Volume Regulation in *Poterioochromonas*¹

ININVOLVEMENT OF CALMODULIN IN THE CA²⁺-STIMULATED ACTIVATION OF ISOFLORIDOSIDE-PHOSPHATE SYNTHASE

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ABSTRACT

In *Poterioochromonas malhamensis Peterfi* (syn. *Ochromonas malhamensis* Pringsheim) osmotically induced shrinkage is reversed by an accumulation of isofloridoside. Addition of CA²⁺ ions to homogenates from standard volume cells initiates an enzyme system for the activation of isofloridoside-phosphate synthase. This process is stimulated in the presence of CA²⁺ by calmodulin, isolated from the same alga or from bovine brain, and requires the presence of membranes. The stimulation observed when CA²⁺ is added without exogenous calmodulin is inhibited by the calmodulin-binding substance R 24571. These results show that the effect of CA²⁺ is mediated by calmodulin. The CA²⁺/calmodulin-dependent activation is enhanced when fluoride or molybdate ions are present in the homogenization buffer. This might indicate the involvement of a phosphorylated compound in the activation mechanism.

An increase in external solutes in a suspension of the golden brown wall-less alga *Poterioochromonas malhamensis* causes the cells to shrink. The lost water is regained subsequently due to an internal accumulation of solutes, mainly IF.² These cells represent a good model system for studying the mechanisms by which cell volume changes affect biochemical reactions which in turn result in osmoregulation (5, 6, 8, 9). It has been shown (9) that IFP-synthase, a soluble key enzyme in the biosynthesis of IF, is more active 2 min after cell shrinkage and thus appears to be a major site of regulation. The enzyme is almost inactive in suitably prepared (6, 8, 9) homogenates from cells of standard volume. The IFP-synthase can be rendered active *in vitro* by addition of 50 mM PPi (9); it remains unclear whether this mode of activation is connected with the physiological role of the enzyme. We could, however, also demonstrate an enzymatic activation mechanism which appears to be of physiological significance as it can be reversibly triggered *in vivo* by cell shrinkage (6, 9, 10). In crude homogenates, this activation mechanism, which requires the presence of membranes, can be started by addition of CA²⁺ ions (6, 10). Indirect evidence (8) suggested that the ultimate activation may involve limited proteolysis either of the IFP-synthase molecule itself or an associated protein inhibitor. This idea has recently been substantiated by the isolation and partial purification of an activating enzyme, which is inhibited by 50% by chymostatin (2 µg/ml), antipain (37 µg/ml), or the glycoprotein fetuin (100 µg/ml), indicating that it might be a specific endoprotease (D. Köhle and H. Kaus, unpublished results). This protease is not directly stimulated by CA²⁺ ions but is activated and rendered soluble by a CA²⁺-dependent reaction sequence operating in membranes (6, 10).

During attempts to characterize the IFP-synthase activation mechanism, several psychotropic drugs (trifluoperazine, fluphenazine, chlorpromazine) were employed (6). In the presence of CA²⁺, these substances can bind and inactivate calmodulin, an intracellular CA²⁺ receptor widely distributed in eucaryotic cells (2, 11). The results obtained using the above substances were difficult to interpret, however, as activation of IFP-synthase was stimulated in the absence of CA²⁺. The observation that addition of certain detergents (Triton X-100, digitonin) could also initiate the membrane-associated activation mechanism suggested a probable explanation (7, 10). As the dose-response curves for the detergents and the phenothiazine drugs were similar, it appears that the effect of the latter on the IFP-synthase activation system is not due to their calmodulin-binding ability but to their amphi-pathic nature. These results indicate not only a need for caution in interpretation of physiological effects caused by the above drugs but also a possible role for polar lipids in the volume-sensing process (7).

In the present paper, it will be shown that the effect of CA²⁺ ions on the IFP-synthase activation mechanism in homogenates from *P. malhamensis* is indeed mediated by calmodulin. The activation mechanism can now be depicted in the following scheme, part of which is based on established reactions (right side) and the remainder on working hypothesis (left side), supported in part by the present experiments:

\[ \text{polar lipids} \rightarrow \text{Ca²⁺/calmodulin} \rightarrow \text{protease} \rightarrow \text{IFP-synthase, inactive} \]

\[ \text{membrane altered} \rightarrow \text{protease active} \rightarrow \text{IFP-synthase, active} \]

\[ \text{cell shrinkage} \]

\[ \text{polar lipids} \rightarrow \text{Ca²⁺/calmodulin} \rightarrow \text{protease} \rightarrow \text{IFP-synthase, inactive} \rightarrow \text{IFP-synthase, active} \rightarrow \text{cell shrinkage} \]

The crucial event in the volume-sensing step might be a change in the level of cytoplasmic or membrane-associated CA²⁺ and/or a change in the activity of an unknown membrane enzyme, possibly induced by an alteration in the fluidity or local concentration of polar lipids in the membrane resulting from cell shrinkage (7, 10).

MATERIALS AND METHODS

Methods for growth, preparation of cell suspensions, and homogenization of *P. malhamensis* Peterfi (syn. *Ochromonas malhamensis* Pringsheim) were described previously (6, 8, 9). An
important change was that prior to homogenization the cells were suspended in ice-cold and freshly prepared 100 mM Mes/NaOH (pH 6.2) containing 3.3 mM 2-mercaptoethanol, 2 mM EDTA-Na₂, and 5 mg/ml BSA (Sigma, Cohn fraction V). Under these conditions, the reactions leading to IFP-synthase activation are slowed down significantly (10) enabling the pH 6.2 preincubation step to be performed at 25°C instead of at 6°C as used previously (6). The assay of IFP-synthase was performed as described (6) by measuring the amount of [³²P]IFP produced within 5 min at pH 7.5 from UDP-galactose and sn[¹⁴C]glycerol-3-phosphoric acid. Free Ca²⁺ ions were measured by means of a Ca²⁺-Selective electrode (6).

Calmodulin was kindly provided by K. S. Thomson of this department. The samples were purified by chromatography on DEAE-cellulose at pH 6.5 (elution with 0.5 mM NaCl) followed by affinity chromatography on fluphenazine-Sepharose in the presence of Ca²⁺ (elution with EGTA) essentially as described for plant calmodulin (1). Details on the purification and properties of calmodulin from *P. malhamensis* will be published elsewhere (K. S. Thomson, Ch. Jung, M. Tadros, and H. Kauss, in preparation). The calmodulin-binding substance R 24571 was bought from Janssen Pharmaceutica, Beerse (Belgium), solubilized in dimethyl sulfoxide, and added to the homogenate to give a solvent concentration of 0.1% (v/w). All other materials were as described before (6).

**RESULTS**

The IFP-synthase activation mechanism can be demonstrated by preincubation of the homogenates at pH 6.2 (which is optimal for the activation) followed by determination of the IFP-synthase activity in a second step at pH 7.5 (Fig. 1). The increase in IFP-synthase activity with time reflects the activity of the activating protease (see above scheme). In homogenates from standard volume cells (Fig. 1) without additions or with calmodulin alone, the activation rate is low but is enhanced when Ca²⁺ is also added. Under the improved preincubation conditions used in this paper, Ca²⁺ need only be present for less than 1 min, as addition of EDTA after this time does not affect the subsequent activation (6, 10). This indicates that the protease itself does not require Ca²⁺ but is the product of a Ca²⁺-stimulated reaction sequence. The effect of Ca²⁺ is significantly stimulated on addition of soluble calmodulin isolated from either *P. malhamensis* or bovine brain (Fig. 1). The nonlinearity of the curve with calmodulin after 4 min is due to substrate depletion in the IFP-synthase assay which is linear only up to a total of about 40 × 10⁴ cpm IFP formed.

The increase in the Ca²⁺-stimulated activation is evident if more than about 25 μg/ml calmodulin are added and is maximum at about 500 μg/ml (data not shown). With 100 μg/ml added calmodulin, no stimulation occurs at very low Ca²⁺ ion concentrations (Table I), although the added Ca²⁺ alone significantly enhances the activation. The added calmodulin increased the activation rate at Ca²⁺ concentrations of 25 μM or higher. Table II shows that membranes are necessary for the Ca²⁺/calmodulin-mediated increase in activation rate; about two-thirds of the necessary membrane material can be removed by low speed centrifugation and no Ca²⁺/calmodulin-triggered activation occurs after complete removal of membranes. Addition of fluoride or molybdate ions to the homogenization buffer doubles the enhancement in activation rate mediated by Ca²⁺/calmodulin (Table III). If the two ions were added shortly after the homogenization, an increase of only 1.4- to 1.6-fold was observed (data not shown).

Without exogenous calmodulin, the stimulation of the IFP-synthase activation mechanism by Ca²⁺ is significantly inhibited by the calmodulin-binding substance R 24571 (4) at concentrations where no significant influence on the activation without Ca²⁺ is found (Fig. 2). At a concentration of 0.5 mM without added Ca²⁺, this inhibitor caused some activation, similar to that found

**DISCUSSION**

In the presence of Ca²⁺, the Ca²⁺-binding protein calmodulin can significantly increase the activation rate of IFP-synthase (Fig. 2).
CALMOMULIN IN SENSING VOLUME CHANGES

Table II. Requirement of Membranes for the IFP-Synthase Activation Mechanism

| Conditions as were as in Fig. 1, and the activation rate is given for the 0- to 2-min interval. Calmodulin from Poteriochromonas was used at 500 µg/ml. |
| IFP-Synthase Activation Rate |
| Fraction Used | No addition | +Ca²⁺ | +Ca²⁺, +calmodulin |
| Crude homogenate | 0.5 | 2.9 | 10.7 |
| Supernatant, 10,000g | 0.5 | 0.8 | 3.3 |
| Supernatant, 90,000g* | 0.3 | 0.4 | 0.5 |

* 1 min, Eppendorf 3200 centrifuge, 4°C.

Table III. Influence of Salts Added to the Homogenization Buffer on the Ca²⁺/Calmodulin-Dependent Activation Mechanism for IFP-Synthase

| Conditions as were as in Fig. 1. The activation rate is given for the 0- to 4-min interval. Calmodulin from Poteriochromonas was used at 500 µg/ml. |
| IFP-Synthase Activation Rate |
| Homogenization in | No addition | +Ca²⁺ | +Ca²⁺, +calmodulin |
| × 10⁻³ cpm IFP/5 min/10 µl |
| Standard buffer | 1.0 | 3.0 | 8.5 |
| +50 mM NaCl | 1.0 | 2.8 | 9.2 |
| +50 mM NaF | 0.8 | 6.3 | 20.3 |
| +10 mM Na₂MoO₄ | 2.0 | 5.6 | 19.8 |

1). This suggests that the effect of Ca²⁺ is mediated by an interaction of the Ca²⁺/calmodulin complex with an unknown enzyme which is part of the reaction sequence presumed to operate in membranes. Most of the enzymes or enzyme systems reported to be regulated by the Ca²⁺/calmodulin complex have phosphorylated compounds as their substrates (2, 11). The finding that fluoride, a general phosphatase inhibitor, and molybdate, an inhibitor of phosphoprotein phosphatase (13), have a beneficial effect on the IFP-synthase activation may indicate that one of the unknown constituents of the membrane-bound system may also be a phosphorylated compound, possibly a phosphoprotein. This observation may be of value in further characterizing the activation system. It also appears of interest that a great deal of the necessary membrane material can be spun out at rather low speed (Table II) and that added calmodulin (mol wt about 17,000; Refs. 2 and 11) has access to the system at all. This might be an indication that in the homogenate the IFP-synthase activation system is not located mainly in small closed vesicles but in rather large membrane sheets, possibly derived from the plasma membrane.

Previous attempts (6) to demonstrate a participation of calmodulin in the activation indirectly by using trifluoperazine, fluphenezine, and chlorpromazine failed as these substances show detergent-like properties which cause activation in the absence of Ca²⁺ (7). This effect has obscured their inhibitory action on the calmodulin-dependent step. Fortunately, R 24571 differs in this respect (Fig. 2) as it significantly inhibits the Ca²⁺-dependent activation at concentrations where it shows no detergent-like action (activation without Ca²⁺). The apparent concentration (Fig. 2) needed for 50% inhibition of the Ca²⁺-stimulated IFP-synthase activation is higher than that reported for highly purified enzymes (4). This might be due to nonspecific association of R 24571, a lipophilic substance, with membrane material, similar to that of other lipophilic drugs (3). Thus, the effective concentration of R 24571 may be less than that calculated from the amount added.

The results from the inhibitor experiments and from calmodulin addition suggest that calmodulin is present in the homogenate, possibly still bound to the membranes, and that it mediates the activation mechanism on addition of Ca²⁺. Only if Ca²⁺ (Table I) or calmodulin is added at higher concentrations can an additional stimulation by the exogenous calmodulin be observed. The need for relatively high concentrations of exogenous calmodulin to cause stimulation as compared to purified enzymes regulated by Ca²⁺/calmodulin (2, 4, 11) might partly be due to the presence in the crude homogenate of numerous other enzymes which can also bind soluble calmodulin and thus compete with the IFP-synthase activation mechanism for added calmodulin. In addition, effects of added calmodulin observed with crude membrane preparations (e.g. Ref. 14) or partially purified membrane enzymes (e.g. Ref. 12) appear generally to be significant only when calmodulin is added at high concentrations. This might indicate nonspecific binding of calmodulin to membranes and/or limited access of exogenous calmodulin to the active sites. There remains, however, another hypothetical possibility to explain the observation that calmodulin has to be added at high concentrations to enhance the IFP-synthase activation mechanism. It could be assumed that an unidentified Ca²⁺-binding protein is involved in the activation mechanism and that added calmodulin partially substitutes for it by virtue of a structural similarity. Research on this aspect requires, however, some purification and fractionation of the membrane-associated IFP-synthase activation mechanism.

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