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Activation of plant quinate:NAD^+ 3-oxidoreductase by Ca^{2+} and calmodulin

(carrot cells/cyclic AMP/protein kinase/enzyme phosphorylation)

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ABSTRACT **Quinate:NAD^+ 3-oxidoreductase (EC 1.1.1.24) from carrot cell suspension cultures has previously been shown to be activated by phosphorylation and inactivated by dephosphorylation. Here it is shown that the reactivation of the inactivated quinate:NAD^+ oxidoreductase is an enzyme-mediated process that requires ATP and protein kinase activity. The reactivation is completely inhibited by EGTA and can be restored by the addition of Ca^{2+}. Cyclic AMP at concentrations up to 5 μM did not have any effect on the reactivation either with or without EGTA in the medium. Calmodulin-depleted fractions containing quinate:NAD^+ oxidoreductase were obtained by passage of the crude extracts through an affinity column of 2-chloro-10-(3-aminopropyl)phenothiazine coupled to Sepharose 4B. The enzyme in this calmodulin-deficient fraction could be inactivated but not reactivated even in the presence of ATP and Ca^{2+}. However, addition of bovine brain calmodulin completely restored the activity of the enzyme. Half-maximal activation occurred at 130 nM calmodulin. We conclude from these data that the quinate:NAD^+ oxidoreductase is activated by a Ca^{2+}- and calmodulin-dependent plant protein kinase.**

Post-translational modification of enzymes is of great importance in the control of cellular metabolism (see ref. 1 for review). Reversible phosphorylation is particularly considered to be a mechanism by which hormonal and neural stimuli are transmitted to animal cells by the mediation of second messengers—e.g., cyclic AMP and Ca^{2+} (reviewed in refs. 2 and 3). The best-elaborated example to date is the regulation of glycogen metabolism by phosphatases and cyclic AMP- and Ca^{2+}/calmodulin-dependent protein kinase (1–3). So far no cyclic AMP-dependent protein kinases having a similar regulatory function have been reported to occur in plants. Recently two papers appeared indicating the presence of Ca^{2+}/calmodulin-dependent protein kinase activity (4, 5).

From recent work we conclude that the plant enzyme quinate:NAD^+ 3-oxidoreductase (QORase, EC 1.1.1.24) isolated from carrot cell suspension cultures is activated by phosphorylation and inactivated by dephosphorylation. QORase reversibly converts dehydroquinic acid into quinic acid, a by-product of the shikimate pathway that is accumulated at high concentration in many plants (6). Our experimental data support the assumption that protein kinases and phosphatases are involved in the reversible phosphorylation of this enzyme. The degree of phosphorylation is strictly correlated with the QORase activity (7). QORase activity can therefore be taken as a measure of the enzyme phosphorylation.

In this paper we report on the Ca^{2+}/calmodulin-dependent activation of the QORase. No activation by cyclic AMP could be detected. Our data suggest that the plant QORase is activated by one or several Ca^{2+}/calmodulin-dependent protein kinases.

MATERIALS AND METHODS

Materials. Dehydroquinate was synthesized according to Grewe and Handler (8). 2-Chloro-10-(3-aminopropyl)phenothiazine (CAPP) was obtained from A. A. Manian (National Institute of Mental Health, Rockville, MD). CNBr-activated Sepharose 4B and DEAE-Sephacel were purchased from Pharmacia (Freiburg, Federal Republic of Germany). Calmodulin-deficient adenosine 3′,5′-cyclic monophosphate phosphodiesterase (PDEase) was obtained from Sigma (München, Federal Republic of Germany). All other reagents were the best grades commercially available. Bovine brain was obtained from the local slaughterhouse. Carrot cell suspension cultures were grown and harvested as described in ref. 7.

QORase Assay. Proteins were extracted from lyophilized carrot cells and QORase was partially purified as described in detail by Refeno *et al.* (7). QORase activity was determined spectrophotometrically (7). The specific QORase activity in the partially purified preparation before inactivation was 20 nkat/mg of protein [1 kat (katal) catalyzes conversion of 1 mol of substrate per sec]. This activity was set as 100% (Figs. 1 and 3).

Preparation of a Calmodulin-Deficient Protein Fraction. The proteins from the partially purified fraction were applied to a CAPP-Sepharose 4B column that was equilibrated with 40 mM Tris·HCl buffer, pH 7, containing 1 mM mercaptoethanol, 1 mM EDTA, and 5 mM $CaCl_2$. The coupling of the CAPP to the CNBr-activated Sepharose 4B was performed with 73.2 mg of CAPP and 15 g of CNBr-activated Sepharose 4B according to the instructions given by Pharmacia. The calmodulin was retained by the immobilized CAPP in a Ca^{2+}-dependent manner as described by Jamieson and Vanaman (9).

Purification of Calmodulin from Bovine Brain. Electrophoretically pure calmodulin from bovine brain was prepared as described by Jamieson and Vanaman (9) with some modifications: One kilogram of bovine brain was homogenized in 1 liter of buffer (40 mM Tris·HCl, pH 7.0, containing 1 mM 2-mercaptoethanol and 1 mM EDTA). The homogenate was centrifuged for 1 hr at 10,000 × *g* and incubated with 200 ml of DEAE-Sephacel. The DEAE-Sephacel was washed with 800 ml of extraction buffer containing 100 mM NaCl. Proteins were eluted with 400 ml extraction buffer containing 500 mM NaCl. The eluate was loaded on the CAPP-Sepharose 4B column in the presence of 5 mM $CaCl_2$. The column was washed until all material having absorbance at 280 nm was eluted and then cal-

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Abbreviations: QORase, quinate:NAD^+ 3-oxidoreductase; CAPP, 2-chloro-10-(3-aminopropyl)phenothiazine; PDEase, cyclic AMP phosphodiesterase.

modulin was eluted by adding 10 mM EGTA to the wash buffer instead of $CaCl_2$. The eluate was dialyzed overnight against 1 mM NH_4HCO_3/1 mM 2-mercaptoethanol and lyophilized. Contaminating S 100 protein was removed by ion-exchange chromatography on DEAE-Sephadex A-50. Minor high molecular weight contaminants and salt were removed by Sephadex G-100. The calmodulin obtained was electrophoretically pure.

RESULTS

Inactivation and Reactivation of the QORase. The QORase in a partially purified preparation from carrot cell suspension cultures has been shown to be inactivated in the presence of $MgCl_2$. This inactivation does not occur in the presence of NaF, an inhibitor of phosphatases (7). The inactivated enzyme can be reactivated by the addition of ATP. Fig. 1 illustrates that QORase that was 50% inactivated in 20 min or almost 90% inactivated in 50 min can be fully reactivated by the addition of ATP. The lag phase that occurs before the onset of reactivation can be shortened by exogenously added phosphorylase kinase from rabbit muscle, indicating that the plant protein kinase activity is a limiting factor of reactivation (7).

Reactivation of the QORase Depends on Ca^{2+} and Not on Cyclic AMP. As is shown in Fig. 2 *Left*, the reactivation of the QORase is inhibited by EGTA, a Ca^{2+}-chelating agent. The addition of 2 mM $CaCl_2$ not only allows complete activation of the enzyme (i.e., 100%) but also stimulates the QORase further by about 40%. The dependence of the QORase activity on the Ca^{2+} concentration is shown in Fig. 2 *Right*. Addition of 1 mM $CaCl_2$ is sufficient to reactivate the enzyme to its highest activity level. The additional activation is also observed when $CaCl_2$ is added to the enzyme preparation prior to inactivation, indicating that Ca^{2+} is limiting.

We could also show that cyclic AMP did not cause a reactivation of the QORase. Addition of cyclic AMP at concentrations between 10 and 50 μM did not result in any reactivation. When cyclic AMP at the same concentration was added in the presence of EGTA no reactivation occurred (data not shown). The dependence of the enzyme activity on Ca^{2+} suggests that the activation is mediated by calmodulin. We have therefore examined the possible role of calmodulin on the Ca^{2+}-dependent reactivation.

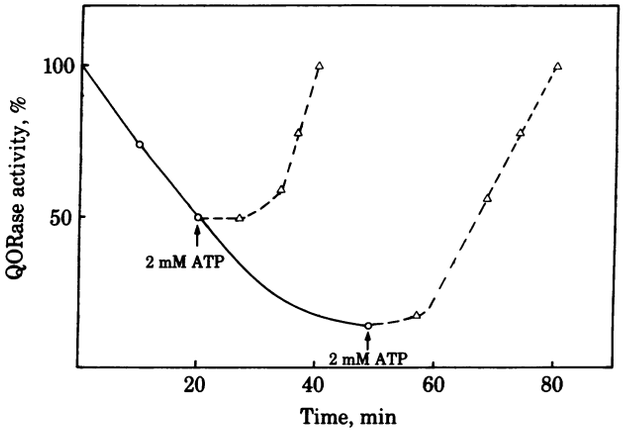

FIG. 1. Inactivation and reactivation kinetics of the QORase. Inactivation (○——○) of the enzyme preparation was performed at 30°C in the presence of 4 mM $MgCl_2$. Reactivation (△---△) was carried out at 20°C and was initiated by the addition of 2 mM ATP. QORase activities (represented in this and the following figures) were measured for 2 min in the presence of the substrate at the end of the different incubation programs.

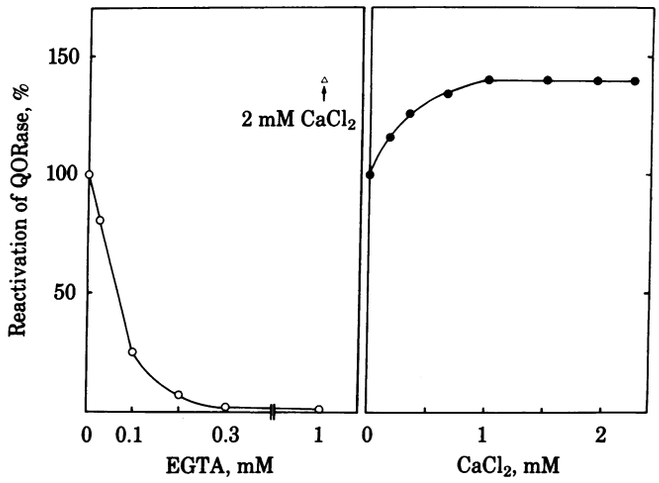

FIG. 2. Effect of EGTA and $CaCl_2$ on the reactivation of the QORase. In all experiments the enzyme was first inactivated for 20 min as described in the legend of Fig. 1, then the reactivation was carried out at 20°C by addition of 2 mM ATP in the presence of various concentrations of EGTA (○) or $CaCl_2$ (●). Reactivation of 140% was obtained in the presence of 1 mM EGTA and 2 mM $CaCl_2$ (△).

Ca^{2+}-Dependent Reactivation of the QORase Is Mediated by Calmodulin. In order to investigate the effect of calmodulin we have passed the protein preparation from carrot cells through a CAPP-Sepharose 4B affinity column. In the presence of Ca^{2+} almost all of the calmodulin remains on the column and the pass-through fraction is essentially calmodulin deficient (unpublished data). However, the pass-through fraction contains all the QORase activity.

The QORase in the pass-through fraction could be inactivated as in the experiment described in Fig. 1. This indicates that the phosphoprotein phosphatases that are responsible for the inactivation (7) are also present in the pass-through fraction. Bar I in Fig. 3 represents the activity of the QORase that had been inactivated for 20 min. No increase of the enzyme activity could be observed when the reactivation was carried out in the presence of ATP but in the absence of Ca^{2+} and calmodulin (bar II) or calmodulin alone (bar III). Full reactivation was achieved when both Ca^{2+} and calmodulin were present (bar IV). Addition of EGTA again inhibits the reactivation (bar V).

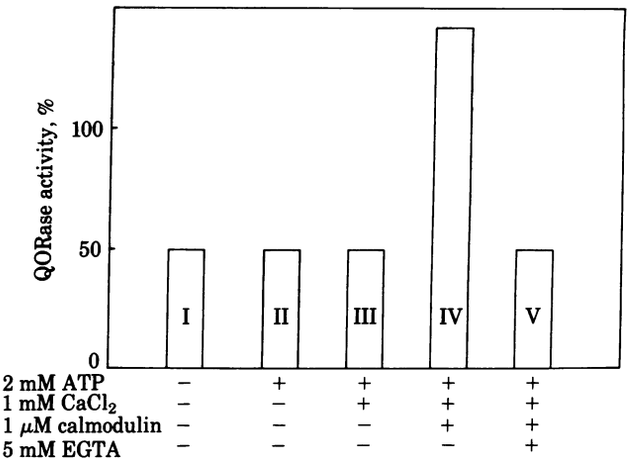

FIG. 3. Reactivation of QORase after removal of calmodulin. The extracts were inactivated for 20 min at 30°C in the presence of $MgCl_2$ (bar I) and then reactivated for 20 min at 20°C in the presence of ATP and the indicated additions (bars II, III, IV, and V).

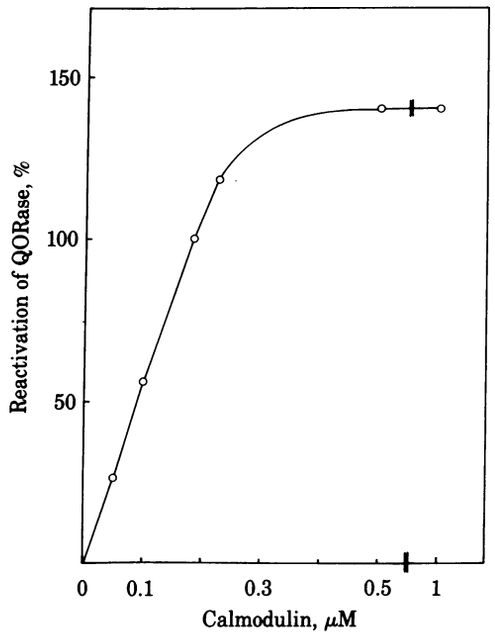

FIG. 4. Effect of increasing amounts of calmodulin on the reactivation of QORase in a calmodulin-depleted preparation. QORase was inactivated at 30°C for 45 min (about 85% inactivation) before reactivation was performed for 20 min at 20°C in the presence of 2 mM ATP, 1 mM $CaCl_2$, and different amounts of calmodulin.

The dependence of the activation on the calmodulin concentration is shown in Fig. 4. Maximal activation is obtained at 500 nM calmodulin. Half-maximal activation occurred at a calmodulin concentration of 130 nM. This concentration is comparable to that necessary to obtain half-maximal stimulation of the plant NAD kinase (unpublished results).

DISCUSSION

The results presented in this paper demonstrate that the activation of the QORase from carrot cells that is mediated by reversible phosphorylation (7) depends on Ca^{2+} and calmodulin. After removal of the endogenous calmodulin and inactivation of the QORase for 20 min at 30°C in the presence of $MgCl_2$ the enzyme cannot be activated. Only when purified bovine brain calmodulin is added to the medium does the QORase become reactivated upon addition of ATP. This indicates that both the phosphatases and protein kinases are still present in the QORase preparation and that the calmodulin is not a tightly bound subunit of the protein kinase as has been reported for the phosphorylase kinase from liver and muscle (3). We also demonstrated that reactivation of the enzyme could not be initiated by the addition of cyclic AMP.

Since the discovery of calmodulin in plants by Anderson and Cormier (10) only two enzymes, the NAD kinase (10–12) and the membrane-associated Ca^{2+} transport ATPase (13, 14), have been reported to be controlled by calmodulin in a Ca^{2+}-dependent manner. In both cases no evidence for protein phosphorylation has been reported. The regulatory mechanism reported here suggests Ca^{2+}- and calmodulin-dependent protein kinase is involved in the activation of the QORase. Our data show that the QORase activity *in vitro* can be varied to a large extent depending on the degree of phosphorylation. Even if the physiological significance of this modulation is not clear at present, this system seems to be suited to study of the mechanism of Ca^{2+}/calmodulin-dependent modulation of plant enzyme activities by protein kinases.

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