**Molecular mechanism underlying transport and allosteric inhibition of bicarbonate transporter SbtA**

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**Author Contributions:** S.F. and X.H. designed the experiments and carried out protein purification and functional analysis, crystallization, X-ray diffraction data collection, and cryo-EM sample preparation; X.Z. carried out cryo-EM data collection and structure determination; M.Z. and L.L. contributed to functional analysis; F.Y., Y.H. and H.G. contributed to protein expression and purification; P.Z. and X.Z. conceived the project, analyzed the data and wrote the manuscript with inputs from other authors.

**Competing Interest Statement:** The authors declare no competing interests.

**Keywords:** Bicarbonate transporter; $CO_2$ concentrating mechanism; SbtA; allosteric inhibition; cryo-EM; crystal structure; photosynthesis

**Abstract**

SbtA is a high-affinity sodium-dependent bicarbonate transporter found in cyanobacterial $CO_2$ concentrating mechanism (CCM). SbtA forms a complex with SbtB, while SbtB allosterically regulates the transport activity of SbtA by binding with adenyl-nucleotides. The underlying mechanism of transport and regulation of SbtA is largely unknown. In this study, we report the three-dimensional structures of the cyanobacterial *Synechocystis* sp. PCC 6803 SbtA-SbtB complex in both the presence and absence of $HCO_3^-$ and/or AMP, at 2.7 Å and 3.2 Å resolution. Analysis of the inward-facing state of the SbtA structure reveals the $HCO_3^-$/$Na^+$ binding site, providing evidence for the functional unit as a trimer. A structural comparison found that SbtA adopts an elevator mechanism for bicarbonate transport. Structural-based analysis revealed that the allosteric inhibition of SbtA by SbtB occurs mainly through the T-loop of SbtB, which binds to both the core domain and the scaffold domain of SbtA and locks it in an inward-facing state. T-loop conformation is stabilized by the AMP molecules binding at the SbtB trimer interfaces and may be adjusted by other adenyl-nucleotides. The unique regulatory mechanism of SbtA by SbtB makes it important to study inorganic carbon uptake systems in CCM, which can be used to modify photosynthesis in crops.

**Significance Statement**

SbtA is a sodium dependent high affinity bicarbonate transporter in cyanobacterial CCM. The transport activity of SbtA is regulated by SbtB which is additionally influenced by adenyl-nucleotides. We determined the 3D structures of SbtA in complex with SbtB in two different conformations: A model summarizing the molecular mechanism of transport and allosteric inhibition of SbtA was established based on structural and biochemical data. The transport and regulatory mechanism revealed by our study represents a valuable case to investigate allosteric regulation of membrane transporters, and more importantly, will benefit plant photosynthesis improvement using the CCM system.

**Main Text**

**Introduction**

Cyanobacteria have evolved a unique $CO_2$ concentrating mechanism (CCM) in carbon fixation. This increases the intracellular concentration of $CO_2$ feeding RuBisCO (Ribulose Bisphosphate Carboxylase-Oxygenase) that is encapsulated in carboxysomes, improving photosynthetic performance (1, 2). The CCM contains five distinct uptake systems that transport dissolved inorganic carbon (Ci), $HCO_3^-$ and $CO_2$ into the cell (3). Among them, SbtA and BicA are sodium-dependent $HCO_3^-$ transporters with high affinity and medium affinity, respectively (4, 5). BCT1 or CmpABCD is a $HCO_3^-$ transporter complex powered by ATP hydrolysis (6), while $NDH-I_3$ and $NDH-I_4$ complexes are responsible for $CO_2$ uptake (7, 8). These Ci uptake systems are ideal targets for enhancing photosynthesis in C3 plants (9-14).

Ci uptake systems are regulated at different levels to adapt to a changing environment. CmpABCD was first identified as a cyanobacterial Ci transporter whose expression level is induced by Ci limitation (6), as are SbtA and $NDH-I_3$ which are regulated by the LysR-type transcription factor NdhR (15). The molecular mechanism underlying transcriptional regulation has previously been studied (16). As a transcription repressor, NdhR controls the expression levels of *ndh-I3*, *BicA*, and *SbtA/B*, while the Ci limitation metabolite 2-phosphoglycerate (2-PG) can bind to NdhR to alter its conformation and release repression.

Ci transporter activity is allosterically or post-translationally regulated. SbtA forms a complex with SbtB, while SbtB regulates the transport of SbtA via binding with AMP or cAMP (17).The active form of BicA is a dimer stabilized by the C-terminal STAS domain, however, this activity could not be reconstructed in a heterologous expression system (18, 19). This suggests that transporter activity could require additional regulatory proteins or post-translational modification. In addition. it is considered that the CmpABCD transporter activity is regulated by the cytoplasmic $HCO_3^-$ concentration since the CmpC subunit contains an extra substrate-binding domain in addition to the nucleotide-binding domain (3). The molecular mechanisms of transport and activity regulation of Ci transporters remain largely unknown, which to a large extent limits their application in photosynthesis improvement (20-22).

86   This study focuses on the structural and mechanistic analysis of the bicarbonate transporter

87   SbtA by determining its complex structure with the regulatory subunit SbtB, both in the

88   presence and absence of $HCO_3^-$ and/or AMP. Structure based analysis suggests molecular

89   mechanisms underlying transport and regulation.

90   **Results**

91   **SbtA activity and allosteric inhibition by SbtB**

92   *Can* encodes the carbonic anhydrase (CA), which is required for the growth of *E. coli*

93   under normal air conditions; a *can* paralog, *cynT*, can replace *can* for normal growth when

94   induced with azide (23). This finding was used successfully for active bicarbonate

95   transporter screening (24). We generated the *can* knockout strain using *E. coli* C43(DE3)

96   [C43(DE3)-Δ*can*], which was used to characterize the activity of *Synechocystis* sp. PCC

97   6803 SbtA, SbtA-SbtB, and its derivative mutations. Consistent with previous results, the

98   C43(DE3)-Δ*can* strain cannot grow under normal air conditions, but can grow in the

99   presence of 1 mM azide (*SI Appendix*, Fig. S1). The transfer of the SbtA gene into

100   C43(DE3)-Δ*can* partially complements growth failure under normal air conditions, but the

101   co-transfer of SbtA and SbtB genes fails to do so (Fig. 1 *A and B*). This suggests that

102   *Synechocystis* sp. PCC 6803 SbtA alone possesses bicarbonate transporter activity when it

103   is expressed in *E. coli* C43(DE3), while SbtB allosterically inhibits transporter activity,

104   which is consistent with previous studies (24).

105   **Overall structure of SbtA in complex with SbtB**

106   To obtain SbtA and SbtB protein complex for structural analysis, we co-expressed the

107   *Synechocystis* sp. PCC 6803 originated *SbtA* and *SbtB* in *E. coli* C43 (DE3). We found that

108   addition of 2 mM AMP or ADP but not cAMP or ATP in the purification buffer was

109   necessary for a stable 1:1 molar ratio complex formation (Fig. 2*A*). This is consistent with

110   previous results suggesting that the membrane association of SbtB depends on the presence

111   of AMP/ADP, but not on cAMP (17). The purified SbtA-SbtB complex protein sample was

112   used for both cryo-EM and crystallization analysis. The cryo-EM structure of SbtA-SbtB

113   complex was obtained in lipid nanodiscs at 2.7 Å resolution (SbtAB$^{EM}$) (Fig. 2 *B and C*, *SI*

114   *Appendix*, Fig. S2 and Table S1). The map quality was high and numerous additional

115   densities around SbtA were assigned as annular membrane lipids (*SI Appendix*, Fig. S3),

116   allowing us to build an accurate model. The resulting model was then used as a template to

determine the crystal structure by molecular replacement, resulting in the 3.2 Å resolution crystal structure of the SbtA-SbtB complex (SbtAB$^{Xtal}$) (Fig. 2D and *SI Appendix*, Table S2). The overall structures of SbtAB$^{EM}$ and SbtAB$^{Xtal}$ are similar, which form a three-fold complex, with a root mean squared deviation (RMSD) of 0.635 Å over 1178 Cα atoms. SbtA and SbtB form homotrimers in the membrane and cytoplasm, respectively, and each SbtB monomer binds to the cytoplasmic surface of a SbtA monomer to form a heterodimer (Fig. 2 *B-D*). Similar trimeric transporter structures have been reported in the bacteria ammonium transporter AmtB (25, 26), the glutamate transporter homologue Gltph (27), the human excitatory amino acid transporter EAAT1 (28), and the human concentrative nucleoside transporter CNT3 (29), suggesting that the trimer is the functional unit of SbtA as proposed in other studies (24).

The SbtA molecule in both SbtAB$^{EM}$ and SbtAB$^{Xtal}$ structures is comprised of 10 transmembrane helices (TMs). Of these, TMs 1, 4, 6, and 9 are interrupted by short loops (Fig. 2 *C and E*). The long loop connecting TM5 and TM6 (residues 165 to 207 in SbtAB$^{EM}$ and residues 170 to 207 in SbtAB$^{Xtal}$), which may be involved in activity regulation (30), is missing in both structures. Both the N and C termini face the periplasm, which confirms the previous experimental results (30). SbtA contains a scaffold domain, consisting of TMs 1-2 and 6-7, that mediates the interactions between SbtA molecules formed along the trimer. It also contains a core domain consisting of TMs 3-5 and 8-10 (Fig. 2 *C, E and F*). A narrow cleft is present between two domains (Fig. 2*F*). The SbtB structure is a typical PII fold, characterized by a four-antiparallel-stranded β-sheet inserted by two helices, and the long loop comprising residues 40 to 58 (previously named T-loop) is inserted between β2 and β3 (Fig. 2*C*).

SbtA in both the SbtAB$^{EM}$ and SbtAB$^{Xtal}$ structures is positioned in an inward-facing state. However, the HCO$_3^-$ substrate is clearly defined in each SbtA molecule of the SbtAB$^{Xtal}$ structure, but not in the SbtAB$^{EM}$ structure. Three AMP molecules are well-defined in the SbtB trimer interfaces of the SbtAB$^{EM}$ structure, but are disordered in the SbtAB$^{Xtal}$ structure. Accordingly, the T-loop in the SbtB structure is well-defined in the SbtAB$^{EM}$ structure but disordered in the SbtAB$^{Xtal}$ structure (Fig. 2 *C and D*). Metal ion binding sites, which are supposed to be sodium, are observed in both the SbtAB$^{EM}$ and SbtAB$^{Xtal}$ structures (Fig. 3*F* and *SI Appendix*, S4 *A and B*).

**Substrate binding site**

149   Seen from the SbtAB$^{Xtal}$ structure, the substrate $HCO_3^-$ binding site is located at the

150   interface of the core domain and the scaffold domain (Fig. 3*A*). Two discontinuous

151   transmembrane helices, TM4a/4b and TM9a/9b, are surrounded by other transmembrane

152   helices and form a TM cross. $HCO_3^-$ binds to the pericentral side of the cross (Fig. 3 *A and*

153   *B*, and *SI Appendix*, Fig. S4*C*). The $HCO_3^-$ binding site is accessible from the cytoplasm

154   through a cavity comprised of the TMs 2, 6a, 7, and 9b (Fig. 3*A*). The side chains of

155   residues Ser114 and Ser116 from TM4b, and Asp325, Ser327 from TM9b form hydrogen

156   bonds with $HCO_3^-$ either directly or through a water molecule; the main chain oxygen of

157   Ser324 and two water molecules form hydrogen bonds with $HCO_3^-$ (Fig. 3*C*). Notably,

158   residues Ser116, Asp325 and Ser327 are subjected to conformational changes in the SbtA

159   molecule of the SbtAB$^{EM}$ structure, which may distort the binding of $HCO_3^-$ (Fig. 3*D* and

160   *SI Appendix*, Fig. S4 *C and D*). Mutation of the $HCO_3^-$ binding residue could significantly

161   impair the complementary function of SbtA (Fig. 3*E* and *SI Appendix*, Fig. S5). On the

162   peripheral side of the TM cross, there was a clear electron density peak that supposed to be

163   a $Na^+$ binding site (Fig. 3*B* and *SI Appendix*, Fig. S4 *A and B*); it forms five bonds (usually

164   seen in sodium-dependent transporters (19, 31))with the main chain oxygens of residues

165   Phe110, Gly111 and Ala112 from TM4a, and Ala 320 and Ser322 from TM9a (Fig. 3*F*).

166   Most of the residues involved in $HCO_3^-$ and $Na^+$ binding are conserved among

167   cyanobacterial SbtA proteins (*SI Appendix*, Fig. S6), suggesting a conserved binding mode.

168   In summary, the SbtA structures captured in SbtAB$^{EM}$ and SbtAB$^{Xtal}$ complexes represent

169   substrate-free and substrate-binding conformations of the inward-facing state, respectively.

**Interactions between SbtA and SbtB and functional role of AMP**

171   While SbtA and SbtB form similar heterodimers in the SbtAB$^{EM}$ and SbtAB$^{Xtal}$ structures,

172   the interaction surfaces are significantly different. In the SbtAB$^{EM}$ structure, the interaction

173   surface of SbtA and SbtB is clearly defined and buries about 807.4 $Å^2$ (Fig 4*A*); it is

174   constituted by the β1-α1 loop, the α2-β4 loop, and the T-loop from SbtB as well as TM2, 7,

175   9b, and 10 from SbtA. The T-loop of SbtB inserts itself into the cytoplasmic cavity of SbtA

176   constituted by TMs 2, 6a, 7, and 9b, and the interactions are primarily van der Waals'

177   contacts (Fig. 4*A*). Additionally, hydrogen-bonding interactions are found between the side

178   chains of residues Glu265, Ser268, Arg269, and Arg333 from SbtA and the side chains of

179   residues Glu13, Asn52, and Tyr87, the backbones of residues Arg46, Thr53, and Asp86

180   from SbtB (Fig 4 *B and C*). However, in the SbtAB$^{Xtal}$ structure, the T-loop of SbtB is

181  disordered and the interactions between SbtB and SbtA are restricted to the β1-α1/α2-β4

182  loops and TM9b, while the interaction surface area is reduced to 127.6 Å$^2$ (*SI Appendix*,

183  Fig. S7*A*).

184  We analyzed the determinants stabilizing the SbtB T-loop conformation in the SbtAB$^{EM}$

185  structure and found that the AMP molecule bound in SbtB plays a critical role (Fig. 4*A*).

186  The AMP binds at the intermolecular cleft of two neighboring SbtB molecules (Fig. 4*D*

187  and *SI Appendix*, Fig. S8), which is similar to the previous SbtB trimeric structure (17, 32).

188  However, a detailed analysis revealed more extensive hydrogen bonds surrounding AMP in

189  our structure (Fig. 4*D*). In particular, the residues Ser42 and Arg43 from T-loop and the

190  residue Gly89 from b4 form four hydrogen bonds with the phosphate group of AMP.

191  Additionally, the guanidine group of Arg43 forms a hydrogen bond with the main chain

192  oxygen of residue Asp86 from the α2-β4 loop; Arg46 forms hydrogen bonds with the

193  Arg43 main chain oxygen and Asn59. These interactions dictate and stabilize the T-loop

194  conformation of SbtB when binding with SbtA.

195  We identified key residues involved in SbtA-SbtB interactions and AMP binding to

196  perform mutation-based functional analyses. Pull-down results show that the mutant

197  SbtA(R333A)-SbtB  almost  abolishes  the  SbtA-SbtB  complex  formation,  while

198  SbtA-SbtB(E13D) impairs complex formation (Fig. 4*E*). Point mutations aiming to reduce

199  the SbtB T-loop interactions with SbtA, such as SbtA(R269A)-SbtB, SbtA(E265A)-SbtB

200  and SbtA-SbtB(V45L) have relatively minor effects on complex formation which may be

201  due to their extensive interaction surface areas. However, SbtA-SbtB(S47Q) mutation

202  aiming to introduce steric confliction at the interaction surface disrupts the complex

203  formation (*SI Appendix*, Fig. S7*B*). Furthermore, mutations of residues involving AMP

204  binding, SbtA-SbtB(S42A/R43A) and SbtA-SbtB(R46A), significantly reduce the complex

205  formation (Fig. 4*E*). Accordingly, mutations that abolish the complex formation, such as

206  SbtA-SbtB(S47Q) and SbtA(R333A)-SbtB, could significantly reduce the growth failure of

207  C43(DE3)-Δ*can-SbtAB,* while mutations that impair the complex formation, such as

208  SbtA-SbtB(E13D), SbtA-SbtB(S42A/R43A), SbtA-SbtB(R46A), and SbtA(E269A)-SbtB,

209  have positive effect on the growth (Fig. 4*F* and *SI Appendix*, Fig. S9). Therefore, we

210  conclude that the inhibition of SbtA activity by SbtB relies on the SbtA-SbtB complex

211  formation, which is greatly strengthened by AMP binding, as also proposed in other work

212  (17, 24).

213 These results provide a molecular explanation for why the addition of AMP during the

214 protein purification greatly enhances the complex formation of SbtA-SbtB (Fig. 2*A*), and

215 also well explain why the association of SbtB to the membrane largely depends on the

216 presence of AMP (17). To investigate why the addition of cAMP impairs the SbtA-SbtB

217 complex formation, we superimposed the cAMP molecule to the AMP binding site of the

218 SbtAB$^{EM}$ structure (Fig. 4*G*). Our results suggest that the binding mode of the adenine

219 moiety of cAMP overlaps with AMP, while the hydrogen bonds formed via residues Ser42

220 and Arg43 may lost in the presence of cAMP. Additionally, cAMP displays a steric clash

221 with the sidechain of Arg46. These processes could distort the T-loop conformation and

222 diminish SbtA-SbtB interactions.

**Transport and regulatory mechanism**

224 The TM cross is a typical structural feature of the substrate-binding site in the SbtA protein

225 (Fig. 3*B*), and resembles the structure of some solute carrier (SLC) family transporters. A

226 protein structure comparison via DALI server (33) produced four matches (Z-score over

227 10), all of which were sodium-dependent SLC family transporters [NhaA, NhaP, NapA,

228 and ASBT; respective corresponding PDB accession codes 4atv, 4cz8, 4bwz, and 3zux

229 (34-37)]. These not only share a similar structure feature at the substrate-binding site, but

230 similar overall topology (Fig. 5*A*). Of particular interest is the ASBT$_{NM}$, which is a

231 bacterial homolog (*Neisseria meningitidis*) of the animal sodium-dependent bile acid

232 symporter ASBT (34). While SbtA and ASBT$_{NM}$ only share approximately 10% sequence

233 identity, both structures contain 10 transmembrane helices, and over 80% of their structural

234 elements were aligned. All structures of these transporters contain two domains: a core

235 domain for substrate binding and a scaffold domain for oligomerization. The transport

236 mechanism of these transporters has been proposed based on their structures (34-38),

237 which could take the form of a rocking bundle or elevator mechanism involving rigid body

238 movement of the core domain between inward-facing and outward-facing states. SbtA

239 could follow a similar transport mechanism (Fig. 5*B*) and the core domain may undergo

240 rigid movement to translocate HCO$_3^-$ into the plasma membrane.

241 Current and previous results both demonstrate that SbtB can inhibit the bicarbonate

242 transporter activity of SbtA when heterologous expressed in *E. coli* (24) (Fig. 1 *A and B*),

243 suggesting that SbtB allosterically inhibits the transport activity of SbtA by forming

244 complexes under certain physiological conditions. Analysis of our structural data reveals

245 the underlying molecular mechanism of the allosteric inhibition of SbtA by SbtB. In the

246 SbtAB$^{EM}$ structure, the SbtA-SbtB interaction surface involves both the core domain and

247 the scaffold domain of SbtA; in particular, the T-loop of SbtB inserts itself into the

248 cytoplasmic cavity formed between the two domains of SbtA. This could preclude the core

249 domain movement during transport and lock the SbtA at the inward-facing substrate-free

250 conformation (Fig. 5*B*). However, in the SbtAB$^{Xtal}$ structure, the intermolecular

251 interactions are restricted to the core domain of SbtA due to the disordered T-loop or the

252 absence of AMP in the SbtB molecule (Fig. 5*B*) and the SbtA protein is located in the

253 inward-facing substrate-binding conformation. This implies that the SbtAB$^{Xtal}$ structure

254 could represent a pre-step of allosteric inhibition. Therefore, we conclude that high AMP

255 concentrations stabilize the SbtB T-loop conformation and help insert it into the

256 cytoplasmic cavity of SbtA, which locks the conformation of the scaffold domain and core

257 domain of SbtA in the inward-facing state and inhibits bicarbonate transporter activity.

258 Additionally, the presence of cAMP can compete with AMP to bind with SbtB, inducing

259 conformational change in the T-loop and precluding its interaction with SbtA, relieving

260 SbtB inhibition toward SbtA. Therefore, the T-loop of SbtB could regulate the transporter

261 engine SbtA in response to environmental AMP or cAMP concentration.

262 **Discussion**

263 The mechanism of allosteric regulation of SbtA by SbtB is similar to the regulation of the

264 ammonium transporter AmtB by GlnK in bacteria, where GlnK also forms a trimer with

265 ADP molecules binding at the neighboring dimeric interface to allosterically inhibit the

266 transporter activity of AmtB (39). Both SbtB and GlnK belong to the PII family of proteins,

267 which help regulate various aspects of nitrogen assimilation and carbon homeostasis via

268 binding with adenyl-nucleotides (21). This regulation mechanism is found in many

269 different species, despite long term evolution.

270 Cyanobacterial CCM contains three bicarbonate transporters. Of these, SbtA can be

271 allosterically inhibited by SbtB. The inhibitory effect relies on the StbA and SbtB complex

272 formation and is adjusted by the adenyl-nucleotides binding with SbtB. The presence of

273 AMP stabilizes the SbtA-SbtB complex, while cAMP disrupts the complex (Fig. 2*A*) as

274 also shown in other studies (17). The T-loop of SbtB could be involved in the interaction

275 between SbtA and SbtB, but is disorganized in structures where SbtB is associated with

276 AMP, ADP, or cAMP (17, 32). Therefore, the structure of how SbtA-SbtB complexes bind

277     with AMP explains the inhibitory mechanism of SbtA by SbtB. A recently reported

278     $Ca^{2+}$-ATP:SbtB structure from *Cyanobium* sp. 7001 revealed that $Ca^{2+}$ could stabilize the

279     T-loop conformation, which is required for allosteric regulation of SbtA (32). However,

280     additional structural analysis demonstrates that the T-loop conformation in $Ca^{2+}$-ATP:SbtB

281     structure is different from the structure we outlined, and conflicts with SbtA when aligned

282     to the SbtAB$^{EM}$ structure (*SI Appendix*, Fig. S10). These results are consistent with our

283     biochemical data, which demonstrates that the presence of ATP decreases the SbtA-SbtB

284     complex formation (Fig. 2*A*). The SbtA-SbtB complex was also destabilized by cAMP

285     both *in vivo* and *in vitro*, and cAMP was considered a physiologically high carbon signal

286     (21). This seems to be conflict with the notion that Ci transporter activity can be activated

287     at low carbon levels and inhibited at high carbon levels, however, it could accommodate

288     specific physiological environmental transitions (24, 32). The unique regulatory

289     mechanism of SbtA by SbtB makes it important to investigate Ci uptake systems, which

290     could facilitate the photosynthetic modification of CCM in crops (40).

291

## Materials and Methods

### Gene cloning and protein purification.

The genes encoding SbtA and SbtB were amplified by PCR from *Synechocystis* sp. PCC 6803 genomic DNA. The fragment *SbtA* and *SbtB* were digested with NcoI/SalI and NdeI/XhoI, respectively, and were subsequently ligated into the MCS1 and MCS2 of pRSFDuet plasmid, respectively. The recombinant plasmid pRSFDuet-*SbtA*-*SbtB* including a C-terminal His-tag on *SbtA* was used to transform *E. coli* C43(DE3) for expression. Bacterial cells were grown at 37°C in Luria broth (LB) medium with 50 μg/mL kanamycin and protein expression was induced by 0.25 mM β-d-thiogalactopyranoside (IPTG) at around $OD_{600}=1.2$.

After 14 h at 37°C, the cells were collected and homogenized in buffer A (100 mM NaCl, 20 mM Tris-HCl, pH 8.0, 5% (v/v) glycerol and 2 mM AMP), and lysed using a French press. Cell debris was removed by centrifugation. The supernatant was collected and centrifuged using ultracentrifugation at 150,000 g for 1 h. The membrane fraction was incubated with 1% (w/v) n-dodecyl-β-d-maltopyranoside (DDM; Bluepus) for 2 h at 4°C. After another centrifugation step at 20,000g for 45 min, the supernatant was loaded onto an $Ni^{2+}$-NTA affinity column (Qiagen) and then washed with buffer B (100 mM NaCl, 20 mM Tris-HCl, pH 8.0, 0.018% DDM and 2 mM AMP) supplemented with 25 mM imidazole. The protein was eluted from the column using buffer B supplemented with 250 mM imidazole and was then concentrated to around 10 mg/mL before further purification by gel filtration (Superdex-200) in buffer C [100 mM NaCl, 20 mM Tris-HCl, pH 8.0, 0.18% n-decyl-β-d-maltoside (DM; Anatrace) and 2mM AMP]. The peak fraction was collected and concentrated to approximately 5 mg/mL for crystallization.

### Construction of *can* deletion mutant.

The *can* deletion in the chromosome of *E. coli* was constructed. The upstream and downstream fragments of *can* operon was amplified and overlapped together by PCR, resulted in the replacement fragment Overlap-*can*. The sgRNA plasmid targeting gene *can* pCB003_N20_*can* was obtained from pCB003 by PCR. The plasmid pCB006 was transformed into *E. coli* C43(DE3). Arabinose was added to the culture when preparing the *E. coli*/pCB006 competent cell for recombination. 4 μL Overlap-*can* and 4 μL pCB003_N20_*can* were electroporated into the *E. coli*/pCB006 competent cells. Cells

323     were recovered at 30 °C for 2 h, then spread onto LB agar plates containing kanamycin and

324     spectinomycin, and incubated at 30 °C for 24 h. The right colonies were confirmed by

325     DNA sequencing. pCB003 was cured by adding IPTG, and pCB006 was cured by growing

326     at 37°C overnight.

**Growth Assay.**

328     C43(DE3) and C43(DE3)-Δ$can$ strains were first grown on LB medium supplemented with

329     50 μg/mL kanamycin and 0.1 mM sodium azide at 37°C overnight. Seed cells were

330     prepared in LB medium to $OD_{600}$ of 0.1. 1 mL of seed cells was added to 20 mL of LB

331     medium containing 0.2 mM IPTG and 50 μg/mL kanamycin. Growth was measured at

332     different time slots. For dilution spotting assay, seed cells were diluted by $10^1$, $10^2$, $10^3$, $10^4$

333     and $10^5$ folds, respectively. 2 μL aliquot of each dilution was spotted onto the LB agar plate

334     containing 0.2 mM IPTG and 50 μg/mL kanamycin. Plates were incubated at 37°C

335     overnight. All tests were repeated at least three times independently.

***In vitro* pulldown assays.**

337     To analyze the SbtA-SbtB complex formation with the addition of adenyl nucleotides, 2

338     mM ATP, 2 mM ADP, 2 mM AMP or 2 mM cAMP was added to the purification buffer,

339     respectively. Wild type SbtA-SbtB or mutations were co-expressed in *E. coli* C43(DE3).

340     After cell disruption and centrifugation, the supernatants were loaded to $Ni^{2+}$-NTA affinity

341     resin. Nonspecific bound protein was washed off. Target protein was eluted from the resin

342     and examined with SDS–PAGE and visualized with Coomassie Blue staining.

**Nanodisc reconstitution.**

344     POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol, Avanti) was solubilized in

345     chloroform, dried under argon gas to form a thin lipid film and stored under vacuum

346     overnight. The lipid film was hydrated and re-suspended at a concentration of 10 mM in a

347     buffer containing 20 mM Tris, pH 8.0, 100 mM NaCl and 100 mM sodium cholate.

348     SbtA-SbtB complex proteins, MSP2N2 membrane scaffold protein, and POPG were mixed

349     at a molar ratio of 1:3:150 in a buffer containing 20 mM Tris, pH 8.0, 100 mM

350     NaCl,15 mM sodium cholate and 2 mM AMP, and incubated at 4°C for 1 h. Extra DDM

351     detergent was removed by incubation with 0.6 mg/mL Bio-Beads SM2 (Bio-Rad) at 4°C

352     overnight. Nanodisc-embedded SbtA-SbtB was purified using a Superdex 200 column in a

353    buffer containing 20 mM Tris, pH 8.0, 100 mM NaCl and 2 mM AMP.

354    **Cryo-EM sample preparation and data acquisition.**

355    To prepare samples for cryo-EM analysis, 3 μL of purified nanodisc-embedded SbtA-SbtB

356    complex at a concentration of 1 mg/mL was applied to glow-discharged carbon grids

357    (Quantifoil Cu R1.2/1.3). Grids were blotted for 3 s and plunge-frozen in liquid ethane

358    cooled by liquid nitrogen using a Vitrobot Mark IV (Thermo Fisher) at 8 °C and 100%

359    humidity. The prepared grids were transferred to a Titan Krios electron microscopy

360    operating at 300 kV equipped with Gatan K3 detector and GIF Quantum energy filter. The

361    movie stacks were recorded in the super-resolution mode at nominal magnification of

362    81,000× with a calibrated pixel size of 0.539 Å. The defocus range was from -1.8 to -1.5

363    μm. Each stack of 32 frames was exposed for 2.56 s with a total dose rate of ~50 e$^-$/Å$^2$.

364    AutoEMation was used for automated data collection (41). All 32 frames in each stack

365    were aligned using MotionCor2 (42) and binned to a pixel size of 1.0773 Å. The defocus

366    value of each image was determined by Gctf (43).

367    **Data processing.**

368    A total of 3,186,493 particles were automatically picked using RELION 3.0 (44) from

369    2,971 micrographs. After two rounds of 2D classification, ~20% of the selected particles

370    were used to generate initial model by RELION 3.0. With particles rescaled to 128 pixel,

371    one good reference and three bad references were generated after 3D classifications. The

372    good class from the last four iterations yielded dataset containing 268,921 particles, giving

373    rise to reconstruction at 3.4 Å resolution with C3 symmetry applied. The dataset was then

374    subject to local search multi-reference 3D classification, and the multi-reference models

375    were generated using the reconstruction low-pass filtered to 5-30 Å. Particles from good

376    classes were merged and duplicated particles were removed, and another round of

377    multi-reference classification using bin1 particles yielded dataset containing 125,998

378    particles, resulting in reconstruction at 3.1 Å. Application of a solvent mask for further

379    post-processing improved resolution to 2.7 Å. The overall resolutions were estimated based

380    on the gold-standard Fourier shell correlation (FSC) = 0.143 criterion. The details related

381    to data processing are summarized in *SI Appendix*, Fig. S2 and Table S1.

382    **Model building and structure refinement.**

383  The atomic coordinate of SbtAB complex was generated by combining homology

384  modeling and *de novo* model building. The initial structure model of SbtA was predicted

385  by protein structure prediction server trRosetta (45) and the crystal structure of SbtB (17)

386  (PDB code: 5O3S) was used. The structure of SbtAB complex was docked into the density

387  map and manually adjusted and re-built by COOT (46). The resulting model was refined

388  by phenix.real_space_refine program in PHENIX (47) with secondary structure and

389  geometry restraints. The structure was validated with MolProbity (48) , and refinement

390  statistics is shown in *SI Appendix*, Table S1. All structure figures were prepared in

391  ChimeraX (49) .

392  **Crystallization, data collection and determination of structure.**

393  SbtA-SbtB complex was concentrated to 5 mg/mL and crystallized via vapor diffusion at

394  20°C in 96-well sitting-drop plates. The best crystals were grown in 0.05 M magnesium

395  chloride ,0.1 M glycine pH 9.0 and 22% polyethylene glycol (PEG) 400 (the protein:

396  reservoir volume in a ratio of 1:1), and were directly flash-frozen in liquid nitrogen for data

397  collection. All data were collected at BL19U1 beamline of the Shanghai Synchrotron

398  Radiation Facility (SSRF) under 100 K liquid nitrogen stream (wavelength = 0.9798 Å )

399  and processed using HKL-3000 (50).The SbtA-SbtB complex crystallized in space group

400  H32 with cell parameters a= 107.2 Å, b= 107.2 Å, and c= 352.2 Å.

401  The complex structure was solved by molecular replacement with PHENIX, using the

402  SbtAB[EM] structure (PDB code 7EGK) as an initial model. The model was manually built in

403  COOT, refined with PHENIX and validated using MolProbity. A summary of data

404  collection and refinement statistics is provided in *SI Appendix*, Table S2.

405  **Data availability**

406  The atomic coordinate of SbtAB[Xtal] have been deposited in the Protein Data Bank with

407  accession code 7EGL. The cryo-EM map and atomic coordinate of SbtAB[EM] have been

408  deposited in the EMDB (EMD-31135) and the Protein Data Bank (7EGK).

409  **Acknowledgements**

420

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534

**Figure Legend**

535 **Figure Legend**

536 **Figure 1.** Functional characterization of SbtA and SbtB.

537 Complementation of Δ*can* strain was conducted by expression of *SbtA*, *SbtA-SbtB* or empty

538 vector as a control. Wild-type strain was used as a positive control. Growth curve (*A*) and

539 dilution spotting assay (*B*) are shown, respectively. The error bars in (*A*) represent standard

540 deviation (s.d.) and n = 4 technical replicates.

541 **Figure 2.** Overall structure of SbtA-SbtB complex.

542 (*A*) Pull-down assay results show the interaction between SbtA and SbtB with/without

543 addition of different adenyl nucleotides. 2 mM ATP, 2 mM ADP, 2 mM AMP or 2 mM

544 cAMP was added to the purification buffer in the assay, respectively. (*B*) Cryo-EM map of

545 SbtAB$^{EM}$ in side view colored by molecules. (*C*) Structure of SbtA-SbtB heterodimer

546 formed in SbtAB$^{EM}$ complex. Structure elements of SbtB are labeled. Scaffold domain of

547 SbtA, slate blue; Core domain of SbtA, light blue; SbtB, green; T-loop of SbtB, orange red.

548 (*D*) Crystal structure of SbtAB$^{Xtal}$ presented as a trimer. SbtA, gold; SbtB, green. (*E*)

549 Topology of SbtA. The transmembrane helices (TMs) are numbered from 1 to 10. Numbers

550 indicate the beginning/ending residue positions. (*F*) Extracellular view of SbtA trimer.

551 Scaffold domain and core domain are displayed as cylinder and colored accordingly, the

552 narrow cleft between two domains is indicated by grey shadow.

553 **Figure 3.** Substrate-binding site of SbtA.

554 (*A*) Intracellular view of substrate-binding site in the SbtAB$^{Xtal}$ structure. Scaffold domain

555 and core domain are shown in electrostatic surface and ribbon, respectively. Blue and red

556 colors represent positive and negative charges. Bicarbonate and sodium ion are indicated as

557 yellow and purple spheres, respectively. (*B*) TM cross constituted by TM4a/b and TM9a/b.

558 (*C*) Bicarbonate binding site in the SbtAB$^{Xtal}$ structure. Bicarbonate and residues involved

559 in coordination are labeled and shown as sticks. Water molecules are indicated as red

560 spheres. 2Fo-Fc electron densities of bicarbonate and water molecules are contoured at 1.5

561 σ. (*D*) Comparison of the bicarbonate binding site in SbtAB$^{Xtal}$ (gold) and SbtAB$^{EM}$ (slate

562 blue). (*E*) Growth assay of substrate-binding site mutants in Δ*can* strain. Complementation

563 of Δ*can* strain was conducted by expression of four mutants involved in bicarbonate

564 binding, or empty vector as a control. Wild-type strain was used as a positive control. The

565 error bars represent standard deviation (s.d.) and n = 3 technical replicates. (*F*) Sodium

566 binding site in the SbtAB$^{Xtal}$ structure. Residues involved in coordination are labeled and

567 shown as sticks. 2Fo-Fc electron densities of sodium ion are contoured at 1.5 σ.

568 **Figure 4.** Interaction between SbtA and SbtB.

569 (A) Side view of SbtA-SbtB interface in SbtAB$^{EM}$ structure. SbtA and SbtB are shown in

570 electrostatic surface and ribbon, respectively. Structure elements involved in SbA-SbtB

571 interaction are labeled. (B-C) Hydrophilic interactions between SbtA and T-loop (B) or

572 b1-a1/a2-b4 loop (C) of SbtB. Residues involved are labeled and shown as sticks. The

573 hydrogen bonds are indicated by dash lines. (D) A detailed view of AMP binding pocket.

574 The neighboring SbtB molecule is shown and colored by grey. (E) Pull-down assay of

575 SbtAB mutants involved in SbtA-SbtB interaction and AMP binding. (F) Growth assay of

576 SbtAB mutants in Δ*can* strain. Complementation of Δ*can* strain was conducted by

577 expression of six SbtAB mutants involved in SbtA-SbtB interaction and two in AMP

578 binding. Wild-type strain was used as a positive control. The error bars represent standard

579 deviation (s.d.) and n = 4 technical replicates. (G) Binding pocket of adenyl nucleotide

580 superimposed with cAMP (pink). The dash circle shows the conflict between cAMP and

581 SbtB, with key residues indicated.

582 **Figure 5.** Transport and regulatory mechanism of SbtA.

583 (A) Structure comparison of SbtA with topological homologs. Monomer of SbtA, NhaA,

584 NhaP, NapA and ASBT are displayed in intracellular view. Scaffold domain and core

585 domain are colored by slate blue and light blue, respectively. (B) Proposed transport and

586 regulatory mechanism of SbtA. Distinct conformations are indicated. The top panel

587 illustrates key conformational states of SbtA during the transport of substrate. In the

588 outward state, the TM cross is exposed to the periplasm, and Na$^+$-binding may facilitate the

589 binding of substrate. Converting to the inward state, the core domain undergoes rigid

590 movement in order to translocate HCO$_3^-$ into the plasma membrane. The bottom panel

591 illustrates the allosteric inhibition of SbtA by SbtB. In the presence of AMP, T-loop of

592 SbtB inserts into the cytoplasmic cavity formed between the two domains of SbtA, which

593 locks SbtA at inward substrate-free state and inhibits the bicarbonate transporter activity.

**A**

WT/pRSFDuet
Δ*can*/pRSFDuet
Δ*can*/SbtA
Δ*can*/SbtA-SbtB

**B**

| | 1 | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ | $10^{-5}$ |

WT/pRSFDuet

Δ*can*/pRSFDuet

Δ*can*/SbtA

Δ*can*/SbtA-SbtB

**A**

MW (kDa)

+ATP +ADP +AMP +cAMP −

55
43 — SbtA
34

26 — SbtB
17

**B**

**C**

Extracellular

SbtA

T-loop

β4
β3          α2
SbtB      β1
β2  α1

Intracellular

**D**

**E**

N-terminus                                                    C-terminus

3    60   72  127      134  236    240  306  310      370

1a        4b           6b              9a
2      3           5        7      8          10
1b      4a                6a              9b

32   39   90        99       212   268  279  335  341
                         163

**F**

scaffold domain

6b
2   6a
7   5   3
4   9   8
1b
1a  10

cleft

core domain

**A**

TM7

TM2

TM9b

TM6a

**B**

TM4b

TM9a

TM9b

TM4a

**C**

BCT

S116

S114

S324

S327

D325

**D**

BCT

S116

S327

D325

**E**

Absorbance at 600 nm

Time (hrs)

- WT/pRSFDuet
- Δ*can*/pRSFDuet
- Δ*can*/SbtA
- Δ*can*/SbtA(S114A)
- Δ*can*/SbtA(S116A)
- Δ*can*/SbtA(S324A)
- Δ*can*/SbtA(S327A)

**F**

A320

S322

Na⁺

F110

G111

A112

**A**

SbtA

NhaA
(PDB 4atv)

NhaP
(PDB 4cz8)

NapA
(PDB 4bwz)

ASBT
(PDB 4zux)

**B**

Outward

Inward substrate-binding

Inward substrate-free

core
domain
movement

transport

SbtA-scaffold domain
SbtA-core domain
SbtA-helices cross
SbtB-T loop
SbtB

AMP

AMP

AMP

allosteric
inhibition

SbtAB^Xtal

SbtAB^EM