Oxidation of Reduced Pyridine Nucleotide by a System Using Ascorbate and Hydrogen Peroxide from Plants and Algae

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Received for publication February 16, 1981 and in revised form June 20, 1981

ABSTRACT

A NAD(P)H oxidizing system (NAAP) was detected and partially purified from leaves of spinach and Sedum praealtum, seeds and leaves of pea and cells of the green and red algae which oxidized NAD(P)H in the presence of ascorbate and H₂O₂.

The partially-purified spinach system had substrate Kᵣ values of 5 micromolar for NADH, 50 micromolar for H₂O₂, and 300 micromolar for 1-ascorbic acid at the pH optimum of 6.8. NADH was a better electron donor than NADPH. Among other electron donors, isoascorbic acid had considerable activity but hydroquinone and resorcinol had only weak activities. The enzyme was inhibited by cyanide, a,a’-dipyridyl, and mono- di-thiol reagents. Inhibition by thiol-reagents was partially restored by Fe²⁺ as was enzymic activity lost following dialysis against buffer.

Subcellular localization studies with spinach and S. praealtum leaves indicated that a portion of the cell’s NAAP was in the chloroplast fraction. Photosynthetic conditions resulted in a decrease in this activity solubilized from spinach and S. praealtum chloroplasts. The presence of 3-(3,4-di-chlorophenyl)-1,1-dimethyurea or Fe²⁺ in the incubation medium eliminated the light-mediated inhibition of NAAP.

NAAP may function in the recycling of NAD(P)H generated in the dark within the chloroplast. Inasmuch as all preparations of NAAP contained ascorbate peroxidase activity, the data do not rule out the possibility that NAAP is the same protein as ascorbate peroxidase or, alternatively, a combination of ascorbate peroxidase and some other enzyme.

Of the soluble peroxidases, the most characterized is the Euglena gracilis enzyme which is a hemoprotein (21) like many other plant peroxidases (18). Euglena AAP is an unusual hemoprotein in that it is stabilized by sucrose and by ferrous sulfate. In addition, the enzyme has high affinities for ascorbate and H₂O₂, with Kᵣ values of 410 and 56 µM, respectively (21). The insoluble spinach enzyme has similar substrate affinities and also appears to be a hemoprotein.

We report here on a peroxidative activity similar to AAP in peas, spinach, Sedum praealtum and algae. This enzyme system designated as NAAP uses NAD(P)H, ascorbate, and H₂O₂ as substrates. Furthermore, this system can be solubilized from the chloroplast and is subject to photoregulation.

MATERIALS AND METHODS

Plants and Algae. S. praealtum and peas (Pisum sativum var. Progress No. 9) were grown in the greenhouse. Sedum and peas were grown in a vermiculite-soil mixture or in vermiculite, respectively. Spinach (Spinacia oleracea) was cultivated under controlled conditions (12 h light at 22°C and 12 h dark at 18°C) in a vermiculite-soil mixture. The algae with the exception of Chlamydomonas reinhardtii F-60 (9) and Euglena gracilis (10) were grown photoautotrophically with CO₂ as the carbon source.

Cell-Free Preparations. About 2 kg of fully expanded spinach leaves or pea leaves taken from 2-week-old plants were washed, cut into segments after the midribs were removed, and homogenized with a Waring Blender in 50 mM Hepes-NaOH (pH 7.0) at 4°C. Pea seed which were imbibed in water overnight was also disintegrated in the blender. After the homogenate was filtered through cheesecloth, the homogenate was centrifuged at 10,000g for 10 min to remove debris. The homogenate was then fractionated by solid (NH₄)₂SO₄ precipitation. The portion of the homogenate insoluble in 50 to 80% (NH₄)₂SO₄ was resuspended in 50 mM Hepes-NaOH (pH 7.0) and absorbed onto calcium phosphate gel. After elution from the gel with 0.1 M K-phosphate, this solution free of catalase was used as the source of higher plant ascorbate peroxidases. The result of treatment with (NH₄)₂SO₄ and gel was an approximate 5-fold purification. Cell-free algal preparations were made by grinding the organisms with an equal weight of aluminum oxide for 5 min in a chilled mortar and pestle. About 5 to 10 ml of 50 mM Hepes-NaOH buffer (pH 7.0) was added and the slurry was centrifuged at 25,000g for 10 min to remove the cellular debris. The clear supernatant fluid was used for enzyme assays.

Enzyme Activity. NAAP was assayed at 25°C as the oxidation of NAD(P)H at 340 nm. The 1-ml assay volume contained 50 mM Hepes-NaOH (pH 7.0), 0.2 mM NAD(P)H, and 5 mM ascorbate. After a baseline of absorbance change was established, a reading was taken 1 min after the addition of 0.18 mM H₂O₂. AAP was assayed at 25°C as the oxidation of ascorbate at 265 nm (15). The 1-ml assay volume contained 50 mM Hepes-NaOH (pH 7.0) and 0.1 mM ascorbate. The reaction was started by adding 0.18 µM

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1 Supported by National Science Foundation Grant PCM 79-22612 and Department of Energy Grant EY-76-S-3231-14.
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4 Abbreviations: AAP, ascorbate peroxidase which catalyzes the reaction involving ascorbate and H₂O₂; NAAP, reoxidation of reduced pyridine nucleotide with a preparation involving ascorbate, H₂O₂ and NAD(P)H. Most experiments were carried out with NADH-NAAP.
H₂O₂. Malate dehydrogenase and triose-P dehydrogenase were measured as the oxidation of NADPH at 340 nm. Malate dehydrogenase assays contained, in a final 1.1 ml volume: 50 mM Hepes-NaOH (pH 7.0), 0.11 mM NADPH, and 0.22 mM oxaloacetate to initiate the reaction. Triose-P dehydrogenase assays contained in a final 1.0 ml volume: 50 mM Hepes-NaOH (pH 7.6), 0.2 mM NADPH, 1 mM glyceraldehyde-3-P, 1 mM MgCl₂, 1 mM DTT, and 30 units glyceraldehyde-3-P kinase. Glucose-6-P dehydrogenase was measured as the reduction of NADPH at 340 nm. The 1.0-ml assay medium contained 50 mM Hepes-NaOH (pH 7.6), 1 mM MgCl₂, 2 mM glucose-6-P, and 0.5 mM NADPH. Cytochrome oxidase was measured as the oxidation of reduced Cyt c at 550 nm (24). Catalase was monitored as the breakdown of H₂O₂ at 240 nm (16).

Chloroplast Isolation. Spinach leaves were chopped into small pieces and ground (1.5 s) with a Virtis model 45 in a grinding medium of 50 mM Hepes-NaOH (pH 6.8), 330 mM sorbitol, 2 mM disodium EDTA, 1 mM MgCl₂, and 1 mM MnCl₂. The homogenate was filtered through two layers of Miracloth (Chicopee Mills, Inc., Milltown, NJ) with the filtrate being centrifuged at 750g for 1 min. The chloroplast pellet was resuspended in fresh grinding medium and recentrifuged. The final washed chloroplast pellet was resuspended in a minimal volume of grinding medium and used for chloroplast incubations. Chloroplasts were at least 50% intact as measured by ferricyanide-stimulated O₂ evolution.

In some experiments, spinach chloroplasts were prepared from protoplasts according to the procedure of Nishimura et al. (17). In this procedure, the protoplasts were disrupted by passage through a syringe into the grinding medium used for the spinach leaves.

S. praealtum leaves were sliced and digested enzymatically to yield protoplasts as described by Spalding and Edwards (23). The protoplasts were gently broken by passage through a syringe and the homogenate obtained was centrifuged in a flotation gradient to yield a chloroplast fraction. These chloroplasts were at least 90% intact as measured by ferricyanide-stimulated O₂ evolution.

Chemical Assays. Protein content was determined according to Bradford (5), using standards of BSA. Chl was determined according to Arnon (4).

RESULTS

Comparison of NAAP and AAP Activities. NAAP and AAP (Table I) coexisted in roughly equal levels in the leaf and algal preparations with the possible exception of pea seed, spinach thylakoids, and C. vulgaris (light and dark grown). Only a trace of AAP was detected in pea seed while the particulate matter from the spinach chloroplast was free of NAAP and with respect to Chlorella, the ratio of NAAP:AAP averaged about 2:1. NADH at 0.2 mM was twice as effective as NADPH at an equal concentration. In general, NADH was twice as effective as NADPH. Solubilized activities of AAP and NAAP from spinach and pea leaves could be concentrated but not separated by (NH₄)₂SO₄ fractionation and treatment with calcium phosphate gel, indicating the two enzyme activities may be associated with the same protein. Further attempts to resolve the higher plant enzymes by alcohol precipitation or by DEAE chromatography resulted in a total loss of peroxidase activity. Attempts to determined stoichiometry for NAAP were unsuccessful due to the presence of AAP activity.

Effect of Substrate Concentration on the Rate of Spinach NAAP. The apparent Km values were 5 μM for NADH, 50 μM for H₂O₂, and 300 μM for ascorbate (Fig. 1). The Vmax for ascorbate was 1.07 μmol/mg protein · min, a value which contrasts with 0.47, 0.17, and 0.06 when ascorbate was replaced by 5 mM isascorbate, hydroquinone, or resorcinol.

pH Profile of Spinach NAAP. The pH optimum for enzyme activity was around 6.8, both for NADH (Fig. 2) and NADPH (data not shown)-linked activity. At pH 8.0 enzyme activity was less that 25% of the rate at pH 6.8.

Effect of Temperature on the Stability of NAAP and AAP.

Table I. Activity of NAAP and AAP From Different Sources

<table>
<thead>
<tr>
<th>Plant Sources</th>
<th>NADH</th>
<th>NADPH</th>
<th>AAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol NAD(P)H/mg protein·min</td>
<td>μmol ascorbate/mg protein·min</td>
<td></td>
</tr>
<tr>
<td>Pea leaf</td>
<td>2.95</td>
<td>2.01</td>
<td>4.32</td>
</tr>
<tr>
<td>Spinach leaf</td>
<td>1.59</td>
<td>0.64</td>
<td>2.51</td>
</tr>
<tr>
<td>S. praealtum leaf</td>
<td>2.13</td>
<td>0.90</td>
<td>5.32</td>
</tr>
<tr>
<td>Pea seed</td>
<td>0.25</td>
<td>0.14</td>
<td>0.03</td>
</tr>
<tr>
<td>Spinach chloroplast particles</td>
<td>ND</td>
<td>ND</td>
<td>1.16</td>
</tr>
<tr>
<td>E. gracilis Z</td>
<td>0.15</td>
<td>0.04</td>
<td>0.32</td>
</tr>
<tr>
<td>Scenedesmus obliquus</td>
<td>0.23</td>
<td>0.14</td>
<td>0.16</td>
</tr>
<tr>
<td>Chlamydomonas reinhardii F-60</td>
<td>0.16</td>
<td>0.20</td>
<td>0.68</td>
</tr>
<tr>
<td>Chlamydomonas reinhardii</td>
<td>1.00</td>
<td>0.16</td>
<td>1.77</td>
</tr>
<tr>
<td>Chlorella vulgaris (dark)</td>
<td>0.19</td>
<td>0.15</td>
<td>0.09</td>
</tr>
<tr>
<td>Chlorella vulgaris (light)</td>
<td>0.12</td>
<td>0.10</td>
<td>0.04</td>
</tr>
<tr>
<td>Porphyridium cruentum</td>
<td>1.01</td>
<td>0.71</td>
<td>2.17</td>
</tr>
</tbody>
</table>

* Not detected.

Fig. 1. Lineweaver-Burk plot of spinach NADH-NAAP as a function of ascorbate, H₂O₂, and NADH concentrations. Reaction mixtures were as described in Figure 2 using Hepes-NaOH as a buffer. The concentration of ascorbate (upper), H₂O₂ (middle), and NADH (lower) were varied as indicated.

NAAP isolated from the spinach leaf or from the spinach chloroplast was unaffected by heating for 5 min at 40°C. Heating at 60°C for 5 min completely inactivated both preparations. On the other hand, only 50% of the chloroplasts membrane-bound AAP was destroyed after 5 min at 60°C.

Inhibition Studies with Spinach and Pea NADH-NAAP. Several
Fig. 2. pH profile of spinach NADH-NAAP. Reaction was monitored by following the oxidation of 0.2 mM NADH in the presence of 5 mM ascorbate and 0.18 mM H$_2$O$_2$ in the appropriate buffer (100 mM) as indicated: (●), phosphate; (□), Hepes buffer; (△), Tricine buffer.

Table II. Inhibitors of Spinach NADH-NAAP

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>KCN, 0.1 mM</td>
<td>30</td>
</tr>
<tr>
<td>2,3-Dimercaptopropanol, 25 μM</td>
<td>30</td>
</tr>
<tr>
<td>L-Cysteine, 0.2 mM</td>
<td>50</td>
</tr>
<tr>
<td>Mercaptoethanol, 0.3 mM</td>
<td>30</td>
</tr>
<tr>
<td>DTT, 0.3 mM</td>
<td>90</td>
</tr>
<tr>
<td>α,α'-Dipyridyl, 0.2 mM</td>
<td>105</td>
</tr>
<tr>
<td>Tiron, 0.2 mM</td>
<td>99</td>
</tr>
<tr>
<td>Diethylthiocarbamate, 0.2 mM</td>
<td>18</td>
</tr>
<tr>
<td>Pyrophosphate, 10 mM</td>
<td></td>
</tr>
</tbody>
</table>

Organizations with the 5-fold purified spinach NADH-NAAP (Table II). Cyanide at 0.1 mM inhibited enzyme activity by 70%. Incubation with sulfhydryl reagents such as 2,3-dimercaptopropanol, DTT, cysteine, and mercaptoethanol reduced enzyme activity but 2 mM reduced glutathione had no effect. The degree of inhibition by these —SH containing compounds was dependent upon the order of substrate addition. For example, 25 μM 2,3-dimercaptopropanol and 300 μM 2-mercaptoethanol inhibited the peroxidase by 70% if the thiol compound was incubated for 5 min with the enzyme preparation prior to the addition of 5 mM ascorbate. If the ascorbate was added first, the inhibitory effect of the thiol reagents were reduced to about 10%.

Several oxidized compounds were tested to determine if they could reverse the dithiol inhibition of NADH-linked ascorbate peroxidase. Dehydroascorbate, oxidized glutathione, or oxidized DTT at 1 mM had no effect on the peroxidase. When NAAP was incubated for 5 min with 0.1 mM DTT it was inhibited 60% and the inhibition value was reduced to 30% on addition of 1 mM dehydroascorbate but 1 mM oxidized DTT or 1 mM oxidized glutathione was without effect.

The pea leaf NADH-linked peroxidase showed similar responses to the reagents listed in Table II. Furthermore, the pea leaf peroxidase was unaffected by inhibitors like arsenite (1.0 mM), iodoacetamide (5 mM), and N-ethylmaleimide (5 mM) indicating that a sulphydryl group is not required for enzyme activity. The enzyme was inhibited 50% by a 5 mM Na$_3$O$_4$ but little affected by 1 mM Na$_2$S.

The Fe$^{2+}$ chelator, α,α'-dipyridyl at 0.2 mM inhibited by 70% spinach NAAP while the chelators of Fe$^{3+}$ (Tiron) and of Cu$^{2+}$ (diethyldithiocarbamate) were completely ineffective at 0.2 mM levels. The addition of 10 mM pyrophosphate inhibited the enzyme by 80%.

Effect of Fe$^{2+}$ on Spinach NADH-NAAP. Since inhibition by pyrophosphate and cyanide indicated participation of a metal ion and the finding with α,α'-dipyridyl specified Fe$^{2+}$, this property of NAAP was studied in some detail.

Half of the NADH-linked peroxidase activity was lost after 1 h dialysis of 1 ml enzyme against 2 liters of 50 mM Hepes-NaOH (pH 7.0). This loss was increased to 90% if 0.1 mM DTT was included in the dialyzing fluid. The activity of the dialyzed enzyme was completely restored on addition of 10 to 25 μM Fe$^{2+}$. Higher concentrations (50–100 μM) of Fe$^{2+}$ severely inhibited. Bound iron in the form of ferrodoxin or ferrocyanide was not active in restoring activity lost during dialysis. Other ions such as Zn$^{2+}$, Mg$^{2+}$, Co$^{2+}$, Ni$^{2+}$, MoO$_4^{2-}$ at 25 μM had either no effect or were inhibitory.

Cellular Localization of NAAP and AAP. The data recorded in Table I indicated the presence of AAP but not to NAAP bound to the spinach chloroplast thylakoids. On lysis of spinach protoplasts, about 20 to 30% of the total NADH-linked ascorbate peroxidase and AAP was found to be associated with a 650 g pellet which contained the bulk of the NADPH-glyceraldehyde-3-P dehydrogenase, a chloroplast marker (Table III). Another interesting feature of Table III is the finding that repeated washing of this fraction in an isosmotic medium did not change the specific activity on a Chl basis of either NADPH-glyceraldehyde-3-P dehydrogenase or the ascorbate specific activity of catalase, the microbody marker and Cyt c oxidase, the mitochondrial marker decreased several fold.

Photoregulation of Ascorbate Peroxidases. The amount of NAAP and AAP solubilized from a chloroplast preparation was

![Table III. Enzyme Activities Associated with Particles Sedimenting at 650 g](image-url)
found to be dependent upon whether the chloroplasts were photosynthesizing or kept in the dark prior to organelle rupture. For example, NADH-NAAP (Fig. 3A) and AAP (Fig. 3B) declined to 20 to 30% in photosynthesizing spinach chloroplasts when compared to the enzymic activity in dark-kept chloroplasts. The inhibitory effect of light was reversible inasmuch as NADH-NAAP and AAP increased once the light was turned off. The light-dark response was the same regardless of whether chloroplast incubation and lysis was carried out at pH 7.1 or 8.1.

Light treatment also reduced the level of NADH-NAAP (Fig. 4A) and AAP (not shown) solubilized from S. praealtum chloroplasts. NADPH-dependent malate dehydrogenase (Fig. 4B) was assayed as a control in order to demonstrate parallel photoregulation of a chloroplastic enzyme which is activated in the light and inhibited in the dark.

Several compounds eliminated the effect of light on spinach chloroplastic NADH-NAAP. Enzymic activity was not inhibited in the light when chloroplasts were incubated with 10 μM DCMU, a concentration which completely blocked photosynthesis. The addition of 25 μM FeSO₄ to the chloroplast incubation mixture also eliminated the light-mediated inhibition of NADH-NAAP. In a typical experiment with spinach chloroplasts, the rates of μmol/mg Chl·h of NADH-NAAP were: chloroplasts in dark for 10 min, 4.9; chloroplasts photosynthesizing for 10 min, 2.5; chloroplasts kept in dark for 10 min + 25 μM FeSO₄, 11.2; and chloroplasts photosynthesizing for 10 min + 25 μM FeSO₄ 12.9.

**DISCUSSION**

In every plant assayed, whether the test material was leaf, seed, chloroplast or four green and one red alga, both AAP and NAAP were found together. The results of our study do not rule out the possibility that NAAP is the same protein as AAP, or alternatively, a combination of AAP and some other protein. Thus, it is a possibility that we are measuring ascorbate peroxidase followed by a reoxidation of NAD(P)H with the resulting dehydroascorbate. Several features of the NAAP enzyme may be mentioned which distinguished it from other peroxidase preparations which have been reported.

Beecroft (6) has described a NAD oxidation system in cucumber extracts which is stimulated by ascorbate, extremely sensitive to sulphydryl reagents and cyanide, but lacks of inhibition by catalase (data similar to NAAP. Horseradish peroxidase has been reported to oxidize reduced pyridine nucleotide in the presence of catalytic amount of certain phenols and Mn²⁺ and the reaction is sensitive to cyanide and catalase (1). NAAP clearly differs from horseradish peroxidase since ascorbate and Fe²⁺ do not substitute for phenol and Mn²⁺ in the reaction described by Akazawa and Conn (1) and furthermore, their preparation was insensitive to pyrophosphate.

Our preparation of spinach NAAP has properties very similar to those reported for the spinach chloroplastic membrane-bound enzyme of Groden and Beck (11), and the soluble, cytoplasmically-located enzymes of Kelly and Latzko (15) and Skigeoka et al. (21, 22) obtained from peas and E. gracioliss, respectively. For example, in addition to reasonably similar Km values for ascorbate and H₂O₂, NAAP mimics the spinach, pea and *Euglena* AAP with respect to sensitivity to cyanide (11, 15, 21, 22), insensitivity to sulphydryl inhibitors (21) and the role of Fe²⁺ (21). In contrast, the pH profiles differ inasmuch as the optimum for spinach NAAP is 6.5 which compares with 6.2 for *Euglena* (21) and 7.5 to 8.0 for spinach chloroplastic membrane-bound peroxidase (11).

NAAD-NAAP is presumably a hemoprotein like other plant peroxidases (18) and this is indicated by cyanide inhibition. But loss of enzymic activity by the Fe³⁺ chelator, a,a-dipyridyl, and restoration of activity by Fe³⁺ following dialysis indicates that noncovalently-bound iron may also play a role. It is also possible that the inhibition of NAAD-NAAP by DTT may, in part, be associated with the heavy metal chelation property of the dithiols. The AAP purified from *Euglena* by Shigeoka et al. (21) has the interesting property of extreme lability in the absence of Fe³⁺. This instability would appear to be associated with the loss of Fe³⁺ during purification of their enzyme and is similar to the instability we find for NAAD-NAAP. The ferrous form of iron has been implicated in a number of plant enzymes, notably ascorbate and γ-aminolevulinate synthase but its cofactor or structural role in those enzymes (19) and in NAAD-NAAP remain to be determined.

A portion of the cells’ NAAD-NAAP is located in the chloroplasts of spinach and *S. praealtum* (Table III, Fig. 4). Both NAAD-NAAP and its AAP activity are lower following incubation of
spinach and S. praealtum chloroplasts under photosynthesizing conditions. An inhibition by light has been reported for glucose-6-P dehydrogenase from spinach chloroplasts (3) and pea leaf phosphofructokinase, a cytoplasmic enzyme (13). The photo-regulation observed here for NADH-NAAP is consistent with the hypothesis advanced by others (2, 7) that light-generated reducing agents regulated metabolism by inhibiting and stimulating certain enzymic steps. Light-mediated activation or inactivation of enzyme activity appears to involve a thiol-disulfide reaction on the modulatable protein and can usually be duplicated in vitro by DTT. Thus, inactivated glucose-6-P dehydrogenase (3), phosphofructokinase (13), and NADH-NAAP reported here are all inhibited by DTT but at least with respect to NADH-NAAP, inhibition by the dithiol does not seem to be compatible with the ineffectiveness of iodoacetamide, N-ethylmaleimide, and arsenite.

Light-mediated inhibition of NADH-NAAP is abolished when the photosynthesizing chloroplasts are incubated with 10 μM DCMU. This finding is readily explained on the need of a reductant generated during the photochemical act. On the other hand, the apparent regulatory role of Fe²⁺ on NADH-NAAP is not readily explained on the basis of current concepts of interaction between the photochemical act and enzymic activity.

Finally, inasmuch as H₂O₂ has been shown to inhibit photosynthetic activity in isolated chloroplasts (14, 20), then chloroplast-localized ascorbate peroxidase activity may be a protective mechanism to counter H₂O₂ production which is not destroyed by diffusion into other cellular compartments. Indeed, Groden and Beck (11) have proposed that the physiological role of plastocianic peroxidatic activity is H₂O₂ detoxification which is produced in the chloroplast by photosynthetic O₂ reduction. Our observations that NADH-NAAP functions optimally at a pH (Fig. 2), similar to that of the stromal pH of darkened chloroplasts (12) and that the soluble chloroplast peroxidatic activity is inhibited during photosynthesis (Figs. 3 and 4) are consistent with the notion that this enzyme functions primarily during dark metabolism in the chloroplast. We suggest that a possible role for NAAP in the chloroplast would be to recycle reduced pyridine nucleotide generated from such metabolic activity as starch breakdown either by glycolysis or the oxidative pentose-P cycle.

Acknowledgments—We thank Nancy O'Donoghue, René Gfeller, and Dwight Peavey for growing the algal cultures and to Dr. J. A. Schif for the culture of E. gracilis.

LITERATURE CITED


