Essential Arginine Residues in the Nitrate Uptake System from Corn Seedling Roots

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ABSTRACT

Three dicarbonyl reagents were used to demonstrate the presence of an essential arginine residue in the NO$_3^-$ uptake system from corn seedling roots (Zea mays L., Golden Cross Bantam). Incubation of corn seedlings with 2,3-butanedione (0.125–1.0 millimolar) and 1,2-cyclohexanedi none (0.5–4.0 millimolar) in the presence of borate or with phenylglyoxal (0.25–2.0 millimolar) at pH 7.0 and 30°C resulted in a time-dependent loss of NO$_3^-$ uptake following pseudo-first-order kinetics. Second-order rate constants obtained from slopes of linear plots of pseudo-first-order rate constants versus reagent concentrations were $1.67 \times 10^{-2}$, $0.68 \times 10^{-2}$, and $1.00 \times 10^{-2}$ millimolar per minute for 2,3-butanedione, 1,2-cyclohexanedione, and phenylglyoxal, respectively, indicating the faster rate of inactivation with 2,3-butanedione at equimolar concentration. Double log plots of pseudo-first-order rate constants versus reagent concentrations yielded slope values of 1.031 (2,3-butanedione), 1.004 (1,2-cyclohexanedi none), and 1.067 (phenylglyoxal), respectively, suggesting the modification of a single arginine residue. The effectiveness of the dicarbonyl reagents appeared to increase with increasing medium pH from 5.5 to 8.0. Unaltered $K_m$ and decreased $V_{max}$, in the presence of reagents indicate the inactivation of the modified carriers with unaltered properties. The results thus obtained indicate that the NO$_3^-$ transport system possesses at least one essential arginine residue.

In natural and agricultural soils, nitrate is the most prevalent form of inorganic nitrogen and is believed to be absorbed by plant roots through protein channels or carriers, which may be coupled to the proton gradient generated by the plasma membrane H$^+$-ATPase (3, 15, 16, 23). The capacity of nitrate uptake can be induced several fold from a constitutive low level by supplying nitrate to previously nitrate-starved plants such as corn (8), wheat (9), and barley (19). Inhibitors of RNA and protein synthesis prevent induction of nitrate uptake capacity (8), thus formation of the putative nitrate carrier apparently requires protein synthesis.

The function of a number of amino acid residues in proteins has been studied by chemical modification techniques and much useful information has been obtained. It has been clearly demonstrated that the positively charged guanidinium groups of arginine residues are involved in anion recognition and binding for proteins acting on anionic substrates and cofactors (18, 21). Most of the evidence has been obtained through the use of arginine-specific reagents which react co-

valently and cause changes in biological functions. Such a probe, PGO$^2$, has been found to inhibit sulfate and chloride equilibrium exchange across the red blood cell membrane (27, 29). The dicarbonyl compounds, BD and CHD, in the presence of borate, have been reported to inactivate sulfate transport in resealed erythrocyte ghosts (10, 28). Recently, PGO has been shown to inhibit nitrate transport in corn roots (5, 15, 16). In previous experiments, we have further confirmed the involvement of arginy1 residues in nitrate uptake by using two other arginine-specific reagents, BD and CHD, in the presence of borate (17).

The inhibition of sulfate exchange across red cell membrane by both PGO and BD exhibited pseudo-first-order kinetics with a reaction order close to one for PGO (10, 30), indicating that an arginine residue is important for the binding of substrate anions. In the case of Neurospora crassa H$^+$-ATPase, experiments with PGO and BD have provided evidence for a single essential arginine residue located at or near the nucleotide binding site (12). Recently, kinetic analyses of the inactivation by BD and PGO have been used to identify essential arginine residues associated with plasma membrane ATPase from red beet (Beta vulgaris L.) storage tissue (6) and mung bean (Vigna radiata L.) seedling roots (11).

In the experiments described below, we have used three dicarbonyl reagents to chemically modify arginine residues and inhibit nitrate uptake in the roots of corn seedlings (Zea mays L., Golden Cross Bantam). In addition, the optimal pH condition for the inactivation by the reagents of nitrate uptake and the influence of inhibitors on kinetic parameters of the nitrate uptake system have been examined. Kinetic analyses have been used to study the mechanism of inactivation of nitrate uptake by the dicarbonyl reagents. The results obtained indicate that the nitrate transport system possesses at least one essential arginine residue.

MATERIALS AND METHODS

Chemicals

PGO, BD, CHD, and nitrate reductase (EC 1.9.6.1 from Aspergillus) were purchased from Sigma. All other reagents were of analytical grade. Stock solutions of BD, CHD, and PGO were freshly prepared just before use.

Plant Materials

Corn caryopses (Zea mays L., Golden Cross Bantam) were germinated on two layers of paper towels saturated with 0.6

2 Abbreviations: PGO, phenylglyoxal; BD, 2,3-butanedione; CHD, 1,2-cyclohexanedi none; RFW, root fresh weight.
mm Ca(H₂PO₄)₂ at 25°C in the dark for 2 d in a growth chamber. Uniform seedlings were selected and placed for an additional 3 d on two layers of cheesecloth stretched over a 2-L polyethylene beaker containing N-free medium (0.5 mm KH₂PO₄, 0.5 mm K₂HPO₄, 0.25 mm K₂SO₄, 0.4 mm MgSO₄, 0.6 mm Ca(H₂PO₄)₂, 46.4 μM H₂BO₃, 9.2 μM MnSO₄, 0.3 μM CuSO₄, 0.8 μM ZnSO₄, 0.1 μM Na₂MoO₄, and 5 μg/mL FeEDTA). The nutrient solution was aerated and maintained at 25°C in the dark. On the 5th d, the seedlings were induced for 4 h with 5 mm Ca(NO₃)₂ by adding 0.5 μM stock to the growth medium.

Incubation with Inhibitors and Uptake Experiment

The induced seedlings were selected and placed into aerated 0.1 mm Ca(H₂PO₄)₂ solution. Batches of 10 seedlings about 1.5 g RFW were wrapped with cotton and inserted into 50 mL test tubes containing 0.1 mm Ca(H₂PO₄)₂, and then into test tubes containing aerated 0.5 mm Ca(NO₃)₂ solution for about 20 min. Uptake was started by transferring the seedlings to 44 mL standard uptake or incubation solution (prewarmed to 30°C from room temperature) containing 0.5 mm Ca(NO₃)₂ in 5 mm borate/Hepes (BD and CHD) or 2 mm Mes/Tris (PGO) pH 7.0, and various concentrations of inhibitors as indicated in the figure legends. The tubes were kept, with gentle aeration, in a water bath maintained at 30°C and 0.6 mL aliquots were removed from duplicate tubes at intervals as indicated in the figure legends. In an alternative two-step procedure, batches of seedlings were selected into 50 mL test tubes containing 0.1 mm Ca(PO₄)₂ and then transferred to 44 mL aerated solution that contained 0.5 mm Ca(PO₄)₂, 5 mm borate/Hepes, or 2 mm Mes/Tris (pH 7.0), and various concentrations of inhibitors for 20, 40, 60, and 80 min. The seedlings were rinsed and transferred to 0.5 mM Ca(NO₃)₂ solution for 20 min and uptake was started by transferring to 44 mL solution containing 0.5 mM Ca(NO₃)₂ and 5 mm borate/Hepes or 2 mM Mes/Tris (pH 7.0) for 20 to 30 min. At the end of the experiment, roots were excised below the seeds for RFW determination. All the experiments were repeated at least twice with similar trends exhibited and a representative set of data is shown in this report.

Effects of pH on Inhibition of Uptake

To assay the pH effect on inhibition, the incubation or uptake medium contained 0.5 mm Ca(NO₃)₂, 5 mm borate/Mes (pH 5.5–6.5), or 5 mm borate/Hepes (pH 7.0–8.0) for incubation with BD and CHD or 2 mm Mes/Tris (pH 5.5–8.0) for incubation with PGO, and various concentrations of inhibitors as indicated in the figure legends. Aliquots were taken for nitrate assay at the end of the incubation.

Influence of Inhibitors on Kinetic Parameters of Uptake System

After pretreatment in 0.5 mm Ca(NO₃)₂ solution for 20 min, batches of corn seedlings were transferred to 44 mL of uptake medium containing 5 mm borate/Hepes (pH 7.0), 0 and 0.5 mm BD or 1.0 mm PGO, and various concentrations of Ca(NO₃)₂ (0–1.0 mM) for 80 min at 30°C. Aliquots were taken for nitrate assay at the end of the incubation.

Measurement of Nitrate Uptake

For experiments involving BD and CHD or the reagents used in two-step procedure, the disappearance of nitrate between successive samplings was determined using a modification of the spectrophotometric procedure of Cawse (1).

Briefly, 0.2 mL uptake solution was made to 0.45 mL with deionized H₂O and 0.05 mL of 10% (w/v) sulfamic acid was added. After 2 min, 0.75 mL of 7% (v/v) perchloric acid was added and the absorption was measured at 210 nm. For samples from the PGO incubation medium, nitrate was assayed with nitrate reductase supplied by Sigma. In this procedure, nitrate is converted to nitrite which was determined using a combination of the procedures of Ida and Morita (7) and Stewart and Orebamjo (22). The assay mixture contained in a final volume of 1 mL, 0.4 mL of 0.8 mm fresh-made methylviologen in 250 mm potassium phosphate buffer (pH 7.0), 0.1 unit of nitrate reductase in 0.1 mL of 100 mm potassium phosphate (pH 7.0), 0.2 mL of nitrate uptake solution or the desired standards, 0.3 mL of 34.5 mm sodium dithionite in 71.4 mm sodium bicarbonate in open test tubes. The reaction was started by adding the freshly prepared sodium dithionite in sodium bicarbonate and incubated at 30°C for 1 h, at which time the reaction was terminated by vigorously vortexing. One mL each of 1% (w/v) sulfanilamide in 1.5 N HCl and 0.02% (w/v) N-1-naphthylethylenediamine dihydrochloride was then added and the absorption of the diazo color was read at 540 nm after standing for 20 min. Zero time samples from medium without inhibitors (control) or with inhibitors exhibited identical OD readings and fivefold dilution resulted in no possible inhibition of nitrate reductase from Aspergillus. Net uptake was calculated and expressed as μmol/g RFW.

RESULTS AND DISCUSSION

Optimal Pretreatment

To ensure reproducibly high depletion rates during the relatively short sampling intervals, seedlings were exposed to 10 mm nitrate for 4 h before commencing the studies. This exposure period has been shown to be adequate to induce the nitrate uptake system (5, 8). Excess nitrate on the surface and in the free space of the induced roots was depleted by incubating the seedling roots in calcium phosphate solution. When these induced and washed seedlings were transferred to the 1 mm nitrate uptake medium, there was a rapid influx of nitrate during the first 20 min of exposure to the uptake medium. Similar rapid entry of nitrate into corn roots has been observed by Jackson et al. (8). The transient rapid uptake is attributed to the entry of nitrate into the cell wall matrix and thus does not necessarily represent cellular uptake. To eliminate the confounding influence of the rapid influx on the study of uptake, it was necessary to preexpose the induced and washed roots of the corn seedlings to 1 mm nitrate solution for 20 min before transferring to uptake incubation medium.

Influence of pH on Inhibition of Uptake

The reaction rates of the dicarboxyl inhibitors with arginine increase as the pH is raised. It is generally concluded that this
Influence of pH on inhibition of corn seedling nitrate uptake by BD and CHD (A) or PGO (B). The seedlings were incubated for 100 min at 30°C and various pH values in medium containing 0.5 mM Ca(NO₃)₂, 5 mM borate/Mes (pH 5.5–6.5), or 5 mM borate/hepes (pH 7.0–8.0) for incubation with BD and CHD or 2 mM Mes/Tris (pH 5.5–8.0) for incubation with PGO, and 0 and 1 mM BD or 4 mM CHD or 2 mM PGO.

The kinetic properties of the nitrate uptake system were studied by incubating the seedlings with various concentrations of nitrate in the presence or absence of 0.5 mM BD or 1.0 mM PGO. In the presence of inhibitors, the $K_m$ values for nitrate were not altered in comparison to the controls, but the $V_{max}$ values decreased from 4.242 (control) to 2.299 (0.5 mM BD) and 1.823 (1.0 mM PGO) µmol/g RFW/hr, respectively (Table I). This indicates that the binding of the dicarboxyl reagents to arginine residues results in complete inactivation of the modified carrier proteins rather than the formation of a modified protein having different properties. A similar observation has been found in mung bean plasma membrane H⁺-ATPase when modified by BD (11). Within the concentration range used, we only observed one-phase kinetics with a progressive levelling off of the nitrate uptake rate above 0.5 mM nitrate. Chantarotwong et al. (2) have made similar observations with nitrate uptake in barley.

**Inactivation of Nitrate Uptake by BD and CHD**

BD and CHD have been demonstrated to act as arginine-specific reagents by very similar reaction mechanisms. Borate buffer enhances the rate of reaction and also stabilizes the product (20). We have demonstrated in previous experiments that 2 mM borate greatly increased the effectiveness of BD

**Table I. Influence of Inhibitors on Kinetic Parameters of Nitrate Uptake System**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$K_m$</th>
<th>$V_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.159</td>
<td>4.242</td>
</tr>
<tr>
<td>0.5 mM BD</td>
<td>0.152</td>
<td>2.299</td>
</tr>
<tr>
<td>1.0 mM PGO</td>
<td>0.170</td>
<td>1.823</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$k_1$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD</td>
<td>1.674</td>
<td>1.031</td>
</tr>
<tr>
<td>CHD</td>
<td>0.682</td>
<td>1.004</td>
</tr>
<tr>
<td>PGO</td>
<td>1.004</td>
<td>1.067</td>
</tr>
<tr>
<td>PGO (two-step method)</td>
<td>1.070</td>
<td>1.024</td>
</tr>
</tbody>
</table>

For 1 mM BD, 4 mM CHD, and 2 mM PGO, respectively. It was found that in control roots increasing the pH above 7.0 decreased the uptake of nitrate, and similar patterns of pH profiles were exhibited regardless of different buffer systems used. To eliminate the deleterious influence of high pH on nitrate uptake, all subsequent kinetic studies were conducted at pH 7.0.

**Influence of Inhibitors on Kinetic Parameters of Uptake System**

The nitrate uptake system was incubated with various concentrations of nitrate in the presence or absence of 0.5 mM BD or 1.0 mM PGO. In the presence of inhibitors, the $K_m$ values for nitrate were not altered in comparison to the controls, but the $V_{max}$ values decreased from 4.242 (control) to 2.299 (0.5 mM BD) and 1.823 (1.0 mM PGO) µmol/g RFW/hr, respectively (Table I). This indicates that the binding of the dicarboxyl reagents to arginine residues results in complete inactivation of the modified carrier proteins rather than the formation of a modified protein having different properties. A similar observation has been found in mung bean plasma membrane H⁺-ATPase when modified by BD (11). Within the concentration range used, we only observed one-phase kinetics with a progressive levelling off of the nitrate uptake rate above 0.5 mM nitrate. Chantarotwong et al. (2) have made similar observations with nitrate uptake in barley.
and CHD (17), and therefore, 5 mM borate used in the present studies was stoichiometrically sufficient to obtain inhibition. For the types of kinetic analyses, protein modification is usually carried out as a two-step procedure (12). In the first step, the protein is modified by the reagents for an appropriate time. In the second step, treatment with the reagents is stopped and the effect of modification on the initial rate of an activity is examined. In our experiments, similar kinetic parameters were obtained using the two-step method or procedures with simultaneous derivitization and uptake (Table I). The majority of the studies were conducted using the latter method. When corn seedlings were incubated with BD (0.125–1.0 mM) or CHD (0.5–4.0 mM) in borate buffer at pH 7.0 and 30°C, the time-dependent loss of nitrate uptake activity followed pseudo-first-order kinetics, as indicated by semilog plots of percent residual activity versus time (Fig. 2, A and B). The pseudo-first-order rate constants were then estimated from the slopes of the plots according to the equation 

\[ \ln A = \ln A_0 - k_1 t \]

where \( A \) is the percent residual activity at time \( t \) and \( k_1 \) the pseudo-first-order constant. After exposure for 100 min, the activity of the transport system was reduced to 6.5% and 8.6% (the actual data before constructing the line of best fit) of the control value at 1 mM BD and 4 mM CHD, respectively. Further incubation with the inhibitors resulted in complete inactivation of the uptake system (or at least to a level lower than the sensitivity range of our assay). Half-maximal inhibition with 1 mM BD and CHD occurred at 37.6 and 97.7 min, respectively, indicating the faster rate of inactivation with BD at equimolar concentrations.

According to Kasher et al. (12), the second-order rate constant (\( k_2 \)), which represents the rate of inactivation on a molar basis, can be obtained from slopes of linear plots of pseudo-first-order rate constants (\( k_1 \)) versus reagent concentrations. When data from Figure 2 were plotted this way, the second-order rate constants were found to be 1.674 \( \times \) 10^{-2} and 0.682 \( \times \) 10^{-2} mm^{-1} min^{-1} for BD and CHD, respectively (Table I). The reaction order with respect to the inhibitor was determined from double log plots of 1000/\( t^2 \) as a function of reagent concentration as described by Marcus et al. (14) and Kasher et al. (12). In such a case, a straight line should be obtained with a slope value equal to \( n \), the average number of molecules of inhibitor reacting with or binding to each active unit of the enzymes or proteins to produce an inactive complex. When the data from Figure 2 were analyzed in this way, slope values of 1.031 (BD) and 1.004 (CHD) were obtained through regression analysis (Fig. 3, A and B). The same \( n \) values were also obtained using the methods described by Levey and Ryan (13) and Gildensoph and Briskin (6). The results obtained with the two reagents indicate that at least one molecule of BD or CHD was necessary to cause the inactivation of the uptake system under the above incubation condition. Similar reaction order values of approximately 1.0 with respect to BD have also been reported for enzymes with anionic substrates such as plasma membrane H^+ -ATPase from Neurospora crassa (12), red beet storage tissue (6), and mung bean roots (11).

**Inactivation of Nitrate Uptake by PGO**

PGO is a dicarbonyl reagent which unlike BD and CHD does not require borate for its reactivity and forms an irreversible complex with the arginine residues. As with BD and CHD, inactivation with PGO also resulted in pseudo-first-order time course kinetics (Fig. 4A). Half-maximal inactivation was obtained at 66.7 min when corn seedlings were treated with this reagent at 1 mM. Linear primary plots of pseudo-first-order rate constant versus PGO concentration yielded a second-order rate constant of 1.004 \( \times \) 10^{-2} min^{-1} (Table I), which is slower than that of BD but faster than that of CHD.

When pseudo-first-order rate constants were used to construct double log plots, a slope value of 1.067 was obtained for corn seedlings (Fig. 4B), thus confirming the observations with BD and CHD that inactivation of the uptake system resulted from the modification of one arginine residue in the putative nitrate transport protein. The anion transport protein of red blood cells (30) and H^+ -ATPase from Neurospora
the stoichiometry of the binding of PGO and other dicarbonyl reagents to the nitrate transporter. On the basis of the presented kinetic data, it appears that if two PGO molecules bind to each essential arginine residue then the association of the first molecule of PGO is the rate limiting step in controlling inhibition (20, 24). The fact that the binding of the second PGO molecule to the arginine is not critical to inhibition is supported by the impairment of reaction observed at low PGO concentration when the stoichiometry of reaction with arginine apparently falls to 1:1 (12, 20).

It has been suggested that anion transport may be coupled to the proton gradient generated by the plasma membrane H+-ATPase (3, 15, 16, 23). Others have questioned the involvement of H+-ATPase in anion transport. Thus, in early studies performed with corn roots (25) and Chara corallina crassa (12) and ATPase from red beet storage tissue (6) also show inactivation with PGO exhibiting a reaction order of approximately unity.

The results of double log plots of log 1000/t' for a as a function of reagent concentration produced straight lines for all of the inhibitors. The slopes (n) of the plots for BD, CHD, and PGO were 1.031, 1.004, and 1.067, respectively. As discussed by Marcus et al. (14), the value for n represents the average number of molecules of inhibitor binding to each active site during inactivation. Therefore, these analyses indicate that one molecule of the respective inhibitors react with one essential arginine residue of the nitrate carrier protein. Similar observations on the bases of kinetic analyses have been reported for H+-ATPase (6, 11, 12) and the anion transporter in red blood cells (30). However, labeling has indicated that PGO reacts with arginine residue in a 2:1 stoichiometry except in the presence of borate or at low PGO concentration (12, 20). Unfortunately, the unavailability of purified carrier precludes the conducting of labeling experiments to determine

\[
\log 1000/t'_1 = n \log([I] - \log k_1'), \quad n = 1.031, r = 0.984
\]

\[
\log 1000/t'_1 = n \log([I] - \log k_1'), \quad n = 1.004, r = 0.979
\]

\[
\log 1000/t'_1 = n \log([I] - \log k_1'), \quad n = 1.067, r = 0.994
\]
H+-ATPase by diethylstilbestrol. More recently, McClure et al. (15, 16) reported that with longer exposure to the inhibitor nitrate uptake was prevented and the hyperpolarization associated with anion uptake eliminated. Fluorescein isothiocyanate was more inhibitory (85%) than PGO (35%) at 1 mM on H+-ATPase from corn root plasma membrane fraction, but fluorescein isothiocyanate in contrast to PGO did not inhibit nitrate uptake (5). Despite these contradictions, the investigations of McClure et al. (15, 16) show a consistent relationship between the cotransport of nitrate and protons. The dicarboxyl reagents, BD and PGO, have been demonstrated to inhibit plasma membrane H+-ATPase activity (6, 11, 12). It is therefore possible that the inhibition of nitrate uptake by BD, CHD, and PGO observed in the present study may be due to direct interaction with the nitrate transporter or an indirect effect in response to an impairment of the H+-ATPase or by action at both sites.

The concentrations of BD and PGO required to inhibit H+-ATPase in isolated vesicles (6) or solubilized from the plasma membrane (6, 11) are 10-fold greater than those found to effectively inhibit nitrate uptake in the present study. However, McClure et al. (15, 16) reported 1 mM PGO which inhibited nitrate uptake also abolished electrical responses. It was suggested that PGO could bind initially with arginine in the plasma membrane, with longer exposure it was reasoned that sufficient PGO could accumulate in the cytoplasm to bind to residues of the plasma membrane H+ pump that are exposed to the cytoplasm. Such an explanation does not appear to apply in the present investigation. In other experiments, we have isolated microsomal pellets from intact roots which were incubated with 0.5 mM BD and 1 mM PGO in borate buffer for 2 h. Although the treated roots showed restricted nitrate uptake, the total ATPase and plasma membrane vanadate-sensitive H+-ATPase or K+-stimulated ATPase activities from homogenates and microsomal pellets were not inhibited (17). Significantly, Wieth et al. (27) reported that in red blood cells the membranes are readily permeable to PGO. Thus, the accumulation of PGO to levels to be inhibitory to the H+-ATPase is unlikely. Furthermore, the stoichiometry of inhibition of nitrate uptake and binding of the inhibitors determined from n values suggest the involvement of only one inhibitory site rather than the two that would be required for interaction with both the carrier protein and the H+-ATPase.

In conclusion, the inhibitory effects of the three dicarboxyl reagents on nitrate uptake are consistent with an involvement of an essential arginine residue in nitrate transport in plant cells. The kinetic analyses indicate the modification of a single arginine residue by all of the reagents. The information demonstrating the involvement of an arginine residue in nitrate uptake is consistent with studies on anion transport which have identified an essential arginine residue required for anion transport in red blood cells. It is likely that an arginine residue is essential for nitrate uptake by the, so far unidentified, nitrate carrier of plant roots.

LITERATURE CITED


