The BADC and BCCP subunits of chloroplast acetyl-CoA carboxylase sense the pH changes of the light–dark cycle

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Acetyl-CoA carboxylase (ACCcase) catalyzes the first committed step in the de novo synthesis of fatty acids. The multisubunit ACCase in the chloroplast is activated by a shift to pH 8 upon light adaptation and is inhibited by a shift to pH 7 upon dark adaptation. Here, titrations with the purified ACCase biotin attachment domain-containing (BADC) and biotin carboxyl carrier protein (BCCP) subunits from Arabidopsis indicated that they can compete independently of biotin carboxylase (BC) but differ in responses to pH changes representing those in the plastid stroma during light or dark conditions. At pH 7 in phosphate buffer, BADC1 and BADC2 gain an advantage over BCCP1 and BCCP2 in affinity for BC. At pH 8 in KCl solution, however, BCCP1 and BCCP2 had more than 10-fold higher affinity for BC than did BADC1. The pH-modulated shifts in BC preferences for BCCP and BADC partners suggest they contribute to light-dependent regulation of heteromeric ACCase. Using NMR spectroscopy, we found evidence for increased intrinsic disorder of the BADC and BCCP subunits at pH 7. We propose that this intrinsic disorder potentially promotes fast association with BC through a “fly-casting mechanism.” We hypothesize that the pH effects on the BADC and BCCP subunits attenuate ACCase activity by night and enhance it by day. Consistent with this hypothesis, Arabidopsis badc1 badc3 mutant lines grown in a light–dark cycle synthesized more fatty acids in their seeds. In summary, our findings provide evidence that the BADC and BCCP subunits function as pH sensors required for light-dependent switching of heteromeric ACCase activity.

In most plants (dicots and nongrass monocots), a multisubunit ACCase in plastids to generate the malonyl-CoA required for de novo synthesis of fatty acids (1, 2) (Fig. 1A). This first committed step of fatty acid synthesis controls carbon flow into the pathway and, thus, is highly regulated (1, 2). Newly synthesized FAs are processed into glycerolipids for plant cell membranes or storage triacylglycerols (oils) within the seed or mesocarp. The varied markets for vegetable oils, including cooking and dietary oils, biodiesel, and chemical feedstocks, motivates ongoing interest in the engineering of oilseeds (3, 4) and the regulatory properties of hetACCase (5–7).

The formation of malonyl-CoA by ACCase was reported to be the only light-regulated step for de novo FA synthesis in plants (8). The activity of hetACCase in chloroplasts is increased in the light by the conditions created by the photosynthetic light reactions, namely, high ATP levels, a shift to pH 8 in the stroma, and a pool of reduced thioredoxin that enhances hetACCase by reducing disulfide bonds within the carboxyltransferase (CT) subunits (9–12). The first half-reaction of hetACCase is catalyzed by the biotin carboxylase (BC) subcomplex, which uses bicarbonate and ATP to carboxylate the biotin cofactor of a biotin carboxyl carrier protein (BCCP; Fig. 1A) (2, 5, 13–15). The carboxyltransferase subcomplex of α-CT and β-CT subunits catalyzes the second half-reaction that transfers the carboxyl group from biotin to acetyl-CoA to form malonyl-CoA (2, 5, 13–15). The structural bases of the functions of the BC, BCCP, and CT subunits were reviewed (15). An oligomeric complex of the folded region of BCCP and BC subunits from the ACCase from Escherichia coli was elucidated by crystallography (16). In plants, the malonyl-CoA pool in the plastid for de novo FA synthesis is distinct from the cytosolic malonyl-CoA pool used for FA elongation, which is generated by a separate homomeric ACCase (2).

A BCCP-like biotin attachment domain-containing protein (At1g52670) was first described in 2009 (17) and subsequently named biotin attachment domain-containing 2 (BADC2) (6). The BADC1 and BADC3 members of this gene family were identified and were demonstrated to associate with the subcomplex containing BCCP1, BCCP2, and BC subunits of Arabidopsis (6). The mature BADC subunits at 22 to 24 kDa in mass (6) are slightly larger than the BCCP subunits of 18 to 21 kDa and less than half the mass of the BC and CT subunits (Table S1). Although BADC subunits retain the sequence similarity of the biotin/lipoic attachment domain, BADC1, BADC2, and BADC3 specifically lack the canonical four-amino-acid sequence motif for biotinylation in BCCPs and were experimentally shown to lack a covalently bound biotin prosthetic group (6). The addition of excess BADC1, BADC2, or BADC3 to extracts from developing Arabidopsis siliques inhibited hetACCase activity by about 37%, 33%, and 24%, respectively (6). The addition of BADC1 to leaf extracts inhibited ACCase activity up to ~27% in a concentration-dependent fashion (6). BADC subunits’ lack of biotinylation and partial inhibition of hetACCase support the hypothesis that BADC subunits inhibit by competing with BCCP subunits for access to BC (6). Partial

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RNAi silencing of BADC1 in Arabidopsis seeds increased seed oil content (6). Comparison of T-DNA knockout badc1 badc3 Arabidopsis with the WT found ACCase activity to be higher and seeds to contain 33% more triacylglycerols in badc1 badc3 lines (7). This suggests that the relief of inhibition by BADC1 and BADC3 results in higher hetACCase activity and carbon

Figure 1. In chloroplast ACCase, the sequences of the BADC and BCCP subunits suggest both folded and disordered regions. A, the cartoon of hetero-ACCase indicates the types of subunits in the biotin carboxylase and carboxyltransferase subcomplexes, as well as the overall reaction. B and C, homology models of the folded domains of BCCP2 (B) and BADC1 (C) from A. thaliana. The ribbon is colored with the spectrum from purple at the N-terminal end to red at the C terminus. D–H, plot order predictions by MetaDisorderMD2 (using multiple algorithms at the GeneSilico server) with black squares versus sequence numbering of the mature forms of the BCCP1 (D), BCCP2 (E), BADC1 (F), BADC2 (G), and BADC3 (H) subunits. Values of >0.5 are considered disordered. ANCHOR2 (open circles) predicts potential regions of protein binding where the values exceed 0.5. The location of β-strands predicted by homology modeling using the EXPASY server are marked with arrowheads. T marks residues predicted to be part of the loop called the thumb. B represents the lysine that becomes biotinylated. A histidine is marked H in the folded region or h in an unfolded region.
flux through de novo FA synthesis (7). Potential mechanisms for BADC competition with BCCP subunits remain unclear. Results in Arabidopsis suggest that BCCP and BADC are capable of binding to BC somewhat independently (6, 7). However, this has yet to be demonstrated in quantitative fashion.

More roles for the BADC subunits in Arabidopsis have emerged recently. An important but unidentified role in seed development is indicated by the inability to obtain badc2 badc3 mutant seeds as well as by the smaller seeds, roots, and rosettes of badc1 badc2 mutants (7, 18). The BADC1 and BADC3 subunits were implicated in long-term response to oversupply of FAs by feedback inhibition (7). When Arabidopsis BC was coexpressed in E. coli with Arabidopsis BCCP1 or BCCP2, a high level of coexpression with a BADC subunit boosted BCCP recruitment of BC by more than 10-fold to nearly stoichiometric ratios in each of the BCCP-BADC-BC subcomplexes (18). The in vitro catalytic efficiencies and maximum catalytic velocities of the BCCP-BADC-BC subcomplexes reconstituted in E. coli were similarly elevated over those of their BCCP-BC counterparts, with BADC2 and BADC3 providing the biggest enhancement (18).

Assembly and stoichiometry of macromolecular assemblies, such as hetACCase, is complex and often difficult to ascertain, given the need for absolute quantitation in vivo. The MS approach known as absolute quantitation of multiplexed reaction monitoring (AQUA-MRM) provided the molar protein quantities of hetACCase subunits in Arabidopsis siliques when the seeds are actively filling with oil. The molar quantities of the subunits rank in the order β-CT > BC > BADC1 > BADC3 ≈ BCCP1 > α-CT ≈ BCCP2 > BADC2 (19). During oilseed development, BADC1 and BCCP1, being the most highly expressed of the nonbiotinylated and biotinylated smaller subunits, respectively, merit study of their behaviors.

Illumination and the photosynthetic light reactions pump protons out of the chloroplast stroma, raising its pH from 7 to 8 (20). We focus here on investigation of the hypothesis that the light-dependent swings in pH of the plastid stroma regulate association of BCCP and BADC subunits with the biotin carboxylase subunit, which forms and alters the subcomplexes catalyzing carboxylation of biotinylated BCCP1 or BCCP2. We considered the possibility that the physiological pH range affects the BCCP and BADC subunits in terms of (i) their affinities for BC and (ii) their structural scaffolds in solution. The BADC and BCCP subunits associate with BC directly and independently with $K_D$ of $<10 \mu M$ under all solution conditions evaluated. The affinities are greater at pH 7 than at pH 8. The intrinsic disorder detected by NMR in pH titrations of BCCP1, BCCP2, and BADC1 is also increased at pH 7. At pH 7, enhanced BADC occupation of BC predicts that biotin carboxylation should be slower at pH 7. At pH 8, enhanced BCCP subunit occupation of BC predicts enhanced biotin-mediated transfer of carboxyl groups between the active sites of BC and CT subunits. Thus, the BCCP and BADC subunits sense pH and respond in a differential manner that may help to (i) inhibit the biotin carboxylase half-reaction in the neutral pH of dark and low-light conditions and (ii) accelerate biotin carboxylation in pH 8 light adaptation and thereby enhance the light-dependent regulation of hetACCase in plastids. Consistent with these predictions and with the abundance of the BADC1 and BADC3 subunits, the seeds produced by Arabidopsis badc1 badc3 lines accumulate more total lipid when the lines are grown with a daily light/dark cycle.

**Results**

**Predictions of folded and disordered regions of plastid BCCP and BADC subunits**

The ~90 residues at the C-terminal end of the BCCP1, BCCP2, BADC1, BADC2, and BADC3 sequences from A. thaliana exhibit homology to structures in the Protein Data Bank. Homology models, using crystallographic coordinates of BCCP from E. coli, contain two anti-parallel β-sheets in each of these subunits of acetyl-CoA carboxylase (Fig. 1, B and C). Homology models of this region from each BADC subunit comprise eight β-strands, seven strands in BCCP2, and six strands in BCCP1, plus a potential but ambiguous seventh strand at its C terminus. At the N-terminal end of this region, the BADC subunits add a β-strand to one β-sheet (Fig. S1 and Fig. 1C, purple), while the corresponding segment of the BCCP1 and BCCP2 models is similar but diverges (Fig. S2B). The exposed “thumb” loop is much longer and enriched in basic residues in the models of the BADC subunits (Fig. 1C and Fig. S2B). The lysine in the β-hairpin loop that becomes biotinylated in BCCP subunits is replaced by glycine in the BADC subunits. Apart from localized differences at the termini and thumb, the backbone coordinates of the homology models are highly similar (Fig. S2).

Due to the small size of the folded regions, we examined the sequences of the mature BCCP1, BCCP2, BADC1, BADC2, and BADC3 subunits from A. thaliana for disorder. The unusual structural, sequence, and functional properties of intrinsic disorder were reviewed (21). Intrinsically disordered regions (IDRs) exhibit elevated mean charge, low mean hydrophobicity, depletion of bulky hydrophobic side chains, and enrichment in disorder-promoting Pro, Glu, Ser, Lys, and Gln residues (21–23). The content of these five disorder-promoting residues ranges from 38% in BADC2 to 45% in BCCP1. We applied the CASP9-winning MetaDisorderMD2 approach that integrates multiple predictions of disorder (24, 25). These predictions suggest that a large IDR lies in the middle of each sequence, preceding the structured domain (Fig. 1D–H). The predictions also feature an N-terminal IDR followed by an ordered region of 20 to 30 residues. The thumb region of BADC1 is predicted to be an IDR (Fig. 1D–H). MetaDisorderMD2 suggests disorder for 59% of the residues of BCCP1, 56% of BCCP2, 62% of BADC1, 55% of BADC2, and 51% of BADC3. The BCCP subunits average a proline content of 14%, while the BADC subunits average 7% proline. The proline residues, which promote extended structure, are concentrated in the large central IDR predicted as well as in the thumb (Fig. S1). Glu and Asp residues comprise 11 to 12% of the amino acids of the BCCP and BADC subunits. The enrichment in negative charge provides electrostatic repulsion opposed to hydrophobic collapse (21).

**Affinities for BC respond to pH and ions**

The question of the pH dependence of the BADC and BCCP subunits’ affinities for the BC subunit arose from the pH
dependence of ACCase activity (10, 12) and from the sensitivity of the folds of BCCP1, BCCP2, and BADC1 to pH (see below). We monitored titrations of BC with BCCP1, BCCP2, BADC1, BADC2, or BADC3 by microscale thermophoresis (MST), which is sensitive to binding interactions. MST senses molecular interactions via the accompanying changes to molecular size, charge, and hydration shell that respond to a temperature jump from an IR laser pulse (26, 27). The changes in thermal molecular movement are detected by fluorescence, which is sensitive to modest amounts of the binding partners. We compared pH conditions representative of daylight (pH 8) and dark (pH 7). Good’s buffers have been popular for assays of hetACCCase activity (6, 18). However, the 1.2 to 2 mM phosphate (Pi) present in the stroma of chloroplasts fully active in photophosphorylation (28) suggests the relevance of phosphate-containing buffers. Hence, we also conducted titrations in PBS containing NaCl or PBK containing KCl. [K\(^{+}\)] in chloroplasts was reported to range from 40 mM in pea to 160 to 200 mM in spinach and sugarbeet chloroplasts and [Na\(^{+}\)] to range from 40 to 70 mM in pea, spinach, and sugarbeet chloroplasts (29). Potassium ions are required for a high rate of photosynthesis (30). Titrations detected by MST suggest that the affinities for BC strongly depend on the ionic milieu (Fig. 2). Comparisons of the Gibbs free energies of association with BC clarify the effects of pH and choice of buffer (Fig. 3).

The drop in pH from 8 to 7 significantly increased BC affinities for each of the small sibling subunits in NaCl-containing solutions of PBS (rightward shifts in Fig. 3A). The BCCP subunits increased in affinity for BC by about 1 kcal/mol due to this change (Fig. 3B). BC affinity for BADC1, BADC2, and BADC3 increased at pH 7 by 2.1, 0.8, and 1.5 kcal/mol, respectively. Consequently, at pH 7 in NaCl and phosphate, BC affinity for BADC1 and BADC3 exceeded that in KCl and HEPEs by 1.4 and 0.9 kcal/mol, respectively (Fig. 3, A and C). BADC1 and BC reached a K\(_D\) of 54 nM at pH 7 in PBS (Table 1, Fig. 2C). These results can be regarded as enhancements of BADC1 and BADC3 affinity for BC at pH 7 by PBS (Fig. 3, A and C). This behavior suggests that the combination of 40 to 70 mM Na\(^{+}\) and 1 to 2 mM phosphate in the plastid (28, 29) with the pH 7 of dark conditions could enhance occupation of BC by abundant BADC1. The likelihood of more occupation of BC by BADC1 at pH 7 could increase inhibition of hetACCCase, because BADC1 lacks a biotin group needed for the biotin carboxylase half-reaction.

In KCl solution buffered by HEPEs, BCCP1 affinity for BC exceeded that in PBS by 1.2 kcal/mol at pH 7 and by 2.2 kcal/mol at pH 8 (Fig. 3, A and C). BCCP2 mimicked this at a lower level. In HEPEs–KCl, BCCP2 affinity for BC exceeded that in PBS by 0.7 kcal/mol at pH 7 and by 1.5 kcal/mol at pH 8 (Fig. 3, A and C). In HEPEs–KCl, the gain in affinity for BC with a drop in pH from 8 to 7 was limited to 1.2, 0.6, and 0.3 kcal/mol for BADC1, BADC2, and BADC3, respectively, and to 0.0 and 0.3 kcal/mol for BCCP1 and BCCP2 (Fig. 3B). In HEPEs–KCl, BCCP1 attained a K\(_D\) of about 45 nM for BC at either pH 7 or 8 (Table 1, Fig. 2A). KCl appeared to enhance the affinity of BCCP1 and BCCP2 for BC relative to NaCl (Fig. 3A). In contrast, KCl seemed to detract from BADC1 affinity for BC and to do likewise at pH 7 to BADC3 (Fig. 3A).

Consequently, it appears as if Na\(^{+}\) ions in the buffer tended to enhance BC affinities for the BADC subunits relative to BCCP1, whereas K\(^{+}\) ions usually appeared to detract from the BC affinities of the BADC subunits relative to BCCP1 (Fig. 3D). This suggests that K\(^{+}\) ions confer a preference of BC binding of BCCPs over BADC1 in the pH 8 of daylight conditions when hetACCCase, FA synthesis, and photosynthesis are active.

Phosphate buffer at pH 8, compared with HEPEs, detracted from BC affinity for BCCP1 by 1.6 kcal/mol and for BCCP2 and BADC1 by 0.4 kcal/mol (purple circles in Fig. 3C). In contrast, phosphate at pH 8 boosted BC affinity for BADC3 by 1.0 kcal/mol (Fig. 3C). This resulted in equivalent BC affinities for BCCP1, BADC1, and BADC3 at pH 8 in PBS (Table 1, Fig. 3, A and D). The combination of KCl, phosphate, and pH 8 was equalizing to the BC affinities of BCCP1, BCCP2, BADC2, and BADC3 but diminished that of BADC1 (Fig. 3D). At both pH 7 and 8, phosphate established more parity (compared with HEPEs) among the BC affinities for BCCP and BADC subunits (triangles in Fig. 3D). The exceptions to the phosphate-promoted convergence in affinity for BC have the potential to regulate hetACCCase activity, i.e. Na\(^{+}\) enhancements of the BC affinities of BADC1 and BADC2 at pH 7 and K\(^{+}\)-diminished BC affinity for BADC1 at pH 8 (Fig. 3C).

Evidence of partial unfolding at neutral pH and lower

The backbone amide region of NMR spectra is excellent for assessing the structural integrity and unfolding of proteins prepared with \(^{15}\)N labeling of the peptide bonds (31, 32). NMR spectra of the BCCP and BADC subunits examined are surprisingly sensitive to pH changes across the neutral range (Fig. 4) and suggest the extent of unfolding as pH drops. The spectra of the BCCP1, BCCP2, and BADC1 subunits in the range of pH 7.5 to 8 show the broad spread of backbone amide peaks expected of a folded domain (Fig. 4, A, E, and I, measured in PBS). In the 12 spectra plotted in Fig. 4, most of the blue-colored peaks represent amide groups in folded parts of the proteins, whereas most of the red-colored peaks represent unfolded regions. The unfolded character associated with the red peaks is suggested by their position in random coil regions of the spectrum (31, 32), their sharp line widths, and growth with acidification of the samples. In contrast, the blue-colored peaks in Fig. 4 probably include 65 or more residues predicted with acidification of the samples. The random coil peaks of solvent-exposed sequences may, however, be broadened away by chemical exchange with the solvent water, and possibly by chemical exchange among conformers, which is likely in the unfolded state; see Fig. 4D for example. Once the pH is titrated to the neutral range, additional amide NMR peaks at random coil chemical shifts appear (red contours in Fig. 4, B, F, and J), suggestive of partially unfolded forms present.

We sought to compare the pH dependence of partial and complete unfolding of BCCP1, BCCP2, and BADC1 quantitatively, based on the pH titrations monitored with NMR spectra. The size, extent, and unknown direction of the peak shifts upon acidification (Fig. 4) impede manual quantification. Fortunately, the pH dependences of protein folding transitions have
Figure 2. Affinities for BC of BADC and BCCP subunits measured by microscale thermophoresis (MST). The titrations were measured in HEPES buffer with 140 mM KCl (left) or phosphate buffer with 140 mM NaCl or KCl (right), with \( n = 3 \) technical replicates providing the S.D. of each point. Recombinant BC (19.5 nM) was titrated by recombinant BCCP1 (A), BCCP2 (B), BADC1 (C), BADC2 (D), or BADC3 (E). Black squares mark titrations at pH 7.0. Red circles mark titrations at pH 8.0. Green triangles mark titrations in phosphate buffer, named PBK, that replaces NaCl with KCl. Vertical gray lines mark the strongest and weakest \( K_D \) in each column of panels.
Apparent affinities for the BC subunit measured in selected solution conditions by microscale thermophoresis

Table 1

| Parameter | HEPES, pH 7, KCl | HEPES, pH 8, KCl | PBS, pH 7 | PBS, pH 8 | PBK, pH 8 |
|-----------|------------------|------------------|-----------|-----------|-----------|
| BCCP1     |                  |                  |           |           |           |
| $K_{D, M}$ | $4.4 \pm 0.9E-8$ | $4.6 \pm 0.5E-8$ | $3.6 \pm 0.6E-7$ | $1.9 \pm 0.3E-6$ | $6.9 \pm 0.6E-7$ |
| $\Delta G_{NaCl/PBS}$ | $-100 \pm 0.1$ | $-100 \pm 0.1$ | $-8.8 \pm 0.1$ | $-7.8 \pm 0.1$ | $-8.4 \pm 0.1$ |
| BCCP2     |                  |                  |           |           |           |
| $K_{D, M}$ | $1.5 \pm 0.4E-7$ | $2.6 \pm 0.8E-7$ | $4.6 \pm 0.5E-7$ | $3.3 \pm 0.2E-6$ | $4.7 \pm 0.9E-7$ |
| $\Delta G_{NaCl/PBS}$ | $-9.3 \pm 0.1$ | $-9.0 \pm 0.1$ | $-8.6 \pm 0.1$ | $-7.5 \pm 0.1$ | $-8.6 \pm 0.1$ |
| BADC1     |                  |                  |           |           |           |
| $K_{D, M}$ | $5.8 \pm 2.3E-7$ | $4.7 \pm 0.6E-6$ | $5.4 \pm 1.7E-8^b$ | $1.9 \pm 0.5E-6$ | $8.8 \pm 2.2E-6$ |
| $\Delta G_{NaCl/PBS}$ | $-8.5 \pm 0.2$ | $-7.3 \pm 0.1$ | $-9.9 \pm 0.1^b$ | $-7.8 \pm 0.1$ | $-6.9 \pm 0.1$ |
| BADC2     |                  |                  |           |           |           |
| $K_{D, M}$ | $1.85 \pm 0.2E-7$ | $5.1 \pm 2.3E-7$ | $1.5 \pm 0.3E-7$ | $5.8 \pm 1.0E-7$ | $7.1 \pm 1.1E-7$ |
| $\Delta G_{NaCl/PBS}$ | $-9.2 \pm 0.1$ | $-8.6 \pm 0.2$ | $-9.3 \pm 0.1$ | $-8.5 \pm 0.1$ | $-8.4 \pm 0.1$ |
| BADC3     |                  |                  |           |           |           |
| $K_{D, M}$ | $1.7 \pm 0.4E-6$ | $2.9 \pm 0.9E-6$ | $3.6 \pm 0.8E-7$ | $1.8 \pm 0.2E-6$ | $5.0 \pm 1.1E-7$ |
| $\Delta G_{NaCl/PBS}$ | $-7.9 \pm 0.1$ | $-7.6 \pm 0.1$ | $-8.8 \pm 0.1$ | $-7.8 \pm 0.1$ | $-8.6 \pm 0.1$ |

$^a$Uncertainties in the curve fits are listed. The titrations were performed in triplicate.

$^b$Required a second, lower affinity association (not listed) for an acceptable fit; see Fig. 2.
underlying pH-dependent unfolding transitions of BCCP1, BCCP2, and BADC1. The pH-dependent unfolding transitions are suggested by the two biggest principal components (PCs) of change among the spectra (35, 36). One transition suffices to fit PC1 derived from each titration, whereas two pH-dependent unfolding transitions are required to fit PC2 from each of the titrations. The midpoint of the higher conformational transition of BCCP1 is near pH 6.8 in PC1 and pH 7.45 in PC2 (Fig. 5A); this describes the appearance of peaks at random coil chemical shifts in the near-neutral pH range. The midpoint of the appearance of amide NMR peaks of BCCP2 at random coil positions is pH 5.0 in both PC1 and PC2 (Fig. 5B). This indicates that BCCP2 withstood mild acidification more robustly than did the more labile BCCP1. In the case of BADC1, the higher pH transition is resolved in PC2 with the apparent midpoint of pH 5.2 (Fig. 5C). The fit to PC1 of the BADC1 titration also improved by including the pH 5.2 transition (as 17% of the change). The fitted pH titrations suggest the midpoint of partial unfolding of BCCP1 is as much as 1.8 pH units higher than that in BCCP2 or BADC1. However, some partial unfolding of BCCP2 and BADC1 is also present near pH 6.8 (Fig. 4, F and J). The partial unfolding transitions suggest BCCP1 is the most
susceptible to the pH 7 of dark-adapted chloroplast stroma. The relative structural integrity might account for the advantage of BADC1 over BCCP1 in affinity for BC at pH 7 in PBS (Fig. 2 and 3C).

To compare the pH titrations quantitatively, we continued the titrations into the acid range. BCCP1 appeared to be fully unfolded below pH 5.3 (Fig. 4D), BCCP2 by pH 2.6, and BADC1 by pH 2.6. The midpoint fitted to the lower pH-dependent transition captured by PC2 corresponds to the disappearance of the amide NMR peaks of folded protein, i.e. apparent unfolding. The midpoint of pH 5.5 for the unfolding of BCCP1 is higher than the midpoints of unfolding of BCCP2 at pH 3.8 and BADC1 at pH 3.9 (Fig. 5). Thus, the fitting of the NMR spectral changes suggests BCCP1 is the most vulnerable to both unfolding and partial unfolding by decreasing pH.

Increased seed oil in badc1/3 mutant line grown with light–dark cycle

The biophysical results above suggest the hypothesis of BADC subunits dominating complexes with BC and inhibiting hetACCase activity under conditions of neutral pH expected in the plastid stroma under conditions of dark and low light. The comparatively high abundance of BADC1 and BADC3 (19) (Table S1) and recent results on the enhanced seed oil content of badc1 badc3 lines (7, 18) suggested that these two subunits act importantly in the inhibition of hetACCase during night and possibly light-dark transitions, i.e. at dawn and dusk. Therefore, we tested these hypotheses together by comparing Arabidopsis seed oil accumulation in WT, badc1 badc2, and badc1 badc3 double mutants grown under a light-dark cycle or under 24 h of constant light (Fig. 6). In general, plants grown at 24 h of light had higher seed weight and lipid content than those grown with a 16/8 h light-dark cycle, consistent with previous comparisons of seed lipid content from plants grown under constant light versus cycling between day and night conditions (40). When grown under a light-dark cycle, the badc1 badc3 mutant had a higher seed weight, FA content per seed, and FA content as a percentage of seed weight compared with those of the WT (Fig. 6, A–C). However, there was no significant difference in seed weight or seed FA accumulation when the same lines were grown under constant light (Fig. 6, D–H). This result suggests that the enhancement of seed FA biosynthesis in the badc1 badc3 double mutant is related to cycles of light and dark, which also produce cycles in plastid pH from 8 to 7.

Discussion

Light-induced changes in pH and activity

The environment of the heteromeric ACCase in the chloroplast stroma is a scene of remarkable light-induced change in which photosynthetic electron transport drives the pumping of protons to the thylakoid lumen to build the proton motive force with the uncertainties in the fits. The curves fit the data points with $R^2$ of 0.93, 0.99, and 0.98 for BCCP1, BCCP2, and BADC1, respectively. The changes in the appearance of the NMR spectra are attributable to partial unfolding or complete unfolding of the structured domain at lower pH.
Of variance, from 25th to 75th percentile; and 15
to maximum. Significant differences between lines (one-way analysis of variance) are indicated by different letters. 

(i) The BCCP and BADC subunits each bind directly and independently to BC in vitro with KD < 10 μM under all solution conditions evaluated. (ii) Neutral pH, especially in PBS, enhanced the affinities for BC (Fig. 3B). (iii) The affinity of BADC1 for BC increases more than in pH drops to 7 (Fig. 3A). (iv) BCCP1 and BCCP2 each gain at least an order of magnitude more affinity for BC than BADC1 at pH 8 in KCl (Table 1, Fig. S3). (v) More than half of each BCCP and BADC sequence from Arabidopsis appears likely to be disordered (Fig. 1). (vi) Dropping to neutral pH enhances the partial unfolding of the BCCP1, BCCP2, and BADC1 subunits that is predicted across more than half of each sequence (Fig. 1, 4, and 5). BCCP1, BCCP2, and BADC1 show more NMR peaks at random coil chemical shifts near pH 7 (Fig. 4, B, F, and J). The very marginal stability of the folded domains and their sensitivity to solution conditions is consistent with the small folding core (Fig. 1).

**BADC1 should dominate and inhibit BC at neutral pH under low light conditions**

Calculations using the KD values and concentrations of the subunits present in plastids (Table 1 and Table S1) predict that most of each BCCP or BADC subunit should be capable of binding BC (Fig. 7A). The ability of individual BCCP and BADC sibling subunits to bind the BC subunit in vitro with comparable affinities (Table 1, Fig. 3C) is noteworthy, because in vivo concentrations of BC (83 fmol/μg protein) are lower than the collective abundance of BCCP and BADC subunits (121 fmol/μg protein) (Table S1). This suggests the possibility of competition among sibling BCCP and BADC subunits to bind fewer BC subunits. The bacterial ACCase complexes may be regarded as models for the hetACase from plants. A 1:1 ratio of BCCP to BC subunits was proposed in the enzyme from pseudomonas citronellolis (44) but a 2:1 ratio in the enzyme from E. coli (45). However, the crystallographic structure of the BC subcomplex from E. coli displays 4 BCCP subunits bound to 2 BC subunits, as a dimer of dimers (16) (Fig. 7B). If a 1:1 ratio of BCCP to BC generalizes to hetACase in plastids, the almost 50% excess of BCCP and BADC subunits over BC would be regarded as models for the hetACase from plants.

**pH-sensing small subunits of plastid ACCase**

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*Figure 6. Increase in seed oil content of the Arabidopsis badc1 badc3 mutant depends on light conditions during growth.* The WT (Col-0, blue) and the double mutants badc1 badc2 (badc1/2, red) and badc1 badc3 (badc1/3, green) were grown together in a 16/8 h light/dark cycle (A–C) or under 24 h of constant light (D–F). Harvested seeds were analyzed for seed weight (A, D), fatty acid content per seed (B, E), and fatty acid content as a percentage of seed weight (C, F). Each measurement utilized 100 seeds from a single plant and 15–18 separate individual plant replicates. Box-and-whisker plot: box, 25th to 75th percentile; line, median; +, mean; whiskers, the range of minimum to maximum. Significant differences between lines (one-way analysis of variance, p < 0.05) are indicated by different letters.
subunits in siliques from *Arabidopsis* (Table S1) suggests that not all BADC and BCCP subunits can bind concurrently to the smaller pool of BC subunits. We discuss potential implications of pH sensing by these small sibling subunits upon affinities for BC.

The addition of exogenous BADC1 to excess attenuated hetACCase activity (6). Endogenous BADC1 already enjoys enough of a concentration advantage to dominate the binding of BC in PBS relative to its sibling subunits (Fig. 7A). This advantage is extended in two ways. First, the BADC subunits together enjoy an ~2.5-fold concentration advantage over the BCCP subunits in developing siliques (Table S1). Second, BADC1 exhibited a clear advantage in affinity for BC over the BCCP subunits at pH 7 in PBS (Figs. 2, A−C and 3, A and D). Consequently, BADC subunits, led by BADC1 and lacking biotinylation, offer means of inhibiting hetACCase, especially during dark adaptation when the enzyme is less active.

**Figure 7.** Predicted shifts in occupation of BC by BADC1 and BCCP1 between dark conditions of pH 7 and daylight conditions of pH 8. This prediction is based on the BC affinities for BCCP and BADC subunits, protein levels of these subunits during development of seeds in *Arabidopsis* siliques (19), and the simplifying assumption that the small subunit binds independently to BC. A, the saturation of BC by each subunit is calculated using Equation 3, $K_D$ measured in phosphate solution (Table 1), and the subunit concentrations in siliques given in Table S1, scaled up by the 400-mg/ml protein present in plastids (63). The horizontal lines indicate the maximum possible levels of saturation of BC by that subunit based on the concentrations in Table S1.8, the anticipated pH-dependent shift in the equilibrium binding of BC by the BADC1 and BCCP1 subunits that are abundant in plastids is depicted. The crystallographic coordinates (PDB entry 4HR7) of the BC subcomplex from *E. coli* (16) shows a tetramer of BC subunits in shades of blue and the folded domain of the BCCP subunits in green, with the conserved site of biotinylation (sequence of MKM) colored yellow. The homology model of the folded domain of BADC1 (brown) from *Arabidopsis* is superimposed on selected BCCP chains bound to a BC subunit in the crystal structure. *Arabidopsis* BCCP1 chains are represented by the *E. coli* BCCP chains (green) in the crystal structure. This overly simplified representation of the BC subcomplex in *Arabidopsis* plastids does not portray the range of oligomerization states, associations with other BADC and BCCP subunits, their intrinsic disorder, and the anticipated complexity of the mixtures of structural ensembles.
BADC1 also underwent the biggest increases in affinity for BC as pH dropped from 8 to 7, which accompanies dark adaptation of plastids. The 35-fold increase in the BC affinity for BADC1 induced by the pH drop in PBS (Fig. 2, C and D, A and B) corresponds to 2.1 kcal/mol (Fig. 3B). The results in phosphate-containing buffers seem more relevant than those in HEPES, because the plastid stroma may contain up to 2 mM phosphate (28). BADC1 enjoys 1.1 and 1.3 kcal/mol advantages in affinity for BC over those of BCCP1 and BCCP2, respectively, at pH 7 in PBS (Table 1, Fig. 3, A and C). Since BADC3 shares with the BCCPs similar protein expression levels in developing siliques and similar affinities for BC in PBS (Fig. 7A), BADC3 may also compete (or collaborate through heterodimerization [18]) with BCCP subunits in binding BC. This is corroborated by badc1 badc3 Arabidopsis strains grown with cycles of light and dark (Fig. 6, A–C). pH 8 detracted from BADC1 affinity for BC significantly, especially with KCl present (Fig. 2C and 3, A–C). Consequently, at pH 8 in KCl, BADC1 is predicted to lose much of its physiological advantage in saturating BC (Fig. 7A). In HEPES-KCl, BCCP1 and BCCP2 held the advantage in BC affinity over BADC1 and BADC3, especially at pH 8 (Fig. 3, A and C). [K+] being 160 to 200 mM in plastids (29) suggests physiological relevance for these observations. Consequently, in light-adapted plastids at pH 8 or higher, BCCP1 and BCCP2 should become more competitive with BADC1 in populating BC (Fig. 7). This should enhance ACCCase activity, while the proton-pumping activity of the photosynthetic light reactions is active during daylight.

Switching of roles of BADC subunits?

Nearly normal to enhanced synthesis of FAs by badc1 badc2 and badc1 badc3 lines of Arabidopsis implies the assembly of active BC subcomplexes despite a shortfall of BADC subunits (Fig. 6). This supports the view that BADC subunits, which lack biotin, competitively inhibit biotin carboxylase (5–7). However, reconstitution in E. coli of Arabidopsis BC and BCCP subunits together to a stoichiometric degree requires coexpression with a BADC subunit (18). Moreover, E. coli-expressed BC subcomplexes have substantially higher biotin carboxylase activity when reconstituted by coexpressing the BADC2 or BADC3 subunits (18). These observations suggest that BADC subunits recruited BCCP subunits to BC in E. coli. Can the contrasting pieces of evidence for competitive inhibition and activation of Arabidopsis hetACCase by BADC subunits both be accommodated?

The similarity of BC affinities for the BCCP and BADC subunits in phosphate buffer (Fig. 3D, Table 1) suggests that the siblings should bind BC concurrently. Since pH 8 weakens the BC affinity of all five siblings (Fig. 3, A and C), the BADC subunits, being less concentrated in plastids (19), might increase in association with BC at pH 8 if more abundant BADC subunits recruit them. The known heterodimerization of BCCP and BADC subunits (6, 18) makes joint recruitment to BC more plausible. Heterodimerization conceivably has another value in E. coli. The level of BCCP1 expression in E. coli tends to pass through an early peak 2 to 4 h after induction, followed by a drop at around 6 h (Fig. S4). This suggests some degradation of BCCP1 in E. coli. Since the disordered N terminus of BCCP1 interacts with a BADC subunit (18), it is possible that the heterodimerization partially protects BCCP1 from proteolysis and thereby ferries BCCP1 more successfully to its BC partner.

We lack evidence in plastids to evaluate BCCP-BADC heterodimers in physiological regulation of the activity of BC subcomplexes. Our simplified titrations in vitro again suggest that (i) at pH 7 in phosphate buffer, the more abundant BADC1 is dominant in association with BC in dark-adapted plastids, and (ii) at pH 8 in KCl solution, BADC1 loses its dominance in BC subcomplexes to BCCP1 and BCCP2, having one to two orders of magnitude more affinity for BC in light-adapted plastids (Table 1, Fig. 3 and 7, and Fig. S3).

Functional advantages expected from the disorder of BCCP and BADC subunits

The intrinsic disorder of the BCCPs and BADCs and the higher affinities for BC at pH 7 should have biophysical implications. Intrinsic disorder is observed in multiprotein complexes and confers promiscuity to the protein–protein interactions of hub proteins (46). Flexible peptides and intrinsically disordered proteins enjoy fast on-rates and off-rates in their associations with protein partners (46, 47). The speed of their association is attributable to fast zippering up due to the cooperative addition of many weak contacts at the interface (47, 48). The “fly-casting” model argues that intrinsic disorder increases the radius for capturing the partner with weak contacts to begin a molecular encounter complex (49, 50). The multiple weak protein–protein contacts typically combine with folded domains to accumulate affinity for protein partners (46). The IDRs of the BCCP and BADC subunits also may confer affinities for other subunits of hetACCase. Consider the two biggest predicted IDRs in each BCCP and BADC, i.e. the central sequence of 50 residues and the N terminus (Fig. 1). The N-terminal 70 residues of BCCP1 is required for its heterodimerization with BADC3 (18). A region of 48 residues from the disordered central IDR of BCCP1 appears to enhance association with BADC3, while the folded C-terminal region did not participate (18).

Potential protein-binding regions residing in IDRs can be predicted using the ANCHOR method (51, 52). The ANCHOR method predicts that the principal IDR in BCCP1, BCCP2, and BADC1 may have two regions for protein associations but only one such site in the main IDR of BADC2 and BADC3 (Fig. 1, open circles). Additional partial unfolding apparent at near-neutral pH in the amide NMR spectra of Arabidopsis BCCP1, BCCP2, and BADC1 suggests that pH 7 enhances their capture radii and exposure of protein binding regions beyond that at pH 8. This might increase the number of multivalent contacts possible with BC. Such enhancements might account for the higher affinities of the BCCP and BADC subunits for BC at pH 7 than at pH 8.

While NMR spectra reveal partial unfolding of the folded domain by neutral pH (Fig. 4), how much of the chains of BCCP and BADC subunits sense pH changes is less clear. The folded domains could sense the pH changes directly. In the homology models, basic and acidic side chains appear to be interspersed for favorable attractions. Partial protonation of these acidic
side chains might decrease these stabilizing interactions, yet the folded domains also could be influenced indirectly by the IDR. The IDR may sense pH changes because IDR is highly charged and responsive to pH and counter-ions (21, 53). Since the anionic IDR may be attracted to the basic thumb of BADC in each BADC subunit, IDR may indirectly stabilize the folded domains in a pH-dependent fashion. The interaction of the N-terminal 71 residues of BCCP1 with BADC3 (18) might be compatible with the basic thumb of BADC3 attracting the net negative charge of this disordered part of BCCP1.

Under conditions of high activity at pH 8 in KCl, how can BC select BCCP1 and BCCP2, needed for the activity, over BADC1, which is more abundant in plastids? At pH 8 in KCl, BC affinities for BCCP1 and BCCP2 were as little as 1.5 kcal/mol better than that of BADC1 (Table 1). A theory of promiscuous protein–protein associations pointed out that discrimination among alternative partners with small differences in affinity is maximized by modest affinities; the discrimination is maximal when $K_D$ lies within an order of magnitude of the concentrations of the partners (54). Fig. S3 suggests that this regime optimizes the BC preference for BCCP1 and BCCP2 over BADC1 or BADC3 at pH 8 in KCl, because the $K_D$ values approach the physiological concentrations.

Agreement of biophysical data with seed oil in badc1/3 lines grown in light–dark

The badc1 badc3 line grown in a day/night cycle had an ~18% increase in FAs per seed and an ~8% increase in seed weight, producing an ~10% increase in seed lipid content by dry weight (Fig. 6). Previously, the badc1 badc3 mutant also grown under a day/night cycle was reported to have an ~9% increase in FAs per seed and an ~17% decrease in seed weight, producing an ~30% increase in seed lipid content by dry weight (7). The reasons for the differential increase/decrease in seed weight are not clear but are likely due to other differences in growth conditions, such as fertilizer, humidity, and especially light intensity, which is known to have a large effect on oilseed development (40). However, both studies indicate the badc1 badc3 mutant has a higher FA content when grown under a day/night cycle.

Comparison of affinities for BC suggests BADC subunits (Fig. 2, Table 1) should be more effective inhibitors at neutral pH conditions expected in chloroplasts under dark, dawn, and dusk conditions. High protein levels of BADC1 and BADC3 during seed development (19) draw attention to them. pH 8-enhanced competitiveness of BCCP1 and BCCP2 for BC (Fig. 3D and Fig. S3) may facilitate the higher catalytic turnover of carboxylated BCCP by BC during high activity at pH 8 of light adaptation. The increased seed oil content of badc1 badc3 mutant Arabidopsis grown with a light-dark cycle is in accord with these expectations. A low level of BADC2 protein expression may indirectly stabilize the folded domains also could be influenced indirectly by the IDR. The IDR may sense pH changes because IDR is highly charged and responsive to pH and counter-ions (21, 53). Since the anionic IDR may be attracted to the basic thumb of BADC in each BADC subunit, IDR may indirectly stabilize the folded domains in a pH-dependent fashion. The interaction of the N-terminal 71 residues of BCCP1 with BADC3 (18) might be compatible with the basic thumb of BADC3 attracting the net negative charge of this disordered part of BCCP1.

**Conclusions**

The BADC and BCCP subunits of hetACCase appear to act as pH sensors. Amid the overall weaker affinities for BC at pH 8, BCCP1 and BCCP2 gained more than 10-fold advantages in affinity for BC over BADC1 (in KCl solutions). The pH 8 boost to BC preferences for BCCP1 and BCCP2 seems favorable for the activity of hetACCase in daylight. The decrease of pH from 8 to 7 increased the affinities of BADCs and BCCPs for the BC subunit of hetACCase, with BADC1 undergoing the greatest boost to affinity in PBS. The high physiological concentration and high BADC1 affinity for BC at pH 7 in PBS suggest BADC1 is a principal inhibitor of biotin carboxylase activity in the dark. The intrinsic disorder of the BADC and BCCP subunits is likely to promote fast association with BC. If BADC subunits mediate inhibition by night and if BCCP subunits are enhanced in their interactions with BC under daylight conditions, that would implicate BADCs in light- and pH-dependent switching of hetACCase activity. Consistent with this switching hypothesis, the badc1 badc3 double mutant of Arabidopsis grown with a daily cycle of light and dark synthesizes more FAs in its seeds.

**Experimental procedures**

**Disorder predictions**

The mature sequences of the BCCP1, BCCP2, BADC1, BADC2, and BADC3 subunits of the hetACCase of A. thaliana were submitted to the GeneSilico server and its MetaDisorder-MD2 algorithm for a series of disorder calculations (25). MetaDisorder-MD2 integrates predictions from 13 disorder predictors with several structure-based predictions to enhance the sensitivity and specificity of the disorder prediction (25). The sequences of the subunits were also submitted to the IUPred2A server to obtain ANCHOR2 predictions of disordered binding regions for proteins (51, 52).

**Sequence alignment and structural modeling**

Multiple-sequence alignment of the BADC and BCCP sequences from Arabidopsis was performed using Clustal Omega (55). The homology models were constructed with SWISS-MODEL (56, 57) at RRID: SCR_018123. Crystallographic coordinates of E. coli BCCP (PDB entry 4HR7) (16) served as the template for building the homology models of BCCP1, BCCP2, BADC1, BADC2, and BADC3 from A. thaliana. Hypothetical BADC1 docking into tetrameric BC subcomplexes exploited structural alignment of its homology model with the BCCP coordinates in the crystal structure of the BC subcomplex from E. coli (16) using the align function in PyMOL (58). The locations of the secondary structures predicted by the homology models were rendered on the multiple-sequence alignment using the ESPript server (59).
Protein samples for MST

E. coli BL21 harboring pET28A expression vectors with inserts for the coding frames for Arabidopsis BC, BCCP1, BCCP2, BADC1, BADC2, or BADC3 were prepared previously (6). Each protein was expressed in Luria broth containing either kanamycin. Expression was induced with 100 μM IPTG when the optical density at 600 nm reached around 0.6. After a 6-h induction period, the cultures were pelleted and resuspended with 10 ml of lysis buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4; designated PBS). After cell disruption by French press and centrifugation to pellet the cell debris, the supernatant was incubated with 1 ml of nickel agarose resin (Gold Biotechnology, H-320). After 30 min at 4°C, the protein mixture was loaded onto a column of the nickel agarose and washed with 50 ml of wash buffer (20 mM imidazole in lysis buffer, pH 7.4). After washing, the protein was eluted with 10 ml elution buffer (150 mM imidazole in lysis buffer, pH 7.4). The protein samples were purified to more than 90% purity and concentrated to around 500 μl with a Pierce concentrator PES with a 10,000-Da cutoff (ThermoFisher, 88527) according to the user manual. The buffers of the concentrated samples were changed by dialysis using dialysis vials (Sigma, PURN12030) overnight at 4°C. After dialysis, the protein concentration was measured using the Bradford assay (19). Solutions of 15N-labeled samples of Arabidopsis BC, BCCP1, BCCP2, and BADC1 were prepared by culturing E. coli BL21 with pET28a in PG minimal medium (64) prepared with 15N NH₄Cl (Cambridge Isotope Laboratories). The 15N-labeled samples of Arabidopsis BC, BCCP1, BCCP2, and BADC1 were prepared at 150 to 180 μM in PBS buffer with the composition listed above but set to pH 8. The titration in Fig. 5A used 500 μM BCCP1, however.

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The Km values measured in phosphate were used to estimate the physiological fraction of BC bound, fbd, by a single BCCP or BADC subunit, present at concentration Lt, using Equation 3:

\[ f_{bd} = \frac{K_D + L_t + P_t + \sqrt{(K_D + L_t + P_t)^2 - 4P_tL_t}}{2P_t} \]  

(Eq. 3)

The total molar concentration of BC, Pt, was estimated as the 83-fmol/μg total protein measured in developing siliques (19), multiplied by the 400 mg of total protein/ml observed in plastids (63). Lt, estimates of concentrations of the BCCP or BADC subunits in plastids were estimated similarly using the quantities in Table S1 scaled up by the 400-mg/ml protein present in plastids.

NMR sample preparation

The BADC1, BCCP1, and BCCP2 coding frames were optimized for expression in E. coli using the IDT tool and sub cloned into pET27B(+). 15N-labeled Arabidopsis BCCP1, BCCP2, and BADC1 were prepared by culturing E. coli BL21 with pET28a in PG minimal medium (64) prepared with 15N NH₄Cl (Cambridge Isotope Laboratories). The 15N-labeled samples of Arabidopsis BCCP1, BCCP2, and BADC1 were prepared at 150 to 180 μM in PBS buffer with the composition listed above but set to pH 8. The titration in Fig. 5A used 500 μM BCCP1, however.

pH titrations and NMR and their fitting

BEST-TROSY spectra (65) of the 15N-labeled samples at 25°C in 3-mm tubes (Norell) were collected using a Bruker Avance III 800-MHz NMR spectrometer. Starting with the samples near pH 8, small aliquots of 0.1 N HCl or citric acid were added, pH read using a narrow glass electrode, and BEST-TROSY spectra collected with progressively lower pH as far as pH 2.6. Buffering of the pH in the acidic range was provided by the abundance of carboxylic acids in the protein chain, i.e. 24 in BCCP1, 20 in BCCP2, and 27 in BADC1. The NMR spectra were read and PCA calculated using TRENDNMR.exe, while the response, y, of each PC was fitted to Equations 4 or 5 using TRENDanalysis.exe (34, 35). Each fitted PC from the pH titrations was plotted in OriginLab 2019 or 2020. The pH transitions were fitted to equations similar to those reported previously (66, 67), featuring a midpoint (pKₘid) in each pH transition captured by the PC from the titration. PC1 from each pH titration was monophasic and was fitted to a single term:

\[ y = \frac{y_{max} - y_{min}}{1 + 10^{-(pK_{mid} - pH)}} \]  

(Eq. 4)

Fitting PC2 from the pH titrations required two terms opposite in sign (and yₘₐₓ₁ ≈ yₘₐₓ₂):

\[ y = y_{min} + \frac{y_{max1} - y_{min}}{1 + 10^{-(pK_{min1} - pH)}} + \frac{y_{max2} - y_{min}}{1 + 10^{-(pK_{min2} - pH)}} \]  

(Eq. 5)

Fits of PC2 from the titrations to Equation 5 optimized yₘᵢₙ to -0.47 for BCCP1 and BCCP2 and -0.52 for BADC1.
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**Light–dark cycle versus constant-light-grown seed oil measurements**

Seeds were sterilized (with 70% ethanol for 5 min, 10% bleach with 0.1% SDS for 15 min, and washing 5 times with sterile water) and applied to germination plates (1× MS salts, 0.05% MES-free acid, 1% sucrose, and 0.8% agar, pH 5.7). The plates were incubated at 4°C for 2 days and then placed under 24 h of low-light conditions, of around 100 μmol photons m⁻² s⁻¹ light, until all lines germinated and produced two true leaves (~7–10 days). The seedlings were then transferred to soil and placed in either 1) 16/8 h light/dark cycle with 150–175 μmol photons m⁻² s⁻¹ light intensity at pot height measured across the chamber or 2) 24 h constant light of 180–200 μmol photons m⁻² s⁻¹. Both growth chambers used full-spectrum ceramic MH 315W bulbs and were set at a constant 23°C. All plants were watered as needed and fertilized twice a week with general-purpose mix NPK 20-10-20 (57.4 g/gal). For each light treatment, 18 individual plants of each plant line were grown together to provide seeds for analysis. Their locations were randomized across the growth chamber to minimize the effects of position within the chamber. Seed lipid content was determined by direct conversion to FA methyl esters (FAMES) and quantification by GC with flame ionization detection based on reference 68. In brief, dry seeds were counted (n = 100) and weighed. FAMES were produced in 1.5 ml 5% sulfuric acid in methanol at 85°C for 1.5 h together with 50 μg 17:0 tracyglycerol in 0.2 ml toluene as an internal standard. FAMES were extracted with 0.2 ml hexane and 1.5 ml 0.88% potassium chloride. The hexane phase was analyzed on an Agilent 7890B GC-FID with a DB-FATWAX UI column (30 m, 0.25 mm inner diameter, degrees of freedom of 0.25 μmol) with He carrier gas at constant linear velocity 28.5 cm/s, 100°C for 0 min, increased at 25°C/min to 200°C, and then increased at 10°C/min to 240°C, where it was held for 4.5 min. All calculations were done with Microsoft Excel, and graphing and statistical analysis were done with GraphPad Prism version 8.3.

**Data availability**

All fitted and interpreted data are presented in the manuscript. The following data used for fitting are available upon request: (i) the MST titration data of Fig. 2 in text format and (ii) the raw and Fourier-transformed NMR spectra used in Fig. 4 and 5 in Bruker format. Send requests to the corresponding author.

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**Abbreviations**—The abbreviations used are: hetACCase, heteromeric acetyl-CoA carboxylase; AQUA-MRM, absolute quantitation of multiple reaction monitoring; BADC, biotin attachment domain-containing; BC, biotin carboxylase; BCCP, biotin carboxyl carrier protein; CT, carboxyltransferase; FA, fatty acid; FAME, fatty acyl methyl ester; IDR, intrinsically disordered region; MST, microscale thermophoresis; NMR, nuclear magnetic resonance spectroscopy; PBS, phosphate-buffered saline; PBK, phosphate buffer with KCl; Trend, software for tracking equilibrium and nonequilibrium shifts in data; TROSY, transverse relaxation optimized spectroscopy; PCA, principal component analysis.

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