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Original Contribution

Substrate and inhibitor specificities differ between human cytosolic and mitochondrial thioredoxin reductases: Implications for development of specific inhibitors

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ABSTRACT

The cytosolic and mitochondrial thioredoxin reductases (TrxR1 and TrxR2) and thioredoxins (Trx1 and Trx2) are key components of the mammalian thioredoxin system, which is important for antioxidant defense and redox regulation of cell function. TrxR1 and TrxR2 are selenoproteins generally considered to have comparable properties, but to be functionally separated by their different compartments. To compare their properties we expressed recombinant human TrxR1 and TrxR2 and determined their substrate specificities and inhibition by metal compounds. TrxR2 preferred its endogenous substrate Trx2 over Trx1, whereas TrxR1 efficiently reduced both Trx1 and Trx2. TrxR2 displayed strikingly lower activity with dithionitrobenzoic acid (DTNB), lipoamide, and the quinone substrate juglone compared to TrxR1, and TrxR2 could not reduce lipoic acid. However, Sec-deficient two-amino-acid-truncated TrxR2 was almost as efficient as full-length TrxR2 in the reduction of DTNB. We found that the gold(I) compound auranofin efficiently inhibited both full-length TrxR1 and TrxR2 and truncated TrxR2. In contrast, some newly synthesized gold(I) compounds and cisplatin inhibited only full-length TrxR1 or TrxR2 and not truncated TrxR2. Surprisingly, one gold(I) compound, $[Au(d2pype)_2]Cl$, was a better inhibitor of TrxR1, whereas another, $[(iPr_2Im)_2Au]Cl$, mainly inhibited TrxR2. These compounds also inhibited TrxR activity in the cytoplasm and mitochondria of cells, but their cytotoxicity was not always dependent on the proapoptotic proteins Bax and Bak. In conclusion, this study reveals significant differences between human TrxR1 and TrxR2 in substrate specificity and metal compound inhibition *in vitro* and in cells, which may be exploited for development of specific TrxR1- or TrxR2-targeting drugs.

The thioredoxin system consists of isoenzymes of thioredoxin reductase (TrxR)[1] and thioredoxin (Trx) that are responsible for a wide range of cellular functions, including redox regulation, antioxidant defense, and synthesis of deoxyribonucleotides [1]. The major cytosolic forms of TrxR and Trx are known as TrxR1 and Trx1 and those present in mitochondria are known as TrxR2 and Trx2 [1]; the four proteins are encoded by distinct genes that are all essential, as their respective deletions are embryonically lethal in mice [2–4]. All mammalian TrxR isoenzymes are selenoproteins with a redox-active selenocysteine (Sec) residue in their active sites and belong to the pyridine nucleotide-disulfide oxidoreductase family of proteins that catalyze NADPH-dependent reduction of their native Trx substrates [5,6]. They are homodimers containing a flavin adenine dinucleotide (FAD) redox cofactor and a redox-active disulfide within a conserved CVNVGC motif in one subunit, which interacts with a redox-active selenenylsulfide/selenolthiol motif at the C-terminus of the other subunit [6–9]. In addition to Trx, mammalian TrxR isoforms also accept a range of low-molecular-weight disulfide-containing substrates, including 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), lipoic acid, and lipoamide, as well as nondisulfide substrates such as selenite, quinone compounds, and ascorbic acid [1,6,7,10,11]. This

Abbreviations: DTNB, dithionitrobenzoic acid; DTT, dithiothreitol; TrxR, thioredoxin reductase; TrxR1, cytosolic thioredoxin reductase; TrxR2, mitochondrial thioredoxin reductase; Trx1, cytosolic thioredoxin; Trx2, mitochondrial thioredoxin; PAO, phenylarsine oxide; auranofin, 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosato-*S*-triethylphosphine gold(I); $[Au(d2pype)_2]Cl$, bis[1,2-bis(dipyridylphosphino)ethane] gold(I) chloride; $[Au(d2pypp)_2]Cl$, bis[1,3-bis(di-2-pyridylphosphino)propane] gold(I) chloride; $[(iPr_2Im)_2Au]Cl$, bis(1,3-diisopropylimidazol-2-ylidene) gold(I) chloride.

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broad substrate specificity of mammalian TrxRs is usually attributed to two unique features of the enzymes, the easily accessible C-terminal selenenylsulfide/selenolthiol active center and the ability of the N-terminal FAD/–CVNVGC redox center to directly reduce certain small substrate compounds independent of the canonical Sec-containing active site [12–15].

Most of the current knowledge about mammalian TrxR enzymes is based upon extensive studies of the cytosolic TrxR1 purified from bovine [16], rat [17], or human tissues [5,18,19], as well as mouse and rat proteins expressed in recombinant form [7,15]. Although the gene for mitochondrial TrxR2 has been cloned from a range of mammals including rat, mouse, cow, and human and has been purified from rat liver (reviewed in [12]), there is still limited knowledge about its exact role in mitochondria. Much of what is known about the biochemical properties of TrxR2 stems from the work of Bindoli and Rigobello with co-workers studying the purified enzyme from rat [20] and studies of semisynthetic variants produced by Hondal and co-workers [15,21]. Moreover, the crystal structure of mouse TrxR2 has been solved [10], as well as that of both rat [7,11] and human TrxR1 [22]. Because of evident structural similarities between TrxR2 and TrxR1, particularly regarding the redox-active centers and broad substrate specificities of both enzymes, it is generally thought that TrxR2 has redox-regulating and antioxidant functions in mitochondria that are similar to those TrxR1 would have in the cytosol [7,10,11]. A functional analogy to this would be found in *Drosophila melanogaster*, in which the mitochondrial and cytosolic forms of TrxR are encoded by the same gene [23]. Although mammalian TrxR1 and TrxR2 have some substrates in common, the different subcellular localizations of the isoenzymes have probably given rise to some differences, including specific protein substrates such as Trx2, glutaredoxin 2, and cytochrome *c* for TrxR2 [24,25] and thioredoxin-related protein 14 for TrxR1 [26]. The important question of whether TrxR2 may have some distinct biochemical features differing from those of TrxR1 has not yet been well addressed, but clues for such differences can be found in the literature. For example, it was recently reported that several TrxR-catalyzed substrate reactions might be selenium-independent, which was found using TrxR2-derived enzyme scaffolds [15], whereas such activity with similar substrates was not found using a TrxR1-derived protein [27]. Furthermore, it was suggested recently that TrxR2 might prefer Trx1 as a substrate [28]. However, the substrate specificities of human TrxR1 and TrxR2, with their natural Trx1 and Trx2 substrates or with other low-molecular-weight compounds, have not been investigated previously in a direct side-by-side comparison using well-defined enzyme preparations. Here we have compared the activities of pure human recombinant TrxR1 and TrxR2 using their endogenous human Trx1 and Trx2 substrates and small-molecule dithiol compounds and found that human TrxR2 has different substrate affinities compared to human TrxR1. We also compared the inhibition patterns of human TrxR1 and TrxR2 with metal-based inhibitors and found pronounced differences in inhibition between TrxR1 and TrxR2 using different recently synthesized gold(I) compounds. These compounds inhibited TrxR activity in cells and caused cell death via mitochondria, but by different pathways, some of which required Bax and Bak, whereas others did not.

Materials and methods

Materials

[$(iPr_2Im)_2$Au]Cl, [Au(d2pype)$_2$]Cl, and [Au(d2pypp)$_2$]Cl were synthesized and used as reported elsewhere [29–31]. Platinol (cisplatin) was from Mayne Pharm Pty Ltd. Native 4–16% polyacrylamide gels were from Invitrogen. 2′,5′-ADP Sepharose was from GE Healthcare. All other reagents used in this study were from Sigma, including the gold(I) compounds auranofin and aurothioglucose. Full-length human TrxR1 was amplified from a testes cDNA library (Clontech) by PCR with primers that introduced flanking NcoI and BseAI sites. NcoI and BseAI digestion was used to remove the rat TrxR1 insert along with the engineered SECIS from pET-TRSter [32] and replace it with the hTrxR1 PCR fragment. The engineered SECIS from pET-TRSter was reinserted subsequently into the human TrxR1-encoding plasmid as a BseAI fragment. DNA sequencing confirmed that the TrxR1 sequence was identical to the human TrxR1 isoform 2, Entrez Gene reference sequence NM_182729.1, in which the second amino acid, asparagine, was changed to aspartic acid owing to the insertion of an NcoI restriction site. Human TrxR2 was amplified from HeLa cell cDNA by PCR. The fragment corresponding to amino acids 37–623 of TrxR2 followed by an engineered bacterial SECIS [33] was flanked by NcoI and PacI sites. The PCR product was digested with NcoI and PacI and inserted into NcoI- and PacI-cut pETDuet-1 (Novagen) to make pET-hTrxR2. DNA sequencing showed that the hTrxR2 sequence was identical to the Entrez Gene reference sequence NM_006440.3. Trx1 was a kind gift from Professor A. Holmgren (Karolinska Institutet, Stockholm, Sweden). Trx2 (amino acids 59–166, NP_036605.2) was cloned into pGEX-4T-2 and expressed as a GST fusion protein in *Escherichia coli* Rosetta2, purified using glutathione–Sepharose (GE Healthcare), eluted by thrombin cleavage according to the manufacturer's instructions, and further purified by size-exclusion chromatography in PBS.

Expression and purification of human recombinant TrxR1 and TrxR2

Recombinant human TrxR1 or TrxR2 was expressed in either *E. coli* ER2566 (New England Biolabs) or *E. coli* BL21(DE3) cells (New England Biolabs), transformed with the corresponding pET-hTrxR1 or pET-hTrxR2 plasmids, and in both cases the pSUABC plasmid [32] according to the previously established protocol [27], in growth medium containing 10 g NaCl, 10 g peptone, and 10 g yeast extract per liter water, supplemented with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside, 100 μg/ml L-cysteine, and 5 μM sodium selenite. Cells were harvested and lysed for 1 h using lysozyme, snap-frozen in liquid nitrogen, and sonicated (six 15-s pulses at setting 20 using a Sonifier 150; Branson). The initial purification on 2′,5′-ADP Sepharose (GE Healthcare Life Sciences) and subsequent purification on PAO Sepharose were performed as described previously [27]. The full-length enzymes were further purified by an ÄKTA-Explorer system (GE Healthcare) using a Superdex 200 10/300 column (GE Healthcare) with a total bed volume of 24 ml. The two-amino-acid Sec-deficient truncated human TrxR2 was deliberately expressed as such in *E. coli* ER2566 cells by transformation with a plasmid lacking a bacterial-type SECIS element and having a UAA replacing the original UGA stop codon, thus expressing the enzyme without its last two amino acids (resulting in a C-terminal Gly-Cys-COOH instead of Gly-Cys-Sec-Gly-COOH). This enzyme was purified using 2′,5′-ADP Sepharose, followed by gel filtration on an ÄKTA Purifier system using a Superdex 200 10/300 column (GE Healthcare).

Mass spectrometry

Mass spectrometry analyses were performed on a 4000 Q-TRAP (Applied Biosystems, Foster City, CA, USA) operating with an ion spray voltage of 5500 V and an ion source gas 1 at 30 and scanning over a mass range from 600 to 2000 *m/z* and detection in Q3 scanning mode. Initial *m/z* peaks were deconvoluted with the Bayesian Protein Reconstruct tool to provide the intact protein masses within the Analyst 1.5.1 software (Applied Biosystems).

Gel electrophoresis

We used either 4–16% Bis–Tris native polyacrylamide gels or 10% Tris–Glycine SDS denaturing polyacrylamide gels to resolve the