Phosphoenolpyruvate-Dependent Fructose Phosphotransferase System of *Rhodopseudomonas sphaeroides*: Purification and Physicochemical and Immunochemical Characterization of a Membrane-Associated Enzyme I†

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ABSTRACT: The phosphotransferase system (PTS) of the phototrophic bacterium *Rhodopseudomonas sphaeroides* consists of a component located in the cytoplasmic membrane and a membrane-associated enzyme called "soluble factor" (SF) [Saier, M. H., Feucht, B. U., & Roseman, S. (1971) *J. Biol. Chem. 246*, 7819–7821]. SF has been partially purified by a combination of hydrophobic interaction and ion-exchange and gel-permeation chromatography. SF is similar to *Escherichia coli* enzyme I in its molecular characteristics and enzymatic properties. It has a molecular weight of 85 000 and readily dimerizes. Phosphoenolpyruvate and Mg^{2+} stabilize the dimer. The enzyme catalyzes the conversion of phosphoenolpyruvate into pyruvate and becomes phosphorylated in the process. The phosphoryl group is subsequently transferred to fructose in the presence of *R. sphaeroides* membranes. SF substitutes for *E. coli* enzyme I in fructose or glucose phosphorylation with *E. coli* enzyme II and HPr. The activities of SF with the *R. sphaeroides* PTS and the *E. coli* PTS reside on structurally distinct molecules as shown by their response to limited proteolytic digestion and by immunochemical studies. The activity of SF with the *E. coli* PTS arises during the isolation procedure and is most likely due to the removal of HPr-like protein from SF.

Obligate and facultative anaerobic bacteria accumulate many sugars by the vectorial phosphotransferase system (PTS),[1] which is genetically and biochemically well characterized (Postma & Roseman, 1976; Saier, 1977; Rosen, 1978). Analysis of the PTS from *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Spirochaeta aurantia* (Roseman, 1969; Kundig & Roseman, 1971a,b; Saier, 1977) showed that phosphoryl-group transfer occurred in a sequential fashion using at least three protein fractions.

$$\text{phosphoenolpyruvate} + \text{HPr} \xrightarrow{\text{enzyme I}} \text{phospho-HPr} + \text{pyruvate}$$

$$\text{phospho-HPr} + \text{sugar (out)} \xrightarrow{\text{enzyme II}} \text{sugar-P (in)} + \text{HPr}$$

Enzyme I and HPr are considered to be cytoplasmic proteins that lack sugar specificity, whereas the enzyme II complex is a sugar-specific membrane component.

It is generally believed that phototrophic bacteria were the progenitors of bacteria capable of aerobic respiration (Gest, 1980). Thus, the observations of Saier et al. (1971) that the prototrophic bacteria *Rhodospirillum rubrum* and *Rhodopseudomonas sphaeroides* both possessed a novel phosphotransferase system capable of transporting only fructose were particularly interesting. The system differs from other PTS identified up until now in that it appears to lack HPr but contains a large membrane-associated enzyme (SF, soluble factor) that is responsible for phosphorylating E_{II} directly from phosphoenolpyruvate. In *E. coli*, enzyme I is responsible for the first step in the phosphoryl-group transfer from phosphoenolpyruvate. Although it is located almost exclusively in the soluble fraction of cell extracts, recent purification procedures demonstrated that it possesses very hydrophobic surface regions (Robillard et al., 1979), suggesting that it too may be membrane associated in intact cells.

These limited similarities between enzyme I from *E. coli* and SF from *R. rubrum* prompted us to investigate, in more detail, the molecular mechanism of a PTS in a phototrophic bacterium, the ultimate goal being to gain a deeper insight into the evolution of PTS-mediated transport and its control mechanism.

The studies reported in this paper have demonstrated that the PTS of *R. sphaeroides* is a membrane-bound complex consisting of a membrane-associated E_I/HPr type of molecule and an integral membrane E_{II}-type component.

The membrane-associated component has been solubilized, purified, and characterized. It is functionally equivalent to E_I of *E. coli*, and it is partially cross-reactive with antibodies against *E. coli* enzyme I. It is associated with an HPr-type molecule that can be replaced by *E. coli* HPr subsequently catalyzing α-methylglucose phosphorylation in conjunction with *E. coli* enzyme II.

Materials and Methods

Materials. Hexyl-agarose, lot 30F-9550, butyl-agarose, lot 99C-9690, phosphoenolpyruvate (cyclohexylammonium salt), dithiothreitol, and soybean trypsin inhibitor were purchased from Sigma. Trypsin dicyclohexylcarbodiimide was purchased from Miles Laboratories, Ltd. D-[U-^{14}C]Fructose (301 mCi/mmol), methyl α-D-[U-^{14}C]glucopyranoside (180 mCi/mmol), and ^{14}C-labeled PEP (monocyclohexylammonium salt) (12.5 mCi/mmol) were purchased from Amersham.

Rhodopseudomonas sphaeroides, strain 2.4.1, was grown anaerobically in the light in the medium described by Sistrom (1960) at 30 °C. The cells (20 mL) were then transferred to a 1-L flask with 300 mL of a medium containing 0.4% yeast extract, 0.6% casamino acids, and 0.2% fructose and were grown aerobically in the dark at 30 °C. Cells were harvested

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[1] Abbreviations used: PTS, phosphoenolpyruvate-dependent phosphotransferase system(s); PEP, phosphoenolpyruvate; DFP, diisopropylfluorophosphate; E_{II}, enzyme II; E_I, enzyme I; EDTA, ethylenediaminetetraacetic acid; Pyr, pyruvate.

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at an OD of 2–2.5 at 663 nm and stored at −20 °C.

Salmonella typhimurium ptsI mutant, strain SB 1690, and ptsHI mutant, strain SB 2950, were grown as described earlier (Dooijewaard et al., 1979). Components of the *S. typhimurium* and *E. coli* PTS are completely homologous and can be used interchangeably. Therefore, in the interest of readability, *E. coli* and *S. typhimurium* PTS components are all referred to as *E. coli* components or the *E. coli* system.

Enzyme I was purified from *E. coli* P 650 by the method of Robillard et al. (1979) with the following modification. The E_I fractions obtained after the second octyl-Sepharose chromatography were directly applied to a DEAE-cellulose column (2 × 23 cm) equilibrated with 20 mM Na_2HPO_4, 100 mM KCl, 1 mM DTT, and 1 mM NaN_3, pH 7.6. After washing the column with 90 mL of buffer, enzyme I was eluted with a 700-mL linear gradient of 100–400 mM KCl. The flow rate was 20 mL/h. This modification was required because the results of the phenyl-Sepharose step, as described in the original purification procedure, were dependent on the lot number of the hydrophobic resin.

HPr was purified from *E. coli* P 650 as described by Dooijewaard et al. (1979).

Sodium dodecyl sulfate–polyacrylamide disc gel electrophoresis was performed in the buffer system described by Laemli (1970). Samples were denatured by heating at 90 °C for 5 min in the presence of 3% sodium dodecyl sulfate and 5% 2-mercaptoethanol. Gels were calibrated with phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and α-lactalbumin (Pharmacia Fine Chemicals). Regular disc gel electrophoresis was carried out by using the same buffer system without sodium dodecyl sulfate.

Protein concentrations were determined by the procedure of Lowry et al. (1951). Bovine serum albumin was used as a standard.

Measurement of the Phosphotransferase Activity. Soluble factor "activity" was measured by using D-[U-^{14}C]fructose as the phosphate acceptor. The phosphorylation reaction was carried out at 37 °C in a final volume of 200 μL containing the following components: 5 μmol of potassium phosphate, pH 7.5, 2.5 μmol of KF, 0.25 μmol of DTT, 1 μmol of PEP (cyclohexylammonium salt), 0.375 μmol of D-[U-^{14}C]fructose (800 000 cpm/μmol of fructose), 0.5 μmol of $MgCl_2$, 25 μL of a membrane fraction of *R. sphaeroides* (5 mg of protein/mL; see Results for preparation) or 50 μL of a crude cell extract (48000*g* supernatant) of the *S. typhimurium* ptsI mutant, strain SB 1690 (Robillard et al., 1979), and the specified amounts of the soluble factor. Radioactive fructose phosphate was processed and counted as described by Misset et al. (1980).

Determination of Soluble Factor Concentration with ^{14}C-Labeled PEP. SF (300 μL), containing 250 μg of protein, was incubated at 37 °C with 35 μL of 62.5 μM ^{14}C-labeled PEP in a 10 mM sodium phosphate buffer, pH 7.5, containing 2.5 mM $MgCl_2$. Samples of 50 μL were taken at several time intervals. PEP and pyruvate were separated and radioactivity was counted according to the procedure described by Hoving et al. (1981). The concentration of SF was calculated from the amount of [^{14}C]pyruvate present at $t = 0$ (see Results).

Antibodies against *R. sphaeroides* membrane vesicles and enzyme I of *E. coli* were prepared according to Elferink et al. (1979). The *R. sphaeroides* vesicles were prepared by osmotic shock followed by low-speed centrifugation.

Crossed immunoelectrophoresis of soluble factor or enzyme I against anti enzyme I immunoglobulins was performed as described by Smyth et al. (1978).

Limited Proteolytic Digestion of SF and Enzyme I with Trypsin. SF (500 μg/mL) was incubated with trypsin at 37 °C in 5 mM sodium phosphate and 1 mM DTT, pH 7.5; a similar incubation was carried out in the presence of 10 mM PEP and 5 mM $MgCl_2$. Enzyme I of *E. coli* (500 μg/mL) was incubated with trypsin under identical conditions. The trypsin:SF(E_I) ratio was 1:50 (w/w) in all experiments. Trypsinolysis was inhibited at several time intervals by addition of a 2-fold excess (w/w) of soybean trypsin inhibitor over trypsin. SF samples were then assayed as described above and subjected to sodium dodecyl sulfate electrophoresis. E_I samples were assayed as described by Robillard et al. (1979).

Results

Purification of SF. Ten grams of *R. sphaeroides* cells was suspended in 50 mL of 5 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, and 1 mM NaN_3, pH 7.5. The suspension was brought to a final concentration of 1 mM DFP by using a stock solution of 0.1 M DFP in isopropyl alcohol and then immediately ruptured by passage through a French press at 10 000 psi at 4 °C. The solution was centrifuged for 20 min at 40000*g* at 4 °C.

Hexyl-agarose Chromatography. The supernatant (40 mL) was brought to 20% in ethylene glycol by addition of pure ethylene glycol. The solution was loaded at room temperature at 25 mL/h on a 1.6 × 25 cm column of hexyl-agarose equilibrated in 20% ethylene glycol containing 5 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, and 1 mM NaN_3, pH 7.6. NaN_3 is used in all isolation procedures because lower yields of *E. coli* E_I activity were previously observed to coincide with bacterial growth. After loading, the column was rinsed, at 20 mL/h, with 65 mL of equilibration buffer. The membrane fraction containing E_{II} activity eluted between 33 and 87 mL and was centrifuged at 200000*g* for 135 min. The pellet was redissolved in 20 mL of 5 mM Na_2HPO_4, 1 mM DTT, and 2.5 mM $MgCl_2$, pH 7.5, homogenized with a tissue homogenizer, and kept at −20 °C in 1-mL portions. The membrane preparation obtained in this way was used as the E_{II} fraction in the fructose phosphorylation assay (see Materials and Methods). SF activity was eluted from the column, at 20 μL/h, with a 400-mL linear gradient from 20 to 90% ethylene glycol, containing the buffer components mentioned before. Fractions were tested for SF activity, and the ethylene glycol gradient was determined by measuring the index of refraction. Ninety percent of the SF activity eluted at approximately 65% ethylene glycol (Figure 1A).

Butyl-agarose Chromatography. The peak of SF activity from the hexyl-agarose column was pooled and diluted to a final concentration of 20% ethylene glycol, and the salt concentration was raised to 20 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, and 1 mM NaN_3, pH 7.5. The solution was then loaded on a 1.5 × 25 cm column at room temperature with a flow rate of 25 mL/h. After loading, the column was rinsed with 20 mL of equilibration buffer at 20 mL/h. SF activity was eluted with a 400-mL linear gradient of 20–80% ethylene glycol containing the same buffer components. SF activity eluted at approximately 42% ethylene glycol (Figure 1B). All attempts to reuse the hexyl- and butyl-agarose after regeneration were unsuccessful. Each chromatography was done with new resin.

DEAE-cellulose DE-52 Chromatography. The peak of SF activity was pooled, diluted twice, and loaded on a 1 × 43 cm DEAE-cellulose column equilibrated with 20 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, and 1 mM NaN_3, pH 7.5, at 4 °C with a flow rate of 24 mL/h. After loading, the column

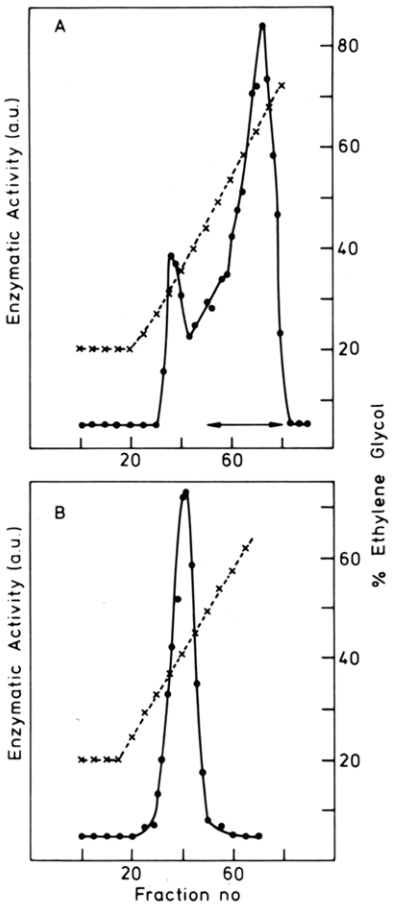

FIGURE 1: Hydrophobic interaction chromatography of SF. (A) Elution profile from a 1.6 × 25 cm hexyl-agarose column eluted at room temperature with a 400-mL gradient of 20–90% ethylene glycol in 5 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, and 1 mM NaN_3, pH 7.5. The flow rate was 20 mL/h and the fraction volume was 5 mL. Fractions 50–80 were collected and chromatographed on butyl-agarose. (B) Butyl-agarose (1.5 × 25 cm) eluted at room temperature with a 400-mL gradient of 20–80% ethylene glycol in 20 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, and 1 mM NaN_3, pH 7.5. The flow rate was 20 mL/h and the fraction volume was 5 mL. Fractions 32–46 were collected and loaded on DEAE-cellulose. (●) Enzymatic activity; (×) % ethylene glycol.

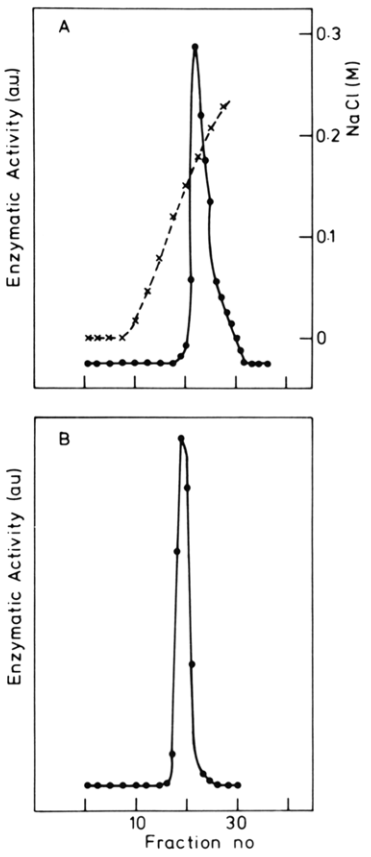

FIGURE 2: (A) DEAE-cellulose (1 × 43 cm) chromatography at 4 °C of the SF pool from the butyl-agarose chromatography in Figure 1B. The column was eluted with a 300-mL linear gradient of 0–300 mM NaCl in 20 mM Na_2HPO_4, 1 mM DTT, 1 mM EDTA, and 1 mM NaN_3, pH 7.6. The flow rate was 22 mL/h and the fraction volume was 5.4 mL. Fractions 42–58 were collected, concentrated, and subjected to gel filtration on Sephacryl S-200. (B) Gel filtration of the DEAE-cellulose pool on Sephacryl S-200 (1.2 × 100 cm) at 4 °C in 5 mM Na_2HPO_4 and 1 mM DTT, pH 7.5. The flow rate was 11 mL/h and the fraction volume was 2.75 mL. Fractions 17–21 were collected.

was rinsed with 50 mL of equilibration buffer. SF activity was eluted with a 300-mL linear gradient of 0–300 mM NaCl in equilibration buffer. SF eluted at approximately 0.15 M NaCl (Figure 2A). The pooled material was concentrated on an Amicon ultrafiltration apparatus equipped with a UM-20 membrane.

Sephacryl S-200 Chromatography. Concentrated SF (3 mL) was chromatographed on a 1.2 × 100 cm column of Sephacryl S-200 at 4 °C. The column was eluted with 5 mM Na_2HPO_4 and 1 mM DTT, pH 7.5, at 11 mL/h. The pooled material (Figure 2B) was concentrated by ultrafiltration to a final volume of 2.5 mL and stored in 100-μL portions at −20 °C.

Polyacrylamide Gel Electrophoresis. The results of regular and detergent polyacrylamide gel electrophoresis are shown in Figure 3. Regular gels show one main component, which can be extracted from the gel and phosphorylate fructose in the presence of *R. sphaeroides* membranes. The pattern obtained after detergent polyacrylamide gel electrophoresis is rather complex. A main component with a molecular weight of 83 000 is observed together with bands corresponding to molecular weights of 58 000, 54 500, 52 000, 47 000, 42 500, and 34 000. Subsequent experiments will show that soluble factor is associated with the 83 000 band.

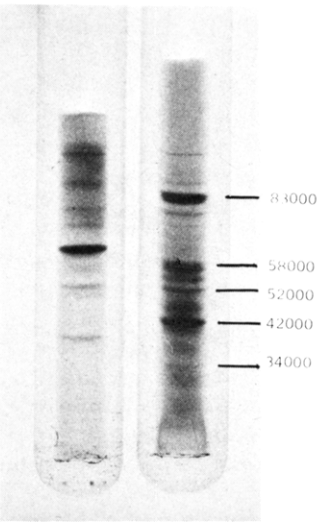

FIGURE 3: Polyacrylamide disc gel electrophoresis of partially purified SF: (left) 10% polyacrylamide gel; (right) 7.5% polyacrylamide gel containing 0.1% sodium dodecyl sulfate. The cathode is at the bottom in both cases. The protein was stained with Coomassie brilliant blue.

Gel Filtration Studies. A Sephacryl S-200 column was calibrated with the following proteins: bovine serum albumin (monomer and dimer), ovalbumin, soybean inhibitor, and cytochrome *c*. The elution position of SF was strongly de-

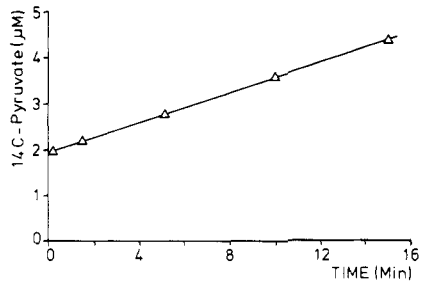

FIGURE 4: Conversion of [^{14}C]PEP into [^{14}C]pyruvate catalyzed by SF. See Materials and Methods for details.

pendent on the protein concentration, and the estimated molecular weight ranged from 90 000 to 160 000. Since *E. coli* E_I dimerizes in the presence of PEP and Mg^{2+} (Misset et al., 1980), this same property was examined for soluble factor. In the presence of PEP and Mg^{2+}, SF eluted as a single peak with a molecular weight of about 160 000. Apparently SF occurs in a monomer–dimer (80 000 ↔ 160 000) equilibrium that is shifted to the dimer in the presence of PEP and Mg^{2+} in a manner similar to that observed for *E. coli* E_I.

Determination of SF Concentration. When SF is incubated with ^{14}C-labeled PEP, an initial burst of [^{14}C]pyruvate can be demonstrated. Therefore SF catalyzes the following reaction:

$$SF + PEP \leftrightarrow SF\text{-}P + pyruvate$$

This property of SF can now be used to determine its concentration, assuming that the equilibrium is established rapidly and is far to the right, as has been demonstrated for the same reaction catalyzed by enzyme I of *E. coli* (Hoving et al., 1981). If this assumption is correct, the concentration of SF should equal the concentration of pyruvate at $t = 0$ and remain constant, provided that SF-P is stable. As can be seen from Figure 4, there is an initial burst of pyruvate when SF and PEP are mixed, representing the formation of SF-P, followed by a slow increase of the pyruvate level, indicative of the slow hydrolysis of SF-P. As long as PEP is present in sufficient concentrations, the rate of the backward reaction will be negligible, and SF-P will be in a steady state. Hence, $dPyr/dt = K_h[SF\text{-}P]$, in which K_h is the first-order rate constant for the reaction

$$SF\text{-}P \xrightarrow{H_2O} SF + P_i$$

The concentration of SF can be calculated from the intercept of the plot in Figure 4. This calculation assumes that the SF dimer binds only one phosphoryl group as has been found for *E. coli* E_I (Hoving and Misset, personal communication). Comparing the SF concentration and protein content of a purified SF preparation demonstrates that SF corresponds to about 40% of the protein. This agrees reasonably well with the gel electrophoresis pattern (Figure 3). K_h, calculated from the slope of Figure 4, is 0.095 min^{-1}.

Catalytic Properties of SF. SF catalyzes fructose phosphorylation in the presence of *R. sphaeroides* membranes. The catalytic activity of the different SF preparations ranged from about 20 μmol of fructose min^{-1} (μmol of SF)$^{-1}$ to 170 μmol of fructose min^{-1} (μmol of SF)$^{-1}$. SF also catalyzes the fructose or glucose phosphorylation in the presence of *E. coli* HPr and E_{II}. HPr was absolutely essential for this activity. The activity of the different SF preparation ranged from about 1 μmol of fructose min^{-1} (μmol of SF)$^{-1}$ to 65 μmol of fructose min^{-1} (μmol of SF)$^{-1}$. Even though SF can, under certain conditions, substitute for *E. coli* E_I in the *E. coli* system, *E. coli* E_I and HPr could not substitute for SF in the *R. sphaeroides* PTS.

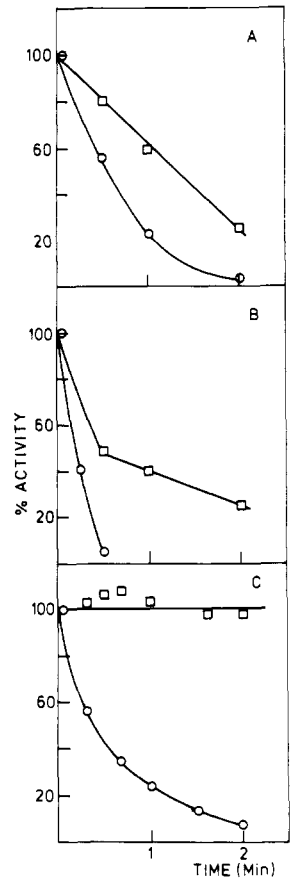

FIGURE 5: Effect of limited tryptic digestion on SF and *E. coli* enzyme I: (A) SF activity with the *R. sphaeroides* PTS; (B) SF activity with the *E. coli* PTS; (C) *E. coli* enzyme I activity with the *E. coli* PTS. (○) 5 mM Na_2HPO_4 and 1 mM DTT, pH 7.5; (□) 5 mM Na_2HPO_4, 1 mM DTT, 10 mM PEP, and 5 mM $MgCl_2$. See Materials and Methods for details.

Limited Proteolytic Digestion of SF and Enzyme I. The response of SF and E_I to limited tryptic digestion is shown in Figure 5. SF activity with the *R. sphaeroides* PTS disappears gradually within 2 min (Figure 5A), whereas SF activity with the *E. coli* PTS abruptly decreases within 30 s of incubation (Figure 5B). SF is protected against proteolytic digestion by PEP and Mg^{2+} (Figure 5A,B), suggesting that the dimer may be a more stable conformation. E_I is fully protected by PEP and Mg^{2+} against trypsinolysis (Figure 5C). The decrease in activity of SF is paralleled by a decrease of the 83 000 molecular weight band observed on sodium dodecyl sulfate gels. The other bands do not decrease. New bands appear around 55 000 and 33 000. Proteolytic surgery does not influence the pattern observed on regular gels. *E. coli* enzyme I is initially degraded as follows: 67 000 to 43 000 and 24 000.

Immunochemical Experiments with Antibodies against R. sphaeroides Membranes. The *E. coli* PTS activity observed with E_I or SF could be inhibited by preincubation of E_I or SF with antibodies against *R. sphaeroides* membranes. Increasing amounts of antibodies in the preincubation step led to increasing levels of inhibition [see Figure 6A,B, (○)]. If the antibodies were first exposed to 50 μL of ptsI mutant extract and the assays were subsequently started by addition of E_I or SF, hardly any inhibition was observed. The ptsI mutant extract contains normal levels of a mutated form of E_I with only 1% of the wild-type activity. E_I-type antibodies treated with the extract appeared to become saturated with this mutated E_I and were not available to complex active E_I or SF that was subsequently added. Therefore, we can conclude that the inhibition of fructose phosphorylation by antibodies against

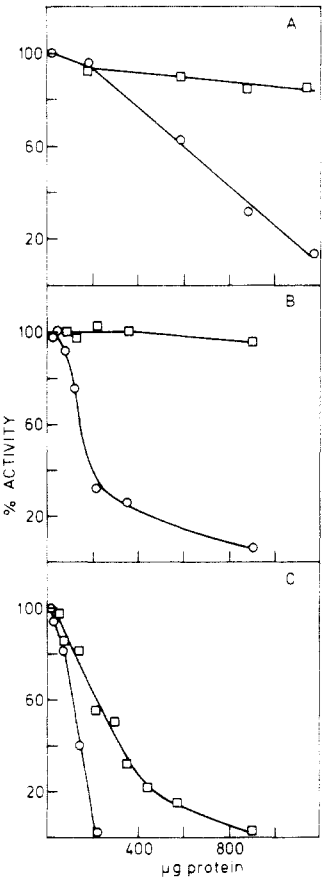

FIGURE 6: Inhibition of *E. coli* E_{II} + HPr-dependent fructose phosphorylation by antibodies against *R. sphaeroides* membranes and by *E. coli* enzyme I antibodies. (A) (○) 7.5 μg of protein containing approximately 1.5 μg of SF was preincubated for 5 min at 37 °C with 290, 580, 870, and 1160 μg of antibodies against *R. sphaeroides* membranes in a volume of 100 μL. Fructose phosphorylation was then started by addition of 50 μL of ptsI mutant extract and 50 μL of buffer containing [^{14}C]fructose, PEP, and Mg^{2+}. (□) Antibodies were preincubated with 50 μL of ptsI mutant extract for 5 min at 37 °C in a final volume of 100 μL. The assay was then started by addition of 100 μL of buffer containing 1.5 μg of SF, [^{14}C]fructose, PEP, and Mg^{2+}. (B) 1.5 μg of enzyme I incubated with *R. sphaeroides* membrane antibodies. Conditions are as described under (A). (C) 1.5 μg of enzyme I incubated with enzyme I antibodies. Conditions are as described under (A).

R. sphaeroides membranes resulted from the interaction between E_I or SF and anti E_I or anti SF-specific immunoglobulins that were present in the antibody preparation. It is not clear why high amounts of antibodies are required to inhibit SF preparations (Figure 6A) as compared to an equivalent amount of *E. coli* E_I (Figure 6B). Since the binding of SF to the membrane is stronger than the binding of E_I to the membrane, higher antibody concentrations might be necessary to remove it before the phosphorylation reaction can be inhibited.

The *R. sphaeroides* PTS activity observed with SF could also be inhibited by *R. sphaeroides* membrane antibodies. When the phosphorylation was carried out under conditions where *R. sphaeroides* membranes were in excess and SF was the rate-limiting factor, preincubation of SF and antibodies gave rise to an inhibition of fructose phosphorylation. Addition of SF restored the activity. This inhibition was not released by preincubation of the membrane antibodies with ptsI mutant extract (Table I). When SF was present in excess and the membrane fraction was rate limiting, fructose phosphorylation was also inhibited by *R. sphaeroides* membrane antibodies. The activity could then be restored by addition of the membrane fraction. It can therefore be concluded that antibodies against *R. sphaeroides* membranes recognize SF, *E. coli* E_I, and the membrane-bound complex of the *R. sphaeroides* PTS as well.

Table I: Effect of *E. coli* Enzyme I Antibodies (E_I AB) and *R. sphaeroides* Membrane Antibodies (RhM AB) on the Fructose Phosphorylation Catalyzed by E_I of *E. coli* or SF

enzyme + antibodies	assay system	inhibition	release of inhibition[a]	molecular level of inhibition
E_I + E_I AB[b]	E_I^- mutant	yes	yes	E_I
E_I + RhM AB[c]	E_I^- mutant	yes	yes	E_I
SF + E_I AB	E_I^- mutant	yes	yes	SF
SF + RhM AB	E_I^- mutant	yes	yes	SF
SF + E_I AB	RhM	no		
SF + RhM AB	RhM	yes	no	SF and RhM

[a] Release of inhibition by preincubation of the antibodies with E_I^- mutant extract of *S. typhimurium* SB 1690. [b] E_I AB, antibodies against enzyme I of *E. coli*. [c] RhM AB, antibodies against the cytoplasmic membrane of *R. sphaeroides*.

Immunochemical Experiments with E. coli E_I Antibodies. The *E. coli* PTS activity found with E_I and SF could be inhibited by antibodies against pure *E. coli* E_I. Again, this specific effect could be released by preincubation of E_I antibodies with ptsI mutant extract (Figure 6C and Table I).

The *R. sphaeroides* PTS activity could not be inhibited by preincubation with E_I antibodies. The fructose phosphorylation catalyzed by SF and *R. sphaeroides* membranes could only be inhibited by *R. sphaeroides* membrane antibodies. The results of the experiments with antibodies are summarized in Table I and will be interpreted under Disucssion.

Crossed Immunoelectrophoresis. Cross-reaction electropherograms in two-dimensional immunoelectrophoresis of E_I and SF against anti E_I immunoglobulins are shown in Figure 7. Enzyme I and SF show precipitation lines on the immunoplates at the same position. Moreover, when a mixture of E_I and SF is subjected to crossed immunoelectrophoresis, SF comigrates with E_I, resulting in an electropherogram identical with that of Figure 7A. E_I and SF are, therefore, immunochemically related proteins. It should be mentioned here that different SF preparations showed different reactivities to the E_I antibodies. Best results were obtained with SF preparations that showed high activity with the *E. coli* PTS. This matter will be taken up again under Discussion.

Discussion

When a crude cell extract (40000*g* supernatant) of *R. sphaeroides* is subjected to high-speed centrifugation, 200000*g* for 2 h, 40% of the PTS activity remains associated with the membrane fraction. Recombination of pellet and supernatant restores full activity. When the membrane fraction is homogenized in H_2O containing 1 mM DTT and centrifuged again, the particulate fraction is essentially devoid of PTS activity. The membrane-associated component that could be extracted by resuspension and centrifugation will be called SF (soluble factor).

Enzyme I of the *E. coli* PTS can be purified completely by hydrophobic interaction chromatography on octyl-Sepharose and phenyl-Sepharose (Robillard et al., 1979). Given our initial observations on the hydrophobic nature of SF, we tried the same purification procedure. However, our attempts to

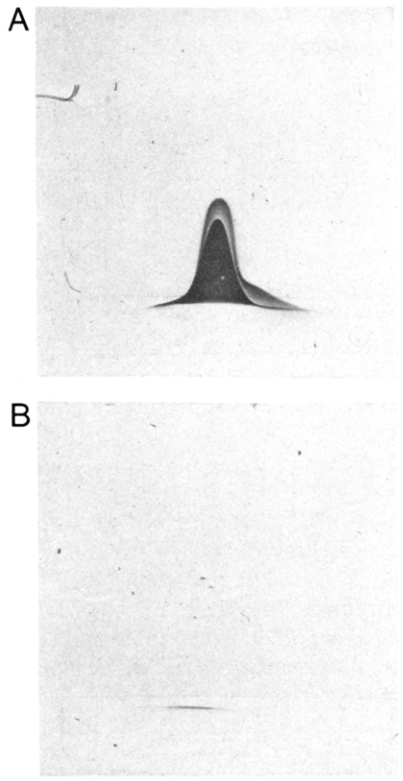

FIGURE 7: Crossed immunoelectropherograms of *E. coli* enzyme I (A) and *R. sphaeroides* soluble factor (B) against anti *E. coli* enzyme I antibodies. Per assay 5 μL containing 5 μg of protein was applied on the application spot. 250 μL of anti *E. coli* enzyme I antiserum was incorporated in the second-dimension gel. After electrophoresis the immunoplates were stained with Coomassie brilliant blue. The anode was at the left and at the top of the figures.

purify SF with these resins failed because SF was bound too strongly by octyl-Sepharose. Instead, resins containing shorter hydrophobic side chains were used (Shaltiel & Er-El, 1973). Chromatography of a crude cell extract of *R. sphaeroides* on hexyl-agarose showed that SF activity was distributed in two species eluting around 32 and 65% ethylene glycol (Figure 1A). Since the second peak contained about 90% of the SF activity, this material was collected, whereas the first peak was discarded. With 10 g of cells (wet weight, giving a crude cell extract containing 620 mg of protein), the isolation procedure resulted in 1–1.6 mg of SF (determined with ^{14}C-labeled PEP) with an average yield of around 10%. The enzyme isolated in this way was still not completely pure (Figure 3). The band on the sodium dodecyl sulfate gels corresponding to SF was inferred from the results obtained by subjecting SF to limited proteolytic digestion. Only the 83 000 molecular weight band disappeared during the time course of digestion, concomitant with a loss in fructose phosphorylation activity. The molecular weight obtained in this indirect way agrees well with our gel-filtration studies, which showed the existence of a monomeric, 80 000-dalton and a dimeric, 160 000-dalton species. The sodium dodecyl sulfate gels of partially purified SF show molecular weight species around 55 000 and 34 000, which are probably proteolytic fragments of SF, since trypsin cleaves the native molecule in 55 000- and 34 000-dalton fragments.

The actual amount of SF in our preparations could be determined with ^{14}C-labeled PEP (Figure 4). Per milligram of protein, 0.4 mg of SF that could be phosphorylated was present. SF is functionally equivalent to enzyme I of *E. coli*. Both enzymes catalyze the conversion of PEP into pyruvate, becoming phosphorylated in the process. Both phosphorylated enzymes transfer their phosphoryl moiety to *E. coli* HPr. Both enzymes occur in a monomer–dimer equilibrium that is shifted to the right by PEP. Yet there is an important difference between E_I and SF. Enzyme I is only active with the *E. coli* PTS, whereas SF catalyzes two activities: (1) fructose phosphorylation with the *E. coli* membranes, for which *E. coli* HPr is required; (2) fructose phosphorylation with the *R. sphaeroides* membranes, for which no extra HPr seems to be required. (Attempts to show the presence of a membrane-bound HPr have been unsuccessful.) These two activities reside on structurally distinct proteins as judged by their response to limited tryptic digestion and to enzyme I antibodies. From Figure 5A,B it can be seen that SF with *E. coli* PTS activity is much more susceptible to proteolytic digestion than SF with *R. sphaeroides* PTS activity. In Table I we see that only the SF active with the *E. coli* PTS is sensitive to inhibition by enzyme I antibodies. The SF active with the *R. sphaeroides* PTS is not inhibited by enzyme I antibodies at all. For ease of discussion we shall call the structurally distinct SF molecules SF-R, active with the *R. sphaeroides* PTS, and SF-E, active with the *E. coli* PTS. As already mentioned under Results, the different SF preparations varied in their activities with both PTS. In different isolations SF preparations that were highly active with the *R. sphaeroides* PTS invariably showed very low activities with the *E. coli* PTS. Conversely, SF preparations that showed low activities with the *R. sphaeroides* PTS were highly active with the *E. coli* PTS. The conversion of SF-R into SF-E occurred during the isolation procedure. An SF preparation obtained after the DEAE-cellulose chromatography showed an activity of 170 μmol of fructose min^{-1} (μmol of SF)$^{-1}$. Activity with the *E. coli* PTS could not be detected. After the concentration step on a UM-20 membrane, SF-R activity was decreased to 90 μmol of fructose min^{-1} (μmol of SF)$^{-1}$. SF-E activity had increased to 12 μmol of fructose min^{-1} (μmol of SF)$^{-1}$. The mechanism for the conversion of SF-R into SF-E has not yet been elucidated. However, since SF-E recognizes and phosphorylates *E. coli* HPr, the simplest explanation is that an HPr-like protein is removed from SF-R, leaving an HPr binding site accessible to *E. coli* HPr. This hypothesis is supported by the following experiments. Twenty milliliters of a very diluted SF preparation (9 μg of SF/mL in buffer used for the DEAE-cellulose chromatography) was concentrated on a UM-20 membrane (molecular weight cutoff 20 000) to a final volume of 1.1 mL. The UM-20 filtrate was then concentrated on a UM-2 membrane (molecular weight cutoff 2000) to a final volume of 1.1 mL. Addition of 10 μL of this UM-2 concentrate to an assay containing 20 μL of the UM-20 washed SF and 25 μL of *R. sphaeroides* membranes led to a 20% stimulation of fructose phosphorylation. On the other hand, these results could be explained by an FPr-type molecule. The fructose-induced PTS in *E. coli* and *A. aerogenes* phosphorylate fructose at the 1-hydroxyl position just as it does in *R. sphaeroides* (Hanson & Anderson, 1968; Walter & Anderson, 1973; Waygood, 1980). In both the *E. coli* and *A. aerogenes* fructose PTS, HPr is replaced by another phosphoryl-group carrier, FPr, with a molecular weight of 45 000. In spite of the difference in size, HPR and FPr must be partially homologous since both proteins can be phosphorylated by the same E_I. The same might be expected for the *R. sphaeroides* enzyme. We have looked for an HPr-type protein but have never found a low molecular weight component on sodium dodecyl sulfate–polyacrylamide gels of partially pure SF. However, one of the components between 35 000 and 50 000 could be an FPr-type protein. Further experiments are in progress to distinguish between these two possibilities.

The immunochemical experiments described in this paper are now easily understood. It is SF-E that precipitates with *E. coli* E_I antibodies during crossed immunoelectrophoresis. SF-R does not cross-react. Before looking in more detail at the data presented in Figure 6 and Table I, we still have to explain why the inhibitory effect of enzyme I antibodies and in some cases of *R. sphaeroides* antibodies could be released by incubation of the antibodies with ptsI mutant extract. *S. typhimurium* ptsI contains a leaky enzyme I that still has the capacity to dimerize with itself or with wild-type enzyme I but has only 1% of the catalytic activity of the wild-type enzyme (O. Misset, personal communication). This leaky enzyme I is apparently still able to bind enzyme I and SF-E antibodies. This explanation for the effect of ptsI mutant extract is supported by the fact that preincubation of antibodies with extract from a deletion mutant ptsHI does not protect against inhibition at all. The results summarized in Table I can be explained as follows: Antibodies against *R. sphaeroides* membranes contain antibodies against SF-R and SF-E. Only the latter bind to E_I and the mutated E_I form in ptsI mutant extract. In addition, SF-E antibodies "inhibit" SF-E and *E. coli* enzyme I. *E. coli* enzyme I antibodies inhibit *E. coli* E_I and SF-E but do not inhibit SF-R. SF-R antibodies only inhibit SF-R. This inhibition is not released by the ptsI mutant extract. It should be emphasized here that SF-E and *E. coli* enzyme I are not identical. *E. coli* enzyme I, for example, is completely protected against proteolysis when in the dimeric form. Under the same conditions SF-E is almost completely inactivated after 2 min of incubation (Figure 5B,C).

Summarizing, we may say that the *R. sphaeroides* PTS deviates much less from the *E. coli* PTS than was originally expected (Saier et al., 1971). It is composed of a tightly associated, possibly covalently linked, E_I and HPr-type molecule as well as a membrane-bound E_{II}. The major difference with respect to the *E. coli* PTS is that the *R. sphaeroides* PTS is membrane associated. It may even exist as a ternary E_I–H(F)Pr–E_{II} complex.

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