FIRST DEMONSTRATION OF UDP-GAL: *sn*-GLYCERO-3-PHOSPHORIC ACID 1-α-GALACTOSYL-TRANSFERASE AND ITS POSSIBLE ROLE IN OSMOREGULATION

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1. Introduction

The osmotic balance in cells of the flagellated alga *Ochromonas malhamensis* is mediated by elements of carbohydrate metabolism. When any osmotic pressure raising substance like inorganic salts, glucose, mannitol or polyethyleneglycol are added to the nutrient solution a concentration of JF is built up rapidly inside the cell high enough for direct osmotic balance. Decreasing the external osmotic pressure results in a transformation of JF back to β-1-3-glucans [1–3]. This offers a simple model system to study biophysical and biochemical steps involved in the regulation of the osmotic balance as the biochemistry and regulation of carbohydrate metabolism seems fairly well understood.

Among the numerous enzymes presumably necessary in the above system only an AMP-stimulated β-1-3-glucan phosphorylase has been studied in some detail up to now [4, 5]. The isolation from Ochromonas cells of JF bearing a phosphate group in the glycerol part of the molecule led to the suggestion that JF may be synthesized by transfer of a galactosyl unit to glycerophosphate followed by dephosphorylation [6]. Experiments which demonstrate the existence of an enzyme responsible for this reaction will be reported in this communication. Some data will be presented, also, which indicate that the osmotic balance in Ochromonas may be regulated at the level of this enzyme.

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Abbreviations:
JF = isofloridoside = 0-α-D-galactopyranosyl-(1⟶ 1)-glycerol;
JFP = isofloridoside-phosphate
JFP-synthase = UDP-galactose: *sn*-glycero-3-phosphoric acid 1-α-galactosyl-transferase
Glyc-3-P = *sn*-glycero-3-phosphoric acid = L-α-glycerophosphate (for nomenclature see [8])
Glyc-1-P = *sn*-glycero-1-phosphoric acid = D-α-glycerophosphate
Glyc-2-P = *sn*-glycero-2-phosphoric acid = β-glycerophosphate
BSA = bovine serum albumin
DTE = dithioerythritol
HSA = human serum albumin
BSG = bovine serum γ-globulin

2. Materials and methods

2.1. *Extracts*

Ochromonas malhamensis was grown for 2 days as described before [1], the glucose content of the growth medium, however, was increased to 10 g/l. The cells were suspended in the respective fluid in a way that a 1:50 dilution of the suspension corresponded to an absorbance of 0.6–1.0 (1 cm cuvette, 510 nm, Beckman B). Extracts were prepared therefrom as described in the legends by freezing in a mixture of dry ice and ethanol or by sonication for 10 sec at 0°. Both methods resulted in the same transferase activity. Alternatively, the algae were sedimented by centrifugation, mixed with 50 μl toluene

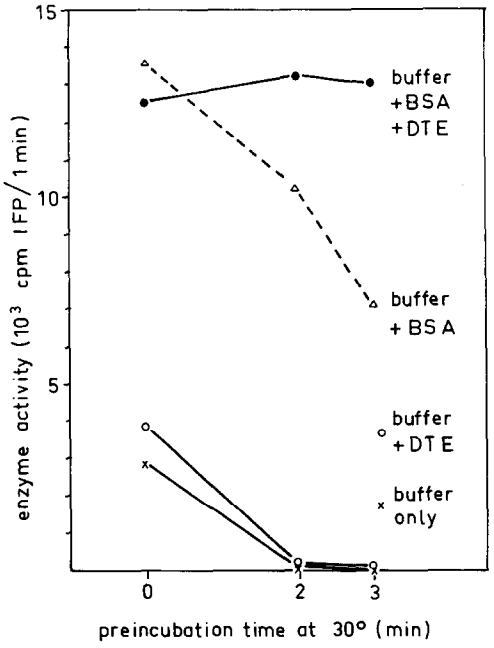

Fig. 1. Stability of the JFP-synthase at 30° in the crude extract as influenced by BSA and DTE. The algae were suspended in 0.1 M Na-pyrophosphate, pH 7.5, containing BSA (5%, w/v) and/or DTE (10^{-2} M) as indicated. Extracts were prepared by sonification and placed in a water bath at 30°, 5 µl aliquots were taken out after the indicated preincubation times and assayed for transferase activity for one additional minute as described in sect. 2.2.

for about 30 sec with a Vortex mixer and 1 ml of 0.1 M Na-pyrophosphate, pH 7.5, added per g algae (fresh weight).

2.2. *Assay*

The incubation mixture for the assay of the transferase contained 5 µl crude extract + 20 µl 0.1 M Na-pyrophosphate (pH 7.5, 0.15 M NaF, 2% BSA) + 1.6 µM UDP-Gal + 60,000 cpm ^{14}C-glycerophosphate (about 2.2×10^{-5} M) in a total volume of 40 µl. After incubation at 30° for the time indicated 30 µl of 10% TCA at 0° were added and the tube centrifuged for 10 min at 10,000 *g*.

A 50 µl aliquot of the supernatant was applied on paper strips of Whatman No. 1, wetted with buffer (water/pyridine/conc. acetic acid: 890/10/100, pH 3.6) and subjected to paper electrophoresis at 3,000 V until a marker spot of picric acid had run about 20 cm. The radioactivity was located with a F + H strip scanner and the area corresponding to JFP counted in 10 ml of 5 g PPO/l toluene in a Beckman LS 100 liquid scintillation system. The $R_{picrate}$ values were found to be about 1.2 for glyc-3-P and 0.9 for JFP. For each determination two identical assays were run and the results averaged.

2.3. *Materials*

Glyc-3-P was prepared from UL-^{14}C-glycerol (Amersham, 20 mCi/mmole) by incubation of the following mixture: 10^7 cpm ^{14}C-glycerol + 6 µM ATP + 50 µl glycine-buffer (pH 9.8, 0.2 M) + 10^{-2} M $MgCl_2$ + 50 µg glycerokinase (Boehringer & Söhne, 85 U/mg) in a total volume of 0.25 ml for 1 hr at 30°. The glyc-3-P was isolated by paper electrophoresis as described in sect. 2.2.

Part of the glyc-3-P was heated in 0.25 N HCl for 3 hr at 100° in a sealed tube to give by phosphate migration a mixture of glyc-3-P, glyc-2-P and glyc-1-P besides some free glycerol. The mixture was chromatographed for 3 days in n-butanol/pyridine/water/acetic acid (60/40/30/3) to separate glyc-2-P ($R_{glyc\text{-}3\text{-}P}$ = 1.25) from its 1- and 3-isomers which move together.

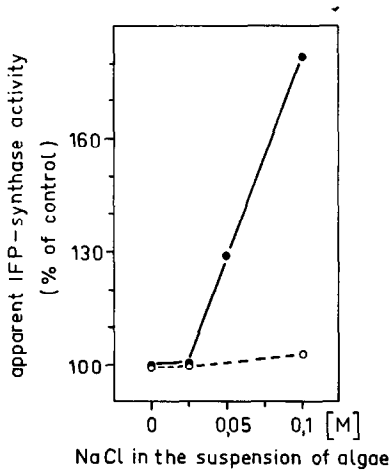

Fig. 2. Influence of osmotic treatment of the algae on apparent JFP-synthase activity (measured under non-optimal conditions). The algae were suspended in nutrient solution (dil. with 4 vol. water) containing the indicated concentration of NaCl. The cells were sedimented after 5 min, disintegrated with toluene and assayed as described in sect. 2.2 (solid line). In some of the experiments (broken line) the NaCl was added after the disintegration together with the Na-pyrophosphate buffer.

Table 1
Activity of the JFP-synthase in the crude extract as influenced by the addition of various proteins.

Protein added	IFP formed (cpm/min/5 μl extract)
– (control)	143
BSA (cryst., lyophil, Sigma)	3289
BSA (95%, Cohn Frac. V, Serva)	3310
HSA (98%, Serva)	3615
α-lactalalbumin (Koch-Light)	1900
egg albumin (90%, Serva)	1198
BSG (98%, Cohn Frac. II, Serva)	842

The algae were suspended in 0.1 M Na-pyrophosphate, pH 7.5. 0.2 ml of this suspension were added to 0.2 ml of the same buffer but containing 5×10^{-3} M DTE and 5% (w/v) of the different proteins. The mixtures were frozen to prepare crude extracts which were assayed as described in sect. 2.2.

Table 2
Substrate specifity for the formation of JFP by the crude extracts.

Substrates present		^{14}C-JFP formed/2 min/ 5 μl extract	
^{12}C (donor)	^{14}C (acceptor)	(cpm)	(%)
UDP-gal	Glyc-3-P	3257	100
UDP-gal	Glyc-2-P	1551	46
UDP-gal	Glyc-1-P	87	2
–	Glyc-3-P	23	0.7
Gal-1-P	Glyc-3-P	265	8
Gal	Glyc-3-P	179	5

Extracts were prepared as described in the legend of table 3 and assayed as described in sect. 2.2.

The latter mixture was incubated for 30 min at 25° with GDH to oxydize the glyc-3-P: 150 μl hydrazine–glycine-buffer (according to [7]) + 50 μl NAD (40 mg/ml) + 10 μl GDH (Boehringer & Söhne, 2 mg/ml, 40 U/mg) + 5×10^5 cpm of a mixture of labelled glyc-3-P and glyc-1-P in a total volume of 220 μl. The mixture was again chromatographed as above and the remaining glyc-1-P checked for purity by dephosphorylation [6] followed by chromatographic comparison with glycerol and dihydroxyacetone in the above mentioned solvent. All the ^{14}C-glycerophosphates were taken up in water to give 60,000 cpm/10 μl. All other materials were commercial preparations.

3. Results and discussion

When crude extracts of Ochromonas cells were incubated in the presence of NaF with labelled glyc-3-P and unlabelled UDP-gal a new radioactive substance was readily formed which moved slower than picric acid in paper electrophoresis. This product was identified as JFP by dephosphorylation followed by chromatography in different solvent systems as well as by acid and enzymic hydrolysis [6]. Under these conditions, however, the reaction rate was not found to be proportional to time and extract amount. As the aim of our studies was possibly to find correlations between the physiological state of the cells and enzyme activity, we had to work out conditions for the determination of enzyme activities in the crude extracts.

It was recognized that the nonlinearity of the assay was due to a rapid inactivation of the JFP-synthase in the crude extracts. Even at 0° the extract lost about half of its activity within 30 min and at 30° was almost inactive after some minutes (fig. 1). In preincubation experiments similar to those reported in fig. 1 we tried to stabilize the enzyme by addition of various substances such as diisopropylfluorophosphate, phenylmethylsulfonylfluoride EDTA, inorganic ions, NaF, SH-reagents and proteins.

Fully satisfying results were obtained only when a combination of DTE and BSA was present during the destruction of the cells (fig. 1). DTE can be replaced by high concentrations of β-mercaptoethanol (1%, v/v). Under this condition the activity was found also to remain constant on storage of the extracts at 3° for at least 1 hr. Although the different serum albumins brought best results, they could be replaced to some extent by other albumins or even γ-globulin (table 1). This suggests that the proteins may function in a relatively nonspecific way by protecting against protein inactivating enzymes.

Using the combination of BSA and DTA during the preparation of the extracts, we were able to determine exact enzyme activities as indicated by the

Table 3
Influence of osmotic treatment of algae on JFP-synthase activity measured under optimal conditions.

JFP-synthase activity (cpm JFP/min/5 μl algae)	samples withdrawn	
4195		before treatment
4163		
4257	2 min	after osmotic treatment
3954	6 min	
3973	10 min	
3883	30 min	

The algae were suspended in growth medium (diluted by addition of 2 vol. of water) and placed for 30 min under the conditions where they had been grown. Two 0.25 ml samples of algae were taken followed by addition of one volume of growth medium but containing 0.2 M sucrose. Subsequently samples were withdrawn at the times indicated. All samples were mixed with 0.25 ml of 0.1 M Na-pyrophosphate (pH 7.5, 5% BSA, 5×10^{-3} M DTE) and frozen. Assay methods as described in sect. 2.2.

fact that the amount of JFP formed was now fully proportional to the time of incubation and to the amount of extract added as long as not more than about 10,000 cpm of JFP were formed.

Details on the substrates necessary are given in table 2. The data show that UDP-gal functions as donor of galactosyl-units. The very weak incorporation found without UDP-gal or with gal-1-P or gal may be due to the presence in the crude extract of traces of substrates and/or enzymes for their interconversion. From the different glycerophosphates the glyc-3-P was by far the best acceptor whereas its optical antipode, glyc-1-P, was not used at all. To our surprise we found that a product migrating like JFP in electrophoresis was formed in significant amounts when glyc-2-P was used as the acceptor. The identity and significance of this product is not clear up to now.

In recent studies on the physiology of JF-formation it was found that after a step-up of the osmotic value in the surrounding fluid the level of JF in the cells increases rather rapidly. The higher rate of JF formation is reached already 1–3 min after the addition of a substance increasing the osmotic pressure and this increase is not affected when the cells were treated by cycloheximide or actinomycin D [3]. Both facts suggest that the regulation may occur by activation of an already existing enzyme rather than by the induced formation of new enzyme molecules. It was of interest in this regard to see whether or not the activity of the JFP-synthase, which obviously is a key enzyme in the formation of JF, changes after an increase in the outside osmotic pressure.

The JFP-synthase activity was determined, therefore, in algae before and after addition of 0.1 M sucrose, an amount sufficient to raise the JF-content from 0.2 to about 12 μM/ml packed cells. The data in table 3, on the one hand, show that the assay method is sufficiently reproducible and on the other that, when measured with BSA and DTE, there is no difference in the activity of the JFP-synthase due to osmotic treatment of the cells. If the algae, however, were disintegrated without BSA and DTE (fig. 2) and the JFP-synthase activity determined under this condition, where it is decreasing with time (see fig. 1), we definitely find a higher apparent activity in osmotically-treated algae. The effect can be found only when the osmotic pressure substance was added before disintegrating the cells, which means that the "activation" of the enzyme due to the osmotic stress somehow requires the metabolic activity of the living cell. The higher activity of the transferase could be the cause for the JF-production observed to increase under this condition [1–3].

The discrepancy between the data in table 3 and fig. 2 may be explained by a working hypothesis. On addition of the SH-reagents and BSA we may artificially bring all the enzyme present into an active state. We may thus measure under this condition (table 3) the total potency of the JFP-syntahse. In contrast fig. 2 may reflect the *in vivo* situation where only part of the enzyme may be active and the proportion of active enzyme be controlled in the manner of a chemical modification of an as yet unknown nature.

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