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ACTIVATION OF NITRATE REDUCTASE BY CALCIUM AND CALMODULIN

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Key Word Index—Nitrate reductase; calcium; calmodulin.

Abstract—Nitrate reductase prepared from the leaves of *Amaranthus* is activated by calcium and a small M_r protein factor prepared from spinach by the procedures used for calmodulin preparation. The activation is considerably enhanced if both Ca^{2+} and the protein factor are present. This activation is inhibited by EGTA, a Ca^{2+} specific chelator and by anticalmodulin compounds like chlorpromazine. The effect of EGTA is reversed by Ca^{2+}. The protein factor was identified as calmodulin. The enzyme is also activated by commercially available calmodulin. Calmodulin activation seems to be manifested in the $FMNH_2$-NR moiety of the enzyme molecule.

INTRODUCTION

The requirement of calcium by plants for several metabolic functions is well known [1]. However, there is very little information on the specific Ca^{2+} requirement by plant enzymes and its precise role in enzyme function. In the past few years many reports have appeared regarding the calcium regulation of enzymes in animals through a small M_r protein designated as calmodulin [2–4]. In plants also calmodulin has been shown to be involved in the regulation of NAD kinase [5–7], calcium ATPase [7] and plant protein kinases [8]. The microsomal Ca^{2+} uptake in plants has been shown to be under calmodulin control [9]. Recently we have reported the activation of aspartate kinase from different plants by calmodulin [10, 11].

Dekock *et al.* [12] have previously demonstrated that the reduction of nitrate in the leaves is dependent upon the presence of Ca^{2+} ions and that a metabolic connection exists between nitrate assimilation and the calcium content of leaves. These studies suggested a possible role of Ca^{2+} in nitrate assimilation.

In this communication we report the regulation of nitrate reductase (EC 1.6.6.1) by calcium and calmodulin. The data presented in this paper show that calmodulin can stimulate the activity of nitrate reductase in the presence of Ca^{2+} and that the activity of the activated enzyme can be inhibited by EGTA which chelates Ca^{2+} specifically, or by anticalmodulin compounds like chlorpromazine.

RESULTS AND DISCUSSION

The protein factor used in these studies was isolated from spinach leaves by the procedures used for the purification of calmodulin (see Experimental) and has been referred to as calmodulin-like protein factor or simply factor. An identical factor can also be prepared from the leaves of other plants such as *Amaranthus*, winged bean, etc. Figure 1 shows that the nitrate reductase

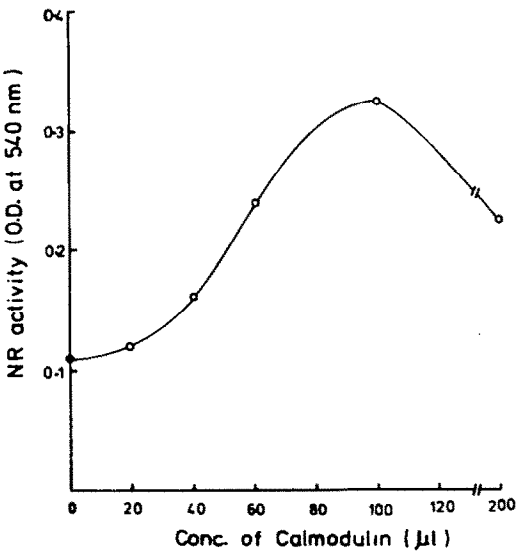

Fig. 1. Effect of varying concentrations of calmodulin-like protein factor on the nitrate reductase activity isolated from *Amaranthus*. Maximum activation was obtained when 100 μl (20 μg protein) was added to 1 ml reaction mixture. The specific activity of the enzyme used was 1.2 μmol nitrite produced/mg protein/min.

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Abbreviations: EDTA, ethylene diamine tetra acetic acid; EGTA, ethylene glycol-bis(α-amino ethyl ether *N,N,N*-tetraacetic acid; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NAD, nicotinamide adenine dinucleotide; MES, 4-(*N*-morpholino)ethane-sulphonic acid; Tris, tris(hydroxymethyl)aminomethane.

activity is stimulated by a calmodulin-like protein factor showing maximum stimulation when it was added to the extent of 20 μg protein/ml reaction mixture. The enzyme used had a specific activity of about 1.2 μmol nitrite produced per mg protein per min. The activation observed is around 2–2.5-fold.

Table 1 shows the stimulation of nitrate reductase activity by the protein factor and calcium. The factor alone can stimulate the activity to about 2-fold. This stimulation can be further increased by the addition of Ca^{2+} to almost 3.5-fold. Calcium alone also stimulates the enzyme activity but the maximum stimulation obtained is a little over 2-fold. The data show that both Ca^{2+} and calmodulin like factor are necessary for the maximum stimulation of the enzyme activity.

The requirement of calcium for the activity of the nitrate reductase is further demonstrated by the data in Table 2. EGTA, which preferentially chelates calcium, inhibits the enzyme activity in a dose dependent manner (Table 2). This inhibition is reversed by the addition of Ca^{2+}. The percentage activity of the enzyme reduced to about 40% is stimulated to 150% by 5 mM Ca^{2+}. This reversal is calcium specific, as other divalent metal ions could not effectively reverse the inhibition caused by EGTA (data not included). Since calmodulin stimulation of enzyme has been known to require Ca^{2+}, it was expected that EGTA would inhibit the stimulatory effect of the protein factor if the protein factor was calmodulin. This was indeed observed. The data presented in Table 3 show that 2-fold stimulation of the enzyme activity by the protein factor is inhibited by EGTA which in turn can be reversed by the addition of Ca^{2+}. It is therefore concluded that the activation by protein factor is calcium dependent. The protein factor, as we have shown earlier, was isolated by the procedure used for calmodulin isolation [10, 11]. It is also eluted from G-200 column in a M_r range which corresponds to calmodulin. Further, the protein factor is able to stimulate the activities of both the NAD kinase [5, 6] and aspartate kinase [10, 11] like calmodulin. These considerations suggest that the factor stimulating nitrate reductase is probably identical with calmodulin. If this is so, its activation should be inhibited by anticalmodulin compounds such as chlorpromazine. At low concentrations (2.4 mM) of chlorpromazine the enzyme activity was inhibited to 31% and that in the presence of factor it was brought down from 140% to 57%. These observations further support the suspicion that the activating protein factor under study is calmodulin.

Table 1. Effect of Ca^{2+} and calmodulin-like protein factor on nitrate reductase activity from *Amaranthus*

Treatment	Activity (%) −Factor	+Factor
Enzyme	100	254
Enz + Ca^{2+} (50 μM)	163	323
Enz + Ca^{2+} (100 μM)	232	345
Enz + Ca^{2+} (200 μM)	209	277
Enz + Ca^{2+} (500 μM)	145	254

The specific activity of the enzyme was 1.2 μmol nitrite produced/mg protein/min.

Table 2. Inhibition of nitrate reductase activity from *Amaranthus* by EGTA and its reversal by Ca^{2+}

Treatment	Ca^{2+} (mM)	Activity
Enzyme	Nil	100
Enz + EGTA (0.1 mM)	Nil	97
Enz + EGTA (0.5 mM)	Nil	93
Enz + EGTA (1.0 mM)	Nil	83
Enz + EGTA (5.0 mM)	Nil	45
Enz + EGTA (5.0 mM)	0.2	89
Enz + EGTA (5.0 mM)	0.5	92
Enz + EGTA (5.0 mM)	1.0	129
Enz + EGTA (5.0 mM)	5.0	150

The specific activity of the enzyme was 1.0 μmol of nitrite produced/mg protein/min.

As a final proof for the involvement of calmodulin in the stimulation of nitrate reductase activity, the commercially available calmodulin from Sigma Chemical Co. was used. The activity of the depleted enzyme in the presence of Ca^{2+} could be stimulated around two fold by the authentic sample of calmodulin (data not presented). This result confirms the conclusion that nitrate reductase is under the regulatory control of calmodulin.

The nitrate reductase complex consists of two parts in plants, *viz.* NADH dehydrogenase and $FMNH_2$-NR which reduces NO_3^- to NO_2^- from reduced flavin mononucleotide (FMN). It was considered of interest to study both the partial reactions to pinpoint the site of calmodulin activation. These studies have shown that the NADH dehydrogenase part of the reaction is not affected by calmodulin. However, the terminal part ($FMNH_2$-NR) concerned with the conversion of NO_3^- to NO_2^- is activated about two times—almost as much as the activation of the total nitrate reductase, i.e. NADH-NR. It therefore appears that the site of action of calmodulin is in the $FMNH_2$-NR portion of the enzyme molecule.

The conclusion that nitrate reductase is regulated by calmodulin and Ca^{2+} has several interesting implications. It explains the general observation that nitrate reductase loses considerable activity during the purification procedures. Although this may partly be ascribed to the loss of molybdenum (Mo) or of the flavin component of the enzyme [13], it is tempting to propose that even under conditions that prevent the loss of these two cofactors, the enzyme activity may be reduced if calmodulin is separated from the enzyme during purification.

Table 3. Effect of addition of calmodulin-like protein factor on EGTA inhibition of the nitrate reductase from *Amaranthus* in the presence and absence of Ca^{2+}

Treatment	−Factor	+Factor
Enzyme	100	191
Enz + EGTA (5 mM)	45	96
Enz + EGTA (5 mM) + Ca^{2+} (5 mM)	125	230

The specific activity of the enzyme used was 1.0 μmol nitrite produced/mg protein/min.

EXPERIMENTAL

Nitrate reductase was isolated from the leaves of *Amaranthus tricolor* using the procedure of ref. [14]. The leaves were homogenized in 50 mM NaPi buffer (pH 8.9) containing 5 mM EDTA and 1 mM dithiotreitol (DTT). The homogenate was filtered through 8 layers of cheese cloth and centrifuged at 10000 *g* for 30 min. The supernatant was subjected to $(NH_4)_2SO_4$ fractionation. The ppt. formed between 30 and 60% saturation, was resuspended in the extraction buffer and was desalted by filtration through Sephadex G-15. The desalted enzyme was bound to the blue sepharose. The bound enzyme was eluted with 0.3 M KNO_3 soln as described in ref. [15] and used for the studies. The enzyme activity was determined by following NO_2^- production [14]. In the reactions where Ca^{2+} was used, 20 mM Tris–HCl, pH 7.5, was used instead of NaPi buffer. The enzyme was pre-incubated for 10 min with the individual chemicals or the protein factor for studying their effects. Partial activities of the NR complex, i.e. NADH dehydrogenase and $FMNH_2$-NR, were determined by the method of ref. [16], except that instead of FAD reduction, DCIP reduction from NADH was monitored in the case of NADH dehydrogenase.

The calmodulin-like factor was prepared by the procedure described earlier [10]. The leaves of spinach (or winged bean or *Amaranthus*) were homogenized in 20 mM Tris–HCl buffer, pH 7.5, containing 1 mM EDTA. The extract after filtration and centrifugation was heated to 80° for 2 min. It was then cooled rapidly and centrifuged. The supernatant was stirred with DEAE cellulose for 1 hr and centrifuged to sediment the DEAE cellulose. The DEAE cellulose was resuspended in Tris–MES buffer (20 mM, pH 7.5) containing 0.1 mM EGTA and 0.6 M NaCl and was eluted with the saline buffer. The eluate, dialysed overnight against Tris–MES buffer to remove NaCl, was concd. The factor was further purified by chromatography on Sephadex G-200. The fractions eluting around 16–18 k M_r were found to be active. The preparation was tested for its calmodulin activity by examining the stimulation of NAD-kinase [6, 17] and aspartate kinase [10] activities.

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