Broadened Substrate Specificity of 3-Hydroxyethyl Bacteriochlorophyllide a Dehydrogenase (BchC) Indicates a New Route for the Biosynthesis of Bacteriochlorophyll a*

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Background: Bacteriochlorophyll biosynthesis is fundamental for the photosynthetic capture of solar energy by photosynthetic bacteria.

Results: 3-Hydroxyethyl chlorophyllide a was identified as a novel substrate of 3-hydroxyethyl bacteriochlorophyllide a dehydrogenase (BchC).

Conclusion: The broadened substrate specificity of BchC would allow for a novel branched pathway for bacteriochlorophyll a biosynthesis.

Significance: The observed plasticity of the pathway might be relevant for the engineering of photosynthetic organisms.

Bacteriochlorophyll a biosynthesis requires formation of a 3-hydroxyethyl group on pyrrole ring A that gets subsequently converted into a 3-acetyl group by 3-vinyl bacteriochlorophyllide a dehydratase (BchF) followed by 3-hydroxyethyl bacteriochlorophyllide a dehydrogenase (BchC). Heterologous overproduction of Chlorobaculum tepidum BchF revealed an integral transmembrane protein that was efficiently isolated by detergent solubilization. Recombinant C. tepidum BchC was purified as a soluble protein-NAD⁺ complex. Substrate recognition of BchC was investigated using six artificial substrate molecules. Modification of the isocyclic E ring, omission of the central magnesium ion, zinc as an alternative metal ion, and a non-reduced B ring system were tolerated by BchC. According to this broadened in vitro activity, the chlorin 3-hydroxyethyl chlorophyllide a was newly identified as a natural substrate of BchC in a reconstructed pathway consisting of dark-operative protochlorophyllide oxidoreductase, BchF, and BchC. The established reaction sequence would allow for an additional new branching point for the synthesis of bacteriochlorophyll a. Biochemical and site-directed mutagenesis analyses revealed, in contrast to theoretical predictions, a zinc-independent BchC catalysis that requires NAD⁺ as a cofactor. Based on these results, we are designating a new medium-chain dehydrogenase/reductase family (MDR057 BchC) as theoretically proposed from a recent bioinformatic analysis.

The capturing of the global energy demand vitally depends on the biosynthesis of chlorophylls (Chls) and bacteriochlorophylls (BChls). These tetrapyrrole pigments are essential for light capturing, the transfer of the obtained excitation energy, and the primary charge separation process of photosynthesis (1). The light absorption and redox properties of BChls and Chls are modulated by the peripheral substituents of the macrocycle. However, the most important diversification concerns the aromatic system (Fig. 1B): the chlorin ring structure characteristic for Chls and e.g. BChl c (reduced D ring at C17/C18; \( \lambda_{max}, \text{Qy-band} \approx 650–700 \text{ nm} \)) and the bacteriochlorin ring structure specific for BChls (additionally reduced B ring at C7/C8; \( \lambda_{max}, \text{Qy-band} \approx 770–800 \text{ nm} \)) (2).

The biosynthesis of the core ring structure of Chls and BChls starts with the ATP-dependent magnesium insertion into the fully conjugated protoporphyrin IX molecule. The following two reaction steps are then responsible for the formation of the fifth, isocyclic ring E. The propionate side chain at ring C is methylated, and the resulting magnesium protoporphyrin IX monomethyl ester is subsequently converted into 8-vinyl protochlorophyllide in a sophisticated oxidation/cyclization reaction. Then the reduction of the peripheral vinyl group at C8 results in the formation of protochlorophyllide a (Pchlide; compound 11; see Fig. 5) (for a review of Chl biosynthesis, see Ref. 2).

Subsequently, reduction of the C17/C18 double bond of ring D leads to the formation of the chlorin ring structure of chlorophyllide a (Chlide; compound 12). Two unrelated enzymes have developed for the regio- and stereospecific reduction of the fully conjugated Pchlide ring system: light-dependent protochlorophyllide oxidoreductase performs a light-driven, NADPH-dependent reduction, whereas the ATP-dependent multisubunit enzyme dark-operative protochlorophyllide a oxidoreductase (DPOR) uses nitrogenase-like biochemistry (3–6).

At this point, BChl a and Chl a biosynthesis pathways diverge. Chlide is the immediate precursor of Chl a, which is

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2The abbreviations used are: Chl, chlorophyll; BChl, bacteriochlorophyll; BChlide, bacteriochlorophyllide; BPhide, bacteriopheophorbide, Chlide, chlorophyllide a; COR, chlorophyllide a oxidoreductase; DPOR, dark-operative protochlorophyllide a oxidoreductase; MDR, medium-chain dehydrogenase/reductase; Pchlide, protochlorophyllide a; Pheide, pheophorbide; 3'-OH-Chlide, 3-hydroxyethyl chlorophyllide a.
formed via esterification of a polyisoprene alcohol to the C17 propionic acid moiety (7).

The synthesis of BChl \( \alpha \) requires three additional enzymatic steps involving the following enzymes: (i) chlorophyllide \( \alpha \) oxidoreductase (COR), the second enzyme of the pathway, that performs ATP-dependent nitrogenase-like catalysis (8, 9); (ii) 3-vinyl bacteriochlorophyllide \( \alpha \) hydratase (BchF), which is responsible for the hydroxylation of the 3-vinyl group; and (iii) 3-hydroxyethyl bacteriochlorophyllide \( \alpha \) dehydrogenase (BchC), which catalyzes the subsequent oxidation of the 3-hydroxyethyl group into a 3-acetyl moiety.

Pioneering work for the elucidation of the \( bchf \) and \( bchC \) gene functions was the spectroscopic characterization and identification of accumulated BChl \( \alpha \) precursor molecules in the respective mutants of different \textit{Rhodobacter} strains (10–15). Whole cell isotope labeling experiments revealed that the oxygen atom at C3\(^1\) was derived from a water molecule using the purple bacteria \textit{Rhodobacter sphaeroides} and \textit{Roseobacter denitrificans} (16). Based on the spectroscopic analysis of the \textit{Rhodobacter capsulatus} \textit{bchF} mutant strains MB1003, KZK8G9, and CB1200 (10, 11, 17, 18) and the \textit{bchC} mutants \textit{R. capsulatus} CW100 (13) and \textit{Sphaeroides} T127 and TB34 (12, 19), the following two enzyme order variants of the BChl \( \alpha \) biosynthetic pathway were concluded (compare also Fig. 5).

\[
\text{DPOR} \to \text{COR} \to \text{BchF} \to \text{BchC}
\]
\text{Reaction Sequence I}

\[
\text{DPOR} \to \text{BchF} \to \text{COR} \to \text{BchC}
\]
\text{Reaction Sequence II}

Accordingly, the metabolite Chlide is located at the branching point of the pathway, implying extended substrate specificity for the enzymes COR and BchC.

Plasticity of the late steps of BChl/Chl biosynthesis was also proposed with respect to the 8-vinyl reduction step and for the formation of the 7-formyl group of Chl \( b \) on the basis of \textit{in vitro} experiments using chemically modified substrate variants. An 8-vinyl derivative (instead of the 8-ethyl substrate) and the 7-formyl analog (instead of the 7-methyl substrate) were efficiently converted by DPOR (20).

The green sulfur bacterium \textit{Chlorobaculum tepidum} is a strict anaerobe and an obligate photoautotroph. It is a well established model organism for the investigation of anoxygenic photosynthesis, in particular under conditions of very low light intensity (21–23). The main photosynthetic pigments of \textit{C. tepidum} are BChl \( c \), Chl \( a \), and BChl \( \alpha \). The term “BChl \( c \)” names a group of pigments differing from other BChls and Chls: BChl \( c \) molecules contain a chiral hydroxylated center at C3\(^1\), lack a 13\(^2\)-methylcarboxylate group, and possess a characteristic C20 methyl group together with a varying number of additional methyl groups at C8\(^2\) and C12\(^1\) (for a review, see Ref. 24). Despite the established designation as a BChl, all BChl \( c \) pigments possess a chlorin instead of a bacteriochlorin ring system (Fig. 1B). BChl \( c \) molecules are the major light-harvesting pigments that are present in large antenna structures called chlorosomes where they self-assemble into tubular nanostructures that are stabilized by interpigment magnesium ligation and \( \pi-\pi \) and H-bonding interactions (25, 26).

Chl \( a \) acts as the primary electron acceptor in the photochemical reaction centers of \textit{C. tepidum}. BChl \( \alpha \) also plays a fundamental role in the reaction center, and it is also found in antenna proteins of the chlorosome envelope (for a review, see Ref. 27).

With respect to the late steps of BChl \( \alpha \) biosynthesis, only the sophisticated enzymology of the multisubunit COR enzyme has been well characterized (8, 9, 28–31). Besides this, the \textit{bchF} gene from \textit{C. tepidum} has been shown to complement a \textit{bchF}-deficient \textit{R. capsulatus} mutant (32). No further biochemical characterization of any BchF or BchC enzyme is available from the literature to date.

In the present investigation, we made use of recombinant DPOR, BchF, and BchC proteins to analyze the BChl biosynthesis pathway of \textit{C. tepidum}. A series of artificial BchC substrates allowed for the characterization of the substrate specificity of BchC. These results, in combination with a DPOR-BchF-BchC pathway reconstitution assay, would allow for a novel branched biosynthetic route via 3-hydroxyethyl chlorophyllide \( a \) (3\(^1\)-OH-Chlide; compound 12) as a BchC substrate. In addition, the cofactor specificity and metal requirement of BchC were characterized.

**Experimental Procedures**

**Cloning and Production of \textit{C. tepidum} BchF and BchC—**

Genes \textit{bchf} and \textit{bchC} from \textit{C. tepidum} strain TLS were PCR-amplified using primers GCAGGATCCGATGCGCTTTATACACC and GAAGGTGCAGCTCAGGCTCCCGCTG (\textit{bchf} pACYC_Duet-1), GCAGGATCCATGGAAGCGAGAAGATCC and GCAGGATCTAGTCAGTGGCTGGGCGTGGTTC (\textit{bchC} pGEX6P-1), or GACCATATGGAAAGCGAGAATAATCAAAGCC and GACCTCGAGTTGCGCAGTGGCG (\textit{bchC} pGEX6P-1; integrated restriction sites are shown in italics). PCR products were ligated into the BamHI and SalI sites of pACYC_Duet-1 (\textit{bchf}, first multiple cloning site) (Merck Millipore Novagen\textsuperscript{®}) and pGEX6P-1 (\textit{bchC}) (GE Healthcare) to yield plasmids pACYC-F (coding for a His\(_{16}\)-BchF fusion protein) and pGEX-C (coding for a GST-BchC fusion protein). Subsequently, the \textit{bchC} gene was cloned into the second multiple cloning site of pACYC-F via restriction sites Ndel and Xhol to yield plasmid pACYC-FC. The three plasmids were individually transformed into \textit{Escherichia coli} BL21(DE3). Overnight cultures were used to inoculate 500 ml of LB medium containing the respective antibiotics. Protein production in the strains containing plasmids pACYC-F and pACYC-FC (or pGEX-C) was induced at an \( A_{\text{growth}} \) of ~0.5 by the addition of 300 \( \mu \text{M} \) (50 \( \mu \text{M} \)) isopropyl \( \beta\)-D-thiogalactopyranoside. After 3 h (14 h) of cultivation at 37 °C (17 °C) at 200 rpm (180 rpm), the cells were harvested, resuspended in 10 ml of ice-cold buffer 1 composed of 50 mM Tris-HCl, pH 8.0 (20 ml/liter of culture buffer 2 composed of 50 mM Tris-HCl, 500 mM NaCl, pH 8.0), and stored at −20 °C.

Production of a Cell-free Extract Containing \textit{C. tepidum} DPOR—**Production of \textit{C. tepidum} DPOR subunits N, B, and L was mainly performed as described earlier (plasmid pGEX-bchNBL (5)) using 500 ml of LB medium containing 1 mM L-cysteine and 1 mM Fe(III) citrate to improve iron-sulfur cluster
formation. All remaining steps were performed under anoxic conditions at an oxygen partial pressure below 1 ppm (oxygen detector, Coy Laboratories, Grass Lake, MI). Cells were harvested and resuspended in 10 ml of anoxic buffer 3 (100 mM HEPES-NaOH, 150 mM NaCl, 10 mM MgCl₂, pH 7.5). The cell-free extract containing all three DPOR subunits was obtained by a single French press passage (16,000 p.s.i.) and subsequent ultracentrifugation in sealed anoxic tubes (4 °C, 65 min, 130,000 × g). The supernatant was stored as 500-μl aliquots in anoxic bottles at −20 °C for up to 3 months.

Production of a C. tepidum BchF- and BchC-containing Extract—The resuspended pellet from 500 ml of an E. coli culture containing pACYC-FC was passed through a French press (16,000 p.s.i.), and the crude cellular extract was stored on ice.

Solubilization of BchF—The resuspended pellet from 500 ml of E. coli culture overproducing His₆-BchF (pACYC-F) was disrupted by a single French press passage (16,000 p.s.i.). For the BchF activity assays, this crude cellular extract was stored on ice for up to 6 h. His₆-BchF was solubilized from the host membrane fraction. For this purpose, the crude cellular extract was subjected to low speed centrifugation (4 °C, 60 min, 4,000 × g). The resulting supernatant was then subjected to ultracentrifugation (4 °C, 60 min, 146,000 × g). The obtained supernatant was discarded, and the membrane pellet was washed twice with 3 ml of ice-cold buffer 4 (50 mM Tris-HCl, 20 mM MgCl₂, pH 8.0). Subsequently, membrane proteins were solubilized from the membrane fraction by stirring at 4 °C for 2 h in 7 ml of solubilization buffer (buffer 4 containing 0.6% (v/v) Triton™ X-100). After another ultracentrifugation step (4 °C, 60 min, 146,000 × g), the supernatant containing the solubilized membrane proteins was stored on ice for subsequent activity measurements. Integrity of the His₆-BchF protein fraction was confirmed by N-terminal sequencing.

Purification of C. tepidum BchC—The resuspended pellet from 1 liter of E. coli cell culture overproducing GST-BchC (from plasmid pGEX-C) was thawed on ice for 12 h, 2 μl Benzonase® (Merck Millipore) was added, and the cells were disrupted by a single passage through a French press at 16,000 p.s.i. Cell debris was removed by ultracentrifugation (4 °C, 65 min, 130,000 × g), and the supernatant was applied to 1 ml of ProRPC agarose (Merck Millipore) and subjected to BchC activity assays. The liberated BchC protein was eluted with 3 ml of buffer 2 and stored at 4 °C.

Determination of Protein Concentration—The concentration of purified protein fractions was determined using the Bradford reagent (Sigma-Aldrich) according to the manufacturer’s instructions with bovine serum albumin dissolved in buffer 2 as a standard.

N-terminal Amino Acid Sequence Determination—Automated Edman degradation was used to confirm the identity of the solubilized BchF and the purified BchC protein.

Chelator Treatment of BchC—Purified BchC samples were incubated in the presence of EDTA or EGTA at concentrations of 10 or 100 mM (75 min at 48 °C). After a centrifugation step (10 min, 12,100 × g, room temperature), protein solutions were buffer-exchanged to buffer 2 using an “illustra” NAP-5 column (GE Healthcare) according to the manufacturer’s instructions. Protein samples were concentrated using an Amicon® Ultra-0.5 centrifugal filter device (10,000 nominal molecular weight limit; Merck Millipore) and subjected to BchC activity assays.

Artificial Substrates of BchC—Compounds 1–6 (Fig. 1, compare B and C) were dissolved in DMSO. Their respective synthesis and/or isolations are cited in Fig. 1C.

BchC Activity Assays in the Presence of Artificial Substrates—Qualitative in vitro activity assays containing 3.2–8.4 μM BchC were performed in the presence of 1 mM NAD⁺ (or alternatively 1 mM NADH) and 750 mM–23 μM substrates 1–6 in a total volume of 250 μl of buffer 1. Reactions were incubated for 30 min at 34 °C in the dark and subsequently stopped by addition of 500 μl of acetone. Insoluble assay components were sedimented (two times, 17,000 × g, 10 min, 4 °C), and the supernatant was analyzed by UV-visible light absorption spectroscopy as described elsewhere (9).

The specific activity of BchC in the presence of compounds 1–3, 5, and 6 was determined in the linear range of the activity assay (100 nM–1 μM purified enzyme and 8 μM substrate) in the presence of 1 mM NAD⁺ or NADH in a volume of 550 μl. Compounds 1 and 5 were epimeric 3'R/S mixtures; hence an effective substrate concentration of ~4 μM was implied (60). At selected time points, 100-μl samples were taken and mixed with 400 μl of acetone. Samples were analyzed as described above. The following extinction coefficients were used to calculate specific activities: Zn-3-acetyl-Pheide α, ε = 65.2 mm⁻¹ cm⁻¹ (33); Zn-3-acetyl-132-OH-Pheide α, ε = 65.2 mm⁻¹ cm⁻¹ (33); BPheide α, ε = 67.5 mm⁻¹ cm⁻¹ (34); Zn-BPheide α, ε = 67.7 mm⁻¹ cm⁻¹ (34). It is worth noting that esterified pigments (at C173) show an extinction coefficient of the Q band identical to that observed for the non-esterified derivatives. Chelator-treated BchC samples (3.2 μM) were assayed in the presence of compound 1 (10 μM) or 5 (2.6 μM) for 30 min.

Single Turnover BchC Experiments—The purified BchC-NAD⁺ complex was assayed in 250 μl of buffer 1 in the presence of 2.9 μM substrate 5. A negative control (without protein) and experiments with increasing concentrations of BchC (0.8, 2.0, 4.0, 6.0, and 8.0 μM) were incubated in the absence of external cofactors for 3 h at 34 °C in the dark. A control assay was analogously performed in the presence of 4 μM BchC and 1 mM NAD⁺.

Isolation of Pchlide—Pchlide was isolated from R. capsulatus strain ZY5 (35) as described elsewhere (36) using PY medium (3 g/liter peptone, 3 g/liter yeast extract) containing 1 mM MgCl₂, 0.2% (w/v) l-lysine, 0.3% (w/v) l-tryptophan, and 5 μg/ml kanamycin. Pchlide was dissolved in DMSO.

Isolation of Chlide—Chlide was isolated from R. capsulatus strain CB1200 (11) as outlined before using RCV 2/3 PY medium containing 12 mg/liter FeSO₄ under dimmed light conditions (37). The pigment was dissolved in DMSO and stored at −20 °C.

Reconstitution of the DPOR-BchF-BchC Pathway—Coupled enzymatic assays were initiated in an anoxic chamber (Coy Laboratory Products Inc.). DPOR activity from 40 μl of the cell-free
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E. coli extract (preparation described above) was analyzed in the presence of 5 μM Pchlide, 0.72 mM dithionite as an artificial electron donor, 5 mM dithiothreitol, 2 mM ATP, and an ATP-regenerating system (20 mM creatine phosphate and 20 units of creatine phosphokinase) in 250 μl of buffer 3 (34 °C, 600 rpm, 60 min, in the dark). Coupled DPOR-BchF assays were subsequently completed (under oxic conditions) by the addition of 100 μl of a crude cellular BchF extract or alternatively 100 μl of a purified, detergent-solubilized BchF sample (as described above). Coupled DPOR-BchF-BchC experiments were either supplemented with 1.6–3.9 μM purified BchC, or alternatively, 100 μl of a crude cellular extract from a BchF/BchC-overproducing E. coli strain were used. As cofactor, 500 μM NADP⁺ (or alternatively NADP⁺) were used. For all experiments, buffer 1 was added to a final volume of 500 μl, and samples were incubated for another 30 min at 34 °C. Assays were stopped by the addition of 1 ml of acetone, and the pigment composition was analyzed as described above.

Low Temperature Fluorescence Spectroscopy—Coupled DPOR-BchF-BchC activity assays were analyzed by low temperature fluorescence spectroscopy (at 77 K) using a FP-8500 fluorometer equipped with a PMU-830 cryoholder (Jasco, Gross Umstadt, Germany). Samples of 750 μl in NMR vials (5-mm outer diameter) were cooled to liquid nitrogen temperature, and the characteristic excitation and emission maxima of Chlide, 3'-OH-Chlide, and 3-acetyl-Chlide were determined.

HPLC Analysis of Intermediates of the Reconstituted Pathway—HPLC analyses in combination with multiwavelength fluorescence detection were used to further characterize the intermediates of the reconstituted pathway. Samples from all pathway reconstitution experiments were analyzed on a Jasco HPLC system equipped with an FP-1520 multichannel fluorescence detector using an UltraSep ES RP18 column (SEPSERV). GC-mediates of thereconstituted pathway. Samples from all path-

Metal Determination—The zinc content of the recombinant purified BchC protein was determined by inductively coupled plasma mass spectrometry (Currenta GmbH and Co. OHG, Leverkusen, Germany). In a parallel affinity purification, a metal-independent GST-protein (alanyl-phosphatidylglycerol synthase (40)) was purified and analogously analyzed by mass spectrometry to assess the background level of the experimental approach used.

Results

C. tepidum BchF Is an Integral Membrane Protein—In the current literature, a 3-vinyl bacteriochlorophyllide a hydratase activity is ascribed to the BchF protein (ORF CT1421) due to the observation of substrate accumulation in the respective R. capsulatus mutant strains (10, 11). Bioinformatics analysis of the BchF amino acid sequence using the TMpred program (41) revealed four putative membrane-spanning helices comprising the transmembrane segments Ile23–Tyr42, Thr56–Glu77, Phe94–Gln113, and His118–Gln140 according to C. tepidum numbering. The recombinant His₆-BchF protein was solubilized from the total membrane fraction (Fig. 1A, left). Bacterial cells were disrupted (Fig. 1A, lane 2), and residual unbroken cells (and potential inclusion bodies) were removed by low speed centrifugation. The supernatant was then subjected to high speed centrifugation (supernatant; lane 3), and the sedimented (membrane) fraction was solubilized. After another high speed centrifugation, the final purification step revealed a single dominant BchF protein band (lane 4, arrow) that was confirmed by N-terminal amino acid sequencing. The SDS-PAGE mobility of BchF (Mᵣ ~ 25,000) significantly differed from the calculated molecular mass of 20,008 Da as observed for other trans-

Purification of the Soluble BchC Protein from C. tepidum—The subsequent enzyme of the pathway has been annotated as BchC (ORF CT1422) due to substrate accumulation in mutants of different Rhodobacter species (11–15). The recombinant GST–BchC fusion protein was localized in the soluble cytosolic fraction of the E. coli host used (Fig. 1A, right, lane 6). The GST-tagged protein was purified by affinity chromatography (lanes 7–9), and subsequently the native BchC protein was liberated by on-column PreScission protease treatment (lanes 10–12). Overall, 3 mg of an almost homogeneous protein were obtained from 1 liter of cell culture. The integrity of this protein with a relative molecular weight of 37,000 (calculated molecular mass, 37,068 Da) was confirmed by N-terminal amino acid sequencing. The purified BchC was stable for several weeks when stored at 4 °C.

Artificial Substrates of BchC—Synthetic modified substrate molecules were tested to identify important determinants for substrate recognition (20, 33, 43, 44). Six chemically synthesized BchC substrates (containing various modifications at rings A, B, and E as well as different central metal ions) were analyzed in in vitro experiments using purified BchC. All pigments were characterized on the basis of the respective Qᵥ absorption band (red-most absorption maximum; compare cited literature of individual compounds listed in Fig. 1C). Specific activities for all substrates were determined in the linear
range of the enzyme assay by measuring specific substrate depletion or product formation.

In the present literature, the bacteriochlorin 31-OH-BChlide (16) is described as the natural substrate of BchC (this substrate has a chiral center at C31, but stereochemical aspects have not been considered in the present investigation). We first tested the demetallated equivalent of this substrate for enzymatic activity. As shown in Fig. 2A (bottom left), the NADH-dependent oxidation of 31-OH-BPheide 3 resulted in a bathochromic absorption shift of 36 nm that gave rise to a new QY band at 753 nm, which is indicative of the formation of BPheide a. A specific activity of 1.1 nmol min\(^{-1}\) mg\(^{-1}\) was observed. This result indicated that the presence of the central magnesium ion was not a prerequisite for BchC substrate recognition.

Consequently, compounds with an altered central metal ion (e.g. zinc or palladium) were also considered as artificial substrates of BchC. Compounds Zn-BPheide a (2) and Pd-BPheide a (3), both carrying a 3-acetyl group, were subjected to in vitro activity assays in the presence of 1 mM NADH to assess the reverse BchC reaction. For Zn-BPheide a, a hypsochromic absorption shift of 56 nm and a specific activity of 64.9 nmol min\(^{-1}\) mg\(^{-1}\) were observed due to the formation of Zn-31-OH-BPheide a (Fig. 2A, bottom right). By contrast, experiments in the presence of Pd-BPheide a (3) did not reveal BchC activity above the detection limit of the assay used (0.4 nmol min\(^{-1}\) mg\(^{-1}\)). Obviously, the presence of the Pd\(^{2+}\) central metal ion

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**FIGURE 1.** Localization and purification of BchF and BchC analyzed by SDS-PAGE and spectral characteristics of artificial BchC substrates and related products. A, left, the heterologously produced BchF membrane protein was isolated from the total E. coli membrane fraction by Triton X-100 solubilization. Lane 1, molecular mass markers (relative molecular mass (×1,000) are indicated); lane 2, crude cellular extract; lane 3, supernatant after first high speed ultracentrifugation step; lane 4, supernatant after Triton X-100 solubilization and subsequent high speed ultracentrifugation containing the solubilized BchF protein with an apparent molecular mass of 25 kDa (arrow). A, right, affinity purification of BchC. GST-tagged BchC was immobilized using Protino glutathione-agarose 4B, and the target protein BchC was liberated by on-column PreScission protease treatment. Lane 5, molecular mass markers; lane 6, cell-free E. coli extract; lanes 7 and 8, flow-through of the GST affinity chromatography; lane 9, wash fraction; lanes 10–12, elution fractions containing untagged BchC. B, core structures of porphyrin (P), chlorin (C), and bacteriochlorin (B) pigments. Ring systems A–E are indicated. Substituents R1–R4 and the chelated central atom are indicated in C. C, structural and spectroscopic details of artificial substrates (left) and the resulting BchC reaction products (right). Substrates 2, 3, and 6 were used to analyze the BchC-catalyzed back-reaction in the presence of NADH. Efficient substrate utilization for compounds 1, 2, 5, and 6 was determined. a, mixture containing R and S stereoisomers; b, this study, measured in acetone, 50 mM Tris-HCl, pH 8.0 (volume ratio, 2:1); c, in diethyl ether; d, in acetone; n.a., not available. Note that the 3-(1-hydroxy)-ethyl substituent is often referred to as 3-hydroxyethyl or 31-OH in the present literature. -, no product formation.
hampers the BchC catalyzed (back) reaction. When 3'-OH-BChl a (4) was analyzed in the presence of NAD⁺⁻, no change in the spectral properties was observed. It was concluded that the presence of a bulky hydrophobic ester side chain on ring D abolished enzymatic substrate oxidation.

The conjugated π system of bacteriochlorins (Fig. 1, B and C, compounds 1–4) is mainly responsible for the absorption properties of BChl a and its derivatives. The subsequent BchC activity assays make use of chlorins showing Qy bands at ~100-nm-shorter wavelengths (compounds 5 and 6; Fig. 1, B and C). Until now, no biosynthetic chlorin intermediates have been described as a substrate of BchC (Fig. 5).

However, when a BchC in vitro activity assay was performed in the presence of Zn-3'-132-di-OH-Pheide a (5) and 1 mM NAD⁺⁻, a bathochromic absorption shift of 24 nm and a specific activity of 21.4 nmol min⁻¹ mg⁻¹ were observed (Fig. 2A, top left). Obviously, the chlorin ring structure of compound 5 did not impede BchC catalysis. Furthermore, neither the presence of an additional 132-hydroxyl on ring E nor the central zinc ion hampered BchC catalysis. This result was substantiated by using the chlorin Zn-3-acetyl-Pheide a (6), which might represent a natural chlorin reaction product of BchC (with magnesium replaced by zinc). In this reverse BchC assay, Zn-3-acetyl-Pheide a (6) was reduced in the presence of NADH to Zn-3'-OH-Pheide a (10), showing an almost identical Qy band at 653 nm as the above mentioned Zn-3'-132-di-OH-Pheide a (Qy band at 652 nm; see Fig. 2A, top right). A specific activity of 172.5 nmol min⁻¹ mg⁻¹ was determined.

In summary, the chlorin substrates Zn-3-acetyl-Pheide a (6) and Zn-3'-132-di-OH-Pheide a (5) showed a significantly increased specific activity when compared with the related bacteriochlorin compounds Zn-BPheide a (2) and 3'-OH-BPheide a (1) of the present investigation. Based on these results, it was proposed that 3'-OH-Chlide (13) might also be a natural substrate of BchC. Such additional substrate utilization would reflect an alternative biosynthetic route for the synthesis of BChlide as depicted in the lower part of Fig. 5A. These results are further supported by a C. tepidum mutagenesis study. A ΔbciC mutant impaired in the removal of the 132-COOCH₃ substituent accumulated 3-acetylphophorbide a together with bacterioopherphorbide a (plus their monomethyl derivatives; Ref. 45).

Reconstitution of the DPOR-BchF-BchC Pathway—To substantiate this alternative biosynthetic pathway further, coupled activity assays in the presence of the highly oxygen-labile DPOR enzyme were performed in an anoxic chamber. All pathway reconstitution experiments made use of an E. coli strain for the well established polycistrionic overproduction of subunits BchN, BchB, and BchL of DPOR (5). The corresponding DPOR

**FIGURE 2. BchC activity assays in the presence of artificial substrates.** A, left, BchC catalysis in the presence of 1 mM NAD⁺⁻. Right, back-reaction in the presence of 1 mM NADH. Dotted lines show control experiments without enzyme, and continuous lines show BchC catalysis. Top left, 4.2 μM Zn-3'-132-di-OH-Pheide a (5) tested with 6.2 μM BchC; top right, 750 nM Zn-3-acetyl-Pheide a (6) with 4.0 μM BchC; bottom left, ~10 μM 3'-OH-BPheide a (1) with 3.2 μM BchC; bottom right, 15.3 μM Zn-BPheide a (2) with 4.8 μM BchC. Characteristic Qy bands of reaction products and the cited literature are given in Fig. 1 C, B, determination of specific activities in the linear range of the enzyme assay. Abs, absorption.
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FIGURE 3. Reconstitution of the DPOR-BchF-BchC pathway, cofactor specificity, and single turnover experiments of BchC. A, coupled DPOR-BchF-BchC assay. The DPOR activity assay with the Pchlide substrate (Q<sub>v</sub>, 628 nm) revealed the formation of Chlide (Q<sub>v</sub>, 667 nm; dotted line). The coupled DPOR-BchF assay revealed the formation of 3′-OH-Chlide (Q<sub>v</sub>, 662 nm; dashed line; using a crude cellular extract of BchF). The coupled DPOR-BchF-BchC assay resulted in 3-acetyl-Chlide formation (Q<sub>v</sub>, 680 nm; continuous line; using a crude cellular extract of BchF and BchC). B, coupled activity assay as in A using DPOR and Triton X-100 solubilized BchF (dashed line) and using DPOR, crude cellular BchF extract, and purified BchC (continuous line). C, coupled DPOR-BchF-BchC assay in the presence of different redox cofactors (using BchF crude cellular extract and purified BchC). Long dashed line, no additional cofactor; continuous line, 500 μM NAD<sup>+</sup>; short dashed line, 500 μM NADP<sup>+</sup>; dotted line, DPOR control experiment. D, BchC single turnover activity assay. Different amounts of purified BchC (0.8, 2.0, 4.0, 6.0, and 8.0 μM) were incubated with a 2.9 μM concentration of the artificial substrate Zn-3′,13′-di-OH-Pheide α (5) in the absence of additional cofactor (dotted lines; increasing amounts of BchC are indicated by the arrowhead). Control experiments without BchC (dashed line) or containing 4 μM BchC, 2.9 μM substrate 5, and 1 mM NAD<sup>+</sup> (continuous line) are shown.

activity assay (containing an ATP-regenerating system and the DPOR substrate Pchlide; Q<sub>v</sub> band at 628 nm) clearly revealed formation of Chlide as indicated by a new Q<sub>v</sub> peak at 667 nm (Fig. 3A, dotted line).

This specific Q<sub>v</sub> Chlide peak was no longer present in coupled DPOR-BchF assays using either a crude cellular extract from a BchF-overproducing E. coli strain (Fig. 3A, dashed line) or alternatively the solubilized BchF membrane protein (Fig. 3B, dashed line). A new absorption maximum at 662 nm was indicative for the formation of the proposed 3′-OH-Chlide (13) (Fig. 3A, dashed line; for references of Q<sub>v</sub> absorption maxima see Table 1).

In extending this reaction pathway, DPOR-BchF-BchC reconstitution experiments were carried out using either crude cellular BchF and BchC extracts (Fig. 3A, solid line) or alternatively a BchF extract and the purified BchC protein (Fig. 3B, solid line). Obtained results revealed a new Q<sub>v</sub> band at 680 nm indicative of the formation of 3-acetyl-Chlide (14). In low temperature fluorescence experiments (at 77 K), this compound revealed a characteristic fluorescence at 694 nm upon excitation at 470 nm. These determined fluorescence parameters allowed for the specific detection of 3-acetyl-Chlide (14) in mixtures containing Pchlide, Chlide, and/or 3′-OH-Chlide (13).

The individual steps of the reconstituted pathway were further analyzed by reversed phase HPLC and multiwavelength fluorescence detection. DPOR catalysis resulted in Chlide formation (λ<sub>ex</sub> = 439 nm and λ<sub>em</sub> = 670 nm; eluting at 21.8 min) as depicted in Fig. 4A. Subsequently, the DPOR-BchF-dependent formation of 3′-OH-Chlide (13) (λ<sub>ex</sub> = 439 nm and λ<sub>em</sub> = 662 nm; double peak eluting at 10.0 and 10.8 min) is indicated in Fig. 4B. This metabolite was efficiently oxidized due to the presence of BchC and NAD<sup>+</sup>, resulting in the formation of 3-acetyl-Chlide (14) (λ<sub>ex</sub> = 470 nm and λ<sub>em</sub> = 694 nm; eluting at 14.4 min) as indicated in Fig. 4C. Identical results were observed in experiments using crude cellular BchF extract, solubilized His<sub>6</sub>-BchF, or purified BchC (Fig. 3B).

Based on the in vitro assays in the presence of artificial substrates and the series of analyses from the pathway reconstitution experiments, we conclude that BCH a biosynthesis in C. tepidum might include an additional branching point that is based on the BchC-catalyzed conversion of 3′-OH-Chlide. This route is implemented in the reaction scheme depicted in Fig. 5 (7–14, 19, 35, 61–63).

Bioinformatic Analysis of BchC—Theoretical analyses related C. tepidum BchC to the medium-chain dehydrogenase/reductase (MDR) protein superfamily (46–49) comprising an
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**TABLE 1**

**Spectroscopic characterization of the reconstituted pathway intermediates**

| Pigment | $\lambda_{\text{max}, \text{Ex}}$ | $\lambda_{\text{max}, \text{Em}}$ (literature) | $\lambda_{\text{max}, \text{Ex}}$ | $\lambda_{\text{max}, \text{Em}}$ (literature) |
|---------|----------------|----------------------|----------------|----------------------|
| Chlide (11) | 628 | 626 | 629 | 637 |
| Chlide (12) | 667 | 667 | 670 | No emission |
| 3′-OH-Chlide (13) | 662 | 659 | 662 | No emission |
| 3′-Acetyl-Chlide (14) | 680 | 682 | 681 | 694 |

| Pigment | $\lambda_{\text{em}}$ | $\lambda_{\text{em}}$ |
|---------|----------------|----------------|
| Chlide | 670 nm | 670 nm |
| 3′-OH-Chlide | 682 nm | 681 nm |
| 3′-Acetyl-Chlide | 682 nm | 681 nm |

| Pigment | $\lambda_{\text{em}}$ | $\lambda_{\text{em}}$ |
|---------|----------------|----------------|
| Chlide | 670 nm | 670 nm |
| 3′-OH-Chlide | 682 nm | 681 nm |
| 3′-Acetyl-Chlide | 682 nm | 681 nm |

\(a\) This study; measured in acetone, 50 mM Tris-HCl, pH 8.0 (volume ratio, 2:1).

\(b\) In 80% acetone.

\(c\) In 50% acetone.

\(d\) In diethyl ether.

\(e\) In acetone.

**Activity Analysis of BchC Mutant Proteins—** Highly conserved amino acid residues of BchC were deduced from an extended sequence alignment of BchC sequences (compare conservation pattern depicted in the BchC/MDR alignment shown in Fig. 6). A potential role of residues Ser39, Ser42, Glu46, Tyr67, Glu68, and His141 as alternative zinc ligands was hypothesized. Accordingly, BchC was subjected to site-directed mutagenesis to identify key catalytic amino acid residues.

**Zinc Content of Recombinant BchC—**

Samples of purified BchC were preincubated in the presence of up to 100 mM EDTA or EGTA (75 min at 48 °C). Subsequently, coupled BchC activity experiments and assays in the presence of substrate 3′-OH-BPheide \(a^2(1)\) or \(a^2(5)\) did not reveal reduced BchC activities when compared with the respective control experiment (not shown). These results did not reflect the outcome of related experiments for well characterized zinc-dependent MDR enzymes: chelation of the zinc ions of yeast alcohol dehydrogenase (53) and \(a^2(5)\) resulted in enzymatic inactivation. Accordingly, a zinc-independent BchC catalysis was considered.

**Chelator Treatment of BchC—**

Samples of purified BchC were preincubated in the presence of up to 100 mM EDTA or EGTA (75 min at 48 °C). Subsequently, coupled BchC activity experiments and assays in the presence of substrate 3′-OH-BPheide \(a^2(1)\) or \(a^2(5)\) did not reveal reduced BchC activities when compared with the respective control experiment (not shown). These results did not reflect the outcome of related experiments for well characterized zinc-dependent MDR enzymes: chelation of the zinc ions of yeast alcohol dehydrogenase (53) and \(a^2(5)\) resulted in enzymatic inactivation. Accordingly, a zinc-independent BchC catalysis was considered.

**Zinc Content of Recombinant BchC—**

The zinc content of the purified BchC enzyme and of an analogously purified zinc-independent reference protein (40) was analyzed by inductively coupled plasma mass spectrometry (MS). No metal content above the background level of the experimental approach used was determined.

Inductively coupled plasma MS analyses in combination with the chelator treatment experiments used were indicative of a...
zinc-independent BchC catalysis. These results were not in agreement with the initial bioinformatics analysis. However, the observed zinc-independent catalysis of BchC clearly reflects the absence of highly conserved ligands of a catalytically relevant metal ion (cysteine, histidine, and glutamate; Fig. 6). With respect to these results, it was not possible to clearly assign BchC to one of the established MDR families described in the literature (for a review, see Refs. 46 and 56).

Cofactor Specificity of BchC—

NADPH is a cofactor that commonly acts as a stereospecific electron acceptor within the framework of anabolic pathways. Therefore, a coupled BchC activity assay was also performed in the presence of 500 μM NADPH. This experiment revealed a low degree of product formation when compared with the related NADP−-containing experiment (absorption spectrum depicted in Fig. 3C, short dashes). However, a related control experiment without externally added nucleotide cofactor indicated an almost identical absorption spectrum (Fig. 3C, long dashes, overlapping the spectrum of the NADP+ -containing experiment).

Single turnover in vitro experiments containing purified BchC in the presence of Zn-31-132-di-OH-Pheide a (without external addition of nucleotide cofactor) also revealed product formation (as judged from the absorption at 676 nm). This enzymatic activity was efficiently stimulated by addition of 1 mM NADPH. No such effect was observed in the presence of 1 mM NADP+.

In agreement with these findings, a tightly bound NADPH cofactor of BchC was hypothesized. The presence of this co-purified nucleotide was subsequently confirmed by single turnover experiments with increasing BchC concentrations. In Fig. 3D, a control assay containing 2.9 μM Zn-31-132-di-OH-Pheide a in the absence of BchC did not indicate BchC product formation (dashed line). Supplementation of this experiment with increasing amounts (0.8, 2.0, 4.0, 6.0, and 8.0 μM)
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of BchC revealed gradually increasing product concentrations (0.1, 0.3, 0.5, 0.6, and 0.8 μM) as judged by absorption measurements at 676 nm (dotted lines). A control experiment with NAD⁺ excess is shown in Fig. 3D (continuous line). From these results, we conclude that the overproduced C. tepidum BchC is purified (partially) in complex with its specific cofactor NAD⁺.

Discussion

Fundamentals of the BChl a biosynthetic pathway were elucidated more than 40 years ago based on the spectroscopic analysis of accumulated precursors from different Rhodobacter mutant strains (57, 58). This methodology already revealed that the DPOR reaction product Chlide is located at a central hub of the following branched biosynthetic pathways.

DPOR → COR → BchF → BchC

Relevant Sequence I

DPOR → BchF → COR → BchC

Relevant Sequence II

Recently, evidence for Reaction Sequence I was also obtained from a thorough COR characterization (9). The present study...
Summary

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clearly reveals the BchF-dependent synthesis of 3′-OH-Chlide via Reaction Sequence II in C. tepidum. These results might indicate that biosynthesis of Bchl a is parallel in C. tepidum and e.g. R. capsulatus with respect to Reaction Sequences I and II.

In the present investigation, the analysis of chemically modified BchC substrates allowed for the determination of the following additional route for the synthesis of Bchl a.

\[ \text{DPOR} \rightarrow \text{BchF} \rightarrow \text{BchC} \rightarrow \text{COR} \]

Reaction Sequence III

BchC from C. tepidum is able to efficiently oxidize the bacteriochlorin substrate 3′-OH-BPheide a and the chlorin Zn-3′-13\(^2\)-di-OH-Pheide a. In our subsequent pathway reconstitution experiments, 3′-OH-Chlide was identified as a BchC substrate that is located at this newly identified branching point. These findings are in agreement with a C. tepidum mutant study (45) and are further supported by a recent investigation for the COR enzyme from the purple bacterium R. denitrificans. An artificial substrate carrying a 3-acetyl group (instead of a 3-hydroxyethyl or a 3-vinyl group) was efficiently converted by COR (9). These findings might indicate that Reaction Sequence III is also relevant for Bchl a biosynthesis in purple bacteria. The observed plasticity of the BChl biosynthetic pathways might be the evolutionary clue for the broad diversification of pigments under different environmental light conditions.

The present investigation clearly indicates that C. tepidum BchC is an unusual zinc-independent dehydrogenase specifically using NAD\(^+\) as a redox cofactor. These findings are strongly supported by a recent bioinformatics investigation that assigns BchC to a newly defined family of MDRs using an automated algorithm for the refinement of the hidden Markov models used (47). According to these findings, BchC from C. tepidum might be loosely related to the zinc-independent enone oxidoreductase from Fra
caggia × ananassa (strawberry) that catalyzes the NADH-dependent reduction of a polar C=C double bond (Protein Data Bank code 4IDA (59) and Fig. 6). Based on the present literature, it was not possible to conclude a common reaction mechanism for the different zinc-independent MDRs characterized so far. In this study, critical involvement of residues Glu\(^{66}\), Tyr\(^{67}\), Glu\(^{68}\), and His\(^{141}\) in BchC catalysis was demonstrated. However, the comparison of BchC with members of different MDR families (depicted in Fig. 6) does not reveal the conservation of these key catalytic BchC residues. Therefore, a differing substrate ligation/activation pattern in the active site of BchC must be concluded.

Nevertheless, the present study reveals an initial picture of C. tepidum BchC substrate recognition. The enzyme tolerates artificial substrates containing zinc instead of the central magnesium and the absence of a central metal ion. Furthermore, the presence of an additional 13\(^2\)-OH group on ring E is accepted. However, a bulky side chain attached to ring D clearly abolishes BchC activity. These findings might indicate that mainly pyrrole rings A and D are involved in substrate recognition. Hydrogenation of a chlorin ring B to a bacteriochlorin changes the spatial orientations of the C7 and C8 substituents. The observed BchC activity with both chlorins and bacteriochlorins (compounds 1 and 5) might be indicative for an active site not covering the B ring system of the natural substrates. Last but not least, the coupled assays developed in this work are a basis for elucidating the details of BchC catalysis including the stereoselectivity for the configuration of C3.

Author Contributions—J. M., D. J., and H. S. designed the study. J. M., D. J., C. L., and H. S. wrote the paper. C. L., S. K., S. P., and S. V. characterized BchF and BchC. H. S. supplied the artificial substrates. All authors analyzed the results and approved the final version of the manuscript.

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