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The Activity of Barley NADPH-Dependent Thioredoxin Reductase C Is Independent of the Oligomeric State of the Protein: Tetrameric Structure Determined by Cryo-Electron Microscopy

Ragna Peterson Wulff,[†,‡] Joakim Lundqvist,[†] Gudrun Rutsdottir,[†] Andreas Hansson,[§] Anne Stenbaek,[§] Dominika Elmlund,[∥,⊥] Hans Elmlund,[⊥] Poul Erik Jensen,[§] and Mats Hansson*[,†]

[†]Carlsberg Laboratory, Gamle Carlsberg vej 10, DK-1799 Copenhagen V, Denmark

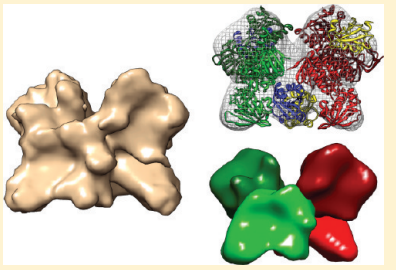
[‡]Department of Biochemistry and Structural Biology, Center of Molecular Protein Science, Lund University, Box 124, SE-22100 Lund, Sweden

[§]VKR Research Centre "Pro-Active Plants", Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, 40 Thorvaldsensvej, DK-1871 Frederiksberg C, Denmark

[∥]Department of Biotechnology, Royal Institute of Technology, Novum, SE-14186 Huddinge, Sweden

[⊥]Department of Structural Biology, Fairchild Building, Stanford University School of Medicine, Stanford, California 94305, United States

ABSTRACT: Thioredoxin and thioredoxin reductase can regulate cell metabolism through redox regulation of disulfide bridges or through removal of H_2O_2. These two enzymatic functions are combined in NADPH-dependent thioredoxin reductase C (NTRC), which contains an N-terminal thioredoxin reductase domain fused with a C-terminal thioredoxin domain. Rice NTRC exists in different oligomeric states, depending on the absence or presence of its NADPH cofactor. It has been suggested that the different oligomeric states may have diverse activity. Thus, the redox status of the chloroplast could influence the oligomeric state of NTRC and thereby its activity. We have characterized the oligomeric states of NTRC from barley (*Hordeum vulgare* L.). This also includes a structural model of the tetrameric NTRC derived from cryo-electron microscopy and single-particle reconstruction. We conclude that the tetrameric NTRC is a dimeric arrangement of two NTRC homodimers. Unlike that of rice NTRC, the quaternary structure of barley NTRC complexes is unaffected by addition of NADPH. The activity of NTRC was tested with two different enzyme assays. The N-terminal part of NTRC was tested in a thioredoxin reductase assay. A peroxide sensitive Mg-protoporphyrin IX monomethyl ester (MPE) cyclase enzyme system of the chlorophyll biosynthetic pathway was used to test the catalytic ability of both the N- and C-terminal parts of NTRC. The different oligomeric assembly states do not exhibit significantly different activities. Thus, it appears that the activities are independent of the oligomeric state of barley NTRC.



Cells adjust enzymatic activities in response to external signals. Thioredoxin and its partner protein NADPH thioredoxin reductase (NTR) can mediate such signals and are part of a growing number of known redox regulatory components.[1,2] Both thioredoxins and NTRs contain the characteristic CXXC amino acid sequence in the active site. Through the cysteine residues of the active site, thioredoxins are able to reduce cysteine residues in target proteins and thereby modulate their activity.[1,3] The target proteins of plant thioredoxins include enzymes involved in the Calvin cycle, ATP synthesis, fatty acid biosynthesis, starch metabolism, and stress-coupled reactions.[2] The tetrapyrrole biosynthetic pathway has also been thought to contain target proteins such as magnesium chelatase, glutamate 1-semialdehyde aminotransferase, and uroporphyrinogen decarboxylase.[1,4,5] Recently, it has been reported that an *Arabidopsis thaliana* thioredoxin−NTR system can reduce the I subunit of magnesium chelatase in vitro, and the ATPase activity of this subunit is increased in the presence of thioredoxin and NTR.[6,7]

NADPH thioredoxin reductase C (NTRC) is a newly discovered protein in plants, green algae, and some cyanobacteria. It contains an N-terminal NTR domain fused with a C-terminal thioredoxin domain.[8] The two domains are separated by a linkage region of ∼35 amino acid residues. Analytical gel filtration of rice (*Oryza sativa*) NTRC showed that the protein formed large oligomeric complexes with a molecular mass of >669 kDa.[9] In the presence of NADPH or DTT, rice NTRC migrated with a mass of 153 ± 13 kDa. Given the theoretical mass of 52.5 kDa and the experimentally determined mass of 66 kDa for the monomeric protein, it was suggested that the 153 kDa NTRC corresponded to a dimer. In light of the observation that NTRC has different oligomeric states in the

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absence or presence of its NADPH cofactor, it was suggested that different oligomeric states may have different activities.[9]

Although NTRC has the characteristic enzymatic activities of NTR and thioredoxin, its specific role is less well understood. 2-Cys peroxiredoxin has been identified as a target protein.[10−13] This suggests that NTRC plays an important role in the extensive and finely balanced scavenging system for reactive oxygen species in chloroplasts.[14,15] *A. thaliana* chloroplasts contain at least four peroxiredoxins.[16,17] 2-Cys peroxiredoxins are antioxidant enzymes of 17−20 kDa, which use a Cys-thiol-based mechanism to reduce peroxides, including H_2O_2.[18,19] NTRC can donate electrons from NADPH to 2-Cys peroxiredoxins.[11,12] In this way, 2-Cys peroxiredoxin can be regenerated. Thus, NTRC could possibly have several different functions that would affect cell metabolism in different ways: via removal of H_2O_2 and via redox regulation of disulfide bridges. We have previously found that NTRC might play a role in the Mg-protoporphyrin IX monomethyl ester (MPE) cyclase enzyme system of the chlorophyll biosynthetic pathway because NTRC in combination with 2-Cys peroxiredoxin can stimulate MPE cyclase activity in vitro, although their exact relation to the MPE cyclase reaction is not clear.[20] Our interest in chloroplast metabolism in general and chlorophyll biosynthesis in particular prompted us to characterize the barley NTRC further. Different oligomeric forms of barley NTRC were observed, which were stable in the presence of NADPH. The oligomeric complexes were tested for thioredoxin activity and their ability to stimulate MPE cyclase activity.

EXPERIMENTAL PROCEDURES

Recombinant Barley NTRC. The *NTRC* gene of barley (*Hordeum vulgare*) was cloned into the pET-15b expression vector (Novagen) to produce a recombinant NTRC protein with an N-terminal His tag. High Fidelity polymerase (Fermentas) was used in combination with primers that would introduce *Nde*I and *Bam*HI restriction sites into the proximal and terminal parts of the amplified *NTRC* gene (5′-TCCCCGCCGCCCATATGTCCAAGGCCCT-3′ and 5′-CCATTACACTTCAAAACCCTGGGATCCACAATGAGCTC-3′), respectively. The resulting plasmid, named pET15bNTRC1, was used to transform *Escherichia coli* BL21 pLysS competent cells and plated onto an LB-agar plates supplemented with 50 mg/L ampicillin and 20 mg/L chloramphenicol. A baffled flask containing 1 L of LB medium (100 mg/L ampicillin and 30 mg/L chloramphenicol) was inoculated to an OD_{600} of 0.1 with fresh transformants from one plate grown overnight at 37 °C. The cells were grown (37 °C and 200 rpm) until an OD_{600} of 0.5 was reached. Isopropyl β-D-thiogalactopyranoside was added until a final concentration of 0.5 mM was reached, and the culture was cooled to 22 °C (130 rpm) and left overnight. Cells were harvested by centrifugation at 5000*g* for 15 min at 4 °C. The cell pellet was washed with 250 mL of binding buffer consisting of 20 mM sodium phosphate, 500 mM NaCl, and 20 mM imidazole and finally suspended in 50 mL of binding buffer supplemented with 10 μM FAD. The cells were disrupted using a cell disrupter system (Constant), divided into 12.5 mL aliquots, and stored at −80 °C until they were used. Aliquots were loaded on HisTrap FF crude columns (GE Healthcare), and NTRC was purified according to the manufacturer's instructions with the exception that 10 μM FAD was added to both the binding buffer and the elution buffer. The protein was eluted in 500 μL fractions. The most yellow fractions were collected and desalted in 30 mM Tris-HCl (pH 8.0) buffer containing 150 mM NaCl and 10 μM FAD using NAP-10 columns (GE Healthcare). Antibodies were produced in rabbits against recombinant barley NTRC.

Gel Filtration of Recombinant NTRC. Samples were loaded on a Superose 6 gel-filtration column (GE Healthcare) and run on an ÄKTA system with a flow rate of 0.4 mL/min. The running buffer consisted of 30 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 10 μM FAD. The elution profile was monitored at 280 nm, and 0.5 mL fractions were collected. The size standard proteins (GE Healthcare) were thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), and conalbumin (75 kDa).

Preparation and Fractionation of Barley Etioplasts. Barley was grown for 8 days at 20 °C in the dark. The top 5−8 cm of the etiolated seedlings was collected. Plastids were isolated as previously described,[20] except that the lysis buffer contained 20 mM Tricine (pH 8), 10 mM HEPES, 1 mM $MgCl_2$, and 1 mM EDTA. All operations were performed under green dim light and generated a crude plastid lysate (termed TP). To eliminate intrinsic protochlorophyllide, NADPH was added to the TP until a final concentration of 1 mM was reached, and the sample was left in room light for 5 min. After this step, all operations could be performed in the light. The TP was centrifuged for 10 min at 13000*g* and 4 °C. Approximately 90% of the supernatant (termed TPS) was collected. The remaining supernatant was discarded. The pellet (TPP) was suspended in lysis buffer and centrifuged again to remove soluble proteins. The TPP fraction was finally suspended in lysis buffer supplemented with 0.25% (w/v) Triton X-100 and left on ice for 10 min. After another round of centrifugation, the supernatant (TPPS) was collected.

Assays for Cyclase and Thioredoxin Reductase Activity. Cyclase activity assays were performed as described previously.[20] MPE was isolated from the *bchE* kanamycin-resistant transposon mutant *Rhodobacter capsulatus* DB575.[21] Thioredoxin reductase activity assays followed the method described in ref 22. Thioredoxin reductase reduces DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] to TNB (5′-thionitrobenzoic acid), which has a yellow color that can be monitored spectrophotometrically as an increase in absorbance at 412 nm. An Infinite M1000 plate reader (Tecan Trading AG) was used to follow the fluorescence in the cyclase assay and the absorbance of the thioredoxin reductase assay.

Sample Preparation and Electron Microscopic Imaging. NTRC was purified via HisTrap chromatography followed by gel filtration using a Superose 6 column. The chromatographic steps were conducted in the presence of FAD but in the absence of NADPH and DTT. NTRC (200 kDa) was diluted with 30 mM Tris-HCl (pH 8.0) to a final concentration of 0.1 mg/mL. The sample was centrifuged for 10 min at 10000*g* and 4 °C to remove contaminating precipitates from the solution. A vitrified specimen for cryo-electron microscopy was prepared by applying 4 μL aliquots of the solution to glow-discharged copper grids coated with a thin holey carbon film (Quantifoil, Jena) that was subsequently plunge-frozen with a Vitrobot environment control unit (FEI) in liquid ethane. Samples were imaged under low-dose conditions using a JEOL 2100F transmission electron microscope equipped with a field-emission gun, operated at an acceleration voltage of 120 kV. Image data were recorded using a TemCam-F415 4K × 4K CCD camera (Tietz Video and Image Processing System) at a magnification of 60000×, giving a sampling distance of 2.5 Å/pixel at the specimen level. Defocusing of the images varied from 0.6 to 8.0 μm to avoid systematic loss of information because of the contrast transfer function.

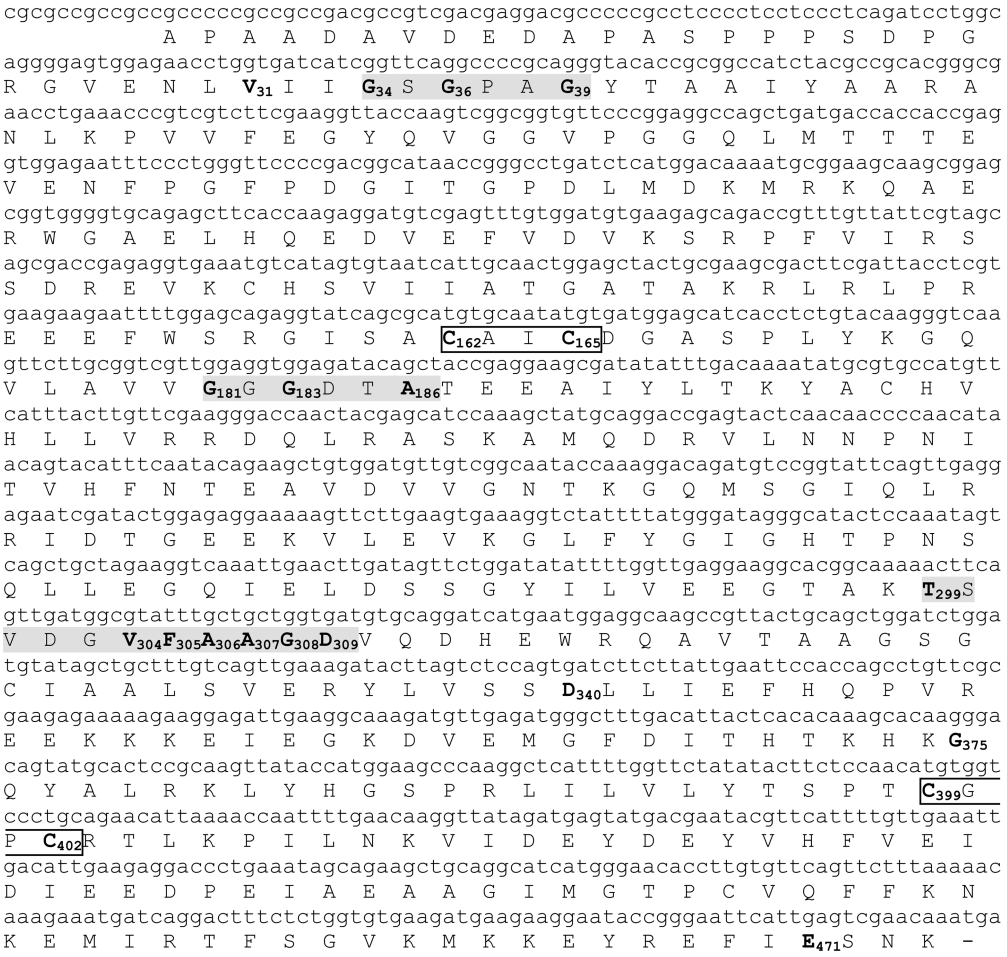

Figure 1. Barley *NTRC* cDNA sequence and deduced polypeptide of the mature protein. The mature NTRC polypeptide of 470 amino acid residues (51614 Da) can be deduced from base pairs 13−1422. NTRC is a fusion protein consisting of an N-terminal NTR domain (residues 31−340) and a C-terminal thioredoxin domain (residues 375−471). The conserved active sites of the NTR domain ($C_{162}XXC_{165}$) and the thioredoxin domain ($C_{399}XXC_{402}$) are boxed. The FAD binding site ($G_{34}XG_{36}XXG_{39}$ and $T_{299}XXXXV_{304}FAAGD_{309}$) and the NADPH binding site ($G_{181}xG_{183}xxA_{186}$) are highlighted in gray. The DNA sequence is GenBank accession number EU360810.

Single-Particle Processing. The contrast transfer function was parametrized individually for each CCD frame in Ctfit[23] by using the averaged power spectrum calculated from the set of 256 × 256 pixel images periodically boxed over the micrograph with a 50% overlap between each image. Contrast transfer function correction was done by applying a Wiener filter with careful up-weighting of high-frequency components to each defocus group using the commands "tf c" and "tf cts" in Spider.[24] In total, 20606 single particles were selected from the 11 CCD images with best contrast in a defocus range of 3.5−8.0 μm using Boxer.[23] These high-contrast particle images were used to generate starting reconstructions in the SIMPLE program package for ab initio reconstruction.[25] Refinement of the initial reconstruction was conducted in EvolAlign[26] using 54230 particles chosen from 20 low-defocus (0.6−3.5 μm) CCD images. Altogether, 34990 particle images were used for calculation of the final reconstruction.

RESULTS

Barley NTRC Sequence. To clone the *NTRC* gene from barley, we used the *A. thaliana* NTRC polypeptide (At2g41680) in a TBLASTX search against the Expressed sequence EST database (http://blast.ncbi.nlm.nih.gov/). The unigen contig Hv_5276 showed the highest level of similarity. Oligonucleotides were designed according to the Hv_5276 consensus sequence and used for polymerase chain reaction (PCR) amplification. First-strand cDNA obtained from total RNA from the barley cultivar Svalöf's Bonus was used as a template. A cDNA fragment of 1686 bp was obtained and sequenced with overlapping reads in both directions (Figure 1). The DNA sequence was deposited in GenBank as accession number EU360810. The cleavage site of the transit peptide was estimated with ChloroP.[27] The mature protein consists of 470 amino acid residues, corresponding to a theoretical molecular mass of 51614 Da. The mature polypeptide is 86% identical to the rice *NTRC* gene product (GenBank accession number AJ582621). Because of the high degree of identity, the two proteins are expected to have very similar properties. The barley sequence showed the unusual NTRC arrangement with an NTR domain in the N-terminal part (amino acid residues V31−D340) and a thioredoxin domain in the C-terminal part (G375−E471) of the same polypeptide. The NTR region includes the conserved cysteine residues $C_{162}XXC_{165}$ in the active site. We also found the sites for binding NADPH ($G_{181}XG_{183}XXA_{186}$) and FAD ($G_{34}XG_{36}XXG_{39}$ and $T_{299}XXXXV_{304}FAAGD_{309}$). NTRC uses NADPH as a reducing agent,

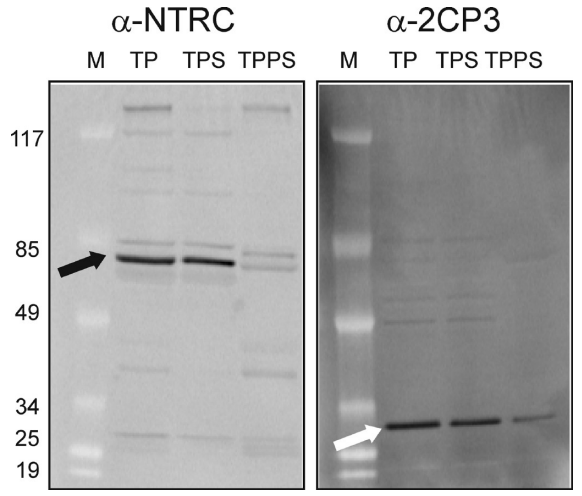

Figure 2. Presence of NTRC (black arrow) and 2-Cys peroxiredoxin (white arrow) in barley etioplast fractions as determined by Western blot analysis. A barley etioplast extract (TP) was centrifuged, and a soluble fraction (TPS) and a pellet-derived fraction (TPPS) were prepared. Five micrograms of protein was loaded in each lane, separated by SDS−PAGE, blotted to a membrane, and detected with rabbit polyclonal antibodies raised against *A. thaliana* 2-Cys peroxiredoxin[16] and barley NTRC. Both proteins were mainly found in the soluble TPS fraction.

and electrons are shuffled via the cofactor FAD to the active site cysteines in the NTR domain, which reduce the disulfide bond in the active site of the thioredoxin domain ($C_{399}XXC_{402}$). The two regions, NTR and thioredoxin, are separated by a linker region consisting of approximately 35 amino acid residues (L341−K374).

NTRC Is a Soluble Protein in Barley Etioplasts. NTRC may constitute an alternative H_2O_2-scavenging system particularly important during the night-time or early stages of plant development, in which reduced ferredoxin is limited by the lack of photosynthetic activity but NADPH can be regenerated via the oxidative pentose phosphate pathway.[12] We therefore analyzed whether NTRC is present in plastids of barley seedlings grown in the dark. A total plastid lysate (named TP) was isolated from etiolated barley seedlings. The lysate was centrifuged, resulting in a supernatant (TPS) and a pellet (TPP). The pellet was subsequently treated with 0.25% Triton X-100 to release bound membrane-associated proteins and centrifuged again to generate a new supernatant (TPPS). Western blot analysis showed that both NTRC and its target protein 2-Cys peroxiredoxin are present as soluble proteins in barley etioplasts, i.e., early in development (Figure 2).

Purification of Recombinant NTRC. A DNA fragment corresponding to that encoding the predicted mature NTRC protein from barley was amplified by PCR and cloned into the pET-15b expression vector carrying an N-terminal His tag sequence (Novagen). The resulting plasmid was named pET15bNTRC1; it allowed expression of the barley *NTRC* gene in *E. coli* BL21(DE3) pLysS. The recombinant protein thus had an N-terminal His tag, which was used in a one-step purification procedure by standard Ni^{2+}-affinity chromatography protocols. The eluted protein was bright yellow, indicating that barley NTRC contained an FAD cofactor. This was in accordance with the DNA sequence analysis, which suggested one FAD binding site. A light absorption spectrum of the

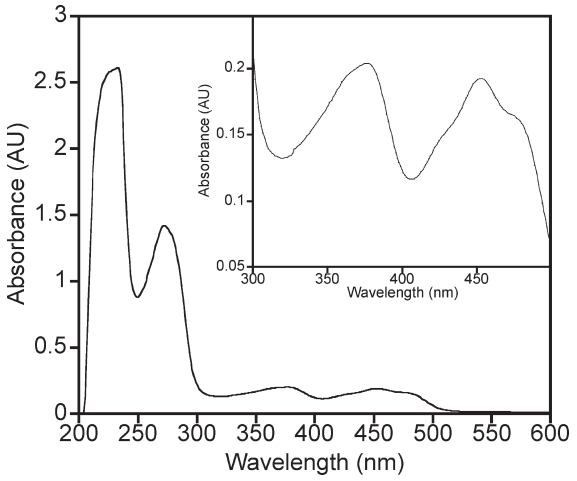

Figure 3. Absorption spectrum of recombinant NTRC from barley. The peaks at 380 and 450 nm show that NTRC is a flavin-containing protein.

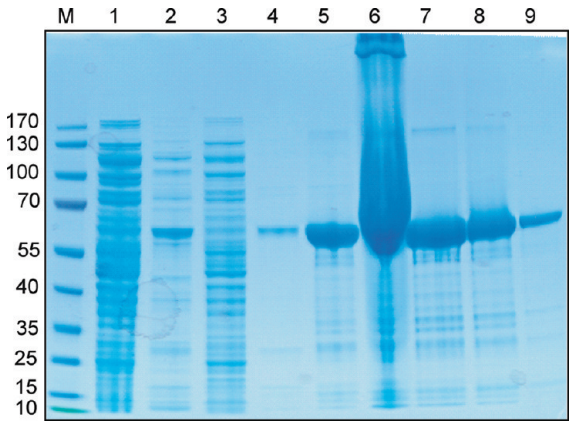

Figure 4. SDS−PAGE analysis of different purification fractions of recombinant NTRC. The His-tagged NTRC from barley was purified on a HisTrap FF crude column and migrated as a 63 kDa protein: lane M, molecular mass markers; lane 1, supernatant of an *E. coli* BL21 pLysS/pET15bNTRC1 lysate; lane 2, wash fraction; lane 3, run-through fraction; lanes 4−9, elution fractions 1−6, respectively.

purified protein showed peaks at 280, 380, and 450 nm and a shoulder at 470 nm (Figure 3). The peaks at 380 and 450 nm are characteristic of flavin-containing proteins.[28]

Determination of the Size of NTRC. The purified recombinant protein migrated as a 63 kDa protein via SDS−PAGE (Figure 4). This was considerably larger than the expected value of 54662 Da deduced from the *NTRC* DNA sequence including the His tag provided by the pET-15b expression vector. It should be noted that rice NTRC, with a theoretical mass of 53 kDa, behaved like a 66 ± 5 kDa protein under similar conditions.[9] Although the 63 kDa form of NTRC predominated in SDS−PAGE gels, there were several other less abundant proteins, especially a protein of 130 kDa that was often clearly visible in the gel preparations (compare lanes 4−9 of Figure 4 with lane N of Figure 5B). Analysis by mass spectrometry showed that they were all derived from NTRC. The NTRC band at 130 kDa is most likely a dimer, although SDS−PAGE was conducted under

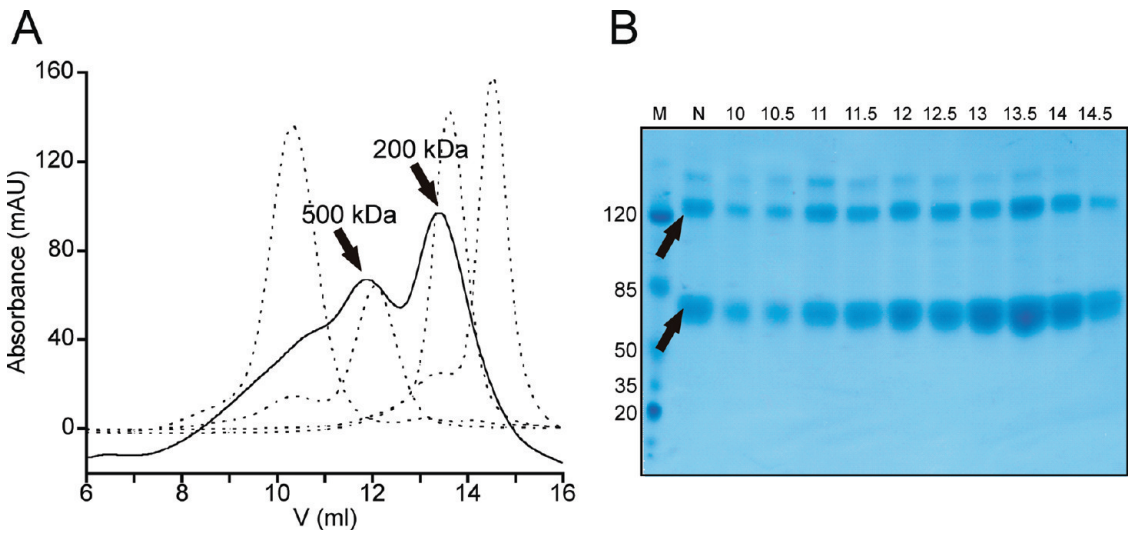

Figure 5. Superose 6 gel-filtration chromatography of purified NTRC from barley. (A) Chromatogram of recombinant NTRC (—) and standard proteins (···). Two peaks (indicated by arrows) appeared at 13.4 and 11.9 mL and correspond to molecular masses of 200 and 500 kDa, respectively. The four size standard proteins, thyroglobulin (10.3 mL, 669 kDa), ferritin (12.0 mL, 440 kDa), aldolase (13.6 mL, 158 kDa), and conalbumin (14.5 mL, 75 kDa), are shown as dotted lines. (B) SDS−PAGE analysis of the NTRC fractions in panel A. SDS−PAGE confirmed that the peaks observed in panel A were from NTRC. The lanes are named after the retention volumes in the chromatogram (A). NTRC appeared as monomers and dimers on the SDS−PAGE gel, although the same denaturing conditions described in the legend of Figure 4 were used. Lane N contained HisTrap-purified NTRC that was loaded on the Superose 6 column. Lane M contained molecular mass markers.

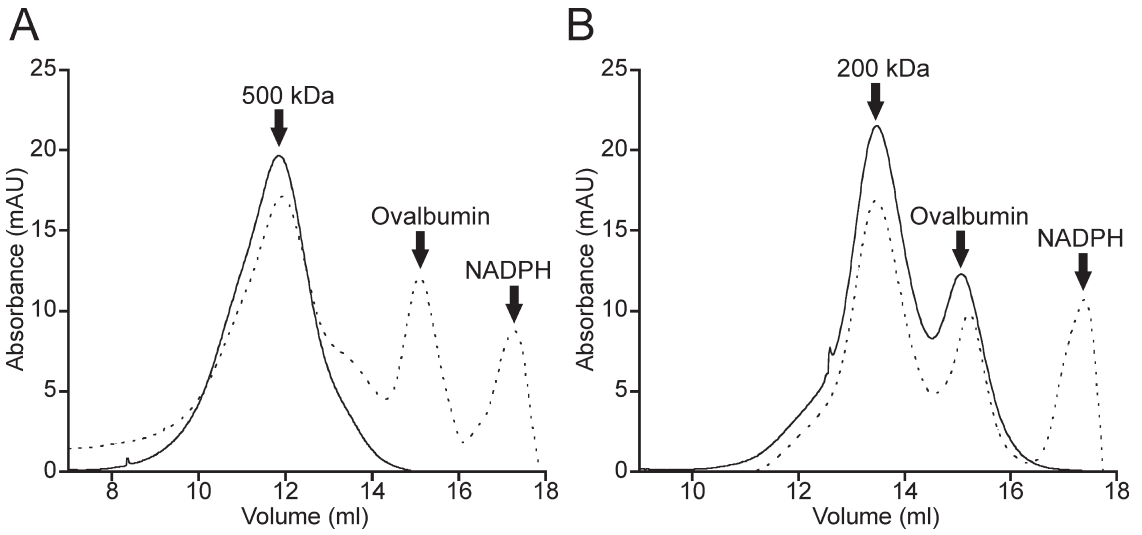

Figure 6. Second round of Superose 6 gel filtration of 500 (A) and 200 kDa NTRC (B) obtained from the experiment shown in Figure 5. One pool was made of fractions that eluted between 11.0 and 12.5 mL (500 kDa peak in Figure 5). The other pool contained protein eluted between 13.0 and 14.5 kDa (200 kDa peak in Figure 5). Each pool was divided into two samples. NADPH was added until a final concentration of 250 μM was reached to one sample of each of the pools and incubated for 90 min. The running buffer used for the analysis of the NADPH-treated samples was supplemented with 250 μM NADPH. The elution profiles of NTRC treated and not treated with NADPH are shown as dotted and solid lines, respectively. Ovalbumin (0.05 mg, 45 kDa, peaks at 15 mL) was used as an internal size standard in three of the four experiments. The equilibrium between the 200 and 500 kDa complexes appeared to be relatively slow, and the isolated oligomeric forms remained in this second round of gel filtration. This was true also in the presence of NADPH (peaks at 17.5 mL).

denaturing conditions in the presence of DTT. The smaller proteins corresponded to C-terminal truncations.

To further determine the oligomeric state of barley NTRC under native conditions, we performed an analytical gel-filtration analysis. Size exclusion chromatography using a Superose 6 gel-filtration column clearly showed that recombinant barley NTRC is a mixture of large oligomers. Two peaks of 200 and 500 kDa were detected (Figure 5A). SDS−PAGE of the eluted fractions confirmed that the observed peaks corresponded to NTRC (Figure 5B). The peak at 200 kDa predominated in the mixture, and it was approximately 3 times greater in magnitude than the peak at 500 kDa. Prediction of the composition of protein complexes via gel-filtration analysis is difficult. However, considering the theoretical mass of 54662 Da for monomeric NTRC

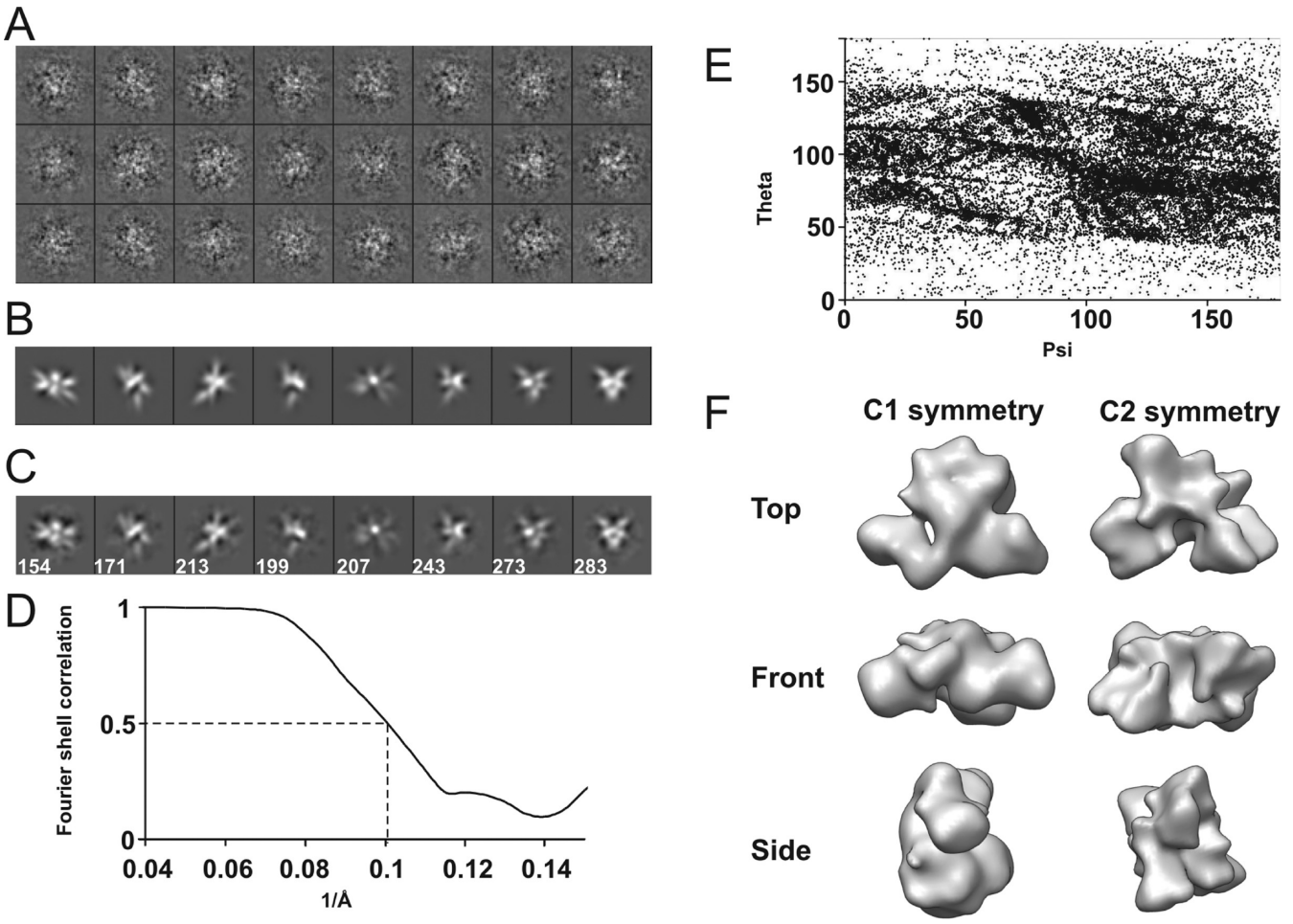

Figure 7. Validation data for the single-particle three-dimensional reconstruction. (A) Example of raw particles used for refinement. (B) Projections of the final reconstruction. (C) Class averages in corresponding orientations with the number of particles indicated. (D) Fourier Shell Correlation plot, with the resolution determined to 10.0 Å according to the Fourier Shell Correlation = 0.5 criterion. (E) Scatter diagram over the two nonazimuthal Euler angles showing that data points are present in the entire Euler angle space. (F) Data for symmetry determination. The left panel shows the asymmetric (*C*1) reconstruction of the NTRC complex. The right panel shows the *C*2 symmetric reconstruction. The similarity between the *C*1 and *C*2 reconstructions indicates that the NTRC complex has 2-fold symmetry.

including the His tag, gel-filtration analysis would suggest that the 200 kDa peak at least corresponds to a trimeric form of NTRC.

We then performed a stability test with the two different assembly forms of barley NTRC. Fractions containing 500 kDa NTRC in the first round of gel filtration were subjected to a second round of gel filtration. The assemblies remained as a 500 kDa complex in the second round, and no pronounced peak corresponding to 200 kDa NTRC was visible (Figure 6A). Similarly, fractions containing the 200 kDa complex in the first round of gel filtration did not show a pronounced peak of the 500 kDa complex in the second round (Figure 6B). Isolated complexes maintained their oligomerization state for at least 1 week on ice, but not for 2 months. It has been shown previously that oligomeric forms of rice NTRC fully disaggregate into smaller units when treated with NADPH (250 μM) or DTT (5 mM) for 3 min.[9] Surprisingly, the barley oligomers were unaffected by a similar treatment. We also included 250 μM NADPH or 5 mM DTT in the running buffer for the analytical gel filtration, but this had no effect on the oligomeric states of barley NTRC. Figure 6 shows the experiment in which the protein had been treated for 90 min with 250 μM NADPH and 250 μM NADPH was included in the running buffer.

Structural Analysis of NTRC. The peak corresponding to 200 kDa in the gel-filtration experiment represented the most abundant oligomeric form of NTRC. This was therefore selected for structure determination using cryo-electron microscopy. The 200 kDa fraction from a chromatography run with FAD present but without any reducing agent was used for preparation of a cryo-specimen as previously described.[29] In total, 34990 single-particle images were collected from 31 CCD images acquired under low-dose conditions (10−15 electrons/Å^2) in a defocus range of 0.6−3.5 μm using a transmission electron microscope equipped with a field-emission gun (Figure 7). To generate a first reconstruction of NTRC, we used an ab initio reconstruction method designed for macromolecules coexisting in different functional states.[26] We attempted to partition the data into two-, three-, and four-state groups, but only a single stable partition could be found, indicating that the NTRC fraction was homogeneous in composition as well as in conformation. No internal symmetry of the NTRC complex had been assumed up to this point. Nevertheless, the asymmetrical reconstruction

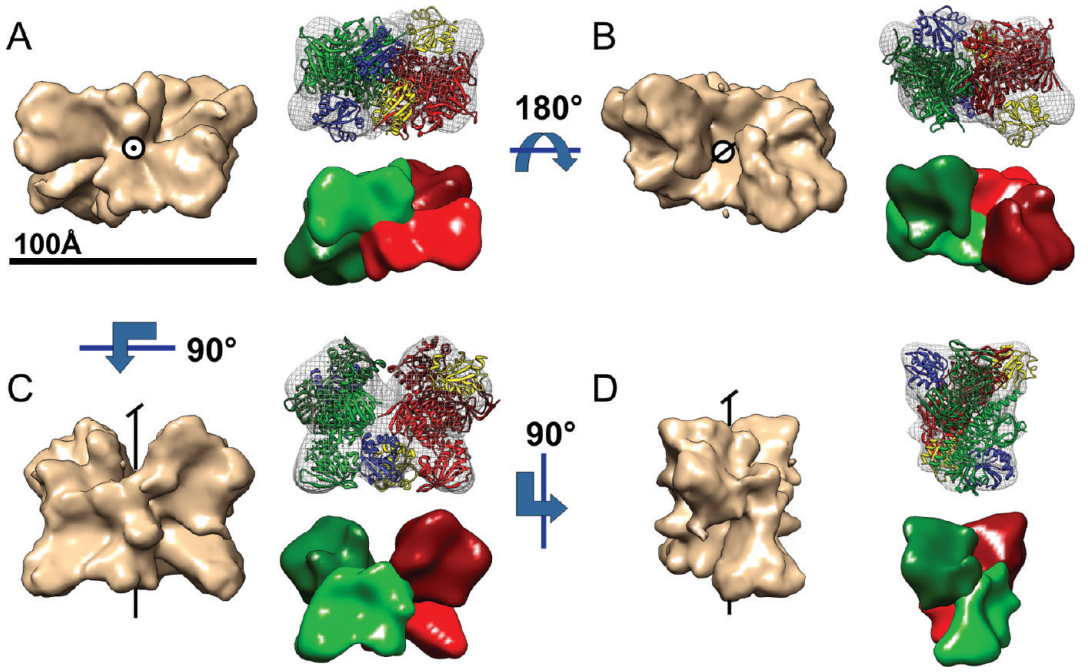

Figure 8. Three-dimensional reconstruction of the 200 kDa NTRC complex as determined by cryo-electron microscopy and single-particle reconstruction (EMDataBank accession code EMD-1857). The reconstitution shows a tetrameric complex formed by two homodimers. The structure has four ear-shaped domains arranged as a dimer of dimers. The two dimers are colored green and red. One monomer within a dimer has a light green or light red color, while the other monomer has a dark green or dark red color. Panels A−D show the structure in four different views. In the top right corners of panels A−D, the crystal structure of a dimeric complex of NTR, thioredoxin, and the $NADP^+$ analogue $AADP^+$ from *E. coli* (Protein Data Bank entry 1f6m) is docked into the density of the electron microscopic reconstruction. In the ribbon representations, the two thioredoxins in each homodimer are colored blue and yellow.

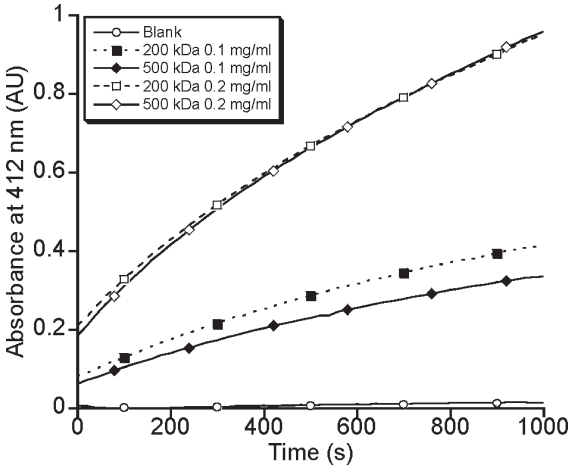

Figure 9. Thioredoxin reductase activity of barley NTRC. The activities of the NTRC forms of 200 and 500 kDa were tested at two different concentrations (0.1 and 0.2 mg/mL). The reduction of DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] to TNB (5′-thionitrobenzoic acid) was followed as an increase in absorbance at 412 nm. No major difference in activity could be detected between the oligomeric forms.

showed clear 2-fold symmetry [*C*1 symmetry (Figure 7F)]. We repeated the ab initio reconstruction procedures assuming *C*2 point group symmetry. The resulting *C*2 symmetric reconstruction was very similar to the asymmetric reconstruction, indicating that the NTRC complexes have 2-fold symmetry (Figure 7F). The *C*2 symmetric reconstruction was used as a starting point for 18 rounds of spectrally self-adaptive orientation refinements[25] of the individual particle images. The resolution of the final density map was assessed by Fourier Shell Correlation to ∼10 Å according to the Fourier Shell Correlation = 0.5 criterion, and the map was low-pass filtered to 10 Å (Figure 7D). We compared projections in eight evenly distributed orientations (Figure 7B) with the corresponding projection averages (Figure 7C), which showed excellent agreement with the projections.

The dimensions of the NTRC complex are 100 and 70 Å in the directions perpendicular to the *C*2 symmetry axis and 60 Å along the axis (Figure 8). The 200 kDa NTRC complex from barley is a tetramer composed of four ear-shaped domains, with two domains placed on each side of the symmetry axis. To facilitate the visual interpretation, we colored the two dimers (green and red volumes in Figure 8). Each monomer of the dimer is highlighted in dark and bright colors. Two domains form a closely entangled pair that crosses the central symmetry axis (light green and light red in Figure 8A). The other two domains point toward the solution (dark green and dark red in Figure 8B). No crystal structure of NTRC is available. We therefore used the dimeric asymmetric unit of the homologous crystal structure of a complex of NTR, thioredoxin, and the $NADP^+$ analogue $AADP^+$ from *E. coli* (Protein Data Bank entry 1f6m) to assign NTRC monomers to the domains identified in the density map (ribbon representations in the top right corners of Figure 8). Docking of the crystal structure was done in both hands of the density map, and the best-fitting hand was selected. One monomer fits into each ear-shaped domain. The dimeric asymmetric unit of the crystal structure fits well into the dimers segmented from the density map (bright and dark red and green in Figure 8),

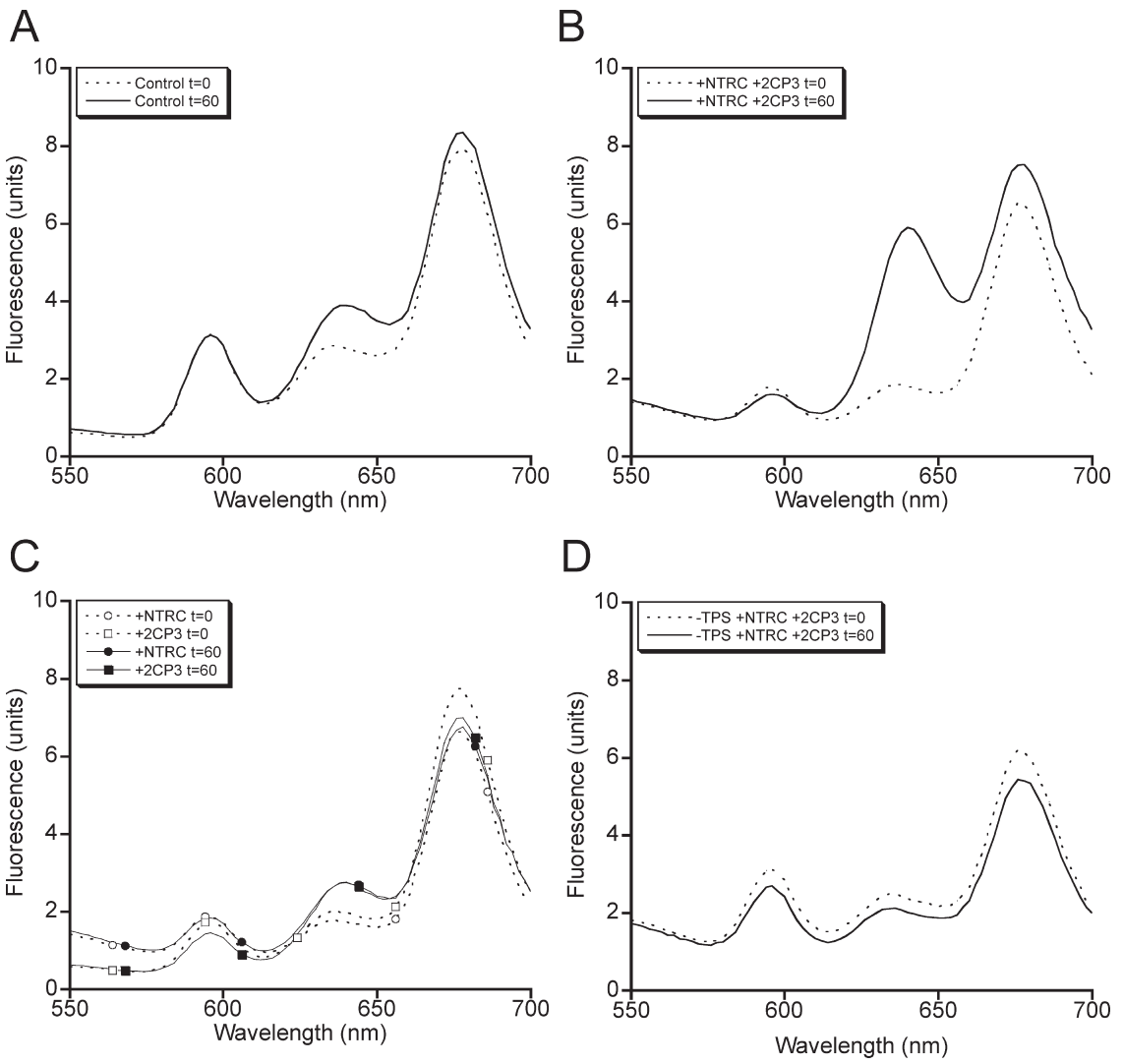

Figure 10. Ability of NTRC to stimulate cyclase activity. Active cyclase components were separated into a soluble fraction (TPS) and a pellet-derived fraction (TPPS). (A) Low cyclase activity could be detected if TPS and TPPS were combined in an assay. (B) The activity could be stimulated 4-fold by addition of NTRC in combination with 2-Cys peroxiredoxin. (C) No stimulation of activity was seen with addition of only NTRC (circles) or 2-Cys peroxiredoxin (squares). (D) The combination of recombinant NTRC and 2-Cys peroxiredoxin could not substitute for TPS in the assay. The product of the cyclase reaction is protochlorophyllide, which has an emission maximum at 640 nm. The MPE substrate peaks at 595 nm. Chlorophyllide associated with the TPPS peaks as 670 nm. The dotted and solid lines show the reactions at 0 and 60 min, respectively.

and only minor structural modifications would be needed for an optimal fit. Thus, our structural reconstitution shows that the 200 kDa NTRC complex is indeed a tetrameric complex formed from two homodimers. The crystal structure mentioned suggests that the monomers within the homodimer are stabilized by hydrophobic interactions between the NTR parts of the complex. Our reconstitution supports this observation and further suggests that the two homodimers within the tetramer mainly interact with surfaces assigned to the larger NTR parts, and to a lesser extent with the smaller thioredoxin parts (Figure 8).

NTRC Oligomers Have Similar Activity. It was previously demonstrated that rice NTRC disaggregates from large oligomeric complexes to dimeric structures in the presence of its NADPH cofactor.[9] Consequently, it was hypothesized that the different oligomeric forms of NTRC might have different activities, which would indicate that the redox status of the chloroplast affects the oligomeric state of NTRC and could thereby provide a mechanism for regulating the activity of NTRC. In contrast to these findings, the barley NTRC remained as a stable tetramer and as a stable 500 kDa complex in the presence of NADPH. We could therefore determine whether the oligomerization state of NTRC had an influence on the activity of the enzyme.

The thioredoxin reductase activity of NTRC was monitored by reduction of DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] into TNB (5′-thionitrobenzoic acid).[22] The formation of TNB can be detected spectrophotometrically as an increase in absorbance at 412 nm. In the assay, we used the same mass of tetrameric and 500 kDa complexes. Fresh fractions were always used. Assuming that both assembly forms have one active site per monomer, the assay demonstrated that the NTRC oligomers have the same level of thioredoxin reductase activity (Figure 9).

To study full chain electron transport through NTRC all the way from NADPH to reduction of disulfide bridges in the thioredoxin part of

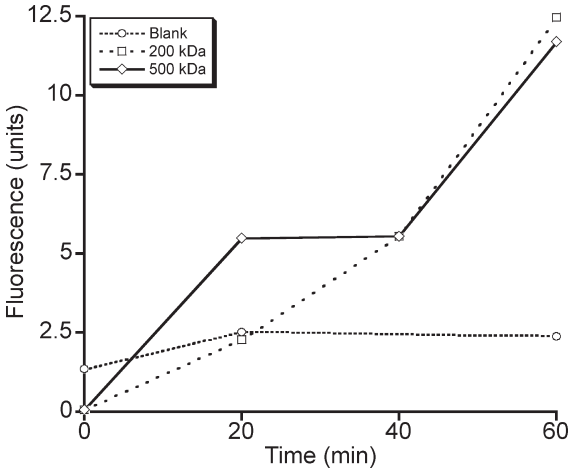

Figure 11. Ability of NTRC of different oligomeric forms to stimulate cyclase activity. NTRC forms of 200 and 500 kDa were isolated by Superose 6 gel-filtration chromatography. The different oligomeric NTRC fractions were used in cyclase activity assays with TPS, TPPS, and 2-Cys peroxiredoxin. The activity is seen as an increase in the fluorescence of the enzymatic product protochlorophyllide at 640 nm, measured between 0 and 60 min. The different oligomeric forms of NTRC stimulated cyclase activity to the same extent. The blank was performed without any NTRC added.

NTRC, we exploited the fact that NTRC in combination with 2-Cys peroxiredoxin stimulates MPE cyclase activity.[20] A soluble fraction (TPS) and a pellet-derived fraction (TPPS) of a barley etioplast lysate were used for the MPE cyclase activity measurements. A combination of TPS and TPPS showed a small but reproducible amount of cyclase activity (Figure 10A). The activity was stimulated 4-fold by supplementation with NTRC and 2-Cys peroxiredoxin (Figure 10B). The same stimulation could be achieved by addition of catalase (data not shown). No stimulation of activity was observed when NTRC or 2-Cys peroxiredoxin was added separately to the assay containing TPS and TPPS (Figure 10C). The combination of recombinant NTRC and 2-Cys peroxiredoxin could not substitute for TPS in the assay (Figure 10D). This suggests that TPS contains a cyclase component that is not identical to NTRC or 2-Cys peroxiredoxin. Combined with 2-Cys peroxiredoxin, the tetrameric and 500 kDa NTRC complexes stimulated the cyclase activity to similar extents (Figure 11).

In conclusion, both the thioredoxin reductase assay and the MPE cyclase assay demonstrate that transformation into different oligomeric forms does not appear to be a mechanism of regulation of barley NTRC.

DISCUSSION

The redox level of the chloroplast varies with the photosynthetic activity, which depends on the availability of light. The redox level is therefore an obvious regulatory mechanism to explore with regard to regulation of chloroplast metabolic pathways, which requires different activities during conditions in light and dark such as that of the chlorophyll biosynthetic pathway.[30] NTRC may be a key player in this process. In this study, we detected NTRC in barley etioplasts. This suggests that NTRC is needed at an early stage of plant development or during the dark period of the day−night cycle. Transcript profiling has shown that an *A. thaliana ntrc* mutant has altered expression of genes involved in chlorophyll biosynthesis (particularly *CHLH* and *HEMA1*), chloroplast biogenesis, stomatal development, and circadian clock-linked light protection systems.[31] Furthermore, *A. thaliana* NTRC by itself can stimulate the ATPase activity of the *Synechocystis* magnesium chelatase I subunit (A. Stenbaek and P. E. Jensen, unpublished results). The overall magnesium chelatase activity was, however, not stimulated, and addition of dithiothreitol (DTT) was still required. NTRC has also been reported to be important in starch anabolism via redox regulation of ADP-glucose pyrophosphorylase.[32] In view of the versatile functions of NTRC, we cloned the *NTRC* gene from barley, expressed it in *E. coli*, and characterized the gene product. Like other NTRC proteins, it contains an FAD cofactor and has thioredoxin reductase activity. Active site cysteines in both the thioredoxin and the NTR part of the barley protein are fully conserved. Both the thioredoxin and the NTR part of barley NTRC are active as demonstrated by stimulation of MPE cyclase activity when NTRC is combined with 2-Cys peroxiredoxin. Rice NTRC has been shown to form large oligomeric complexes with a molecular mass of >669 kDa. The rice complexes break into 153 kDa units in the presence of NADPH or DTT.[9] These observations suggest that the redox status of the chloroplast could influence the aggregation state of NTRC, which may provide a mechanism for regulation of NTRC activity in vivo. The heterologously produced barley NTRC generates a mixture of two dominating oligomers, 200 and 500 kDa in size. The 200 kDa complex predominates in the recombinant NTRC preparation. By repeated gel-filtration chromatography of the isolated assemblies, we have shown that the NTRC oligomers form stable complexes. In addition, the oligomers are unaffected by addition of NADPH or DTT, and the ratios between the oligomers are also unaffected when NADPH or DTT is present in the running buffer. This contrasts with the corresponding results using the NTRC oligomer from rice, which disassembles into 153 kDa units (supposedly dimers) upon treatment with NADPH or DTT.[9] Because of the uncertainty of the structural composition of the barley NTRC, we performed a structural analysis of the complex by cryo-electron microscopy and single-particle reconstruction. The predominant peak of 200 kDa from gel filtration was selected for analysis. The reconstructed volume showed a dimeric arrangement of two NTRC homodimers into a tetrameric structure. The four characteristic ear-shaped domains were identified as NTRC monomers by docking of the related structure of NTR in complex with thioredoxin. We note the slow transition between the tetramer and the 500 kDa complex but suggest that structural analysis of the 500 kDa complex would be required to determine whether the transition involves large structural rearrangements of homodimers or a plain docking of two tetramers into an octamer. An assay of the 200 and 500 kDa NTRC complexes from barley for thioredoxin reductase activity and MPE cyclase activity did not reveal any pronounced differences in activity between the different assembly forms. It should be noted that the thioredoxin reductase assay involves only the NTRC protein, whereas the MPE cyclase assay is a much more complex reaction involving crude plastid extracts. However, the MPE cyclase assay reveals the functional activity of the entire NTRC protein, from accepting electrons from NADPH to reductions of disulfide bridges in the thioredoxin part of the protein. We conclude that the activities are independent of the oligomeric state of barley NTRC, at least in vitro, and that the aggregation state of barley NTRC is unlikely to provide a mechanism for regulation.

Accession Codes

The barley *NTRC* DNA sequence was assigned GenBank accession number EU360810. The map of the tetrameric barley NTRC protein complex has been deposited in the EMDataBank under accession code EMD-1857.

AUTHOR INFORMATION

Corresponding Author

*Carlsberg Laboratory, Gamle Carlsberg vej 10, DK-1799 Copenhagen V, Denmark. Fax: +4533274708. Telephone: +4533275299. E-mail: mats.hansson@carlsberglab.dk.

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ABBREVIATIONS

DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MPE, Mg-protoporphyrin IX monomethyl ester; NTR, NADPH thioredoxin reductase; NTRC, NADPH-dependent thioredoxin reductase C; TNB, 5′-thionitrobenzoic acid; TP, total plastid extract; TPP, pellet of TP; TPPS, supernatant obtained from Triton X-100 treatment of TPP; TPS, supernatant of TP; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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