Oxidation of NAD(P)H in a Reconstituted Spinach Chloroplast Preparation Using Ascorbate and Hydrogen Peroxide

Received for publication July 14, 1981 and in revised form October 12, 1981

Yoke Wah Kow, Douglas A. Smyth, and Martin Gibbs
Institute for Photobiology of Cells and Organelles, Brandeis University, Waltham, Massachusetts 02254

ABSTRACT

The conversion of fructose-1,6-bisphosphate to glyceraldehyde-3-phosphate (PGA) was studied in a reconstituted spinach (Spinacia oleracea L.) chloroplast preparation to determine whether a chloroplast-localized NAD(P)H-oxidizing system (Kow, Smyth, Gibbs 1982 Plant Physiol 69: 72-76) is present. By adding NADPH and ascorbate, both NAD(P)H and H2O2 could be used as a coupling enzyme in the recycling of NAD(P)H. The rate of PGA formation was monitored as an indicator of NAD(P)H generation. With NAD as a cofactor, ascorbate enhanced PGA formation, and an additional increase resulted upon addition of glucose-glucose oxidase, a H2O2-generating enzyme. This increase in PGA formation due to H2O2 was eliminated by the addition of catalase. With NADP and ferredoxin as cofactors, the recycling of NADPH apparently was catalyzed both by ferredoxin-NADP reductase coupled to O2 and by the NAD(P)H-oxidizing system.

It was concluded that the oxidation of NAD(P)H by a system using ascorbate and H2O2 can serve as a means of recycling NAD(P)H but that another reaction involving ascorbate and NAD(P)H may also function in the spinach chloroplast.

RESULTS AND DISCUSSION

The recycling of the reduced pyridine nucleotides by the NAD(P)H-oxidizing system was monitored using the chloroplast pellet suspension fortified with substrate levels of fructose-1,6-bisP and catalytic amounts of NAD and NADP per ferredoxin. The chloroplast pellet was shown in previous publications (8, 9) to be a particulate preparation free of chloroplast envelope but containing the enzymes of the reductive pentose-P cycle, including fructose-1,6-bisP aldolase and triose-P dehydrogenase. We have also shown previously (10) that the NAD(P)H-oxidizing system is chloroplastic, and, here, we demonstrate that it is an integral part of the particle resulting from rupturing the intact chloroplasts in 15 mM MgCl2. Thus, we utilized this preparation both for the conversion of NAD(P) to NAD(P)H through the oxidation of glyceraldehyde-3-P generated from fructose-1,6-bisP to PGA and for the reoxidation of the reduced pyridine nucleotides. To satisfy the substrate requirements of the NAD(P)H-oxidizing system, ascorbate was added to both NAD- or NADP-dependent preparations, but the means of supplying H2O2 differed with the cofactor. The reoxidation of reduced ferredoxin by O2 generated H2O2 in the reaction mixture with NADP was the cofactor, but glucose-glucose oxidase was utilized when NAD was the cofactor, since NADH, in contrast to NADPH, is known not to couple effectively with NADPH-ferredoxin reductase. Therefore, the rate of PGA formation reflects the rate of NAD(P)H oxidation. Finally, inasmuch as the activity of glucose-6-P dehydrogenase was negligible

1 Supported by National Science Foundation Grant PCM 7682157 and Department of Energy Grant DE-AC02-76-ER03251.
2 Present address: Department of Biochemistry, University of Wisconsin, Madison, WI 53706.
3 Present address: Department of Agronomy and Soils, Washington State University, Pullman, WA 99164.
Table 1. Factors Affecting PGA Formation in NAD- and NADP-Dependent Systems

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Rate of PGA Formation ((\mu mol/mg \text{ Chl-h}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>With NAD</td>
<td>With NADP</td>
</tr>
<tr>
<td>Control</td>
<td>0.9</td>
</tr>
<tr>
<td>+ Glucose oxidase, glucose</td>
<td>1.0</td>
</tr>
<tr>
<td>+ Ascorbate</td>
<td>1.6</td>
</tr>
<tr>
<td>+ Ascorbate, catalase</td>
<td>3.2</td>
</tr>
<tr>
<td>+ Ascorbate, glucose oxidase, glucose</td>
<td>2.4</td>
</tr>
<tr>
<td>+ Ascorbate, glucose oxidase, glucose, catalase</td>
<td>1.8</td>
</tr>
</tbody>
</table>

in our chloroplast preparation, the oxidative pentose-P pathway was eliminated as a source of NAD(P)H.

Table I shows the rate of PGA formation in the NAD-dependent system. Addition of glucose-glucose oxidase did not increase PGA formation over the control, indicating that NADH is stable in the presence of \(H_2O_2\) under these conditions. Addition of ascorbate, alone, roughly doubled the rate over that of the control. The combination of ascorbate and the \(H_2O_2\)-generating mixture resulted in an approximate tripling of the rate over the control, and inclusion of catalase to this reaction mixture partially eliminated the effect of glucose-glucose oxidase, confirming the requirement of \(H_2O_2\) for NADH oxidation. The enhancing effect of ascorbate, alone and in the presence of catalase, on PGA formation suggests that the NAD(P)H-oxidizing system may function with an oxidant other than \(H_2O_2\) or that our chloroplast preparation contains another means of oxidizing NADH which requires ascorbate.

It has been reported from this laboratory (9) that the oxidation of NADPH by a particle resulting from rupturing the intact chloroplast in 15 mM \(MgCl_2\) could be coupled to \(O_2\) through NADP-ferredoxin reductase, since reduced ferredoxin is nonenzymically oxidized by atmospheric \(O_2\) with the formation of \(H_2O_2\). Inasmuch as this pathway of \(O_2\) reduction is of possible physiological significance (12), it seemed important to compare its contribution (pathway a) with respect to the NAD(P)H oxidizing system (pathway b) in the recycling of NADPH. As seen in Table I, the control rate of PGA formation is increased 3-fold when NAD is replaced by ferredoxin and NADP, apparently reflecting the functioning of pathway a. The addition of ascorbate resulted in a 25% increase in PGA formation over the control rate (pathway b), while inclusion of catalase lowered the ascorbate-dependent rate to 10% over the control rate, indicating a limited requirement for \(H_2O_2\). The presence of glucose-glucose oxidase did not affect ascorbate-dependent PGA formation, suggesting a sufficient supply of \(H_2O_2\) generated by reduced ferredoxin or perhaps a limiting level of the pyridine nucleotide-oxidizing system in our chloroplast preparation. The latter is probably valid, since the specific activity of glyceraldehyde-3-P dehydrogenase is at least 15- to 20-fold in excess of that of the NAD(P)H-oxidizing system (8).

Finally, the data presented in the table support the hypothesis that the NAD(P)H-oxidizing system represents a means of oxidizing NADH and, perhaps to a lesser extent, NADPH. The relative contribution of the NAD(P)H-oxidizing system as compared to other enzymes, such as ferredoxin-NADP reductase and malate dehydrogenase, in recycling reduced pyridine nucleotides remains unresolved. In contrast to malate dehydrogenase, which requires a shuttle mechanism linking the chloroplast with the mitochondrion (5), the NAD(P)H-oxidizing system, ascorbate (4), and \(H_2O_2\) are available within the organelle.

We present (Fig. 1) a tentative scheme to account for the cycling of chloroplastic pyridine nucleotides required for substrate oxidation such as starch (13). The presence of reduced glutathione and glutathione reductase within the chloroplast (3) provides a reaction for recycling dehydroascorbate, an endproduct of the NAD(P)H-oxidizing reaction. The chloroplast appears to lack dehydroascorbate reductase, but the nonenzymic reaction between dehydroascorbate and reduced glutathione has been described (3). Finally, the scheme seems adequate to explain NAD(P)H oxidation within the darkened chloroplast, since the NAD(P)H-oxidizing system is light-inactivated and functions optimally at pH 7 (10), the pH of the stroma in the dark (7).

LITERATURE CITED

3. FEVER CH, B HALLWELL 1976 The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133: 21–25
8. KOW KW 1980 The study of regulatory processes in intact and reconstituted chloroplast systems. PhD thesis. Brandeis University, Waltham, MA