

Molecular Recognition in the Interaction of Chloroplast 2-Cys Peroxiredoxin with NADPH-Thioredoxin Reductase C (NTRC) and Thioredoxin x

Pilar Bernal-Bayard¹, Valle Ojeda¹, Manuel Hervás¹, Francisco J. Cejudo¹, José A. Navarro^{1*}, Adrián Velázquez-Campoy² and Juan M. Pérez-Ruiz¹

¹Instituto de Bioquímica Vegetal y Fotosíntesis. CSIC and Universidad de Sevilla. Avda. Américo Vespucio 49, 41092-Sevilla, Spain, and ²Institute of Biocomputation and Physics of Complex Systems (BIFI), Joint Unit IQFR-CSIC-BIFI, Universidad de Zaragoza, C/ Mariano Esquillor, Zaragoza-50018, Spain; Department of Biochemistry and Molecular and Cell Biology, University of Zaragoza, Zaragoza, Spain; and Fundacion ARAID, Government of Aragon, Spain.

*To whom correspondence should be addressed: José A. Navarro. Instituto de Bioquímica Vegetal y Fotosíntesis, CSIC and Universidad de Sevilla, cicCartuja, Avda. Américo Vespucio 49, 41092-Seville, Spain. Tel.: 34-954-489-515; Fax: 34-954-460-065; E-mail: jnavarro@ibvf.csic.es

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ABSTRACT

In addition to the standard NADPH thioredoxin reductases (NTRs), plants hold a plastidic NTR (NTRC), with a thioredoxin module fused at the C-terminus. NTRC is an efficient reductant of 2-Cys peroxiredoxins (2-Cys Prxs). The interaction of NTRC and chloroplastic thioredoxin *x* with 2-Cys Prxs has been confirmed *in vivo*, by bimolecular fluorescence complementation (BiFC) assays, and *in vitro*, by isothermal titration calorimetry (ITC) experiments. In comparison with thioredoxin *x*, NTRC interacts with 2-Cys Prx with higher affinity, both the thioredoxin and NTR domains of NTRC contributing significantly to this interaction, as demonstrated by using the NTR and thioredoxin modules of the enzyme expressed separately. The presence of the thioredoxin domain seems to prevent the interaction of NTRC with thioredoxin *x*.

Highlights:

Plant NTRC is a key regulatory enzyme that, in addition to a flavin and a disulfide group, contains an extra thioredoxin module. NTRC is an efficient reductant of 2-Cys Prxs, thus being involved in the plant response to oxidative stress and in maintaining plastids redox homeostasis.

We have used BiFC and ITC methodology to carry out an analysis of the interaction of NTRC and Trx *x* with 2-Cys Prxs. In comparison with canonical NTRB and chloroplastic Trx *x*, NTRC binds 2-Cys Prx with higher affinity. Both the Trx and NTR domains contribute significantly to the interaction of NTRC with 2-Cys Prx, as established by using both the native proteins and the NTR and Trx modules of NTRC expressed separately. The Trx domain prevents the interaction with chloroplastic Trx *x*.

INTRODUCTION

Photosynthesis involves the transport of electrons in an aerobic environment and, thus, harmful reactive oxygen species (ROS), including hydrogen peroxide, are inevitably formed [1]. Hydrogen peroxide, however, has also an important function in plant signalling [1,2]. Therefore, the control of the level of this molecule in chloroplasts is of great relevance in order to balance its toxic and signalling activity. In this regard, chloroplasts are equipped with different antioxidant systems, of which peroxiredoxins (Prxs), in particular, are among the most abundant proteins in these organelles [3]. Prxs are thiol-based peroxidases encoded by a gene family formed by 9-10 genes in plants [4].

The *Arabidopsis* chloroplast harbours two almost identical typical 2-Cys Prxs, termed 2-Cys Prx A and 2-Cys Prx B, which undergo overoxidation of the peroxidatic cysteine residue in the active sites [5]. Overoxidation leads to the inactivation of the peroxidase activity, but switches on the chaperone activity of these enzymes [6], thus allowing an important signalling activity of hydrogen peroxide [7].

Another enzymes involved in the defence against oxidative stress in plants are NADPH-dependent thioredoxin-reductases (NTRs). These enzymes contain a flavin cofactor and a dithiol group, operating as an intramolecular electron transfer (ET) chain that drives electrons from NADPH to their disulfide-containing thioredoxin (Trx) substrates [8,9]. In its turn, Trxs can transfer reducing equivalents to Prxs, among other targets [10]. Plants contain two canonical NTRs, termed NTRA and NTRB. While NTRB shows a predominant cytosolic localisation, NTRA is mainly present in the mitochondria [11]. The structure of *Arabidopsis* NTRB has been solved, the enzyme being similar to the previously reported *E. coli* protein [8,12]. The structure reveals a

dimeric enzyme, each monomer comprising two domains, one enclosing the flavin FAD cofactor and the other containing both the NADPH binding site and the active 2-Cys group [12].

In addition to NTRA and NTRB, a plastidial NTR, termed NTRC, has been identified, which is exclusive of oxygenic photosynthetic organisms [13,14]. NTRC is a non-canonical NTR, as it contains a C-terminal Trx module, the catalytic unit being also a homodimer [13,15-17]. The enzyme shows both NTR and Trx activities [13], which are conjugated to efficiently reduce 2-Cys Prxs [18-21]. However, although chloroplast 2-Cys Prxs reduction is mainly performed by NTRC [22], other plastidial Trxs, such as Trx *x* [23] or CDSP32 [24], have been also proposed to reduce 2-Cys Prxs. Furthermore, in addition to its antioxidant role as reductant of Prxs, NTRC exerts a relevant function in redox regulation of the biosynthesis of starch [25] and chlorophyll [26, 27]. Moreover, its localization in both chloroplasts and non-green plastids [28] has led to propose NTRC as a key component of redox regulation in plants [16,17].

The catalytic mechanism of the canonical NTR from *E. coli* has been intensively studied [29], the redox cycle of the enzyme involving large protein motions and conformational movements. In the case of the NTRC homodimeric enzyme, the transfer of electrons occurs from the NTR module of one subunit to the Trx module of the other subunit [15, 21]. A recent kinetic study suggests that NTRB and NTRC share the large protein dynamics associated with the process of catalysis in canonical NTRs [21], in agreement with the structural similarities shared by the different NTR moities, as deduced from sequences comparison [9]. However, NTRC displays additional dynamic motions and restricted configurations associated with the ET to the extra Trx module, which are altered in the presence of 2-Cys Prx [21].

In this study, we have investigated the interaction of 2-Cys Prxs with NTRC and Trx *x* by a combination of *in vivo* and *in vitro* approaches. Bimolecular fluorescence complementation (BiFC) methodology was used to carry out a comparative analysis of the interaction of NTRC and Trx *x* with 2-Cys Prxs *in vivo*. Furthermore, we have used isothermal titration calorimetry (ITC) to extend our previous kinetic studies towards the interaction of NTRC and Trx *x* with 2-Cys Prx. Additionally, the contribution of both NTR and Trx domains of NTRC to 2-Cys Prx binding has been investigated.

MATERIALS AND METHODS

BiFC assay and confocal microscopy

The full length open reading frame (ORF), excluding the stop codon, encoding NTRC, Trx *x*, 2-Cys Prx A and 2-Cys Prx B were amplified by RT-PCR from total RNA of *Arabidopsis* seedlings using primers (Supplementary Table 1) that added attB recombination sites at the 5' and 3' ends, respectively. PCR products were gel-purified, cloned in the Gateway pDONR207 vector (Invitrogen) and sequenced in both strands. The cloned cDNAs were then transferred to the BiFC vectors pSPYNE-35S_*GW* and pSPYCE-35S_*GW*, respectively [30], using the LR Clonase (Invitrogen) following manufacturer's instructions. Resulting plasmids, pSPYNE:NTRC, pSPYNE:Trx *x*, pSPYCE:2-Cys Prx A and pSPYCE:2-Cys Prx B, were then transformed into the *Agrobacterium tumefaciens* strain GV301. It should be noted that we were unable to transfer neither 2-Cys Prx A nor 2-Cys Prx B cDNAs to the pSPYNE-35S_*GW* vector, indicating that these constructs may be toxic to the *E. coli* cells. For BiFC assays, *Agrobacterium* strains carrying individual constructs were mixed at 1:1 ratio or individually infiltrated into leaves of 4-week-old *Nicotiana benthamiana* plants. Leaf sections were analyzed 4 days later by confocal microscopy performed with a Leica SP/2 inverted microscope. Image analysis was performed with the Leica SP/2 software package and the ImageJ bundle provided by the Wright Cell Imaging facility.

Expression and purification of recombinant proteins

Expression and purification from *E. coli* cells of recombinant NTRB and Trx *h1* (cytoplasmic) from wheat, and NTRC, 2-Cys Prx and Trx *x* (chloroplastic) from rice, were carried out as previously described [13,18,21,31]. The truncated NTR (NTR_M) and

Trx (Trx_M) domains of NTRC were generated **introducing a termination codon in the interdomain region**, and purified as previously described [13, 18, 21]. In all cases proteins were produced as N-terminal His-tagged forms in their oxidized state. NTRs concentrations were determined spectrophotometrically using absorption coefficients of 12.2 (NTRB) and 15.2 (NTRC and NTR_M) mM⁻¹ cm⁻¹ at 456 nm [21]. Concentrated protein solutions were stored at -80° C until use.

Isothermal titration calorimetry experiments

ITC experiments were carried out in 5 mM phosphate buffer, pH 7.5, supplemented with 0.02% (w/v) Triton X-100, using an Auto-ITC200 instrument (Microcal, GE Healthcare) at 25 °C and a stirring speed of 1000 rpm. The reference cell was filled with distilled water. Protein solutions (20 μM) were titrated with successive additions (2 μL injections) of concentrated protein partner solutions. All solutions were degassed before titrations. Titrant was injected at appropriate time intervals to ensure that the thermal power signal returned to the baseline prior to the next injection. Control experiments of the dilution of individual injected proteins were carried out and **these reference heat values were subtracted from measuring values of test titrations when appropriate** (e.g. titration of Trx_M with NTR_M). The normalized heat per injection as a function of the molar ratio was analyzed with Origin 7 (OriginLab). In all cases the heat evolved during titrations could be well fitted to a 1:1 binding stoichiometry, and the association constant, K_A (and the dissociation constant, K_D), and the binding enthalpy (ΔH) and entropy (ΔS) values for the interaction process were estimated [32]. The heat capacity change (ΔC_p) in the NTRC/2-Cys Prx interaction was estimated from experiments at different temperatures (15, 20 and 25°C) [32]. Estimated errors in the

determined values were 15% for the equilibrium constants, 5% for the binding enthalpy and entropy, and 2% for the binding Gibbs energy.

RESULTS AND DISCUSSION

***In vivo* analysis of the interaction of NTRC and Trx *x* with 2-Cys Prxs A and B**

Although initial analysis of the plastidial Trxs, based on *in vitro* activity assays, identified type-*x* Trx as a potential reductant of 2-Cys Prxs [23], further studies revealed that NTRC acts as a more efficient reductant of these sulfoproteins [18]. These results are in line with *in vivo* data showing interaction of NTRC, but not Trx *x*, with 2-Cys Prx based on Förster resonance energy transfer (FRET) analyses [33]. Moreover, the redox status of 2-Cys Prxs is severely affected in NTRC deficient plants, in contrast with the Trx *x* knockout mutant [22], lending support to the notion that NTRC is the predominant reductant of chloroplast 2-Cys Prxs.

In this study we have investigated the interaction of 2-Cys Prxs with NTRC and Trx *x* by a combination of *in vivo* and *in vitro* approaches, **the rationale for such analyses being to arrive at a quantitative understanding of the thiol-disulfide redox regulatory network of the chloroplast**. BiFC experiments show the *in vivo* interaction of NTRC and Trx *x* with both 2-Cys Prx A and B in *N. benthamiana* chloroplasts (Fig. 1), whereas control experiments with individual constructs did not show fluorescence signals (Supplementary Fig. 1). While the Trx *x*/2-Cys Prxs interaction produced a uniform fluorescence within chloroplasts, patches were observed in the interaction of NTRC with either 2-Cys Prx A or 2-Cys Prx B (Fig. 1). This pattern is similar to that previously reported for the interaction of NTRC with the CHL-I subunit of the Mg-chelatase complex [27], indicating the tendency of NTRC to form speckles in the interaction with its chloroplast targets. This seems to be a specific feature of NTRC since it is not observed in the 2-Cys Prxs/Trx *x* interaction (Fig. 1). Although this behaviour may be related with the tendency of NTRC to form aggregates [15, 34], it is

not yet known the functional significance of the formation of speckles of this protein and its interacting partners.

***In vitro* molecular recognition of 2-Cys Prx with NTRC and Trx x**

Once established the *in vivo* interaction of NTRC and Trx x with 2-Cys Prxs, the molecular recognition between these proteins was analysed *in vitro* by ITC. This matter is of particular interest in the case of NTRC, as it has been previously reported that this enzyme shows additional dynamic motions associated with the extra Trx module, which are altered in the presence of 2-Cys Prx [21]. Consequently, in order to understand the catalytic behaviour of this enzyme, it is of interest to establish the contribution of the NTR and Trx modules to the interaction with 2-Cys Prx, for which experiments using the truncated NTR (NTR_M) and Trx (Trx_M) modules of NTRC were also carried out.

In plants both NTRC and 2-Cys Prxs can exist as oligomers [15, 34, 35], and thus dilution effects affecting the oligomerization equilibrium could disturb the ITC results. As oligomerization was shown to specifically affect reduced 2-Cys Prxs [35], the oxidized form of the enzyme was used in our analyses. In the case of NTRC, although oligomeric species could be still formed at the low concentration used (20 μM), dilution effects were minimized by placing this protein in the calorimeter cell (see also inset in Figure 2 as a control of NTRC dilution). Moreover, it was previously proven that NTRC activity is independent of its oligomeric state, indicating that accessibility of the flavin and the disulfide active sites are not affected in the oligomeric species [34]. Therefore, interferences from dilution/oligomerization effects can be **excluded** for both proteins. In addition, in all cases where interaction was observed the dependence of the heat evolved during titrations could be well fitted to a 1:1 binding stoichiometry (Figures 2 and 3),

confirming the adequacy of the experimental approach used, and values for K_A (and K_D and ΔG) as well as the ΔH and $-T\Delta S$ for the binding process were estimated (Table 1).

Figure 2 shows titration experiments for the NTRC/2-Cys Prx and Trx *x*/2-Cys Prx native partner interactions. In agreement with previous kinetic data [21], no interaction was detected for the NTRC/Trx *x* couple (Figure 2, *inset*), thus confirming that NTRC does not act as reductant of Trx *x*. As shown in Table 1, K_D and free energy values for the binding process are of the same order of magnitude for the NTRC/2-Cys Prx and Trx *x*/2-Cys Prx couples, although lower K_D , and thus higher affinity, was observed for the NTRC/2-Cys Prx system. The data also indicate similar interaction forces in the 2-Cys Prx binding to either NTRC or Trx *x*. Thus, the binding process, as deduced from the ΔH and $-T\Delta S$ values (Table 1), is mainly driven by entropic factors in both cases, whereas the enthalpic contributions are rather weak. The favorable entropic contribution to binding in these two redox couples probably indicates solvent desorganization, arising from the release of water molecules from the protein-protein interaction area upon complex formation. It is worthy to note that although protein-protein interactions in redox reactions must be transient and usually show the moderately low K_A values here determined, a previous ITC study on Trx/Prx interaction of mitochondrial proteins identified a extremely high-affinity binding ($K_D = 126$ pM) driven by enthalpic factors, able to largely counterbalance unfavourable entropic contributions [36].

The ΔC_P value for the NTRC/2-Cys Prx interaction (-80 cal·K⁻¹·mol⁻¹; not shown), estimated from experiments at different temperatures, is relatively small but similar to that of other transient interactions between redox binding partners, indicating a major contribution of the hydrophobic effect to binding [37]. Moreover, ΔC_P is considered to mirror changes in the exposure to solvent during the interaction process,

and a small value of ΔC_p would suggest both a small interaction interface and the occurrence of small conformational changes during partners binding [38]. However, it has been previously suggested that NTRC has a "closed" conformation of the two interacting NTR and Trx modules, which "opens" when interacting with 2-Cys Prx [21]. This would be compatible with a small ΔC_p if the solvent exposure of the binding interface compensates for the conformational change upon binding.

By using the isolated NTR_M and Trx_M truncated modules of NTRC, we have then analysed the involvement of the NTR and Trx domains in the interaction of this enzyme with 2-Cys Prx. Figure 3 shows the titrations of the NTR_M/Trx_M and Trx_M/2-Cys Prx couples, the thermodynamic parameters determined from the data fitting being shown in Table 1. Both Trx_M (Figure 3) and NTR_M (not shown) modules are able to bind 2-Cys Prx with an equivalent affinity but somewhat lower than for the full NTRC enzyme ($K_D \approx 13\text{-}14 \mu\text{M}$ vs. $9 \mu\text{M}$, respectively; Table 1). This fact strongly supports that both the NTR and Trx domains contribute to 2-Cys Prx binding. This is not unexpected, as the modeled NTRC structure shows that the active cysteine groups of the Trx domain locate at the interface with the NTR domain and in close contact with the latter [17]. The forces involved in the interaction of 2-Cys Prx with each one of the two domains are, however, different: the strong entropic contributions that drive the NTR_M/2-Cys Prx binding markedly contrast with the strong favourable enthalpic character of the interaction of the isolated Trx module with 2-Cys Prx. Based on the experimental approach used here, however, it is difficult to elucidate the relative contribution of each NTRC domain to substrate binding, as separate Trx_M and NTR_M modules may expose additional protein surfaces that are hindered in the complete NTRC enzyme, thus having influence on the ITC experiments.

The truncated NTR_M and Trx_M polypeptides show NTR and Trx activity,

respectively [13]. Therefore, although NTRC might have NTR activity, it seems not to be the case since, as stated before, no NTRC/Trx *x* interaction was detected neither by kinetic nor ITC analysis (Figure 2, Table 1; and see Bernal et al., 2012). Interestingly, the removal of Trx_M allows the NTR module of NTRC to interact with Trx *x*, although with reduced affinity (K_D ca. 53 μM ; Table 1). Remarkably, the thermodynamic parameters for the interaction of NTR_M with either Trx *x* or the isolated Trx_M are equivalent, with similar low K_D values of ca. 50 μM ($K_A \approx 2 \times 10^4 \text{ M}^{-1}$; $\Delta G \approx -24 \text{ kJ mol}^{-1}$; Table 1). A possible explanation for this behaviour is that in NTRC the “Trx substrate” is already bound to the NTR module, and there is no strong selective pressure for a high affinity towards Trxs, although in any case the interaction of NTR_M with Trx *x* and Trx_M is similar. The data also indicate that the interaction of NTR_M with either Trx *x* or the isolated Trx_M is mainly driven by enthalpic interactions, indicating that the NTR module of NTRC might behave as a standard NTR. To test this possibility, we analysed the interaction of the NTRB/Trx *h1* couple, which was previously isolated from wheat [39,40], as an example of standard NTR/Trx system. Interestingly, NTRB/Trx *h1* binding is also mainly driven by enthalpic factors (Table 1), suggesting the occurrence of strong polar specific interactions (electrostatic and hydrogen bonds) between the two partners, in a similar manner to the NTR_M interaction with either Trx *x* or the Trx_M module of NTRC (Table 1). Thus the interaction between plant NTRs and their Trx substrates seems to be mainly determined by electrostatic forces and hydrogen bonds, either when interacting with a free Trx (in NTRB) or with a Trx fused module (in NTRC). Although the structure of NTRC is not yet solved, a modeled 3-D structure of the NTRC dimer has been recently reported [17]. The model shows that the linker region connecting the NTR and Trx domains would allow enough flexibility for the repositioning of the latter, and that extensive interactions involving electrostatic groups

between both domains may stabilize the dimeric NTRC structure [17]. Moreover, the analysis of different 2-Cys Prx mutant variants indicates that the C-terminus of 2-Cys Prx is decisively involved in the interaction with NTRC, and that a variant mimicking the hyperoxidized form shows a reduced interaction [41].

To conclude, our results demonstrate the interaction of 2-Cys Prx with both NTRC and Trx *x in vivo*, as shown by the BiFC experiments, and indicate that these interactions exhibit the moderately low affinity typical of transient complexes, as observed by ITC. In addition, both the NTR and Trx domains of NTRC contribute significantly to the interaction with 2-Cys Prx. Finally, our results support the proposal that 2-Cys Prx can be reduced in the chloroplast by two parallel, and complementary, pathways [18,23], involving either NADPH-dependent reduction by NTRC, or an alternative pathway associated to a ferredoxin-thioredoxin reductase and Trx *x*, and thus dependent of ferredoxin. However, it should be noted that NTRC binds 2-Cys Prx with higher affinity, thus confirming the predominant role of NTRC in the control of the redox status of 2-Cys Prx as previously suggested by the analysis of the *ntrc* and the *trxx* knockout mutants [22].

Abbreviations: BiFC, bimolecular fluorescence complementation; ET, electron transfer; FAD, flavin-adenine-dinucleotide; ITC, isothermal titration calorimetry; K_A and K_D , equilibrium association and dissociation constants, respectively; NTR, NADPH-dependent thioredoxin reductase; NTRB, NADPH-dependent thioredoxin reductase B; NTRC, NADPH-dependent thioredoxin reductase C; NTR_M, Trx_M, truncated NTR and Trx domains of NTRC, respectively; Prx, peroxiredoxin; Trx, thioredoxin; ΔC_p , heat capacity change; ΔG , ΔH , ΔS , free energy, binding enthalpy and entropy values, respectively, for the bimolecular interactions.

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FIGURE LEGENDS

FIGURE 1. Confocal microscopy micrographs of mesophyll cells of *Nicotiana benthamiana* leaves that were agro-infiltrated with the indicated constructs. Leaf sections were analyzed 4 days after infiltration. Red, chlorophyll autofluorescence; yellow, YFP fluorescence.

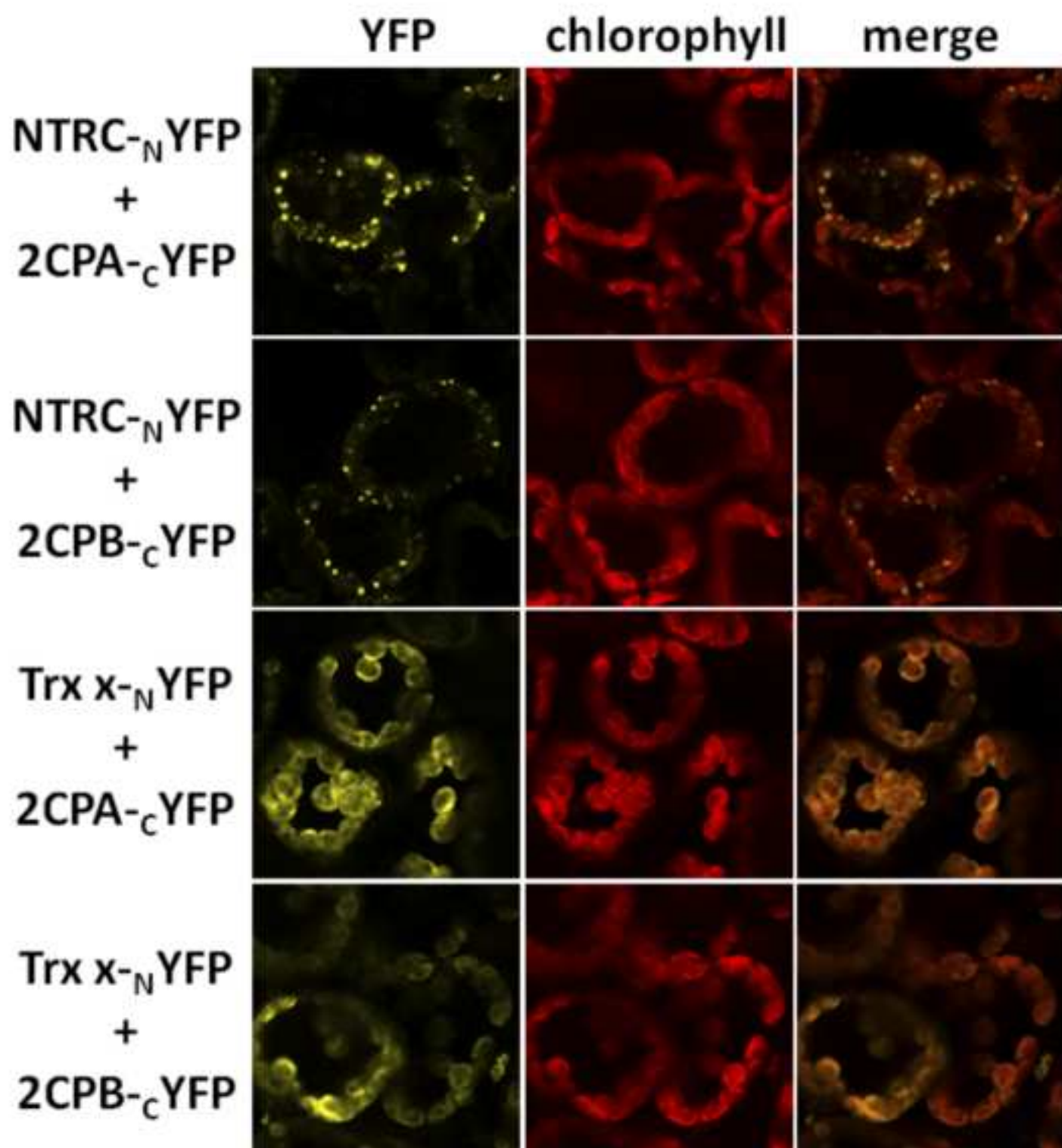
FIGURE 2. (*Left*) Calorimetric titrations for the binary complexes formed between NTRC and 2-Cys Prx or (*inset*) Trx *x*. (*Right*) Calorimetric titration for the binary complex formed between Trx *x* and 2-Cys Prx. (*Upper*) Raw data thermogram of titrations. (*Lower*) Binding isotherm and data fitting to the standard single binding site model with no cooperativity allows an estimation of the binding stoichiometry, the K_A and the binding enthalpy and entropy values for the interaction process. NTRC or Trx *x* solutions (20 μ M in the calorimetric cell) were titrated with successive 2 μ L additions of 300 μ M solutions of the protein partners. Other experimental conditions were as described in Materials and Methods.

FIGURE 3. Calorimetric titrations of the binary complexes formed between the Trx module of NTRC and NTR_M (*left*) or 2-Cys Prx (*right*). (*Upper*) Raw data thermogram of titrations. (*Lower*) Binding isotherm and data fitting to the standard model. Trx_M solutions (20 μ M in the calorimetric cell) were titrated with successive 2 μ L additions of 300 μ M solutions of the protein partners. Other experimental conditions were as described in Figure 2.

Table 1. Affinity constants and thermodynamic parameters for the interaction of the different protein:protein couples as determined by isothermal titration calorimetry.

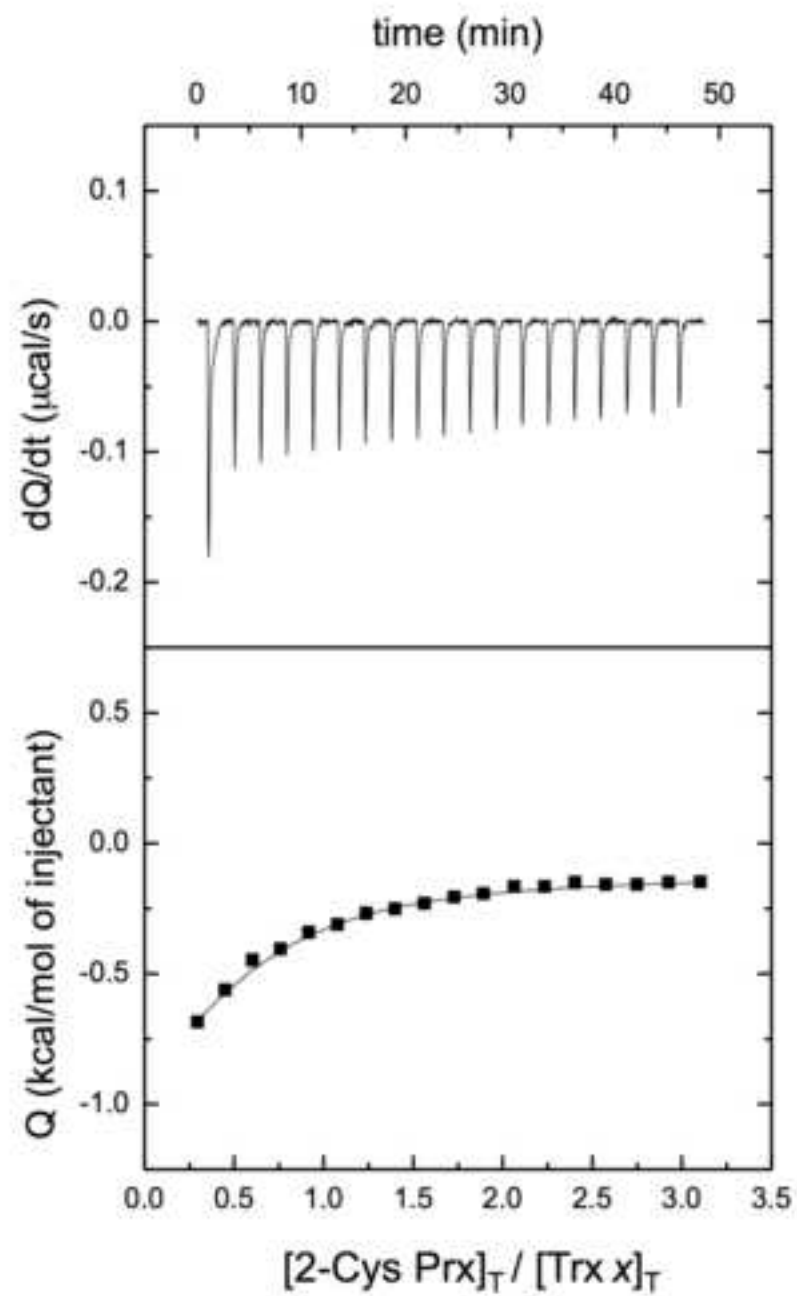
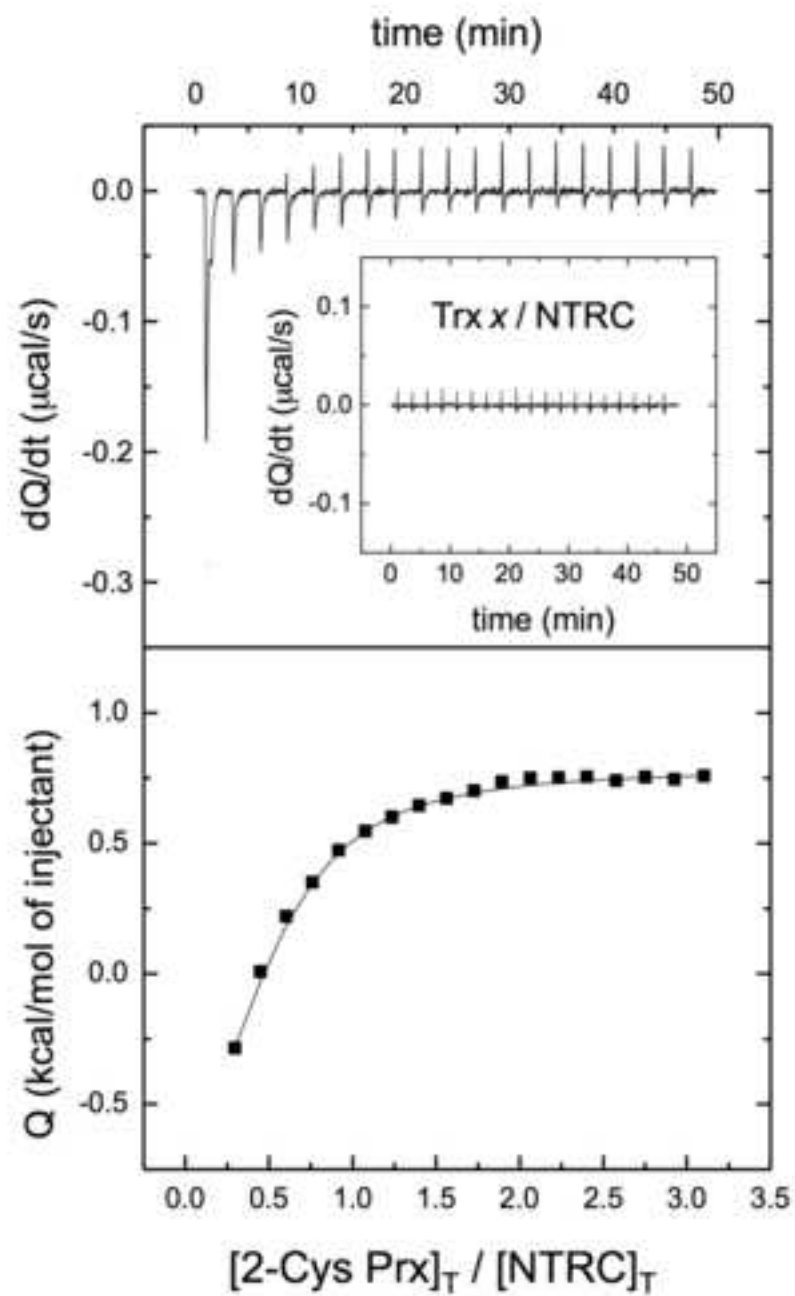
Proteins	K_A^a (M^{-1})	K_D^a (μM)	ΔG^b (kJ/mol)	ΔH^b (kJ/mol)	$-T\Delta S^{b,c}$ (kJ/mol)
NTRC / 2-Cys Prx	1.1×10^5	9	-28.8	-8.7	-20.0
Trx_x / 2-Cys Prx	5.9×10^4	17	-27.2	-5.4	-21.8
NTRC / Trx_x	No interaction	-	-	-	-
Trx_M / 2-Cys Prx	7.1×10^4	14	-27.7	-29.2	+1.5
NTR_M / 2-Cys Prx	7.7×10^4	13	-27.9	-8.3	-19.6
NTR_M / Trx_x	1.9×10^4	53	-24.4	-15.4	-9.0
NTR_M / Trx_M	2.0×10^4	50	-24.6	-23.7	-0.8
NTRB / Trx_{h1}	5.3×10^4	19	-26.1	-18.8	-7.4

^a K_A and K_D , equilibrium association and dissociation constants, respectively; ^b ΔG , ΔH , ΔS , free energy, binding enthalpy and entropy values, respectively, for the bimolecular interactions; ^cT, absolute temperature.



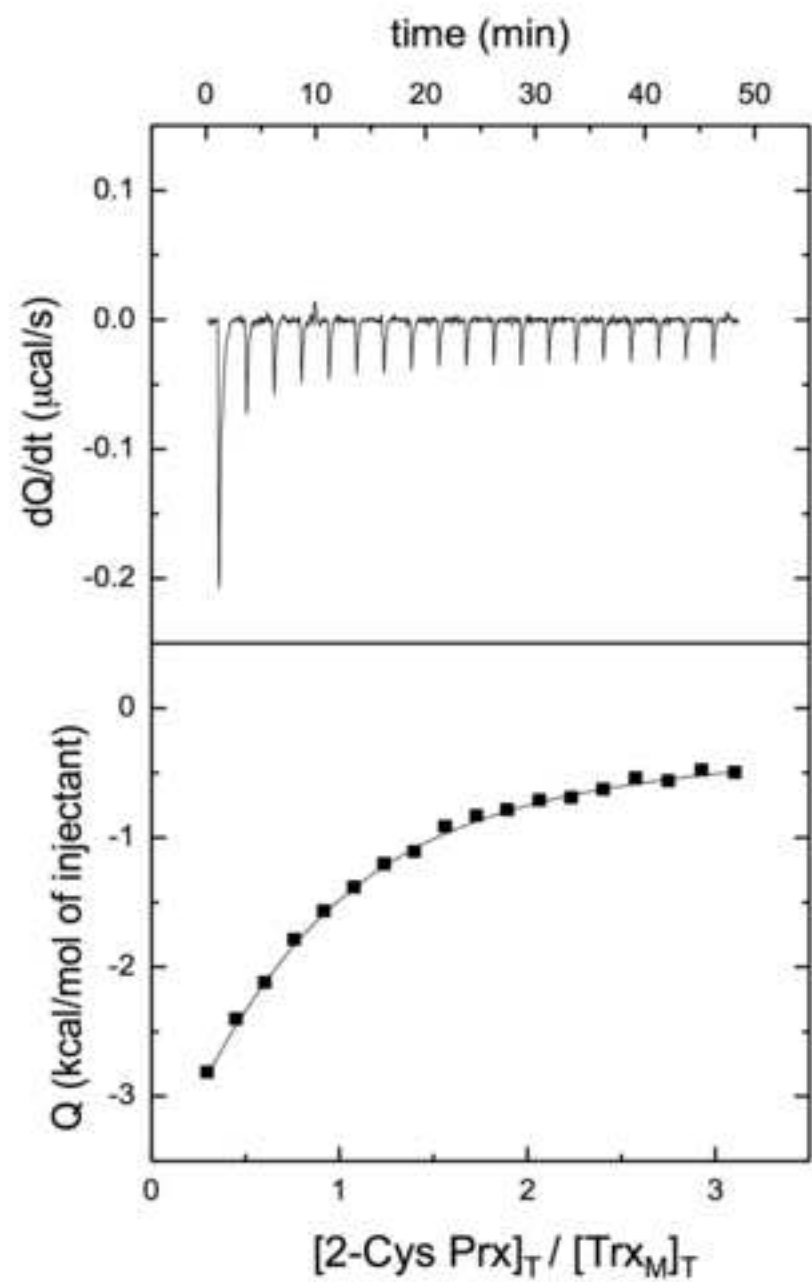
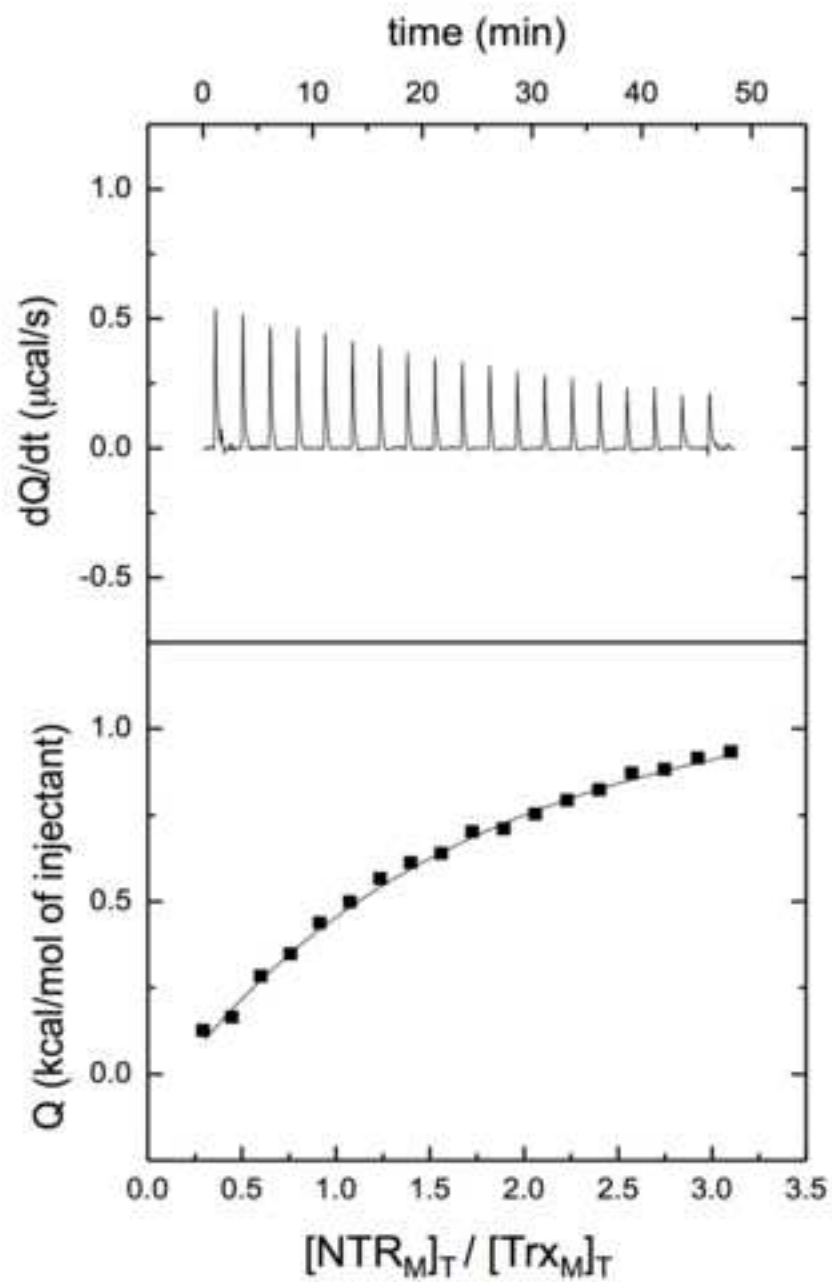
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Figure 2
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Figure 3
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