FEBS Letters 584 (2010) 3376–3380

journal homepage: www.FEBSLetters.org

Kinetic and thermodynamic properties of two barley thioredoxin h isozymes, HvTrxh1 and HvTrxh2

Kenji Maeda [a,1], Per Hägglund [a], Olof Björnberg [a], Jakob R. Winther [b], Birte Svensson [a,*]

[a] Enzyme and Protein Chemistry, Department of Systems Biology, Søltofts Plads, Building 224, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark
[b] Department of Biology, University of Copenhagen, Ole Maaløes Vej 5, DK-2200 Copenhagen N, Denmark

ARTICLE INFO

Article history:
Received 30 April 2010
Revised 10 June 2010
Accepted 15 June 2010
Available online 23 June 2010

Edited by Peter Brzezinski

Keywords:
Thioredoxin
Dithiol/disulfide exchange
Tryptophan fluorescence
Redox potential
Thiol pK_a

ABSTRACT

Barley thioredoxin h isozymes 1 (HvTrxh1) and barley thioredoxin h isozymes 2 (HvTrxh2) show distinct spatiotemporal distribution in germinating seeds. Using a novel approach involving measurement of bidirectional electron transfer rates between *Escherichia coli* thioredoxin, which exhibits redox-dependent fluorescence, and the barley isozymes, reaction kinetics and thermodynamic properties were readily determined. The reaction constants were ~60% higher for HvTrxh1 than HvTrxh2, while their redox potentials were very similar. The primary nucleophile, Cys_N, of the active site Trp-Cys_N-Gly-Pro-Cys_C motif has an apparent pK_a of 7.6 in both isozymes, as found by iodoacetamide titration, but showed ~70% higher reactivity in HvTrxh1, suggesting significant functional difference between the isozymes.

1. Introduction

Thioredoxin (Trx) is a ubiquitous protein disulfide reductase of ~12 kDa [1] containing the so-called Trx-fold with a common βαβαββα topology [2]. The Trx superfamily includes proteins with diverse functions and redox potentials ranging from Trx and glutaredoxin acting as reductants in the cytosol, to the eukaryotic endoplasmatic protein disulfide isomerase and prokaryotic periplasmic DsbA both involved in disulfide bond formation [3].

The cysteines in the active-site motif, Trp-Cys_N-Gly-Pro-Cys_C, form an intramolecular disulfide bond in oxidized Trx. Trx receives reducing equivalents from NADPH-dependent Trx reductase (NTR) or, in plant chloroplasts, from ferredoxin-dependent Trx reductase [4]. The Cys_N thiol acts as a nucleophile and attacks target disulfide bonds to form a transient mixed disulfide; the buried Cys_C thiol completes the reaction by attacking Cys_N with formation of oxidized Trx and reduced target disulfide. The Cys_N pK_a among Trx superfamily members ranges from >6.3 for Trx [5–8] to <4 for Grx [9] and DsbA [10].

Higher plants possess numerous Trx isozymes which are grouped based on sequence similarity [11]. Trx h constitutes the largest group of isozymes suggested to have distinct biological roles [12]. Trx h is proposed to be important for seed germination by solubilising storage proteins and inactivating inhibitors of proteases and carbohydrate-degrading enzymes through disulfide reduction [13]. Barley seeds contain at least two Trx h and two NTR isozymes (barley thioredoxin h isozyme 1 (HvTrxh1), barley thioredoxin h isozyme 2 (HvTrxh2), HvNTR1 and HvNTR2) with distinct spatiotemporal distribution patterns representing one of the best characterized Trx systems from higher plants [14,15]. Here, redox properties of the two barley Trx h isozymes are characterized and compared in details involving a novel approach based on reaction with *Escherichia coli* thioredoxin (EcTrx).

2. Materials and methods

2.1. Protein production

Untagged and his-tagged HvTrxh1 and HvTrxh2 were produced as described [14,16]. The tag did not affect activity in a Trx reductase assay (data not shown). EcTrx was from Promega (Madison, WI, USA). Protein concentrations were determined by amino acid analysis.

Abbreviations: $E^{o\prime}$, standard redox potential; EcTrx, *E. coli* thioredoxin; HvTrxh1, barley thioredoxin h isozyme 1; HvTrxh2, barley thioredoxin h isozyme 2; IAM, iodoacetamide; NTR, NADPH-dependent thioredoxin reductase; TCEP, Tris(2-carboxyethyl)phosphine; Trx, thioredoxin

* Corresponding author. Fax: +45 4588 6307.
E-mail address: bis@bio.dtu.dk (B. Svensson).
[1] Present address: EMBL Heidelberg, Meyerhofstraße 1, 69117 Heidelberg, Germany.

0014-5793/$36.00
doi:10.1016/j.febslet.2010.06.028

2.2. Insulin reduction assay

The reaction mixtures contained his-tagged HvTrxh1 or HvTrxh2 (0.1 μM) in 0.1 M potassium phosphate pH 7.0, 2 mM EDTA, 0.1 mg/ml BSA, 0.16 mM NADPH and insulin in a range of concentrations up to 260 μM. The reactions were initiated by addition of HvNTR2 to 0.2 μM, and the oxidation of NADPH was monitored spectrophotometrically at 340 nm for 4 min. A background ΔA340 of 0.0014 min^{-1} obtained in the absence of insulin was subtracted from the data which were fitted to the Michaelis–Menten equation using Kaleidagraph (Synergy Software, Reading, PA, USA).

2.3. Fluorescence emission spectra of Trxs

The assay buffer (100 mM Hepes pH 7.0, 1 mM EDTA) was degassed, thoroughly purged with argon, and throughout the experiment exposure of Trxs to oxygen was avoided. Fluorescence spectra of 12 μM untagged Trx in the assay buffer (pre-incubated for at least 1 h in 100 μM Tris(2-carboxyethyl)phosphine (TCEP) for reduced Trx) were acquired using a Perkin–Elmer Luminescence Spectrometer LS55 with a thermostated single-cell. The excitation wavelength and emission wavelengths were 280 nm (5 nm slit width) and 300–400 nm (8 nm slit width), respectively.

2.4. Fluorescence Trx assay

Reduced Trx was obtained by incubating 60 μM Trx with 1.0 mM TCEP in 100 μL for at least 1 h at RT. TCEP was removed using a NAP-5 desalting column (GE Healthcare). When Trx was omitted no significant fluorescence increase was observed for the mixture of the desalted solution and the oxidized EcTrx (data not shown), confirming complete removal of TCEP. The temperature of the Trx solutions and the cuvette was adjusted to 25 °C prior to assays. Assays at 25 °C were initiated by mixing reduced and oxidized Trxs to a final concentration of 6.0 μM each in a volume of 150 μl. The initial value of fluorescence intensity was recorded 1 min after mixing, with excitation and emission wavelengths of 280 and 380 nm (8 nm slit width), respectively, and the fluorescence intensity was followed with 2–5 min intervals.

2.5. Alkylation kinetics

His-tagged Trxs (12 μM) were pre-incubated with 0.5 mM TCEP in 5 mM HEPES pH 7.0, 50 mM NaCl at RT for at least 1 h. Alkylation of 4.0 μM reduced Trx was performed in duplicates at RT with 20 μM iodoacetamide (IAM) in 1.0 mM EDTA, 200 mM NaCl and 30 mM MES (pH 5.5–6.6), HEPES (pH 7.0–7.8), and Tris–HCl (pH 8.0–8.6). The reactions were quenched at appropriate time points by adding 40% acetic acid (50 μl) to the assay mixture (150 μl). Unmodified and carbamidomethylated Trxs were separated and quantified using a C18 RP-HPLC column on an ICS-3000 Ion Chromatography System (Dionex, Sunnyvale, CA, USA) at 30 °C. The column was pre-equilibrated with solvent A (0.1% trifluoroacetic acid) and proteins were eluted by a linear 37–54% gradient with solvent B (0.1% trifluoroacetic acid, 90% acetonitrile) for 25 min at 1.0 ml min^{-1}. Using CurveExpert v. 1.3 (D.G. Hyams, Hixson, TN, USA) the second order reaction constants, k, were obtained by fitting to Eq. (1):

$$kt = \frac{1}{[\mathrm{Trx}]_0 - [\mathrm{IAM}]_0} \ln \frac{[\mathrm{Trx}][\mathrm{IAM}]_0}{[\mathrm{IAM}][\mathrm{Trx}]_0} \quad (1)$$

k values were fitted to the Henderson–Hasselbalch equation (Eq. (2)):

$$k = \frac{k_{max}}{1 + 10^{(pK_a - pH)}} \quad (2)$$

3. Results

3.1. Enzymatic properties

We previously reported that HvTrxh1 and HvTrxh2 with 51% sequence identity are reduced by HvNTR2 with similar kinetic parameters – K_m of 1.12 ± 0.04 and 1.29 ± 0.25 μM and k_{cat} of 3.26 ± 0.09 and 2.98 ± 0.16 s^{-1} for HvTrxh1 and HvTrxh2, respectively [15]. Here, reduction of bovine insulin by HvTrxh1 and HvTrxh2 was assayed in a coupled reaction with HvNTR2 as electron donor. Measurement of NADPH oxidation rates (340 nm) yielded limiting rates of 0.0395 ± 0.00079 and 0.0334 ± 0.001 Δ340/min, corresponding to turnovers of 1.1 and 0.90 disulfides per second for HvTrxh1 and HvTrxh2, respectively (Fig. 1). Apparent K_m values of 26.1 ± 1.8 and 37.8 ± 4.8 μM obtained for HvTrxh1 and HvTrxh2, respectively by fitting to the Michaelis–Menten equation were comparable to the K_m of 11 μM found for EcTrx [17]. HvTrxh1 thus showed significantly higher catalytic efficiency than HvTrxh2 with k_{cat}/K_m being $0.41 \cdot 10^5$ and $0.24 \cdot 10^5$ s^{-1} M^{-1}, respectively. This difference was not rate-limited by the reductase (data not shown) and can be compared to the k_{cat}/K_m ratio of 1×10^5 M^{-1} s^{-1}reported for EcTrx with insulin as substrate [17].

3.2. Oxidoreduction kinetics and thermodynamics

To elucidate the oxidoreduction kinetics and thermodynamics of HvTrxh1 and HvTrxh2, EcTrx was employed both as electron donor and acceptor in the electron transfer reactions:

$$\mathrm{EcTrx_{red}} + \mathrm{HvTrxh1_{ox}}\ (\text{or } \mathrm{HvTrxh2_{ox}}) \leftrightarrow \mathrm{EcTrx_{ox}} + \mathrm{HvTrxh1_{red}}\ (\text{or } \mathrm{HvTrxh2_{red}})$$

The reactions can be continuously monitored as the fluorescence intensity of EcTrx is redox-sensitive owing to the non-conserved tryptophan at position −4 from Cys_N [18]. Indeed, complete disulfide reduction by TCEP increased the fluorescence intensity >fivefold for EcTrx (Fig. 2A), but had only minor effect on HvTrxh1 and HvTrxh2 which only are 22% and 23% sequence identical to EcTrx, respectively, and lack this tryptophan (Fig. 3). When oxidized EcTrx (6.0 μM) and equimolar amounts of the reduced HvTrxh1 or HvTrxh2 were mixed, the fluorescence intensity increased due to EcTrx reduction and reached steady state after ~5 and ~10 h, respectively (Fig. 2B). The increases in fluorescence

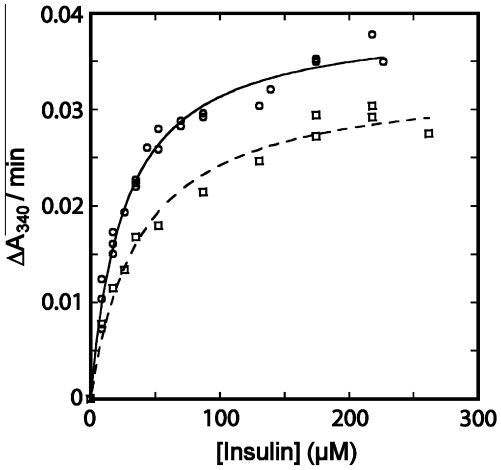

Fig. 1. Reduction of bovine insulin by the barley Trx system. Rates of NADPH oxidation with 0.1 μM HvTrxh1 (circles) and HvTrxh2 (squares) in the presence of 0.2 μM thioredoxin reductase are shown and fitted to the Michaelis–Menten equation.