Light Activation of Purified Aconitase by Washed Thylakoid Membranes of Pea (*Pisum sativum* L.)

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**ABSTRACT**

Purified aconitase, an iron-sulfur protein, from either beef heart mitochondria or pig heart can be activated fully by light when combined with washed thylakoid membranes from pea (*Pisum sativum* L.) chloroplasts. The light activation of the enzyme does not require any other additive or cofactor and is sensitive to 3-(3,4-dichlorophenyl)-1,1-dimethyl urea, 2,6-dichlorophenol-indophenol, ferricyanide, and methyl viologen, indicating that the photoreceptor transport system of the thylakoid membranes, and in particular, photosystem I, is involved in the process of activation. Light activation of the enzyme is also markedly inhibited when the thylakoid membranes are treated with sulfite or arsenite, and abolished totally when the membranes are treated with Zwittergent, suggesting that the light effect mediator involved in the light modulation of chloroplastic enzymes mediates the activation of purified aconitase also.

Light modulates the activity of several enzymes in plants (1, 3, 6). These are mostly chloroplastic, but at least two are cytoplasmic. Light modulation is a reductive process involving posttranslational modification of enzyme molecules. Mechanistically, in peas, light modulation is mediated by the LEM system, in which the thylakoid-bound protein, namely the LEM, catalyzes the transfer of electrons from the photoelectron transport chain to the soluble protein mediating this reaction. The enzyme probably undergoes a reductive covalent modification and/or perhaps a disulfide-sulfhydryl conversion, which alters its catalytic property (2). The precise mechanism by which light modulates the activity of the enzymes outside the chloroplast remains to be elucidated.

Aconitase (EC 4.2.1.3) is a HiPIP (non-heme) of mitochondria (10, 13). The enzyme as isolated exhibits an EPR signal (G = 2.01) typical of a [3Fe-3S] center (9), and possesses acid labile sulfur in iron-sulfur clusters (8, 10, 13). The reversibly inactivated protein can be activated by FeEDTA and a reducing agent, cyanide or DTT. The precise mechanism of activation of aconitase is not clear. It has been suggested that the reduction of the iron-sulfur cluster of aconitase (by either dithionite or ferrous ion) leads to a conformational change which exposes the thiol group(s), and that the actual activation of the enzyme involves disulfide-sulfhydryl conversion at the active site (5, 13, 14). More recently, it has been suggested that the [3Fe-3S] cluster is converted to a [4Fe-4S] cluster during activation (9).

We report here data to show that purified aconitase is activated by visible light in a thylakoid-enzyme combined system without the addition of FeEDTA or DTT.

**MATERIALS AND METHODS**

The isolation of chloroplasts from 12- to 14-d-old pea (*Pisum sativum* L., cv Little Marvel) shoots and the preparation of washed thylakoid membranes was done as described elsewhere (12). The thoroughly washed thylakoid membranes were finally resuspended in 50 mM Hepes(Na+), pH 7.2, containing 2 mM MgCl2, 1 mM EDTA(Na+), and 10 mM KCl (the Hepes buffer), which had been bubbled with N2.

Purified aconitase from beef heart mitochondria was kindly provided by Professor H. Beinert. Aconitase (type I) from pig heart was obtained from Sigma Chemical Co. The beef heart aconitase, which had been kept frozen in liquid N2, was thawed and supplemented with BSA (final concentration, 1 mg protein/ml) and stored frozen at −70°C. The enzyme was diluted, whenever needed, with the cold Hepes buffer which had been bubbled with N2. The pig heart aconitase was dissolved in the Hepes buffer supplemented with BSA (1 mg protein/ml) and stored in small aliquots at −70°C.

**Chemical Activation of the Enzyme.** The enzyme (final concentration, 12.5 mg protein/ml) was incubated with 1 mM ferrous ammonium sulfate (freshly prepared) and 5 mM DTT at 25°C. Small aliquots were withdrawn at the indicated time intervals and added directly to the enzyme assay medium.

**Light Activation of the Enzyme.** The enzyme was combined with washed thylakoid membranes of pea (500 μg protein of purified beef heart enzyme and 80 μg total Chl equivalent of thylakoids in a volume of 40 μl) and exposed to white light (6,000 ft-c) at 25°C. Aliquots were withdrawn at different time intervals and added directly to the enzyme assay medium.

**RESULTS AND DISCUSSION**

Light and pea leaf thylakoids can elicit activation of purified beef heart mitochondrial aconitase as do Fe2+ and DTT (Fig. 1).
Fig. 1. Kinetics of light activation of purified aconitase by washed thylakoid membranes of pea. The purified beef heart mitochondrial aconitase was combined with washed thylakoid membranes and illuminated (6,000 ft-c) at 25°C (in control assay, 500 μg of enzyme protein was combined with 80 μg total Chl equivalent of membranes in a total volume of 40 μl). The solution was made 3 μM in DCMU; 2 mM in methyl viologen, 250 μM DCPIP, or 1 mM in ferricyanide 1 min prior to illumination. The control and DCMU samples were 2% in ethanol. The washed thylakoid membranes were treated with 5 mM Zwittergent as described earlier (12). For chemical activation, the enzyme was incubated with ferrous ammonium sulfate and DTT at 25°C in air. The final concentration of aconitase was 12.5 mg protein/ml. The spectrophotometric assay was done under an atmosphere of air. Chemical activation of the enzyme: (●), in 1 mM Fe²⁺ and 5 mM DTT; (□), in 1 mM Fe²⁺, 5 mM DTT, and 1 mM ferricyanide. Light activation of the enzyme: (Δ), light control; (○), light in 2 mM methyl viologen; (×), light in 250 μM DCPIP; (●), light in 1 mM ferricyanide; (V), light in 3 mM DCMU; (★), light with Zwittergent-treated thylakoid membranes supplemented with 8 mM sodium ascorbate plus 0.5 mM DCPIP; (▲), dark; (○), enzyme alone (no activation). Similar results were obtained with pig heart aconitase.

This is the first time that an enzyme from a non-plant source has been shown to be activated by a chloroplastic activation system. This is also the first report of activation of a purified, nonchloroplastic enzyme by a thylakoid-enzyme recombined system.

Light activation of aconitase is totally abolished by DCMU, suggesting that the Chl of the thylakoid membranes is the photosensitizer and the photoelectron transport chain is involved in the reductive process of activation of the enzyme. This is corroborated by the considerable inhibition of light activation of aconitase by DCPIP, methyl viologen, and ferricyanide. Although ferricyanide, an oxidant, inhibited chemical activation of the enzyme about 30%, it totally abolished the light-mediated activation of the enzyme, as did DTNB and NEM (Figs. 1 and 2). While the inhibition of light activation of the enzyme by the Hill oxidants DCPIP and ferricyanide is suggestive of the involvement of photoelectron transport in general, the inhibition by methyl viologen (redox, −0.65 V) points out that the reducing side of PSI, in particular, is involved in the light activation of the purified aconitase.

The LEM which is involved in the light activation and inactivation of various enzymes (2, 3) is apparently involved in the photoactivation of aconitase also, as the activation, after 2 min, of the purified enzyme was inhibited 43% when the thylakoid membranes were treated with 20 μM sulfite in the dark (Fig. 2). Likewise, 2 min activation was inhibited 51% when the thylakoids were exposed to 1 mM arsenite in the light (Fig. 2). Further, the washed thylakoid membranes totally lose the property of photoactivating the purified aconitase after treatment with Zwittergent, which removes the LEM from the membrane (12) (Fig. 1).

Similar light activation by the washed thylakoid membranes of pig heart aconitase (type I, Sigma) could be observed when the enzyme concentration was about 10 times more than that of the beef heart mitochondrial aconitase reported here. Light activation...
of the enzyme from both sources could not be observed at all if the enzyme protein concentration was lowered by a factor of three, the significance of which is not understood.

It is pertinent to note that the thioredoxin, thioredoxin-reductase system, a proposed alternative system for light activation of chloroplastic enzymes (6), does not activate purified beef heart mitochondrial aconitase (5). The washed thylakoid membranes used in the present studies do not contain measurable Fd, a necessary component of that system, although they do contain some Fd-NADP reductase (4; AH Mohamed, LE Anderson, unpublished). It should be noted that, while deazariboflavin-mediated anaerobic photoactivation of aconitase is possible (14), the occurrence of deazariboflavin in the thylakoid membranes is unknown.

Within the chloroplasts, a soluble stromal factor, the protein modulating factor, acts as a mediator between the activated membrane-bound LEM and the light-modulatable stromal enzymes (4). A somewhat similar, and possibly identical, protein has been isolated and is known to be a HiPIP (7) as is aconitase. Our attempts to replace the protein modulating factor with aconitase in the recombined assay system for light modulation (4) were not successful.

In conclusion, the phenomenon of activation of purified aconitase by washed thylakoid membranes and light, reported here, could provide meaningful clues to the understanding of the activation of aconitase as well as the operation of the LEM system. It seems possible that (a) some modulatable enzymes of the chloroplast might be directly modified by the LEM without the need for a soluble protein modulating factor, and (b) there are LEM analogs in the mitochondria of eukaryotic plants and animals which are reductively activated and which control the activity of aconitase and other mitochondrial enzymes.

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LITERATURE CITED