EFFECT OF LINOLENATE ON PHOTOSYNTHESIS BY INTACT SPINACH CHLOROPLASTS

Conditions for the inhibition of orthophosphate uptake

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1. Introduction

Linolenate has been shown [1] to inhibit photosynthesis in intact spinach chloroplasts by inhibiting the influx of orthophosphate and the efflux of 3-phosphoglyceraldehyde. The inhibitory effect of linolenate was shown to depend on the level of the internal pool of metabolites [2] as a result of experiments which on the one hand used chloroplasts from preilluminated leaves or chloroplasts which were artificially loaded with 3-phosphoglyceric acid ('high pool' chloroplasts) and on the other hand chloroplasts from darkened leaves ('low pool' chloroplasts). This suggested that the inhibition of photosynthesis by linolenate is due to an inhibition of the 'phosphate translocator' which is located in the envelope and mediates the exchange of orthophosphate and internal phosphorylated metabolites [3,4]. This investigation is an attempt to elucidate the mechanism by which linolenate inhibits the function of the 'phosphate translocator', in particular with respect to the uptake of orthophosphate.

2. Material and methods

Intact chloroplasts from spinach (*Spinacia oleracea*, var. Nobel) were isolated from leaves as in [1] and the chlorophyll content was determined by the method in [5]. Intact chloroplasts were isolated from preilluminated leaves as in [2]. The orthophosphate concentration was estimated according to [6] and related to curves using readings obtained from standards in the presence of sorbitol or sucrose. Oxygen evolution was measured polarographically and CO_2 fixation was assayed as in [1]. Experimental conditions and reaction mixtures are described in the figure legends.

3. Results and discussion

Figure 1 shows that in the presence of 0.7 mM phosphate in the reaction mixture the rates of O_2 evolution and of phosphate uptake (disappearance of P_i from the medium) with intact chloroplasts were not stoichiometrically related (as is also illustrated in fig.2A). Addition of increasing concentrations of linolenate affected the two activities differently; whilst the rate of phosphate uptake decreased progressively with increasing linolenate concentration, O_2 evolution was first activated and then inhibited. Under the experimental conditions used, the external P_i concentration (0.7 mM) was too high for maximum rates of O_2 evolution as discussed [2,7]. The addition of low concentrations of linolenate inhibited P_i uptake (fig.1 and [1]) and therefore reduced it to a value nearer to the optimum for O_2 evolution: this would explain the stimulatory effect of linolenate on O_2 evolution. With further additions of linolenate chloroplasts were depleted in P_i and consequently O_2 evolution diminished.

Figure 1 shows that the addition of NH_4Cl inhibited

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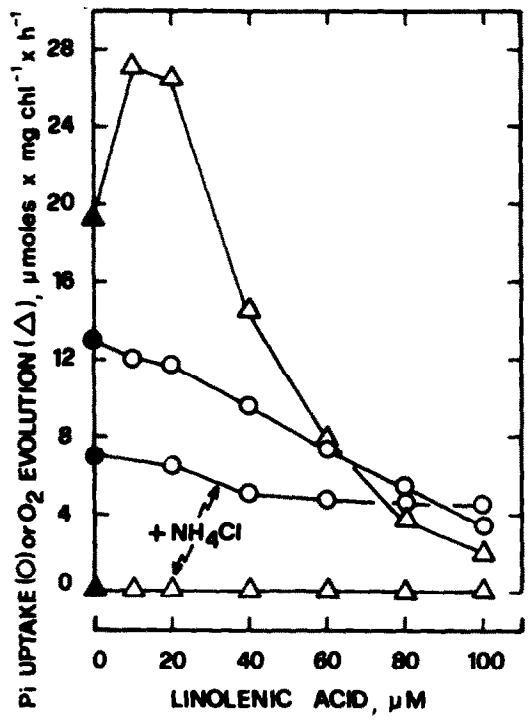

Fig.1. Influence of linolenate on oxygen evolution and phosphate uptake in the light by intact chloroplasts. O_2 evolution and inorganic phosphate uptake were assayed simultaneously in the same vessel containing: 350 mM sorbitol, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) adjusted to pH 7.6 with NaOH, 0.2 mM Na-isoascorbate, 6.6 mM $KHCO_3$, 0.7 mM K_2HPO_4 and chloroplasts (100 μg chl./ml). Linolenic acid (dissolved in ethanol) and 6 mM NH_4Cl were added as indicated. Reactions in the light (white light at ~5 × 10^6 ergs . cm^{-2} . s^{-1}) were carried out at 20°C and stopped after 10 min. The P_i concentrations were estimated in the supernatants, from aliquots removed at the beginning and end of the experiment and centrifuged immediately. Symbols are indicated along the ordinate.

the rate of phosphate uptake by ~50% and completely inhibited O_2 evolution. Added NH_4Cl was shown [8] to affect CO_2-supported O_2 evolution in a complex manner which depended upon the pH and the concentration of phosphate in the chloroplast suspension medium and also on the concentration of NH_4^+ [8]. The inhibition of O_2 evolution by 6 mM NH_4Cl can be explained in two ways:

(i) The uncharged amine (NH_3) penetrates the envelope and with an effectiveness dependent upon the internal pH consumes H^+ to form the charged species (NH_4^+); this would deplete the stromal region of H^+ and raise its pH and this would result in a decrease of CO_2-dependent O_2 evolution by affecting several enzyme activities of the Calvin-Benson cycle [9];

(ii) NH_4Cl uncouples the chloroplasts and by causing a shortage of ATP inhibits completely CO_2-dependent O_2 evolution.

These NH_4Cl-treated chloroplasts were, however, still able to take up P_i and this uptake was only marginally inhibited by linolenate (fig.1). This interesting observation suggests that in the presence of NH_4Cl, phosphate can still partially move across the envelope by a mechanism which is not dependent on its consumption in photophosphorylation.

In fig.2A, O_2 evolution and phosphate uptake by intact chloroplasts were plotted as a function of exogenous phosphate concentration. Whilst the rate of O_2 evolution reached a maximum at about 0.2 mM P_i and decreased rapidly above this concentration, as reported [1,7], phosphate uptake increased significantly up to 2 mM and approached a saturation plateau at higher values of P_i. The latter curve was of the Michaelis type; indeed the double reciprocal plot of the concentration dependence of the rate of phosphate uptake was linear thus indicating an approach to saturation of a carrier mechanism, as illustrated in fig.2B (black symbols). It appears therefore that there is no simple correlation between the rate of P_i uptake and O_2 evolution in the light. The form of the P_i uptake curve, resembling Michaelis-Menten kinetic curves, suggests that P_i is transported inside the chloroplast by a specific carrier named phosphate translocator [3,4].

Addition of linolenate inhibited phosphate uptake and affected the slope and the intercept of the curve with the ordinate (fig.2B). Thus V_m decreased in value with increasing concentrations of linolenate but on the other hand the app. K_m value remained the same. The absolute values of K_m and V_m varied with different market spinach leaves. In spite of this variability, the inhibition by linolenate was always of a non-competitive type. Since it has been demonstrated that the uptake of orthophosphate is mediated by a phosphate translocator located in the envelope of intact chloroplasts [3,4] these results indicated that linolenate did not change the affinity of the translocator for phosphate. The absence of any

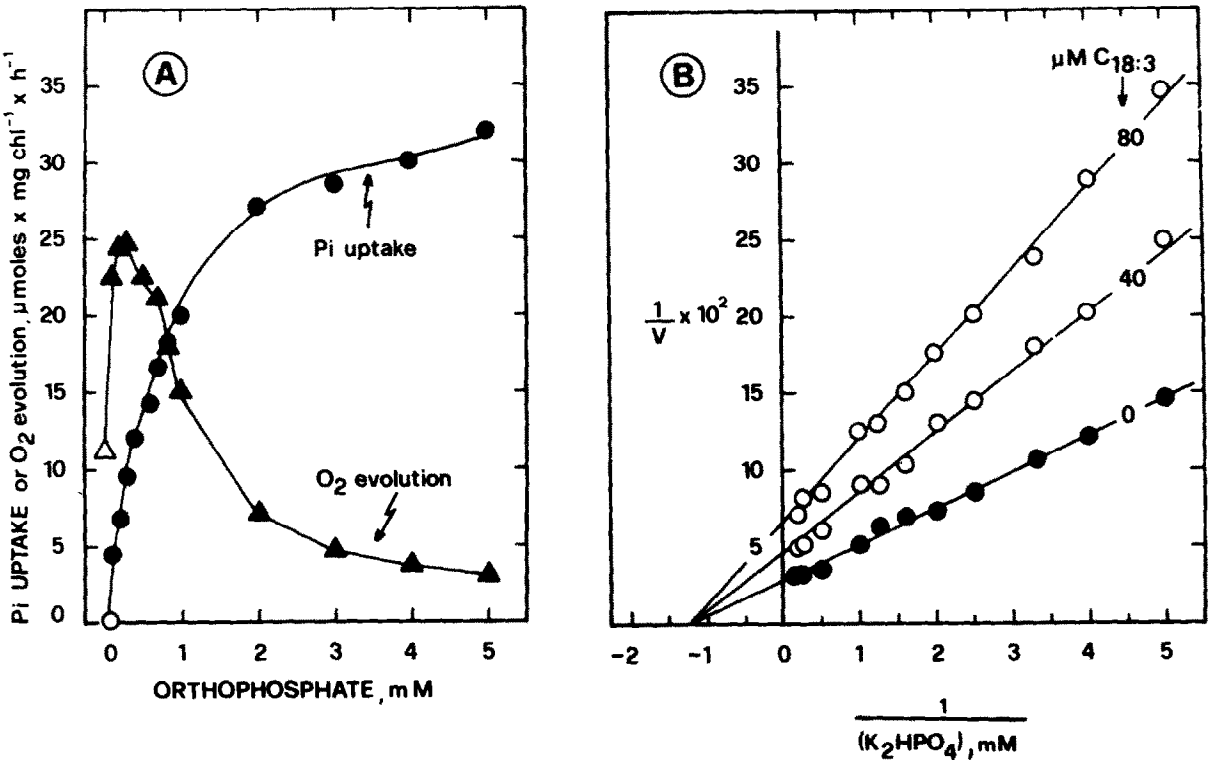

Fig.2. Phosphate uptake and oxygen evolution by intact chloroplasts as a function of external P_i concentration (2A). Conditions and reaction mixtures were the same as in fig.1 except that 2 mM EDTA, 1 mM $MgCl_2$ and 1 mM $MnCl_2$ were added in the reaction medium and chloroplasts were extracted from non-preilluminated leaves. The concentration of inorganic phosphate was varied as indicated and linolenate was at 40 μM. Lineweaver-Burk plots showing the inhibition of inorganic phosphate uptake in intact chloroplasts by 40 μM linolenate (2B). Experimental conditions and reaction mixtures were the same as in fig.2A. The K_m for orthophosphate was 0.8 mM in the presence and in the absence of linolenate. K_i and V_{max} were 60 μM and 39.6 μmol P_i . mg chl.$^{-1}$. h^{-1}.

structural similarity between P_i and $C_{18:3}$ and the considerable difference in their solubility characteristics mean that competitive inhibition would be unlikely in any case. It is therefore suggested that linolenate does not bind specifically at the active site of the phosphate translocator with respect to phosphate.

In further experiments phosphate uptake by intact chloroplasts in the light was assayed as a function of external pH (fig.3). The rates of phosphate uptake presented a broad maximum from pH 7.5–9.0. Outside this range, the activities dropped abruptly. Linolenate inhibited phosphate uptake between pH 7.5 and 9.6 (with a peak which was repeatedly observed near pH 8); on the other hand at higher pH values linolenate stimulated phosphate uptake. The pH profile of P_i uptake for the control without linolenate suggests that near the two extremities of the plateau (i.e., pH 7.5 and 9.0) the ionisation constants for the substrate (phosphate) and for chemical groups at the active site of the translocator are favourable for the formation of a translocator–P_i complex. This indicates the possible existence of two sites with different pK values which bind phosphate in the active region of the translocator, as is often the case for an isolated enzyme [10]. The pK_2 of P_i is 7.2, therefore at pH 7.5 the two ion species $H_2PO_4^-$ and HPO_4^{2-} are at about the same concentration, whereas at pH 9.0 the divalent ion species is predominant. Since P_i uptake is not influ-

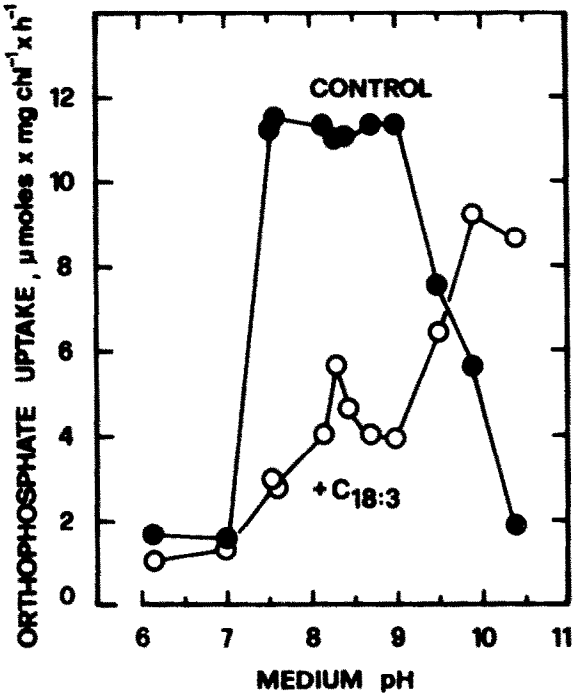

Fig.3. The pH dependence of inorganic phosphate uptake by intact chloroplasts in the presence and absence of linolenate (av. 9 expts). The reaction mixtures were as for fig.1 except that sorbitol was replaced by sucrose and the initial inorganic phosphate was 1 mM. Where indicated, linolenate ($C_{18:3}$) was present at 66 μM. The different buffers used all at 50 mM were 2-(*N*-morpholino)-ethanesulfonic acid (MES, pH 6.15), imidazole–HCl (pH 7.05), Hepes–NaOH (pH 7.55, 7.60 and 8.3), *N*-tris(hydroxymethyl) methyl glycine (tricine, pH 8.15), glycylglycine (pH 8.4, 9.0), glycine–HCl (pH 8.7 and 9.5, 9.9 and 10.4). All the assays were carried out in the light simultaneously for 10 min in 5 ml flasks mounted on the shaking frame of a Gilson Respirometer GRP 14, which imparted a swirling motion to the chloroplasts. Other conditions as in fig.1 and [1].

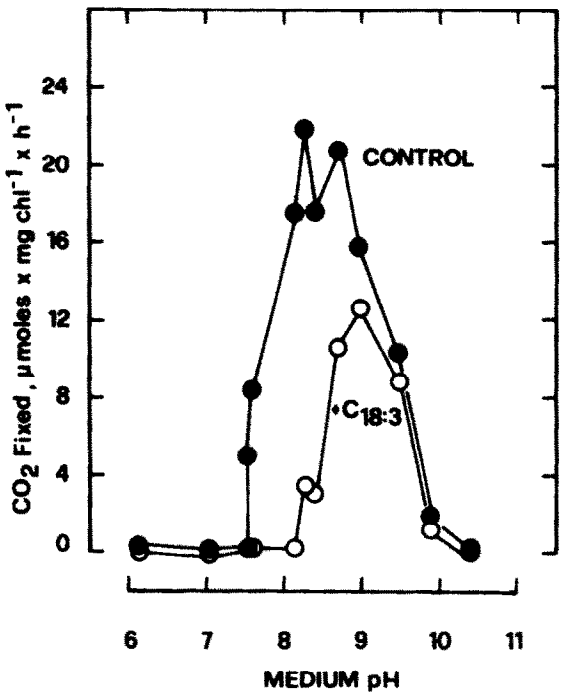

Fig.4. pH-dependence of CO_2 fixation by intact chloroplasts and inhibition by linolenate. Experimental conditions were the same as in fig.3 except that the reaction mixture was supplemented with 6.6 mM $NaH^{14}CO_3$ (0.1 μCi/μmol). The acid-stable labelling was estimated as in [1].

enced by pH between pH 7.5 and 9.0 it is reasonable to assume that HPO_4^{2-} is the ionic species which is mostly transported by the carrier. This assumption was also made [11] from different data. It would seem that the drop in the rate of P_i uptake on each side of the 'plateau' cannot be related directly to a change in the anionic form of P_i but rather to a change in the ionisation of the chemical groups involved near the 'active' or binding site(s) of the translocator or of the translocator–P_i complex. It is likely to be amino groups of the carrier which are positively charged between pH 7.5 and 9.0 which bind phosphate at the active site. As a result of specifically labelling the carrier protein with inhibitors the suggestion has been made that arginine and lysine could be involved at the catalytic site(s) [11–13]. The addition of linolenate appears to inhibit P_i uptake at the pH values corresponding to the two presumed binding sites. A rapid increase in the rate of phosphate uptake at alkaline pH values suggests either a conformational change of the carrier protein which might expose a third site towards P_i or a non-specific phosphate entry in the presence of linolenate and some membrane changes.

In order to compare the pH dependence of phosphate uptake and the rate of photosynthesis, the rate of CO_2 fixation by intact chloroplasts was measured in parallel experiments to the above as a function of the pH of medium. As can be seen in fig.4, the rates of CO_2 fixation presented two maxima, at pH 8.3

and 8.7, under our experimental conditions, with 1 mM phosphate which is known to be above the optimal value for photosynthesis [1,7]. On all sides of the peaks, the activities decreased. Linolenate was a potent inhibitor of CO_2 fixation in the region of the first peak (pH 8.3) and less effective near the second peak (pH 8.7). In the more alkaline range, linolenate had no effect on the rapid decline in activity shown in the control. The shape of the pH profile characterized by two maxima reflects the variability of pH optima reported by others [14–17]. Variations in the reaction mixture [14], growth conditions for the spinach [17] and regulation phenomena [15] are reasons put forward to explain the differences in the pH optima for CO_2 fixation or CO_2-dependent O_2 evolution. In other experiments the dependence of CO_2 fixation by intact chloroplasts on the pH of the medium was found to be affected by the P_i concentration in the reaction mixture (L. M. A., P. A. S., in preparation). In the more alkaline pH range linolenate did not stimulate the CO_2 fixation (fig.4) as was found for P_i uptake (fig.3) and indeed it had no effect on the decline in photosynthesis above pH 9. This would suggest that the improved rate of phosphate entry in the presence of linolenate in this region, whether or not it is due to the translocator, is not effective for photosynthesis.

The results presented give further support to the conclusion [1,2] that linolenate affects the phosphate/triosephosphate translocator in the envelope of chloroplasts. In addition they give evidence relevant to the mode of phosphate binding to the translocator with two possible points of attachment and reveal an additional effect of linolenate on phosphate entry at high pH values.

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