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Regulation of nitrate assimilation by light in higher plants

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Abstract

Assimilation of nitrate in plants is strictly light dependent. Light regulates the activity of the first enzyme, namely, nitrate reductase, indirectly by blocking the mitochondrial oxidation of NADH, thus making it available for nitrate reduction. Subsequent assimilation of nitrite is carried out in the chloroplasts by nitrite reductase, glutamine synthetase and glutamate synthase which are all light dependent. Recent evidence also suggests that NADH for nitrate reduction is generated by the mitochondrial citric acid cycle dehydrogenases and NAD^+-malic enzyme. It has also been shown that in light, carbon skeleton for citric acid cycle turnover is perhaps provided by 2-carbon compounds generated during photorespiration. Thus, combined activities of chloroplasts, mitochondria, cytoplasm and perhaps peroxisomes regulate nitrate assimilation in plants.

Key words : Nitrate assimilation, regulation by light ATP and mitochondria, source of NADH, higher plants.

1. Introduction

Nitrate is an important source of nitrogen for almost all higher plants[1]. It is assimilated in two steps. The first reaction, *i.e.*, reduction of nitrate to nitrite is catalysed by the enzyme nitrate reductase and the second step, the conversion of nitrite to ammonia is catalysed by the enzyme nitrite reductase. The ammonia thus formed is incorporated into amino acids *via* carbon skeleton derived from CO_2 fixation in light[2,3]. The first step in the nitrate assimilation process is considered to be the rate limiting step and is thus likely to be regulated at different levels.

Several reviews on various aspects of nitrate assimilation have appeared in the past[2,4,5]. However, the precise role of light was not well understood until recently. The physiological reductant for nitrate reduction in plants is also better understood today. The present article reviews the recent developments in these fields.

Abbreviations used : NR, nitrate reductase ; NiR, nitrite reductase ; GS, glutamine synthetase; GOGAT, glutamine-2-oxoglutarate amino-transferase.

2. Localizations of nitrate assimilation

The enzymes involved in the assimilation of nitrate into amino acids are nitrate reductase (E.C. 1.6.6.1), nitrite reductase (E.C. 1.7.7.1), glutamine synthetase (E.C. 6.3.1.2) and glutamine: 2-oxoglutarate amino transferase (E.C. 2.6.1.53). It has been shown that nitrite reductase in green leaves is located in the chloroplasts and the reductant required for its activity is reduced ferredoxin generated in the photosynthetic reactions[6,7]. GS and GOGAT, also chloroplastic enzymes, depend on ATP and reduced ferredoxin, generated during photosynthesis for their activity[8]. Thus, the three enzymes responsible for the assimilation of nitrite into glutamate are present in the chloroplasts and are largely light dependent.

As regards nitrate reductase, it is generally believed that it is present in the cytoplasm[9,10]. However, there are some reports which suggest that it may be loosely associated with the outer membrane of the chloroplast[11]. In spite of these conflicting reports about the localisation of nitrate reductase in plant cells it is generally agreed that NADH present in/or supplied to the cytoplasm is used as a reductant for this enzyme.

3. Inhibition of *in vivo* nitrate reduction by oxygen

The assay of nitrate reductase developed by Klepper *et al*[12] depends on incubation of leaf discs under anaerobic conditions in the dark. Nitrite accumulates in the tissues, because in the chloroplast, nitrite reductase which depends on reduced ferredoxin for activity cannot function in the dark. Sawhney *et al*[13] observed that reduction of nitrate to nitrite during dark incubation is extremely sensitive to oxygen and is completely inhibited at less than one per cent of the normal atmospheric oxygen concentration. It was also observed by them that even in air, NO_3^- could be reduced to NO_2^- provided mitochondrial electron transfer chain is inhibited by specific inhibitors, such as rotenone and amytal. They also found that a brief exposure of wheat leaves to carbon monoxide, an inhibitor of mitochondrial electron transfer chain, at the cytochrome oxidase step, is sufficient to obtain rapid reduction of nitrate to nitrite even in the presence of air. Carbon monoxide effect could be easily reversed by a brief exposure of wheat leaves to light, which dissociates carbon monoxide cytochrome oxidase complex. Subsequently, Canvin and Woo[14] also observed that inhibition of mitochondrial respiration by antimycin A stimulates nitrate reduction in the dark aerobic conditions. The above observations show that inhibition of oxidation of NADH *via* the mitochondrial electron transfer chain is essential for nitrate reduction. In other words there is a competition for the reducing equivalents from NADH between oxygen and NO_3^-. Recently Mann *et al*[15] also observed that oxygen suppresses nitrate reduction and for *in vivo* nitrate reductase assay anaerobic conditions are necessary. They also observed that some respiratory inhibitors and uncouplers are able to partially overcome the oxygen inhibition of nitrate reduction. They suggested that the reversal of oxygen inhibition by these inhibitors and uncouplers could be due to their effect on membranes rather than on respiration as such.

4. Role of light in nitrate assimilation

As mentioned earlier, three enzymes namely, NiR, GS and GOGAT involved in nitrate assimilation are chloroplastic and are strictly dependent on light. Several reports are available which implicated light as a crucial factor for the activity of nitrate reductase also[2,3,4]. Evans and Nason[16] isolated from soybean an NADPH-dependent nitrate-reductase and demonstrated that a reconstituted system, consisting of the enzyme, grana and NADP could reduce nitrate in light. On this basis, they concluded that light is coupled to nitrate reduction *via* an electron transfer system. This implied the location of nitrate reductase within the chloroplast, which is contrary to present knowledge. Later, it was consistently observed that nitrate reductase from almost all higher plants is NADH-specific[12]. The problem was further complicated by the observation of Klepper *et al*[12] that nitrate could be reduced to nitrite by leaf discs even in the dark under anaerobic conditions. They also observed that under dark anaerobic conditions several glycolytic intermediates could stimulate nitrate reduction. They concluded that triosephosphates synthesized during active photosynthesis are metabolised in the cyto-plasm to produce NADH through the glycolytic dehydrogenase. According to them, this NADH is utilized for nitrate reduction. They argued that nitrate reduction is essentially a dark reaction. Free nitrite is rarely detected in leaves kept in dark under aerobic conditions. Thus it was suggested that either nitrate is not reduced in dark or the nitrite formed under physiologcal conditions is rapidly assimilated. The exact role of light was still unknown. However, it was the pioneering work of Canvin and Atkins[17], which conclusively demonstrated that nitrate assimilation in wheat, barley, corn and bean leaves is strictly light dependent. Using $^{15}NO_3^-$ they observed that reduction of $^{15}NO_3^-$ to $^{15}NO_2^-$ ceases abruptly when the light is extinguished. The relation between light and nitrate reduction was rather quite direct and as the enzyme levels in these leaves as such undergo only a slight change in dark the authors were unable to explain the exact mode of action of light in the regulation of nitrate reduction. If nitrate reduction was a dark reaction as suggested by Klepper *et al*[12], the instanta-neous effect of switching off light could not be explained because the reduction of nitrate would have continued even in dark, till the triosephosephates accumulated during photo-synthesis were exhausted.

Under normal physiological conditions when plants are grown in air, free nitrite is rarely detected in either light or dark. Thus reduction of nitrate to nitrite and its sub-sequent reduction to ammonia are tightly coupled. In the dark nitrite cannot be reduced further and nitrate is also not reduced. This type of control mechanism ensured that nitrite, which is highly toxic to plants, does not accumulate to any significant level in the cells.

In light, rapid assimilation of NO_3^- to NO_2^- commences as was observed by Canvin and Atkins[17]. Hence a mechanism must exist which triggers nitrate reduction even in the presence of oxygen (air). This suggests that oxidation of NADH in the mito-chondria is inhibited in light. Recent work reviewed by Heber[18] has clearly shown that

during photosynthesis the cytoplasmic ATP concentration increases considerably on account of transport of triosephosphates synthesized in the chloroplast. It is known that very little direct transport of ATP across the chloroplast membrane takes place since they are almost impermeable to adenine nucleotides. However, as shown by Heber[18], a very rapid indirect transport occurs by the export of triosephosphates, particularly, dihydroxy-acetone phosphate from the chloroplasts. The glycolytic dehydrogenase namely glyceraldehyde-phosphate dehydrogenase generates ATP in the cytoplasm which results in a dramatic increase in the cytoplasmic adenylate energy charge. This in turn is known to inhibit mitochondrial oxidation of NADH. On the basis of this knowledge Sawhney *et al*[12] proposed a switch mechanism to explain the role of light. According to them as mitochondrial respiration is in progress in the dark, NADH is not available for nitrate reduction. In light, when increased adenylate energy charge brings about inhibition of mitochondrial electron transfer chain, nitrate reduction starts, and as such NADH is not oxidised in the mitochondria and is thus available for cytoplasmic nitrate reduction.

5. Physiological source of reductant for nitrate reduction

The source of reductant (NADH) for *in vivo* nitrate reduction has always been a controversial issue. However, the widely accepted view is that of Klepper *et al*[12]. Based on their observation that glycolytic intermediates stimulated dark anaerobic nitrate reduction they had suggested that NADH generated during the reaction catalysed by the cytoplasmic glyceraldehyde-3-phosphate dehydrogenase is utilised for *in vivo* nitrate reduction. They had also observed that citric acid cycle intermediates did not stimulate nitrate reduction *in vivo*. But later Sawhney *et al*[19] reported that pyruvate, phosphenol pyruvate and some citric acid cycle intermediates did stimulate dark *in vivo* nitrate reduction in wheat leaves. The difference in observation of these two groups of workers arose probably because of the different pH of the infiltration medium used by them. Sawhney *et al*[19] used a more acidic pH (pH 5·0), at which the entry of organic acids in the leaf tissue is significantly higher. At pH 7·0, the pH used by Klepper *et al*[12], organic acids do not enter into the leaf tissues in significant amount. Sawhney *et al*[19] also reported that malonate-inhibited dark anaerobic *in vivo* nitrate reduction and this inhibition was partially reversed by fumarate. Based on these observations they suggested that NADH generated subsequent to glycolysis by the citric acid cycle dehydrogenases, in the mitochondria, is also utilised for *in vivo* nitrate reduction. Malonate inhibition of *in vivo* nitrate reduction in sorghum leaves was also reported by Kadam *et al*[20]. These workers and Subbalakshmi *et al*[21] reported that leaves subjected to freezing in liquid nitrogen completely lost *in vivo* nitrate reductase activity, but *in vitro* activity was not affected. They suggested that probably NADH utilized for nitrate reduction is of mitochondrial origin. Thus, inhibition of nitrate reduction by freezing in liquid nitrogen could be explained on the basis of the disruption of mitochondrial membrane structure during freeze thaw treatment, which inhibits NADH generation and supply. Similar conclusions were also drawn by Srinivasan and Ramarao[22] based on their fluoride inhibition studies of nitrate reduction. They observed that

fluoride inhibits *in vivo* nitrate reduction considerably but *in vitro* activity is unaffected. They suggested that fluoride could bring about this inhibition by adversely affecting NADH supply required for *in vivo* nitrate reduction. Further, based on the observation that pyruvate could partially overcome fluoride inhibition they suggested that fluoride could inhibit pyruvate supply for the citric acid cycle by its well-known action on enolase, thus indirectly blocking NADH supply required for *in vivo* nitrate reduction[23].

Some workers[24–26] have also suggested that cytoplasmic malate dehydrogenase could function as a source of reductant for *in vivo* nitrate reduction. But this seems unlikely on two accounts. Firstly, the equilibrium of this reaction lies far towards the formation of malate and thus significant amount of NADH could not be generated unless the oxaloacetate formed is rapidly removed. Secondly, this reaction is not known to be inhibited by malonate, which completely inhibits *in vivo* nitrate reduction[27].

Recent observation of Ramarao *et al*[27] have further substantiated the view that NADH utilised for *in vivo* nitrate reduction is exclusively generated in the mitochondria. These conclusions were based on the following two important observations:

(i) *In vivo* nitrate reduction could be completely inhibited by malonate.

(ii) CO_2 evolution during *in vivo* dark anaerobic reaction is directly related to the extent of nitrate reduction. In order to study CO_2 evolution during nitrate reduction, leaves were allowed to assimilate $^{14}CO_2$ both in dark and in light so that endogenous substrates were labelled. Evolution of $^{14}CO_2$ from these leaf discs during dark anaerobic conditions depended on *in vivo* nitrate reduction. When nitrate reduction was inhibited by either tungstate or cyanide, $^{14}CO_2$ evolution was also correspondingly inhibited. Conversely, malonate, which inhibits citric acid cycle and thus CO_2 evolution also, inhibited nitrate reduction.

Although these studies do provide evidence that NADH for the *in vivo* nitrate reduction is of mitochondrial origin, it was not clear which reactions are involved. Sawhney *et al*[28] reported that under anaerobic conditions significant levels of succinate accumulate in wheat leaves. Recent unexpected observations of Ramarao *et al*[29] showed that succinate itself significantly inhibited dark *in vivo* nitrate reduction. Thus, malonate inhibition of nitrate reduction could not be explained on the basis of its known inhibition of citric acid cycle at the succinate dehydrogenase step. It was also found that D-malate inhibits *in vivo* nitrate reduction and that this was partially overcome by pyruvate and fumurate. Inhibition of NAD^+-malic enzyme (decarboxylating) by malonate and D-malate has been reported[30, 31]. Thus it was suggested that NAD^+-malic enzyme (decarboxylating) present in the mitochondria could be a probable source of NADH for *in vivo* nitrate reduction[29]. CO_2 evolution studies also could be explained if NAD-malic enzyme is involved in the supply of NADH for *in vivo* nitrate reduction, because besides NADH, CO_2 is also produced in this reaction.

Thus it is becoming increasingly clear that NADH generated in the mitochondria is an important source of NADH for nitrate reduction. Mitochondrial membranes are not permeable to NADH. Hence, a mechanism must exist for the transport of the reducing equivalents of NADH to cytoplasm. Canvin and Woo[14, 26] proposed that dicarboxylate shuttle (malate/oxaloacetate) could perform this function. Alternatively, plant mitochondria have been shown to be capable of transmembrane hydrogen transfer.[31] Day and Wiskich[32] have shown that plant mitochondria can oxidise external NADH and similarly internal NADH generated by citric acid cycle dehydrogenases can also be made available to the cytoplasm, because these enzymes can reduce external NAD^+.

6. Effect of photosynthesis on dark mitochondrial respiration

There are conflicting reports about the effect of light on mitochondrial respiration which is also known as dark respiration, in order to distinguish it from photorespiration. Inhibition of dark respiration in actively photosynthesising tissue has been reported by Heber[18]. According to him the light dependent increase in the adenylate energy change which is transmitted to mitochondria inhibits electron transfer from NADH to oxygen. Inhibition of mitochondrial respiration by ATP in light has also been observed in bean leaves[24]. Similarly, it was shown that in moss spores the cytochrome oxidase pathway does not function under high light intensities as it is under the control of ATP/ADP ratio[25]. These reports thus show that oxidation of NADH *via* cytochrome oxidase pathway is inhibited in light. However, various other reports have shown that citric acid cycle does function in light[36, 37]. It has been demonstrated that individual enzymes of the citric acid cycle are active in light[36]. Hence the turnover of the citric acid cycle which results in the generation of NADH and evolution of CO_2 is not inhibited in light. The rate of turnover would obviously depend on the rate of recycling of NADH under conditions when its oxidation *via* oxygen is blocked. One of the reactions by which NADH could be oxidised is by the cytoplasmic nitrate reductase. Hence, the rate of turnover of citric acid cycle would depend on the rate at which NADH is oxidised by nitrate reductase. As explained in the previous section, evolution of CO_2 during *in vivo* nitrate reduction is strictly dependent on the functioning of nitrate reduction. Thus it appears that citric acid cycle can turnover in light by making use of NO_3^- as an electron acceptor for the oxidation of NADH.

7. Carbon source for citric acid cycle in light : Possible role of photorespiration

In the dark, pyruvate generated during glycolysis is the source of carbon for citric acid cycle. However, Kent[38, 39] has shown that export of triosephosphates from chloroplast may not at all be a significant source of carbon for citric acid cycle during active photosynthesis. Since these are mostly utilised for sucrose synthesis in the cytoplasm, he also suggested that serine or some unknown anapleurotic carboxylation reactions may provide carbon for citric acid cycle, which is an important source of keto acids, independent of ATP synthesis. Palmer[37, 40] has suggested that a modified citric acid cycle

functions in light, making use of malate as a source of both pyruvate and oxaloacetate. He argued that mitochondrial NAD^+-malic enzyme (decarboxylating) generates pyruvate which can subsequently give rise to acetyl CoA. Oxaloacetate is also generated within the mitochondria by malate dehydrogenase. Thus the citric acid cycle can function as long as malate is available. Plant cells are known to accumulate large quantities of malate. Thus, it is clear that this modified citric acid cycle can function independent of glycolytic source of pyruvate. However, during prolonged operation of citric acid cycle in light, *de novo* synthesis of malate is essential. Alternatively, some other source of carbon would be necessary to replenish the carbon skeletons consumed in amino acid synthesis. The source of the carbon is not yet clear. It is known that two carbon compounds such as glycolate and glyoxylate are produced in large quantities in C–3 plants during photorespiration, also known as glycolate oxidation pathway. In these reactions, phosphoglycolate is synthesized in the chloroplast by the action of ribulose 1,5 biphosphate oxygenase. Subsequent oxidative decarboxylation of glycolate in the peroxisomes or mitochondria is assumed to be the source of photorespiratory CO_2. It is suggested by Tolbert[41] that glycine synthesized in the peroxisomes by transamination of glyoxylate is transported to the mitochondria where 2 moles of glycine generate one each of serine, CO_2, NH_3 and NADH. Photorespiration is considered to be a wasteful process and no definite physiological role has yet been indicated. Naik and Singh[42] suggested that photorespiration could function as a source of carbon for citric acid cycle during photosynthesis. Two mechanisms are possible. Firstly, glyoxylate and acetyl CoA can form malate by the action of malate synthetase. This could be a source of *de novo* synthesis of malate making use of glyoxylate derived from photorespiration. However, the presence of malate synthetase in plant mitochondria has not been reported. The second possible mechanism is the reversal of reaction catalysed by isocitrate lyase. This enzyme is present in the leaf mitochondria[43]. Although the equilibrium of this enzyme reaction favours cleavage of isocitrate to form succinate and glyoxylate, it is known that this reaction is reversible in the presence of high concentration of succinate and glyoxylate[44]. Thus it is likely that glyoxylate could be used for the synthesis of isocitrate which can be metabolised further in the citric acid cycle.

It thus appears that the reduction of nitrate to nitrite in the leaves is regulated by light indirectly by inhibiting mitochondrial oxidation of NADH. It is demonstrated that citric acid cycle dehydrogenase including NAD^+- malic enzyme (decarboxylating), which generate NADH and CO_2 simultaneously, are involved in nitrate reduction as a source of NADH. Thus citric acid cycle can turnover in light by making use of nitrate as an oxidant for NADH. The source of carbon for citric acid cycle in light is, however, not known with certainty. It is suggested that a 2-carbon compound such as glycolate derived from photorespiration could be a source of carbon for citric acid cycle. If this is correct then this pathway indicates a possible physiological function for photorespiration in the sense that glycolate could serve as a source of carbon in light for the generation of carbon skeleton in the citric acid cycle. It thus appears that assimilation of nitrate in plants is regulated by the integrated activities of chloroplasts, mitochondria, cytoplasm and perhaps peroxisomes. This is schematically depicted in Fig. 1,

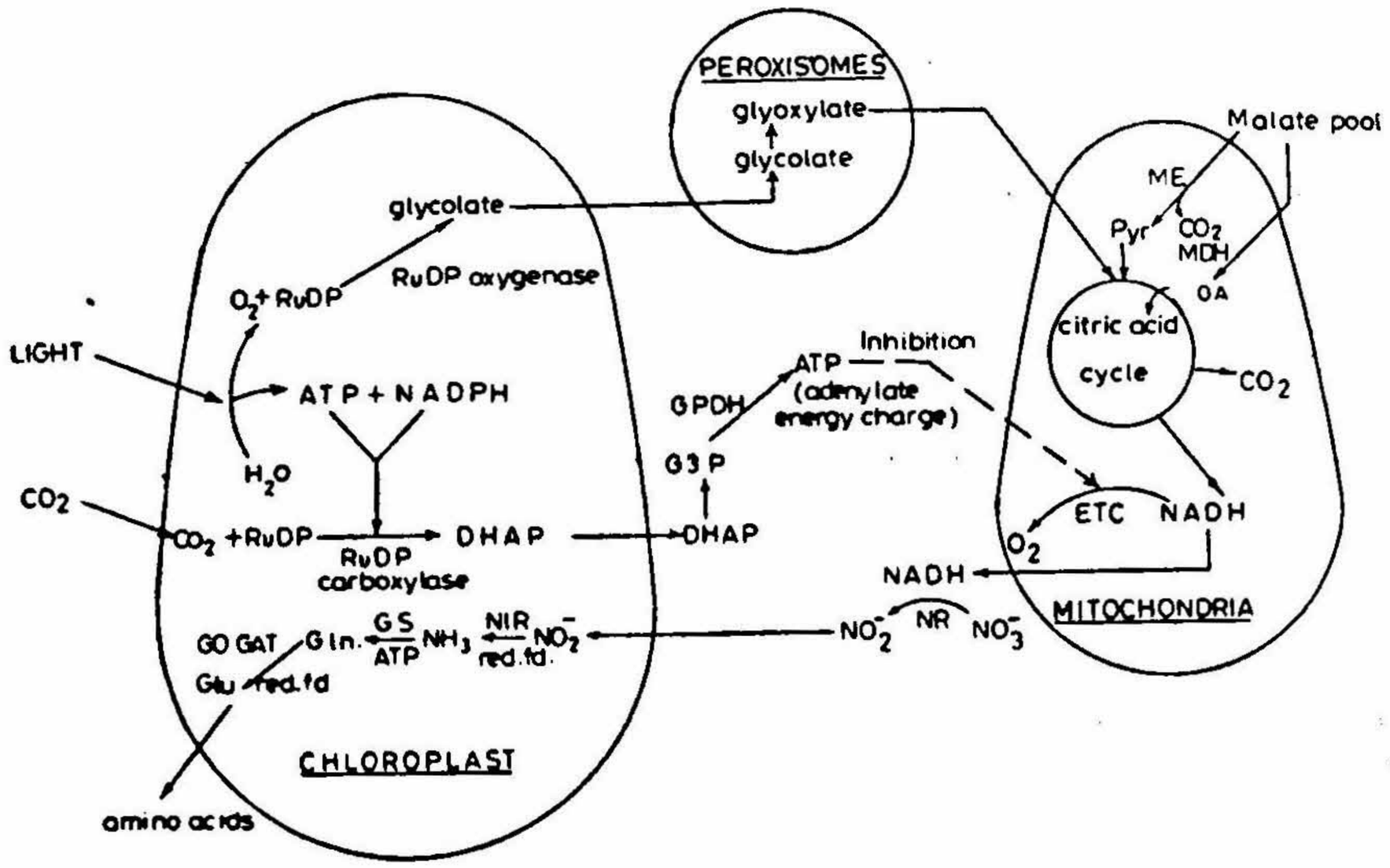

FIG. 1. **Role of chloroplasts, mitochondria and peroxisomes in the assimilation of nitrate in plant cells.**

RuDP—Ribulose 1,5 biphosphate ; DHAP—Dihydroxyacetone phosphate ; G3P—Glyceraldehyde-3-phosphate ; GPDH—Glyceraldehyde-3-phosphate dehydrogenase ; ME—NAD^+- Malic Enzyme (decarboxylating) ; MDH—Malate dehydrogenase ; ETC—Electron transfer chain ; OA—Oxaloacetate ; Pyr—Pyruvate ; Gln—Glutamine ; Glu—Glutamate.

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