Plant Physiol. (1978) 61, 35-37

Proteolytic Activation of a Galactosyl Transferase Involved in Osmotic Regulation[1]

Received for publication July 19, 1977 and in revised form September 13, 1977

HEINRICH KAUSS, KLAUS S. THOMSON, MARTA TETOUR, AND WOLFGANG JEBLICK
University of Kaiserslautern, Department of Biology, Postfach 3049, 6750 Kaiserslautern, GFR

ABSTRACT

Osmotic regulation in the flagellate *Ochromonas malhamensis* Pringsheim is mainly mediated by changes in the pool size of α-galactosyl-(1 → 1)-glycerol (isofloridoside). Isofloridoside phosphate synthase, a regulated key enzyme responsible for the formation of isofloridoside phosphate, appears to exist as an inactive proenzyme which can be activated by incubation of crude cell extracts with endogenous or exogenous proteases.

The osmotic situation of any plant cell plays a major role in its physiological network (4, 6). However, the biochemistry of osmotic regulation is only vaguely understood. In *Ochromonas* osmotic regulation occurs mainly by formation and degradation of IF[2] (5, 6). This system offers the opportunity to study the mechanism of osmotic regulation at the enzyme level. One of the regulated enzymes of IF metabolism is a galactosyl transferase (8, 11). It was recently observed (7) that the activity of this enzyme in crude extracts is strongly enhanced by a reaction that is time-, temperature-, and pH-dependent, suggesting a chemical modification of the enzyme occurs that is due to the action of another enzyme.

The experiments reported here demonstrate that limited proteolysis of an inactive proenzyme is the most likely explanation for the activation process observed *in vitro*.

MATERIALS AND METHODS

The culture of *Ochromonas malhamensis* Pringsheim (syn. *Poterioochromonas malhamensis* PETERFI) (12) and preparation of suspensions have been described previously (7). The buffer (pH 7.8) in which cellular disintegration was performed (7) contained 33 mM tris-maleate, 1 mM Na_2-EDTA, 3.3 mM β-mercaptoethanol, and 5 mg/ml of BSA. The suspension was transferred quickly into a cooled cylinder of a Yeda press and homogenized under 100 atm of N_2. Cell debris was removed by centrifugation for 5 min at 50,000*g* and 4 C in a Sorvall RC 2-B centrifuge. Samples of the supernatant crude extract were adjusted with 1 M maleic acid or 2 N NaOH to the desired pH values as indicated in the legends. The assay mixture for the IFP-synthase contained 25 μl of 0.1 M HEPES-NaOH (pH 7.5) containing 5 mg/ml of BSA, 5 μl of 5 mM UDP-galactose (Boehringer-Mannheim), 10 μl of sn-U[^{14}C]glycerol-3-P (56,000 cpm, 0.32 mM, New England Nuclear Corp., diluted with the respective [^{12}C]substance obtained from Sigma). The reaction was initiated by the addition of 10 μl of extract. After 1 min at 25 C, 30 μl of cold 10% (w/v) trichloroacetic acid was added and the tube centrifuged for 2 min at 12,000*g* in an Eppendorf centrifuge.

For separation of the [^{14}C]glycerol-P and [^{14}C]IFP a 40-μl aliquot of the reaction mixture was applied to 1.5-cm strips (prepared by cutting 0.3- × 40-cm intersections into a sheet of Whatman 3MM paper) which were wetted with buffer (pyridine-acetic acid-water, 10:100:890, v/v, pH 3.6). Electrophoresis was performed at 4 kv in a Savant apparatus until a reference spot of picric acid had run about 25 cm. Under these conditions [^{14}C]glycerol-P is positioned slightly behind picric acid and about 7.5 cm ahead of [^{14}C]IFP, as monitored for two strips out of each sheet with a paper strip scanner. The amount of [^{14}C]IFP formed was counted directly using a liquid scintillation cocktail which contains 5 g of 2,5-diphenyloxazole/liter of toluene.

Most materials were as described before (7). Crystallized and lypophilized BSA (Sigma) was used in most experiments but recently was replaced by fraction V BSA (Sigma) with identical results. Trypsin was purchased from Boehringer (Mannheim), chymotrypsin from Serva (Heidelberg) and soybean trypsin inhibitor from Sigma.

RESULTS AND DISCUSSION

IFP-synthase activity was found to be elicited in crude extracts of *Ochromonas* by a reaction dependent on time, temperature, and pH (optimum pH 6.2) and to disappear again by reactions also dependent on time and temperature but optimal at pH values lower (5) or higher (8.6) than the optimal activation pH. We thought that this might indicate an activation mechanism involving chemical modification of the enzyme (7). When the extracts were first activated at pH 6.2 followed by inactivation at pH 8.6 there was no subsequent reactivation possible. These preliminary experiments (data not shown) also indicated that activation might not be caused by a reversible aggregation of subunits but suggested instead that indeed a permanent chemical modification might take place during the activation and deactivation processes.

Many examples of enzyme regulation by chemical modification have been demonstrated in recent years in animal or bacterial systems (3). One type employs the enzymatic transfer of a modifying group (*e.g.* phosphate, AMP, ADP-ribose) as a means of enforcing the active conformation of an enzyme. The operation of such a mechanism in the crude extracts of *Ochromonas* would require the presence of nucleotide coenzymes. However, after dialysis of the cell extract against 33 mM tris-maleate, mM EDTA-Na_2, 3.3 mM β-mercaptoethanol, the activation process at pH 6.2 still occurred to some extent, although a 30 to 60% decrease of the maximal values resulted after 5 or 19 hr dialysis, respectively. These observations render a group transfer mechanism unlikely.

An alternative type of enzyme activation by chemical modifi-

[1] This work was supported by the Deutsche Forschungsgemeinschaft.

[2] Abbreviations: IF: isofloridoside or α-galactosyl-(1 → 1)-glycerol; IFP: α-galactosyl-(1 → 1)-glycerol-3-phosphoric acid; IFP-synthase: UDP-galactose:sn-glycerol-3-phosphoric acid 1-α-galactosyl transferase.

cation involves peptide bond cleavage by specific proteases. Numerous physiological reactions including metabolic regulation are mediated by limited proteolysis (9, 10). The only plant systems known to involve such activation appear to be restricted to yeast (*e.g.* chitin synthase [1]; tryptophan synthase [2]).

The first observation which indicated that limited proteolysis might be activating the IFP-synthase in *Ochromonas* is demonstrated in Figure 1. When BSA was included in the buffer used during cell disintegration we observed several effects. The velocity of activation was slowed down and the inactivation which we always observed to some extent even at pH 6.2 (7) was now only significant after incubations of more than 60 min. This indicated that an endogenous protease with a pH optimum at 6.2 might be responsible for the activation which was slowed down when BSA was present as competing substrate. In addition, the activity found in crude extracts from unstressed cells prepared with BSA at pH 7.8 was by far lower (*e.g.* about one-fifth comparing the zero time values of Fig. 1 ref. 7, with the figures in the present report). Evidently most of the transferase activity observed in unstressed cells during previous studies (7, 8) was a result of proteolytic activation which to some extent is also taking place at pH 7.8 and 0 C during cell disintegration and extract handling (see also control of Fig. 2). This is in accordance with an observation (unpublished results) that a rather high but transient enzyme activity is found when disintegration is performed at pH 6.2 instead of 7.8. In such experiments subsequent activation *in vitro* is only by a factor of 2 to 3 but with maximal activities similar to those attained in parallel samples in which the cells were disintegrated at pH 7.8.

Further evidence for a proteolytic type of activation came from experiments using exogenous proteases and protease inhibitor (Table I). At pH 6.2 addition of high concentrations of soybean trypsin inhibitor partly diminished the activation process presumably effected by an endogenous protease (control). Addition of trypsin and chymotrypsin both elevated the initial rate (15 min). Longer incubation (60 min) with trypsin resulted in a rapid decrease of activity, whereas chymotrypsin appears to operate more specifically, leading to values which in most cases were higher than those gained by incubation at pH 6.2 without exogenous proteases.

The effect of exogenous proteases was even more pronounced when working in a pH range in which the presumed endogenous activating protease is inactive, namely at pH 7.8 (Fig. 2). In these experiments increasing amounts of proteases gave increasing activation rates. Chymotrypsin again resulted in more stable enzyme activity. At both pH values, however, the curves for chymotrypsin were also found to decrease at longer incubation times. Quite similar results were found at both pH 6.2 and 7.8 with extracts made in the absence of BSA. In the latter case the activating effects of trypsin and chymotrypsin could best be followed by incubation at 8 C instead of 25 C. The nature of the presumed endogenous protease responsible for the activation observed *in vitro* is still unknown. It appears not to be a serine-protease as in the presence of phenyl-methyl sulfonylfluoride (PMSF, 2 mg/ml to give a saturated solution) activation was still possible.

The suggestion that the IFP-synthase occurs in crude extracts of unstressed cells in an inactive form which *in vitro* can be rendered active by limited proteolysis may offer an entrance into the understanding of osmotic regulation in *Ochromonas*. In addition, it is of a practical consequence: all attempts to purify IFP-synthase in its active form were handicapped by the fact that its activity was lost even after partial purification. Preliminary experiments show that when crude cell extracts of pH 7.8 and containing BSA are kept at 0 C the presumable proenzyme was fully stable even over 20 hr, whereas activation was followed by rapid inactivation. This observation strongly resembles the studies with yeast in which metabolic enzymes were found to be especially vulnerable to proteolysis in their active form (2). We are now attempting to purify the inactive precursor of the IFP-synthase to understand further the biochemical events involved in osmotic regulation in *O. malhamensis*.

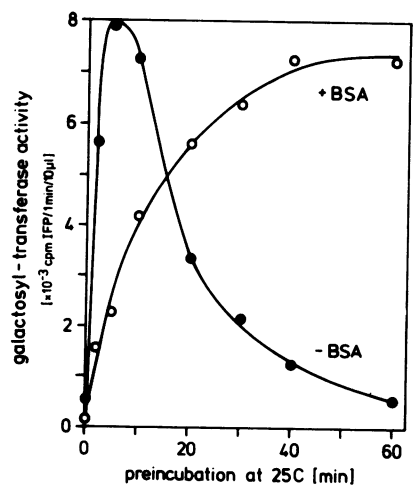

FIG. 1. Influence of BSA on the time course of IFP-synthase activation. Crude extract was brought to pH 6.2 and incubated at 25 C. Cell disintegration was performed in buffer with (○——○) or without (●——●) BSA.

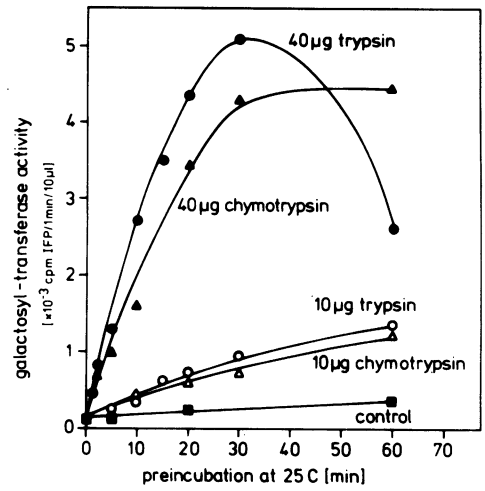

FIG. 2. Activation of the IFP-synthase at pH 7.8 by proteases. Crude extracts (400 µl) were incubated at 25 C and 100 µl of either buffer (control) or different proteases in buffer were added and samples taken to follow the increase in activity. Addition of 40 µg of trypsin inactivated by 200 µg of soybean trypsin inhibitor resulted in values similar to the control without trypsin.

Table I.

Activation of the IFP-synthase at pH 6.2 in the crude extract of Ochromonas as influenced by proteases and soy bean trypsin inhibitor.
Activation procedure was as in figure 2. The activity before incubation (zero time) was 190 [cpm IFP/min/10 µl extract].

additions	IFP-synthase activity after incubation for	
	15 min	60 min
	cpm IFP/min/10 µl	
none (control)	1564	4527
+ soy bean trypsin inh. (600 µg)	964	3050
+ trypsin (40 µg)	3219	635
+ chymotrypsin (40 µg)	4590	8419

LITERATURE CITED

1. CABIB E 1976 The yeast primary septum: a journey into three-dimensional biochemistry. Trends Biochem Sci 1: 275-277
2. HOLZER H 1976 Characteristics and functions of proteinases and proteinase inhibitors in yeast. *In* S Shaltiel, ed, Metabolic Interconversion of Enzymes 1975. Springer-Verlag, Berlin, pp 168-174
3. HOLZER H, W DUNTZE 1971 Metabolic regulation by chemical modification of enzymes. Annu Rev Biochem 40: 345-374
4. HSIAO TC 1973 Plant responses to water stress. Annu Rev Plant Physiol 24: 519-570
5. KAUSS H 1973 Turnover of galactosyl glycerol and osmotic balance in *Ochromonas*. Plant Physiol 52: 613-615
6. KAUSS H 1977 Biochemistry of osmotic regulation. *In* DH Northcote, ed, Internat Rev Biochem, Plant Biochem II, Vol 13. University Park Press, Baltimore, pp 119-140
7. KAUSS H, H QUADER 1976 *In vitro* activation of a galactosyl tranferase involved in the osmotic regulation of *Ochromonas*. Plant Physiol 58: 295-298
8. KAUSS H, B SCHOBERT 1971 First demonstration of UDP-Gal: sn-glycerol-3-phosphoric acid 1-α-galactosyl transferase and its possible role in osmoregulation. FEBS Lett 19:131-135
9. NEURATH H, KA WALSH, A GERTHER 1974 Initiation of physiological function by limited proteolysis. *In* EH Fischer, EG Krebs, H Neurath, ER Stadtman, eds, Metabolic Interconversion of Enzymes 1973. Springer-Verlag, Berlin, pp 301-312
10. PONTREMOLI S, E MELLONI, A DE FLORA, BL HORECKER. 1976 Specific proteolytic modification of rabbit liver fructose bisphophatase under gluconeogenic conditions. *In* S Shaltiel, ed, Metabolic Interconversion of Enzymes 1975. Springer-Verlag, Berlin, pp 175-184
11. QUADER H, H KAUSS 1975 Die Rolle einiger Zwischenstoffe des Galaktosylglyzerinstoffwechsels bei der Osmoregulation in *Ochromonas malhamenisis*. Planta 124: 61-66
12. SCHNEPF E, G DEICHGRÄBER, R RÖDER, W. HERTH 1977 The flagellar root apparatus, the microtubular system, and the associated organelles in the chrysophycean flagellate *Poterioochromonas malhamensis* PETERFI (syn *Poteriochromonas stipitata* Scherffel and *Ochromonas malhamensis* Pringsheim). Protoplasma 92: 87-107