A key step in bacteriochlorophyll biosynthesis is the reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide), catalyzed by dark-operative protochlorophyllide oxidoreductase (DPOR). DPOR is made of electron donor (BchL) and acceptor (BchB) component proteins. BchNB is further composed of two subunits each of BchN and BchB arranged as an α2β2 heterotetramer with two active sites for substrate reduction. Such oligomeric architectures are found in several other electron transfer (ET) complexes, but how this architecture influences activity is unclear. Here, we describe allosteric communication between the two identical active sites in *Rhodobacter sphaeroides* BchNB that drives sequential and asymmetric ET. Pchlide binding to one BchNB active site initiates ET from the pre-reduced [4Fe-4S] cluster of BchNB, a process similar to the deficit spending mechanism observed in the structurally related nitrogenase complex. Pchlide binding in one active site is recognized in trans by an Asp-274 from the opposing half, which is positioned to serve as the initial proton donor. A D274A variant DPOR binds to two Pchlide molecules in the BchNB complex, but only one is bound productively, stalling Pchlide reduction in both active sites. A half-active complex combining one WT and one D274A monomer also stalled after electron was transferred in the WT half. We propose that such sequential electron transfer in oligomeric enzymes serves as a regulatory mechanism to ensure binding and recognition of the correct substrate. The findings shed light on the functional advantages imparted by the oligomeric architecture found in many electron transfer enzymes.

Many oligomeric enzymes that transfer electrons for catalysis or substrate reduction have two identical active sites and their subunits are arranged with a head-to-head or head-to-tail symmetry. The electron acceptor component proteins of nitrogenase and the nitrogenase-like class of enzymes, such as the dark-operative protochlorophyllide oxidoreductase (DPOR) and chlorophyllide oxidoreductase, are arranged as α2β2 tetramers (Fig. 1a) (1–4). A similar architecture is also seen in the nitric oxide synthase (5) and ribonucleotide reductase family of enzymes (6). Given the evolutionary and functional significance of these enzymes, a mechanistic significance must exist behind such structural assemblies. In gymnosperms, cyanobacteria, and all photosynthetic eubacteria, DPOR catalyzes the reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) in the dark, a key step in the chlorophyll and bacteriochlorophyll biosynthetic pathways (7).

DPOR consists of electron donor (BchL) and electron acceptor (BchN-BchB; BchNB) component proteins (Fig. 1a) (8, 9). BchL is a homodimer containing one [4Fe-4S] cluster ligated at the dimer interface by two cysteine residues per monomer and possesses one ATP binding site per monomer (10). BchNB is an α2β2 tetramer carrying one [4Fe-4S] cluster and substrate (Pchlide) binding site per half of the tetramer (3). ATP binding to BchL drives the assembly of the BchL and BchNB proteins and the transient assembly of this complex promotes electron transfer (ET) (8). The electron is transferred from the [4Fe-4S] cluster of BchL, to the [4Fe-4S] cluster of one half of BchNB and finally to the C17=C18 double bond of Pchlide. Two rounds of electron and proton transfer are required to reduce Pchlide to Chlide (Fig. 1b). In DPOR, one proton required for reduction originates intrinsically from within the C17 propionate of Pchlide, and the second proton is donated in trans from an Asp-274 of the opposing BchB subunit (Fig. 1c) (11, 12). Chlide is subsequently reduced by the structurally homologous chlorophyllide oxidoreductase (COR) in the bacteriochlorophyll biosynthetic pathway (13, 14). These reductive steps fine-tune the spectral and reactive properties of bacteriochlorophyll to be optimally suited for photosynthesis.

An overarching question about these enzymes is centered on their conserved architectural complexity: Why are these enzymes assembled as two functional halves, and how do they cooperatively function during substrate reduction? Because two rounds of ET (per half) are required for substrate reduction, multiple BchL binding and dissociation cycles occur at each BchNB half. The two BchL binding interfaces on BchNB are situated ~100 Å apart (4). If their binding events are coordinated, long-range, intersubunit allosteric communication is necessary. Here, we explored such fundamental mechanistic questions using DPOR components from *Rhodobacter sphaeroides*. We show that the two halves indeed communicate, and that perturbation of substrate reduction activity at one-half abolishes ET at the other, thus stalling the entire DPOR complex. The findings explain key functional advantages of the oligomeric architecture found in many enzymes that catalyze electron transfer reactions.
Results

A half-reactive DPOR complex is defective for substrate reduction

To test the functional importance of the $\alpha_2\beta_2$ architecture, we first generated half-reactive versions of DPOR and tested overall Pchlide reduction activity. BchN and BchB are expressed as separate open reading frames and form a tetramer. To generate a half-reactive BchNB complex, we used a dual-affinity tag approach where two BchB open reading frames were generated, coding for either a poly-histidine or Strep-affinity tag on the N terminus. N-terminal positioning of either affinity tag does not affect Pchlide reduction activity, as their rates of Pchlide reduction are similar (Fig. 2, a and b). Reduction of Pchlide to Chlide results in a spectral shift with appearance of an absorption maximum at 666 nm. We note that placing affinity tags on the C terminus of BchB perturbs Pchlide reduction activity (data not shown). These tagged BchB constructs were co-expressed along with an untagged version of the BchN polypeptide. This strategy generated an ensemble of BchNB tetramers carrying either two poly-histidine–tagged BchB, two Strep-tagged BchB, or one of each in the context of a BchNB tetramer (Fig. 2c). This dual-tagged approach enabled us to sequentially fractionate the mixed population of BchNB proteins over Ni$^{2+}$-nitrilotriacetic acid (NTA) and Strep-Tactin agarose resins. The final purified BchNB complex had one BchB subunit carrying a poly-histidine tag and the other a Strep-tag (Fig. 2e). The presence of the appropriate affinity tags over the sequential purification steps were further confirmed by Western blotting using anti-His and anti-Strep antibodies (Fig. 2, g and i and Fig. S1).

To generate a half-active BchNB complex, we introduced an Asp-274 to Ala substitution only in the BchB subunit carrying the poly-histidine tag. Asp-274 acts as one of the necessary proton donors for Pchlide reduction, the other coming from the Pchlide itself (Fig. 1c). Asp-274 interacts with the Pchlide molecule and substitution of Asp-274 to Ala has been shown to perturb substrate reduction in DPOR (3). When expressed along with WT BchN and WT Strep-tag BchB, a mixture of BchB complexes were generated (Fig. 2d) carrying either entirely WT BchNB (strep-B:[BchN]$_2$:strep-B); variant BchNB (his-B$^{D274A}$:[BchN]$_2$:his-B$^{D274A}$); or half-active BchNB (his-B$^{D274A}$:[BchN]$_2$:strep-B). Using the dual-affinity purification...
strategy we were able to isolate the half-reactive BchNB (his-B\(^{D274A}\):[BchN]_{2}:strep-B) (Fig. 2f) and confirmed the presence of both affinity tags by Western blotting using the above-described antibodies (Fig. 2, h and j and Fig. S1). We next monitored Pchlide reduction activity of the dual-tagged WT and half-active BchNB complexes by mixing them with Pchlide, BchL, and ATP. Whereas WT BchNB carrying either affinity tag reduces Pchlide, the half-active BchNB is defective for Pchlide reduction (Fig. 2, a and b). These data show that two functional halves in the context of the \(a_{2}b_{2}\) BchNB tetramer are required for substrate reduction.

The predicted sequence of Pchlide-reduction events in DPOR involve binding of Pchlide to BchNB, the binding of BchL to BchNB, and the respective electron transfer events. In addition, ATP binding and hydrolysis within the BchL complex are also elemental steps in substrate reduction (15, 16). Because the half-active BchNB proteins were defective for overall Pchlide reduction, we next sought to identify where the enzyme stalled in the catalytic cycle. Thus, we focused on measuring the electronic properties of the [4Fe-4S] cluster of BchNB using EPR spectroscopy. Unfortunately, the yields of the half-active BchNB protein were minimal after the dual-affinity purification and hence precluded us from performing detailed EPR studies. Hence, we investigated the spectral properties of WT and variant (BchNB\(^{D274A}\)) DPOR complexes to capture the steps in the electron transfer cycle.

The [Fe-S] cluster of BchNB can be pre-reduced by BchL and re-primed for sequential reactions—a “deficit spending” model

The Asp-274 residue in BchB is unique in its role as a proton donor to Pchlide (3). Structurally, it functions in \(\text{trans}\), where Asp-274 from one half of the BchNB tetramer serves as the proton donor to the Pchlide molecule bound to the active site of the opposing half BchNB (Fig. 1c) (3, 4). Thus, this residue probably serves as a key communication element between the two halves of the \(a_{2}b_{2}\) BchNB tetramer. The [4Fe-4S] cluster of BchNB is ligated by three Cys residues from BchN (Cys-29, Cys-54 and Cys-115) and one Asp residue from BchB (Asp-36; \(R.\ sphaeroides\) numbering) (Fig. 3a). The EPR spectral properties of the BchNB [4Fe-4S] cluster have not been thoroughly characterized, but a small EPR signal has been observed and described by an \(S = 3/2\) spin state (17). We measured the EPR
spectra of BchNB alone, in complex with its substrate (Pchlide), and upon addition of BchL (± ATP). As described before (17), the [4Fe-4S] cluster of BchNB is EPR silent (Fig. 3b, red trace). In comparison, the [4Fe-4S] cluster of BchL produces a large signal in the ~350 mT range as reported previously (Fig. 3b, gray trace) (18). Even at higher BchNB concentrations (90 μM tetramer) no EPR signature is observed for the [4Fe-4S] cluster of BchNB (Fig. 3c, red trace). When Pchlide is added to BchNB, we do not observe a change in signal either (Fig. 3c, blue trace). When BchNB, BchL, and Pchlide are mixed together in the absence of ATP, the signal for the [4Fe-4S] cluster for BchL is observed, but no new signals for BchNB are observed (Fig. 3c, green trace). This finding is not surprising as ATP binding to BchL is required to drive complex formation between BchNB and BchL. When we introduce BchL and ATP to the reaction containing BchNB in the absence of Pchlide, a peak appears at ~130 mT (Fig. 3, b and c, black traces), which resembles the EPR spectra previously reported (17). This suggests that ATP-bound BchL forms a complex with BchNB in the absence of Pchlide and donates an electron to the [4Fe-4S] cluster of BchNB. When the entire reaction is reconstituted with BchL, BchNB, Pchlide, and ATP, the steady-state reaction results in the presence of the ~130 mT peak (Fig. 3c, orange trace).

Previous models for DPOR suggest that the [4Fe-4S] cluster of BchNB exists in an oxidized state. The mechanism of action includes BchNB binding to Pchlide, followed by complex formation with BchL, and the transfer of the first electron from BchL to BchNB and then onto Pchlide. In our experiments, because ATP-bound BchL can reduce BchNB in the absence of Pchlide, we suggest an alternate possibility where BchNB could be pre-reduced in the cell followed by Pchlide binding, and the subsequent transfer of the first electron to Pchlide. This model is congruent with the deficit spending mechanism proposed for nitrogenase (19), wherein the first electron donation to substrate from the pre-reduced MoFe protein is accomplished before ATP-dependent reduction from the Fe-protein (electron donor component).

We next measured the kinetics of substrate reduction in DPOR by following the appearance of the absorbance signal at 666 nm (Fig. 4a), and the appearance of the ~130 mT EPR signal (Fig. 4b). Reduction reactions containing BchL, BchNB, and Pchlide were initiated by adding ATP and quenched samples were assessed for electron transfer (EPR) and Pchlide formation (absorbance). The appearance of the EPR signal correlates with the formation of Chlide, albeit at a slower rate (Fig. 4c), suggesting that (a) the EPR signal represents electron transfer to the [4Fe-4S] cluster of BchNB. (b) Because rate of appearance of the EPR spectra lag behind the Chlide formation (absorbance signal), the EPR signal likely reflects the re-reduced cluster on BchNB. Thus, during the reaction, the BchNB cluster is always in a reduced state and primed to donate an electron to Pchlide during the next substrate reduction cycle, as would be expected in the deficit spending model. This agrees with reduction of the BchNB by BchL (+ATP) in the absence of Pchlide (Fig. 3c).

Asp-274 to Ala substitution reveals unresolved electron transfer intermediates in BchNB and a sequential substrate reduction mechanism within the two halves

Establishment of the order of Pchlide binding to BchNB is necessary to understand the mechanism of ET. From the half-reactive BchNB experiments we deciphered that multiple rounds of ET do not occur when one or both BchNB halves carry a substitution in the proton donor residue, Asp-274. Thus, we proposed that a stalled BchNB intermediate could be identified through EPR for the BchNB<sup>D274A</sup> complex. Interestingly, the EPR signature of BchNB<sup>D274A</sup> (in the absence of Pchlide, BchL, or ATP) showed the presence of a peak at ~130 mT, indicative of the presence of a reduced [4Fe-4S] cluster (Fig. 5a, orange trace). This is in stark contrast to WT BchNB that is EPR silent (Fig. 5a, red trace). This suggests that the...
substitution at Asp-274 influences the electronic properties of the [4Fe-4S] cluster either by physically promoting structural changes in the protein that make the cluster more accessible to the reductant (dithionite) in the reactions, or by propagating/perturbing a network of amino acid interactions that change the reduction potential of the cluster, making it EPR visible.

Analysis of the crystal structures of the Pchlide-free (20) and Pchlide-bound BchNB (3) crystal structures show large-scale conformational changes that might likely explain this difference (Fig. S2). The collapse of the binding pocket in WT BchNB, in the absence of Pchlide, likely serves as a protective mechanism for the [4Fe-4S] cluster as the cluster is not reduced when recombinantly purified. Binding of Pchlide or BchL (or both) likely triggers the necessary conformational changes and primes the [4Fe-4S] cluster for accepting electrons. BchNB can bind to Pchlide in the absence of BchL; similarly, BchL can bind to BchNB in the absence of Pchlide and donate an electron in the presence of ATP (Fig. 5b, black trace). Thus, Pchlide or BchL binding to BchNB are likely not mutually exclusive events and a specific order of binding may not dictate overall substrate reduction activity.

Because BchNB<sup>D274A</sup> is defective for Pchlide reduction but is preloaded with electrons, we next tested whether ET occurs. When incubated with Pchlide, the EPR spectra for the [4Fe-4S] cluster of BchNB<sup>D274A</sup> disappears (Fig. 5a, yellow trace). Thus, BchNB<sup>D274A</sup> donates its preloaded electrons to Pchlide. The complete disappearance of the EPR signal implies that the first ET event occurs in both halves. Interestingly, when BchNB<sup>D274A</sup> is incubated with BchL, Pchlide, and ATP, the EPR signal at $-130$ mT is restored (Fig. 5b, blue trace). However, the intensity of the EPR peak is almost exactly 50% of the amplitude of the signal intensity observed for WT BchNB under similar conditions (Fig. 5c). This is indicative of a stabilized intermediate where re-reduction of only one of the two [4Fe-4S] clusters of BchNB has occurred. The data also affirm our interpretation that the EPR signal at $-130$ mT for WT BchNB (and the BchNB<sup>D274A</sup> without Pchlide) reflects two total electrons per tetramer, one per BchNB half.

These findings further support the sequential ET model in DPOR where events in one half control activity in the other. If the two halves were to act independently, (a) the half-reactive DPOR complex should have been able to retain partial Pchlide reduction activity, but this is not the case (Fig. 2b). (b) Similarly, in the EPR analysis, for an independent model, the amplitude of the spectra for BchNB<sup>D274A</sup> should have been either around 100 or 0% of WT BchNB, but this again is not the case (Fig. 5c). Thus, similar to our observations for nitrogenase (21), we propose that an intrinsic functional asymmetry exists in DPOR.

### Direct measurements of Pchlide binding to BchNB reveal a substrate sensing mechanism that establishes functional asymmetry

Because the two halves in the BchNB complex appear to transfer electrons to Pchlide in a sequential manner, we next tested how asymmetry is established. We explored two potential models: 1) BchNB, when synthesized, is intrinsically endowed with asymmetric Pchlide binding properties through differences in active-site conformations between the two halves. 2) Alternatively, Pchlide binding to either active site is stochastic and changes in the active site upon Pchlide binding then sets asymmetry and allosterically controls Pchlide binding to the other half. To test these models, we directly monitored Pchlide binding by capturing the changes in fluorescence upon binding to BchNB.

A scan of the fluorescence properties of Pchlide shows an excitation and emission maxima at 440 and 636 nm, respectively (Fig. S3). Based on the spectral features, we excited a sample of Pchlide at 440 nm and measured the change in fluorescence in the absence and presence of BchNB (Fig. 6a). When BchNB is added to the reaction, the fluorescence emission of Pchlide increases $\sim700$-fold (Fig. 6a). The utility of Pchlide fluorescence to investigate its binding to other proteins has been controversial. One such study using protochlorophyllide reductase showed no significant changes in Pchlide fluorescence upon binding to Pchlide, as nonspecific binding to BSA also
Mechanism of action of DPOR

Figure 5. BchNB$^{D274A}$ is synthesized with electrons preloaded and reveals a deficit-spending–like mechanism for the first electron transfer event. a, comparison of EPR spectra of WT BchNB (red) versus BchNB$^{D274A}$ (orange) reveals a pre-reduced [4Fe-4S] cluster in BchNB$^{D274A}$. When Pchlide is added to BchNB$^{D274A}$, the electron is donated, and the cluster becomes EPR silent (yellow). b, EPR spectra of WT BchNB and BchNB$^{D274A}$, in the presence and absence of Bchl and ATP reveal electron transfer properties of the DPOR complex. No ET is observed between Bchl and BchNB in the absence of ATP, but presence of Pchlide (green). ET from Bchl to BchNB is observed in the presence of ATP, and in the absence of Pchlide (black). Steady-state ET is observed when all components of the reaction are present (orange). The preloaded electron in BchNB$^{D274A}$ is transferred to Pchlide in the presence of Bchl, and in the absence of ATP (purple), thus supporting evidence for a deficit-spending mechanism. When all the reaction components are present with BchNB$^{D274A}$ (blue), the EPR intensity is half that observed for WT BchNB. c, overlay of the EPR spectra for WT BchNB (orange) and BchNB$^{D274A}$ (blue) in the presence of Bchl, Pchlide, and ATP, show that the amplitude of the BchNB$^{D274A}$ intensity is ~50% of the WT reaction (insert). Thus, BchNB and Bchl form an ATP-dependent complex in the absence of Pchlide and can donate an electron from Bchl to BchNB and then onto Pchlide, followed by re-reduction of the BchNB [4Fe-4S] cluster.

Discussion

The oligomeric $\alpha_2\beta_2$ structural arrangement observed in the BchNB complex of DPOR is found in a host of other protein complexes, especially in enzymes that catalyze ET reactions. These enzymes have two diametrically situated, identical active sites. In DPOR, extensive structural contacts between the two active sites are a prominent feature, with one half contributing a key proton donor (Asp-274) in trans to the Pchlide molecule bound in the active site of the opposing subunit (3, 4).

generated similar changes in fluorescence (22). Thus, such changes in Pchlide fluorescence were ascribed to nonspecific solution partition effects. This is not the case for Pchlide binding to the BchNB complex. Under our reaction conditions, BSA does not produce a change in Pchlide fluorescence (Fig. 6b). An increase in Pchlide fluorescence is observed as a function of BchNB concentration (Fig. 6b). The signal plateau when a stoichiometric amount of BchNB is present to bind all the Pchlide molecules in the reaction. Curiously, when an excess of BchNB is added to the reaction the fluorescence does not plateau. Instead, a drop in the Pchlide fluorescence is observed (Fig. 6b). As the concentration of BchNB is increased in the reaction, the equilibrium likely shifts from a 2-Pchlide–bound BchNB complex (higher fluorescence) to a 1-Pchlide–bound complex (lower fluorescence). The quantum yield of the 2-Pchlide–bound complex is likely different from the 1-Pchlide–bound complex. Given the asymmetry in BchNB activity, we propose that one Pchlide is bound in a conformation different from the other. The conformational differences likely contribute to two different quantum yields for Pchlide fluorescence when bound to BchNB.

When similar binding experiments are performed with the BchNB$^{D274A}$ variant, we observe a similar overall profile where an increase in Pchlide fluorescence is observed. The signal saturates stoichiometrically, as observed for WT BchNB. Thus, both active sites are Pchlide bound (Fig. 6b). However, for BchNB$^{D274A}$, the fluorescence quantum yield upon reaching stoichiometry is half that observed for WT BchNB. These data suggest that the conformational positioning and/or electronic landscape of the bound Pchlide molecules are different between BchNB$^{D274A}$ and WT BchNB. Because the recombinantly generated BchNB$^{D274A}$ protein is pre-reduced, the loss in Pchlide fluorescence for BchNB$^{D274A}$ could also arise from the electron being transferred to Pchlide.

Extrapolating back to the EPR results, the spectra obtained for the WT BchNB likely reflect one electron transferred to each Pchlide molecule. This could function as a sensing step to recognize and lock in the Pchlide within the active site. The EPR spectra for BchNB$^{D274A}$, measured under the same conditions, also show ET to the bound Pchlide molecules (Fig. 5a). In BchNB$^{D274A}$, the ability to donate the first proton from Asp-274 to Pchlide is perturbed. In the presence of Bchl and ATP, complex formation between Pchlide-bound BchNB and ATP-bound Bchl occurs. This re-reduces the [4Fe-4S] cluster on BchNB. The extent of re-reduction is 50% in the BchNB$^{D274A}$ variant and thus Pchlide reduction is not achieved in either active site. This evidence points to asymmetric and sequential ET in the DPOR system and the Asp-274 residue serves as a key sensor in communicating the electron transfer status between the two halves.
allosteric and direct communication between the two halves likely controls the catalytic steps in the ET and Pchlide reduction mechanisms. Our findings support this model and show communication between the two active sites. In the half-reactive engineered version of BchNB, where one site is WT and the other carries a D274A substitution, Pchlide reduction activity in both halves is abolished. Thus, in this scenario, the half-reactive enzyme does not go through multiple rounds of ET required for Pchlide reduction. The proposed sequence of catalytic events for DPOR activity involves the following.

1) Pchlide binding to both active sites of BchNB: One possibility with respect to Pchlide binding to BchNB, where one site is WT and the other carries a D274A substitution, Pchlide reduction activity in both halves is abolished. Thus, in this scenario, the half-reactive enzyme does not go through multiple rounds of ET required for Pchlide reduction. The proposed sequence of catalytic events for DPOR activity involves the following.

   a. Pchlide binding to both active sites of BchNB: One possibility with respect to Pchlide binding to BchNB is an inherent built-in asymmetry when synthesized in the cell and the binding sites are structurally asymmetric. This could dictate the order of Pchlide binding to the two active sites. Alternatively, the active sites are identically poised to accept Pchlide and stochastic binding of Pchlide to one or the other site establishes asymmetry within BchNB. Contrary to previous models, our data show that the [4Fe-4S] cluster of BchNB exists in a reduced state and is able to donate the first electron to Pchlide upon binding in the absence of Bchl. In the steady state experiments, the EPR spectra corresponding to the [4Fe-4S] cluster of BchNB shows a persistent reduced state well after the time course of Pchlide reduction (Fig. 4). This mechanism is similar to the deficit spending model proposed for nitrogenase, where the first ET in the MoFe-protein occurs before binding of the Fe protein (19). Whether this first ET occurs sequentially in both active sites of BchNB or just within one site (thus maintaining asymmetry) is yet to be determined. Such sequential ET occurs in nitrogenase where ET in one half allosterically suppresses catalytic events in the other (21, 23). Another interesting observation is the difference in pre-reduced states of our WT BchNB and the BchNB^D274A variant. The [4Fe-4S] cluster of BchNB^D274A is always pre-reduced and is visible in EPR (Fig. 5a). The WT BchNB protein is EPR silent (Fig. 3c). This difference suggests conformational differences around the [4Fe-4S] cluster between the WT and variant BchNB complexes. However, both proteins bind to two Pchlide molecules (Fig. 6b).

   We propose that the Asp-276 residue is used to sense and communicate the presence of Pchlide molecule within the active site and the deficit spending mechanism might be an integral part of this process. This sensing and selection of Pchlide likely serves two roles: (a) it correctly positions the porphyrin-ring structure of Pchlide within the active site, and (b) it helps DPOR differentiate between Pchlide and Chlide. Chlide is the reduced product of Pchlide and must dissociate from the active site of DPOR. Chlide then binds to the active site of COR, the next enzyme in the pathway. COR is proposed to be structurally similar to DPOR, but the difference between Pchlide and Chlide is one double bond in the C17=C18 position of the porphyrin ring. Thus, the initial positioning and sensing of Pchlide by the Asp-274 residue likely serves as a substrate check sensing mechanism within the active site of BchNB.

2) The next step in the DPOR mechanism is the binding of Bchl to BchNB: We do note here that although it is logical to think of Pchlide binding to BchNB as the first step, followed by complex formation with Bchl, these steps need not be sequential or dependent on one other. Bchl forms a complex with
BchNB in the absence of Pchlide and transfers electrons (Fig. 3c), thus, a direct measure of the kinetics and thermodynamics of complex formation between BchNB and BchL. The influence of Pchlide, would be required to better understand the complexities underlying complex formation in DPOR. Similarly, when BchL binds to BchNB, these events can occur on both halves of the BchNB complex. Whether these binding events are stochastic and cooperative/independent and how they contribute to overall asymmetry remains to be investigated. The crystal structure of DPOR bound to the ATP-analog ADP-αF stabilized a transition-state complex with BchNB bound to BchL on both halves (4). In the absence of ATP, there is no ET from BchL to BchNB (Fig. 3c). Thus, at a minimum, complex formation between BchL and BchNB is transient and coupled to ATP binding and hydrolysis within BchL.

3) ET from the [4Fe-4S] cluster of BchL to the [4Fe-4S] cluster on BchNB and then onto Pchlide occurs upon complex formation: As stated above, whether ET occurs independently within the two halves remains to be established. Our data provide strong evidence that communication regarding ET from one half is relayed to the other through Asp-274. Substitution of Asp-274 in just one half of BchNB stalls Pchlide reduction. Thus, the two halves are synchronized with respect to the steps in their Pchlide reduction cycles. In nitrogenase, we showed negative cooperativity and allosteric communication between the two halves (21). ET occurs first in one half, and this process is suppressed in the other, thus establishing a sequential ET mechanism between the two halves. Because the half-active DPOR complex fails to reduce Pchlide in both halves, we propose that such asymmetry also exists in the DPOR system.

The commonalities between DPOR and nitrogenase with respect to communication and allostery between the two halves suggest that sequential ET might be a theme embedded in other such α2β2-structured enzymes. Evolutionary functional advantages to such structures could be used to direct the flow of electrons, substrate-binding selectivity, and overall efficiency of ET. Both nitrogenase and DPOR catalyze substrate reduction reactions requiring multiple rounds of ET. The allosteric and sequential ET process could also be utilized to count/calibrate the number of electrons that accumulate at the metal clusters and on the substrate. It would be interesting to explore such mechanistic differences between α2β2 enzyme complexes that catalyze single versus multiple ET-dependent reduction chemistries.

**Experimental Procedures**

**Buffers and reagents**

Chemicals were purchased from Millipore Sigma, Research Products International, and Gold Biotechnology. Chromatography resins were from GE Healthcare Life Sciences. Standard (STD) buffer is composed of 100 mM HEPES, pH 7.5, 150 mM NaCl, and 1.7 mM sodium dithionite. 10 mM MgCl2 is added to the STD buffer where denoted.

**Generation of protein expression constructs**

Plasmids used to recombinantly produce BchL and BchNB are described (24). Briefly, open reading frames for BchL, BchN, and BchB were PCR amplified from genomic DNA extracted from *R. sphaeroides* (a kind gift from Dr. Scott Ensign, Utah State University) and cloned into respective vectors. BchL and BchB open reading frames were engineered to carry an N-terminal poly-histidine (His6) tag and 3C protease recognition sites and were cloned into pRSF-Duet1 using BamH1/Not1 and SacI/SalI restriction sites, respectively. BchN contained no modifications and was cloned into a pET-Duet1 vector using Nde1/Kpn1 restriction sites. The BchB-pRSF parent plasmid was used as a template to engineer N- or C-terminal poly-histidine or Strep-tags onto BchB using Q5 site-directed mutagenesis (New England Biolabs).

**Generation of Pchlide**

Pchlide was generated as described (24) using a *R. capsulatus* ZY-5 strain harboring a deletion of the BchL gene (a kind gift from Dr. Carl Bauer, Indiana University) (8).

**Protein expression and purification**

WT/variant BchNB and BchL protein complexes were purified as described (24). The following modified procedure was used for purification of the half-reactive BchNB complex. Plasmids encoding BchN, His-BchB, and Strep-BchB were co-transformed into BL21(DE3) SufeScient cells. The transformants were grown, and protein overproduction was induced as described for the WT BchNB complex. After cell lysis, the clarified lysates from His-tagged or dual-tagged constructs were loaded onto a Ni2+-NTA (1.6 ml suspended beads/liter growth) column (Thermo Scientific), equilibrated with 10 column volumes (CV) STD buffer (100 mM HEPES, pH 7.5, 150 mM NaCl, 1.7 mM sodium dithionite). Nonspecifically bound proteins were washed off with 10 CV STD buffer containing 20 mM imidazole. Bound proteins were eluted into a septum-sealed bottle using 30 ml STD buffer containing 250 mM imidazole. The clarified lysates from Strep-tagged BchNB were loaded onto a Strep-Tactin Sepharose column (IBA Scientific, Germany) (1.6 ml resuspended beads/liter growth) equilibrated with 10 CV STD buffer. Post washing with 10 CV STD buffer, bound proteins were eluted using 30 ml STD buffer containing 25 mM desthiobiotin (IBA Scientific). Asymmetric or dual-tagged BchNB constructs followed the methods described for Ni2+-NTA columns and for Strep columns but performed sequentially. Eluted BchNB proteins were concentrated using a spin concentrator (30 kDa molecular weight cut-off). Proteins were aliquoted in the glove box into 1.2 ml cryo-tubes which have a gasket sealed cap (Corning Scientific). Closed tubes with protein were removed from the glove box and flash frozen using liquid nitrogen and stored under liquid nitrogen. Protein concentrations were determined using Bradford reagent with BSA as reference.

**Western blotting**

Western blotting was performed on samples after separation on 10% SDS-PAGE. Proteins were transferred (100 mA for 90 min) onto nitrocellulose membranes. Membranes were washed thrice with TBS plus 0.1% (v/v) Tween-20 (TBST) and rocked for 15 min after the third rinse. Membranes were blocked with...
**Mechanism of action of DPOR**

5% (w/v) milk in TBST for 1 h at 25°C. Membranes were then washed thrice with TBST and exposed to primary antibody. Membranes were exposed to StrepMAB-Classic HRP conjugate (IBA Scientific) primary antibody (1:34,000 dilution in TBST) for 1 h while rocking at 25°C and/or anti–poly-histidine antibody, HRP conjugate (Invitrogen) (1:1000 dilution in TBST plus 1% (w/v) milk) overnight (~17 h) while rocking at 4°C. Membranes were then washed, rocked for 3 h after the third rinse before adding detection reagent (Pierce Fast Western Blotting Kit, ECL Substrate, Thermo Scientific). Blots were imaged using an Al 600 imager (GE Healthcare).

**Assay for substrate reduction by DPOR**

Reduction of Pchlide to Chlide was measured spectrophotometrically by mixing BchNB (3 μM tetramer), BchL (9 μM dimer), and 35 μM Pchlide, in the absence or presence of ATP (3 mM) in STD buffer containing 10 mM MgCl₂ and then incubated for 20 min before being transferred to an airtight 2 ml SEPTA screw cap cuvette (Firefly Scientific). Fluorescence emission spectra were recorded on a PTI fluorometer (Photon Technology International) excited at 440 nm, with excitation and emission slit widths of 1.5 mm and 3.0 mm, respectively. PMT voltage and slit widths were maintained between replicates. Data were fit to a 2-site independent binding model using Origin: 

\[
y = \left( \frac{B_{\text{max1}}^* x}{K_1 + x} \right) + \left( \frac{B_{\text{max2}}^* x}{K_2 + x} \right)
\]

where \(B_{\text{max1}}^*\) and \(B_{\text{max2}}^*\) are the respective amplitude of the fluorescence signal, and \(K_1\) and \(K_2\) are the associated binding constants for the two binding sites in the BchNB complex.

**Data availability**

All data are contained within the manuscript. Plasmids used for protein overexpression are available upon request.

**Author contributions**—E. I. C., B. B., and E. A. formal analysis; E. I. C., B. B., and E. A. investigation; E. I. C. and E. A. visualization; E. I. C., B. B., and E. A. methodology; E. I. C., B. B., and E. A. writing-original draft; E. I. C., B. B., and E. A. writing-review and editing; B. B. and E. A. supervision; B. B. validation; E. A. conceptualization; E. A. funding acquisition; E. A. project administration.

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: ET, electron transfer; DPOR, dark-operative protochlorophyllide oxidoreductase; Pchlide, protochlorophyllide; Chlide, chlorophyllide; ; COR, chlorophyllide-stabilized protochlorophyllide oxidoreductase complex. Membranes were exposed to StrepMAB-Classic HRP conjugate (IBA Scientific) primary antibody (1:34,000 dilution in TBST) for 1 h while rocking at 25°C and/or anti–poly-histidine antibody, HRP conjugate (Invitrogen) (1:1000 dilution in TBST plus 1% (w/v) milk) overnight (~17 h) while rocking at 4°C. Membranes were then washed, rocked for 3 h after the third rinse before adding detection reagent (Pierce Fast Western Blotting Kit, ECL Substrate, Thermo Scientific). Blots were imaged using an Al 600 imager (GE Healthcare).

**EPR spectroscopy**

EPR spectra were obtained at 10 K on an updated Bruker EMX-AA-TDU/L spectrometer equipped with an ER4112-SHQ resonator (9.48 gigahertz) and an HP 5350B microwave counter for precise frequency measurement. Temperature was maintained with a ColdEdge/Bruker Stinger S5-L recirculating helium refrigerator, and an Oxford ESR900 cryostat and MercuryITC temperature controller. Spectra were recorded with either 0.3 gauss (3 μT) or 1.2 gauss (0.12 mT) digital field resolution with equal values for the conversion time and the time constant, 5.2 milliwatt incident microwave power, and 12 gauss (1.2 mT) magnetic field modulation at 100 kHz. EPR simulations were carried out using Easyspin (25). Samples for EPR spectroscopy contained various combinations of BchL, BchNB, Pchlide, and ATP. 200 μl EPR samples contained 1.7 mM dithionite, and, where indicated in the figure legends, 40 μM BchL, 20 μM BchNB, 40 μM Pchlide, and 3 mM ATP. Experiments shown in Fig. 5a contained 60 μM BchNB and/or 120 μM Pchlide. All samples were incubated in the glove box for 60 min unless otherwise denoted. Protein samples were prepared and transferred to the EPR tubes in the glove box and capped with a butyl rubber stopper. Samples were removed from the glove box and immediately flash frozen in liquid nitrogen and analyzed by EPR.

**Steady-state pchlide binding fluorescence titrations**

2.0-ml mixtures of BchNB (various concentrations) and Pchlide (20 μM) were made in the glove box in STD buffer with 10 mM MgCl₂ and then incubated for 20 min before being

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