Identification and Characterization of a Factor Which Is Essential for Assembly of Transcarboxylase†

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ABSTRACT: Transcarboxylase (TC) from *Propionibacterium shermanii* is a biotin-containing enzyme which catalyzes the reversible transfer of a carboxyl group from methylmalonyl-CoA to pyruvate. It is composed of a central, hexameric 12S subunit with six outer, dimeric 5S subunits held in a stable 26S complex by twelve 1.3S biotinyl subunits. Each of these subunits has been cloned from the *P. shermanii* genome and expressed in *Escherichia coli*. The purified, expressed recombinant proteins are all indistinguishable from their authentic counterparts except for the recombinant 5S subunit (termed 5S WT), which does not form TC complexes or catalyze the overall transcarboxylase reaction. Circular dichroism and isoelectric focusing suggested differences existed between the authentic and *E. coli*-expressed 5S proteins. HPLC gel filtration was used to separate the authentic 5S dimer from additional components in the preparation. 5S dimer thus purified was unable to form TC complexes or catalyze the overall reaction, behaving identically to the recombinant 5S WT subunit. Fractions from the HPLC gel-filtration purification of authentic 5S were then added to 5S WT or 5S dimer, and one fraction was identified which catalyzed the assembly of TC complexes with either 5S preparation. This assembly activity was shown to be dependent on the concentration of this HPLC fraction. Assembly-promoting factor (APF) is heat-stable and probably a protein, on the basis of its protease susceptibility. Studies with APF and the other TC subunits demonstrate its ability to promote complex formation with 12S and 1.3S subunits. Since the APF was purified from crystals of 26S TC, we believe it to be a novel, previously unidentified subunit of transcarboxylase.

Transcarboxylase is a complex multisubunit enzyme composed of three types of subunits: 12S or central subunit (M_r 360 000), 5S or outer subunit (M_r 120 000), and 1.3S biotinyl subunit which is the carboxyl carrier protein (M_r 12 000) (Wood & Zwolinski, 1976). The 5S subunit is a dimer of identical monomers, contains metals Co^{2+} and Zn^{2+}, and forms a complex with two 1.3S subunits termed the 6S subunit. The 12S subunit is a hexamer composed of six identical monomers and combines with 6S subunits to form active transcarboxylase (TC)[1] (Wood & Zwolinski, 1976; Wood & Barden, 1977). The 1.3S subunit, which will combine with isolated 5S subunits but not isolated 12S subunits, is required to attach the 5S and 12S subunits in TC. Active TC consists of a 12S subunit with one to six 6S subunits attached to one or both faces of 12S subunit. There are a maximum of three on each face (26S form), which appear to be located on opposite ends of the cylindrical hexameric 12S subunit (Wood & Zwolinski, 1976; Wood & Barden, 1977). TC is unique among the biotin enzymes as it catalyzes a transcarboxylation rather than CO_2 fixation (Wood, 1976). The enzyme can be dissociated into its constituent 12S, 5S, and 1.3S subunits and then assembled to reform active enzyme. The transcarboxylation reaction 3 is the result of the two partial reactions 1 and 2 which occur at active sites located on different subunits as follows:

$$CH_3CH(COO^-)COSCoA + 1.3S \overset{12S}{\rightleftharpoons} CH_3CH_2COSCoA + 1.3S{\cdot}COO^- \quad (1)$$

$$CH_3COCOO^- + 1.3S{\cdot}COO^- \overset{5S}{\rightleftharpoons} {}^-OOCCH_2COCOO^- + 1.3S \quad (2)$$

$$\text{SUM:} \quad \text{methylmalonyl-CoA} + \text{pyruvate} \rightleftharpoons \text{propionyl-CoA} + \text{oxalacetate} \quad (3)$$

Reaction 1 occurs at the CoA ester binding sites of the 12S subunit and reaction 2 occurs at the keto acid sites of the 5S subunit (Chuang et al., 1975; Ahmad et al., 1972). There are twelve CoA ester sites per 12S subunit and two keto acid sites per 5S subunit. In addition to its role in linking 5S and 12S subunits, the 1.3S subunit also contains the biotin required to transfer the carboxyl group from the CoA ester site to the keto acid site. Free biotin cannot replace the 1.3S subunit in the partial reactions with either isolated 5S or 12S subunits or in the complete reaction in the presence of both 5S and 12S subunits.

The 519-residue 5S subunit monomer has been cloned, sequenced, and expressed in *Escherichia coli* in active form (Thornton et al., 1993). The 5S subunit can form 6S subunits by binding with two 1.3S subunits. The 5S sequence shows a considerable amount of homology with yeast pyruvate carboxylase and *Klebsiella pneumonia* oxalacetate decarboxylase (Samols et al., 1988). The N-terminal region

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[1] Abbreviations: TC, transcarboxylase; WT, wild type; APF, assembly promoting factor; HPLC, high-performance liquid chromatography; NADH, nicotine adenine dinucleotide (reduced); DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone; EM, electron microscopy; TFA, trifluoroacetic acid; CD, circular dichroism; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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(residues 64–153) of the 5S subunit is 74% identical with oxalacetate decarboxylase and 50% identical with yeast pyruvate carboxylase. Kumar et al. (1988) have shown that Trp residues of the 5S subunit are involved in the binding of 5S to the 1.3S subunit and also binding to pyruvate at the keto acid sites.

The recombinant 5S subunit expressed in *E. coli*, 5S WT, was purified to homogeneity and has been shown to be identical to the 5S subunit of *Propionibacterium shermanii* in many respects (Xie et al., 1993). The recombinant 5S subunit was fully active in partial reaction 2 but showed less than 5% activity in the overall reaction and failed to form TC complexes when incubated with 12S and 1.3S subunits.

Here we describe a factor/protein which is necessary for the assembly of TC complexes and which is not the one of the traditional subunits of the enzyme.

MATERIALS AND METHODS

Materials. Methylmalonyl-CoA (MMCoA), malate dehydrogenase, lactate dehydrogenase, pyruvate, reduced nicotinamide adenine dinucleotide (NADH), rose bengal, dithiothreitol (DTT), reduced glutathione, phenylmethanesulfonyl fluoride (PMSF), *N*-ethylmorpholine, pronase, phosphoenolpyruvate, propionyl-CoA, glutaraldehyde, uranyl acetate, and TPCK-trypsin were from Sigma Chemical Co. Ultropac TSK G3000SW HPLC gel-filtration column was from Pharmacia-LKB. V8 protease was from Miles Scientific. [^{14}C]-Biotin is from Amersham Life Sciences. All other chemicals were of reagent grade.

Isolation of the 12S, 5S, and 1.3S Subunits. The 12S subunit was isolated from *P. shermanii* as previously described (Bahler et al., 1981; Hennessey et al., 1982) and the 1.3S wild-type subunit (1.3S WT) was isolated from *E. coli* as described by Shenoy et al. (1988). The 5S subunit from *P. shermanii* was isolated as described by Shenoy et al. (1992) and the recombinant 5S WT subunit was isolated from *E. coli* as described by Xie et al. (1993).

Crystallization of TC. TC was precipitated using 70% $(NH_4)_2SO_4$ and the supernatant was removed by centrifugation at 4 °C, 47800*g* for 15 min. The TC pellet was dissolved in a minimum volume of 250 mM acetate buffer, pH 5.5, and the undissolved TC was removed by centrifugation at 4 °C, 12000*g* for 5 min. The supernatant was then dialyzed against 20.0% $(NH_4)_2SO_4$ in 250 mM acetate buffer, pH 5.5, overnight at 4 °C. The precipitate formed was removed by centrifugation. The supernatant was dialyzed against 24.3% $(NH_4)_2SO_4$ in 250 mM acetate buffer, pH 5.5, overnight at 4 °C. The TC crystals formed at this stage.

Protein Determination. The protein content of the fractions containing the TC complex was estimated as described by Layne (1957). The protein content of the 1.3S subunit was determined using rose bengal according to Elliott and Brewer (1978), since it contains only one tyrosine and no tryptophan.

Biotin Determination. A modification of the method of Rylatt et al. (1977) was used to estimate the amount of biotin in the 1.3S subunit purified from *E. coli* as described previously (Shenoy et al., 1992).

Assays. The synthesis of [4-^{14}C]oxalacetate and measurement of partial reaction 2 with the 5S subunit have been previously described (Xie et al., 1993). TC was assayed in the forward direction as previously described (Shenoy et al., 1992; Wood et al., 1969) by detecting the formation of oxalacetate via a coupled assay system using malate dehydrogenase and measuring the decrease in NADH absorbance spectrophotometrically. The assays for the forward reaction contained the following in a total volume of 0.3 mL: pyruvate, 2.26 μmol; malate dehydrogenase, 4.5 units; NADH, 0.09 μmol; potassium phosphate buffer, pH 6.5, 75 μmol; methylmalonyl-CoA, 90 nmol; and the assembled enzyme. Specific activities are expressed as micromoles of oxalacetate formed in the forward reaction per minute per milligram of TC.

High-Performance Liquid Chromatography. HPLC analyses were performed with a Shimadzu HPLC system equipped with a computer interface and software for the integration and analysis of the peaks in the chromatogram. An Ultropac TSK G4000SW or G3000SW (7.5 × 300 mm) gel-filtration column was used for subunit separation. The elution of protein was achieved using 0.5 M potassium phosphate buffer, pH 6.5. The protein elution was monitored at 220 nm with a flow rate of 0.5 mL/min.

Electron Microscopy. For electron microscopy, samples were dissolved in 0.1 M potassium phosphate buffer, pH 6.5, and cross-linked with glutaraldehyde (0.4%) in order to prevent dissociation during staining with uranyl acetate. The grids for EM were prepared as described by Wrigley et al. (1977). All EM samples contained 100 μg/mL enzyme, a concentration previously shown to yield the best results. Plates were made of the images at a magnification of 65000×.

Fluorescence Measurements. Fluorescence spectroscopy was carried out on a Perkin-Elmer LS-5B spectrofluorometer equipped with a temperature control device and microprocessor-controlled photomultiplier gain. Routinely, emission and excitation slit widths of 3 and 5 nm, respectively, were used. All spectral determinations were made at 25 ± 0.1 °C. The tryptophanyl fluorescence was recorded with an excitation wavelength of 295 nm and the emission was monitored from 300 to 400 nm.

Assembly of Active Enzyme from Its Subunits. TC assembly was achieved by incubating the recombinant 5S subunit or *P. shermanii* 5S subunit with the 12S and 1.3S subunits at 4 °C in 0.5 M potassium phosphate buffer, pH 6.5, overnight. It was necessary to maintain the phosphate concentration at 0.5 M in order to achieve assembly. The molar ratio of the 12S:5S:1.3S subunits used for the reconstitution was 1:6:12 unless otherwise stated. This is the stoichiometry of 12S to 5S to 1.3S subunits in 26S TC which contains the full complement of six 5S subunits.

Reverse-Phase HPLC. Reverse-phase HPLC analysis was performed with a Shimadzu HPLC system equipped with computer interface and software for the integration and analysis of the peaks in the chromatogram. A C4 reverse-phase column was used for the peptide isolation. Linear gradient elution of the peptides was achieved using a solvent system composed of 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). The peptide elution was monitored at 220 nm.

Circular Dichroism Studies. The circular dichroism (CD) spectra were recorded with a Jasco Model J 600 spectropolarimeter (Jasco, Inc., Easton, MD), equipped with a microprocessor-controlled data collection and processing system. The instrument was calibrated with *d*-10-camphorsulfonic acid. All measurements were made with protein solutions of concentration 100 μg/mL using quartz cells with a path length of 1 mm. The relative proportions of α-helix, β-sheet, and random structure were estimated using the computer program SSE (Japan Spectroscopic Co., Ltd.).

Isoelectric Focusing of 5S Subunit. IEF was performed on a flat-bed LKB multiphor unit attached to a circulating

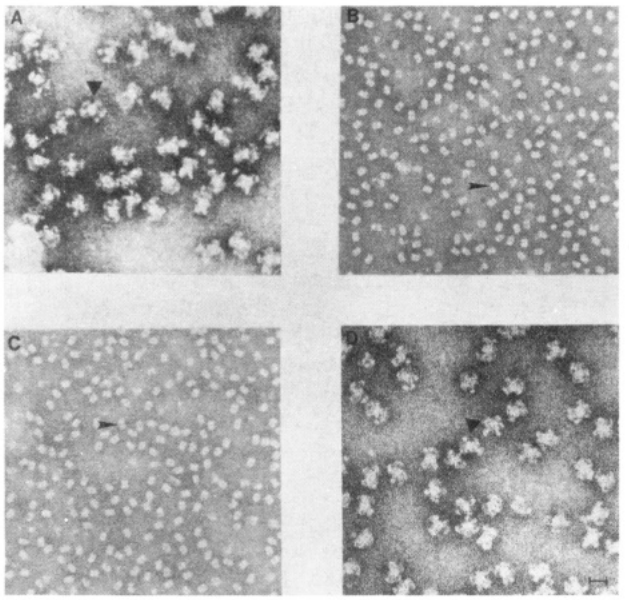

FIGURE 1: Electron micrographs of reconstituted TC from 5S WT subunit or *P. shermanii* 5S subunit with 12S and 1.3S subunits. (A) TC, 26S form; (B) Reconstituted mixture containing 12S, 5S WT, and 1.3S; (C) control mixture of 12S and 5S subunits; (D) TC-5S WT reconstituted from 5S WT, 1.3S, and 12S in the presence of APF. The following symbols are used: ▼, TC complex (26S form); ➢, 12S subunit.

water bath maintained at 4 °C according to the manufacturer using Servalyt Precotes of pH 3–10. The gel was prefocused before applying the samples. After focusing, the gels were stained with Serva Blue W according to Serva.

RESULTS

We have previously shown that the 5S subunit (5S WT), when purified after expression in *E. coli*, was fully active in partial reaction 2 (Xie et al., 1993). *E. coli*-expressed 5S and *P. shermanii* 5S subunits were indistinguishable by N-terminal sequence, amino acid composition, substrate binding, and binding to the 1.3S subunit. Both subunits had similar Co^{2+} and Zn^{2+} contents as well as similar native molecular weights (M_r 120 000) and subunit molecular weights (M_r 60 000). However, the 5S WT differed from the authentic form in that 5S WT could not support catalysis of the TC overall reactions.

Electron Microscopy of Reconstituted TC from Wild-Type 5S, 1.3S, and 12S Subunits. HPLC gel filtration of the reconstituted TC mixtures of 5S WT, 1.3S, and 12S subunits failed to show any stable TC complexes (Xie et al., 1993). It is possible that such enzyme complexes might have formed but dissociated during gel filtration. To rule out this possibility, TC complexes were assayed by electron microscopy, which employs milder conditions and glutaraldehyde to stabilize complexes if present. TC assembly was achieved by incubating the 5S WT or authentic 5S with 12S and 1.3S subunits at 4 °C in 0.5 M potassium phosphate buffer, pH 6.5, for 24 h. The reconstituted TC complexes were then tested for the presence of assembled TC under electron microscopy as described in Materials and Methods. The results are shown in Figure 1. The electron micrographs of reconstituted TC from 5S WT subunit showed only free 5S and 12S subunits (Figure 1B). In contrast, reconstituted TC from authentic 5S subunits showed 26S and 18S forms of TC as well as free 12S and 5S subunits (Figure 1A).

Activity of 5S WT from Crude Extract in the Overall Reaction. The inability of the 5S WT subunit to form TC complexes may be due to its inactivation during purification.

Table I. Secondary Structure of 5S WT and *P. shermanii* 5S Estimated from Their Respective CD Spectra[a]

	α-helix (%)	β-sheet (%)	β-turn (%)	random (%)
P. shermanii 5S	47.5	21.4	7.4	23.7
5S WT	64.9	10.8	3.2	21.1

[a] Calculated using a protein structure estimation program as described in Materials and Methods.

To rule out this possibility, a crude extract of *E. coli* containing the 5S WT subunit was tested in the overall reaction of TC after reconstitution with 12S and 1.3S subunits for 24 h at 4 °C as described in Materials and Methods. It is possible to test the overall activity in crude extracts since *E. coli* does not have any methylmalonyl-CoA utilizing enzymes. A crude extract of *E. coli* containing 5S WT was prepared in potassium phosphate buffer, pH 6.5. *P. shermanii* 5S is known to be stable at this pH. This crude extract showed only 5% of the TC activity of the *P. shermanii* 5S subunit whether the latter was used in a standard reconstitution assay or added to a crude extract of *E. coli* (which did not contain 5S WT subunit). Thus, we conclude that the buffer conditions and the purification procedures were not the cause of the inability of the 5S WT subunit to form TC complexes with 1.3S and 12S subunits.

Global Conformation of Wild-Type 5S Subunit. To determine whether the failure of 5S WT to support TC complex formation was the result of conformational differences between 5S WT and *P. shermanii* 5S subunits, the global conformation of the subunits was compared using circular dichroism as described in Materials and Methods. The 5S WT subunit had a greater α-helical content (64.9%) and reduced β-structure content (14%) compared to the *P. shermanii* 5S subunit (α-helix 47.5%, β-structure 28.8%) (Table I). These results indicate that the conformation of 5S WT subunit is different from that of authentic 5S subunit. This conformational difference was not due to folding differences as judged by sensitivity of the two 5S preparations to the disulfide bond-promoting agent Connecta-SH Purothionin (Wada & Buchanan, 1987) (data not shown).

Isoelectric Focusing of 5S WT Subunit and Authentic 5S from P. shermanii. Although 5S WT subunit was comparable to authentic 5S subunit in many of its characteristics, its conformation was different. To determine whether the 5S subunit from *P. shermanii* contained more than one protein, isoelectric focusing of 5S WT subunit and *P. shermanii* 5S subunit was carried out as described in Materials and Methods and is shown in Figure 2. The 5S WT subunit showed a single band corresponding to a p*I* of 4.2 (Figure 2, lane C). The authentic 5S from *P. shermanii* under similar conditions showed two bands corresponding to p*I*s of 4.2 and 4.0 (Figure 2, lane B). One of the bands of *P. shermanii* 5S subunit comigrated with the 5S WT subunit. The results indicate that the *P. shermanii* 5S subunit preparation contained more than one component.

Overall Reaction 3 and Partial Reaction 2 after HPLC Gel Filtration of P. shermanii 5S Subunit. Since the authentic 5S from *P. shermanii* showed two bands of differing isoelectric points and showed a different conformation when compared to 5S WT by circular dichroism, it was further purified by gel filtration. Gel filtration of the authentic 5S subunit was carried out using a TSK G3000SW HPLC gel-filtration column in 0.5 M potassium phosphate buffer, pH 6.5, as described in Materials and Methods. The HPLC profile is shown in Figure 3. The HPLC peaks were analyzed by SDS–PAGE and Western blotting for molecular weight and immunoreactivity.

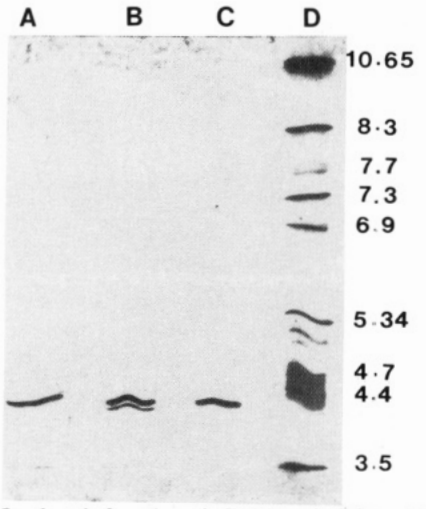

FIGURE 2: Isoelectric focusing of 5S WT and 5S from *P. shermanii*. Isoelectric focusing was carried out using Servalyt precotes of pH 3–10 as described under Materials and Methods. Lane A, 5S dimer after purification on HPLC gel-filtration column; lane B, authentic 5S from *P. shermanii*; lane C, *E. coli* 5S WT; lane D, standard protein mixture.

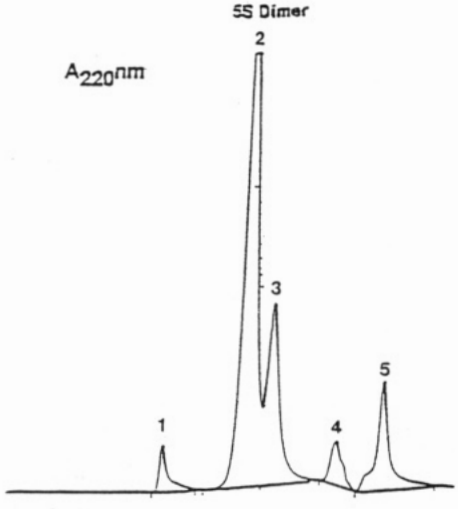

FIGURE 3: HPLC gel-filtration profile of *P. shermanii* 5S subunit. *P. shermanii* 5S subunit was fractionated on a HPLC TSK G3000SW column as described under Materials and Methods. Peak 2 is the dimer of the 5S subunit with a molecular weight of 120 000.

Peak 2 was shown to correspond to the 5S subunit and termed "*P. shermanii* 5S dimer" in order to differentiate it from *P. shermanii* 5S preparations which had not been passed through HPLC gel filtration. The activity of the 5S dimer was tested in partial reaction 2 and overall reaction 3. The results indicated that the *P. shermanii* 5S dimer was fully active in partial reaction 2. However, it was no longer active (0–14%) in overall reaction 3 when compared to the unfractionated *P. shermanii* 5S subunit preparation.

In order to test whether the *P. shermanii* 5S dimer was able to form TC complexes with 12S and 1.3S subunits, reconstitution was carried out in 0.5 M potassium phosphate buffer, pH 6.5, containing 12S, 1.3S, and *P. shermanii* 5S dimer in a molar ratio of 1:12:6 for 24 h at 4 °C. The reconstitution sample was then passed through the HPLC gel-filtration column as described in Materials and Methods to separate the free 12S, 1.3S and 5S dimer from TC. The results shown in Figure 4B indicate that the *P. shermanii* 5S dimer was unable to assemble with 12S and 1.3S subunits to form TC complexes, thus behaving in a similar fashion to the 5S WT subunit. In parallel, the *P. shermanii* 5S subunit which had

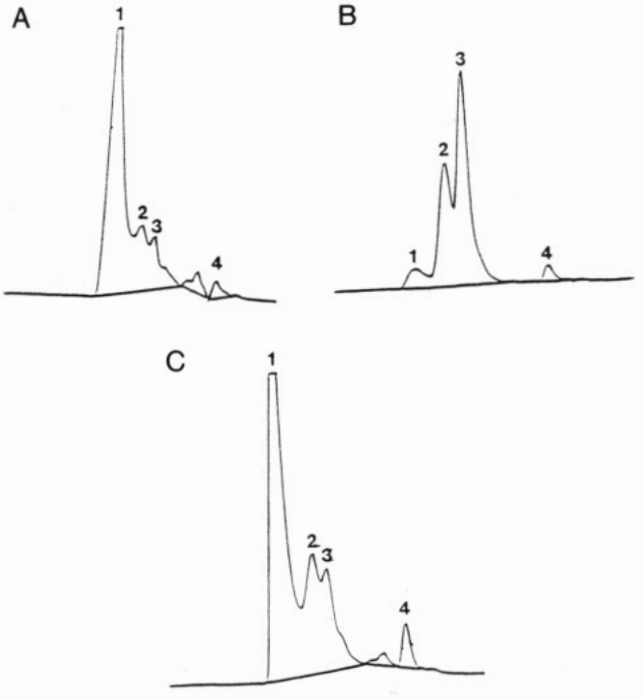

FIGURE 4: HPLC gel-filtration profile of TC reconstitution mixtures. TC reconstitution reactions were carried out as described under Materials and Methods and complexes were separated from free subunits by HPLC gel filtration. The reaction mixtures contained (A) *P. shermanii* 5S (prior to HPLC) with 12S and 1.3S subunits, (B) 5S dimer with 12S and 1.3S subunits, and (C) 5S dimer, 1.3S, and 12S subunits in the presence of APF. Peak 1 = TC, peak 2 = 12S, peak 3 = 5S, and peak 4 = buffer + 1.3S.

not been passed thromgh the HPLC gel-filtration column formed TC complexes with 12S and 1.3S subunits (Figure 4A).

Properties of HPLC-Purified P. shermanii 5S Dimer. The *P. shermanii* 5S dimer showed properties similar to those of the 5S WT subunit (data not shown). The secondary structure as determined by CD was comparable to that of 5S WT subunit, as were the fluorescence properties and binding titrations with 1.3S subunit, oxalacetate, and pyruvate. The *P. shermanii* 5S dimer also has a molecular weight of 120 000 and by metal analysis showed the presence of metals Co^{2+} and Zn^{2+}.

Assembly-Promoting Factor. In order to determine the difference between *P. shermanii* 5S before and after gel filtration, we employed a mixing experiment with fractions from the HPLC column in reconstitution assays. *P. shermanii* 5S dimer (peak 2) was mixed with peak 1, 3, 4, or 5 (Figure 3), incubated with 1.3S and 12S subunits at 4 °C for 24 h in 0.5 M potassium phosphate buffer, pH 6.5, and assayed for activity in the overall reaction. A control experiment was carried out simultaneously in which *P. shermanii* 5S which was not fractionated by HPLC gel filtration was used in reconstitution with 1.3S and 12S subunits. The results, summarized in Table II, show that the addition of peak 4 to the reconstitution mixture (5S dimer + 12S + 1.3S) resulted in the recovery of greater than 90% of the TC activity. The combination of peak 2 with any other peak or peaks, other than peak 4, did not result in any increase in activity.

To test whether the activity increase was due to TC assembly, the reconstituted mixture from *P. shermanii* 5S dimer (peak 2), peak 4, and 1.3S and 12S subunits was fractionated by HPLC gel filtration. A peak corresponding to TC was observed along with decreases in the amounts of free 12S and free 5S dimer (Figure 4C). Thus, it is clear that peak 4 can

Table II. Effect of Assembly-Promoting Factor on TC Activity

reconstitution mixture	specific activity[a] (overall reaction 3) (units/mg of 12S)
5S + 1.3S + 12S (before separation)	48.0
5S dimer + 1.3S + 12S	6.7
5S WT + 1.3S + 12S	2.3
5S dimer + 1.3S + 12S + peak 1	6.8
5S dimer + 1.3S + 12S + peak 3	6.3
5S dimer + 1.3S + 12S + APF (peak 4)	46.0
5S WT + 1.3S + 12S + APF (peak 4)	43.2
5S dimer + 1.3S + 12S + peak 5	6.6

[a] The values presented are an average for five different preparations and with standard error of ±2%.

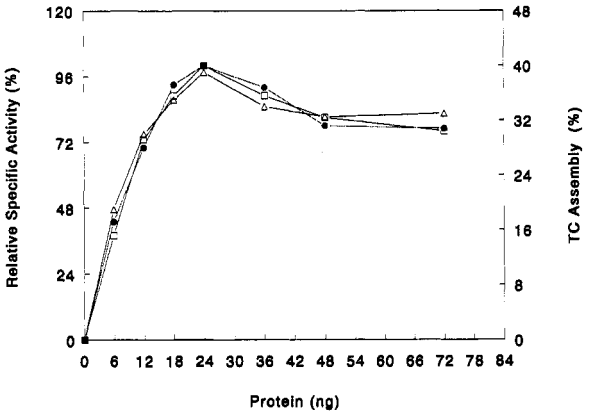

FIGURE 5: Effect of APF concentration on TC assembly and activity. Reconstitution reactions containing 5S WT or *P. shermanii* 5S + 1.3S and 12S were incubated with varying amounts of APF for 24 h in 0.5 M phosphate buffer, pH 6.5, at 4 °C followed by overall reaction and HPLC gel filtration. □, 5S WT, specific activity (%); ●, *P. shermanii* 5S, specific activity (%); △, TC complex (%).

promote the assembly of TC and the resulting activity increase is due to the assembly of the enzyme. The component present in peak 4, which was responsible for activity and assembly of TC reconstituted from *P.shermanii* 5S dimer (peak 2) and 1.3S and 12S subunits, was named "assembly-promoting factor" or APF.

Effect of APF on 5S WT Subunit. To test whether the APF can promote assembly of TC from 5S WT, 1.3S, and 12S subunits, the factor was added to a reconstitution mixture as described in Materials and Methods. The results shown in Figure 1D demonstrate that APF can promote assembly of complexes which appear similar to authentic TC as judged by electron microscopy. Table II shows data demonstrating that reconstitutions which include 5S WT and APF are able to catalyze the TC overall reaction. Thus it is clear that the 5S WT subunit by itself was not the cause for the lack of assembly or activity in the TC reconstitution experiments.

Effect of APF Concentration on TC Assembly and Activity. The concentration dependency of APF on TC activity/assembly was tested by reconstituting *P. shermanii* 5S dimer or 5S WT subunit with 12S and 1.3S subunits in a stoichiometric ratio of 6:1:12 at 4 °C for 24 h in 0.5 potassium phosphate buffer, pH 6.5, with various amounts of APF. A linear increase in activity and assembled TC was observed up to a protein concentration of 12 ng of the APF-containing fraction (Figure 5). The maximum activity/assembly was obtained at 24 ng of this fraction. Further additions of APF inhibited the activity and assembly. These results indicate that activity of APF is concentration-dependent.

Effect of APF on the Assembly of 12S and 1.3S Subunits. It has been shown previously that free 12S and 1.3S cannot

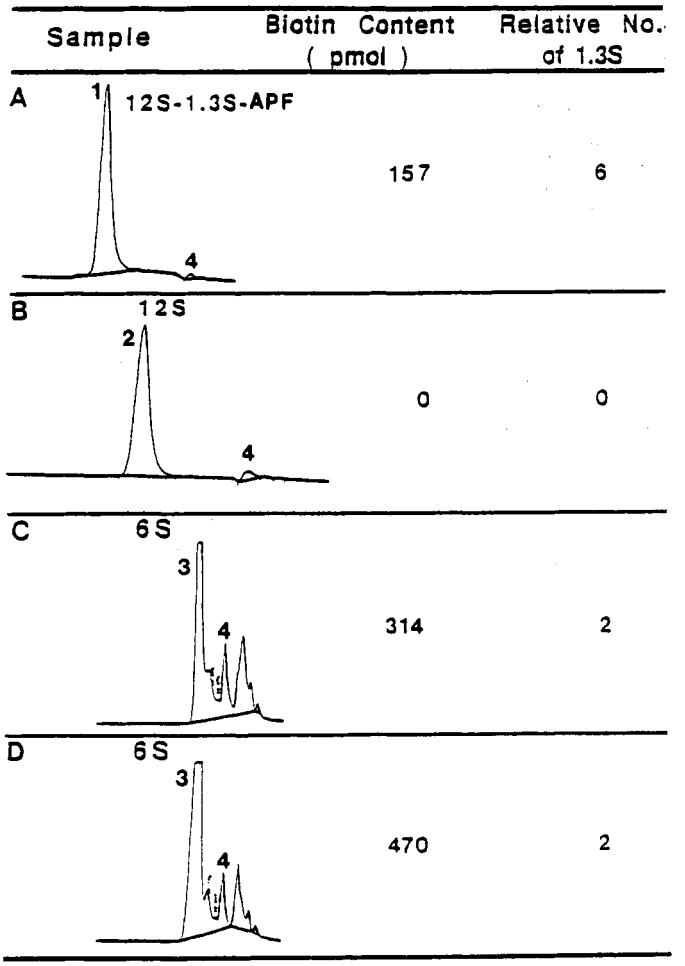

FIGURE 6: Effect of APF on the assembly of 12S + 1.3S and 5S + 1.3S subunits. Reconstitution reactions containing 12S + 1.3S (in a ratio of 1:12) or 5S + 1.3S (in a ratio of 1:2) were incubated in 0.5 M phosphate buffer, pH 6.5, overnight at 4 °C and fractionated on a HPLC gel-filtration column. The biotin content of the largest peak was determined as described under Materials and Methods and the relative number of biotins in each complex was calculated. Peak 1, 12S–1.3S–APF complex; peak 2, 12S; peak 3, 6S; peak 4, 1.3S.

form stable complexes unless the 5S subunit is present in the reconstitution mixture (Shenoy et al., 1992). We therefore determined whether the APF could promote the formation of a complex between 12S and 1.3S subunits. The association reaction was carried out in a stoichiometric ratio of 1:12 at 4 °C for 24 in the presence of APF. The reconstituted mixture was fractionated by chromatography on a HPLC gel-filtration TSK G3000SW column as described in Materials and Methods. The results shown in Figure 6 suggest that the APF can promote complex formation of 12S and 1.3S subunits in the absence of 5S subunits (Figure 6A; elution time of complex, 12.6 min). However, in the absence of APF, no complex of 12S and 1.3S subunits was formed (Figure 6B; elution time of 12S subunit, 13.8 min).

If 12S and 1.3S subunits do form a complex with the help of APF, the peak eluted at 12.6 min should contain biotin. The biotin content of this peak was measured as described in Materials and Methods. The results indicate that there were six 1.3S subunits bound to one 12S subunit. This correleated well with the fact that the preparation of 12S used in this experiment formed only the 18S form of TC (six 1.3S subunits). The peak eluted at 13.8 min, corresponding to 12S, did not contain any biotin. These results confirm that the 12S and 1.3S subunits can only form complexes in the presence of APF.

Table III. Heat Stability and Susceptibility to Various Proteases of Assembly-Promoting Factor

reconstitution mixture	specific activity[a] (%)
control (12S + 5S dimer + 1.3S + APF)	100
APF boiled at 100 °C for 15 min	98
APF treated with trypsin	18
APF treated with V8 protease	0
APF treated with pronase	2

[a] The values presented are an average for five different preparations and with standard error of ±2%.

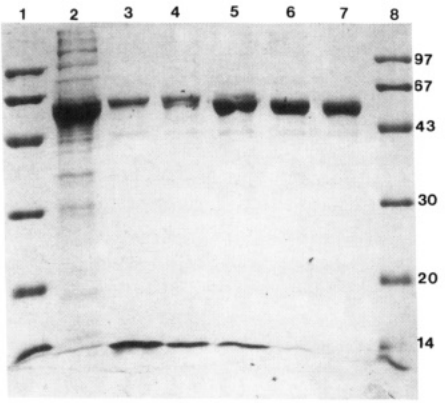

FIGURE 7: SDS–PAGE of APF after HPLC gel-filtration column. SDS–PAGE was performed as described under Materials and Methods. Lanes 1 and 8, molecular weight standards; lane 2, authentic 5S subunit; lanes 3 and 4, APF; lane 5, 5S dimer plus APF; lanes 6 and 7, 5S dimer.

Effect of APF on the Assembly of 5S and 1.3S Subunits. Wood et al. (1975) have shown that *P. shermanii* 5S subunit can form a 6S complex with two 1.3S subunits. APF was tested to determine whether it was necessary for 6S formation from 5S and 1.3S subunits. The reconstitution of *P. shermanii* 5S dimer and 1.3S subunits was carried out in a stoichiometric ratio of 1:2 (5S:1.3S) at 4 °C in 0.5 M potassium phosphate buffer, pH 6.5, for 24 h in the presence and absence of APF. The reconstitution mixture was fractionated by chromatography on a HPLC gel-filtration column as described in Mateerials and Methods and the results are shown in Figure 6C,D. The 5S dimers and 1.3S subunits formed 6S subunits with or without APF, indicating that the APF was not necessary for 6S formation. These results were further confirmed by estimating the biotin contents of the peak eluted at 14.8 min (6S subunit). The peaks contained 2 biotins/6S subunit in both cases, i.e., in the presence and absence of APF. The 5S dimer itself under the above conditions eluted at 15.2 min. We conclude that APF is not necessary for 6S formation.

Properties of APF: (*A*) *Heat Stability.* The APF, in 0.5 M potassium phosphate buffer, pH 6.5, was boiled for 15 min at 100 °C and immediately cooled on ice at 4 °C. It was then added to a reconstitution mixture containing 1.3S, 12S, and 5S dimer (stoichiometric ratio 12:1:6) and incuated for 24 h at 4 °C in 0.5 M potassium phosphate buffer, pH 6.5. The activity of TC formed was then measured and is shown in Table III. The results indicate that the APF is heat-stable and showed no detectable loss in TC activity when boiled and subsequently incubated with 5S dimer and 1.3S and 12S subunits.

(*B*) *SDS–PAGE.* The APF, which had been purified by HPLC gel filtration, showed two major bands by SDS–12.5% polyacrylamide gel electrophoresis (Figure 7). One protein band ran near the dye front (M_r 8000), while the other protein ran with M_r = 60 000. Sequence analysis of the M_r 8000 band indicated that it was the COOH-terminus of the 12S subunit. As full-length *E. coli*-expressed 12S requires APF for TC complex formation, it is unlikely that this band is the assembly factor.

(*C*) *Resistance to Proteolysis.* The APF was digested separately with V8 protease, pronase, and trypsin at a ratio of enzyme to APF (w/w) of 1:50 in 0.1 M potassium phosphate buffer, pH 6.5, at 37 °C for 24 h. The reactions were terminated either by using leupeptin in the case of trypsin (5 times the concentration of trypsin added) or by boiling at 100 °C for 15 min for V8- and pronase-treated samples. All the protease-treated samples were then reconstituted with 5S dimer and 1.3S and 12S subunits, and the activity of assembled TC was measured as described in Materials and Methods. The results are shown in Table III. All the proteases tested inactivated the APF activity to different extents. Control experiments in which the proteases were inactivated at 0 time showed no loss in activity. These results imply that APF is a protein or a molecule closely associated with a protein.

Preparation of APF from Crystallized TC. In order to demonstrate that APF is not a contaminating protein eluted along with TC during purification from *P. shermanii* and further copurified along with 5S subunit during subunit preparation, the APF was purified from crystallized TC. TC crystallization and subsequent 5S preparation were carried as described in Materials and Methods. The APF purified from this 5S subunit preparation was fully active in TC assembly and overall reaction catalysis, indicating that the APF is a part of TC and not a contaminating protein.

DISCUSSION

5S WT subunit, expressed in *E. coli* and purified by CM-52, DE-53, and HPLC gel-filtration columns, was found to be indistinguishable from the *P. shermanii* 5S subunit with respect to dimer formation of molecular weight 120 000, subunit molecular weight of 60 000, binding to 1.3S or substrates (pyruvate and oxalacetate), presence of metals (Co^{2+} and Zn^{2+}), and activity in partial reaction 2 (Xie et al., 1993). However, 5S WT failed to form TC complexes when incubated with 12S and 1.3S subunits, in contrast to *P. shermanii* 5S, which formed such complexes. Moreover, 5S WT subunit showed a different secondary structure and global conformation when compared to the *P. shermanii* 5S subunit. Their isoelectric focusing patterns were also distinct. These differences led us to suspect that *P. shermanii* 5S preparations contained an additional component. This component was separated from *P. shermanii* 5S by HPLC gel filtration. The recovered *P. shermanii* 5S dimer more closely resembled *E. coli* 5S WT than the unfractionated *P. shermanii* 5S preparation.

The APF-containing fraction from HPLC gel filtration contains two proteins as judged by its SDS–PAGE banding pattern. It is capable of promoting two types of complexes: active TC from 12S, 5S, and 1.3S as well as a stable association between 12S and 1.3S. The activity showed concentration dependence with respect to APF and, in addition, was heat-stable. APF activity is lost after digestion with trypsin, V8 protease, and pronase implying that the factor is a protein. Presently, we are unable to estimate the stoichiometric amount of APF in TC because of the low yield of the factor in 5S preparations and contaminating proteins in our current preparations. We believe APF to be part of TC and not a copurifying contaminant because it can be purified from crystallized TC. It is possible that APF is a chaperonin-like molecule which may be found associated with proteins other

than transcarboxylase in *P. shermanii*. However, its activity is ATP-independent, which is not a characteristic of "classical" chaperonins (Mizobata et al., 1992). It is still formally possible that APF is a small molecule tightly associated with a protein. If so, its activity can survive boiling but not proteolysis. We favor the more likely possibility that APF is a protein and probably the 60 000 dalton species we detect in our preparations. APF appears to be a new component of transcarboxylase distinct from the three traditional subunits and one that is essential for TC complex formation but not catalysis. We speculate that it directly interacts with the 12S subunit to promote association with 1.3S during enzyme formation or reconstitution. Further purification and characterization of APF are underway.

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