The reversible association of quinate:NAD$^+$ oxidoreductase from carrot cells with a putative regulatory subunit depends on light conditions

Annick Graziana, Raoul Ranjeva, Bharathi P. Salimath* and Alain M. Boudet

Centre de Physiologie Végétale (L.A. C.N.R.S. No. 241), Université Paul Sabatier 118, route de Narbonne, 31062 Toulouse Cédex, France and **Institüt für Biologie III, Universität Freiburg Schänzlestr. 1, D-7800 Freiburg, FRG*

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The transfer of carrot cell-suspension cultures from light to dark conditions provoked considerable and reversible changes in the regulatory and structural properties of quinate:NAD$^+$ oxidoreductase. Thus, the enzyme became directly activatable by Ca^{2+} and its M_r shifted from 42 kDa to 110 kDa. The analysis of the dark enzyme, purified to homogeneity, showed that an additional subunit of 60–63 kDa was associated to QORase. The homogeneous native enzyme retained the sensitivity to calcium. Preincubation with trypsin had no effect on sensitivity to Ca^{2+} while a chaotropic agent mimicked the action of Ca^{2+}. It is concluded that light–dark transitions provoke the association of QORase with a putative regulatory subunit which may be a calciprotein.

Ca^{2+} activation *Protein assembly* *Dark-control* *Plant calciprotein* *Enzyme regulation*

1. INTRODUCTION

Quinate:NAD$^+$ oxidoreductase (EC 1.1.1.24) reversibly converts dehydroquinate into quinate, a by-product of the shikimate pathway which accumulates at high concentration in many plants [1]. Our previous work with carrot cell-suspension cultures has established that QORase is activated through phosphorylation by Ca^{2+}-CaM-dependent protein kinase(s) [2–4]. Using a specific Ca ionophore, it has also been shown that cellular Ca^{2+} concentrations control the ratio of activated over inactivated forms of the enzyme [5].

Increasing experimental evidence supports the assumption that light triggers altered levels of cytoplasmic Ca^{2+} in plant cells and leads to the activation of CaM-dependent enzymes [6]. Light acts also on the phosphorylation of chloroplastic proteins, especially the light-harvesting complex [7]. Therefore we have studied the effect of light conditions on QORase.

We report here unexpected changes in the properties and structure of the enzyme when carrot cells are submitted to light–dark transitions.

Abbreviations: QORase, quinate:NAD$^+$ oxidoreductase; CaM, calmodulin; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(aminoethyl ether) *N,N'*-tetraacetic acid

2. MATERIALS AND METHODS

2.1. *Chemicals*

All the chemicals were analytical grade when available and were purchased from: Pharmacia (Uppsala), Sephadex G-25, Sephacryl S-200, Polybuffer exchanger 94, Polybuffer 74, Blue Sepharose; Biorad (France); phenothiazine-affigel, Chellex 100; Sigma (MO); EGTA; IBF (France) CaM-ultrogel; UCB (Brussels), CaM antibody.

Dehydroquinate was synthesized as in [8]; Fluphenazine was a gift from J. Demaille (CNRS, Montpellier) and CaM was extracted from bovine brain [4].

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Other chemicals and biochemicals were purchased from Merck, Darmstadt.

2.2. *Buffers*

Buffers were pretreated with Chellex 100 and are referred to as: buffer A – 0.1 M Tris–HCl (pH 7.5) containing 0.1% mercaptoethanol; buffer B – 0.05 M Tris–HCl (pH 8); buffer C – 0.5 M Tris–HCl (pH 8.5); buffer D – 0.025 M imidazole–HCl (pH 7.4); buffer E – Pharmacia polybuffer 74–acetic acid (pH 4).

2.3. *Plant material*

Carrot cell-suspension cultures were grown either under continuous light (285 μmol photons m^{-2} s^{-1}) [2] or in the dark. Other conditions were as in [2]. QORase extracted from light-grown cells (L-cells) will be referred to as L-QORase, that from dark-grown cells (D-cells) as D-QORase.

2.4. *Enzyme extraction and assays*

The harvested cells (6–day-old) were frozen in liquid nitrogen; the proteins were extracted as in [2] but the ratio of plant material over extraction medium was 1:10 instead of 1:1. QORase activity was measured spectrophotometrically. The assays contained 6 mM dehydroquinate, 0.2 mM NADH, buffer C and protein extract in a final volume of 1 ml. The control was done without dehydroquinate. The reactions were run at 30°C and decreases in absorbance at 340 nm monitored with a double-beam spectrophotometer (Jobin & Yvon, model Duospac). Eventual additions of compounds were simultaneously done in reference and assays cuvettes.

2.5. *Purification of the enzyme*

Partial purification of L- and D-QORase was performed by ammonium sulphate precipitation (50% saturation) followed by gel filtration through a Sephadex G-25 column in buffer B. The macromolecular fraction was chromatographed through a Sephacryl S-200 column either in the same buffer (D-QORase) or in buffer B supplemented with 50 mM NaF for L-QORase.

D-QORase was purified to homogeneity after the following steps; ammonium sulphate precipitation (0–50%), gel filtration through Sephadex G-25 equilibrated in buffer D, chromatofocussing and elution by buffer E. The most active fractions were pooled, concentrated by ammonium sulphate precipitation (80%) and desalted again through Sephadex G-25 in buffer B. The macromolecule fraction was then loaded onto a blue Sepharose column in buffer B and eluted with the same buffer.

2.6. *PAGE*

The purified enzyme was concentrated by dialysis under vacuum. PAGE in native conditions was performed with 7.5% acrylamide, SDS-PAGE in a 5–20% gradient acrylamide [9]. Proteins were stained with Coomassie blue R-250.

2.7. *Protein determinations*

Protein determinations were done as in [10].

3. RESULTS

3.1. *Light–dark transitions provoke changes in the regulatory properties of QORase*

Addition of Ca^{2+}, Ca^{2+}-CaM or Ca^{2+}-EGTA to enzyme assays performed with partially purified preparation of L-QORase, did not modify the enzyme activity [5] (fig. 1). In contrast, D-QORase was immediately stimulated by a factor of 3-fold on addition of Ca^{2+} while severe inhibition occurred when the assays were supplemented with EGTA. Excess calcium reversed the inhibition and a slight additional stimulation was observed when

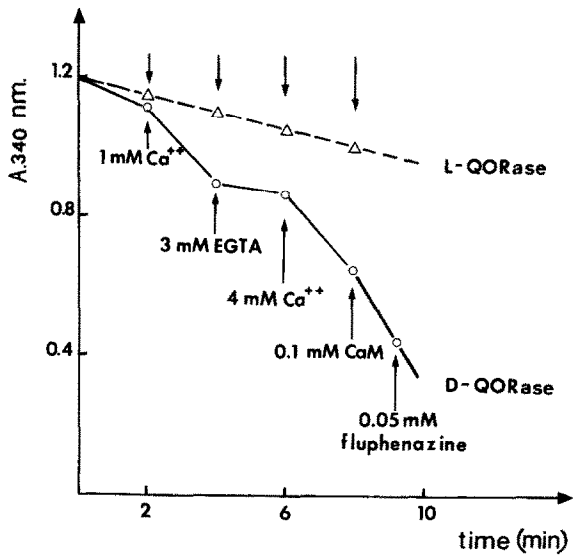

Fig. 1. Action of Ca^{2+}, EGTA and CaM on D-QORase and L-QORase activities (enzyme source: proteins precipitated by $(NH_4)_2SO_4$ and desalted through Sephadex G-25).

CaM was included in the medium. However, the CaM-induced activation was not inhibited by fluphenazine or R 24 571. The Ca^{2+} effect was lost if D-cells were grown in the light and reappeared on transfer of L-cells to dark conditions. The illumination conditions (up to 1 h) have no effect on cell-free preparations from L- or D-cells even in the presence of reducing agent (mercaptoethanol) or a cross-linking compound (dimethyl suberimidate). Therefore, it appears that transfer to darkness provokes changes in the regulatory properties of QORase due to an in vivo intrinsic process.

3.2. *Changes in regulatory properties and parallel structural modifications of QORase*

As shown in fig. 2, the behaviour of enzymes extracted from L- and D-cells differed on gel filtration through Sephacryl S-200. L-QORase exhibited an M_r of 42 kDa [2] whereas D-QORase eluted as a larger molecule. In the latter case the activity was associated with a 110 kDa molecule. Interestingly, the activation by calcium was retained even if the enzyme preparation was freed from CaM by affinity chromatography. The M_r of QORase depended on the last light environment conditions and the shift was reversible in vivo.

In contrast to L-QORase which is stable only in the presence of phosphatase inhibitor [2–4], partially purified D-QORase may be stored at 0–4°C without any particular care. Such a property

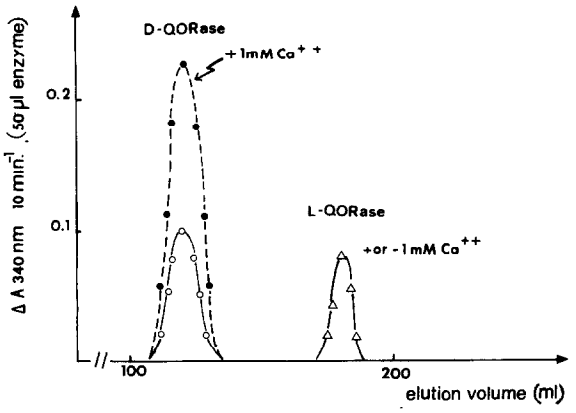

Fig. 2. Comparative elution pattern of D-QORase and L-QORase through gel filtration. Parameters of the column: 121 × 1.75 cm; Flow rate; 30 ml h^{-1}; Fraction volume, 3.5 ml.

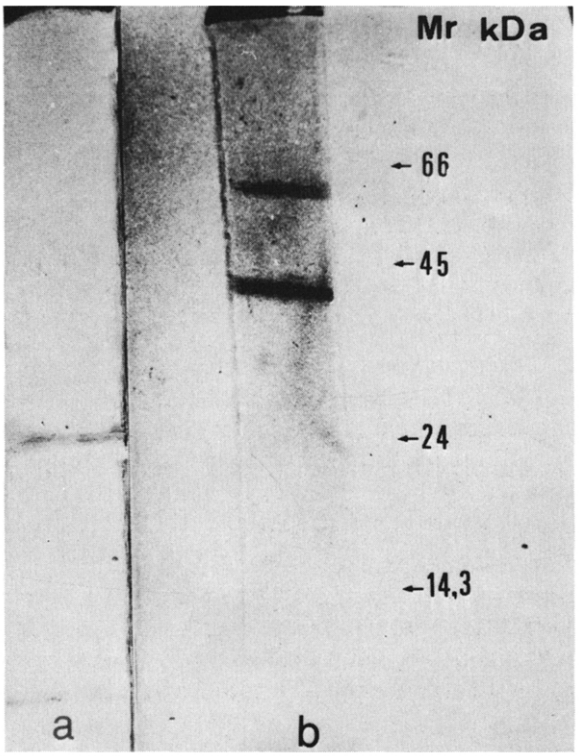

Fig. 3. PAGE of native and denatured D-QORase. (a) PAGE, native D-QORase; (b) SDS-PAGE, denatured D-QORase.

allows the homogeneous enzyme to be isolated (fig. 3a) with a high yield (table 1).

When treated by SDS and analyzed by gradient PAGE, pure D-QORase appeared to be composed of two subunits (fig. 3b). The first behaved as L-QORase the M_r of which was 42 kDa; the second was heavier and migrated as a 60–63 kDa protein. No peptide corresponding to a CaM-like molecule was visible on the electrophoregram.

Therefore, the L–D transition leads to the association of L-QORase with an additional subunit.

3.3. *Ca^{2+} sensitivity is intrinsic to D-QORase*

The pure preparation of D-QORase was sensitive to Ca^{2+} activation and EGTA inhibition (table 2). Thus, it is possible that the native D-QORase contains a recognition site for the cation. When submitted to affinity chromatography either on phenothiazine-affigel or on CaM-ultrogel in the presence or absence of Ca^{2+}, D-QORase was not

Table 1

Purification of D-QORase

Purification step	Total activity (nkat)	Specific activity (nkat mg^{-1} protein)	Yield (%)	Purification (-fold)
Desalted ammonium sulphate preparation	3.4	0.027	100	1
Chromatofocusing	2.8	6.5	82	241
Affinity chromatography (Blue Sepharose)	2.2	17.5	65	648

retarded at all and was completely recovered in the pass-through fraction. Likewise, CaM antibody did not affect the enzyme activity nor its sensitivity to Ca^{2+}. Consequently, CaM does not appear as the main Ca^{2+}sensor in the D-QORase system since it is not a subunit of the enzyme and is not recognized clearly by D-QORase. In these respects, D-QORase differs from animal phosphorylase kinase which contains CaM as a subunit and is able to bind additional CaM molecules [11].

Since trypsin treatment may mimic the Ca^{2+} activation in different experimental models [12,14] experiments were performed to check if this was also the case with QORase (table 2).

However, no convincing evidence was obtained, the enzyme losing in parallel its activity and activability (table 2). In contrast, a chaotropic agent (Triton X-100) mimicked partially the effect of calcium, suggesting that hydrophobic residues may be involved in the activation process.

Table 2

Effects of different Ca^{2+}-related compounds on the activity of pure D-QORase

	Relative activity on sequential addition of				
	–	1 mM Ca^{2+}	3 mM EGTA	4 mM Ca^{2+}	0.1 mM CaM
D-QORase	100	375	40	360	418
D-QORase + CaM antibody*	100	360	35	370	400
D-QORase + 1% Triton X-100	175	350	50	360	400
D-QORase + 1% Triton X-100 + CaM antibody	160	370	55	370	410
D-QORase** + trypsin TPCK	75	300	35	290	300

50 pkat QORase activity was set as 100%

* D-QORase was combined with anti-CaM in different proportions and controls were performed by substituting the antibody by the equivalent volume or bufffer. The vortexed preparations were allowed to stand for 15 min at 30°C and centrifuged for 5 min at 12 000 × *g*. The supernatants were used as enzyme source. Identical figures were obtained for each set of assays irrespective of the ratio of anti-CaM over D-QORase

** D-QORase was preincubated for 2–10 min with 2–20 μg trypsin at different pH values, then leupeptin was added and D-QORase activity measured

4. DISCUSSION

Our results demonstrate that light–dark transitions provoke profound changes in the regulatory property of QORase which becomes directly activatable by calcium. Such a modification parallels structural alteration since the enzyme binds an additional subunit.

In plants, light–dark transitions have been shown to control the organization of complex proteins. Thus, the association of the light-harvesting protein to the grana (containing photosystem II) or to the stroma (containing photosystem I) in chloroplasts depends on light; the protein moves reversibly from photosystem I on illumination [7]. Likewise the assembly of the large and the small subunits of ribulose-1,5-diphosphate carboxylase is effective only if plant cells are illuminated [15]. In the latter case, the rationale of the association remains unclear. The data reported here are the first to describe the reversible association of a plant enzyme to an additional subunit and the subsequent gain in Ca^{2+} sensitivity.

Such results give a more complex picture of the QORase system and its dependence on calcium:

in the light, Ca^{2+} stimulates QORase via the activation of Ca–CaM-dependent protein kinase(s),

in the dark, Ca^{2+} directly activates the enzyme by a process which does not essentially involve CaM. The slight effect of CaM was not inhibited by fluphenazine and QORase did not contain CaM nor recognize the protein modulator.

These data fit well with the assumption of the role of Ca^{2+} as a secondary messenger in plants. Up to now, the cation has been shown to act through phospholipids or CaM [16]. It is suggested here, that other molecules, presumably calciproteins, may also act as transducer. The molecular characterization of such compounds is in progress.

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