment encoding the soluble domain of TlpA(P109H) was isolated from pRJ3580 and (after filling in the ends) ligated into the vector pMal-p that had been digested with *Stu*I. The translational *malE′-′tlpP109H* fusion in the resulting plasmid, pRJ3581, was confirmed by sequencing.

Plasmid pRJ3556 was constructed to create the *tlpA* deletion mutant, Bj3556. For this purpose, the 540-bp *Bam*HI–*Nru*I fragment of *tlpA* was first removed from plasmid pRJ3548 and replaced by the 1398-bp *Bam*HI–*Nru*I fragment from plasmid pUC4-KIXX carrying the *aphII* gene for kanamycin resistance. This resulted in plasmid pRJ3552 with the *aphII* gene being transcribed in the same direction as *tlpA*. The construct was then transferred into vector pSUP202pol3 by isolating the *Xba*I–*Hpa*I fragment from pRJ3552, filling in the ends and ligating it into pSUP202pol3 that had been digested with *Eco*RI and *Xho*I (ends made blunt by filling in). The resulting plasmid, pRJ3556, was mobilized into *B. japonicum* 110*spc*4 cells, and kanamycin-resistant, tetracycline-sensitive clones were selected. The replacement of the chromosomal wild-type *tlpA* gene by the *tlpA::aphII* gene due to double crossover was then confirmed by appropriate Southern blot analysis.

Plasmids pRJ3582 and pRJ3583 were obtained by isolating the corresponding 1.6-kb *Xho*I fragments from pRJ3580 and pRJ3548, respectively, and ligating them into *Xho*I-digested pSUP202pol3. Strains Bj3582 and Bj3583 were then created by introducing pRJ3582 and pRJ3583, respectively, into the *tlpA* deletion mutant Bj3556 and selecting for kanamycin- and tetracycline-resistant clones. The correct co-integration of the plasmids upstream of the *tlpA* deletion in the *B. japonicum* chromosome was confirmed by Southern blot analysis.

2.5. Purification of $TlpA_{sol}$(P109H)

$TlpA_{sol}$(P109H) was expressed as a MalE fusion protein in *E. coli* BL21. After preparing the periplasmic fraction according to Loferer et al. [3], the fusion protein was purified by affinity chromatography on an amylose column. $TlpA_{sol}$(P109H) was then cleaved off by digestion with factor Xa and separated from MalE by ion exchange chromatography using a DEAE Sepharose fast-flow column as described previously for wild-type $TlpA_{sol}$ [2].

2.6. Determination of protein concentration

The concentration of purified $TlpA_{sol}$ and $TlpA_{sol}$(P109H) was determined by the absorbance at 280 nm and using an extinction coefficient of $\varepsilon_{280nm} = 17{,}270\ M^{-1}\ cm^{-1}$ for the native, oxidized protein [3]. The protein concentration of membrane preparations was measured by using the Bradford reagent from Bio-Rad Laboratories (Richmond, CA).

2.7. Insulin disulfide reduction assay

The $TlpA_{sol}$-catalyzed reduction of insulin disulfides by dithiothreitol was analyzed according to the method of Holmgren [24], that was slightly modified as follows. $TlpA_{sol}$ and $TlpA_{sol}$(P109H) were preincubated with the reaction buffer (0.1 M potassium phosphate buffer, pH 7.0, 2 mM EDTA) containing 1 mM DTT at 25°C for 45 min to allow reduction of the proteins by DTT. The reaction was then started by addition of insulin to a final concentration of 0.13 mM. Thioredoxin from *E. coli* (Promega, Madison, WI) was used as a positive control.

2.8. Kinetics of $TlpA_{sol}$ and $TlpA_{sol}$(P109H) reduction by DTT

The kinetics of the reduction of $TlpA_{sol}$ and $TlpA_{sol}$(P109H) by DTT were examined by the increase of tryptophan fluorescence using a SX-17MV Stopped-Flow Reaction Analyser (Applied Photophysics, Leatherhead, UK) with an excitation wavelength of 295 nm and an emission cut-off filter of 320 nm. The reaction was performed at 25°C under pseudo first-order conditions in 0.1 M potassium phosphate buffer, pH 7.0, 1 mM EDTA (filtered) with a final protein concentration of 1 μM and final DTT concentrations of 1 mM for wild-type $TlpA_{sol}$ and 100 μM for the P109H variant. For calculation of the second-order rate constant (K_2), the exact concentration of reduced DTT was determined as described by Ellman [25].

2.9. Determination of the redox potential

The redox potential of the active-site cysteines of $TlpA_{sol}$(P109H) was calculated from the equilibrium constant with glutathione at pH 7.0 and 30°C as described previously for the wild-type protein [3].

2.10. Enzyme assays

Cytochrome *c* oxidase activity was determined from membrane preparations of aerobically grown *B. japonicum* cells. The membrane proteins were solubilized by 1% dodecylmaltoside in 50 mM HEPES/NaOH, pH 7.5, and oxidation of reduced cytochrome *c* (from horse heart) was followed at 550 nm essentially as described by Zufferey et al. [26].

2.11. Plant infection test

Soybean seedlings (*Glycine max* L. Merr. cv Williams) were inoculated with the corresponding *B. japonicum* strains and grown as described by Hahn and Hennecke [27]. After 21 days, nitrogen fixation activities of the root nodules were determined by the acetylene reduction assay [28], and the number, size, colour and dry weight of the nodules were recorded. With each *B. japonicum* strain, 8–10 soybean plants were tested.

3. Results

Previous work from different groups has shown that the redox potential of protein thiol:disulfide oxidoreductases is influenced by the amino acid residues located between the two active-site cysteines [13,15]. To further characterize the in vivo function of TlpA from *B. japonicum*, we substituted Pro^{109} for His by site-directed mutagenesis of the *tlpA* gene.

Bj TlpA	103-WATW**CV**P**C**RKEM-114	[1]
Rs Thio	26-WAEW**CG**P**C**RQIG-37	[4]
Bs Thio	25-WAEW**CG**P**C**RQIG-36	[5]
Ec TrxA	28-WAEW**CG**P**C**KMIA-39	[6]
Sc Thi1	27-FATW**CG**P**C**KMIA-38	[7]
Hs Thio	27-SATW**CG**P**C**KMIK-38	[8]
Ec DsbA	26-FSFF**CP**H**C**YQFE-37	[9]
Hi DsbA	26-FSFY**CP**H**C**YAFE-37	[10]
Av DsbA	40-FWYG**CP**H**C**YQFE-51	unpubl.
Sc PDI1(1)	57-FAPW**CG**H**C**KNMA-68	[11]
(2)	402-YAPW**CG**H**C**KRLA-413	[11]
Hs PDI(1)	49-YAPW**CG**H**C**KALA-60	[12]
(2)	393-YAPW**CG**H**C**KQLA-404	[12]

Fig. 1. Comparison of the active-site sequences of different members of the protein thiol:disulfide oxidoreductase family. The sequences of thioredoxin, DsbA and PDI are from the following organisms: *Bradyrhizobium japonicum* (*Bj*), *Rhodobacter sphaeroides* (*Rs*), *Bacillus subtilis* (*Bs*), *Escherichia coli* (*Ec*), *Saccharomyces cerevisiae* (*Sc*), *Homo sapiens* (*Hs*), *Haemophilus influenzae* (*Hi*) and *Azotobacter vinelandii* (*Av*). The two thioredoxin-like domains of PDI are referred to as (1) and (2). References [1,4–12] are indicated in brackets behind the sequences. The sequence of DsbA from *A. vinelandii* was derived from the Swiss Prot data base (accession number L76098). The two active-site cysteines and the amino acid residue preceding the second cysteine are shown in bold face letters.

The soluble domain (residues 36–221) of the TlpA(P109H) variant was expressed in *E. coli* and purified, yielding 4 mg pure protein from a 2-l culture. Wild-type $TlpA_{sol}$ was also purified with the same yield. The fluorescent properties of oxidized and dithiothreitol-reduced $TlpA_{sol}$(P109H) were similar to those of the wild-type protein [3]. The increase of the intrinsic tryptophan fluorescence upon reduction of the active-site disulfide was used to determine the $TlpA_{sol}$(P109H):glutathione equilibrium constant at pH 7.0 and 30°C. For this purpose, oxidized $TlpA_{sol}$(P109H) was incubated in the presence of 20 μM GSSG and varying concentrations of GSH (0.01–10 mM), corresponding to $[GSH]^2/[GSSG]$ ratios of 5 μM to 1.4 M. Fig. 2 shows the fraction of reduced $TlpA_{sol}$(P109H) at equilibrium (R) that was calculated from the measured fluorescence (F) according to Eq. 1, where F_{red} and F_{ox} are the fluorescence intensities of completely reduced and oxidized protein, respectively.

$$R = (F - F_{ox})/(F_{red} - F_{ox}) \quad (1)$$

The redox equilibrium of $TlpA_{sol}$(P109H) with glutathione is described by Eq. 2–5.

$$TlpA_{red} + GSSG \overset{K_{eq}}{\rightleftharpoons} TlpA_{ox} + 2\ GSH \quad (2)$$

$$K_{eq} = \frac{[TlpA_{ox}] \times [GSH]^2}{[TlpA_{red}] \times [GSSG]} \quad (3)$$

$$R = \frac{([GSH]^2/[GSSG])}{K_{eq} + ([GSH]^2/[GSSG])} \quad (4)$$

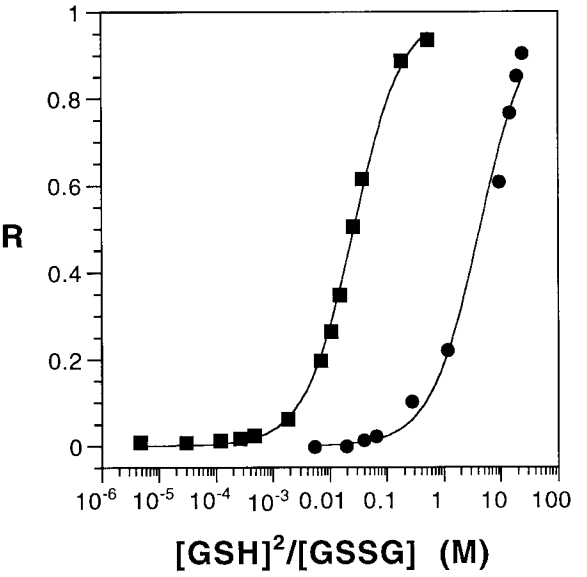
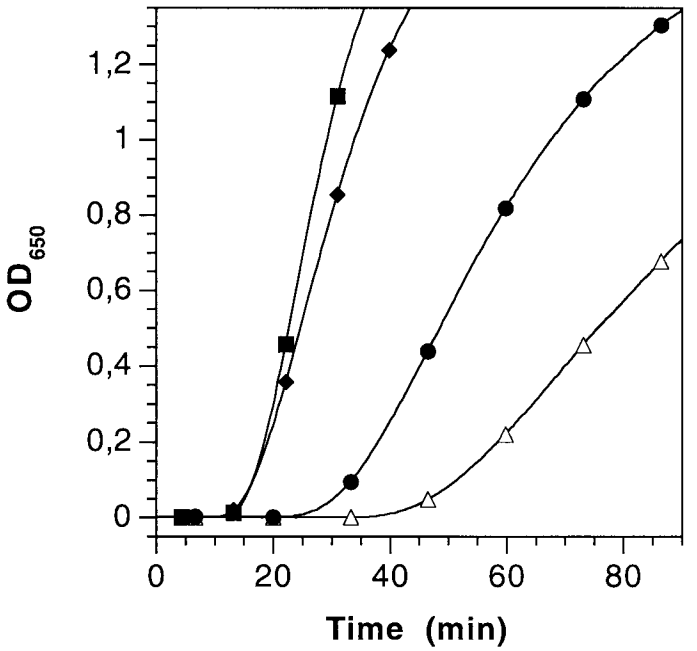


Fig. 2. Redox equilibrium of $TlpA_{sol}$(P109H) and $TlpA_{sol}$ with glutathione. Proteins (1 μM) were incubated at 30°C in 100 mM sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and glutathione as follows: 20 μM GSSG and 0.01–10 mM GSH for $TlpA_{sol}$(P109H) (■) and 10 μM GSSG and 0.25–125 mM GSH for $TlpA_{sol}$ (●). After 20 h, the fraction of reduced protein (R) at equilibrium was measured using the specific fluorescence of the proteins at 355 nm (excitation at 295 nm). The data were fitted according to Eq. 4 (see text).

Table 1
Strains, phages and plasmids

Strain/phage/plasmid	Relevant characteristics	Reference/source
E. coli strains		
DH5α	*supE*44 Δ*lacU*169 (Φ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *relA*1	[16]
TG1	K12, Δ(*lac*, *pro*) *supE thi hsd*Δ5, F′(*tra*D36 *proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*ΔM15)	[17]
S17-1	*pro thi hsdR*, RP4-2 *kan*::Tn*5* *tet*::Mu, integrated in the chromosome	[18]
BL21	F^- *ompT* r_B^- m_B^-	[19]
B. japonicum strains		
110*spc*4	Sp^r (wild type)	[20]
Bj3556	Δ*tlpA*, Km^r	This work
Bj3582	Bj3556, plasmid pRJ3582 (*tlpP109H*$^+$) co-integrated in the chromosome 5′ of Δ*tlpA*	This work
Bj3583	Bj3556, plasmid pRJ3583 (*tlpA*$^+$) co-integrated in the chromosome 5′ of Δ*tlpA*	This work
Phages		
M13mp18		[21]
M13mp18*tlp7*	520-bp *Bam*HI–*Sal*I fragment of *tlpA* in M13mp18	[2]
M13mp18*tlpP109H*	M13mp18*tlp7*, codon 109 of *tlpA* (CCC for Pro) mutated to CAC (for His)	This work
Plasmids		
pUC18	Ap^r	[21]
pRJ3548	Ap^r, 3.2-kb *Eco*RI fragment (*tlpA*$^+$) in pUC18 (250-bp *Sma*I–*Nde*I fragment removed)	[2]
pRJ3580	335-bp *Bam*HI–*Hin*dIII fragment of pRJ3548 replaced by the *Bam*HI–*Hin*dIII fragment from M13mp18*tlpP109H*	This work
pMal-p	Ap^r, *lacI*^{q}P$_{tac}$*malE*-fx-*lacZ*α	New England Biolabs
pRJ3581	620-bp *Ban*I fragment of pRJ3580 cloned into the *Stu*I site of pMal-p	This work
pUC4-KIXX	Ap^r, Km^r, *lacZ*::*aphII*	Pharmacia
pRJ3552	540-bp *Bam*HI–*Nru*I fragment of pRJ3548 (*tlpP109H*$^+$) replaced by the 1.4-bp *aphII* cassette (Km^r) of pUC4-KIXX	This work
pSUP202	Ap^r, Cm^r, Tc^r, *oriT* from RP4	[18]
pSUP202pol3	Tc^r, *Dra*II–*Eco*RI part of the pBluescript II KS+polylinker between *Eco*RI and *Pst*I of pSUP202	T. Kaspar, ETH Zürich, unpublished
pRJ3556	2.06-kb *Xba*I–*Hpa*I fragment from pRJ3552 (Δ*tlpA*::*aphII*) cloned into pSUP202pol3	This work
pRJ3582	1.6-kb *Xho*I fragment from pRJ3580 (*tlpP109H*$^+$) cloned into pSUP202pol3	This work
pRJ3583	1.6-kb *Xho*I fragment from pRJ3548 (*tlpA*$^+$) cloned into pSUP202pol3	This work



Catalyst	Maximal slope [ΔOD_{650}/min]	Onset of aggregation [min]
none	0.019	36.4
$TlpA_{sol}$ (30 μM)	0.032	22.2
$TlpA_{sol}$(P109H) (30 μM)	0.081	11.5
Trx (1 μM)	0.058	10.6

Fig. 3. Insulin reduction assay. $TlpA_{sol}$(P109H) (30 μM) (■), $TlpA_{sol}$ (30 μM) (●) or *E. coli* thioredoxin (Trx, 1 μM) (♦) was incubated with 0.1 M potassium phosphate buffer, pH 7.0, containing 2 mM EDTA and 1 mM DTT for 45 min at 25°C. Buffer with DTT alone served as control for the uncatalyzed reduction of insulin by DTT (△). The reactions were started by addition of bovine pancreatic insulin (final concentration: 0.13 mM) and precipitation of the reduced, free insulin chains was followed by the increase in optical density at 650 nm (OD_{650}). Data were collected every 10 s; only some of the data are indicated by symbols.

$$E'_{0T} = E'_{0G} - \frac{RT}{nF} \times \ln K_{eq} \quad (5)$$

After fitting the normalized fluorescence data by non-linear regression according to Eq. 4, equilibrium constants of 0.027 M and 4.35 M were determined for the $TlpA_{sol}$(P109H)/glutathione and $TlpA_{sol}$/glutathione systems, respectively. Although the latter value is about two times higher than the previously determined one [3], this does not greatly affect the calculation of the redox potential of the wild-type protein (see below). Using the Nernst equation (Eq. 5) and a value of −0.240 V [29] for the glutathione standard redox potential (E'_{0G}), we determined standard redox potentials (E'_{0T}) of −0.193 V for $TlpA_{sol}$(P109H) and −0.259 V for $TlpA_{sol}$. The latter value is in good agreement with that determined previously [3]. (A different value for E'_{0G} had been used in the previous work. For a better comparison, the previous data for $TlpA_{sol}$ were recalculated, resulting in a E'_{0T} value of −0.248 V.) Thus, the P109H exchange in $TlpA_{sol}$ caused an increase in the redox potential by 66 mV.

The disulfide reductase activity of $TlpA_{sol}$(P109H) was then compared to that of $TlpA_{sol}$ with the insulin reduction assay [24]. The three disulfide bridges of insulin are reduced by a slight excess of dithiothreitol in this assay, and the aggregation of reduced insulin is recorded by the increase of the optical density at 650 nm. As shown in Fig. 3, catalytic amounts of $TlpA_{sol}$(P109H) (1/13 at the disulfide level compared to insulin) reduced insulin with an approximately 2.5-fold higher maximal rate (ΔOD_{650} min^{-1}) than the wild-type protein, which was accompanied by a twofold earlier onset of aggregation (see table below the graph in Fig. 3). In view of the more positive redox potential of $TlpA_{sol}$(P109H), this increase in insulin reduction activity was unexpected. However, a possible explanation might be that recycling of the catalytically active, reduced form of $TlpA_{sol}$ by DTT is the rate-limiting step in the catalytic cycle. In this case, reduction of the stronger oxidant $TlpA_{sol}$(P109H) should indeed be faster than the reduction of the wild-type protein.

To test this hypothesis, the reduction of $TlpA_{sol}$(P109H) and $TlpA_{sol}$ by a 100-fold and 1000-fold molar excess of DTT, respectively, was measured in a stopped-flow experi-

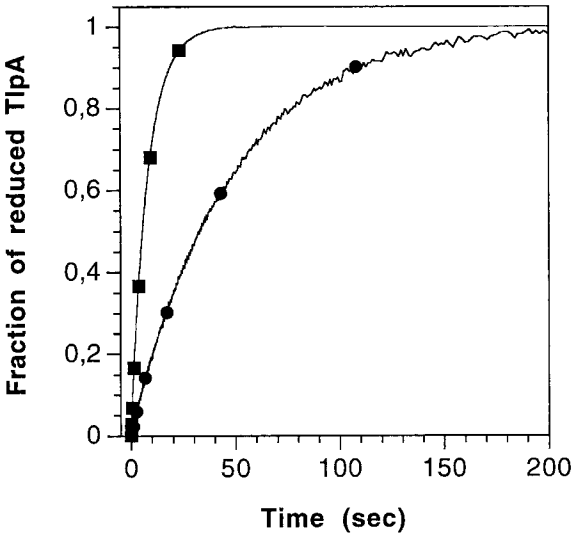


Fig. 4. Kinetics of the reduction of $TlpA_{sol}$(P109H) and $TlpA_{sol}$ by DTT. Proteins (1 μM) were reduced under pseudo first-order conditions using a 1000-fold molar excess of DTT for the wild-type $TlpA_{sol}$ (●) and a 100-fold molar excess for $TlpA_{sol}$(P109H) (■). The reaction was followed by the increase in tryptophan fluorescence in a SX-17MV Stopped-Flow Reaction Analyser (emission cut-off filter of ≤320 nm).

ment using the increase in tryptophan fluorescence (Fig. 4). The pseudo first-order rate constants (K_{app}) were determined to be 0.0211 s^{-1} and 0.126 s^{-1} for $TlpA_{sol}$ and $TlpA_{sol}$(P109H), respectively. Second-order rates ($K_2 = K_{app}$ $[DTT]^{-1}$) of 21 M^{-1} s^{-1} and 1181 M^{-1} s^{-1}, respectively, were calculated from these values. Thus, the increased activity of $TlpA_{sol}$(P109H) in insulin reduction is obviously due to an about 56 times faster recycling of the reduced catalyst by DTT.

These data clearly showed that a Pro^{109}-to-His exchange in $TlpA_{sol}$ causes a significant change of the redox properties of this protein. It was therefore of interest to test whether this change had an effect on the in vivo functions of the protein. To prevent undesirable recombination events between the chromosomal *tlpA* gene and mutated *tlpA* genes that were to be introduced into the cells, we constructed a defined *B. japonicum tlpA* deletion mutant, Bj3556, in which 545 nucleotides (81.8%) of the chromosomal *tlpA* gene were substituted by the kanamycin resistance cassette from plasmid pUC4-KIXX. The phenotype of this mutant was similar to that of the *B. japonicum* COX64 mutant carrying a Tn5 insertion in *tlpA* [1]. The plasmids containing either *tlpA* or *tlpP109H* were then mobilized into strain Bj3556 (Δ*tlpA*) and integrated via homologous recombination upstream of the *tlpA* deletion in the chromosome. The resulting strains were grown aerobically in PSY medium and assayed for cytochrome *c* oxidase activity in the membrane fractions. *B. japonicum* 110*spc*4 (wild type) and Bj3556 (Δ*tlpA*) served as positive and negative controls, respectively. No significant difference was observed between the activities of wild-type, Bj3583 (*tlpP109H*$^+$) and Bj3582 (*tlpA*$^+$) cells (0.786, 0.770 and 0.827 μmol cyt *c* (mg protein)$^{-1}$ min^{-1}, respectively). Almost no activity was detected in strain Bj3556 (Δ*tlpA*) (0.003 μmol cyt *c* (mg protein)$^{-1}$ min^{-1}).

The same strains were used to infect soybean seedlings. Plants infected with Bj3582 (*tlpA*$^+$) developed root nodules that were similar in size and number to those of plants infected with wild-type cells. They were of red colour inside (presence of leghemoglobin) and showed nitrogenase activities similar to those of nodules harbouring bacteroids of Bj3583 (*tlpP109H*$^+$) (265 and 268 μmol ethylene h^{-1} (mg nodule dry weight)$^{-1}$ were produced). For comparison, nodules containing wild-type cells produced 184, those containing Bj3556 (Δ*tlpA*) only 4 μmol ethylene h^{-1} (mg nodule dry weight)$^{-1}$.

4. Discussion

A considerable change in the redox potential of TlpA (66 mV more positive) did not cause an alteration of the in vivo functions of the protein. By contrast, a P34H variant of *E. coli* thioredoxin, possessing a redox potential that was only 35 mV higher than that of the wild-type protein [13], was able to restore viability of a *pdi1* deletion mutant of *Saccharomyces cerevisiae* (lacking protein disulfide isomerase), whereas mutant cells containing the wild-type thioredoxin gene (*trx*) did not grow [30]. In this heterologous system, alteration of the redox potential enabled the variant thioredoxin to replace PDI in vivo. However, the phenotype of a *trxP34H* mutation within *E. coli* has not been reported as far as we know.

An exchange of the amino acid residues between the two active-site cysteines of DsbA from *E. coli* also created variant proteins with different redox potentials. The strains carrying the different *dsbA* mutations were tested for their ability to overcome millimolar concentrations of DTT in the growth media. Although most of the mutants were more sensitive to DTT than the wild-type, the ranking of their sensitivity did not exactly reflect the ranking of their equilibrium redox potential [15].

How important are the intrinsic redox potentials of thiol:disulfide oxidoreductases for their function in vivo? In the most simple case, a thiol:disulfide oxidoreductase (E) is only catalyzing the transfer of electrons from a reductant (R) to an oxidant (O):

$$R \xrightarrow{e^-} E \xrightarrow{e^-} O$$

A change of the redox potential of the enzyme, for instance by a point mutation, would mean that the rate constants of its oxidation and reduction change in opposite directions. However, the overall rate of electron transport and thus the in vivo function of the enzyme would not be affected by the altered redox potential, as long as the rate-limiting reaction (formation of oxidized or reduced enzyme) occurs at about the same rate as in the wild-type protein. This appears to be the case for variants of TlpA and other periplasmic thiol:disulfide oxidoreductases such as DsbA [15].

The situation is completely different in the lumen of the ER of eukaryotic cells. In this compartment, the excess of GSH and GSSG is likely to dictate the fractions of the oxidized and reduced forms of PDI. The GSH/GSSG ratio in the ER was determined to be between 1:1 to 3:1 with an estimated GSH concentration of about 0.5 to 1 mM [31]. From these concentrations and the equilibrium constants between the thioredoxin-like domains of PDI and glutathione (0.7 mM for the *a* domain and 1.9 mM for the *a'* domain; [32]) it follows that the concentrations of oxidized and reduced PDI domains in the ER are practically identical (≈20–60% and 40–80% oxidized molecules in case of the *a* and *a'* domains, respectively).

This suggests that both the oxidized and the reduced form of PDI, i.e. its oxidase and isomerase activity, are very important for the in vivo function of the enzyme and that the PDI_{ox}/PDI_{red} ratio indeed reflects the need for catalysis of formation of native disulfide bonds and reduction of nonnative disulfides in folding proteins:

$$\underset{\sim 50\%\text{ dithiol oxidase activity}}{2\,GSH + PDI_{ox}} \rightleftarrows \underset{\sim 50\%\text{ disulfide isomerase activity}}{GSSG + PDI_{red}}$$

This would explain why the strong reductant thioredoxin is not capable to complement PDI deficiency in yeast, whereas the thioredoxin variant P34H, which has a PDI-like redox potential, restores yeast viability [30]. The general conclusion would be that the intrinsic redox potential of enzymes which are in a permanent, stationary equilibrium with a higher redox system, such as PDI with glutathione or thioredoxin with $NADP^+$/NADPH, are critical for their in vivo function, whereas the overall rate of electron transfer rather than the redox potential is important for bacterial periplasmic thiol:disulfide oxidoreductases. To further assess the mode of TlpA action in the periplasm of *B. japonicum* it will primarily be necessary to identify its target protein(s) and a linked redox system.

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References

[1] H. Loferer, M. Bott, H. Hennecke, EMBO J. 12 (1993) 3373–3383.
[2] H. Loferer, H. Hennecke, Eur. J. Biochem. 223 (1994) 339–344.
[3] H. Loferer, M. Wunderlich, H. Hennecke, R. Glockshuber, J. Biol. Chem. 270 (1995) 26178–26183.
[4] J.D. Clement-Metral, A. Holmgren, C. Cambillau, H. Jörnvall, H. Eklund, D. Thomas, F. Lederer, Eur. J. Biochem. 172 (1988) 413–419.
[5] N.-Y. Chen, J.-J. Zhang, H. Paulus, J. Gen. Microbiol. 135 (1989) 2931–2934.
[6] A. Holmgren, Eur. J. Biochem. 6 (1968) 475–484.
[7] Z.-R. Gan, J. Biol. Chem. 266 (1991) 1692–1696.
[8] E.E. Wollman, L. D'Auriol, L. Rimsky, A. Shaw, J.-P. Jacquot, P. Wingfield, P. Graber, F. Dessarps, J. Biol. Chem. 263 (1988) 15506–15512.
[9] J.C.A. Bardwell, K. McGovern, J. Beckwith, Cell 67 (1991) 581–589.
[10] J.F. Tomb, Proc. Natl. Acad. Sci. USA 89 (1992) 10252–10256.
[11] M. LaMantia, T. Miura, H. Tachikawa, H.A. Kaplan, W.J. Lennarz, T. Mizunaga, Proc. Natl. Acad. Sci. USA 88 (1991) 4453–4457.
[12] T. Pihlajaniemi, T. Helaakoski, K. Tasanen, R. Myllylae, M.-L. Huhtala, J. Koivu, K.I. Kivirikko, EMBO J. 6 (1987) 643–649.
[13] G. Krause, J. Lundström, J. Lopez-Barea, C. Pueyo de la Cuesta, A. Holmgren, J. Biol. Chem. 266 (1991) 9494–9500.
[14] J. Lundström, G. Krause, A. Holmgren, J. Biol. Chem. 267 (1992) 9047–9052.
[15] U. Grauschopf, J.R. Winther, P. Korber, T. Zander, P. Dallinger, J.C.A. Bardwell, Cell 83 (1995) 947–955.
[16] D. Hanahan, J. Mol. Biol. 166 (1983) 557–563.
[17] Amersham International plc (1984) M13 cloning and sequencing handbook. Amersham, Buckinghamshire, UK.
[18] Simon, R., Priefer, U. and Pühler, A. (1983) In: Molecular genetics of the bacteria plant interaction (Pühler, A., Ed.) pp. 98-106, Springer Verlag, Heidelberg, Germany.
[19] F.W. Studier, A.H. Rosenberg, J.J. Dunn, J.W. Dubendorff, Methods Enzymol. 185 (1990) 60–89.
[20] B. Regensburger, H. Hennecke, Arch. Microbiol. 135 (1983) 103–109.
[21] J. Norrander, T. Kempe, J. Messing, Gene 26 (1983) 101–106.
[22] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[23] F. Sanger, S. Nicklen, A.R. Coulson, Proc. Natl. Acad. Sci. USA 74 (1977) 5463–5467.
[24] A. Holmgren, J. Biol. Chem. 254 (1979) 9627–9632.
[25] G.L. Ellman, Arch. Biochem. Biophys. 82 (1959) 70–77.
[26] R. Zufferey, O. Preisig, H. Hennecke, L. Thöny-Meyer, J. Biol. Chem. 271 (1996) 9114–9119.
[27] M. Hahn, H. Hennecke, Mol. Gen. Genet. 193 (1984) 46–52.
[28] Turner, G.L. and Gibson, A.H. (1980) In: Methods for evaluating biological nitrogen fixation (Bergersen, F.J., Ed.) pp. 111-138, John Wiley and Sons, Chichester.
[29] J. Rost, S. Rapoport, Nature 201 (1964) 185.
[30] P.T. Chivers, M.C.A. Laboissière, R.T. Raines, EMBO J. 15 (1996) 2659–2667.
[31] C. Hwang, A.J. Sinskey, H.F. Lodish, Science 257 (1992) 1496–1502.
[32] N.J. Darby, T.E. Creighton, Biochemistry 34 (1995) 16770–16780.