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The SufBCD protein complex is the scaffold for iron–sulfur cluster assembly in *Thermus thermophiles* HB8

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ABSTRACT

Iron–sulfur (Fe–S) clusters are the oldest and most versatile inorganic cofactors that are required to sustain fundamental life processes. Bacteria have three systems of [Fe–S] cluster biogenesis, designated ISC, NIF, and SUF. In contrast, the *Thermus thermophiles* HB8 has only one system, formed mostly by SUF homologs that contain six proteins: SufA, SufB, SufC, SufD, SufS and SufE. The kinetics of SufC ATPase was studied using a linked enzyme assay method. In the presence of SufB, SufD or SufBD complexes, the activity of SufC was enhanced. The cysteine desulfurase activity of SufS was also stimulated by the presence of the SufBCD complex. The results obtained through enzymology revealed that aconitase activity was activated by [Fe–S] clusters reconstituted on the SufBCD complex. Consolidated results from spectral and enzymatic analysis suggest that the SufBCD complex is a novel type of Fe–S scaffold system that can assemble Fe/S clusters de novo.

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1. Introduction

Many proteins depend on cofactors for their function. The ubiquitous and most ancient inorganic cofactors are probably Fe–S clusters, which participate in a wide range of physiological processes including respiration, photosynthesis, DNA repair, metabolism and regulation of gene expression [1,2]. They have been found in all living organisms, including archaea, bacteria and eukarya [3]. Genetic and biochemical studies have so far revealed three common Fe–S cluster biosynthesis pathways in bacteria, all of which are well-characterized. These include the ISC system, the NIF system and the SUF system, which are encoded by the *nif*, *isc* and *suf* operons, respectively [4–6]. The ISC machinery, encoded by the *iscRSUA-hscBA-fdx* gene cluster, is found in the majority of prokaryotes and in mitochondria. Briefly, the ISC system is thought to be the housekeeping biogenesis pathway, as its inactivation leads to drastic reduction in the activity of all [Fe–S] enzymes tested under normal growth conditions [7–11]. The NIF system, first described in *Azotobacter vinelandii*, is responsible for the maturation of nitrogenase, but it is also distributed in some anaerobic organisms lacking nitrogenase. The SUF system, first described in *Escherichia coli*, is adapted to assemble [Fe–S] clusters under environmental conditions such as oxidative stress or iron starvation [3]. The three systems employ a cysteine desulphurase that allows utilization of L-cysteine as a source of sulfur. A scaffold protein then mobilizes sulfur atoms from a cysteine desulphurase and iron atoms from an iron donor to synthesize Fe–S clusters [3].

The SUF machinery has been the focus of intense studies at the biochemical level, especially in *E. coli*. The *suf* operon specifies the SufA, -B, -C, -D, -S, and -E proteins. The SufSE complex serves as the sulfur donor for [Fe–S] cluster assembly. SufS is a cysteine desulfurase that mobilizes the sulfur from L-cysteine. The SufE protein interacts with SufS and greatly enhances its cysteine desulfurase activity [13]. Some studies on bacteria show the importance of SufB, SufC and SufD proteins for iron–sulfur cluster formation. It has been found that the three proteins form a rather tight complex, SufBCD [12]. SufC exhibits ATPase activity, which is enhanced in the presence of SufB and SufD [3,14,16]. However, there is very limited research available on the SUF system in *Thermus thermophiles* HB8, and these details are not well understood in *T. thermophiles* HB8. For example, little is known of the precise function of the SufBCD complex, and the identities of the protein scaffold and iron donor remain unknown. Furthermore, the SUF system is considered the only Fe–S cluster biosynthesis pathway in this thermobacteria. In this study, *in vitro* Fe–S cluster reconstitution experiments have suggested SufBCD can form Fe–S clusters and the three proteins are necessary for Fe–S cluster assembly. We have thus proposed that the SufBCD complex plays the role of a scaffold protein for assembly of Fe–S clusters, in addition to providing the energy during the synthesis of Fe–S clusters.

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2. Materials and methods

2.1. Strains and plasmids

SufC and SufB were amplified by PCR as one DNA fragment using the *T. thermophiles* HB8 chromosomal DNA as a template and the following primers: SufB, 5′-AAGAATTCATGAGCGAGCTGGAGAT-3′ and 5′-TTAAGCTTGCCGACGGAGCCCTCCA-3′; SufC, 5′-TTCCATGGATATGAGCCAGCTGGAG-3′ and 5′-AACATATGTGCGCCCTCCTTCACCT-3′. SufB PCR products were digested with EcoRI and HindIII and cloned into the corresponding sites of pET-28a-c(+), generating recombination plasmid *sufB*-pET-28a. SufC products were then digested with NcoII and NdeI and cloned into the corresponding sites of *sufB*-pET-28a, generating recombination plasmid *sufC*-*sufB*-pET-28a. The recombination plasmids *sufC*-pET-28a and *sufD*-pGEX-6p-1 were constructed and stored in our lab. The sequences of all plasmid inserts were confirmed by DNA sequencing.

2.2. Overproduction and purification of SufBC, SufCD, SufBCD complexes

$(His)_6$-SufBC was produced in *E. coli* BL21(DE3) plysS cells. The SufC protein was co-expressed with the GST-tagged SufD protein from *sufD*-pGEX-6p-1 [10]; the SufBC protein was co-expressed with GST-tagged SufD protein from the *sufD*-pGEX-6p-1 plasmid in *E. coli* BL21(DE3) plysS cells. Expression was induced with 0.5 mM IPTG and incubated for 16 h at 20 °C. Recombinant protein was purified through a Ni-NTA matrix (Qiagen) and eluted with 250 mM imidazole. The protein complex was then further purified by a HiPrep 16/60 Sephacryl S-200 HR column equipped with an ÄKTA explorer system (GE Healthcare) [15].

2.3. ATP hydrolysis

All measurements of SufC's activity were made in buffer A (50 mM Tris–HCl, pH 7.5, 100 mM KCl, 1 mM DTT, 2 mM $MgCl_2$) at 30 °C. The hydrolysis of ATP was monitored using a linked enzyme assay that coupled the formation of ADP to the oxidation of NADH [11]. The solutions (total volume 100 μl) contained 0.16 mM NADH, 1 mM phosphoenolpyruvate, 50 U pyruvate kinase, 50 U lactate dehydrogenase and varying amounts of ATP [17,18]. NADH absorbance was monitored at 340 nm and the rate of NADH oxidation was determined based on its extinction coefficient of 6.2 L $mmol^{-1}$ cm^{-1}.

2.4. Iron–sulfur cluster reconstitution

Two methods were used to determine iron–sulfur cluster reconstitution. For the first method, the purified apo-SufBCD (135 μM) was added to the reconstitution buffer containing a 6-fold excess (810 μM) of ferrous ammonium sulfate solution and an equal amount of Na_2S in 25 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM dithiothreitol (DTT) at 18 °C. After 4 h of incubation, EDTA (135 μM) was added, and after 15 min the mixture was desalted using a desalting-column (GE Healthcare) [5,15,19]. As a control, SufBCD protein was also reconstituted separately in the same manner. The UV-visible spectra scanning of the holo-SufBCD was carried out at 25 °C on a SHIMADZU UV-2450 spectrophotometer. For the second method, SufBCD (135 μM) was incubated with 0.5 μM SufS in the presence of 10 mM cysteine and a 6-fold excess (810 μM) of ferrous ammonium sulfate solution in the reconstitution buffer at 18 °C [20–22]. The final steps were conducted as previously described for the first method.

2.5. Aconitase activity assay

Aconitase activity was assayed by a coupled assay using isocitric dehydrogenase, in which $NADP^+$ reduction is measured [23]. First, we reconstituted and desalted iron–sulfur clusters on SufBCD (0.1–0.5 μM, respectively), according to the methods described previously. Second, aconitase was activated by the reconstitution of various concentrations of iron–sulfur clusters, after incubation at 37 °C for 25 min. Lastly, we added 0.1 ml of the activated aconitase to the assay buffer (Tris–HCl pH 7.4, Citrate 2 mM, iCDH solution, $NADP^+$ 10 mM, $MnSO_4$ 5 mM) and mixed by gentle inversion. We then measured the change in absorbance at 340 nm from 0.5 to 1.5 min. The rate of $NADP^+$ reduction was determined based on its extinction coefficient of 6.2 L $mmol^{-1}$ cm^{-1}.

2.6. SufS activity assay in the presence of SufBCD

All measurements were made in reaction buffer (100 mM Tris–HCl, pH 8.0, 30 mM KCl). The activity of cysteine desulfurase was monitored using a linked enzyme assay that coupled the formation of cysteine to the reduction of NAD. The total reaction solutions contained 40 mM cysteine, 0.5 μM SufS, and varying amounts of SufBCD (0–15 μM) reacting 30 min at 30 °C. The production was boiled 10 min at 100 °C and centrifugated at 12000*g* for 10 min. The supernatant was then transferred into a new tube and 0.1 M NAD, and 20 U alanine dehydrogenase were added [24,25]. NADH absorbance was monitored at 340 nm and the rate of NAD reduction was determined based on its extinction coefficient of 6.2 L $mmol^{-1}$ cm^{-1}.

3. Results

3.1. SufC is an ATPase and interacts with SufB, SufD

To clarify the *in vitro* Fe–S cluster status of the SufBCD complex and to characterize the function of SufBCD in Fe–S cluster assembly, a novel coexpression system based on the pET-28a-c(+) and pGEX-6p-1 protein expression vector was designed. Using the *T. thermophiles* HB8 chromosomal DNA as a template, the soluble complex of SufBC was coexpressed in *E. coli*, and the complexes SufCD and SufBCD were copurified by plasmid cotransformation. The SufBC, SufCD, SufBCD complexes were stable during the purification steps including Ni-NTA chromatography and gel filtration. Unless otherwise noted, all steps were carried out at 4 °C. Initial estimates of molecular weight were obtained from SDS–PAGE in gels of differing composition. The results are shown in Fig. 1A and suggest Mr^{SufBC} = 80.5KD, Mr^{SufCD} = 75.5KD, Mr^{SufBCD} = 154.5KD under the assumption that the proteins were fully denatured. Meanwhile, SufS, SufA and SufU were expressed in *E. coli*. The results are shown in Fig. 1B.

Hydrolysis of ATP was monitored from the linked enzyme system, described in the methods. The rate of hydrolysis by SufC was hyperbolically dependent on ATP concentration. Fitting the data to the Michaelis–Menten equation gave a K_m of 17.70 μM and Vmax of 75.03 μM min^{-1} (Fig. 2). Under the same conditions, and having ensured the same final concentration of protein complexes, the activity of protein complexes SufBC, SufCD, SufBCD were measured (Table 1). The data showed that SufB and SufD can interact with SufC and enhance the activity of SufC. The ATPase activity of SufC alone was 11.76 U, the activity of SufBC and SufCD alone were 27.67 U and 30.19 U, respectively; the activity of SufBCD was 34.83 U. The results clearly showed that the activity of SufC can be doubled by forming the complexes SufBC and SufCD, and can be enhanced further by SufBCD. The results suggest that in the SufBCD complex, the SufC protein can provide energy for

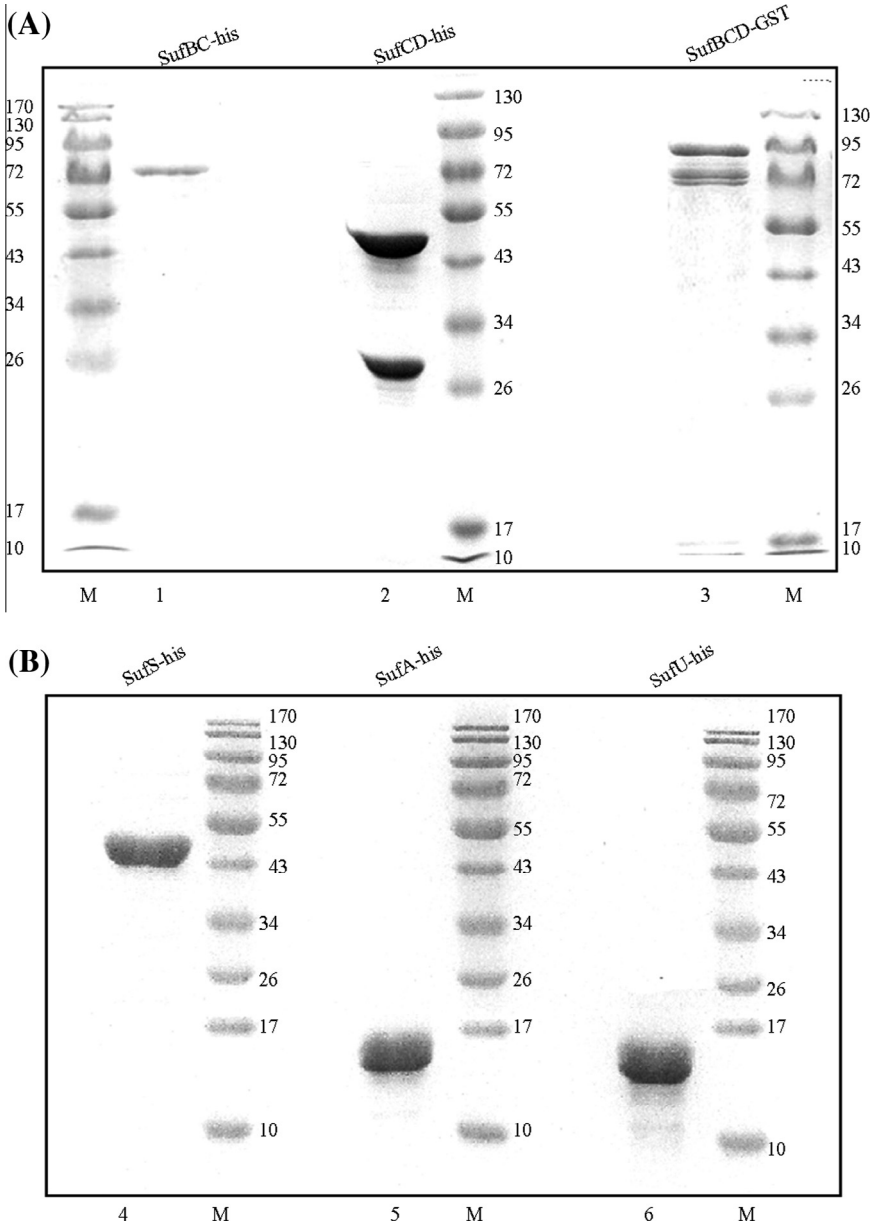

Fig. 1. SDS–PAGE analysis of protein complexes purified using Ni-NTA. Samples from each purification were separated on 12% or 15% SDS–PAGE gel and stained with coomassie blue stain. (A) SufBC, SufCD, SufBCD complexes. M: Protein maker, 1: SufBC (0.1 mg/ml); 2: SufCD (0.6 mg/ml); 3: SufBCD (0.2 mg/ml); (B) SufS, SufA, SufU protein: M: Protein maker; 4: SufS (0.548 mg/ml), 5: SufA (0.513 mg/ml); 6: SufU (0.461 mg/ml).

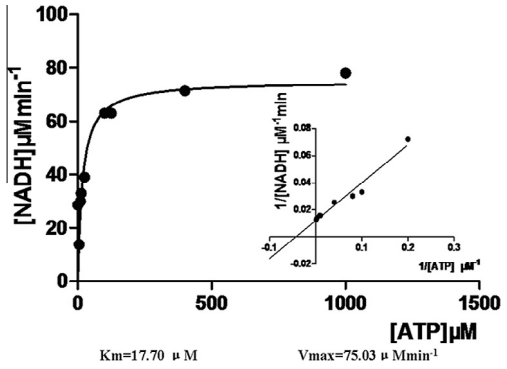

Fig. 2. The dose-dependence of the steady-state rate of hydrolysis of ATP by SufC. The line shows the best fit of data to the Michaelis–Menten equation, giving a K_m of 17.70 μM and Vmax of 75.03 μM min^{-1}.

the synthesis of iron–sulfur clusters. Thus, the ATPase activity of SufC can clearly be increased by the formation of the SufBCD complex.

3.2. Analysis of SufS activity in the presence of SufBCD

In addition to its effect on the activity of ATPase, the potential other features of the SufBCD complex were investigated. In our lab, it was determined that SufS was a cysteine desulfurase that catalyzes the decomposition of the L-cysteine substrate to L-alanine and sulfur. To test whether SufBCD can enhance the activity of SufS, different concentrations of SufBCD (0–15 μM) were added to the reaction buffer. The change in absorbance was then monitored, and the results indicated that a physical interaction between the SufBCD complex and SufS results in further stimulation of the cysteine desulfurase activity of SufS (Fig. 3). Fig. 3 displays how the activity of desulfurase gradually increased with the increased

Table 1
Volume activity of SufC and different protein complexes.

Name	SufC	SufBC	SufCD	SufBCD
Amount of protein (mg/100 μl)	1.5	1.5	1.5	1.5
Volume activity (U/ml)	11.76 ± 0.8446	27.67 ± 0.1236	30.19 ± 1.1329	34.89 ± 1.0335

1U: 1 mmol of substrate converted to a product by a certain amount of enzyme activity per minute.

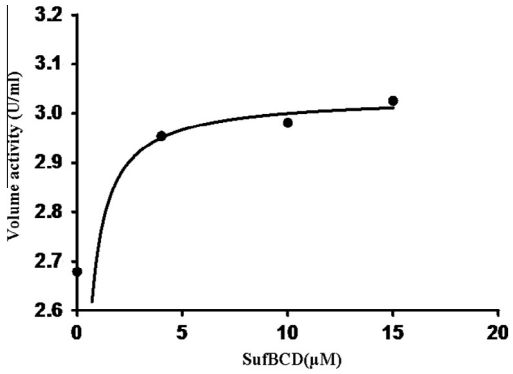

Fig. 3. Modulation of SufS activity by SufBCD at different concentrations. The reactions contain 25 μg SufS, 0.1 M NAD, 20 U alanine dehydrogenase and 40 mM cysteine with increasing concentrations of SufBCD (0–15 μM).

concentration of SufBCD, eventually reaching a steady-state. Thus, the cysteine desulfurase activity of SufS was stimulated by the SufBCD complex.

3.3. Iron–sulfur cluster reconstitution of SufBCD

The stable SufBCD complex used in this research was copurified by plasmid cotransformation directly from an *E. coli* strain. After purification, the SufBCD complex did not contain any cofactor, and, as a consequence, existed in the apo-form. The SufBCD complex was reconstituted with iron and sulfur as described in the experimental procedures section. The protein solution of holo-SufBCD exhibited a very pale red color, suggesting that some Fe–S cluster was associated with the protein. The UV-visible absorption spectrum of holo-SufBCD indicated the presence of an Fe–S cluster with absorption maxima at 450 and 500 nm and a broad shoulder at ~400–430 nm (Fig. 4). Under the same conditions, the SufA and

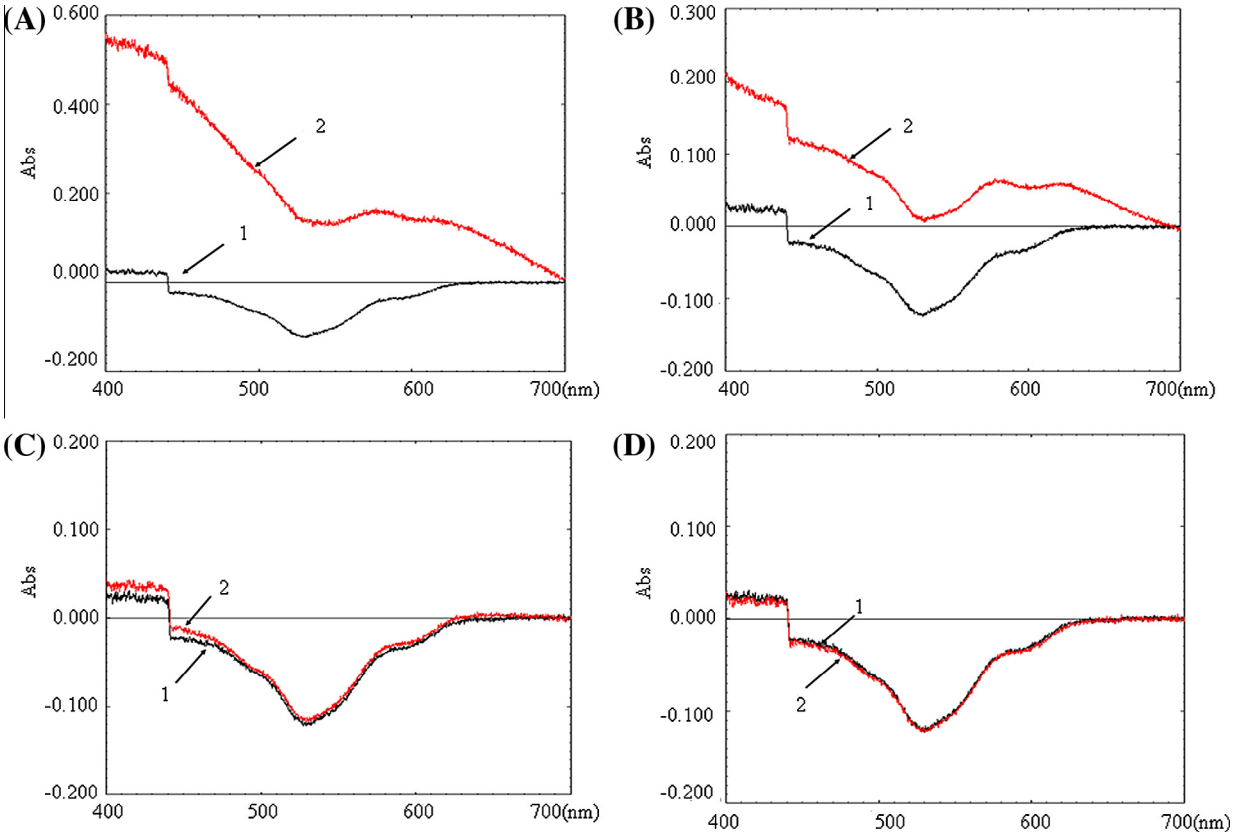

Fig. 4. UV-visible spectra of reconstituted SufBCD-[Fe–S] complexes. (A) 1: The UV-visible absorption spectra of SufBCD (0.135 μM) and DTT (5 mM); 2: The UV-visible absorption spectra of the holoSufBCD complex. ApoSufBCD (0.135 μM) was incubated for 4 h with enough $Fe(NH_4)_2(SO_4)_2$ (0.81 μM, 6-fold molar excess of iron relative to the SufBCD concentration), an equal amount of Na_2S and 5 mM DTT. HoloSufBCD was then separated from the mixture by a desalting column (B) 1: The UV-visible absorption spectra of SufBCD (0.135 μM) and DTT (5 mM); 2: The UV-visible absorption spectra of the holoSufBCD complex. ApoSufBCD (0.135 μM) was incubated for 4 h with enough $Fe(NH_4)_2(SO_4)_2$ (0.81 μM, 6-fold molar excess of iron relative to the SufBCD concentration), 1.2 μM SufS, 40 μM Cys, and 5 mM DTT. HoloSufBCD was then separated from the mixture by a desalting column. (C) 1 the black line: The UV-visible absorption spectra of SufA (0.135 μM) and DTT (5 mM); 2 the red line: The UV-visible absorption spectra of reconstituted SufA–[Fe–S]. ApoSufA (0.135 μM) was incubated for 4 h with enough $Fe(NH_4)_2(SO_4)_2$ (0.81 μM, 6-fold molar excess of iron relative to the SufA concentration), an equal amount of Na_2S and 5 mM DTT. HoloSufA was then separated from the mixture by a desalting column. (D) 1 the black line: The UV-visible absorption spectra of SufU (0.135 μM) and DTT (5 mM); 2 the red line: The UV-visible absorption spectra of reconstituted SufU–[Fe–S]. ApoSufU (0.135 μM) was incubated for 4 h with enough $Fe(NH_4)_2(SO_4)_2$ (0.81 μM, 6-fold molar excess of iron relative to the SufU concentration), an equal amount of Na_2S and 5 mM DTT. HoloSufU was then separated from the mixture by a desalting column. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Volume activity of aconitase.

SufBCD (μM)	0.09	0.135	0.27	0.405	0.54
Volume activity (U/ml)	5.4494 ± 0.09256	8.7623 ± 0.00099	9.1296 ± 0.003394	9.2683 ± 0.009829	11.5940 ± 0.06032

1U: 1 mmol substrate converted to product a desired amount of enzyme activity unit in every minute.

SufU complexes were separately purified and reconstituted with [Fe–S] clusters. The results showed that after incubating for 4 h and desalting, the UV-visible spectra of reconstituted SufA or SufU displayed no significant changes compared to the spectra of SufA or SufU alone. Thus, the SufBCD complex acted as the scaffold rather than SufA or SufU.

3.4. Aconitase is activated by iron–sulfur clusters on SufBCD

Aconitases (Acn) are iron–sulfur proteins that catalyze the reversible isomerization of citrate and isocitrate via the intermediate cis-aconitate in the Krebs cycle. Furthermore, the catalytically active form of aconitase contains a cubane [4Fe–4S] cluster. To assess aconitase catalytic activity, the holo-SufBCD was added to the aconitase and incubated at 37 °C for 25 minutes. From the Table 2, we can see clearly that with increasing SufBCD concentration, the activity of aconitase also increased. Meanwhile, this also proved that iron–sulfur clusters were reconstituted on SufBCD.

4. Discussion

In this study, the SUF system for [Fe–S] cluster biogenesis was identified and characterized as an essential system in *T. thermophiles* HB8. In contrast to the *E. coli* SUF system, which is under the control of an iron-dependent system for regulation, SUF seems to represent the housekeeping [Fe–S] biogenesis system in *T. thermophiles* HB8, since the SUF system was identified as the only complete Fe/S biosynthesis machinery [20]. However, other studies have showed that SufA/IscA type proteins provide an alternative scaffold to the SufU/IscU type proteins for Fe–S cluster biosynthesis in *E. coli* [25–28]. Thus there are two main questions that are currently addressed in our and other laboratories. (1) What is the scaffold protein in [Fe–S] cluster biosynthesis in *T. thermophiles* HB8? (2) What is the main function of the SufBCD complex? We believe that the results reported here provide new insights into the second question.

In our lab, we constructed recombination plasmids *sufC*- pET-28a, *sufD*-pGEX-6p-1 and *sufC-sufB*-pET-28a and obtained SufBC, SufCD and SufBCD complexes. Most of the Fe–S proteins involved in Fe–S biogenesis were usually purified in the apo form when they were expressed. Our experimental results also demonstrated that SufC of *T. thermophiles* HB8 is an ATPase that can interact with SufB and SufD *in vitro*. Meanwhile, the activity of ATP hydrolyase can be enhanced in the presence of SufB, SufD or SufBD. All of the results reported, namely the biochemical and spectroscopic characterization analyses, unambiguously demonstrate that SufB and SufD both interact with SufC to form a SufB–SufC–SufD ternary complex (SufBCD), and that the SufBCD complex can provide energy during the assembling of iron–sulfur clusters.

The SufS complex was a cysteine desulfurase, and the addition of SufBCD to SufS further increased its activity. The second property of the SufBCD complex was its ability to bind, during reaction with iron and the SufS cysteine desulfurase system, an Fe–S cluster. Here, this was characterized by UV spectrophotometry, unambiguously demonstrating that this [Fe–S] cluster was identical to the one found in the reconstituted SufBCD protein. Meanwhile, we reconstituted [Fe–S] clusters on SufA or SufU. However, the results showed that SufA/SufU were not the scaffold proteins which assembled the Fe–S clusters. SufB alone also did not show the function of a scaffold protein. Together these results were consistent with de novo Fe–S cluster assembly on a SufBCD scaffold, further confirming that the SufBCD complex is at the core of the SUF pathway. We also showed that the SufBCD complex interacted with SufS to facilitate sulfur liberation from cysteine, in which the sulfur atom was transferred from SufS to SufBCD. Additionally, the results of enzymology revealed that aconitase was activated by the reconstituted [Fe–S] clusters on the SufBCD complex. Furthermore, Ref. [23] indicated that the activated aconitase contained a cubane [4Fe–4S] cluster. Thus, we speculate that the SufBCD complex binds a [4Fe–4S] during the iron–sulfur cluster assembly process. Taken together these results demonstrate that SufBCD is a type of scaffold protein in the Fe–S cluster synthesis process in *T. thermophiles* HB8, on which iron–sulfur clusters can be assembled; the SufBCD is capable of reconstituting a [4Fe–4S] cluster. Previous studies on the SUF system in *E. coli* suggested that SUF was specifically adapted to synthesize [Fe–S] under environmental stress conditions such as iron starvation and oxidative stress. In contrast, the SUF system for [Fe–S] cluster biogenesis was identified and characterized as an essential system in *T. thermophiles* HB8. Interestingly, the exclusive presence of SUF seems to be a general feature within the *T. thermophiles* HB8 of Gram-positive bacteria, as recently indicated. In bacteria, SufB, SufC and SufD always occur together. In our study, we demonstrated that this was true also in *T. thermophiles* HB8.

In summary, this study presents biochemical evidence *in vitro* that the SufBCD complex can function as an Fe–S cluster scaffold and bind a [4Fe–4S] cluster for the SUF pathway in *T. thermophiles* HB8, and that SufC ATPase activity was required *in vitro* Fe–S cluster assembly.

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