Cell-free Synthesis of Catalytically Active Fructokinase Directed by RNA from *Streptomyces violaceoruber* Grown with Fructose

By MERCEDES MARTIN AND BARTOLOME SABATER*

Department of Plant Physiology, Faculty of Sciences, University of Alcalá de Henares,
Apdo 20, Alcalá de Henares, Madrid, Spain

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Synthesis of fructokinase of *Streptomyces violaceoruber* has been obtained with a preincubated S-30 fraction from *Escherichia coli* and RNA from *S. violaceoruber*. The fructokinase formed was identified by its exceptional catalytic activity at pH 10 and by the similar specificity properties of the native enzyme and the enzyme synthesized in vitro. Its presence was confirmed among the radioactive products of protein synthesis by its transition of molecular weight from 20000 to 80000 in the presence of MgATP and by electrophoresis of purified preparations. The presumptive mRNA of fructokinase contained poly(A) sequences of variable length. Fructokinase mRNA was present in *S. violaceoruber* when grown with fructose but not with glucose, indicating that the induction of fructokinase is controlled at the level of transcription.

INTRODUCTION

In *Streptomyces*, fructose is phosphorylated at the C-6 position by the action of an inducible and highly specific fructokinase (EC 2.7.1.4) (Sabater *et al.*, 1972), whose monomers (mol. wt about 20000) become a tetramer in the presence of MgATP (Sabater & DeLa Fuente, 1975). Under appropriate conditions, *Streptomyces violaceoruber* carries out massive synthesis of fructokinase (Sabater, 1973) which is probably preceded by an accumulation of its mRNA. To study the mechanism of the induction of fructokinase by fructose, we assayed an in vitro system for fructokinase synthesis with an S-30 fraction from *Escherichia coli* and RNA prepared from *S. violaceoruber*. Fructokinase synthesis was detected by the increase of catalytic activity. It was characterized by the similar behaviour both in molecular weight transition and in disc electrophoresis of native fructokinase and of a radioactive product of the protein synthesis.

METHODS

Culture of the micro-organisms, RNA extraction and S-30 fraction preparation. *Streptomyces violaceoruber* was grown as described previously (Sabater & Asensio, 1973). RNA was extracted by the method of Dohan *et al.* (1971) except that lysozyme (EC 3.2.1.17) and incubation at 42 °C were omitted, the rupture of the cells and the phenol extraction being effected in a Sorvall Omni-mixer (setting 5 for 4 min). Samples of the RNA preparation in sterile distilled water (about 1.5 mg ml⁻¹) were stored at -30 °C. About 0.6 mg RNA was obtained from 1 g fresh mycelium. The culture of *E. coli* and the preparation of the S-30 fraction was carried out according to Dohan *et al.* (1971).

Millipore and poly(U) binding assays of RNA. RNA fractions adsorbing to Millipore filters were prepared from crude RNA by Brawerman's method (1974). Poly(U)-Sephadex binding assays of RNA were carried out at 0–5 °C on a column (0.9 × 1.4 cm) in the presence of concentrated salt buffer (0.7 M-NaCl, 50 mM-Tris/HCl, 10 mM-Na₂EDTA, pH 7.5). Adsorbing RNA was eluted at 25 °C with sterile distilled water.

In vitro protein synthesis. Incubations were as described by Dohan *et al.* (1971) except that the S-30 fraction (200 µl) was preincubated at 37 °C with 25.2 µmol Tris/HCl pH 7.8, 1.5 µmol KCl, 20 µmol NH₄Cl and 2.4

μmol mercaptoethanol (final vol. 220 μl). Protein synthesis was stopped by addition of chloramphenicol (0.5 mg ml^{-1} final concentration) and cooling in ice-water. Total protein synthesized with radioactive amino acids was measured according to Dohan *et al.* (1971). Fructokinase activity formed in 0.4 ml of the protein synthesis mixture without radioactive amino acids was assayed by coupling to NADP reduction as described previously (Sabater & DeLa Fuente, 1975), but 1 unit of each auxiliary enzyme was used and the reaction was carried out in 50 mM-glycine/KOH buffer pH 10.0, in which fructokinase is fully active (Sabater *et al.*, 1972) with only minor interference from other activities. Under appropriate conditions, accurate differences of fructokinase activity of 0.2×10^{-3} units could be detected.

Molecular weight transition and polyacrylamide gel electrophoresis. The detection of a molecular weight transition in some radioactive product of the protein synthesis was done by ultracentrifugation in a glycerol gradient in the presence of different effectors (Sabater & DeLa Fuente, 1975). Polyacrylamide gel electrophoresis of the products of protein synthesis was as previously described (Sabater & DeLa Fuente, 1975).

Enzyme activities and other determinations. Bovine pancreas ribonuclease (EC 3.1.27.5) (Sigma type I-A) activity was measured by ultraviolet absorption of the acid-soluble digestion products from *Torula* RNA (Calbiochem) as described for ribonuclease T_1 (EC 3.1.27.3) (Uchida & Egami, 1968). Beef pancreas deoxyribonuclease I (EC 3.1.21.1) (Sigma) activity was measured according to the instructions of the supplier by absorbance increase at 260 nm during hydrolysis of salmon sperm DNA (Calbiochem). Horseradish peroxidase (EC 1.11.1.7) (Calbiochem) activity was measured by continuous recording of absorbance changes at 470 nm due to guaiacol oxidation with hydrogen peroxide (Chance & Maehly, 1955), in 3 ml cuvettes with 40 mM-hydrogen peroxide. One unit of enzyme activity is defined as the amount that transforms 1 μmol of substrate in 1 min.

Cytochrome *c* was determined by absorbance at 407 and 524 nm. Proteins were estimated by absorbance at 260 and 280 nm (Kalckar, 1947). RNA was estimated by absorbance at 260 nm and by the orcinol method (Habermann, 1961).

RESULTS

From a low initial value [$0.008 \text{ units (mg protein)}^{-1}$], fructokinase activity increased to about $0.12 \text{ units (mg protein)}^{-1}$ between 64 and 75 h growth of *S. violaceoruber*. RNA extracted from *S. violaceoruber* after 68 h growth was used in assays of its ability to stimulate fructokinase synthesis with an S-30 fraction from *E. coli*.

Total protein synthesis with an S-30 fraction from E. coli: effect of addition of RNA from S. violaceoruber

The addition of RNA from *S. violaceoruber* stimulated about fivefold the synthesis of proteins by a 10 min preincubated S-30 fraction from *E. coli* (Fig. 1). The protein synthesis stimulated by *S. violaceoruber* RNA depended on Mg^{2+} , the optimal concentration being about 14 mM, as in other systems of protein synthesis (Halbreich, 1979). It was estimated that 3.5–4 nmol amino acids were incorporated into proteins under the optimal conditions. Chloramphenicol (0.5 mg ml^{-1}) inhibited about 90–95% of the synthesis of protein under the conditions shown in Fig. 1. In subsequent assays of protein synthesis we used 10 min preincubation of the S-30 fraction, 20 min incubation for protein synthesis (see below), 14 mM- Mg^{2+} and 1.5 μg Millipore-purified RNA from *S. violaceoruber* (see below). Similar results were obtained when 0.1 mg crude RNA from *S. violaceoruber* was used.

Measurement of catalytic activity of fructokinase synthesized

At pH 10, where fructokinase is fully active (Sabater *et al.*, 1972), only very low fructose-phosphorylating activity was detected in the S-30 fraction from *E. coli*. Table 1 shows the fructose-phosphorylating activity measured after protein synthesis under different conditions. Without RNA from *S. violaceoruber* a basal fructose-phosphorylating activity was observed (7.5×10^{-3} units in the experiment shown). A significant increase (8.3×10^{-3} units) of fructose-phosphorylating activity occurred when the incubation mixture contained RNA from *S. violaceoruber* grown in the presence of fructose. This increase depended on protein synthesis since it was strongly inhibited by chloramphenicol. The RNA from *S. violaceoruber* grown in the presence of glucose did not produce any increase of fructokinase

Fig. 1. Protein synthesis stimulated by RNA from *S. violaceoruber* in an S-30 fraction from *E. coli*. Protein synthesis assays were carried out with 0.05 µCi of uniformly ^{14}C -labelled amino acids: without preincubation of the S-30 fraction and without added RNA (○); after preincubation of the S-30 fraction and without added RNA (△); after preincubation of the S-30 fraction and with addition of 1.5 µg RNA from *S. violaceoruber* grown in the presence of fructose and partially purified by Millipore filtration (●).

Table 1. *Fructose-phosphorylating activity measured after protein synthesis assays*

Conditions of protein synthesis*	$10^3 \times$ Fructose-phosphorylating activity (units)		
	Standard assay for fructokinase	Fructokinase assay with 10 mM-mannose	Increase of fructokinase
I	7.5	3.8	—
II	15.8	12.3	8.3
III	8.1	4.3	0.6
IV	7.3	3.7	0

* I, Without RNA; II, with 1.5 µg Millipore-purified RNA from *S. violaceoruber* grown for 68 h with fructose; III, as II, plus 0.5 mg chloramphenicol ml^{-1} ; IV, with 1.5 µg Millipore-purified RNA from *S. violaceoruber* grown for 68 h with glucose. S-30 fractions were always preincubated for 10 min at 37°C. A zero-time control gave a similar result to that of condition I.

activity. The basal fructose-phosphorylating activity, measured after protein synthesis without addition of RNA to the incubation mixture, was also observed in the S-30 fraction alone; it seems to be due to the mannofructokinase (EC 2.7.1.7) of *E. coli* (Sabastián & Asensio, 1972), which at pH 10 shows a very low activity and is inhibited by mannose. Its value, from the control assays of protein synthesis without RNA, was subtracted from the values for the systems with added RNA to obtain the values for fructokinase synthesized. The increase in activity after protein synthesis with RNA from *S. violaceoruber* was not inhibited by mannose to a detectable extent (Table 1). This agrees with the known high specificity for sugar of *S. violaceoruber* fructokinase (Sabater *et al.*, 1972). Because of the properties of specificity, and since RNA from *S. violaceoruber* grown in glucose did not cause such an increase, it seems that the increase of phosphorylating activity after protein synthesis is due to fructokinase of *S. violaceoruber*.

A specific activity of 300 units $(\text{mg protein})^{-1}$ may be assumed for pure fructokinase (Sabater & DeLa Fuente, 1975). This means that the 8.3×10^{-3} units synthesized *in vitro* would require the incorporation of about 0.25 nmol amino acids in fructokinase, which represents almost 7% of total amino acids incorporated in the newly synthesized proteins.

After 1.5 min of incubation for protein synthesis no newly formed fructokinase activity was detectable, but after 3 min there was substantial fructokinase synthesis (Fig. 2*a*). The

Fig. 2. Effect of the incubation time and of the amount of RNA on fructokinase synthesis. (a) Fructokinase synthesis was carried out with 1.5 μg Millipore-purified RNA from *S. violaceoruber* grown with fructose and the incubation time was varied as indicated. (b) The amount of RNA, either as obtained by the phenol method (O) or after further purification by Millipore filtration (●), was varied as indicated; the incubation time was 20 min.

maximum fructokinase activity was reached at 20 min of incubation. The effects of the amount of both crude RNA and Millipore-purified RNA (see below) on fructokinase synthesis are shown in Fig. 2(b). Both RNA preparations showed an optimal concentration for the stimulation of fructokinase synthesis.

Effects of RNAase and DNAase treatments of RNA on its template activity for fructokinase synthesis

In these assays, 1.5 μg Millipore-purified RNA from *S. violaceoruber* grown in fructose was used for protein synthesis either without previous treatment or after enzyme treatment for 10 min at 30 °C in a total volume of 0.1 ml, with 2 μmol Tris/HCl pH 7.5 and with 4 μg bovine pancreas RNAase Type I-A or beef pancreas DNAase I. While DNAase treatment had no significant effect on the amount of fructokinase formed after protein synthesis, 10 min treatment with RNAase almost entirely suppressed the ability of the RNA preparation to direct the formation of fructokinase. Appropriate controls indicated that the Tris/HCl buffer used had no significant effect on fructokinase synthesis or measurement. Thus, it seems that the ability of RNA preparations to stimulate fructokinase synthesis is due to some RNA component sensitive to bovine pancreas RNAase, corresponding to mRNA of fructokinase.

Molecular weight transition properties of the protein synthesized in vitro

The mixture of radioactive proteins synthesized in vitro by preincubated S-30 fraction and *S. violaceoruber* RNA was ultracentrifuged in a glycerol gradient to see if some of the new proteins exhibited, like fructokinase (Sabater & DeLa Fuente, 1975), the transition of molecular weight from 20000 to 80000 in the presence of MgATP. Figure 3 shows the profile of radioactivity of the different fractions obtained after ultracentrifugation with and without 10 mM-MgATP, and the profile of fructokinase activity resulting from ultracentrifugation of partially purified fructokinase (Sabater & DeLa Fuente, 1975) in the presence of 10 mM-MgATP. Other markers used, but not included in Fig. 3, were: cytochrome *c* (mol. wt 12500); partially purified fructokinase in the presence of 10 mM-fructose and 10 mM-MgCl₂.

Fig. 3. Ultracentrifugation in a glycerol gradient of the products of protein synthesis and of native fructokinase. Protein synthesis (in 0.4 ml) was carried out with 5 μ Ci of uniformly ^{14} C-labelled amino acids after 10 min preincubation of the S-30 fraction. After 20 min incubation, 0.2 mg chloramphenicol was added and the mixture was quickly cooled and added to 5 ml of swollen Sephadex G-25 (previously centrifuged to remove excess water). After 15 min at 0–5 °C this latter preparation was centrifuged at 5000 g for 10 min. The supernatant was centrifuged for 1 h at 110000 g to obtain a new supernatant without polysomes. To 120 μ l fractions of this supernatant, 7.5 μ mol imidazole pH 7.5 and different effectors were added making a final volume of 150 μ l. The mixtures were layered on tubes containing 14.4 ml of a 5–25% linear glycerol gradient with imidazole and effectors as in the samples. After ultracentrifugation for 17 h at 32000 rev. min $^{-1}$ (180000 g at the bottom) in an SW36 rotor, fractions of 0.58 ml were collected in vials with 5 ml of scintillation fluid [120 g naphthalene, 16 g 2,5-diphenyloxazole (PPO), 0.4 g 1,4-di-2(5-phenyloxazoly)benzene (POPOP), 200 ml methanol, 40 ml ethylene glycol and dioxane to 1 l]. Radioactivity was measured with more than 95% efficiency. Ultracentrifugation in the presence of 10 mM-MgATP (●) or without MgATP (○). Two units of partially purified native fructokinase (Sabater & DeLa Fuente, 1975) were ultracentrifuged in the presence of 10 mM-MgATP (△).

(mol. wt 20000); and peroxidase (mol. wt 42000). They showed maxima in the fractions 19, 18 and 17, respectively.

When the products of protein synthesis with RNA from *S. violaceoruber* grown in fructose were ultracentrifuged in the presence of 10 mM-MgATP, a clear maximum of radioactivity was obtained in fraction 15, the same fraction that showed a maximum of the non-radioactive partially purified fructokinase when ultracentrifuged in the presence of 10 mM-MgATP. The values of radioactivity decreased for lower fractions, but were still clearly higher than the radioactivities in the corresponding fractions of the control (consisting of synthesized radioactive proteins ultracentrifuged in the absence of MgATP). The high radioactivity of the upper fractions was presumably due mostly to diffusion of radioactive amino acids not incorporated in proteins and not totally retained in Sephadex G-25. This interference was too large to allow the detection of a peak of radioactivity for the monomer of synthesized fructokinase (presumably between fractions 18 and 19) in the control ultracentrifuged without MgATP. However, it may be significant that fractions 16 and higher of the ultracentrifugation in the absence of MgATP showed higher radioactivity than the corresponding fractions of the ultracentrifugation in the presence of MgATP. The opposite was true for fractions lower than 16.

Other controls performed, but not shown in Fig. 3, were ultracentrifugation in the presence of 10 mM-fructose plus 10 mM-MgCl $_2$ of the products of protein synthesis with RNA from *S. violaceoruber* grown in fructose, and ultracentrifugation in the presence of MgATP of the proteins synthesized without RNA from *S. violaceoruber* and with RNA from *S. violaceoruber* grown in glucose. These all gave results similar to that of the control represented in Fig. 3.

Fig. 4. Polyacrylamide gel electrophoresis of the peak of radioactivity obtained by ultracentrifugation. Fractions 12 to 16 from three ultracentrifugations in the presence of MgATP as in Fig. 3 were pooled and treated with 1 vol. 10% (w/v) trichloroacetic acid. After 1 h the mixture was centrifuged 10 min at 10000 g and the precipitate was resuspended in 0.15 ml of electrophoresis buffer. Parallel electrophoresis of this preparation and of partially purified native fructokinase were carried out as described previously (Sabater & DeLa Fuente, 1975). From two parallel runs of native fructokinase, proteins were recorded after staining of one column and fructokinase activities from 2 mm slices of the other column were measured after elution (Sabater & DeLa Fuente, 1975). The 2 mm slices from the column run with radioactive ultracentrifuged material were treated with 1 ml NCS (Amersham) for 2 h at 65 °C, then with 0.2 ml 30% H_2O_2 for 15 min at 65 °C, and finally with scintillation fluid as in the legend to Fig. 3.

Further characterization as fructokinase of the peak of radioactivity obtained by ultracentrifugation

The proteins of the fractions of the peak of radioactivity obtained by ultracentrifugation (Fig. 3) were precipitated with trichloroacetic acid and separated by polyacrylamide gel electrophoresis. As shown in Fig. 4, most of the radioactivity from the ultracentrifugation peak migrated in gel electrophoresis like the native fructokinase which was the protein of fastest mobility in the partially purified preparation of fructokinase. From the shape of the radioactivity histogram after electrophoresis, it is probable that in the *in vitro* synthesis we obtained more than one form of fructokinase with very similar electrophoretic mobilities.

Millipore and poly(U) binding properties of mRNA of fructokinase

About 1.5% of RNA from the crude preparation was found to be bound by a Millipore filter. This was then eluted with 0.5% (w/v) sodium dodecyl sulphate in 0.1 M-Tris/HCl pH 9 (Brawerman, 1974). However, as shown in Fig. 2(b), this low amount of RNA able to bind to the Millipore filter stimulated fructokinase synthesis in the S-30 fraction of *E. coli* more than the crude RNA did. Amounts up to 100 μg of the RNA that was not retained on the Millipore filter in buffer plus KCl (Brawerman, 1974) failed to support fructokinase synthesis. This indicated the probable presence of poly(A) segments in the mRNA of fructokinase of *Streptomyces*.

Moreover, Fig. 5 shows that although there was a substantial synthesis of fructokinase directed by RNA without apparent affinity for poly(U), there was also a fraction able to support fructokinase synthesis which could only be eluted from poly(U) columns with water at 25 °C, indicating that at least a part of the RNA able to support fructokinase synthesis had

Fig. 5. Fructokinase synthesis stimulating activity of different fractions obtained from poly(U)-treated RNA. Assays for protein synthesis were carried out with 100 μ l samples of the fractions collected by poly(U) binding assays in a column (0.9×1.4 cm) loaded with 50 μ g (in 0.5 ml buffer) of Millipore-purified RNA from *S. violaceoruber* grown with fructose. Fixation of RNA on poly(U)-Sephrose 4B was at $0-5^{\circ}\text{C}$ for 10 min. The column was then washed with 3 ml buffer (0.7 M-NaCl, 50 mM-Tris/HCl, 10 mM- Na_2EDTA , pH 7.5) at the same temperature. The temperature was then raised to 25°C and 5 ml sterile distilled water at 25°C was passed through the column. Fractions of 0.775 ml were collected.

substantial poly(A) sequences. As the RNA preparation was poured only once through the poly(U) column it is possible that the RNA without apparent affinity for poly(U) also contained poly(A) sequences.

DISCUSSION

The hot phenol method (Dohan *et al.*, 1971; Brawerman, 1974) used for preparing RNA from *S. violaceoruber* gave acceptable undegraded RNA preparations as judged by gel electrophoresis (unpublished results) and stimulation of protein synthesis. As *S. violaceoruber* is prokaryotic, its mRNA must be efficiently translated by the 70S ribosomes of *E. coli* (Halbreich *et al.*, 1975; Herrlich & Schweiger, 1978; Sano *et al.*, 1979). In bacterial systems (which are usually assayed with crude RNA preparations) an individual protein synthesized rarely uses more than 1% of the total amino acids incorporated in proteins. The high yield (some 7%) estimated for the synthesis of fructokinase may be due to a high proportion of the mRNA for fructokinase in *S. violaceoruber* at the moment selected for RNA extraction. This high yield is sufficient to measure the newly synthesized enzyme by its catalytic activity, a characteristic found only in a small number of protein synthesis systems (Labarca & Paigen, 1977; Cremer *et al.*, 1978; Pelham, 1978). When we consider the RNA prepared from 1 g *S. violaceoruber* mycelium, the *in vitro* system forms fructokinase at a rate 50% of that of the synthesis *in vivo*. This high yield must be interpreted with caution because *S. violaceoruber* was grown at 30°C and protein synthesis *in vitro* was carried out at 37°C .

As the increase of fructokinase after protein synthesis requires RNA from *S. violaceoruber* grown in fructose, the induction of fructokinase by fructose (Sabater *et al.*, 1972) is mediated by increased levels of its mRNA. In view of the behaviour in poly(U)-Sephrose (Fig. 5), the length of the poly(A) segments of the presumed mRNA for fructokinase seems variable, either because of partial degradation during RNA preparation or, more probably, due to some heterogeneity *in vivo* of its length, as described for globin mRNA (Merkel *et al.*, 1976). The presence of poly(A) sequences has been described in RNA of other bacteria (Ohta *et al.*, 1978; Sarkar *et al.*, 1978) and of plastids (Verdier, 1979). Some of these have also been translated on ribosomes from *E. coli* (Sano *et al.*, 1979).

If the maximum of radioactivity obtained in Fig. 3 corresponds to fructokinase synthesized *in vitro*, the difference in radioactivity between the two conditions represented in Fig. 3 for

fractions 15 and lower must correspond to the minimum amount of radioactivity incorporated into the synthesized fructokinase. When compared with the results represented in Fig. 1, this would mean that a minimum of 3.8% of the total amino acids incorporated into protein synthesized *de novo* was incorporated into fructokinase. Other ultracentrifugation experiments indicated minimum values of amino acids incorporated into fructokinase of up to 5% of total protein synthesized. This minimum estimate agrees with the 7% estimated by the increase of the catalytic activity of fructokinase after protein synthesis. The closeness of the values obtained for fructokinase synthesized when estimated by ultracentrifugation and by catalytic activity are very significant.

The parallel between the ultracentrifugation behaviour in the presence of MgATP of partially purified fructokinase and of some radioactive product after protein synthesis makes it likely that the peak of radioactivity corresponds to fructokinase. Its identity is further corroborated by its behaviour in polyacrylamide gel electrophoresis.

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