Proc. Natl. Acad. Sci. USA
Vol. 78, No. 11, pp. 6667–6669, November 1981
Biochemistry

Reversible unidirectional inhibition of sucrose synthase activity by disulfides

(cleavage suppression/oxidized thioredoxin/glutathione/enzyme oxidation)

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Communicated by Luis F. Leloir, July 8, 1981

ABSTRACT **Sucrose synthase (UDPglucose:D-fructose 2-α-D-glucosyltransferase, EC 2.4.1.13), which catalyzes the synthesis and cleavage of sucrose, exhibits differences in some properties between the two reactions. When enzyme previously incubated with oxidized glutathione or oxidized thioredoxin was used, sucrose cleavage was inhibited whereas sucrose synthesis proceeded at a normal rate. Sucrose cleavage activity could be restored by incubation with dithiothreitol or reduced glutathione. The thioredoxin effect was influenced by the presence of cleavage reaction substrates—i.e., sucrose and UDP. Thioredoxin action was rather slow compared with the catalytic reaction. These findings may have important implications for understanding the metabolic role of sucrose synthase and oxidized thioredoxin. Theoretically, the fact that an enzyme catalyzing a reversible reaction is inhibited in one direction only suggests that a modification in the enzyme affinities for its substrates must have occurred.**

Sucrose synthase (UDPglucose:D-fructose 2-α-D-glucosyltransferase, EC 2.4.1.13) catalyzes the synthesis and cleavage of sucrose according to the following reaction:

$$\text{UDP-Glc} + \text{fructose} \rightleftharpoons \text{UDP} + \text{sucrose}.$$

The reaction has some unusual characteristics that have prompted many studies. Thus, it is the only transglucosylation involving sugar nucleotides that is readily reversible. The enzyme exhibits a wide specificity for the nucleoside base, unlike most enzymes of sugar nucleotide metabolism that are specific for a particular base (1). Besides, the enzyme exhibits differences in cleavage and synthesis pH optima (2–5), buffers (2, 6), temperature (6), and trypsin action (7), as well as activation or inhibition by mercaptoethanol (5), metal ions (5, 8, 9), phenylglycosides (10), and ATP (9, 11).

Attempts to resolve the sucrose cleavage and synthetic activities or to separate the enzyme into different catalytically active units have not been successful (5, 12).

This paper presents evidence that indicates the existence of two forms of sucrose synthase: one oxidized, the other reduced. The oxidized form catalyzes sucrose synthesis almost exclusively, whereas the reduced form preferentially catalyzes cleavage. Both forms are interconvertible through oxidation–reduction reactions.

MATERIALS AND METHODS

All chemicals were purchased from Sigma. Sucrose synthase from wheat germ was obtained completely free of sucrose phosphate synthase activity, according to Salerno and Pontis (13), and was further purified by passage through a Bio-Gel A-1.5 m column. Chloroplast thioredoxin was a gift of R. A. Wolosiuk.

The forward reaction (sucrose synthesis) was assayed by incubating (total vol, 0.05 ml) 0.5 μmol of fructose/0.25 μmol of UDP-Glc/5 μmol of Tris·HCl, pH 8.0/enzyme and compounds to be tested at 30°C. The reaction was stopped by adding 0.2 ml of 0.5 M NaOH and heating in a water bath at 100°C for 10 min (14). The sucrose formed was determined by the thiobarbituric acid method in a total volume of 0.9 ml (15). The activity in the reverse direction (UDP-Glc and fructose formation) was assayed by incubating (total vol, 0.05 ml) 10 μmol of sucrose/0.25 μmol of UDP/5 μmol of Hepes, pH 6.5/enzyme and compounds to be tested at 30°C. The reaction was stopped by adding 0.2 ml of Tris·HCl, pH 8.7, and heating in a water bath at 100°C for 1 min. The UDP-Glc formed was determined with UDP-Glc dehydrogenase by following the reduction of NAD spectrophotometrically (1). The fructose formed was measured by coupling hexokinase, phosphoglucose isomerase, and glucose-6-phosphate dehydrogenase and following the appearance of NADPH spectrophotometrically (1). In this case, the reaction was stopped by adding 0.2 ml of Tris·HCl, pH 8.0, and heating in a water bath at 100°C for 1 min. When any of the substrates was present in a preincubation mixture, an equal amount of that substrate was subtracted from the total amount needed in the incubation mixture used to measure sucrose synthase activity.

Activity measurements were carried out under conditions in which velocity was linear with time and amount of enzyme.

One unit of enzyme (synthesis direction) is defined as the amount that catalyzes the formation of 1 nmol of sucrose/min at pH 8. One unit of enzyme (cleavage direction) is defined as the amount that catalyzes the formation of 1 nmol of fructose or UDP-Glc/min at pH 6.5.

RESULTS

In the course of studies on sucrose synthase, it was found that the enzyme was losing its cleavage activity on standing at 4°C while the synthetic activity remained unaffected.

These observations prompted a study of the effect of mild oxidants on the enzyme activity. Glutathione (GSSG) was tried first. The results presented in Table 1 show the effect of incubating sucrose synthase with GSSG. The enzyme was also incubated in the presence of a reductant, the nonphysiological sulfhydryl reagent dithiothreitol.

When sucrose synthase was incubated in the presence of GSSG (experiment 1A), its ability to catalyze sucrose cleavage was severely impaired while sucrose synthesis still proceeded normally.

On the other hand, enzyme treated with dithiothreitol exhibited high values for cleavage (experiment 1B). This situation

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Abbreviations: GSSG and GSH, glutathione and reduced glutathione, respectively.

Table 1. Effect of GSSG and dithiothreitol on sucrose synthase activity

Exp.	Addition(s)	Cleavage, nmol of fructose formed/10 min	Synthesis, nmol of sucrose formed/10 min	Cleavage/ synthesis ratio
1A	GSSG	3	10	0.3
1B	Dithiothreitol	26	18	1.4
2A	GSSG, then dithiothreitol	26	17	1.5
2B	Dithiothreitol, then GSSG	8	12	0.6

In experiment 1, the enzyme was incubated for 1 hr at 30°C prior to activity determination. Enzyme solutions (0.8 ml) contained 8.4 mg of protein in 25 mM Tris·HCl, pH 7.9/10 mM dithiothreitol or GSSG as indicated. In experiment 2, the enzyme solutions used in experiment 1 were diluted in 25 mM Tris·HCl, pH 7.9/12 mM dithiothreitol (experiment 2A) or GSSG (experiment 2B). The concentrations of GSSG and dithiothreitol carried over from the original solution (1 A and B, respectively) became 2 mM after dilution. Enzyme solutions were again incubated for 1 hr at 30°C prior to activity determination. Enzyme activities were measured by taking the proper volume of solution so the total amount of protein per assay was the same.

was reflected by a modification of the cleavage/synthesis ratio. Enzyme solutions previously used in experiment 1 were diluted in buffer containing either dithiothreitol (experiment 2A) or GSSG (experiment 2B). The enzyme previously oxidized with GSSG recovered the activity for cleavage in the presence of dithiothreitol (Table 1). The cleavage/synthesis ratio changed from 0.3 to 1.5. In contrast, enzyme treated first with dithiothreitol and then with GSSG lost most of its ability to cleave sucrose. As dithiothreitol is a nonphysiological reagent, other reducing substances normally present in the cell were tested. Reduced glutathione (GSH) affected sucrose synthase in the same way as dithiothreitol; i.e., it enhanced cleavage activity (Table 2). On the other hand, ascorbic acid decreased cleavage and synthetic activities equally, producing no change in the cleavage/synthesis ratio.

The fact that GSSG and GSH modified the enzyme activity pointed to a thio–disulfide exchange. In view of this possibility, attention was given to thioredoxin, a dithiol protein whose reduced form activates various enzymes present in chloroplasts (16). The effect of oxidized thioredoxin on sucrose synthase activity was therefore tested. It was found that oxidized thioredoxin from spinach chloroplasts affects sucrose synthase. The rate of inactivation of cleavage activity by thioredoxin is relatively slow and dependent on the concentration of thioredoxin (Fig. 1).

Table 2. Effect of reducing agents on sucrose synthase activity

Addition	Cleavage, nmol of fructose formed/10 min	Synthesis, nmol of sucrose formed/10 min	Cleavage/ synthesis ratio
None (control)	13	12	1.1
GSH, 10 mM	20	11	1.8
Ascorbic acid, 10 mM	6	6	1.0

Sucrose synthase (30 μg) was incubated for 30 min at 30°C in 0.05 ml of 100 mM Hepes, pH 6.5 (cleavage), or 100 mM Tris·HCl, pH 8 (synthesis), containing GSH or ascorbic acid as indicated. The mixture was then completed with the proper substrates for measuring cleavage or synthesis, and the activities were determined after incubation at 30°C for 10 min.

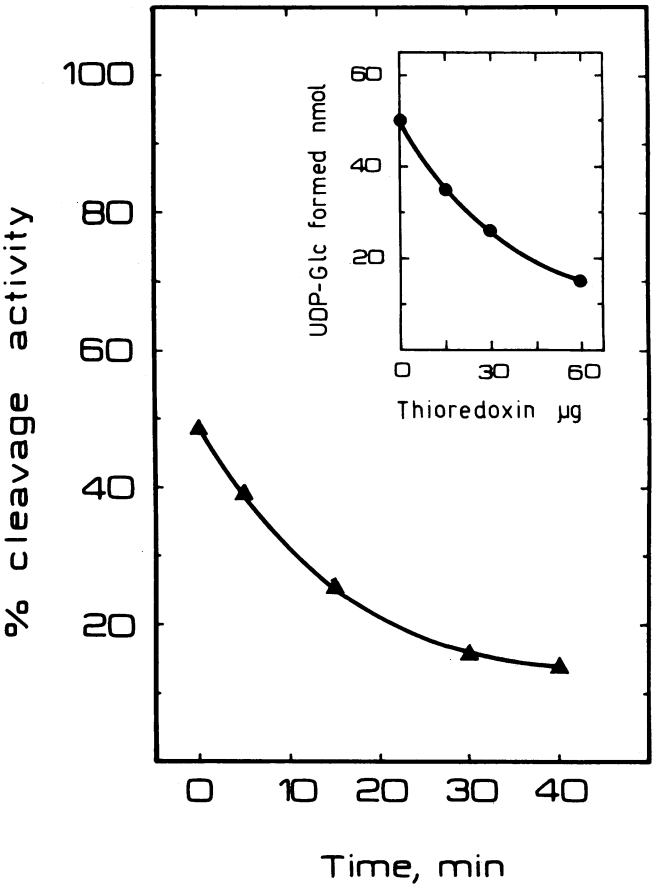

FIG. 1. Variation of cleavage inhibition as a function of time of preincubation with 60 μg of thioredoxin. An activity of 100% corresponds to enzyme activity without thioredoxin. (*Inset*) Variation of cleavage inhibition as a function of amount of thioredoxin. Sucrose synthase, 20 μg per assay; preincubation time, 30 min.

Thioredoxin inhibits cleavage ≈70% without affecting sucrose synthesis (Table 3). The addition of sucrose during the first incubation period increases inhibition to 90%, while the addition of fructose affects synthesis to a lesser degree. These changes are seen more clearly in terms of the cleavage/synthesis ratio. Addition of thioredoxin produces a 3.4-fold change in the ratio while the presence of sucrose increases the ratio 6-fold. The same effect is obtained with UDP (data not shown).

Table 3. Effect of thioredoxin on cleavage and synthetic activities of sucrose synthase

Addition	Cleavage, nmol of UDP-Glc formed	Synthesis, nmol of sucrose formed	Cleavage/ synthesis ratio
None	33	14.0	2.4
Thioredoxin, 60 μg	10	13.8	0.7
Thioredoxin, 60 μg/ sucrose, 5 μmol	4	—	0.4
Thioredoxin, 60 μg/ fructose, 0.5 μmol	—	10	
Sucrose, 5 μmol	34	—	2.5
Fructose, 0.5 μmol	—	13.5	

Sucrose synthase (32 μg) was incubated for 30 min at 30°C in 0.05 ml of 100 mM Hepes, pH 6.5 (cleavage), or 100 mM Tris·HCl, pH 8 (synthesis), containing chloroplast thioredoxin, sucrose, or fructose as indicated. The mixture was then completed with the proper amount of substrates, and the activities were determined after incubation at 30°C for 10 min.

Table 4. Effect of thioredoxin on cleavage and synthetic activities of sucrose synthase at pH 7.0

Addition	Cleavage, nmol of fructose formed/10 min	Synthesis, nmol of sucrose formed/10 min	Cleavage/ synthesis ratio
None (control)	25	12	2.1
Thioredoxin	5	9	0.5

Sucrose synthase (32 μg) was incubated for 30 min at 30°C in 0.05 ml of 100 mM Hepes, pH 7.0, containing chloroplast thioredoxin as indicated. The mixture was then completed with the proper amount of substrates, and the activities were determined after incubation at 30°C for 10 min.

The data presented so far indicate that thiols modify the cleavage/synthesis ratio when both enzyme activities are measured at their optimum pH. However, it may be that modification of the enzyme by a thiol shifts the pH–activity curves for both cleavage and synthesis to lower activity at pH 6.5 but not at pH 8.0. To clear this point, the action of thioredoxin on the enzyme activities was measured at the same pH (7.0). Thioredoxin inhibited cleavage ≈80% while synthesis was reduced ≈27% (Table 4). These results indicate that the thiol effect on sucrose synthase activities is not due to a pH effect.

DISCUSSION

Previous work has shown that sucrose synthase is inhibited by *p*-chloromercuribenzoate (2, 17), but there was no evidence demonstrating direct participation of thiol groups in the reaction mechanism. The results presented here suggest that some thiol groups are involved in the catalytic process. Even more, thiol groups seem to be involved in maintaining a conformation state that facilitates the cleavage reaction.

We do not know which is the oxidant and which is the reductant that react with sucrose synthase in the cell. Although GSSG and GSH switch the enzyme from one state to the other, it does not necessarily follow that they are the natural agents for the oxidation–reduction reaction. The fact that thioredoxin also inhibits cleavage supports this view. Moreover, thioredoxin action is influenced by the presence of sucrose synthase substrates, which is not the case for GSSG. It should be taken into account that the thioredoxin used in this study was isolated from chloroplasts, and it has been shown that different thioredoxins react with different enzymes (18). So far, thioredoxins have been shown to act in their reduced form; the suppression of cleavage activity of sucrose synthase by the action of oxidized thioredoxin is an example of a thioredoxin acting in its oxidized state.

As far as we know, this is the first report of an enzyme that catalyzes a reversible reaction whose activity can be reversibly increased or decreased in one direction only. This is a finding of considerable theoretical and physiological interest. Theoretically, at equilibrium, the rates of the forward and the backward reactions should be equal. A change in the rate of one of the reactions would modify the equilibrium constant, which is invariable and determined by thermodynamic forces. Therefore, unidirectional changes must occur only under nonequilibrium conditions. These changes would require modifications in both the maximum velocity (V_{max}) and K_m for substrates and products. Frieden has discussed this case theoretically and pointed out that "the effect of a particular modifier need not be the same for the forward and reverse directions of a particular enzyme. Not only is it possible for the type of effect to differ, but it is apparent that in certain cases a modifier could enhance the activity in one direction and inhibit the enzyme in the reverse direction" (19).

It should be recalled that sucrose synthase previously treated with trypsin is unable to catalyze sucrose cleavage but still catalyzes synthesis. This tryptic enzyme not only had a decreased affinity for sucrose but also showed sigmoidal rather than hyperbolic saturation curves for sucrose (7).

The simplest interpretation of the data is that oxidation or reduction modifies the conformation of the active center. Moreover, the presence of separate centers on the enzyme for the synthesis and cleavage reactions is unlikely, based on the principle of microscopic reversibility.

The finding that sucrose synthase exists in different states poses the question of their metabolic roles. It is generally believed that the physiological role of sucrose synthase is to cleave sucrose. However, in view of the present results, this conclusion must be reconsidered; it may be that, depending on the conditions, the enzyme can catalyze either cleavage or synthesis. This view is supported by the data of Pontis and Wolosiuk (7), which show that sucrose synthase has a different cleavage/synthesis ratio after isolation from plants under very different physiological situations.

Preliminary work on the isolation and characterization of a thioredoxin-like protein from wheat germ that causes the oxidation and reduction of sucrose synthase suggests that, in wheat seeds and in wheat germ, at least two proteins that modify sucrose synthase cleavage activity are present.

We are indebted to the Instituto de Investigaciones Bioquímicas, Fundación Campomar, for its hospitality. The technical assistance of Miss Clara Fernández is gratefully acknowledged. This investigation was supported by the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires (Grant 2109 7202/78). H.G.P. is a Career Investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina, and J.R.B. is a Fellow of the Comisión de Investigaciones Científicas de la Provincia de Buenos Aires, Argentina.

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