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A hypothesis on the catalytic mechanism of the selenoenzyme thioredoxin reductase

Thioredoxin reductase (EC 1.6.4.5), like lipoamide dehydrogenase, mercuric reductase and glutathione reductase, is a member of the pyridine nucleotide-disulphide oxidoreductase family of dimeric flavoenzymes [1]. It catalyses the NADPH-dependent reduction of the 12 kDa protein thioredoxin [$NADPH + H^+ + \text{thioredoxin-}S_2 \rightarrow NADP^+ + \text{thioredoxin-}(SH)_2$], which provides reducing equivalents for processes like the reduction of ribonucleotides to deoxyribonucleotides by ribonucleotide reductase [2] and is also considered as a pleiotropic regulator of the cellular redox balance [3,4]. The sequences, sizes and catalytic mechanisms of thioredoxin reductases are different between prokaryotes, such as *Escherichia coli*, and mammals [5,6]. The human enzyme exhibits an unusually broad substrate specificity. Redox-active proteins, such as thioredoxin, protein disulphide isomerase, natural killer cell lysin (NK-lysin) and calcium-binding proteins 1 and 2, can serve as substrates, as can smaller compounds like selenodiglutathione, *S*-nitrosoglutathione, lipid hydroperoxides, lipoic acid, L-cystine, alloxan, menadione and 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) ([2,5,7–9] and references therein). Clearly, any hypothesis for the catalytic mechanism of the enzyme must be able to explain this capability of the protein to reduce so many different substrates.

Recently, it has been shown that mammalian thioredoxin reductases are selenoenzymes [6,10–12]. The selenium was identified as a selenocysteine residue, located close to the C-terminus of the protein as the penultimate amino acid [10]. This discovery explains the futile attempts to express active recombinant enzyme in heterologous systems [13]. Mutants lacking the terminal two amino acids do not incorporate the prosthetic group FAD [13]. In a recent study, Arscott et al. [6] have investigated the reductive half-reaction of human placental thioredoxin reductase (hTrxR). Interestingly, not only 2, but almost 3 equivalents of the strong reductant dithionite were required to achieve complete reduction of the enzyme. This observation strongly suggests that, apart from the flavin and the active site thiols, a third redox active site is present in hTrxR. Taking all these data together, we propose the working hypothesis that the penultimate Cys-Sec pair (Sec is selenocysteine), located on a flexible C-terminal arm, is in redox communication with the active site disulphide/dithiol [6], and might even play an essential role in enzyme catalysis [14]. Demonstration of the concomitant loss of the Cys-Sec pair and enzyme activity would very much substantiate this hypothesis.

To study this problem we have chosen the following approach. Human placental thioredoxin reductase (35 units/mg) was purified, essentially as described in [9]. Enzyme activity was determined in the DTNB-reduction assay [5,9]. We then incubated native hTrxR with porcine trypsin in order to remove the last 12
C-terminal amino acids, including the Cys-Sec pair. At different time points, an aliquot was taken, enzyme activity was determined and the fragments were separated from the protein fraction by ultrafiltration (filter cut-off, 10 kDa). Subsequently, the selenium content in the peptide fraction was determined relative to the enzyme selenium content before trypsin digestion (Figure 1). For selenium determination, two distinct methods, namely atomic absorption spectroscopy and neutron activation analysis, were employed and led to very similar data.

The following results were obtained. (1) Only NADPH-reduced hTrxR incubated with trypsin showed a significant loss of enzyme activity over time (Figure 1); the oxidized form of the enzyme as well as trypsin-free controls maintained their full activity over the time of the experiment. (2) The loss of catalytic activity in the reduced and trypsin-digested sample was directly proportional to the increase of selenium in the peptide fraction (Figure 1).

Based on these and the experimental findings cited above, we should like to forward the following hypothesis on the catalytic mechanism of hTrxR. NADPH reduces the active-site disulphide via the flavin. Then the C-terminal redox centre receives the electrons. This is consistent with the results of stopped-flow studies and of static dithionite titrations on the reductive half-reaction of human TrxR [6]. The reduced C-terminal arm then moves to a more exposed position, a view which is supported by the susceptibility of the NADPH reduced enzyme to trypsin digestion, and offers the electrons to the final substrate. That selenium (or the Cys-Sec pair) is essentially involved in catalysis is supported by the fact that loss of selenium leads to a proportional loss in activity. In this context, it should be recalled that disulphide bridges between sequentially adjacent cysteines are strained, due to the comparatively large distance between the unlinked sulphur atoms [15]. Selenium atoms, however, have

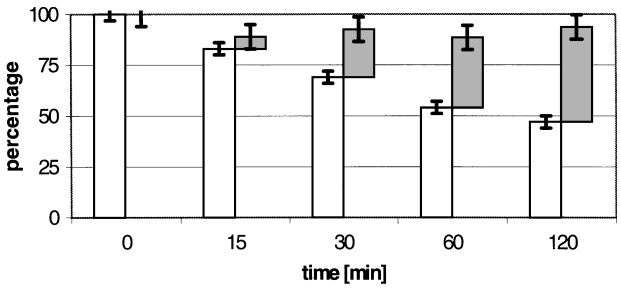

Figure 1 Time-dependent inactivation (white columns) of hTrxR (20 μg/ml in 50 mM ammonium acetate, pH 7.6) by incubation (37 °C) with porcine trypsin (4 μg/ml) in the presence of 400 μM NADPH

The activity of the oxidized enzyme (in the absence of NADPH) was not affected. The dark columns show the increase in selenium content in a filtrate (filter cut-off, 10 kDa) obtained after trypsin digestion, as a percentage of the initial selenium content of the native enzyme sample; error bars indicate the precision of the Se determination. Non-specific digestion of the protein was excluded by SDS/PAGE analysis. As demonstrated, the loss of selenium in the enzyme is directly proportional to the loss of enzyme activity. The bars do not reach exactly 100% because a small fraction of selenium-containing material is trapped in the filter. The results shown are for an experiment in which the selenium content of the filtrates was determined by atomic absorption spectroscopy. An independent experiment using neutron activation analysis yielded almost identical results.

about 15 % longer binding lengths and, as our studies on peptide models indicate, could therefore bridge this gap. After reduction of the substrate, the re-oxidized C-terminal arm would return to its more buried resting position. This is in accordance with our finding that the oxidized enzyme is not affected by trypsin under the experimental conditions chosen.

Our hypothesis is supported by the fact that hTrxR has a very broad substrate specificity, including such bulky molecules as thioredoxin and NK-lysin. A flexible C-terminal extension, carrying reducing equivalents, could explain the ability of the enzyme to serve so many different substrates. Furthermore, the proposed catalytic mechanism of hTrxR is substantiated by comparing the enzyme (C-terminal sequence: LSVTKRSGAS-ILQAG**CU**G; with U representing Sec) with *Staphylococcus aureus* mercuric ion reductase (C-terminal sequence: KLAA-LTFDKDVSKLS**CC**AG). As judged from sequence alignments [13] and spectroscopic data [6], the known three-dimensional structure of mercuric ion reductase resembles closely the proposed structure of hTrxR. The C-terminal extension of mercuric ion reductase is flexible, as suggested for hTrxR, and close to the solvent [16]. It is generally accepted that two adjacent cysteines of the C-terminal extension are supposed to bind the Hg(II)-ion and pass it on to the active centre, where it is reduced to Hg^0 ([1,17] and references therein).

Our working hypothesis that the flexible C-terminal extension of hTrxR carries reducing equivalents from the active site to the substrates requires further experimental substantiation by different techniques. Our approach is to study the structures of the oxidized and the NADPH-reduced enzyme. Crystals of both enzyme species have recently been obtained using Tris buffer and PEG 8000 as precipitant.

If our hypothesis should be supported by further data, interesting perspectives for rational drug design would result. For example, novel antineoplastic and antiphlogistic drugs could be developed by making use of the solvent-exposed essential selenocysteine which is known to exhibit a particular chemistry [18].

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