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Stoichiometry of ATP hydrolysis and chlorophyllide formation of dark-operative protochlorophyllide oxidoreductase from *Rhodobacter capsulatus*

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Abstract

Dark-operative protochlorophyllide (Pchl_{id}) oxidoreductase (DPOR) is a nitrogenase-like enzyme catalyzing the C17=C18 double bond of Pchl_{id} to form chlorophyllide *a* (Chl_{id}) in bacteriochlorophyll biosynthesis. DPOR consists of an ATP-dependent reductase component, L-protein (a BchL dimer), and a catalytic component, NB-protein (a BchN–BchB heterotetramer). The L-protein transfers electrons to the NB-protein to reduce Pchl_{id}, which is coupled with ATP hydrolysis. Here we determined the stoichiometry of ATP hydrolysis and the Chl_{id} formation of DPOR. The minimal ratio of ATP to Chl_{id} (ATP/2e[−]) was 4, which coincides with that of nitrogenase. The ratio increases with increasing molar ratio of L-protein to NB-protein. This profile differs from that of nitrogenase. These results suggest that DPOR has a specific intrinsic property, while retaining the common features shared with nitrogenase.

Keywords: ATPase, BchL, dark-operative protochlorophyllide oxidoreductase, L-protein, nitrogenase, *Rhodobacter capsulatus*

Abbreviations: Chl, chlorophyll *a*; Chl_{id}, chlorophyllide *a*; DPOR, dark-operative protochlorophyllide oxidoreductase; L/NB ratio, L-protein/NB-protein molar ratio; LPOR, light-dependent protochlorophyllide oxidoreductase; Pchl_{id}, protochlorophyllide

1. Introduction

Dark-operative protochlorophyllide (Pchl_{id}) oxidoreductase (DPOR) is a nitrogenase-like enzyme catalyzing a stereospecific reduction of C17=C18 double bond of Pchl_{id} to form chlorophyll_{id} *a* (Chl_{id}) in the late steps of bacteriochlorophyll biosynthesis (Fig. 1) [1–3].

In the chlorophyll *a* (Chl) biosynthesis of cyanobacteria, green algae, and gymnosperms, the reaction by DPOR is the penultimate step since the product Chl_{id} is the direct precursor of Chl [4]. In these oxygenic phototrophic organisms the Pchl_{id} reduction is also catalyzed by light-dependent Pchl_{id} oxidoreductase (LPOR), which belongs to the short-chain dehydrogenase/reductase family [5,6]. DPOR and LPOR are analogous enzymes [7], in which there is no sequence similarity, but they catalyze the same reaction. DPOR is extremely vulnerable to oxygen, like nitrogenase and LPOR requires light for catalysis. Thus, these two Pchl_{id} reductases are partially differentiated in response to environmental light and oxygen levels in oxygenic photosynthetic organisms to complement these intrinsic properties [8].

DPOR consists of two separable components. One is the L-protein, a BchL dimer (BchL)₂, which plays a role as the ATP-dependent reductase component for transferring electrons from ferredoxin (*in vivo*) or dithionite (*in vitro*) to the other component, the NB-protein [9–12]. For ATP-dependent electron transfer, the L-protein possesses one [4Fe–

4S] cluster per dimer and an ATP-binding site in each protomer. The NB-protein, a BchN–BchB heterotetramer (BchN–BchB)₂, is a catalytic component providing the catalytic sites for Pchl_a reduction, and the intermediate metallocenter, a [4Fe–4S] cluster (NB-cluster), is held in the BchN and BchB interface [2,13,14]. The electron from the L-protein reaches Pchl_a via the NB-cluster to convert Pchl_a to Chl_a. The L-protein and the NB-protein are counterparts of the Fe protein (a NifH dimer, (NifH)₂) and the MoFe protein (a NifD–NifK heterotetramer, (NifD–NifK)₂) of nitrogenase, respectively. In nitrogenase, the Fe protein catalyzes ATP-dependent electron transfer to the MoFe protein. The electrons from the Fe protein are transferred to the P-cluster and then transferred to the nitrogen molecule bound in the FeMo-cofactor in the MoFe protein [15].

Genes encoding subunits of DPOR (*bchL*, *bchN* and *bchB*) were identified in the photosynthesis gene cluster of the purple bacterium *Rhodobacter capsulatus* [16,17], and subsequently, their orthologous genes, *chlL*, *chlN* and *chlB*, were identified in the cyanobacteria and chloroplast genomes of green algae, pteridophytes, bryophytes, and gymnosperms [18–23]. The establishment of a reconstitution system with purified L-protein and NB-protein from *R. capsulatus* has enabled further in-depth biochemical analyses of DPOR [24]. Recently, X-ray crystallographic analyses of the individual components [2,11,14]

and the complex of the L-protein and the NB-protein [3] have provided structural insights into Pchlide reduction, the inter-subunit electron transfer, and formation of the complex of the L-protein and the NB-protein. Based on the structure, a reaction mechanism involving the generation of substrate radicals has been proposed [25].

The amino acid sequence of BchL shows approximately 30% identity to that of NifH of nitrogenase. The ATP-binding motif (GKGGIGKS) and the two Cys residues for the chelation of the [4Fe–4S] cluster in NifH are completely conserved in BchL. The X-ray crystal structure of the L-protein is very similar to that of the Fe protein, whereas the surface charge distribution of the docking site for the catalytic component is not the same [11]. Structural comparison between the L-proteins in the “off-state” (the ADP-bound form) and in the “on-state” (the ADP-AlF₄-bound form in the L-protein–NB-protein complex) suggested that upon binding to the NB protein, the [4Fe–4S] cluster of the L-protein is repositioned about 5 Å toward the NB-cluster in the NB-protein [3]. This conformational change of the L protein is very similar to that of the Fe protein in nitrogenase [26]. These overall structural similarities imply that the electron transfer mechanism of nitrogenase from the Fe protein to the MoFe protein is conserved in that of DPOR from the L protein to the NB protein.

The stoichiometry of nitrogenase is as follows:

In this reaction the ATP/2e⁻ ratio is 4, which means that two ATP molecules on the protomers are hydrolyzed when one electron is transferred from the [4Fe-4S] cluster to the P-cluster. However, this ratio is observed only under optimal conditions [27]. The ATP/2e⁻ ratio significantly increases dependent on the molar ratio of the Fe protein to the MoFe protein (the Fe protein/MoFe protein ratio) [28], pH [29], redox potential [30], and temperature [31]. For example, the ATP/2e⁻ ratio reaches 13 in an Fe protein/MoFe protein ratio of 2 [28]. In contrast to nitrogenase, the ATP/2e⁻ ratio has not yet been established since the stoichiometry of DPOR has not been determined.

In this study, we determined the stoichiometry of the ATP hydrolysis and Chlide formation in the DPOR reaction using purified DPOR components from *R. capsulatus*. The minimal ATP/Chlide (ATP/2e⁻) value was 4, which coincides with that of nitrogenase. The ATP/2e⁻ value increased with the increase in the L-protein/NB-protein molar ratio (L/NB ratio), which appears to be a specific intrinsic property of DPOR that differs from the properties of nitrogenase.

2. Materials and Methods

2.1. Purification of the L protein and the NB-protein

The L-protein and the NB-protein of the purple bacterium *R. capsulatus* were purified using an affinity column (Strep-Tactin, IBA, Göttingen, Germany) from the crude extracts of *Escherichia coli* overexpressing Strep-BchL and Strep-BchN–BchB [32]. Pchl_{ide} was prepared from spent culture medium of the $\Delta bchL$ mutant ZY5 of *R. capsulatus*, as described [24].

2.2. DPOR reaction

All procedures of purification and assaying were performed in an anaerobic chamber [24].

The DPOR reaction was performed in a 250- μ l reaction mixture containing 100 mM HEPES-KOH; pH7.4, 5 mM MgCl₂, 5 mM dithiothreitol, 0.01% Triton X-100, 1.4 mM dithionite, 1.2 mM ATP, and 30 μ M Pchl_{ide}. The reaction was initiated by the addition of the purified L-protein and NB-protein and incubated at 34°C for 15 min.

2.3. Determination of ADP and Chlide

Chlide formation was determined using the visible absorption spectra of the acetone extracts

of the reaction mixtures, as described previously [13]. ATP hydrolysis was evaluated by the ADP formation, which was measured using an HPLC system [33]. The reaction mixture was acidified with 125 μ l of 3 M perchloric acid and subsequently neutralized by the addition of 125 μ l of 3 M KOH. Aliquots of neutrilized reaction mixtures were loaded onto a titanium dioxide column (Titansphere TiO 5 μ m 4.6 $\times$ 100 mm, GL Science Inc. Tokyo, Japan). ADP and ATP were eluted at 3.2 min and 4.6 min, which were monitored using the absorbance at 260 nm.

3. Results and Discussion

3.1. Determination of Chlide formation and ATP hydrolysis

First, we examined Chlide formation and ATP hydrolysis in reaction mixtures containing the L-protein, the NB-protein, or both (Fig. 2). As shown previously [24], the reactions containing only the L-protein or only the NB-protein exhibited no detectable activity of Chlide production (Fig. 2A). ATP hydrolysis measured by ADP production was not detected in the reactions containing only one protein component (Fig. 2B), which is consistent with the Chlide production. This result indicated that the ATPase activity of the L-protein is dependent

on the presence of the NB-protein. This NB-protein-dependent ATPase activity was reported in the cyanobacterial DPOR from *Prochlorococcus marinus*, in which ATPase activity was measured as phosphate release [34]. In nitrogenase, the Fe protein shows no ATPase activity in the absence of the MoFe protein [35]. Thus, this property of the L-protein is shared with the Fe protein of nitrogenase.

In the reaction mixture containing both components, the formation of Chlide was detected as the appearance of a 666-nm peak and ATP hydrolysis was detected as a clear increase of the absorbance peak at 3.2 min for ADP in the HPLC profile. Under these conditions, the ratio of ATP to Chlide (the ATP/2e⁻ ratio) was estimated to be 7.8.

3.2. Determination of the minimal ATP/2e⁻ ratio

In nitrogenase, the ATP/2e⁻ ratio varies dependent on the conditions, having a minimal value of 4. The Fe protein/MoFe protein ratio is one of the factors affecting the ATP/2e⁻ ratio [28]. An ATP/2e⁻ ratio of 7 was observed in a reaction with a Fe protein/MoFe protein ratio of 3 in nitrogenase from *Klebsiella pneumoniae* [28]. Thus, Chlide production and ATP hydrolysis activities were determined at the various L/NB ratios from 1 to 512, with a fixed amount of the NB-protein (Fig. 3). The Chlide production activity increased with the increase of the

L/NB ratio in the region from 1 to 4 (Fig. 3A) and reached a plateau at an L/NB ratio of more than about 4 (Fig 3A, C). This profile of Chlide production is consistent with a previous study, in which the activity of Chlide formation was saturated when the ratio L/NB ratio exceeded 3 [13]. The ATP hydrolysis activity also increased with the increase of the L/NB ratio (Fig. 3A, C). In contrast to the Chlide production, the ATP hydrolysis activity continued to increase in the high-L/NB-ratio region (more than 10) (Fig. 3C).

Because the activities of Chlide production and ATP hydrolysis showed biphasic profile, we evaluated the $\text{ATP}/2\text{e}^-$ ratios individually in the first (1 to 10) and second (10 to 512) L/NB-ratio regions (Fig. 3B, D). In the first L/NB-ratio region, the relationship between the $\text{ATP}/2\text{e}^-$ ratio and the L/NB ratio was linear, and the minimal $\text{ATP}/2\text{e}^-$ ratio, 4.6, was observed at an the L/NB ratio of 1 (Fig. 3B), which is very close to 4. This result suggested that the minimal $\text{ATP}/2\text{e}^-$ ratio of DPOR is 4. In the second L/NB-ratio region, the $\text{ATP}/2\text{e}^-$ ratio also linearly increased, albeit at a lower rate than that in the first L/NB-ratio region. It reached 23 at an L/NB ratio of 512.

3.3. Stoichiometry of DPOR

Based on the obtained results, we propose the following stoichiometry of the DPOR reaction:

The minimal requirement of four molecules of ATP for the reduction of one Pchlide molecule is one-quarter of that of the nitrogenase reaction for N_2 reduction, which is consistent with the activation energy of DPOR (22.2 kJ/mol) being about one-quarter of that of nitrogenase (90–97 kJ/mol) [12]. In the nitrogenase reaction, two of the eight electrons are inevitably used for hydrogen production. We attempted to detect hydrogen in the DPOR reaction. However, no significant hydrogen production was detected in any conditions, strongly suggesting that the DPOR reaction is a simple two-electron reduction without hydrogen production. This reaction feature is consistent with the consideration of the activation energy of DPOR. This might also be consistent with the simple arrangement of iron-sulfur clusters in DPOR, namely, the [4Fe–4S] cluster in the L-protein and the NB-cluster (a [4Fe–4S] cluster) in the NB protein, rather than the complex metalloclusters, the P-cluster (a [8Fe–7S] cluster) and the FeMo-cofactor (a [1Mo–7Fe–9S–C–homocitrate] cluster), in nitrogenase.

In this study, a fixed amount of the NB protein was titrated with the L-protein (Fig.

3). The $\text{ATP}/2\text{e}^-$ ratio showed a biphasic profile (Fig. 3). In the first phase (with the L/NB ratio from 1 to 10), the $\text{ATP}/2\text{e}^-$ ratio increased linearly, with a minimal value of 4, with increasing L/NB ratio. For example, at an L/NB ratio of 8, the $\text{ATP}/2\text{e}^-$ ratio becomes about 10 (Fig. 3B). In the cyanobacterial DPOR, the $\text{ATP}/2\text{e}^-$ ratio was reported to be 14 at a single L/NB ratio of 2 [34]. This value is substantially higher than 6, which was estimated from the titration curve that we determined (Fig. 3B). The titration curve may vary for DPOR from different organisms. Alternatively, other unknown experimental factors may result in this difference in the $\text{ATP}/2\text{e}^-$ ratio. Further studies on the $\text{ATP}/2\text{e}^-$ ratio of DPOR under various other conditions are required to explain this difference.

In the second phase (with the L/NB ratio from 10 to 512, Fig. 3D), the linear increase of the $\text{ATP}/2\text{e}^-$ ratio became more gradual and reached 23 at a L/NB ratio of 512. On the other hand, the Chlide production reached its maximal plateau in the second phase. These profiles indicated that the ATP hydrolysis in this second phase was completely decoupled from Chlide production. In contrast, the $\text{ATP}/2\text{e}^-$ ratio of nitrogenase retained its minimal value of 4 in a considerably wider range of the Fe protein/MoFe protein ratio from about 100 to 200 [28]. This difference suggests that the coupling between the substrate reduction and ATP hydrolysis in nitrogenase is considerably tighter than that in DPOR under conditions in which

the reductase component is saturated.

Such a difference in the $ATP/2e^-$ ratio might be caused by a structural difference in the interaction between the reductase component (the Fe protein or the L protein) and the catalytic component (the MoFe protein or the NB protein). Although the individual structures are considerably similar to each other, the interaction between them is apparently unique, particularly in terms of the charge distribution of the docking surfaces of the two components [3,11,26]. Furthermore, a structural comparison between the complexes of DPOR and nitrogenase revealed some unique features [3]. For example, a loop of NifK (β -subunit of the MoFe protein) interacting with NifH (the Fe protein) in the nitrogenase complex corresponds to a loop of BchN interacting with BchL in DPOR, indicating that BchN corresponds to NifK rather than NifD in the docking interface. This feature contrasts to BchN being the counterparts of NifD in the overall structures. In the nitrogenase complex, the protein interaction has been finely tuned up to minimize the $ATP/2e^-$ ratio in a wide range, particularly in the range of excess amounts of Fe protein relative to MoFe protein. Such fine adjustment would be important for survival in oligotrophic environments utilizing the diazotrophic capability with minimal energy loss. In contrast, the DPOR system would not have been subjected to such strong selective pressure because the quantitative demand for

DPOR in the bacteriochlorophyll and Chl biosynthetic pathways in photosynthetic cells is considerably lower than that for nitrogenase in diazotrophic cells. Alternatively, DPOR might have retained an inefficient property of the ancestral nitrogenase-like enzyme.

The results in this study suggest that DPOR has a specific intrinsic property, while retaining the common features shared with nitrogenase. Further detailed biochemical analysis with extensive site-directed mutagenesis should provide insights into the coupling of the substrate reduction in the catalytic component with ATP hydrolysis in the reductase component of nitrogenase-like enzymes.

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Figure Legends

Figure 1. Pchl_a reduction by DPOR. The C17=C18 double bond (shown by yellow box) of the D ring of Pchl_a is stereo-specifically reduced to produce Chl_a. The L-protein is the ATP-dependent reductase component, and the NB-protein is the catalytic component that provides the catalytic site for Pchl_a reduction. The electron transfer from the L-protein to the NB-protein is coupled with ATP hydrolysis by the L-protein. The stoichiometry of ATP hydrolysis (*n*) was determined to be 4 in this study.

Figure 2. Chl_a production (A) and ATP hydrolysis (B) in the DPOR reaction. (A) DPOR assay was performed with purified L-protein only (black line), purified NB-protein only (green line), and both L-protein and NB-protein (red line) in the presence of ATP (1.2 mM), dithionite (1.4 mM), Pchl_a (30 μM), the purified L-protein (2.7 μM), and the purified NB-protein (0.29 μM). The reaction mixtures were incubated for 15 min at 34 °C. After the incubation, pigments were extracted from the reaction mixtures using acetone and their absorbance spectra were recorded. Absorption peaks at 628 nm and 666 nm are derived from Pchl_a and Chl_a, respectively. (B) ATP hydrolysis was evaluated by the ADP production

measured by an HPLC system with a titanium dioxide column. Elution of ATP and ADP was monitored by the absorbance at 260 nm. ADP and ATP were eluted at 3.2 min and 4.7 min, respectively. The dashed lines overlaid in the respective solid trace are the reaction mixtures before the incubation. Note that a trace amount of ADP was contained in ATP, which was added to the reaction mixtures before incubation.

Figure 3. Correlation of the L/NB ratio and the production of Chlide and ADP in DPOR. A fixed amount of the NB-protein (0.039 μM) was titrated with various amounts of the L-protein (0.039 μM –20 μM). **(A)** Chlide formation (blue diamonds) and ATP hydrolysis (ADP production, red circles) were determined in the range of the L/NB ratios from 1 to 10. **(B)** The relationship between the L/NB ratio and $\text{ATP}/2\text{e}^-$ (ATP/Chlide) in the region of panel A. **(C)** Chlide formation and ATP hydrolysis were determined in the range of L/NB ratios from 10 to 512. **(D)** The relationship between the L/NB ratio and $\text{ATP}/2\text{e}^-$ (ATP/Chlide) in the region of panel C.

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Figure 1

Figure 2

Figure 3