Photoactivation of NAD Kinase through Phytochrome

PHOSPHATE DONORS AND COFACTORS

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ABSTRACT

The specificities of phosphate donors and the effects of metal chelating agents and divalent metal ions on NAD kinase activation by phytochrome far-red-absorbing form (Pfr) were examined. ATP was the most efficient phosphorylating agent. Uridine 5'-triphosphate, cytidine 5'-triphosphate (CTP), inosine 5'-triphosphate, and guanosine 5'-triphosphate in this order caused significant phosphorylation in the dark. Under red light, striking photoactivation of NAD kinase was obtained with ATP and subsequently CTP.

In the presence of exogenous Mg²⁺, which is required for NAD kinase activity, α-nitroso-β-naphthol, cyanide, and dimethylglyoxime, strongly inhibited the activation by red light without affecting the level of NAD kinase in the dark.

Of the divalent cations tested with the KCN-treated phytochrome preparation, only Co²⁺ was effective for photoactivation of NAD kinase. Even when Mg²⁺, an essential component of NAD kinase, was added to the assay system, the further addition of Co²⁺ was required for the activation of NAD kinase by Pfr.

NAD kinase (EC 2.7.1.23) catalyzes the formation of NADP from NAD, ATP, and Mg²⁺. The product, NADP, has a different physiological role from NAD (10). Previous studies have revealed that NAD kinase in a partially purified phytochrome preparation is activated by red light and reversed by far red light (4, 5) and that the simultaneous additions of a redox cofactor, PMS²⁻ or FMN or methylene blue, and of kinetin are effective for photoactivation of NAD kinase through phytochrome (6, 10).

The present paper describes efficient phosphorylating agents and the effects of metal chelating agents and exogenous metals on the photoactivation of NAD kinase. Co²⁺ plays a key role in the photoactivation of NAD kinase through phytochrome, while Mg²⁺ is essential for NAD kinase itself. Cobalt ion seems to act on the phytochrome.

MATERIALS AND METHODS

Phytochrome was partially purified from Avena coleoptiles using DTT, as described previously (6). The methods for assay of NAD kinase and light illumination were also as described previously (5, 6).

The assay mixture for measurement of NAD kinase activity in the phytochrome preparation contained 0.8 μmole of NAD, 3.3 μmole of ATP (GTP, ITP, CTP, or UTP in the place of ATP in the experiments of Table I), 7 μmole of MgCl₂, 6 μmole of nicotinamide, 1 μmole of kinetin, 20 μmole of PMS, 100 μmole of tris-HCl buffer, pH 7.6, and the phytochrome preparation (0.25 mg of protein) in a final volume of 1 ml at 0 C. In the experiments of Table III, 10 μmole of metal chloride or MoO₃ was further included. The assay mixtures were preilluminated with far red light for 10 min at 0 C. The assay mixtures were then rapidly warmed on a 30 C water bath to start the reaction and kept under red light for 30 min at 30 C. The control run was kept in the dark at 30 C. The reaction was stopped by heating the mixtures in boiling H₂O for 2 min.

The red and far red light used of 800 and 12,000 ergs cm⁻² sec⁻¹, respectively, were as described previously (5, 6). In the experiments of the far-red far reversibility, the assay mixtures were exposed to alternate 5-min periods of red and far red light at 0 C, the reaction was started by warming rapidly on a 30 C water bath.

The NADP produced was then assayed as follows: 0.5 ml of 0.2 m tris-HCl buffer, pH 7.6, 0.2 ml of 0.1 m MgCl₂, 0.8 ml of 1.2 mm sodium dichlorophenindophenol, 0.20 ml (0.636 mg protein) of isocitric dehydrogenase, and 0.2 ml of NADPH diaphorase (0.102 mg protein) were added. Before the assay reaction was started, no absorption change (λ₁ 690 nm, λ₂ 606 nm) was observed. The assay reaction was started by the addition of 0.1 ml of 20 mm allo-free isocitrate. The reduction of dichlorophenindophenol was followed for 3 min in a Hitachi 356 two wavelength spectrophotometer with λ₁ and λ₂ set a 690 and 606 nm, respectively (5, 6). Then, a definite amount of authentic NADP (0.01 ml of standard solution) was added. The NADP content was calculated from the initial reduction velocity of dichlorophenindophenol and its increment by adding a definite amount of NADP (8).

The sodium salts of ATP, GTP, ITP, CTP, and UTP were purchased from Sigma and chelating agents from Nakarai Chemicals, Kyoto.

RESULTS AND DISCUSSION

Specificity of Phosphate Donor. ATP (final concentration 3.3 mm) was the most efficient phosphorylating agent. However, similar concentrations of UTP, CTP, ITP, and GTP, in this order, caused significant phosphorylation in the dark (Table I). Striking photoactivation of NAD kinase (see red/dark ratio in Table I) was obtained with ATP and with CTP, and subsequently with UTP. No nucleotide tested was without significant effect.

Effects of Chelating Agents on Photoactivation of NAD Kinase,
Table I. Specificity of Phosphate Donors in Photoactivation of NAD Kinase

The reaction mixtures contained 7 mM Mg\(^{2+}\).

<table>
<thead>
<tr>
<th>Phosphorylating Agent</th>
<th>NAD Kinase Activity</th>
<th>Red/Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3 mM ATP</td>
<td>118.6 ± 1.8</td>
<td>2.02</td>
</tr>
<tr>
<td>3.3 mM UTP</td>
<td>118.6 ± 1.8</td>
<td>2.02</td>
</tr>
<tr>
<td>3.3 mM CTP</td>
<td>118.6 ± 1.8</td>
<td>2.02</td>
</tr>
<tr>
<td>3.3 mM ITP</td>
<td>118.6 ± 1.8</td>
<td>2.02</td>
</tr>
<tr>
<td>3.3 mM GTP</td>
<td>118.6 ± 1.8</td>
<td>2.02</td>
</tr>
</tbody>
</table>

Table II. Effects of Chelating Agents on Photoactivation of NAD Kinase

The reaction mixtures contained 7 mM Mg\(^{2+}\).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>NAD Kinase Activity</th>
<th>Red/Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no reagent)</td>
<td>118.6 ± 1.8</td>
<td>2.02</td>
</tr>
<tr>
<td>Sodium diethyldithiocarbamate</td>
<td>118.6 ± 1.8</td>
<td>2.02</td>
</tr>
<tr>
<td>α,α'-Dipyridyl</td>
<td>118.6 ± 1.8</td>
<td>2.02</td>
</tr>
<tr>
<td>Azide</td>
<td>118.6 ± 1.8</td>
<td>2.02</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>118.6 ± 1.8</td>
<td>2.02</td>
</tr>
<tr>
<td>α-Nitroso-β-naphthol</td>
<td>118.6 ± 1.8</td>
<td>2.02</td>
</tr>
<tr>
<td>Cyanide</td>
<td>118.6 ± 1.8</td>
<td>2.02</td>
</tr>
<tr>
<td>Dimethylglyoxime</td>
<td>118.6 ± 1.8</td>
<td>2.02</td>
</tr>
</tbody>
</table>

Sodium diethyldithiocarbamate, α,α'-dipyridyl, azide, and 8-hydroxyquinoline at final concentrations of 10^{-4} M at pH 7.6 did not inhibit the photoactivation. The same concentrations, of α-nitroso-β-naphthol, cyanide, and dimethylglyoxime inhibited the NAD kinase activity under red light to the level in the dark (Table II). Thus the latter chelating agents inhibited the photopotentiated portion of the NAD kinase activity.

Effects of Various Cations. The phytochrome preparation was mixed with KCN at a final concentration of 2 mM. Saturated ammonium sulfate solution, adjusted to pH 7.8 with ammonium hydroxide, was then added to give 40% saturation at 0°C. The mixture was stirred for 15 min and centrifuged for 10 min at 20,000g. The resulting precipitate was dispersed in 20 ml of 20 mM potassium phosphate buffer, pH 7.8, containing 2 mM DTT and 6.5 g of Sephadex G-25 powder were added. The mixture was filtered through two layers of filter paper under a centrifuge force (5,000g, 10 min). This process removed metal essential for the photoactivation (7). The NAD kinase activity of the filtrate was measured under red light and in the dark.

As shown in the control experiment of Table III, even after removing the chelator, KCN, NAD kinase was not activated by red light. Of the metals tested, only cobalt ion was effective for recovery of photoactivation (Table III). The NAD kinase activities in Table III were measured in the presence of 7 mM Mg\(^{2+}\), since NAD kinase is known to require this ion for activity (9).

Even in the presence of Mg\(^{2+}\), Co\(^{2+}\) was required for photoactivation and 10^{-8} M CoCl\(_2\) caused maximal activation by red light without affecting the dark level of NAD kinase in the KCN-treated phytochrome preparation (Fig. 1). In the presence of 20 μM PMS, 1 μM kinetin, 3.3 mM ATP, 10 mM Co\(^{2+}\), and 7 mM Mg\(^{2+}\), photoactivation of NAD kinase in the KCN-treated phytochrome preparation by red light was counteracted by successive far red light illumination. The red-far red reversibility observed with the KCN-treated phytochrome preparation was essentially identical to that reported earlier with the nontreated preparation (6), indicating the participation of phytochrome.

Cobalt Content of Phytochrome Preparation. The cobalt content of the phytochrome preparation was measured using an atomic absorption spectrophotometer (Hitachi Model 208). The cobalt content per mg of protein increased with increase in purification of phytochrome.

Original extract, preparation with ammonium sulfate treatment, and eluate from Sephadex G-200 column in the procedure.

Fig. 1. Effect of Co\(^{2+}\) concentration on red light activation of NAD kinase in the KCN-treated phytochrome preparation in the presence of PMS, kinetin, and Mg\(^{2+}\). NAD kinase activities were assayed under red light (—○—) and in the dark (—●—).

![Graph](image-url)
of phytochrome purification (6) contained 0.008 ± 0.0004, 0.015 ± 0.0015, and 0.020 ± 0.0013 µg of cobalt per mg protein, respectively. Mumford and Jenner (3) found 0.5 to 1 µg of aluminum, 0.05 to 0.1 µg of copper per mg protein in a phytochrome preparation highly purified from oat seedlings. The cobalt content was lower than these metals. Cobalt ion in low level is essential for phytochrome for its effect in enzyme regulation (Table III and Fig. 1). The cobalt content of the phytochrome preparation corresponds approximately to the optimum concentration of cobalt for activation (Fig. 1).

Miller (1, 2) found that expansion of etiolated bean leaf disks was greater after treatment with both cobalt and light than after either treatment alone and suggested a close relation between the effects of light and cobalt. It should be noted that cobaltous ion is significant for the function of phytochrome.

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LITERATURE CITED