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STUDIES ON CHLOROPHYLLASE

THE MECHANISM OF THE ACTION OF LECITHIN LIPOSOMES ON ENZYME ACTIVITY AND THE FUNCTION OF THE CARBOHYDRATE MOIETY OF THE ENZYME

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A sensitive and continuous fluorescence assay of chlorophyllide formation in the presence of lecithin liposomes has been developed. The mechanism of the enhancing effect of lecithin on chlorophyllase-catalyzed hydrolysis of chlorophyll has been elucidated. Using both fluorescence and biochemical assays, the function of the concanavalin A-reactive carbohydrate moiety of chlorophyllase has been investigated. Experiments on the interaction of chlorophyllase with concanavalin A show that the sugar group not only stabilizes the enzyme, but also is essential for the manifestation of enzyme activity. From a comparison of the results obtained with solubilized and membrane-bound enzyme, it is concluded that the active site is situated on the outside of the thylakoid membrane. Mg^{2+}, in combination with dithiothreitol, activates chlorophyllase. In the presence of Mg^{2+}, an abnormal pH dependence of the inhibiting effect of concanavalin A on chlorophyllase-catalyzed chlorophyll hydrolysis and the reversibility of this effect through the addition of α-methyl-D-mannoside have been observed. The cause of this atypical reaction as well as of other Mg^{2+} effects is discussed.

Introduction

Isolated thylakoids of higher plants or algae contain various amounts of chlorophyllase (chlorophyll chlorophyllido hydrolase, EC 3.1.1.14). The enzyme is activated by Mg^{2+} or Ca^{2+}, and reducing substances (e.g. dithiothreitol) greatly enhance the effect of these ions [1]. It was found later [2] that chlorophyllase-catalyzed hydrolysis of chlorophyll can also be enhanced by the addition of lecithin vesicles instead of Mg^{2+} + dithiothreitol to the reaction mixture. In the presence of lecithin, Mg^{2+} or Ca^{2+} exerted a negative instead of a positive influence on chlorophyllide formation. A tentative explanation of the lecithin effect was given.

In later experiments [3], chlorophyllase was shown to combine reversibly with Con A-Sepharose, and it was concluded that the enzyme is a concanavalin A-reactive glycoprotein. Several other enzymes have been found to be glycoproteins. In the cases examined, the saccharide group seems to function as a protein stabilizer, but is not essential for enzyme activity (cf. Refs. 4 and 5). In order to elucidate the function of the saccharide moiety in chlorophyllase, the interaction of this group with concanavalin A in a soluble form was studied. In addition, the influence of both lecithin and Mg^{2+} on the chlorophyllase-concanavalin A interaction was investigated.

Chlorophyllase activity is generally assayed by incubation of the enzyme with chlorophyll and subsequent partitioning of the chlorophyllide formed from the remaining chlorophyll in different organic solvents [1,6,7]. The chlorophyllide is

Abbreviations: Chl, chlorophyll; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

then determined colorimetrically at 663 nm. By this method, however, a relatively large amount of chlorophyllase is required in order to obtain a measurable quantity of chlorophyllide in a short time or, alternatively, with small amounts of chlorophyllase, a long incubation time is needed [1]. In the course of the present experiments it was found that chlorophyllide formation is reflected by increase in the 687 nm fluorescence in a reaction mixture containing lecithin. This provided us with a continuous assay for following the course of chlorophyll hydrolysis even when small amounts of chlorophyllase, similar to those needed with the original long-incubation method, are used.

In this way we found, contrary to our earlier conclusions [2], that chlorophyllase activity is enhanced by Mg^{2+} also in the presence of lecithin. The interaction of chlorophyll(ide) and lecithin is discussed again in the light of these new results.

The experimental results obtained with the concanavalin A-chlorophyllase interaction favor the conclusion that, besides acting as an enzyme stabilizer, the concanavalin A-reactive carbohydrate group in chorophyllase is situated at or near the active site of the enzyme and is essential for its activity.

Materials and Methods

Lecithin (from egg yolk) and Concanavalin A were purchased from Sigma. All other chemicals used were of analytical quality.

Chlorophyllase was prepared from *Phaeodactylum tricornutum*. Cultivation of the cells, preparation of photosynthetic membranes and isolation of chlorophyllase from these membranes were as described in Ref. 3. EDTA ($5 \cdot 10^{-4}$ M) was added to all aqueous media. Generally no separation of active and inactive chlorophyllase by means of Con A-Sepharose adsorption was carried out. If not mentioned otherwise, chlorophyllase was assayed as described in Ref. 1. Chlorophyll was added as an 80% (v/v) acetone extract of spinach chloroplasts; maximum acetone concentration in the reaction medium was 7% (v/v). With reaction mixtures at pH values different from 8, the pH was adjusted to 8 afterwards by addition of 2.5 ml of 2% NaCl in 0.1 M Tris-HCl buffer, pH 8, instead of in water. In this way, the effects that pH has upon partitioning of chlorophyllide between aqueous acetone and petroleum spirit [8] were excluded.

Chlorophyllase activity was expressed in percent chlorophyll converted to chlorophyllide.

Protein was estimated either from 280 nm absorption or by the BioRad micro assay [1].

Lecithin liposomes were prepared from a 10% egg lecithin solution in hexane, evaporated to dryness in an N_2 current. The residue was suspended in 0.02 M Tris-HCl buffer, pH 8.0, or in deionized water (10 mg lecithin/ml). The suspension was passed twice through a French Press needle valve (930 kg/cm^2) [9] and afterwards sonicated for 15–30 minutes in a Bransonic bath sonicator, yielding an opalescent preparation.

Chlorophyll was incorporated in lecithin liposomes by mixing, prior to the evaporation procedure, 1–2 ml of a 0.1–0.2% chlorophyll solution in 80% acetone with 0.5 ml of 10% lecithin solution in hexane.

Fluorescence was measured with a modified apparatus designed by Goedheer [10]. The excitation light, produced by a 150 W halogen lamp in a slide projector, passed through 3 cm of 6% $CuSO_4$ solution in 0.01 N H_2SO_4 and a 448 nm interference filter. The fluorescent emission light was measured at 687 nm after it had passed through a Bausch and Lomb monochromator. The sample (0.5 ml) was placed in a 0.5 cm polystyrene cuvette.

Reaction mixtures of individual experiments are given with the figures. The figures represent typical experiments.

Results and Conclusions

Assay of chlorophyllide by its fluorescence in lecithin liposomes; influence of Mg^{2+}

In the aqueous reaction mixtures used for chlorophyllase-catalyzed chlorophyll hydrolysis, the substrate chlorophyll is in a colloidal form. Consequently, its fluorescence, determined at room temperature at 687 nm, is negligible [11]. The low fluorescence measured is probably due to the presence of a small amount of acetone in the reaction mixture (4%) and/or to impurities in the chlorophyll solution. Chlorophyll that has been artificially incorporated in lecithin liposomes can be highly fluorescent (Table I) [12,13]. However, when

TABLE I

INFLUENCE OF LECITHIN ON CHLOROPHYLL FLUORESCENCE

20 μg chlorophyll. Buffer: 0.02 M Tris-HCl, pH 8.0 in water, containing 9 mM dithiothreitol, lecithin liposomes containing 0.4 mg lecithin, final volume 0.5 ml. Temperature 23°C. $F_{687\ nm}$, fluorescence emission at 687 nm.

Medium	$F_{687\ nm}$ (relative units)
Buffer	1
Lecithin liposomes	40
Buffer, lecithin liposomes added	4

lecithin liposomes are added to a colloidal aqueous chlorophyll solution only a small increase in fluorescence is observed (Table I). Apparently, in the circumstances of our experiments only a few chlorophyll molecules enter the lecithin liposomes. When chlorophyllase is added to an aqueous mixture containing colloidal chlorophyll and lecithin liposomes, a gradual increase in 687 nm fluorescence emission is observed (Fig. 1). It was observed that the fluorescence increase runs roughly parallel with the activity of chlorophyllase in the mixture. Thus, the increase in the fluorescence appears to reflect chlorophyllide formation and the following results were consistent with this hypothesis.

Fluorescence increase in a chlorophyll-chlorophyllase mixture is only observed in the presence of lecithin. The reaction rate increases during the first hour and then remains constant for several hours. If, besides lecithin, Mg^{2+} is added to the reaction mixture the initial reaction rate is increased, but after 2–3 h a decrease is observed. Fig. 1 shows that the optimum Mg^{2+} concentration is below 10 mM.

Previously [2] we found that, in the presence of lecithin, Mg^{2+} inhibits chlorophyllase-catalyzed conversion of chlorophyll to chlorophyllide. If fluorescence really reflects chlorophyllide formation, the results in Fig. 1 imply that Mg^{2+}, in the presence of lecithin, can activate chlorophyllase. Therefore, the influence of Mg^{2+} on chlorophyl-

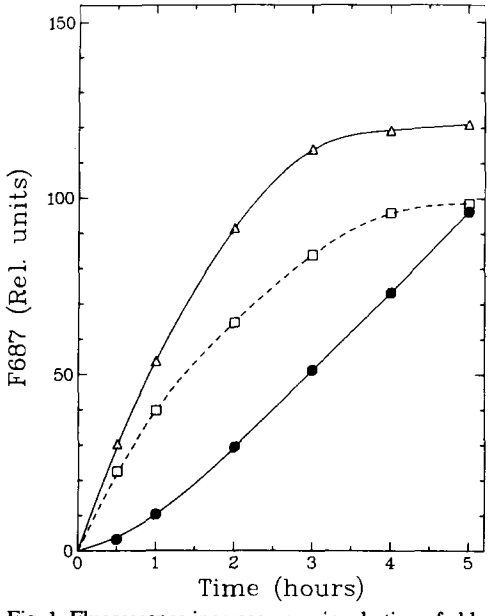

Fig. 1. Fluorescence increase upon incubation of chlorophyllase with colloidal chlorophyll in the presence of lecithin vesicles; influence of Mg^{2+}. Reaction mixtures: 2 μg chlorophyllase, 6.7 mM dithiothreitol, 20 mM Tris-HCl, pH 8.0, 0.08% lecithin, 4 mM $MgCl_2$ (△) or 10 mM $MgCl_2$ (□). Control (●) without $MgCl_2$. Chlorophyll: 10 μg in 0.02 ml of 80% acetone (A_{670} of the reaction mixture 0.45, light path 0.5 cm). Final aqueous volume 0.5 ml. Temperature 23°C. *F*687, fluorescence emission at 687 nm.

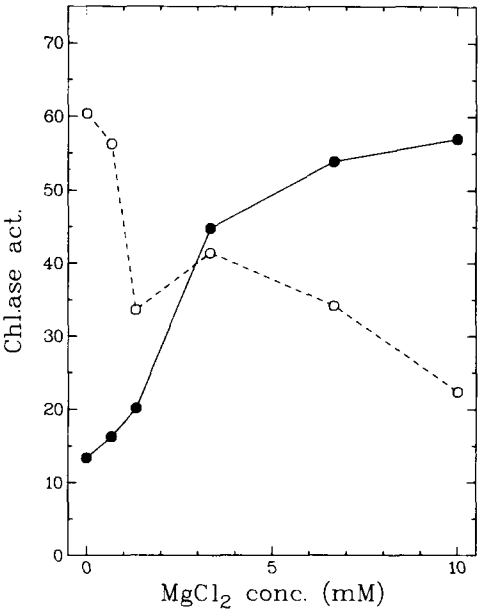

Fig. 2. Influence of Mg^{2+} concentration on chlorophyllase activity (Chl. ase act.) measured with (○) and without (●) lecithin. Reaction mixtures: 2.5 μg chlorophyllase, 6.7 mM dithiothreitol, $MgCl_2$ as indicated, 0.067% lecithin (only in ○) 20 mM Tris-HCl, pH 8.0, 150 μg chlorophyll in about 0.1 ml acetone. Final aqueous volume 1.5 ml. Incubation 17 h at 23°C.

lase-catalyzed chlorophyll conversion, measured with our original assay procedure, was reinvestigated. Chlorophyll hydrolysis, in the presence and absence of lecithin, was measured at various Mg^{2+} concentrations. Whereas a smooth curve is obtained for chlorophyllase activity vs. Mg^{2+} concentration in the absence of lecithin, the curve obtained in its presence is irregular (Fig. 2). The rise in the latter curve between 2 and 4 mM $MgCl_2$ suggests the occurrence of an enzyme activation, which is masked by other Mg^{2+} effects, e.g., on lecithin [2].

From the experiments shown in Figs. 1 and 2, it is concluded firstly that the fluorescence experiments are in accordance with the hypothesis that fluorescence reflects chlorophyllide formation and, secondly that chlorophyllase, both in the presence and absence of lecithin, is stimulated by Mg^{2+} (plus a reducing substance).

Chlorophyllide formation occurs in a mixture of chlorophyll and chlorophyllase, in the presence

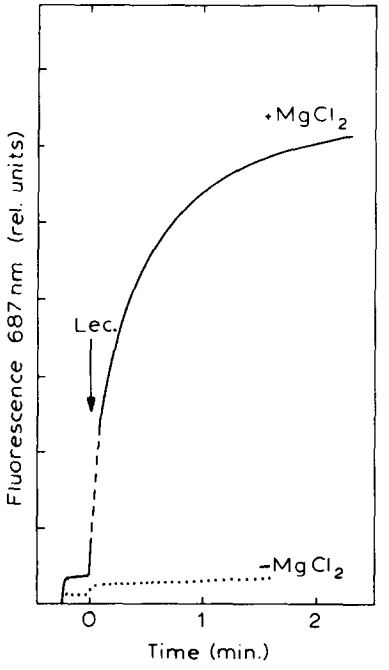

Fig. 3. Fluorescence time course of a chlorophyll-chlorophyllase mixture, preincubated in the presence or absence of $MgCl_2$, upon addition of lecithin. Reaction mixtures: 2 μg chlorophyllase, 6.7 mM dithiothreitol, 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$ (———) or (·····) none, 10 μg chlorophyll in 0.02 ml acetone. Final volume 0.46 ml. Preincubation 2 h at 17°C. 0.04 ml lecithin liposomes (0.1%) were added after preincubation.

of Mg^{2+} and dithiothreitol [1]. Nevertheless, fluorescence is low and no clear increase is observed with time. Addition of lecithin vesicles, after a preincubation time of 2 h, results in a rapid fluorescence increase (Fig. 3). Especially with the highest Mg^{2+} concentration (10 mM) the final fluorescence depends on the presence of dithiothreitol. These experimental results confirm the hypothesis that the 687 nm fluorescence increase in lecithin should be ascribed to chlorophyllide formation.

Inhibition of chlorophyllase by concanavalin A and its reversal by methylmannoside. Effects of pH, Mg^{2+} and lecithin

For investigation of the interaction of soluble concanavalin A and chlorophyllase, the experimental reaction mixtures must be composed in such a way that both proteins will be active. Concanavalin A exhibits maximum carbohydrate binding at pH 6–7, but at pH 9 it is inactive in this respect [14–16]. Concanavalin A needs bivalent metals, preferably Ca^{2+} and Mn^{2+}, or Cd^{2+}, for optimum activity [17,18]. Chlorophyllase was generally assayed at pH 8.0 [1]. The pH optimum depends on the reaction medium; under our conditions it was at pH 8 or lower (Figs. 4 and 5). A further consideration is that below pH 7 the substrate chlorophyll, in an aqueous medium, is unstable.

In a chlorophyllase assay mixture either lecithin or Mg^{2+} is usually present. If lecithin is used, addition of Ca^{2+} or Mn^{2+} for concanavalin A activation would at the same time lower chlorophyllase activity [2]. Fortunately, it was found that in the reaction mixtures used, concanavalin A is active in the absence of added Ca^{2+} or Mn^{2+}; apparently, the concanavalin A preparation contains a sufficient quantitiy of these ions.

The conversion of chlorophyll into chlorophyllide, catalyzed by chlorophyllase, may be enhanced by any protein; proteins, such as bovine serum albumin, often have a protective effect on enzymes in dilute solutions. Concanavalin A may exert such a nonspecific protein effect of this kind. However, the specific action of concanavalin A upon carbohydrates can be recognized by its reversibility upon addition of α-methyl-D-mannoside. Therefore, only the reversible influence of con-

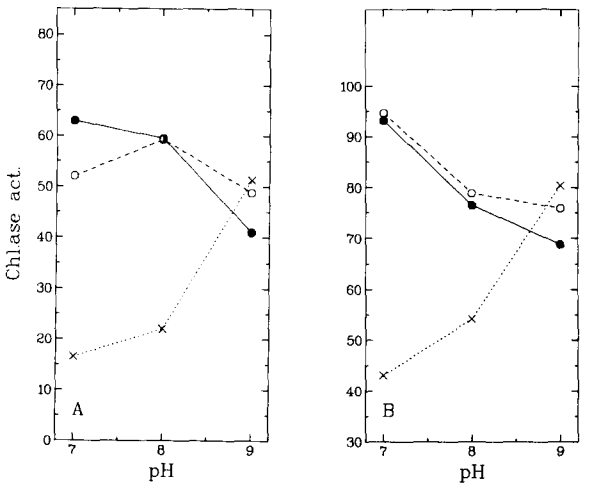

Fig. 4. Influence of pH upon concanavalin A inhibition of soluble and membrane-bound chlorophyllase in the absence of Mg^{2+} but in the presence of lecithin and the reversal of this inhibition by methylmannoside. Reaction mixtures: 1 μg chlorophyllase (A) or washed *Phaeodactylum* photosynthetic membrane fragments (B) containing about 20 μg protein, 6.7 mM dithiothreitol, 20 mM Tris-HCl, pH 8.0 or 9.0, or 20 mM Pipes-NaOH, pH 7.0, 1.5 mg lecithin, 0.1 mg concanavalin A (x and O), 20 mM α-methyl-D-mannoside (O), 150 μg chlorophyll in about 0.1 ml acetone. Final aqueous volume 1.5 ml. (●) Control. Incubation 17 h at 23°C.

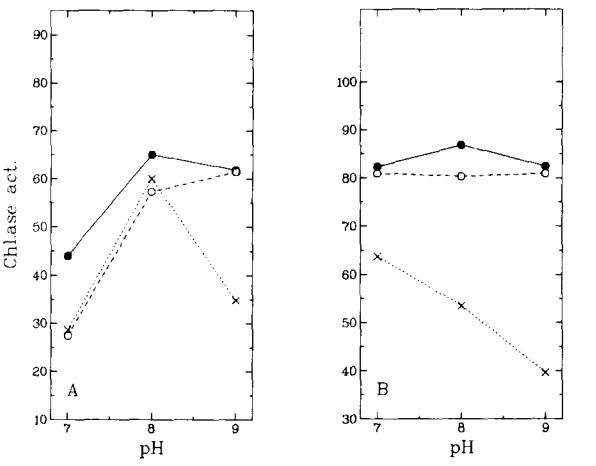

Fig. 5. Influence of pH upon concanavalin A inhibition of soluble and membrane-bound chlorophyllase in the presence of Mg^{2+}, but in the absence of lecithin. Reaction mixtures: See Fig. 4. Lecithin is replaced by $MgCl_2$ (10 mM).

canavalin A should be considered.

Chlorophyllase activity, measured in the presence of lecithin at pH 7, is inhibited by concanavalin A and this inhibition is reversed by α-methyl-D-mannoside (Fig. 4). Inhibition is still clear at pH 8, but absent at pH 9. At pH 9 even a small increase in chlorophyllase activity is observed; this increase, which can also be obtained with bovine serum albumin, is attributed to the nonspecific protective effect of the protein.

The inhibiting effect of concanavalin A is observed with solubilized chlorophyllase (Fig. 4A) as well as with chlorophyllase within membrane fragments (Fig. 4B). That concanavalin A has its maximum effect at pH 7 and its reversible action is absent at pH 9 are in agreement with the known properties of this lectin.

If Mg^{2+} is added to the reaction mixture instead of lecithin, quite a different pattern of chlorophyllase inhibition by concanavalin A is observed between pH 7 and 9. We now see that a maximum reversible effect of concanavalin A is no longer at pH 7 but at pH 9, whereas at pH 7 and 8 a much smaller or even no reversibility of inhibition is found (Fig. 5A and B). This abnormal behavior of concanavalin A in the chlorophyllase reaction at

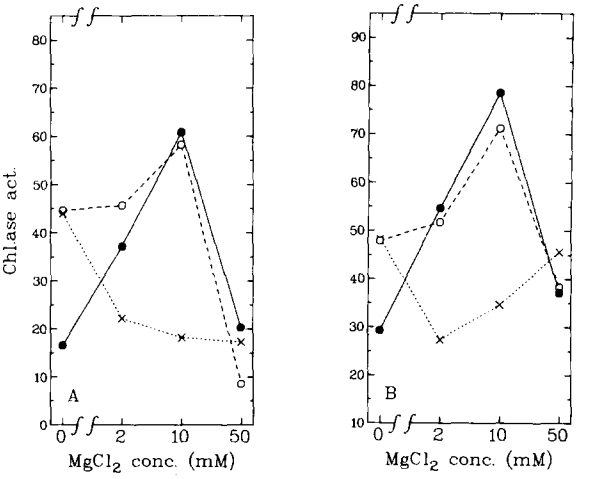

Fig. 6. Effect of Mg^{2+} concentration in the absence of lecithin on chlorophyllase activity and on reversible inhibition of this activity by concanavalin A at pH 9. Reaction mixtures: 1 μg chlorophyllase (A), washed *Phaeodactylum* photosynthetic membrane fragments (B) containing about 20 μg protein, 6.7 mM dithiothreitol, 20 mM Tris-HCl, pH 9.0, $MgCl_2$ as indicated, 0.1 mg concanavalin A (x and O), 20 mM α-methyl-D-mannoside (O). Chlorophyll: 150 μg in about 0.1 ml acetone. (●) Control. Final aqueous volume 1.5 ml. Incubation 17 h at 23°C.

pH 9 depends on Mg^{2+} concentration (Fig. 6): maximum effect was found at 10 mM $MgCl_2$, which is also the optimum Mg^{2+} concentration for chlorophyllase activity. Above 10 mM Mg^{2+} there is no clear effect of concanavalin A. In the absence of $MgCl_2$, when still some chlorophyllase activity is measured, concanavalin A exerts an irreversible activating action, as was also observed in the presence of lecithin at pH 9.

The atypical effect of concanavalin A upon chlorophyllase activity in the presence of Mg^{2+} will be discussed below.

The role of the carbohydrate moiety in chlorophyllase

The concanavalin A-reactive carbohydrate group in chlorophyllase may either constitute part of the active site of the enzyme or it may function only as an enzyme stabilizer. The next experiment was designed to check whether or not the saccharide group exerts a stabilizing function (Table II). Heating of chlorophyllase for 1 h at 37°C leads to enzyme denaturation (cf. A1 and B1 in Table II). This denaturation can be prevented, to a large extent, by the presence of a small amount of chlorophyll (Refs. 2 and 19 and result C1 in Table II). Addition of concanavalin A to preheated enzyme, with or without chlorophyll, results in inactivation of the remaining chlorophyllase (B2, 3 and C2, 3). Upon heating the enzyme in the presence of concanavalin A, the amount of active chlorophyllase is decreased; this result is observed both in the absence (B4, 5) and presence (C4, 5) of chlorophyll during the incubation period. These results indicate that heat denaturation of chlorophyllase is increased in the presence of concanavalin A; in other words, the stability of chlorophyllase decreases upon binding of its saccharide moiety. Further, accelerated heat denaturation is caused not only by concanavalin A but also by concanavalin A + α-methyl-D-mannoside. The latter effect can be explained by the assumption that contrary to the association reaction between chlorophyllase and concanavalin A, the denaturation of chlorophyllase is, in the circumstances of these experiments, irreversible.

The results from Table II are thus in accordance with the hypothesis that the carbohydrate group

TABLE II

INFLUENCE OF CONCANAVALIN A ON CHLOROPHYLLASE ACTIVITY

Reaction mixtures: 1.5 μg chlorophyllase, 6.7 mM dithiothreitol, 20 mM Tris-HCl, pH. 8.0, 100 μg concanavalin (Con A), 20 mM α-methyl-D-mannoside (m.m.), 10 μg chlorophyll, total aqueous volume 1.35 ml. After preincubation for 1 h at the temperature indicated, 0.15 ml lecithin liposomes (1%) in 20 mM Tris-HCl, pH 8.0, and 150 μg (A,B) or 140 μg (C) chlorophyll in about 0.1 ml 80% acetone were added. Incubation 17 h at 23°C.

Series number	Preincubation			Added after preincubation	Relative chlorophyllase activity
	Temperature (°C)	Chlorophyll	Additions		
A1	4	−	−	−	100
2	4	−	−	Con A [a]	44
3	4	−	−	Con A [a] + m.m.	95
B1	37	−	−	−	24
2	37	−	−	Con A [a]	9
3	37	−	−	Con A [a] + m.m.	30
4	37	−	Con A	−	2
5	37	−	Con A + m.m.	−	5
C1	37	+	−	−	99
2	37	+	−	Con A [a]	47
3	37	+	−	Con A [a] + m.m.	97
4	37	+	Con A	−	20
5	37	+	Con A + m.m.	−	35

[a] Preheated 1 h at 37°C.

stabilizes the enzyme. However, this conclusion does not exclude the possibility that the carbohydrate also functions at the active site of the enzyme. If the saccharide group has only a stabilizing function, the effect of concanavalin A can be expected to increase with time. In order to investigate this, it is necessary to study the time course of the chlorophyllase-concanavalin A interaction. An opportunity to do this arose from the fluorescence experiments described in the first paragraph of this section. As was expected, the 687 nm fluorescence in a reaction mixture containing chlorophyll, chlorophyllase and lecithin is lowered by concanavalin A (Fig. 7). In the first stage of the reaction there is no significant difference in the fluorescence of the control and the reaction mixture containing concanavalin A. However, this seems to be due to the absence of a 'lag period' in the fluorescence-time curve in the presence of concanavalin A. Comparison of the curves of mixtures containing either concanavalin A alone or concanavalin A + α-methyl-D-mannoside shows a clear fluorescence difference right from the beginning of the reaction. This result favors the hypothesis that the influence of concanavalin A on chorophyllase is not primarily due to the destabilization of the enzyme, but that concanavalin A inhibits enzyme activity directly by combining with the carbohydrate moiety of the enzyme. This would mean that the concanavalin A-sensitive carbohydrate group of chlorophyllase is situated at or near the active site of the enzyme.

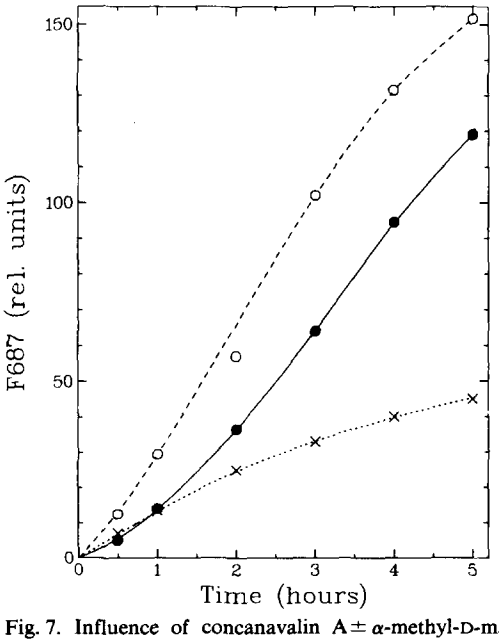

Fig. 7. Influence of concanavalin A ± α-methyl-D-mannoside on fluorescence of a chlorophyll-chlorophyllase mixture in the presence of lecithin. Reaction mixtures: 3 μg chlorophyllase, 6–7 mM dithiothreitol, 20 mM Tris-HCl, pH 8.0, 0.08% lecithin, 25 μg concanavalin A (x and O), 20 mM α-methyl-D-mannoside, 10 μg chlorophyll in 0.02 ml of 80% acetone. Final aqueous volume 0.5 ml. (●) Control. Temperature 23°C.

Discussion

pH dependence of concanavalin A-chlorophyllase interaction

The pH of maximum carbohydrate binding of concanavalin A is between 6 and 7 [13–16]; at pH 9 concanavalin A is inactive [20–22]. In the absence of Mg^{2+} the pH dependence of the interaction between concanavalin A and chlorophyllase is in accordance with the pH optimum of concanavalin A: chlorophyllase inhibition is highest at pH 7, wherease no effect is observed at pH 9 (Fig. 4). In the presence of Mg^{2+}, however, a reversible inhibition of chlorophyllase activity occurs at pH 9, whereas at lower pH values a smaller (with membranes) or no significant effect (with solubilized chlorophyllase) is observed (Fig. 5).

It was shown in Fig. 6 that the reversible inhibition of chlorphyllase activity by concanavalin A at pH 9 is observed only at those Mg^{2+} concentrations which are optimum or suboptimum for chlorophyllase activity. This suggests that concanavalin A inhibits chlorophyllase activity by binding Mg^{2+}. As the protein will be more negatively charged at increasing pH, the effect will be largest at pH 9.

Akedo et al. [23] observed that binding of specific carbohydrates to concanavalin A induced an increase in its net positive charge. This will result in the binding of a smaller amount of Mg^{2+} in the presence of α-methyl-D-mannoside; it may provide an explanation for the reversible inhibition by concanavalin A, in the presence of Mg^{2+}, at pH 9. Our experiments show that interaction of concanavalin A with a protein, even if reversed by specific carbohydrates, may, under certain circumstances, be due to some nonspecific reaction and not to the association of concanavalin A with a carbohydrate group of the protein.

Influence of Mg^{2+} on chlorophyllase-catalyzed chlorophyll hydrolysis

Chlorophyll hydrolysis, in the presence of chlorophyllase, was found earlier to be greatly increased by the addition of either Mg^{2+} + dithiothreitol or lecithin to the reaction mixture. It has now been shown, both from direct chlorophyllide measurements and from fluorescence assays, that Mg^{2+}, in the presence of dithiothreitol, always activates chlorophyllase, even in the presence of lecithin. In the presence of lecithin the favorable effect of Mg^{2+} + dithiothreitol on chlorophyllase activity may be masked by a direct effect of Mg^{2+} on lecithin [24–29].

Still another effect of Mg^{2+} is suggested by a comparison of the shapes of the fluorescence-time curves in the presence of either lecithin or lecithin and Mg^{2+}. With lecithin only, fluorescence steadily increases for at least 4 h. With Mg^{2+}, however, the fluorescence-time curve goes down earlier, especially at high Mg^{2+} concentration (Fig. 1). This may be due to a decreased stability of colloidal chlorophyll in the presence of Mg^{2+}. This may explain why upon prolonged incubation the final amount of chlorophyllide formed in a chlorophyllase-catalyzed reaction in the presence of Mg^{2+} + dithiothreitol does not generally exceed the amount of chlorophyllide formed with lecithin in the reaction mixture, even in the presence of excess substrate.

Chlorophyllide fluorescence in lecithin liposomes

The experimental results show that in the presence of lecithin liposomes the conversion of chlorophyll to chlorophyllide is reflected by increased fluorescence at 687 nm. Chlorophyll in an aqueous medium is not or only slightly fluorescent, whereas its fluorescence has been shown to increase considerably when the chlorophyll is artificially incorporated in lecithin liposomes. Fluorescence of a colloidal chlorophyll solution increases only slightly upon addition of lecithin liposomes; apparently, only a few chlorophyll molecules become incorporated in the liposomes. Unlike chlorophyll, the chlorophyllide formed during the chlorophyllase-catalyzed conversion of chlorophyll spontaneously enters the lecithin liposomes. This may be due to the fact that chlorophyllide is less likely than chlorophyll to form aggregates in an aqueous medium. The experiments thus provide an explanation for the increase in the chlorophyllase-catalyzed conversion of chlorophyll by lecithin liposomes [2]: the reaction product chloropyllide is removed by being taken up in lecithin.

Situation of chlorophyllase in thylakoids

Both chlorophyll and concanavalin A interact with solubilized chlorophyllase; this interaction is dependent on the pH and the Mg^{2+} concentration. It is shown in Figs. 4–6 that chlorophyllase embedded in natural membrane fragments reacts in a way similar to the solubilized enzyme. This means that both chlorophyll and concanavalin A bind to a site on the enzyme which protrudes from the membrane into the aqueous medium. Our results thus show that the active site of chlorophyllase is a hydrophylic one and consists of or is situated close to a specific carbohydrate group.

As suggested earlier [30,31], chlorophyllase, in its inactive form, may constitute a part of the protein-chlorophyll complex of Photosystem I. In the light of the distribution of chlorophyll-protein complexes in photosynthetic membranes and their function in membrane stacking [32–34], an investigation concerning the possible presence of carbohydrate residues in both Photosystem I and II proteins seems to be urgently required.

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