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ORIGINAL RESEARCH

NDH-1L with a truncated NdhM subunit is unstable under stress conditions in cyanobacteria

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Abstract

Cyanobacterial NdhM, an oxygenic photosynthesis-specific NDH-1 subunit, has been found to be essential for the formation of a large complex of NDH-1 (NDH-1L). The cryo-electron microscopic (cryo-EM) structure of NdhM from *Thermosynechococcus elongatus* showed that the N-terminus of NdhM contains three β-sheets, while two α-helixes are present in the middle and C-terminal part of NdhM. Here, we obtained a mutant of the unicellular cyanobacterium *Synechocystis* 6803 expressing a C-terminal truncated NdhM subunit designated NdhMΔC. Accumulation and activity of NDH-1 were not affected in NdhMΔC under normal growth conditions. However, the NDH-1 complex with truncated NdhM is unstable under stress. Immunoblot analyses showed that the assembly process of the cyanobacterial NDH-1L hydrophilic arm was not affected in the NdhMΔC mutant even under high temperature. Thus, our results indicate that NdhM can bind to the NDH-1 complex without its C-terminal α-helix, but the interaction is weakened. NDH-1L with truncated NdhM is more prone to dissociation, and this is particularly evident under stress conditions.

KEYWORDS
cyanobacteria, environmental stress, NDH-1, NdhM

1 | INTRODUCTION

NDH-1 (also referred to as complex I) is an energy-converting oxidoreductase that accepts electrons from NADH or ferredoxin (Fd) and is found in a majority of species from bacteria to mammals. During the evolution from bacteria to mammals, the NDH-1 complex has conserved an L-shaped skeleton and shares a common physiological function, respiration (Laughlin et al., 2020; Peltier et al., 2016). In cyanobacteria, this conserved L-shaped NDH-1 is a large complex (NDH-1L), which is also involved in cyclic electron transport around photosystem I (PSI-CET) (Bernát et al., 2011; Mi, 2022). Recently, the structure of the NDH-1L complex from the cyanobacterium *Thermosynechococcus elongatus* was resolved by several groups by cryo-electron microscopy (cryo-EM) and revealed that the entire NDH-1L consists of 19 subunits (Laughlin et al., 2019; Pan et al., 2020; Schuller et al., 2019; Zhang et al., 2020). Among them, 11 subunits including NdhA-NdhK are conserved among all the NDH-1 complexes from various species. A total of eight subunits, NdhL to NdhQ, NdhS and NdhV, specific to oxygenic photosynthesis (OPS) have been identified in the cyanobacterial NDH-1L complex (Battchikova et al., 2011; Gao, Zhao, Wang, et al., 2016; Ma & Ogawa, 2015; Nowaczyk et al., 2011; Ogawa, 1992; Prommeenate et al., 2004; Wulfhorst et al., 2014; Zhang et al., 2014; Zhao et al., 2014, 2015). While NdhV, NdhS, and NdhO subunits are close to the

Fd-binding site, NdhL is adjacent to the plastoquinone (PQ) cavity. They are involved in different steps of electron transport in PSI CET (cyclic electron transport around PSI). NdhP and NdhQ are subunits with a single transmembrane domain. These two subunits, together with several cofactors such as β-carotene, dipalmitoylphosphatidylglycerol (LHG), sulfoquinovosyldiacylglycerol (SQD), and digalactosyldiacylglycerol (DGD), are required for stabilization of the membrane-spanning domain of the NDH-1 complex (Laughlin et al., 2019; Pan et al., 2020; Schuller et al., 2019; Zhang et al., 2020).

NdhM is an oxygenic photosynthesis-specific NDH-1 subunit, and its deletion leads to a complete collapse of conserved hydrophilic subunits of the NDH-1 in both cyanobacteria and higher plant chloroplasts (He et al., 2016; Shikanai, 2016). The cryo-EM structure of NDH-1L from the cyanobacterium *Thermosynechococcus elongates* and the NDH complex from the higher plant *Hordeum vulgare* show that the N-terminus of NdhM contains mainly three β-sheets while two α-helixes are present in the middle and C-terminal part of NdhM (Shen et al., 2022; Zhang et al., 2020). In Arabidopsis, the absence of NdhM leads to a drastic reduction of NdhK in the chloroplast stroma and impedes the assembly of NdhK (Shikanai, 2016). Vice versa, incorporation of NdhM into assembly intermediates is impaired in the absence of NdhK in the *crr9* mutant (Yamamoto et al., 2016). Thus, NdhM is likely involved in the assembly of NdhK during the biogenesis of the NDH complex. After being assembled, NdhM associates with NdhK mainly through the β-sheets of its N-terminal fragment in the hydrophilic arm of the NDH-1 complex but the role of NdhM in the intact NDH-1 complex is unclear (Laughlin et al., 2019; Pan et al., 2020; Schuller et al., 2019; Zhang et al., 2020).

During the construction of the NDH-1 subunit mutants in cyanobacterium *Synechocystis* 6803, we found, in our great surprise, a mutant expressing normal levels of NDH-1L with a C-terminal truncated NdhM subunit under normal growth conditions (NdhMΔC). Thus, this mutant enables us to study the function of NdhM in the intact NDH-1 complex in cyanobacteria. We showed that NDH-1L with truncated NdhM is unstable under high temperature, high light, and high salt conditions. Moreover, the rate of degradation of NdhK1 in NDH-1L of NdhMΔC is much higher than that of other NDH-1L subunits such as NdhH and NdhI under heat stress. Based on these results, we propose that NdhM in the NDH-1L complex is important for the stabilization of NdhK1 under stress conditions.

2 | RESULTS

2.1 | A truncated NdhM subunit accumulates stably in the NdhMΔC mutant

NdhM, a 14-kDa OPS NDH-1 subunit, is necessary for stabilization of the NDH-1L complex even under normal growth conditions (He et al., 2016). To investigate the functional role of NdhM in *Synechocystis* 6803 further, we replaced the *ndhM* coding region using a spectinomycin resistance marker (Sp^R; Figure 1a). PCR analysis of the *ndhM*

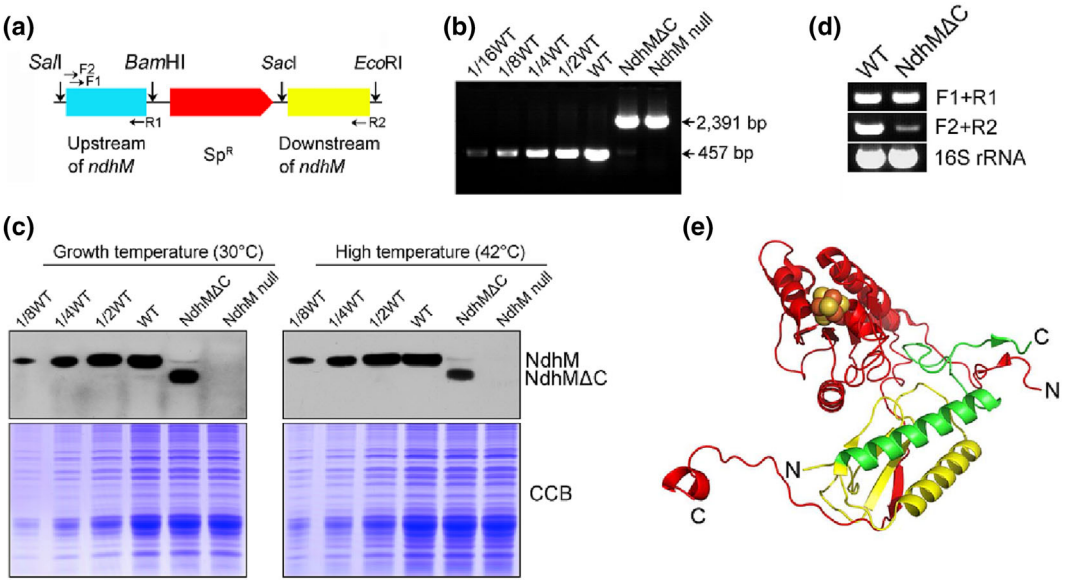

FIGURE 1 Construction and identification of the NdhMΔC and NdhM null mutants. (a) Construction of plasmid (pUC-Δ*ndhM*) used to generate the NdhMΔC and NdhM null mutants. F1/R1 and F2/R2, two pair of primers used for RT-PCR in (d). (b) PCR segregation analysis of the NdhMΔC and NdhM null mutants using the *ndhM*-G and *ndhM*-H primer sequences (Table S2). (c) Immunoblot analysis of NdhM from thylakoid membrane proteins of the WT, NdhMΔC and NdhM null strains grown under conditions of growth temperature (30°C) and high temperature (42°C). Thylakoid membrane proteins corresponding to 1 μg of chlorophyll *a* (100%) were loaded onto each lane. (d) Transcripts levels of NdhM in WT and NdhMΔC mutant. RT-PCR analysis was performed using the F1, R1, F2, and R2 primers indicated in (a). The transcript level of *16 S ribosomal RNA* (*rRNA*) in each sample is shown as a control. (e) Tertiary structure of NdhM and NdhK of the cyanobacterium *Thermosynechococcus elongatus* (PDB ID: 6L7O). NdhK1 subunit is indicated in red color. N-terminal and C-terminal parts of NdhM are shown in yellow and green, respectively. The C-terminal part of NdhM in green is predicated missing in the NdhMΔC mutant.

locus showed an almost complete segregation (more than 90%) of the NdhMΔC mutant allele and a complete segregation of the NdhM null mutant (Figure 1b). Immunoblot analysis using an antibody specifically against intact NdhM demonstrated that the level of intact NdhM in the NdhMΔC strain is reduced to less than 10% of WT cells (Figure 1c). No NdhM was detected in the NdhM null mutant, which is consistent with the complete segregation of the NdhM gene in this strain (Figure 1b,c).

It is noticeable that the antibody against NdhM cross-reacted with a low MW band (hereafter referred to as NdhMΔC) in the NdhMΔC mutant under both growth and high temperature conditions (Figure 1c). Because this band was well recognized by the NdhM antibody, it is likely that the corresponding protein contains at least part of NdhM. To investigate this possibility, RT-PCR analyses were performed using two set of primers, which can amplify the upstream (F1 + R1) and whole length (F2 + R2) of the NdhM gene, respectively (Figure 1d). The results showed that the upstream region of the NdhM gene is normally expressed in the NdhMΔC mutant in comparison with WT cells. In contrast, a very weak signal corresponding to the whole length of NdhM gene was detected in this strain (Figure 1d), consistent with the trace amount of intact NdhM accumulated in the mutant (Figure 1c). Analyses of the sequence of pUC-Δ*ndhM* vector suggest that the main band detected by the NdhM antibody in the NdhMΔC mutant contains the N-terminal part of NdhM and additional nine amino acids translated from the vector (Table S1). From the RT-PCR results and the tertiary structure of the entire NdhM of the cyanobacterium *Thermosynechococcus elongatus* (PDB ID: 6L7O; Pan et al., 2020; Zhang et al., 2020), we found that the C-terminal part of NdhM containing one α-helix was missing in the NdhMΔC mutant (Figure 1e). The remaining N-terminal part of NdhM contains three β-sheets and one α-helix and this part is the main region for the interaction with NdhK1. By contrast, only a short region at the C-terminus of NdhM directly interacts with NdhK1 (Figure 1e).

2.2 | NDH-1L and NDH-1M stably accumulate in the NdhMΔC mutant under normal temperature conditions

In *Synechocystis* 6803, NDH-1L and NDH-1M are two typical NDH-1 types. As observed from the blue native (BN)-PAGE gel of WT thylakoid membranes (Herranen et al., 2004; Prommeenate et al., 2004), both of them are completely absent in the NdhM null mutant (Figure 2a), consistent with previously reported results (He et al., 2016). Unexpectedly, NdhMΔC cells accumulates identical level of the NDH-1L in the thylakoid membranes under growth temperature, as deduced from the abundance of NdhH, NdhI, and NdhK1 (Figure 2a,c). Because only a trace amount of intact MdhM is present in the NdhMΔC cells (Figure 1c), its NDH-1L complex should mainly contain the truncated NdhM. These results indicate that the C-terminus of NdhM is not essential for the stabilization of the two NDH-1 types in the thylakoid membranes of *Synechocystis* 6803 under normal growth conditions. This hypothesis is also supported by structural analyses of NdhM showing that the N-terminal part of NdhM is the main region for the interaction with NdhK1 (Figure 1e).

Thylakoid protein complexes of WT (including indicated serial dilutions) and mutants grown at either 30°C (a) or 42°C (b) were separated by BN-PAGE. Thylakoid membrane extracts corresponding to 9-μg chlorophyll *a* were loaded onto each lane. Red and blue arrows indicate the positions of the NDH-1L and NDH-1M complexes, respectively. Protein complexes separated by BN-PAGE were electroblotted to a polyvinylidene difluoride (PVDF) membrane and were cross-reacted with anti-NdhH, -NdhI, and -NdhK1 antibodies. (c) Signals obtained for NdhH, NdhI, and NdhK1 from NDH-1L in (a) and (b) were quantified from two independent biological repeats and the relative amounts of each subunit were indicated.

2.3 | Intact NdhM is critical for significant increase of NDH-1 activity in response to heat stress

To determine whether the NDH-1 complex with truncated NdhM has NDH-1 activity, we monitored the post-illumination rise in chlorophyll fluorescence during the transfer of WT and NdhMΔC cells from 30°C (growth temperature) to 42°C. As a negative control, the *ΔndhK1* mutant with very low NDH-1 activity was analyzed (Figure 3). The postillumination rise in chlorophyll fluorescence reflects the PQ reduction by cytosolic NAD(P)H, Fd, and other reducing substances through the PSI-CET. Both the slope of the rise and the maximum fluorescence value after the rise correspond to the amount of NDH-1 and thus can be used to indirectly monitor the NDH-1 activity in

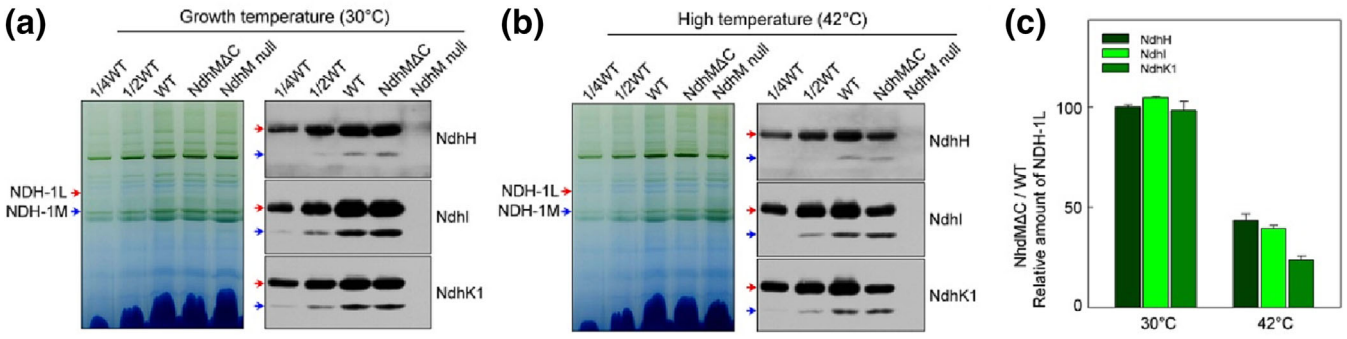

FIGURE 2 Immunoblot analyses of NDH-1L and its degradation product NDH-1M from the WT, NdhMΔC, and NdhM null cells.

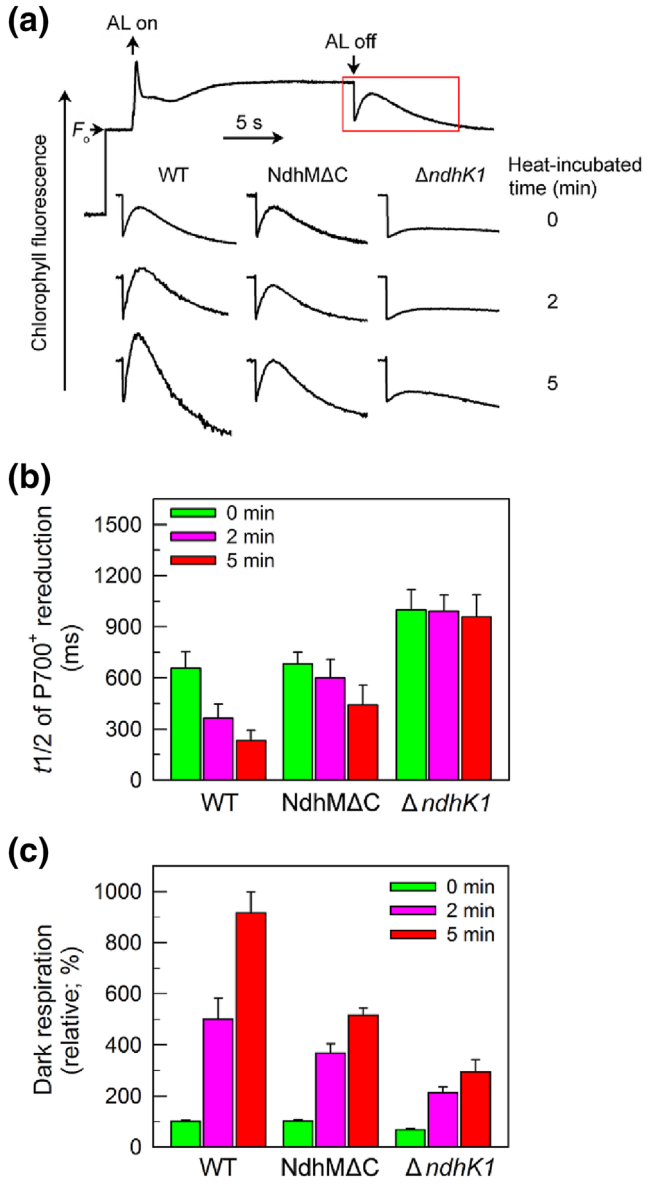

FIGURE 3 Effect of heat stress period on the activity of NDH-1L in WT, NdhMΔC, and Δ*ndhK1* cells. (a) Top curve shows a typical time course of chlorophyll fluorescence in the WT *Synechocystis* 6803. After incubation at 42°C for 0, 2 and 5 min, the cells were immediately exposed to actinic light (AL; 620 nm; 45 µmol photons m^{-2} s^{-1}) for 30 s. AL was turned off, and the subsequent change in chlorophyll fluorescence level was monitored as a measure of NDH-1 activity. The *NdhK1* gene (*Slr1280*) was detected in the Δ*ndhK1* mutants. (b) Half-time of $P700^+$ re-reduction in darkness after actinic light illumination of the WT, NdhMΔC, and Δ*ndhK1* cells incubated at 42°C for 0, 2 and 5 min. Prior to the measurement, 10 µM DCMU was added to the cell suspension. Values are means ± SD ($n = 8$). (c) Dark respiration of the WT, NdhMΔC, and Δ*ndhK1* cells incubated at 42°C for 0, 2 and 5 min. The rate of O_2 uptake was monitored in the dark at 30°C. The value of respiration in untreated WT cells (42°C for 0 min) was 22.3 ± 1.6 µmol O_2 mg^{-1} chlorophyll *a* h^{-1} and was taken as 100%. Values are means ± SD ($n = 5$).

cyanobacteria (Figure 3a; Asada et al., 1993; Mi et al., 1995; Ma & Mi, 2005). We found that the amplitude of the post-illumination rise in chlorophyll fluorescence is comparable in the NdhMΔC and WT cells (Figure 3a), indicating that the C-terminus of NdhM is not essential for the NDH-1 activity under normal growth temperature conditions. However, upon transfer of the cells to 42°C, the height of postillumination rise increased significantly in the WT but the increasing amplitude was markedly suppressed in the NdhMΔC (Figure 3a). As expected, in the *ΔndhK1* mutant, chlorophyll fluorescence was only slightly increased after illumination and heat treatment did not lead to a significant increase in chlorophyll fluorescence as in WT (Figure 3a), suggesting that the increasing amplitude in chlorophyll fluorescence during heat treatment reflects enhanced NDH-1 activity in WT. In the NdhMΔC mutant, the increasing amplitude of fluorescence was suppressed indicating that the increasing of NDH-1 activity is suppressed upon exposure of cells to heat stress.

Next, we measured the activities of cyclic electron transport around photosystem I (PSI CET) and dark respiration during the transfer of WT, NdhMΔC, and *ΔndhK1* cells from growth temperature to the higher temperature. NDH-1 complex donates electron from Fd in stroma or cytosol to PQ in thylakoids. Then the electrons transfers to PSI via Cyt b_6f complex, resulting in a re-reduction of $P700^+$. Thus, the half-time of $P700^+$ re-reduction can be used for evaluation of the activity of PSI CET in the absence of electron transport from PSII. In addition, the NDH-1 complex in cyanobacteria also participates in respiration in the dark. As expected, with longer exposure to high temperature, the activities of PSI CET and respiration gradually and significantly increased in the WT but the increase was evidently suppressed in the NdhMΔC mutant (Figure 3b,c). In the *ΔndhK1* cells, the half-time of $P700^+$ re-reduction was almost unchanged and the increasing in respiration is not as much as in the WT and NdhMΔC cells during heat treatment (Figure 3b,c). Collectively, these results indicate that the C-terminus of NdhM is essential for increasing effectively the activity of NDH-1 upon exposure of cells to heat stress.

2.4 | NDH-1L complex with truncated NdhM is unstable under stress conditions

To investigate whether NDH-1 complexes with truncated NdhM are stable under conditions of heat stress, we compared the levels of NDH-1L in WT, NdhMΔC, and NdhM null cells grown at 42°C (Figure 2b,c). The results indicate that the amount of NDH-1L complex is reduced to more than half of the WT levels in the NdhMΔC mutant and completely absent in the NdhM null cells (Figure 2b,c). We thus conclude that under heat stress conditions, the C-terminus of NdhM is necessary for stabilization of the NDH-1L complex in the thylakoid membranes. We also investigated the stability of NDH-1 under high light and high salt conditions. The results showed that accumulation of NDH-1L complex in the NdhMΔC mutant is reduced to about half of the WT levels under conditions of high light and high salt (Figure 4).

The cells cultured in BG-11 medium were treated with high light (200 µmol photons m^{-2} s^{-1}) (a) and high salt (0.8 M; final concentration) (b) for 24 h. Thylakoid protein complexes corresponding to 9-µg

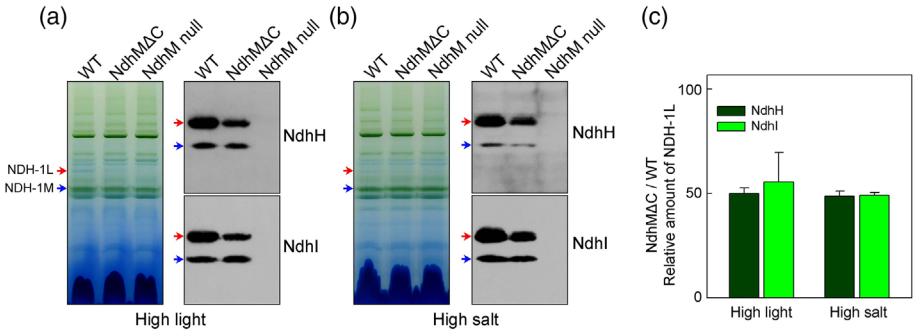

FIGURE 4 Accumulation of NDH-1L from the WT, NdhMΔC, and NdhM null cells grown under stress conditions.

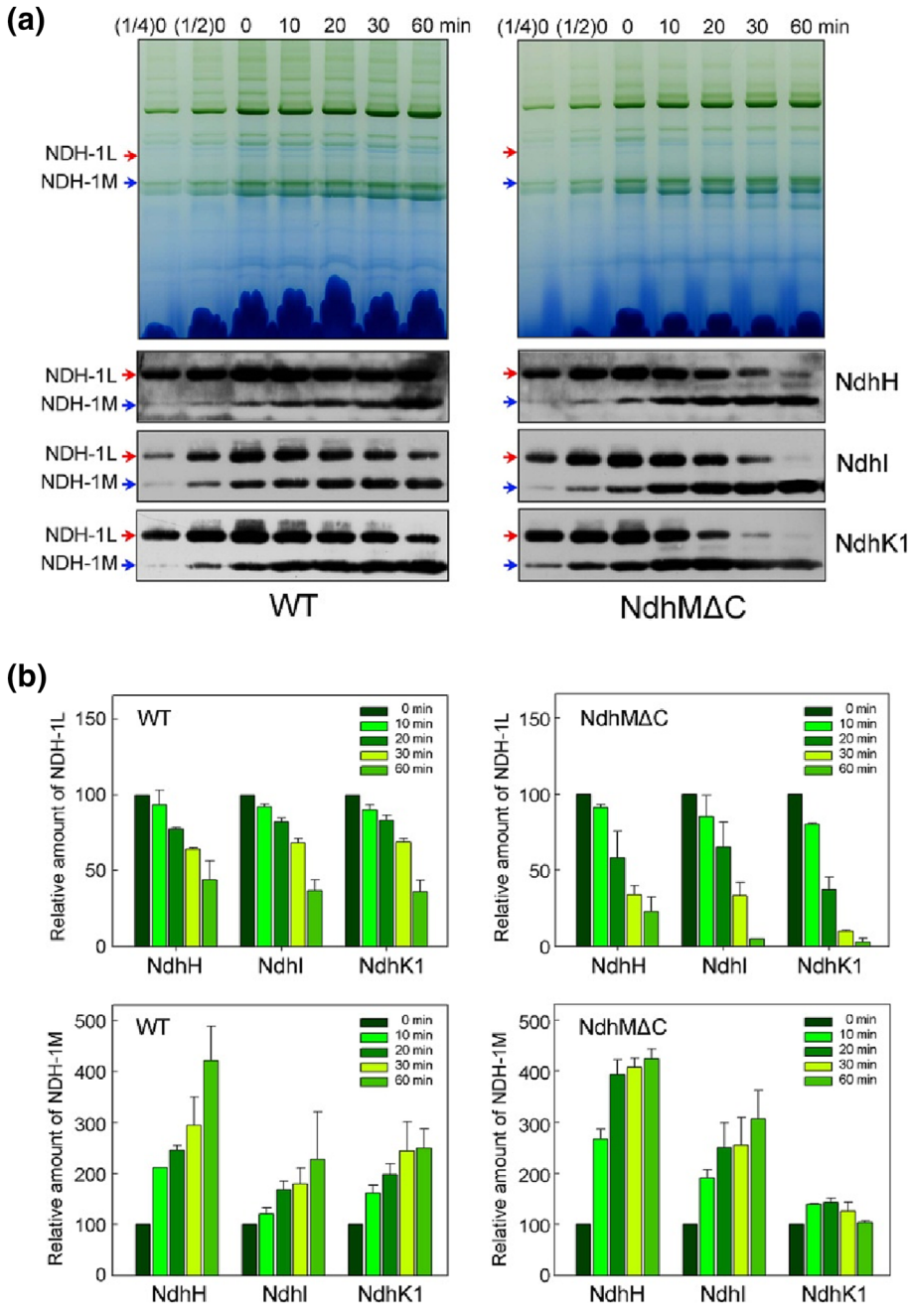

FIGURE 5 Effects of temperature and incubation time on the stability of NDH-1L from the WT and NdhMΔC strains. (a) Thylakoid membranes isolated from the WT and NdhMΔC strains were incubated with 1.5% (*w*/*v*) DM on ice for 60 min (also referred to as at 25°C for 0 min) and then at 25°C for 10, 20, 30, and 60 min. Protein complexes were separated by BN-PAGE. The NDH-1L (red arrows) and NDH-1M (blue arrows) complexes were immunodetected with antibodies against NdhH, NdhI and NdhK1. (b) Signals obtained for NdhH, NdhI, and NdhK1 in (a) were quantified from two independent experiments. Signals at 0 min were set as 100% in each blot.

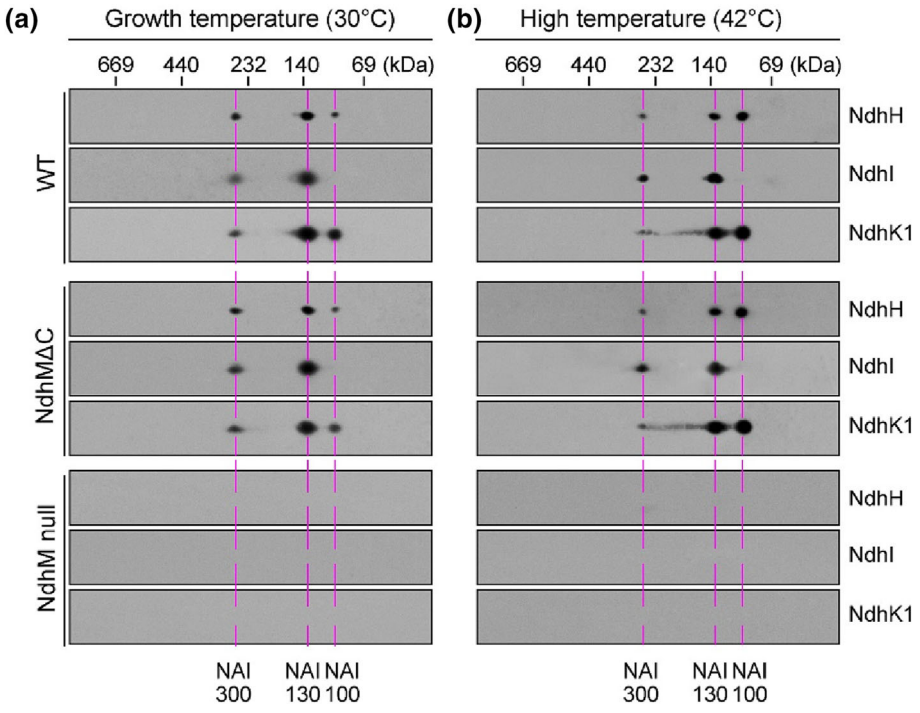

FIGURE 6 Formation of NAIs in the cytoplasm of the WT, NdhMΔC and NdhM null cells.

chlorophyll *a* were separated by BN-PAGE. NDH-1L (red arrows) and NDH-1M (blue arrows) complexes were probed with anti-NdhH and -NdhI antibodies. (c) Signals obtained for NdhH and NdhI from NDH-1L in (a) and (b) were calculated from two independent biological repeats.

The above results suggest that the stability of NDH-1 with truncated NdhM is reduced under stress conditions. For further confirmation of this conclusion, we analyzed the accumulation of NDH-1L complex and its degradation product, the NDH-1M subcomplex, from the DM-solubilized thylakoid membranes of WT and NdhMΔC cells. Thylakoids incubated with DM on ice for 60 min (0 min at 25°C) were further incubated with DM at 25°C for different times (Figure 5a). Qualitative analysis showed that disassembly of NDH-1L was much faster in the NdhMΔC mutant than in the WT when thylakoid membranes were incubated at 25°C (Figure 5b). This indicates that in the presence of DM, the intact NdhM retards the thermal collapse of NDH-1L and its degradation product NDH-1M in the thylakoid membrane, corroborating the conclusion that under stress conditions, the C-terminus of NdhM is essential for stabilizing the NDH-1L complex in the thylakoid membrane of *Synechocystis* 6803 (Figures 2b and 4).

It is worth noting that the decrease in the amount of NdhK1 in NDH-1L was much faster than that of other NDH-1L subunits such as NdhH and NdhI in the NdhMΔC mutant (Figure 5). Moreover, NdhK1 in the NDH-1M subcomplex was also degraded to some extent in the NdhMΔC mutant but not in WT (Figure 5). In contrast, NdhH and NdhI in the NDH-1M subcomplex was not degraded in the NdhMΔC mutant as in WT (Figure 5). These results imply that NdhK1 is the primary target for NdhM. Indeed, as shown in the structure diagram, the N-terminal part of NdhM is closely associated with NdhK1 and a small part of the C-terminus of NdhM also interacts directly with NdhK1 (Figure 1e). Thus, the intact NdhM is likely to play an important role for stabilizing NdhK1 under stress conditions.

2.5 | Assembly of the NDH-1L hydrophilic arm is not affected in the NdhMΔC mutant even under high temperature conditions

Assembly of the cyanobacterial NDH-1L hydrophilic arm was found to mainly undergo a dynamic transition through several assembly intermediates (NAIs) with the aid of various assembly factors (Dai et al., 2013; Ran et al., 2019; Wang et al., 2016). To examine whether the truncated NdhM destabilizes NAIs in the cytoplasm at 42°C when the NDH-1L complex is destabilized in the thylakoid membranes, we analyzed the cytoplasmic protein complexes isolated from WT, NdhMΔC, and NdhM null strains. As deduced from the abundance of NdhH, NdhI, and NdhK1 proteins, accumulation of NAI300 and NAI130 in the cytoplasm of NdhMΔC is not affected under both growth and higher temperature (Figure 6). This indicates that the C-terminal part of NdhM is not essential for the assembly of NAIs even at 42°C. Based on the above results, we propose that NdhM with truncated C terminus affects stabilization of the NDH-1L complex under heat stress.

Cytoplasmic protein complexes isolated from the cells grown at 25°C (normal growth temperature) (a) and 42°C (b) were separated by CN-PAGE and two-dimensional SDS-PAGE. Proteins were immunodetected with three antibodies against NDH-1 hydrophilic subunits NdhH, NdhI, and NdhK1. Dashed lines indicate the positions of several NAIs.

3 | DISCUSSION

Over the past decade, significant progress has been made in elucidating the composition, structure, and function of OPS subunits from the NDH-1L complex in cyanobacteria (Laughlin et al., 2020; Mi, 2022) and higher plants (Shen et al., 2022; Zhang et al., 2023). Among them, multiple OPS subunits are necessary for the stable accumulation of the NDH-1L complex in the thylakoid membrane but their roles in the complex are different. In *Synechocystis* 6803, for example, while NdhP and NdhQ jointly stabilize the variable NDH-1L membrane region, NdhN and NdhM are required for the formation of the mature NDH-1L hydrophilic arm (He et al., 2016; Wang et al., 2016; Zhao et al., 2015). Absence of NdhM, NdhN, and NdhO leads to decreased accumulation of the stroma-faced hydrophilic arm, called NDH subcomplex A in Arabidopsis (Rumeau et al., 2005). In this study, we demonstrated that the NDH-1L complex with a truncated NdhM is unstable under stress conditions in *Synechocystis* 6803.

The cryo-electron microscopic (cryo-EM) structure of NDH-1 from the cyanobactcerium *Thermosynechococcus elongatus* showed that three β-sheets are present in the N-terminal part of NdhM and two α-helixes are present in the middle and C-terminal part of NdhM (Figure 1e; Schuller et al., 2019; Laughlin et al., 2019; Pan et al., 2020; Zhang et al., 2020). NdhM from higher plants *Hordeum vulgare* has a similar tertiary structure (Shen et al., 2022). NdhM directly interacts with NdhK1 mainly through its three β-sheets and the first α-helix (Figure 1e). The second α-helix and C-terminus are located in the peripheral region opposite to NdhK1. In the NdhMΔC mutant, the truncated NdhM protein contains about half of the N-terminal region of the intact NdhM including three β-sheets and one α-helix domain (Figure 1e). The fact that the NDH-1L level was not reduced in the NdhMΔC mutant indicates that the remaining N-terminal part of NdhM confers stability to NdhK under normal growth conditions (Figure 2a,c). Moreover, the activity of NDH-1L in NdhMΔC is comparable with that of WT before heat stress (Figure 3), indicating that electron transport through the [4Fe-4S] clusters in NdhK is not affected under normal growth conditions.

However, the level of NDH-1 is reduced in NdhMΔC under stress conditions, indicating that NDH-1 complex with truncated NdhM is unstable (Figures 2b,c and 4). Because NdhK1 directly interacts with NdhM, NdhK is likely to be one of the initial degradation targets, as the decrease of NdhK1 in NDH-1L in NdhMΔC occurs much faster than that of the NdhH and NdhI subunits of NDH-1L in the thylakoid membranes incubated with DM (Figure 5). Moreover, after heat-stress even for a short time, the increasing in activity of NDH-1L in NdhMΔC is severely suppressed compared with WT cells (Figure 3), suggesting impaired electron transport in NdhMΔC. It is plausible that electron transport is hindered at the NdhK1 subunit that contains one [4Fe-4S] cluster. Intact NdhM may assist NdhK1 in maintaining a correct and optimal protein conformation for efficient electron transport, especially under heat-stress conditions, in which the activity of NDH-1 is significantly enhanced (Figure 3). During prolonged heat stress, impaired electron transfer at NdhK1 may result in the formation of reactive oxygen species (ROS), which in turn cause damage to NdhK and the adjacent subunits of NDH-1. Collectively, we propose that intact NdhM is required for providing optimal complex quaternary structure for NdhK1 and efficient electron transport along the NdhK1 subunit.

Modular assembly of the hydrophilic arm of photosynthetic NDH is initiated in the chloroplast stroma or the cytoplasm of cyanobacteria (Dai et al., 2013; Ran et al., 2019; Wang et al., 2016). In higher plants, CRR1 and CRR9 are specifically required for the biogenesis of NdhK (Shikanai, 2016; Yamamoto et al., 2016). Absence of NdhM leads to a drastic reduction of NdhK1 in the chloroplast stroma and to a complete loss of NdhK1 in the cyanobacterial cytoplasm (He et al., 2016; Shikanai, 2016). Thus, NdhM, like CRR1 and CRR9, may serve as an assembly factor involved in a particular step for NdhK biogenesis. Alternatively, NdhM may be required for the stabilization of NdhK and co-insert with it into assembly intermediates. Why is assembly of the hydrophilic arm unaffected in the NdhMΔC cytoplasm even under heat stress conditions? It is likely that the N-terminal half of NdhM stably interacts with NdhK1 for the subsequent incorporation into NAI intermediates. As electron transport is unlikely to occur within the NAI assembly intermediates in the cytoplasm, no significant degradation of NdhK1 occurs in NdhMΔC during the assembly of the hydrophilic arm of NDH-1.

NdhM is present in all oxygenic photosynthesis-specific NDH-1 complexes. Cryo-EM structure analyses revealed that NdhM from cyanobacteria and higher plant chloroplasts shares a conserved protein structure and a conserved binding site of the NdhK subunit (Laughlin et al., 2019; Pan et al., 2020; Schuller et al., 2019; Shen et al., 2022; Zhang et al., 2020), implying that they fulfill a similar function among different photosynthetic NDH-1 complexes. It is interesting that subunit B17.2 closely interacts with the PSST subunit corresponding to NdhK1 in the *ovine* mitochondrial complex I (Fiedorczuk et al., 2016). The binding site of PSST for B17.2 is similar with that of NdhK for NdhM in photosynthetic NDH. Thus, stabilization of NdhK homologs by an auxiliary protein is probably a common strategy for most of the NDH-1 complexes. Photosynthetic NDH-1 interacts with PSI to form the NDH-PSI supercomplex (Gao, Zhao, Chen, et al., 2016). In mammalian mitochondria, complex I also interacts with another protein complex, complex III (Acín-Pérez et al., 2004). Formation of these supercomplexes is required for enhancement of the stability of NDH/complex I or electron transfer rates. Binding of NdhM with NdhK1 may be another strategy for photosynthetic NDH-1 to reach a high complex stability and high electron transfer activity, especially under stress conditions. Through these strategies, NDH-1 complexes are more resistant to various oxidative stresses in different species.

4 | MATERIALS AND METHODS

4.1 | Culture conditions

A glucose tolerant strain of wild-type (WT) *Synechocystis* 6803 and its mutants, NdhMΔC, NdhM null, and Δ*ndhK1*, were cultured at

30°C or 42°C in BG-11 medium buffered with Tris-HCl (5 mM, pH 8.0) and bubbled with 2% (*v*/*v*) CO_2 in air. Solid medium was the same BG-11 supplemented with 1.5% (*w*/*v*) agar. The mutant strains were grown in the presence of appropriate antibiotics under illumination by fluorescence lamps at 40 μmol photons m^{-2} s^{-1}.

4.2 | Construction of the mutant

The upstream and downstream regions of *ndhM* were amplified by PCR creating appropriate restriction sites using two pairs of primers of *ndhM*-A/B and *ndhM*-E/F, respectively (Table S2). A DNA fragment encoding a spectinomycin resistance (Sp^R) cassette was also amplified by PCR creating *Bam*HI and *Sac*I sites using the *ndhM*-C and *ndhM*-D primers (Table S2). These three PCR products were ligated into the MCS of pUC19 (pUC-Δ*ndhM*; Figure 1a) and used to transform WT cells of *Synechocystis* 6803. The transformants were spread on agar plates containing BG-11 medium and spectinomycin (10 μg mL^{-1}) buffered at pH 8.0, and the plates were incubated in 2% (*v*/*v*) CO_2 in air under illumination by fluorescent lamps at 40 μmol photons m^{-2} s^{-1}. The mutated *ndhM* in the transformants was segregated to homogeneity (by successive streak purification) as determined by PCR amplification using the *ndhM*-G and *ndhM*-H primers (Table S2).

4.3 | RNA extraction and RT-PCR analysis

Total RNA was isolated and analyzed as described previously (McGinn et al., 2003). Reverse transcription polymerase chain reaction (RT-PCR) was performed using the Access RT-PCR system (Promega) to generate products corresponding to *ndhMΔC*, *ndhM*, and *16S rRNA*, with 0.5 μg of DNase-treated total RNA as starting material. RT-PCR conditions were 95°C for 5 min followed by cycles of 95°C, 62°C, and 72°C for 30 s each. The reactions were stopped after 25 cycles. The primers used are summarized in Table S2.

4.4 | Isolation of crude thylakoid membranes

The cell cultures (800 mL) were harvested in the logarithmic phase and washed twice by suspending in 50 mL of fresh BG-11 medium, and the thylakoid membranes were isolated according to Gombos et al. (1994) with some modifications as follows. The cells suspended in 5 mL of disruption buffer (10-mM HEPES-NaOH, 5-mM sodium phosphate, pH 7.5, 10-mM $MgCl_2$, 10-mM NaCl, and 25% glycerol [*v*/*v*]) were supplemented by zirconia/silica beads and broken by vortexing 15 times at the highest speed for 20 s at 4°C with 5 min cooling on ice between the runs. The crude extract was centrifuged at 5000 × *g* for 5 min to remove the glass beads and unbroken cells. By further centrifugation at 20,000 × *g* for 30 min, crude thylakoid membranes were obtained.

4.5 | Electrophoresis and immunoblotting

Blue native (BN)-PAGE of *Synechocystis* 6803 membranes was performed as described previously (Kügler et al., 1997) with slight modifications. Isolated membranes were prepared for BN-PAGE as follows. Membranes were washed with 330-mM sorbitol, 50-mM Bis-Tris, pH 7.0, and 0.5-mM phenylmethylsulfonyl fluoride (PMSF; Sigma), and resuspended in 20% glycerol (*w*/*v*), 25-mM Bis-Tris, pH 7.0, 10-mM $MgCl_2$, 0.1 units RNase-free DNase RQ1 (Promega) at a chlorophyll *a* concentration of 0.3 mg mL^{-1}, and 0.5-mM PMSF. The samples were incubated on ice for 10 min, and an equal volume of 3% *n*-dodecyl β-D-maltoside (DM) was added. Solubilization proceeded for 40 min on ice. Insoluble components were removed by centrifugation at 18,000 × *g* for 15 min. The collected supernatant was mixed with 1/10 volume of sample buffer, 5% (*w*/*v*) Serva Blue G, 100-mM Bis-Tris, pH 7.0, 30% Sucrose (*w*/*v*), 500-mM ε-amino-*n*-caproic acid, and 10-mM EDTA. Solubilized membranes were then applied to a 0.75-mm-thick, 5% to 12.5% (*w*/*v*) acrylamide gradient gel (Hoefer Mighty Small mini-vertical unit). Samples were loaded on an equal chlorophyll *a* basis per lane. Electrophoresis was performed at 4°C by increasing the voltage gradually from 50 V up to 200 V during the 5.5-h run.

Clear native (CN)-PAGE was performed with 0.01% DM and 0.025% deoxycholate additives to the cathode buffer as described in Wittig et al. (2007). Cytoplasmic soluble fractions were applied to a 0.75-mm-thick, 5% to 12.5% acrylamide gradient gel (Hoefer Mighty Small mini-vertical unit; San Francisco, CA). Samples were loaded on an equal protein basis of 80 μg per lane. Electrophoresis was performed at 4°C by increasing the voltage gradually from 50 up to 200 V during the 5.5-h run.

For electrophoresis in the second dimension, several lanes of the CN gel were cut out and incubated in Laemmli SDS sample buffer containing 5% β-mercaptoethanol and 6-M urea for 1 h at 25°C. The lanes were then laid onto a 1-mm-thick 12% polyacrylamide gel with 6-M urea.

For immunoblotting, the proteins were electrotransferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Bedford, MA) and detected by protein-specific antibodies using an ECL assay kit (Amersham Pharmacia, NJ) according to the manufacturer's protocol. Antibodies against NdhH, NdhI, NdhK1, and NdhM were previously described (Ma & Mi, 2005; Zhao et al., 2014). Image J software was used for qualitative analysis.

4.6 | Chlorophyll fluorescence, P700, and dark respiration

After cultured at 30°C for 36 h (A_{730} = 0.5–0.6), WT and NdhMΔC cells were transferred to 42°C for different time periods, the transient rise in chlorophyll fluorescence after actinic light had been turned off was monitored, kinetics of the $P700^+$ re-reduction in darkness after turning off far-red light in the presence of 10-μM DCMU was recorded and oxygen uptake was measured at 30°C in the dark as described previously (Ma & Mi, 2005).

AUTHOR CONTRIBUTIONS

Fudan Gao conceived the study and designed the experiments. Liping Che, Yuecheng Guo, Yanjie Huang, and Fudan Gao performed the experiments. Lianwei Peng and Fudan Gao wrote the manuscript. All authors analyzed the data.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the manuscript and its supporting materials.

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SUPPORTING INFORMATION

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