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Single-molecule studies of disulfide bond reduction pathways used by human thioredoxin

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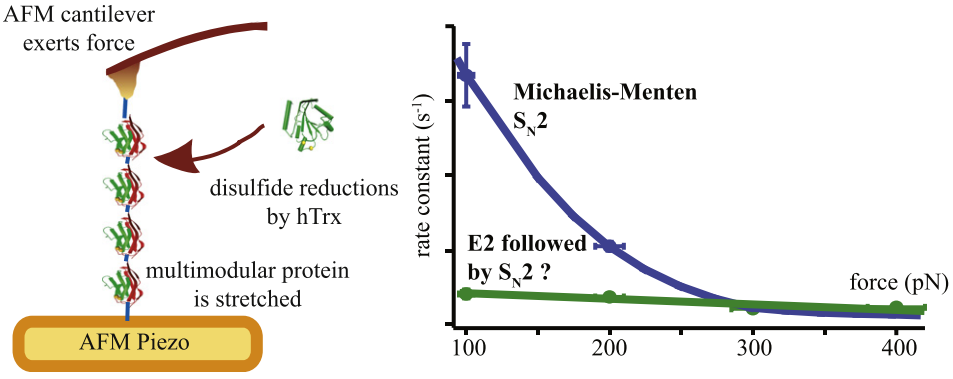
HIGHLIGHTS

- Large set of AFM data resolves enzymatic catalysis by human thioredoxin directly.
- Single molecule AFM data analyzed by exponential fitting and dwell time histograms.
- Several mechanisms of catalysis, including electron tunneling, are reviewed.
- Novel mode of thioredoxin catalysis is proposed.

GRAPHICAL ABSTRACT



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ABSTRACT

Disulfide bond reduction pathways used by human thioredoxin (hTrx) are studied at the single molecule level using a recombinant protein $(I27_{SS})_8$. $(I27_{SS})_8$ contains eight tandem repeats of identical immunoglobulin-like modules with one disulfide bond in each module. Single $(I27_{SS})_8$ molecules are stretched at constant force applied by a cantilever in a force-clamp mode of atomic force microscopy (FC-AFM). Disulfide reduction events are accurately detected from stepwise increases in the end-to-end length of $(I27_{SS})_8$. Earlier FC-AFM studies observed one disulfide reduction pathway used by hTrx and suggested an additional electron tunneling mechanism. Here, a very large set of unbiased FC-AFM data is collected in a range of clamping forces. By analyzing the data using exponential fits and dwell time histograms two disulfide reduction pathways used by hTrx are resolved. Based on previous studies one of these pathways is attributed to force-dependent Michaelis–Menten catalysis. The latter reduction pathway is weakly force-inhibited and occurs sporadically. Bimolecular nucleophilic substitutions (S_N2) and electron tunneling (ET) mechanisms are discussed to explain the second pathway. Direct S_N2 and ET mechanisms cannot be discounted, but a hypothetical E2–S_N2 mechanism involving a hydride reducing a disulfide bond provides an interesting alternative, which needs to be verified in future experiments.

1. Introduction

Enzymatic catalysis outperforms man-made implementations for tightly controlled and stereo-selective chemical reactions with high yields. Molecular details of enzymatic catalysis are still largely unknown.

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Static protein structures, provided by cryo-electron microscopy NMR methods and X-ray crystallography [1–3], do not address enzymatic dynamics. Critical advancements are expected from dynamic NMR methods [4,5] and single molecule methods. For example, recent force-clamp (FC) AFM studies reported on sub-Angstrom enzymatic reconfigurations in force-mediated catalysis of disulfide bond reduction by several kinds of thioredoxins [6,7]. Thioredoxin is a ubiquitous disulfide bond reductase primarily responsible for cell redox homeostasis, anti-apoptotic activity, and signaling [8,9].

The FC-AFM studies of thioredoxin catalyses used a recombinant protein $(I27_{SS})_8$ [6,7,10]. A $(I27_{SS})_8$ molecule contains eight identical modules of the 27th domain from human cardiac titin with precisely one disulfide bond in each module. Each disulfide bond traps a portion of a protein chain, which unfolds after the bond is reduced. $(I27_{SS})_8$ is mechanically clamped between an AFM tip and an arbitrary surface, and reduction events are detected from increases in the protein end-to-end length, i.e., from the FC-AFM length vs. time trace. Using this method, disulfide reduction rate constants were obtained from single exponential fits to the FC-AFM length traces at a given force. In several types of *Escherichia coli* thioredoxins (TRX) disulfide reduction rate constants initially decreased and then increased as clamping force increased [6,7]. In the case of hTrx, which is structurally similar to *E. coli* TRX, disulfide reduction rate constants decreased monotonically with force and remained constant above 300 pN [6]. This has been explained by molecular dynamics simulations, which obtained notable differences in width and depth of an enzymatic substrate binding groove as well as different mobilities of a substrate bound to prokaryotic vs. eukaryotic thioredoxins [7]. Furthermore, electron tunneling mechanism was suggested in thioredoxin catalysis [7].

Overall, earlier experimental data and MD simulations found one disulfide reduction pathway in hTrx and suggested an additional electron tunneling mechanism. To verify whether ET is a viable option and to address whether more than one disulfide reduction pathway is used by hTrx a large number of carefully analyzed FC-AFM data is needed.

Methods for the FC-AFM data analysis include exponential fitting to averaged length vs. time FC-AFM traces and dwell time analysis [11,12]. Exponential fitting was applied extensively in ion channel literature [24,25] and FC-AFM studies [6,10]. Dwell time is the time elapsed from presenting a disulfide bond to a reducing environment to an actual bond rupture. Pioneered by Sigworth and Sine in patch clamp studies, dwell time histograms with a square root ordinate vs. logarithm of dwell times display peaks at each rate constant and their errors are distributed evenly for each bin [13–15]. In a previous FC-AFM study, logarithmic dwell time histograms resolved two catalytic pathways for *E. coli* TRX reducing $(I27_{SS})_8$ at 100 pN clamping force [10].

In this manuscript, disulfide bond reductions by human thioredoxin are addressed using a large number of the FC-AFM data. To compare with previous studies $(I27_{SS})_8$ is used in a range of clamping forces from 100 pN to 400 pN. Rate constants for disulfide bond reductions are obtained from exponential fits and dwell time analysis. The experimental data at 100 pN and 200 pN clamping forces are well fitted using two reaction rate constants, while the data at 300 pN and 400 pN are adequately fitted using one reaction rate constant. Thus, the existence of two disulfide reduction pathways in hTrx is established, and particularly due to the usage of dwell time histograms. One of the pathways is strongly force-inhibited and agrees with previously observed force-dependent Michaelis–Menten type of catalysis by thioredoxins [6,7,10]. The latter pathway is very infrequent and weakly force-inhibited. Direct S_N2 mechanisms and an electron tunneling mechanism are thoroughly discussed to explain the latter pathway. While none of these mechanisms is discounted, they are not very likely in the light of the presented data and literature reports. Instead, a hypothetical E2–S_N2 mechanism involving a hydride reducing a disulfide bond provides an interesting alternative, which needs to be verified in future experiments.

2. Materials and methods

2.1. Proteins and enzymes

Expression and purification of $(I27_{SS})_8$ was described in detail previously [16]. QuikChange site-directed mutagenesis using a kit from Stratagene introduced Gly32Cys and Ala75Cys mutations into each I27 module. Multiple rounds of $I27_{G32C-A75C}$ cloning created an N – C linked, eight-domain polyprotein gene of $(I27_{SS})_8$ [17]. This gene was encoded in the vector pQE30 and expressed in the *E. coli* strain BL21(DE3). The pelleted cells were lysed by sonication and the His_6-tagged protein was purified using an immobilized Talon-Co^{2+} column from Clontech followed by gel filtration with Superdex 200 column from GE Healthcare. Purified protein was stored at 4 °C in a buffer of 10 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.02% NaN_3 (w/v), pH 7.2.

Human thioredoxin was purified as described in detail previously [18]. The pACA/hTrx plasmid was expressed in BL21(DE3) cells. Cell pellets were lysed using a French press and stirred with 7% w/v streptomycin sulfate. Protein was precipitated by adding ammonium sulfate to 85% saturation. Crude extracts were applied to a DEAE 52 column equilibrated with 50 mM Tris–HCl, pH 7.5, 1 mM EDTA and 0.1 mM DTT. Thioredoxin was eluted with a NaCl gradient, concentrated, and purified with a Sephadex G-50 column equilibrated with 50 mM Tris–HCl, pH 7.5, 1 mM EDTA and 0.1 mM DTT. Proper fractions were concentrated and further purified using *E. coli* thioredoxin antibody affinity chromatography. The hTrx concentration was determined spectrophotometrically at 280 nm using a molar extinction coefficient of 8050 M^{-1} cm^{-1}.

For FC-AFM studies a buffer containing 10 μM hTrx, 10 mM HEPES buffer, 150 mM NaCl, 1 mM EDTA, 2 mM NADPH, and 50 nM of eukaryotic thioredoxin reductase from rat liver from Sigma-Aldrich was titrated with 1 M NaOH to pH 7.2. An excess of NADPH and a catalytic amount of thioredoxin reductase were necessary to maintain ~98% of hTrx in an active, reduced form [19,20].

2.2. Force-clamp AFM spectroscopy

A custom built AFM setup [10] with a typical distance resolution in Z-axis of 0.2 nm and feedback time constant of a few ms was used. Commercial V-shaped silicon nitride MLCT-C cantilevers from Bruker, with normal elastic spring constants of 20 pN/nm, were calibrated in liquid prior to each experiment by a thermal method [10,21]. Purified protein was deposited on gold coated glass cover slides from Fisher Scientific.

Data acquisition and data analysis programs were custom developed for Igor Pro 5.0 from Wavemetrics. A following FC-AFM protocol was applied [10]. Initial tip-sample contact was maintained using AFM feedback for 1 s with a contact force of −1 nN. Next, the contact force was reduced to −100 pN and maintained for 100 ms to mark an arbitrary zero extended end-to-end length of $(I27_{SS})_8$. The $(I27_{SS})_8$ molecule was then stretched at 190 pN for 0.15 s in order to quickly expose the disulfide bonds to a bathing solution. Next, a given clamping force was set for 60 s to collect all the reduction events, and not the outliers only. Disulfide bond cleavage was not observed in the absence of hTrx [16]. Any significant changes in the protein end-to-end length occur after the molecule is extended by at least tens of nanometers. Thus, kinetics of disulfide reduction is not expected to depend on water structure on the surface [22], nor on any elasto-adhesive properties of an AFM tip and a substrate [23]. Disulfide reduction events occur after initial $(I27_{SS})_8$ unfolding, so each reduction is spatially isolated by distances of several times the size of a single thioredoxin molecule. This way, the FC-AFM data with long clamping times are unbiased and without any hidden global constraints [24].

2.3. Data analysis

For assessing data fits a standard Pearson (chi-square) test is used; $\chi^2 = \Sigma_i((y_i - f(x_i))/\sigma)^2$, where Σ_i sums over all data points, y_i is an