# A Reexamination of Thioredoxin Reductase from *Thermoplasma acidophilum*, a Thermoacidophilic Euryarchaeon, Identifies It as an NADH-Dependent Enzyme

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ABSTRACT: Flavin-containing Trx reductase (TrxR) of *Thermoplasma acidophilum* (Ta), a thermoacidophilic facultative anaerobic archaeon, lacks the structural features for the binding of 2′-phosphate of nicotinamide adenine dinucleotide phosphate (NADPH), and this feature has justified the observed lack of activity with NADPH; NADH has also been reported to be ineffective. Our recent phylogenetic analysis identified Ta-TrxR as closely related to the NADH-dependent enzymes of *Thermotoga maritima* and *Desulfovibrio vulgaris*, both being anaerobic bacteria. This observation instigated a reexamination of the activity of the enzyme, which showed that Ta-TrxR is NADH dependent; the apparent K_m for NADH was 3.1 μM, a physiologically relevant value. This finding is consistent with the observation that NADH:TrxR has thus far been found primarily in anaerobic bacteria and archaea.
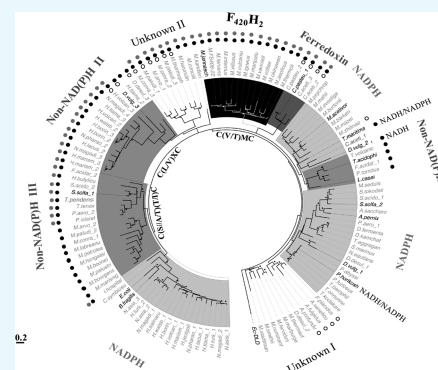
INTRODUCTION

The thioredoxin (Trx) system, which is composed of Trx, Trx reductase (TrxR), and a cognate reductant, is a key component of thiol-based metabolic regulation in almost all living systems. It is often crucial for the survival of these organisms through fluctuations in the availability of energy nutrients and exposure to oxidants in their habitats.[1−5] The Trx system implements this regulation through the oxidation and reduction of specific redox-active cysteine pairs of target proteins by the action of Trx, a small redox protein, thereby influencing their catalytic or physical properties.[1−5] In the process of reducing a target protein, the Cys residues of the redox-active Cys-X-X-Cys motif (X, variable amino acid residue) of Trx are oxidized. The resulting disulfide of Trx is then reduced by TrxR,[1−5] which is of two types: ferredoxin:Trx reductase[5] that carries a [Fe₄-S₄] center and receives electrons from reduced ferredoxin; and flavoprotein Trx reductase that is often dependent on nicotinamide adenine dinucleotide phosphate (NADPH) and consequently known as NADPH:TrxR or NTR.[6] Interestingly, certain flavin-containing TrxRs from anaerobic organisms do not use NADPH,[7−10] and following are examples of this group (electron donor for TrxR, source organism(s)) (Table 1): reduced ferredoxin,[7] *Clostridium pasteurianum*, a fermentative and obligate anaerobic bacterium; NADH,[8,9] *Thermotoga maritima* and *Desulfovibrio vulgaris*, a hyperthermophilic bacterium and a mesophilic sulfate-reducing bacterium, respectively; NADH and NADPH,[10,11] *Pyrococcus horikoshii* and *Deinococcus radiophilus*, a hyperthermophilic archaeon and an aerobic mesophilic bacterium, respectively; reduced coenzyme F₄₂₀ or $F_{420}H_2$,[12] *Methanocaldococcus jannaschii*, a phylogenetically deeply rooted hyperthermophilic methane-producing archaeon. The flavin-containing TrxR of *Thermoplasma acidophilum*, a thermoacidophilic and facultative anaerobic euryarchaeon, has also been identified as one such enzyme, as a recombinant form of this enzyme (Ta-TrxR) has been shown to be unable to utilize either NADPH or NADH as reductant.[16] This heterotroph utilizes peptides and carbohydrates via aerobic or anaerobic respiration. In the absence of oxygen, it uses elemental sulfur as a terminal electron acceptor and generates hydrogen sulfide.[13,14] *T. acidophilum* genome carries only one Trx and one TrxR and thus the electron donor of Ta-TrxR would be a key player in the response of the organism to nutrient limitation and oxidative stress. Curiously, our recent bioinformatics analysis suggested the possibility that Ta-TrxR is NADH dependent (Figure 1). The results of our study with purified recombinant Ta-TrxR as presented in this report indeed supported this hypothesis.

RESULTS

Generation of Homogeneous Recombinant Forms of *Thermoplasma acidophilum* Trx and TrxR (Ta-Trx and Ta-TrxR) Free of *E. coli* NTR. Ta-Trx and Ta-TrxR of *Thermoplasma acidophilum* were expressed in *E. coli* SHuffle T7 Express (NEB, Ipswich, MA) in soluble forms and purified via Ni²⁺−nitrilotriacetic acid (Ni−NTA) affinity chromatography to homogeneity (Figure 2). This *E. coli* strain lacks *trxB* that encodes NTR,[6] and this design eliminated the chance of contamination of *E. coli* NTR in purified proteins. The purified Ta-TrxR preparations exhibited a typical $A_{280/460nm}$ value of 5,

Table 1. Biochemical Properties of NADH-Thioredoxin Reductases (TrxR) in Archaea and Bacteria[a]

host (archaeon, A; bacterium, B; optimal growth temperature, °C)	ORF number for flavin-dependent TrxR (cognate Trx) studied	electron donor	characteristics of flavin-dependent TrxR		k_{cat}/K_m of the electron donor (M^{-1} s^{-1})	alternate substrate	references
			K_m, μM				
			Trx	NADPH, NADH			
Thermoplasma acidophilum (A, 59)	Ta_0984 (Ta_0866)	NADH	ND	-, 3.12	-, 1.3 × 10^6	DTNB (K_m, 2.93 mM)	16 & this study
Desulfovibrio vulgaris (B, 37)	Dvu_0377 (Dvu_0378)	NADH	1.12	ND, 5.6	1.3 × 10^6	ND	10
Pyrococcus horikoshii (A, 98)	Ph_1426 (Ph_0178)	NADPH and NADH	0.6	2.7, 26	ND	-	9
Thermotoga maritima (B, 80)	Tm_0869 (Tm_0868)	NADH and NADPH	ND	780, 73	8.4 × 10^4/8.7 × 10^6	DTNB, benzyl viologen, O$_2$	8, 19
Deinococcus radiophilus (B, 30−37)	ND	NADH and NADPH	ND	12.5, 30.2	ND	DTNB (K_m = 463 μM)	11

[a]ND, not determined; -, activity not detectable; *Thermococcus onnurineus* NA1 carries a NAD(P)H-dependent TrxR which is yet to be analyzed for kinetic constants.[32]
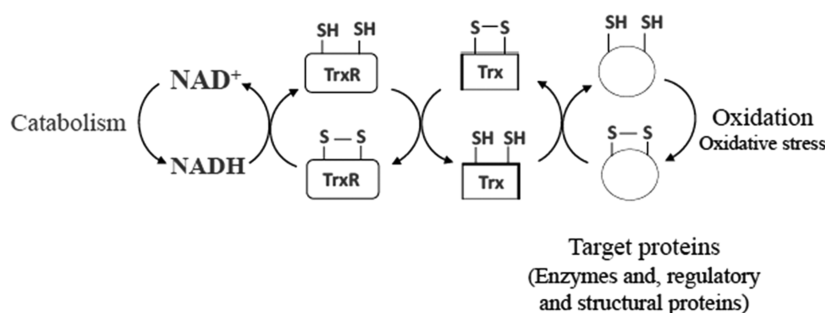

Figure 1. NADH-Thioredoxin system of *Thermoplasma acidophilum*. Trx system of *T. acidophilum* composed of a thioredoxin reductase (TrxR) and a thioredoxin (Trx) encoded by *ta_0984* and *ta_0866 orfs*, respectively, and NADH, the reductant. Trx reduces disulfide bonds of target proteins, and oxidized Trx is reduced by TrxR with NADH.

which is comparable to that of an *E. coli* NTR preparation with full incorporation of FAD.[15] The Ta-Trx preparation was proficient in reducing insulin with dithiothreitol (DTT) as reductant.[16]

Catalytic Properties of Ta-TrxR. The first attempt to assay Trx reductase activity of Ta-TrxR involved coupling it with insulin reduction, as with DTT as reductant for Ta-Trx-reduced insulin. However, likely due to a low activity at 25 °C, Ta-TrxR did not exhibit observable activity with NADH or NADPH as reductant; a high-temperature assay was not possible due to denaturation of insulin. The next effort involved spectrophotometric observation of NADH or NADPH oxidation, and the reduction of Ta-Trx was coupled to the reduction of oxidized glutathione, as described previously.[12] This coupling helped to maintain the level of oxidized Trx constant.[17] In this assay, NADH, but not NADPH, supported activity; the reaction was monitored at 340 nm.[12] With a fixed NADH concentration of 40 μM and at Ta-Trx concentrations of 10 and 20 μM, Ta-TrxR exhibited specific activities of 2.2 ± 0.3 and 3.5 ± 0.2 μmol min^{-1} mg^{-1}, respectively; the activity values represented an average over two measurements with a Ta-TrxR concentration of 50 nM.

For the reasons elaborated in Discussion, the further kinetic analysis of the NADH-dependent activity of Ta-TrxR was performed with 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) as the disulfide substrate in place of Ta-Trx. In this assay, the temperature and pH optima for the activity were found to be 70 °C and 7.5, respectively. With the concentration of NADH varying in the range of 1.6−40 μM and at a fixed concentration of 4 mM DTNB, the apparent values of K_m for NADH and V_{max} were found to be 3.12 ± 0.45 μM and 7.28 ± 0.32 μmol min^{-1} mg^{-1}, respectively (Figure 3A). Similarly, assays at a fixed NADH concentration of 40 μM and in the DTNB concentration range of 0.05−4.5 mM yielded an apparent K_m value of 2.93 ± 0.16 mM for DTNB (Figure 3B), and the corresponding V_{max} value was 10.08 ± 0.29 μmol min^{-1} mg^{-1}. Selenite, lipoic acid, and oxidized glutathione were not reduced by Ta-TrxR with NADH.

Comparative Analysis of Primary Structures of Ta-TrxR and Selected TrxRs. A primary sequence alignment of Ta-TrxR, Ec-NTR, and selected TrxRs of anaerobes is shown in Figure 4. All of these enzymes have been characterized biochemically at the purified enzyme stage,[6,8−10,16] and a comparison of Ta-TrxR with the rest at the amino acid
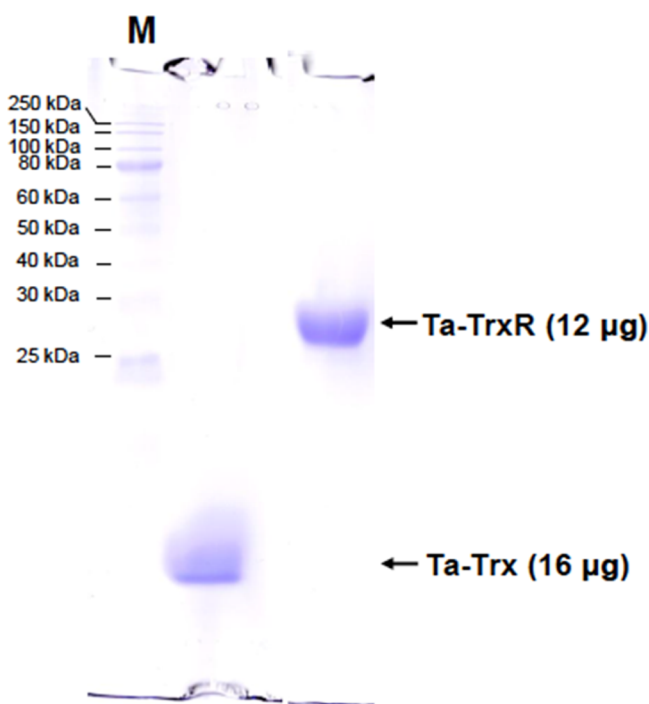
Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profiles of recombinant Ta-Trx and TrxR. A 18% polyacrylamide gel was used. M, Broad Range Protein Ladder (NEB, Ipswich, MA). Ta-Trx and TrxR in a solution containing 100 mM potassium phosphate buffer (pH 7.0) were analyzed.
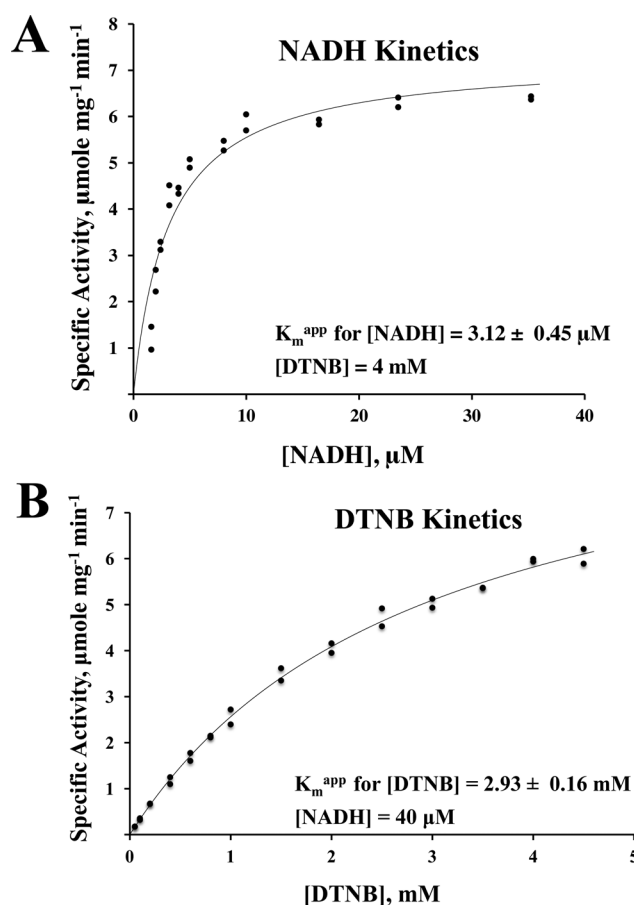

Figure 3. Kinetic analysis of Ta-TrxR. The substrates were 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) and NADH. The assays were performed anaerobically under N_2 atmosphere (1.3×10^5 Pa) at 60 °C in the assay mixture containing 100 mM potassium phosphate buffer (pH 7.0), 2 mM ethylenediaminetetraacetic acid (EDTA), and NADH and DTNB at concentrations as indicated. The assays were performed in duplicate, and in each case, the actual values from both assays have been plotted. (A) NADH kinetics; (B) DTNB kinetics. Each solid curve represents the best fit of the respective initial velocity data to the Henri−Michaelis−Menten hyperbola, $v = V_{max}[S]/(K_m + [S])$, where S is the substrate for which the concentration was varied. ±, standard deviation.

sequence level yielded the following values (source organism, electron donor, %identity, and %similarity to Ta-TrxR): *P. horikoshii*, NADH and NADPH, 40.0 and 53.9%; *T. maritima*, NADH, 39.1, 55.7%; *D. vulgaris*, NADH, 31.3 and 48.8%; and *E. coli*, NADPH, 25.7, 43.7%. Three key structural features of an NTR are the dinucleotide-binding motifs for FAD and NADPH (DBM_{FAD} and DBM_{NADPH}, respectively) and a redox-active Cys-X-X-Cys motif, where X is a variable amino acid residue (Figure 4). The comparison presented in Figure 4 and the X-ray crystallographic structure of the enzyme[16] show that most of the DBM_{FAD}-related sequence features of NTR are conserved in Ta-TrxR. However, this was not the case for DBM_{NADPH},[16,18] and the potential implications of the observed differences are presented in Discussion.

■ DISCUSSION

This study was instigated by the results of our recent phylogenetic analysis of low-molecular-mass flavin-containing TrxRs from several archaea and bacteria (Figure 5).[12] Some of these enzymes do not utilize NADPH, even though they exhibit significant structural similarities to NTR, and thus far NADH, ferredoxin, and coenzyme $F_{420}H_2$ have been identified as alternate electron donors.[10,7,12] In this context, the observed phylogenetic position of Ta-TrxR was intriguing. It belonged to the non-NADPH I group and showed a close relationship with an NTR group of methanogens from the class of Methanomicrobiales and certain bacteria and archaea (Figure 5). Included in this group were TrxRs of *Thermotoga maritima* and *Desulfovibrio vulgaris* (TrxR2).[8,10] Both of these organisms are anaerobic bacteria, and *T. maritima* is a thermophile.[19,20] The *T. maritima* enzyme prefers NADH over NADPH (K_m^{app} NADH = 7.3 μM; K_m^{app} NADPH = 780 μM), whereas TrxR2 of *D. vulgaris* exclusively uses NADH with an apparent K_m of

5.6 μM.[8,10] In the primary sequence, Ta-TrxR was found to be closely related to the *Pyrococcus horikoshii* enzyme, moderately related to the *D. vulgaris* enzyme and distant from *E. coli* NTR. Similar to the *T. maritima* enzyme, the TrxR of *P. horikoshii*, an anaerobic thermophilic archaeon, uses both NADH and NADPH, with a preference for NADH (K_m^{app} NADH = 26 μM; K_m^{app} NADPH = 2.7 μM) (Table 1).[9,11] Taken together, the observations presented above suggested that Ta-TrxR, an enzyme from a facultative anaerobe, might use NADH as electron donor for Trx reduction.

The above hypothesis is consistent with the structural features of Ta-TrxR.[16] The differences between the DBM_{FAD} sequences of Ta-TrxR and *E. coli* NTR were minor[16] (Figure 4). The notable one concerned the first residue of the ATG motif, which was Ala in NTR and Thr in Ta-TrxR. However, there exist major differences between the DBM_{NADPH} features of *E. coli* NTR and Ta-TrxR. The HRR element of the 2′-phosphate-binding motif (VXXXHRRDXXRA) of *E. coli* NTR is absent in Ta-TrxR.[15] In *E. coli* NTR, His175, Arg176, and Arg177 constitute the HRR motif, which is followed by Asp178
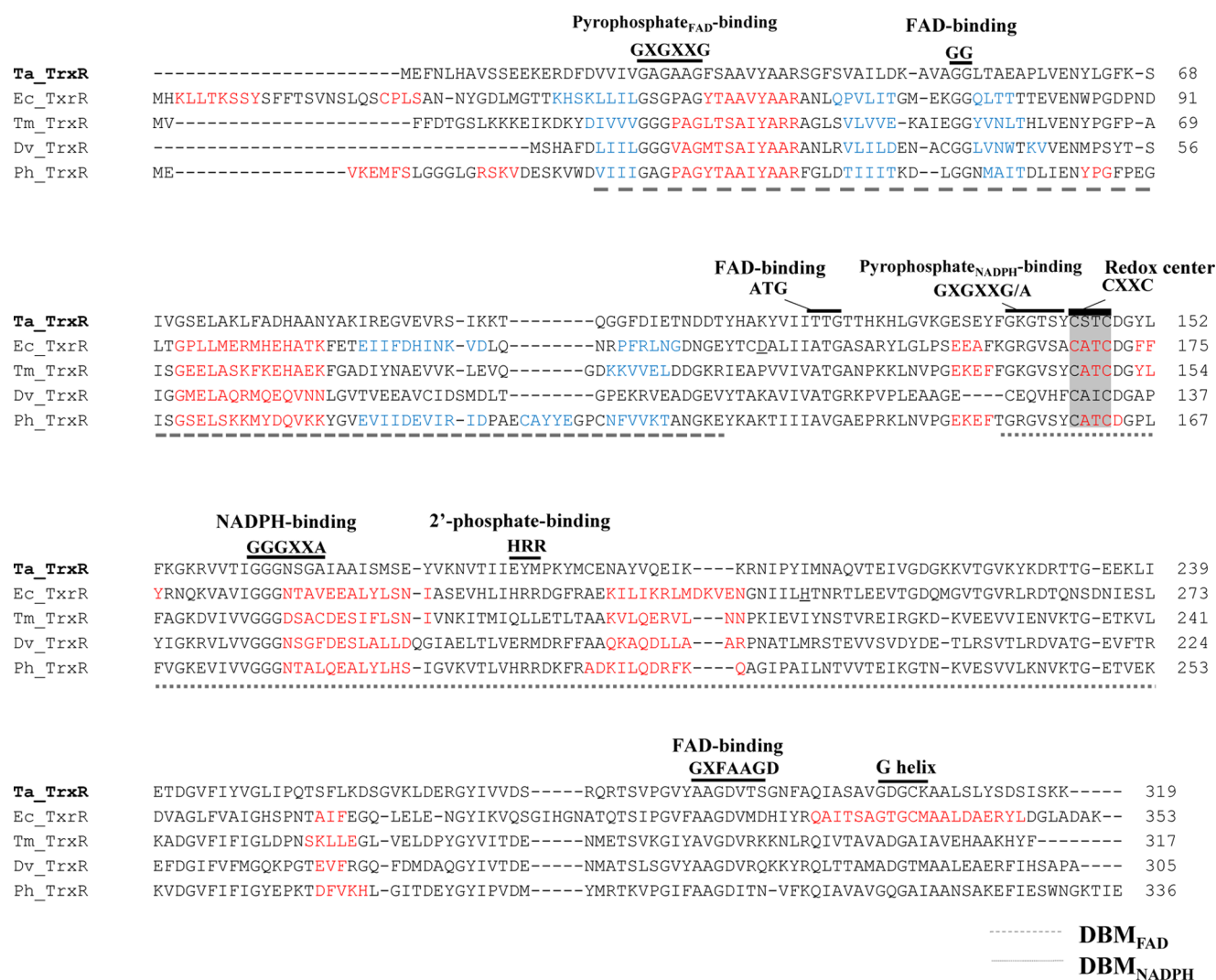
Pyrophosphate$_{FAD}$-binding **FAD-binding**
GXGXXG **GG**

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Ta_TrxR   -----------------------MEFNLHAVSSEEKERDFDVVIVGAGAAGFSAAVYAARSGFSVAILDK-AVAGGLTAEAPLVENYLGFK-S   68
Ec_TxrR   MHKLLTKSSYSFFTSVNSLQSCPLSAN-NYGDLMGTTKHSKLLILGSGPAGYTAAVYAARANLQPVLITGM-EKGGQLTTTTEVENWPGDPND   91
Tm_TrxR   MV--------------------FFDTGSLKKKEIKDKYDIVVVGGGPAGLTSAIYARRAGLSVLVVE-KAIEGGYVNLTHLVENYPGFP-A     69
Dv_TrxR   ---------------------------------MSHAFDLIILGGGVAGMTSAIYAARANLRVLILDEN-ACGGLVNWTKVVENMPSYT-S     56
Ph_TrxR   ME------------VKEMFSLGGGLGRSKVDESKVWDVIIIGAGPAGYTAAIYAARFGLDTIIITKD--LGGNMAITDLIENYPGFPEG          
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FAD-binding **Pyrophosphate$_{NADPH}$-binding** **Redox center**
ATG **GXGXXG/A** **CXXC**

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Ta_TrxR   IVGSELAKLFADHAANYAKIREGVEVRS-IKKT-------QGGFDIETNDDTYHAKYVIITTGTTHKHLGVKGESEYFGKGTSYCSTCDGYL   152
Ec_TxrR   LTGPLLMERMHEHATKFETEIIFDHINK-VDLQ-------NRPFRLNGDNGEYTCDALIIATGASARYLGLPSEEAFKGRGVSACATCDGFF   175
Tm_TrxR   ISGEELASKFKEHAEKFGADIYNAEVVK-LEVQ-------GDKKVVELDDGKRIEAPVVIVATGANPKKLNVPGEKEFFGKGVSYCATCDGYL   154
Dv_TrxR   IGGMELAQRMQEQVNNLGVTVEEAVCIDSMDLT-------GPEKRVEADGEVYTAKAVIVATGRKPVPLEAAGE----CEQVHFCAICDGAP   137
Ph_TrxR   ISGSELSKKMYDQVKKYGVEVIIDEVIR-IDPAECAYYEGPCNFVVKTANGKEYKAKTIIIAVGAEPRKLNVPGEKEFTGRGVSYCATCDGPL   167
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NADPH-binding **2'-phosphate-binding**
GGGXXA **HRR**

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Ta_TrxR   FKGKRVVTIGGGNSGAIAAISMSE-YVKNVTIIEYMPKYMCENAYVQEIK----KRNIPYIMNAQVTEIVGDGKKVTGVKYKDRTTG-EEKLI   239
Ec_TxrR   YRNQKVAVIGGGNTAVEEALYLSN-IASEVHLIHRRDGFRAEKILIKRLMDKVENGNIILHTNRTLEEVTGDQMGVTGVRLRDTQNSDNIESL   273
Tm_TrxR   FAGKDVIVVGGGDSACDESIFLSN-IVNKITMIQLLETLTAAKVLQERVL---NNPKIEVIYNSTVREIRGKD-KVEEVVIENVKTG-ETKVL   241
Dv_TrxR   YIGKRVLVVGGGNSGFDESLALLDQGIAELTLVERMDRFFAAQKAQDLLA---ARPNATLMRSTEVVSVDYDE-TLRSVTLRDVATG-EVFTR   224
Ph_TrxR   FVGKEVIVVGGGNTALQEALYLHS-IGVKVTLVHRRDKFRADKILQDRFK----QAGIPAILNTVVTEIKGTN-KVESVVLKNVKTG-ETVEK   253
```

FAD-binding
GXFAAGD **G helix**

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Ta_TrxR   ETDGVFIYVGLIPQTSFLKDSGVKLDERGYIVVDS-----RQRTSVPGVYAAGDVTSGNFAQIASAVGDGCKAALSLYSDSISKK-----   319
Ec_TxrR   DVAGLFVAIGHSPNTAIFEGQ-LELE-NGYIKVQSGIHGNATQTSIPGVFAAGDVMDHIYRQAITSAGTGCMAALDAERYLDGLADAK--   353
Tm_TrxR   KADGVFIFIGLDPNSKLLEGL-VELDPYGYVITDE-----NMETSVKGIYAVGDVRKKNLRQIVTAVADGAIAVEHAAKHYF--------   317
Dv_TrxR   EFDGIFVFMGQKPGTEVFRGQ-FDMDAQGYIVTDE-----NMATSLSGVYAAGDVRQKKYRQLTTAMADGTMAALEAERFIHSAPA----   305
Ph_TrxR   KVDGVFIFIGYEPKTDFVKHL-GITDEYGYIPVDM-----YMRTKVPGIFAAGDITN-VFKQIAVAVGQGAIAANSAKEFIESWNGKTIE   336
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------- **DBM$_{FAD}$**
.... **DBM$_{NADPH}$**

Figure 4. Amino acid sequence alignment of Ta-TrxR and selected TrxRs. The sequence alignment was constructed employing PROMALS3D[31] with the use of three-dimensional structures of *E. coli* NTR (pdb id 1CL0) and Ta-TrxR (pdb id 3CTY) as guides.[16,18] Abbreviations (organism name, NCBI accession number): Ta, *Thermoplasma acidophilum*, WP_010901395; Tm, *Thermotoga maritima*, AAD35951.1; Dv, *Desulfovibrio vulgaris str. Hildenborough*, AAS94860.1; Ph, *Pyrococcus horikoshii* OT3, BAA30532; Ec, *Escherichia coli*, EGJ07907.1. Conserved amino acids are shown in bold. Consensus predicted secondary structures: red h, α helix; blue e, β strand. Gray shading: the redox-active CXXC motif. Black bar: conserved motifs as found in low-molecular-weight NTR and flavoproteins and labeled as such. DBM: dinucleotide-binding motif.

(Figure 4).[16,18] In Ta-TrxR, the equivalent residues were Glu185, Tyr186, Met187, and Pro188, respectively. The change of His175 (*E. coli* NTR) to Glu185 (Ta-Trx) could create an unfavorable interaction of Ta-TrxR with 2'-phosphate of NADPH. Similarly, the presence of Pro, a nonpolar and structurally constrained residue, in place of Asp, a polar acidic residue, in the vicinity of the HRR element (Pro188 in Ta-TrxR vs Asp178 in *E. coli* NTR), could offer steric hindrance at the NADPH-binding site. In fact, docking studies with the three-dimensional structure of Ta-TrxR have shown that although Glu185 could repel 2'-phosphate of NADPH electrostatically, this unfavorable interaction would be removed with NADH.[15] Additional suggestions along this line come from a comparative analysis of the DBM$_{NADPH}$ sequences of Ta-TrxR (Figure 4). There are subtle structural differences between *E. coli* NTR and Ta-TrxR in the vicinity of the HRR motif. The GXGXXG/A motif of the DBM$_{NADPH}$ of *E. coli* NTR was found in Ta-TrxR; however, in the latter, the terminal residue is Tyr and not Ala, and in other NADH-dependent TrxRs, the respective residue

was either Tyr or Phe (Figure 4). This feature further suggested the use of NADH by Ta-TrxR.

Our biochemical characterizations of the enzyme showed that Ta-TrxR indeed used NADH for the reduction of Ta-Trx. The kinetic analysis of this reaction was impeded by the observation that although a rise of Ta-Trx concentration from 5 to 10 μM increased the activity 1.6-fold, any further increase in the concentration led to the precipitation of this substrate. For this reason, for further studies on the kinetic properties of Ta-TrxR, DTNB was used as the disulfide substrate in place of Ta-Trx. The K_m values for NADH (3.12 μM) and DTNB (2.93 mM) obtained from these assays were typical of flavin-containing TrxRs.[6,8−10] Thus, the thioredoxin reductase of *T. acidophilum* is a bona fide NADH-dependent enzyme. This property is fully supported by the redox potential value of the Ta-TrxR-bound flavin, which was determined to be −305 mV,[21] as it makes NADH a thermodynamically accessible electron donor for this enzyme.

The prokaryotic TrxRs that use solely NADH as an electron donor thus far have been found in obligate and facultative
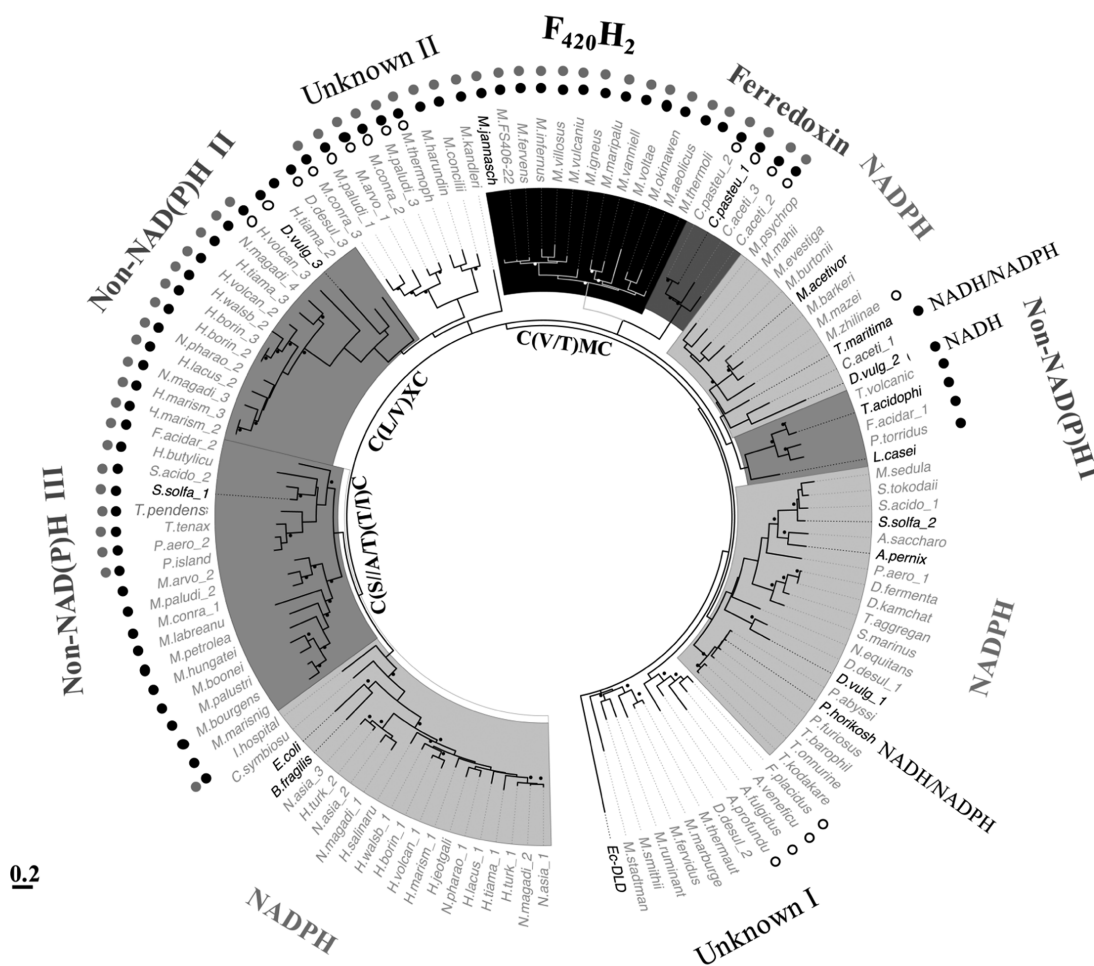
Figure 5. Maximum-likelihood phylogenetic analysis of noncanonical flavin-dependent thioredoxin reductases (TrxR) from archaea and bacteria and selected NTR. This figure has been reproduced from ref 12 with permission. A noncanonical TrxR is defined by the lack of the HRR motif, as shown in Figure 4. Taxa labels in bold, bona fide TrxR validated by direct activity assay. Black and white bullets near branches: bootstrap values >70 (calculated from 100 replicates). Each label appearing on the outer side of the tree: electron donor utilized by the members of the respective clade. Unknown: TrxR with unknown reductant. Non-NAD(P)H: inability to use NAD(P)H as electron donor as determined via direct activity assay. Symbols for the departures in the amino acid sequences at select conserved elements of TrxRs, as described in Figure 4: filled black and gray circles, unrecognizable HRR and GGG motifs, respectively; unfilled circle, absence of the GG motif. Ec-DLD, dihydrolipoamide dehydrogenase of *E. coli* used as an outgroup. Taxa abbreviations, *A. fulgidus, Archaeoglobus fulgidus; A. pernix, Aeropyrum pernix K1; A. profundu, Archaeoglobus profundus; A. saccharo, Acidolobus saccharovorans; A. veneficu, Archaeoglobus veneficus; B. fragilis, Bacteroides fragilis; C. aceti, Clostridium acetibutylicum; C. pasteu, Clostridium pasteurianum; C. symbiosu, Cenarchaeum symbiosum A; D. desul, Desulfovirbrio desulfuricans; D. fermenta, Desulfurococcus fermentans; D. kamchat, Desulfurococcus kamchatkensis; D. vulg, Desulfovibrio vugaris str. Hildenborough; E. coli, Escherichia coli; F. acidar, Ferroplasma acidarmanus; F. placidus, Ferroglobus placidus; H. walsb, Haloquadratum walsbyi; H. borin, Halogeometricum borinquense DSM11551; H. butylicu, Hyperthermus butylicus; H. jeotgali, Halalkalicoccus jeotgali; H. lacus, Halorubrum lacusprofundi; H. marism, Haloarcula marismortui ATCC 43049; H. salinaru, Halobacterium salinarum; H. tiama, Halorhabdus tiamatea; H. turkm, Haloterrigena turkmenica; H. volcan, Haloferax volcanii; I. hospital, Ignicoccus hospitalis; L. casei, Lactobacillus casei; M. mazei, Methanosarcina mazei; M. thermoli, Methanothermococcus thermolithotrophicus; M. acetivor, Methanosarcina acetivorans C2A; M. aeolicus, Methanococcus aeolicus; M. arvoryza, Methanocella arvoryzae MRE50; M. barkeri, Methanosarcina barkeri; M. boonei, Methanoregula boonei 6A8; M. bourgens, Methanoculleus bourgensis; M. burtonii, Methanococcoides burtonii; M. concilii, Methanosaeta concilii; M. conradii, Methanocella conradii HZ254; M. evestiga, Methanohalobium evestigatum; M. fervens, Methanocaldococcus fervens; M. fervidus, Methanothermus fervidus; M. FS406-22, Methanocaldococcus sp. FS406-22; M. harundin, Methanosaeta harundinacea; M. hungatei, Methanospirillum hungatei JF-1; M. igneus, Methanotorris igneus; M. infernus, Methanocaldococcus infernus; M. jannasch, Methanocaldococcus jannaschii; M. kandleri, Methanopyrus kandleri; M. labreanu, Methanocorpusculum labreanum; M. mahii, Methanohalophilus mahii; M. marburge, Methanothermobacter marburgensis; M. maripalu, Methanococcus maripaludis S2; M. marisnig, Methanoculleus marisnigri JR1; M. okinawen, Methanococcus okinawensis; M. paludi, Methanocella paludicola SANAE; M. palustri, Methanosphaerula palustris; M. petrolea, Methanolacinia petrolearia DSM11571; M. psychrop, Methanolobus psychrophilus R15; M. ruminant, Methanobrevibacter ruminantium M1; M. sedula, Methanosphaera sedula; M. smithii, Methanobrevibacter smithii; M. stadtman, Methanosphaera stadtmanae; M. thermaut, Methanothermobacter thermautotrophicus strain Delta H; M. thermop, Methanosaeta thermophila; M. vanniell, Methanococcus vannielii; M. villosus, Methanocaldococcus villosus; M. voltae, Methanococcus voltae; M. vulvaniu, Methanocaldococcus vulcanius; M. zhilinae, Methanosalsum zhilinae; N. asia, Natrialba asiatica; N. equitans, Nanoarchaeum equitans; N. magadi, Natrialba magadii; N. pharao, Natromonas pharaonic; P. abyssi, Pyrococcus abyssi GE5; P. aero, Pyrobaculum aerophilum str. IM2; P. furiosus, Pyrococcus furiosus DSM 3638; P. island, Pyrobaculum islandium; P. torridus, Picrophilus torridus; S. acido, Sulfolobus acidocaldarius; S. marinus, Staphylothermus marinus; S. solfa, Sulfolobus solfataricus; S. tokodai, Sulfolobus tokodai str.7; T. acido, Thermoplasma acidophilum; T. aggregan, Thermosphaera aggregans; T. barophil, Thermococcus barophilus; T. kodakaren, Thermococcus kodakarensis; T. maritima, Thermotoga maritima; T. onnurine, Thermococcus onnurineus; T. pendens, Thermofilum pendens; T. tenax, Thermoproteus tenax; T. volvanic, Thermoplasma volcanicum.*

anaerobic bacteria and archaea.[8−10] However, TrxRs that can use NADH as well as NADPH have been reported in both anaerobes and aerobes (Table 1). The *Deinococcus radiophilus* TrxR is the sole example of such an enzyme from an aerobic bacterium. However, it shows preference for NADPH.[11] Interestingly, a phylogenetic analysis suggested that *D. radiophilus* obtained the enzyme through horizontal gene transfer from anaerobic archaea.[11] These observations suggest that the NADH-dependent TrxRs likely originated in anaerobes.

It is not clear why the anaerobes would prefer NADH to NADPH for Trx reduction. One of the possible reasons is to achieve energy efficiency. Generally, anaerobic metabolism generates less ATP than the aerobic counterparts and the use of NADH avoids investment in the conversion of NADH to NADPH by NAD kinase, which requires ATP.[22] However, the *T. acidophilum* genome encodes both NAD^+/NADH- and $NADP^+$/NADPH-dependent enzymes, which makes the above-mentioned explanation less likely.

Our findings have shown that the investigations on the electron donors of yet-to-be characterized flavin-containing Ta-TrxRs that lack specific structural features for binding of the 2′-phosphate of NADPH could be guided by their relative phylogenetic positions. Our phylogenetic studies have also indicated that there exist at least four more groups of flavin-containing TrxRs that are likely to be non-NADPH types and need to be investigated for their reductants.

■ MATERIALS AND METHODS

Materials. *E. coli* SHuffle T7 Express competent cells and restriction enzymes were obtained from the New England Biolabs (Ipswich, MA). *Thermoplasma acidophilum* genomic DNA and pTev5 were generous gifts from Dr. Dennis G. Searcy of University of Massachusetts Amherst and Dr. Jorge Escalante-Semerena of the University of Georgia, respectively. All chemicals were purchased from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Waltham, MA).

Heterologous Expression of TrxR and Trx of *T. acidophilum* (Ta-TrxR and Ta-Trx) in *E. coli* SHuffle T7 Express. Coding sequences for Ta-TrxR and Ta-Trx were amplified from *T. acidophilum* genomic DNA using Q5 DNA polymerase (NEB, Ipswich, MA), and the respective amplicons were cloned into *Nde*I and *Bam*HI sites of pTev5, a T7-based expression vector,[23] resulting pUL207 and pUL208, respectively. These plasmids were designed to express recombinant proteins with an NH_2-terminal His_6-tag, followed by a TEV protease recognition site. Both plasmids were transformed into *E. coli* SHuffle T7 Express competent cells (NEB, Ipswich, MA) containing pRIL plasmid (Stratagene, La Jolla, CA). The resulting strains were grown at 37 °C in Luria Bertani media containing 100 and 34 μg/mL ampicillin and chloramphenicol, respectively. For the expression of Ta-Trx, the LB media was supplemented with DTT at a final concentration of 0.2 mM. When a growing culture reached an optical density (OD_{600}) of 0.8, as measured with a DU800 UV−visible spectrophotometer (Beckman Coulter, Inc., Brea, CA), IPTG was added to a final concentration of 0.4 mM and the cultivation was continued for additional 5 h at 37 °C for Ta-TrxR overexpression and for 12 h at 15 °C for Ta-Trx overexpression. The cells from these cultures were harvested by centrifugation at 10 000g for 10 min at 4 °C using a Sorvall LYNX 4000 centrifuge (Thermo Fisher Scientific, Waltham, MA), and the cell pellets were stored at −20 °C until used.

Purification of Recombinant Ta-TrxR and Ta-Trx. This task was performed under air and as previously described.[12] Typically, 4 g of frozen cell pellets of *E. coli* SHuffle T7 Express harboring pRIL and either pUL207 or pUL208 were thawed on ice and resuspended in 4 mL of a lysis buffer containing 50 mM sodium phosphate buffer (pH 7.0), 300 mM NaCl, 10 mM imidazole, and a cOmplete EDTA-free Protease Inhibitor Cocktail tablet (Roche Life Sciences, Indianapolis, IN). The cells were then lysed by three passages of the suspension through a French pressure cell operating at a pressure of 1.28 × 10^8 Pa, and the lysate was centrifuged at 18 000g at 4 °C for 30 min. The resulting supernatant was subjected to heating at 80 °C for 10 min to precipitate most of *E. coli* proteins, which were then removed by centrifugation at 18 000g at 4 °C for 30 min. From the clarified supernatant, recombinant protein was purified via Ni−NTA chromatography.[12] Both Ta-TrxR and Ta-Trx were eluted at an imidazole concentration range of 100−200 mM. The column fractions that contained homogeneous proteins were then pooled and concentrated using an Amicon stirred ultrafiltration cell fitted with a membrane with a desired molecular weight cutoff (MWCO) (EMD Millipore, Bedford, MA); the MWCO was 10 kDa for Ta-TrxR and 3 kDa for Ta-Trx. To cleave the NH_2-terminal His_6-tag, the purified protein was incubated overnight at 4 °C with recombinant TEV protease carrying a His_6-tag ($rHis_6$-TEV)[24] at a protein-to-$rHis_6$-TEV ratio of 20:1, following a protocol as described previously.[12] From the resulting mixture, Ta-TrxR carrying intact His_6-tag, cleaved His_6-tag, and $rHis_6$-TEV were removed by passing through a Ni−NTA column. The purities of as-purified and digested proteins were analyzed by SDS-PAGE, and the protein concentrations were determined via Bradford assay using a protein assay kit (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's protocol.[25]

Insulin Disulfide Reductase Assay for Ta-Trx and Ta-TrxR. Disulfide reductase activity of Ta-Trx was measured by insulin reduction assay, as described previously, using DTT as an artificial electron donor.[26,27] A standard assay mixture contained 50 μM Ta-Trx, 0.8 mg/mL insulin, 2 mM EDTA, 100 mM potassium phosphate buffer (pH 7.0), and 1 mM DTT. This assay was also used to test the reactivity of Ta-TrxR toward Ta-Trx, where it was performed under anaerobic conditions,[12] DTT was replaced by NADH (0.1 mM), and Ta-TrxR was added to a final concentration of 100 nM. The assay temperature was 25 °C. The reaction was initiated by the addition of the reductant, DTT or NADH, and the increase of absorbance at 650 nm indicating insulin reduction was monitored using a Beckman Coulter DU800 spectrophotometer (Brea, CA).

Kinetic Assays of Ta-TrxR. The assay was performed anaerobically at 60 °C as described previously[26] using an assay mixture (total volume, 800 μL) that contained 100 mM potassium phosphate buffer (pH 7.0), 2 mM EDTA, and desired levels of NADH and Ta-Trx or an alternate electron acceptor, such as 5′-5′-dithiobis(2-nitrobenzoate) or DTNB. For assays with Ta-Trx, the concentration of this electron acceptor was maintained constant by supplementing the assay mixture with oxidized glutathione to a final concentration of 1 mM.[17] In each case, the reaction was initiated by the addition of the enzyme, and in all cases except with DTNB as electron acceptor, it was monitored at 340 nm for the consumption of NADH; with DTNB, the monitoring was at 412 nm. The initial velocity values were calculated using an extinction coefficient value of 6.2 mM^{-1} cm^{-1} for NADH at 340 nm or 14.15 mM^{-1}

cm^{-1} for 5-thio-2-nitrobenzate or TNB at 412 nm.[28] For pH studies, the potassium phosphate buffer was replaced by constant ionic strength buffers composed of 60 mM MES, 120 mM Tris, and 60 mM glacial acetic acid and adjusted to the desired pH values (4−9.5) with HCl or NaOH.[29] To determine the activities of Ta-TrxR toward alternate substrates, Trx was replaced by one of the following (entity, (final concentration)): DTNB (varied), Na$_2$SeO$_3$ (50 μM), and oxidized L-glutathione (1 mM) and lipoate (1 mM). The apparent kinetic constants were calculated by fitting the initial velocity data to the Henri−Michaelis−Menten equation, $v = V_m [S]/(K_m + [S])$, where S is the substrate for which the concentration is varied and V_m and K_m are maximum velocity and Michaelis constant, respectively; KaleidaGraph 4.5 (Synergy Software Inc., Reading, PA) was used for data fitting.

Bioinformatic Analysis. Primary and secondary structure alignments of Ta-TrxR and its close homologues were performed using MUSCLE[30] and PROMALS3D,[31] respectively. For the latter, three-dimensional structures of *E. coli* NTR and *T. acidophilum* TrxR (pdb id: 1CL0 and 3CTY, respectively) served as references.[16,18]

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D.S. and B.M. designed the research; D.S., U.L., and A.C. performed the research; D.S., U.L., and B.M. analyzed the data; and D.S. and B.M. wrote the article.

Notes

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■ ABBREVIATIONS

Trx, thioredoxin; TrxR, thioredoxin reductase; Ta, *Thermoplasma acidophilum*; NTR, NADPH-thioredoxin reductase; FAD, flavin adenine dinucleotide; DBM, dinucleotide-binding domain; DTNB, 5,5-dithio-bis-2-nitrobenzoic acid

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