The conserved active site tryptophan of thioredoxin has no effect on its redox properties

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Abstract: In *Staphylococcus aureus* thioredoxin (Trx) it has been shown that mutation of the conserved active site tryptophan residue (Trp28) has a large effect on the protein stability, on the pKa of the nucleophilic cysteine and on the redox potential. Since these effects can either be due to the partially unfolding of the Trp28Ala mutant or to the absence of the indole side chain of Trp28 as possible interaction partner for the active site cysteines, the origin of the experimentally observed effects is not known and is beyond experimental approach. With theoretical pKa and density functional theory reactivity analysis on model systems where Trp28 has been replaced by an alanine within the structural environment of Trx it is shown that Trp28 does not affect the redox parameters of Trx. As such, the experimentally observed redox effects of the Trx W28A mutant might be due to structural changes induced by partial unfolding.

Keywords: Density-functional calculations; enzyme catalysis; thioredoxin; HSAB principle

Introduction

Thioredoxin (Trx), a ubiquitous reductase, is one of the most important regulators of the cellular redox balance. The key biological activities of Trx are the promotion of cell growth, the inhibition of apoptosis, the protection against oxidative stress and the modulation of inflammation.¹ All Trx feature a conserved active-site loop with a Trp-Cys-Gly-Pro-Cys^{2–4} sequence motif, numbered as Trp28 to Cys32 in *Staphylococcus aureus* Trx (Trx). In the structure of *S. aureus* Trx (2O7K),⁵ the indole side chain of Trp28 is turned towards the interior of the protein and covers

an important part of the active side. There are no direct hydrogen bond interactions of Trp28 with the catalytic active cysteine residues. Mutation of the conserved active site tryptophan to alanine converts Trx into a domain swapped dimmer (3DIE).⁶ From the thermodynamic stability curves it was shown that both the reduced and oxidized monomeric forms of Trx W28A were severely affected by the mutation.⁶ They seem to be partially unfolded, reminiscent to a molten globule-like population.⁶ We previously showed that the pKa of Cys29 increases from 7.1 in wild type Trx to 8.3 in the monomeric form of Trx W28A,⁵ which is a pKa value more similar to the pKa of a cysteine out of its structural environment. Further, in Trx W28A, the redox potential decreases from –268 mV to –284 mV.⁵ It is not

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Figure 1. Trx reduces oxidized ArsC via an intermediate Trx-ArsC complex: (A) The reaction takes off with a nucleophilic attack of Cys29^{Trx} of reduced Trx on Cys89^{ArsC} of the Cys82^{ArsC}-Cys89^{ArsC} disulfide in oxidized ArsC (1Z2E), leading to the formation of the Cys29^{Trx}-Cys89^{ArsC} mixed disulfide (2IPA). (B) In a second step, Cys32^{Trx} attacks Cys29^{Trx}, leading to the release of reduced ArsC (1Z2D) and oxidized Trx (2GZZ) (C). The figure was generated by using PyMol v099 (Delano Scientific LLC, 2006).

known whether these effects are due to conformational changes induced by the partially unfolding of the Trx W28A mutant or due to the absence of the conserved tryptophan indole side chain as possible interacting partner for the nucleophilic cysteines in the active site. It is not possible to exclude one of the possibilities experimentally, because upon the introduction of the Trp28Ala mutation, Trx shows the tendency to partially unfold.⁵ Therefore, theoretical pKa calculations and density functional theory (DFT)^{7–9} reactivity analysis on model systems with Trp28 replaced by alanine within the structural environment of wild type Trx are used to give insight in the intrinsic role of Trp28 on the redox behavior of Trx.

To explore and quantify the effect of Trp28 on the pKa of Cys29, the linear relationship between the calculated natural population analysis (NPA)¹⁰ charge on the thiol sulfur atoms and the pKa,¹¹ is being used. The more negative the NPA charge on the sulfur atom, the higher the tendency to bind a proton, and the higher the thiol pKa. We have been able to successfully use the NPA-pKa correlation to determine the origin of the pKa perturbation of N-terminal cysteines in α - and 3_{10} -helices,¹² in the study of the activation of the nucleophilic thiolates in the reaction catalyzed by arsenate reductase (ArsC)¹³ and during mixed disulfide complex dissociation of ArsC and Trx.¹¹

In the conserved active site sequence motif Trp28-Cys29-Gly30-Pro31-Cys32 of Trx, we have studied the reactivity of Cys29 and Cys32 towards oxidized ArsC,^{14,15} an endogenous substrate of Trx. Trx reduces oxidized ArsC via the formation of a mixed Trx-ArsC disulfide complex¹⁶ (Fig. 1). To com-

pare the reactivity of Cys29 and Cys32 in the presence and absence of the indole side chain of Trp28, we used the local version of the soft acids and bases principle (HSAB).^{17–19} This principle has been formulated in a conceptual DFT⁹ context and states that hard acids prefer to react with hard bases whereas soft acids prefer to interact with soft bases. Disulfide exchange reactions are soft-soft interactions; as such the softness is used as a reactivity descriptor. The smaller the difference in the local softness (s) between the sulfur atoms of the attacking nucleophilic cysteines and the accepting electrophilic Cys82^{ArsC}-Cys89^{ArsC} disulfide of oxidized ArsC (Fig. 1), the more preferred the interaction is:

$$\Delta s = |s^+ - s^-| \quad (1)$$

with s^+ and s^- the local softness of the electrophilic and the nucleophilic sulfur atoms, here the sulfur atoms of the cysteine residues in respectively the Cys82^{ArsC}-Cys89^{ArsC} disulfide (Fig. 1), and Cys29^{Trx} and Cys32^{Trx}.

The local softness s is obtained as

$$s^+ = S f^+ \quad (2)$$

$$s^- = S f^- \quad (3)$$

in which S is the global softness, a global property that correlates with the system's polarizability.⁹ f is the Fukui function^{8,9} and indicates the orientation effect of a chemical reaction: which nucleophilic sulfur atom (f^-) will attack and which sulfur (f^+) of the disulfide will receive electrons. The $-$ and $+$ sign indicate the reactivity towards nucleophilic and

Table I. *Trp28 has no Effect on the pKa of Cys29 and Cys32 in Reduced Trx and in the Trx-ArsC Complex*

	Model system	PDB	Cysteine residue	q_{NPA}	pKa
Trx	Trx_red_C29	2GZY	C29 ^{Trx}	−0.671	5.5 ¹¹
	Trx_red_C29 W28A	2GZY	C29 ^{Trx}	−0.673	5.5
Trx-ArsC	Trx_ArsC_C32	2IPA	C32 ^{Trx}	−0.791	8.3 ¹¹
	Trx_ArsC_C32 W28A	2IPA	C32 ^{Trx}	−0.791	8.3

Calculated pKa's of the nucleophilic cysteines of Trx and in the Trx-ArsC complex. The pKa values are obtained via the NPA-pKa correlation.¹¹

electrophilic attacks respectively. f^- is related to the electron density of the highest occupied molecular orbital and f^+ to the density of the lowest unoccupied molecular orbital when electrons are received.²⁰ The global and local softness and the Fukui function are well established and extensively used to study generalized acid/base reactions including most of the organic reactions (additions, substitutions, eliminations)⁹ of which disulfide exchange reactions are an example.

In sum, the local softness descriptors are combined with pKa calculations to give fresh insights in the experimentally observed effects of the Trp28Ala mutation in Trx.

Results and Discussion

To get insight into the origin of the experimentally observed effects of the Trp28Ala mutation in Trx, we have performed pKa calculations and reactivity analysis on a model system of reduced Trx in which Trp28 is replaced by alanine.

The pKa of Cys29^{Trx} in wild type reduced Trx was calculated to be 5.5.¹¹ By replacing Trp28 by alanine within the structural environment of wild type reduced Trx (Trx_red_C29 model in Table I), the pKa does not change. Further, in this model, the removal of the indole side chain of Trp28 does not alter the local softness difference between the sulfur atoms of Cys29^{Trx} and the sulfur atoms of the Cys82^{ArsC}-Cys89^{ArsC} disulfide, and thus, no effect on the reactivity of Trx towards ArsC (Table II) has been observed. Both, the Fukui function and the

global softness of Cys29^{Trx} and Cys32^{Trx} in the Trx W28A model are similar to those found in the model of wild type Trx (Table II). In the Trx W28A model, the sulfur atoms of Cys29^{Trx} and Cys32^{Trx} have comparable Fukui function values, corresponding to the same intrinsic reactivity. Cys29^{Trx} has a lower global softness than Cys32^{Trx}, explaining its higher reactivity towards the less soft Cys82^{ArsC}-Cys89^{ArsC} disulfide (Table II). As such, in Trx W28A the experimentally observed regioselectivity of Cys29^{Trx} to nucleophilically attack Cys82^{ArsC} of the disulfide in oxidized ArsC is directed by the difference in global softness between Cys29^{Trx} and Cys32^{Trx}. These results are analogous to the calculations with a model of wild type Trx, and thus within the structural environment of wild type Trx, Trp28 has no effect on reactivity, regioselectivity and on the pKa of Cys29^{Trx}. As such, the experimentally obtained results^{5,6} of an increased pKa of Cys29^{Trx} and a decreased redox potential in Trx W28A most likely originates from the structural effects induced by the partial unfolding of Trx W28A.

Also in the Trx-ArsC complex (Fig. 1), Trp28 has no effect on the pKa and the reactivity. The pKa of Cys32^{Trx} in the model of wild type Trx-ArsC is calculated to be 8.3¹¹ and does not change upon replacing Trp28 by alanine within the structural environment of the complex between wild type Trx and ArsC (Trx_ArsC_C32 model in Table I). Further, in this model, the local softness difference between the sulfur atoms of Cys32^{Trx} and Cys29^{Trx} is similar to this in the complex between wild type Trx and ArsC (Table III). Both, the Fukui function and the global

Table II. *Trp28 has no Effect on the Reactivity of Reduced Trx*

	Model system	Cysteine residue	S	f^+/f^-	s^+/s^-
Trx (2GZY)	Trx_red_C29	C29 ^{Trx}	6.19 ^a	0.866 ^a	5.36 ^a
	Trx_red_C32	C32 ^{Trx}	8.68 ^a	0.858 ^a	7.45 ^a
Trx (2GZY)	Trx_red_C29 W28A	C29 ^{Trx}	6.06	0.866	5.25
	Trx_red_C32 W28A	C32 ^{Trx}	8.73	0.859	7.50
Δs	C29 ^{Trx} -C82 ^{ArsC}	C29 ^{Trx} -C89 ^{ArsC}	C32 ^{Trx} -C82 ^{ArsC}	C32 ^{Trx} -C89 ^{ArsC}	
Trx_red – ArsC_ox	4.73 ^a	4.58 ^a	6.82 ^a	6.66 ^a	
Trx_red W28A – ArsC_ox	4.62	4.47	6.87	6.71	

^a Values obtained from Roos *et al.*¹¹ Global softness (S), Fukui function (f^+ or f^-) and local softness (s^+ or s^-) values of the nucleophilic cysteines in Trx. Reactivity of the sulfur atoms of Cys29^{Trx} and Cys32^{Trx} of Trx towards the sulfur atoms of the Cys82^{ArsC}-Cys89^{ArsC} disulfide of ArsC as measured by the difference in local softness [Δs , Eq. (1)].

Table III. *Trp28 has no Effect on the Reactivity of Trx in the Trx-ArsC Complex*

Trx-ArsC complex (2IPA)	Cysteine residue	<i>S</i>	f^+/f^-	s^+/s^-
Trx_ArsC_C32	C32 ^{Trx}	6.47 ^a	0.865 ^a	5.59 ^a
	C29 ^{Trx}	5.97 ^a	0.522 ^a	3.12 ^a
	C89 ^{ArsC}	5.97 ^a	0.122 ^a	0.73 ^a
Trx_ArsC_C32 W28A	C32 ^{Trx}	6.49	0.865	5.61
	C29 ^{Trx}	5.33	0.517	2.76
	C89 ^{ArsC}	5.33	0.162	0.86
Δs	C32 ^{Trx} -C29 ^{Trx}		C32 ^{Trx} -C89 ^{ArsC}	C82 ^{ArsC} -C29 ^{Trx}
Trx_ArsC_C32	2.48 ^a		4.86 ^a	4.21 ^a
Trx_ArsC_C32 W28A	2.85		4.75	4.59

^a Values obtained from Roos *et al.*¹¹ Global softness (*S*), Fukui function (f^+ or f^-) and local softness (s^+ or s^-) values of the nucleophilic cysteines in Trx-ArsC complex. Reactivity of the sulfur atoms of Cys32^{Trx} towards the Cys29^{Trx}-Cys89^{ArsC} mixed disulfide as measured by the difference in local softness [Δs , Eq. (1)].

softness of Cys32^{Trx} and of Cys29^{Trx} in the Trx-ArsC W28A model do not differ significantly from the values found in the wild type Trx-ArsC model.

All together, as pointed out in the Introduction section, it has been shown that Trp28 has a large effect on the protein structure, on the pKa of the nucleophilic cysteine and on the redox potential of Trx.⁵ Theoretical calculations performed on a model system in which the structural effects of partial unfolding are not present indicate that Trp28 has no influence on the pKa of the nucleophilic cysteine in neither reduced Trx or in the Trx-ArsC complex (Table I). Furthermore, the minimal softness criterion indicates that Trp28 has no effect on the reactivity and on the regioselectivity during Trx-ArsC complex formation (Table II) and dissociation (Table III). As such, the effects of Trp28 on the redox properties of Trx might be assigned to the partially unfolding of thioredoxin when the Trp28Ala mutation is introduced. Equally important we have demonstrated that theoretical calculations can help to rationalize experimental results and can give fresh insight in questions which are currently beyond experimental approach.

Model systems and Computational Details

Model systems of *Bacillus subtilis* Trx (Trx) and *B. subtilis* Trx-ArsC (Trx-ArsC) complex are built based on the structures with PDB code 2GZY and 2IPA. Reduced Trx (2GZY)²¹ is modeled by the W28-C32 active site and the adjacent α 1-helix (K33-E45). In Trx_red_C29, C29 is deprotonated and in Trx_red_C32, both C29 and C32 are deprotonated. The Trx_ArsC complex (2IPA)²² is modeled (Trx-ArsC_C32) by the Trx active site (W28^{Trx}-C32^{Trx}) and the ArsC redox helix (C82^{ArsC}-C89^{ArsC}) and T11^{ArsC} and R16^{ArsC}; C32^{Trx} and C82^{ArsC} are deprotonated. The W28A mutation is built *in silico* starting from the coordinates of Trx_red_C29, Trx_ArsC_C32. In all models, hydrogen atoms are placed and optimized together with the S γ atoms of

the reduced cysteine residues at the B3LYP/6-31G* level. NPA charges, Fukui functions and softnesses are calculated as described.¹¹ All calculations were performed with the Gaussian 03 package.²³

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