

# Characterization of redox properties of FAD cofactor of *Thermotoga maritima* thioredoxin reductase

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The fluorescence properties of FAD of *Thermotoga maritima* thioredoxin reductase (TmTR), taken together with the amino acid sequences and structures of similar TRs, are consistent with the interdomain rotation in the catalysis of TmTR. The standard redox potential of FAD of TmTR,  $-0.230$  V, determined by the reactions with 3-acetylpyridine adenine dinucleotide (APAD<sup>+</sup>/APADH) redox couple, is close to that of *E. coli* TR. During the reduction of duroquinone with TmTR, the transient formation of neutral FAD semiquinone, and, possibly, FADH<sub>2</sub>-NAD<sup>+</sup> complex was observed. This shows that in spite of obligatory two-electron (hydride)-transfer between NADH and physiological disulfide oxidants, the FAD cofactor of TmTR may exist under a stable semiquinone form.

**Keywords:** thioredoxin reductase, *Thermotoga maritima*, FAD, redox potential, semiquinone stability

## INTRODUCTION

NAD(P)H:thioredoxin reductase (TR) and thioredoxin (TRX) systems are ubiquitous in both prokaryotes and eukaryotes, where they mainly perform antioxidant functions [1]. The low molecular weight (m.w.) TRs (~35 kD, L-TRs) of plants, prokarya and many eukarya contain FAD and the catalytic disulfide in the active center [1, 2]. During catalysis, the FAD accepts two electrons (hydride) from NAD(P)H and transfers them to the catalytic disulfide which subsequently reduces the redox active disulfide of TRX. The high m.w. TRs (~55 kD, H-TR) contain an additional redox active – Se-S- moiety

(mammalian TR), or an additional redox active disulfide (*Plasmodium* spp.) [3, 4]. These additional redox groups of H-TRs accept two redox equivalents from the reduced catalytic disulfide adjacent to FAD, and subsequently reduce the corresponding physiological disulfides/oxidants.

TR from the hyperthermophilic bacterium *Thermotoga maritima* (TmTR) with a m.w. of 34.3 kD is a typical representative of L-TRs. It contains the NAD(P)H binding motif, FAD, and redox active cysteines at positions 147 and 150 [5, 6]. In contrast with other L-TRs, TmTR is specific to NADH instead of NADPH [7]. Its physiological oxidants are poorly characterized except for the disulfide protein glutaredoxin-1 (Grx-1) [6, 7], which also possesses a relatively low activity.

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The standard redox potential ( $E^0$ ) of its catalytic disulfide is equal to  $-0.295$  V [8].

In addition to their physiological disulfide oxidants, TmTR and another L-TR, thioredoxin reductase from *Arabidopsis thaliana*, perform mixed single- and two-electron reduction quinones and nitroaromatic compounds [9–11]. These reactions may be important from the ecotoxicological viewpoint, because L-TRs may be potential targets for prooxidant quinoidal and nitroaromatic environmental pollutants ([9, 10], and references therein). These features may be even more important for TmTR in view of the recently emerging interest in the use of hyperthermophilic microorganisms in metabolic engineering platforms for production of fuels and industrial chemicals [12].

Because the FAD cofactor, but not catalytic disulfide, is involved in the above processes [9–11], there is a need for a more thorough characterization of its redox properties. The aim of this work is the characterization of redox potential and electron-transfer properties of FAD of TmTR.

## MATERIALS AND METHODS

*T. maritima* TR and Grx-1 were obtained as described [7, 8]. The concentration of TmTR was determined according to the absorbance of FAD ( $\epsilon_{460} = 10.9$  mM<sup>-1</sup> cm<sup>-1</sup>). NADH, 3-acetylpyridine adenine dinucleotide (APAD<sup>+</sup>), ADP ribose, formate dehydrogenase and other reagents were obtained from Sigma-Aldrich and Fluka.

Steady-state kinetic measurements were performed spectrophotometrically in 0.1 M K-phosphate (pH 7.0), containing 1 mM EDTA, at 25°C, using a PerkinElmer Lambda 25 spectrophotometer. The rates of enzymatic reduction of APAD<sup>+</sup> with NADH were monitored following the formation of APADH ( $\Delta\epsilon_{363} = 5.6$  mM<sup>-1</sup> cm<sup>-1</sup>) [13]. For kinetic studies, APADH was prepared in situ by reduction of APAD<sup>+</sup> with 100 mM formate and 1 mg/ml formate dehydrogenase. APADH concentration was determined according to  $\epsilon_{365} = 7.8$  mM<sup>-1</sup> cm<sup>-1</sup> [14]. TmTR-catalyzed reduction of 0.5 mM ferricyanide by APADH was monitored using  $\Delta\epsilon_{420} = 1.03$  mM<sup>-1</sup> cm<sup>-1</sup>. The steady-state parameters of reactions, the catalytic constants ( $k_{\text{cat}}$ ) and the bimolecular rate constants ( $k_{\text{cat}}/K_m$ ) correspond to the reciprocal intercepts and slopes of Lineweaver-Burk plots,  $[E]/V$  vs  $1/[S]$ , where  $V$  is the reac-

tion rate,  $[E]$  is the enzyme concentration, and  $[S]$  is the concentration of substrate. Throughout this work,  $k_{\text{cat}}$  represents the number of molecules of pyridine nucleotide oxidized or reduced by a single active center of the enzyme per second. NADH substrate inhibition constants ( $K_i$ ) correspond to the opposite intercepts of Cleland plots,  $[E]/V$  vs  $[S]$ , with  $x$ -axis. The kinetic parameters were obtained by fitting kinetic data to the parabolic expression using SigmaPlot 2000 (Version 11.0, SPSS Inc.).

The pre-steady-state kinetic measurements of reduction of TmTR with excess APADH were carried out by using a stopped-flow SX.17 MV spectrophotometer (Applied Photophysics) at pH 7.0 and 25°C. The concentration of the enzyme after mixing was 5.0  $\mu$ M. The kinetics of absorbance decrease at 460 nm were analysed according to the single exponent fit using the SigmaPlot software. Kinetics of 5.0  $\mu$ M TmTR reduction with 50  $\mu$ M NADH and its reoxidation with oxygen or 100  $\mu$ M tetramethyl-1,4-benzoquinone (duroquinone) was carried out analogously. In this case, a second syringe contained NADH and duroquinone.

The fluorescence of TmTR (5.0  $\mu$ M) at 525 nm was recorded using a Hitachi MPF-7 fluorimeter at pH 7.0 and 25°C, excitation wavelength 460 nm.

## RESULTS AND DISCUSSION

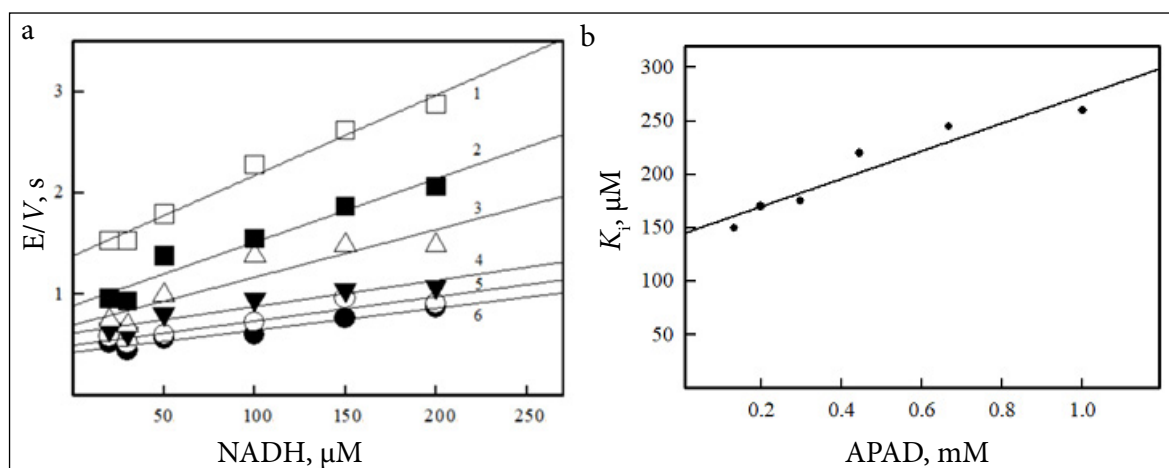
TRs of *E. coli* and *A. thaliana* consist of FAD-binding domain and NADPH/TRX-binding domain, containing catalytic disulfide [15, 16]. When NADPH binds to this domain, its nicotinamide ring is located 17 Å away from the flavin ring [15, 16]. Thus, the access of NADPH to FAD (or access of TRX to catalytic disulfide) requires the rotation of the NADPH/TRX domain with respect to the FAD domain. This transition is accompanied by FAD fluorescence changes, because FAD is fluorescent in flavin-to-NAD(P)H-binding site conformation, and nonfluorescent in the flavin-to-disulfide-conformation [2, 17, 18].

Although the crystal structure of TmTR is not available, there is an indirect evidence that during catalysis, the surroundings of its FAD cofactor are subjected to similar structural changes. First, the interdomain sequence motifs of *E. coli* TR (Gly-244, Pro-247) and *A. thaliana* TR (Gly-298, Pro-301) correspond to Gly-251 and Pro-254 in *T. maritima* TR [11]. Further, in this work we found that

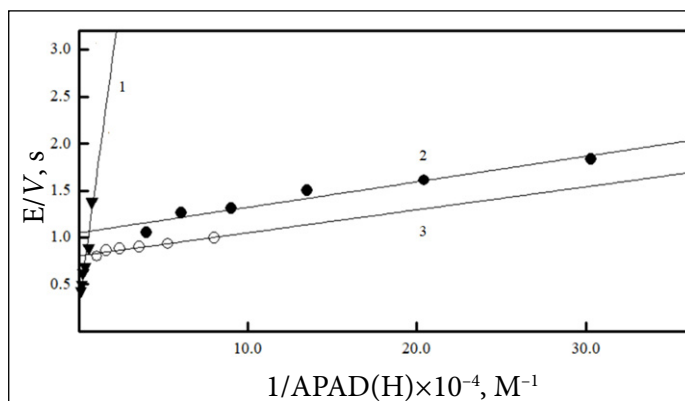
the fluorescence intensity of FAD in TmTR makes up to 20% of that of free FAD, which is close to 25% in *A. thaliana* TR [19]. The addition of ADP-ribose (saturating concentration, 150  $\mu\text{M}$ ) or GRX-1 (saturating concentration, 40  $\mu\text{M}$ ) increased its fluorescence intensity by 30 and 12%, respectively. This is analogous to the enhancement of fluorescence of FAD of *A. thaliana* TR by 2',5'-ADP or TRX [19], which shows that the ligand binding either to NAD(P)H- or to TR-binding site increases the amount of fluorescent flavin-to-NAD(P)H-binding site conformer.

According to the Haldane relationship, the reaction equilibrium constant ( $K$ ) is equal to the ratio of forward and reverse reaction rate constants. This approach was used for the determination of standard redox potentials ( $E^0_7$ ) of glutathione reductase and rat H-TR [20, 21] from their bimolecular reaction rate constants ( $k_{\text{cat}}/K_m$ ) with NADPH and NADP<sup>+</sup>. Because Grx-1 is a slow substrate for TmTR [11], the studies of NAD<sup>+</sup> enzymatic reduction using reduced Grx-1 as a substrate are problematic. For this reason, we examined the reactions of TmTR with 3-acetylpyridine adenine dinucleotide (APAD<sup>+</sup>/APADH) redox couple with  $E^0_7 = -0.258$  V. During the TmTR-catalyzed reduction of APAD<sup>+</sup> by NADH, the substrate inhibition by NADH takes place, i.e. at fixed APAD<sup>+</sup> concentration, the reaction rates decrease with an increasing NADH concentration (Fig. 1a). The data linearization in Cleland plots (Fig. 1a) enables one to determine the substrate inhibition constants ( $K_i$ ) of NADH. Their increase

with APAD<sup>+</sup> concentration (Fig. 1b) demonstrates the competitive character of inhibition. This means that after TmTR reduction, NADH repeatedly binds to the reduced enzyme form thus competing with APAD<sup>+</sup>. At [APAD<sup>+</sup>] = 0,  $K_i$  of NADH is equal to  $140 \pm 15$   $\mu\text{M}$ . The oxidation of TmTR by APAD<sup>+</sup> is characterized by  $k_{\text{cat}} = 2.7 \pm 0.2$  s<sup>-1</sup> and  $k_{\text{cat}}/K_m = 3.8 \pm 0.1 \times 10^4$  M<sup>-1</sup> s<sup>-1</sup> (Fig. 2). The reduction rate of TmTR by APADH obtained in pre-steady-state experiments is characterized by  $k_{\text{cat}} = 1.3 \pm 0.3$  s<sup>-1</sup> and  $k_{\text{cat}}/K_m = 3.3 \pm 0.5 \times 10^5$  M<sup>-1</sup> s<sup>-1</sup>. This reaction is much slower than the reduction of TmTR with NADH,  $k_{\text{cat}} = 82 \pm 3.0$  s<sup>-1</sup> [11]. The steady-state studies of enzymatic APADH oxidation give similar kinetic parameters,  $k_{\text{cat}} = 1.0 \pm 0.2$  s<sup>-1</sup> and  $k_{\text{cat}}/K_m = 3.2 \pm 0.6 \times 10^5$  M<sup>-1</sup> s<sup>-1</sup> (Fig. 2). Finally, using  $K = 8.68 \pm 1.54$ , i.e. the ratio of  $k_{\text{cat}}/K_m$  of forward and reverse reactions, and  $E^0_7 = -0.258$  V for APAD<sup>+</sup>/APADH couple, the Nernst equation gives  $E^0_7 = -0.230 \pm 0.003$  V for FAD of TmTR, with an understanding that the catalytic disulfide/dithiol does not participate in the reactions with APAD<sup>+</sup>/APADH. This value is close, although slightly higher than  $E^0_7$  of FAD of *E. coli* TR,  $-0.242 \div -0.258$  V [22]. On the other hand, it is significantly higher than  $E^0_7$  of catalytic disulfide of TmTR,  $-0.295$  V [8]. Although the mechanism of catalysis of TmTR is not studied in detail, the thermodynamically unfavourable hydride transfer from reduced FAD to catalytic disulfide may be one of the reasons for the slow reduction of Grx-1,  $0.2\text{--}0.3$  s<sup>-1</sup> [11], and other disulfide proteins.



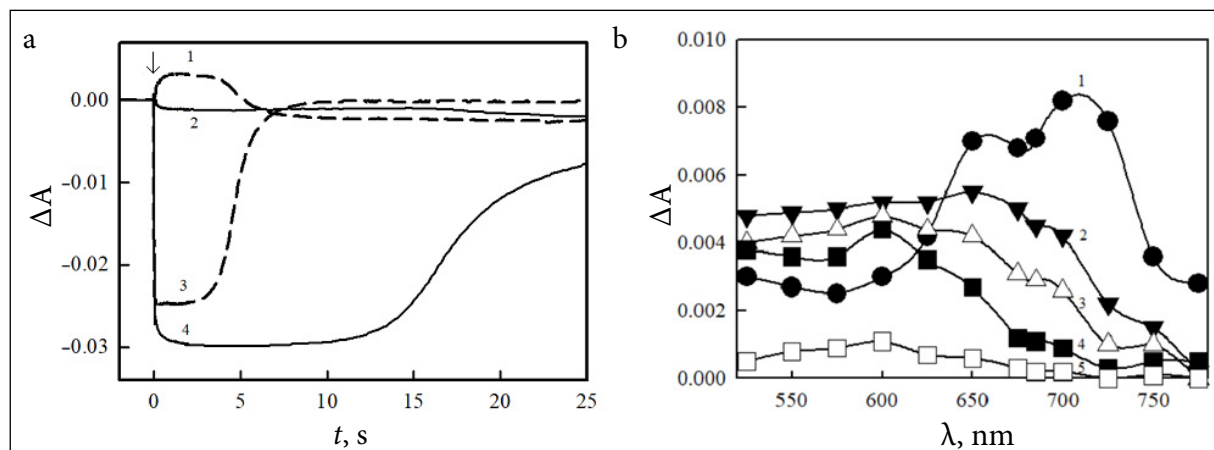
**Fig. 1.** (a) The rates of TmTR-catalyzed reduction of APAD<sup>+</sup> by NADH, presented in Cleland coordinates. The concentrations of APAD<sup>+</sup>: 1.0 mM (1), 0.67 mM (2), 0.44 mM (3), 0.30 mM (4), 0.20 mM (5) and 0.13 mM (6). (b) Dependence of NADH substrate inhibition constant on the APAD<sup>+</sup> concentration



**Fig. 2.** The rates of reactions of APAD<sup>+</sup>/APADH with TmTR: TmTR-catalyzed reduction of APAD<sup>+</sup> with NADH (1), TmTR-catalyzed oxidation of APADH with ferricyanide (2) and rapid reduction of TmTR with APADH (3)

L-TRs catalyze obligatory two-electron (hydride) transfer between NAD(P)H and their physiological disulfide oxidants, however, they perform mixed single- and two-electron reduction of quinones via their FAD cofactor [9, 11]. In order to characterize the redox states of FAD participating in this process, we monitored the multiple turnover of FAD during the TmTR-catalyzed oxidation of NADH by duroquinone. In control experiments performed in the absence of quinone, the initial fast phase of FAD reduction by NADH monitored at 460 nm was followed by a slower reoxidation by oxygen (Fig. 3a). The addition of duroquinone significantly accelerated the reoxidation (Fig. 3a). Importantly, the addition of quinone caused a transient increase in absorbance at 600 nm at the same time

scale, which was almost not evident in the control experiments (Fig. 3a). The data collected at different wavelengths show that the absorbance initially increased in the range of 525–775 nm and above, and was characterized by  $\lambda_{\max} \sim 700$  nm (Fig. 3b). Subsequently, the formation and decay of a secondary absorbance with  $\lambda_{\max} \sim 600$  nm has taken place (Fig. 3b). The latter resembles the absorbance of the neutral (blue) FAD semiquinone (FADH<sup>•</sup>) of *E. coli* TR, which is formed during the enzyme irradiation under anaerobic conditions, but not in physiological reactions [23]. The transient accumulation of FADH<sup>•</sup> during the turnover may indicate that its oxidation is the rate-limiting (slower) step in the reoxidation of two-electron reduced FAD with quinones. However, the other reaction



**Fig. 3.** (a) The TmTR FAD absorbance changes during the enzyme turnover monitored at 600 nm (1, 2) and 460 nm (3, 4) in the presence of 100  $\mu$ M duroquinone (1, 3) or in its absence (2, 4). Enzyme concentration, 5.0  $\mu$ M. (b) Spectra of reaction intermediates formed during the turnover of 5.0  $\mu$ M *T. maritima* TR with 50  $\mu$ M NADH and 100  $\mu$ M duroquinone. Spectra were recorded after 0.2 s (1), 2.0 s (2), 3.0 s (3), 4.0 s (4), and 6.0 s (5)

intermediates, e.g. the  $\text{FADH}_2\text{-NAD}^+$  complex absorbing above 700 nm [18], may be formed at the beginning of the turnover, as shown in Fig. 3(b). It is important to note that although a member of the H-TR group, rat thioredoxin reductase, and a structurally related disulfide reductase, trypanothione reductase, reduce quinones in a mixed single- and two-electron way, FAD semiquinone is not formed in the course of these reactions [21, 24]. In these cases, quinones react with FAD-thiolate charge-transfer complex in the reduced enzyme form [21, 24]. It may follow that in the absence of efficient electronic coupling of FAD with catalytic disulfide/dithiolate, like in L-TRs of *E. coli* [23] and *T. maritima*, the FAD cofactors in flavoenzymes C-S transhydrogenases may possess considerable semiquinone stability, in spite of obligatory two-electron reduction of their physiological disulfide oxidants. However, this problem will be an object of the future studies.

## CONCLUSIONS

Like in other L-TRs, the FAD cofactor of *Thermotoga maritima* TR is surrounded by an apoprotein of dynamic structure. The redox potential of FAD at pH 7.0,  $-0.230$  V, is close to that of *E. coli* TR. The transient accumulation of FAD semiquinone during the enzymatic reduction of duroquinone shows that the limiting step of reaction is the oxidation of semiquinone, and points to its relative stability.

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## THERMOTOGA MARITIMA TIOREDOKSINREDUKTAZĖS FAD KOFAKTORIAUS REDOKSO SAVYBIŲ CHARAKTERIZAVIMAS

### Santrauka

Remiantis *Thermotoga maritima* tioredoksinreduktazės (TmTR) FAD fluorescencijos savybėmis, panašių TR amino rūgščių sekomis ir struktūra, galima teigti, kad TmTR katalizės metu vyksta baltymo domėnų tarpusavio posūčiai. FAD standartinis redokso potencialas, nustatytas reakcijų su 3-acetilpiridino adenino dinukleotido ( $\text{APAD}^+/\text{APADH}$ ) redokso pora,  $-0,230$  V, yra artimas *E. coli* TR FAD redokso potencialui. Durochinono redukcijos TmTR metu stebimas tarpinis neutralaus FAD semichinono ir, greičiausiai,  $\text{FADH}_2\text{-NAD}^+$  komplekso susidarymas. Tai rodo, kad nepaisant privalomo dviejų elektronų (hidrido) perdavimo tarp NADH ir fiziologinių disulfidinių oksidatorių TmTR FAD kofaktorius gali egzistuoti ir stabilioje semichinoninėje būsenoje.