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STUDIES ON THE ACTION MECHANISM OF THE MEMBRANE ENZYME CHLOROPHYLLASE

II. INTERACTION OF CHLOROPHYLLIDE WITH CHLOROPHYLLASE AND WITH CHLOROPHYLLASE COMPLEXED WITH 4,4′-BIS(1-ANILINO-8-NAPHTHALENE SULFONATE) [BIS(ANS)]

JOHANNES W.J. LAMBERS, WILLEMKE TERPSTRA and YEHUDI K. LEVINE

Department of Biophysics, Physics Laboratory, State University of Utrecht, Princetonplein 5, P.O. Box 80.000, 3508 TA Utrecht (The Netherlands)

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The fluorescence of chlorophyllase-bound bis(ANS) (4,4′-bis(1-anilino-8-naphthalene sulfonate) is partly quenched by chlorophyllide. Evidence is obtained that this quenching is due to an interaction of chlorophyllide with bis(ANS) which is bound to a lipid binding site of chlorophyllase. Measurement of fluorescence of both bis(ANS) and chlorophyllide shows that fluorescence excitation energy is transferred from bis(ANS) to chlorophyllide. A similar energy transfer was observed when DGDG liposomes were used instead of chlorophyllase-associated lipids. It is concluded that chlorophyllide migrates from the active site of chlorophyllase to a lipid site on the enzyme. When the chlorophyllase-bis(ANS) complex becomes saturated with chlorophyllide, its residual fluorescence is independent of Mg^{2+} and is due to the binding of bis(ANS) at or near the active site of chlorophyllase. Fluorescence determinations of chlorophyllide formed upon incubation of chlorophyllase with chlorophyll provide evidence that chlorophyllide that has accumulated in enzyme-associated lipids can enter the aqueous phase surrounding the enzyme. A tentative model for the action mechanism of chlorophyllase on a molecular basis is presented.

Introduction

In the preceding article [1], isolated chlorophyllase was shown to contain two different kinds of binding site for the fluorescent probe molecule bis(ANS). One of these binding sites, called the 'protein binding site', has protein characteristics. Since bis(ANS) inhibits chlorophyllase activity competitively, it was concluded that this site is located at or near the active site of the enzyme. The second binding site, called the 'lipid binding site', consists of tightly enzyme-bound lipids. The purpose of the present article is to evaluate the significance of these lipids in the molecular mechanism of chlorophyllase functioning. To this end, the interaction of enzyme-bound bis(ANS) with chlorophyllide was determined by means of fluorescence measurements of both bis(ANS) and chlorophyllide. Chlorophyllide, like chlorophyll, is non-fluorescent in an aqueous medium, but fluoresces in a lipid environment. Unlike chlorophyll, however, chlorophyllide spontaneously binds to

Abbreviations: bis(ANS), 4,4′-bis(1-anilino-8-naphthalene sulfonate); DGDG, digalactosyl diacylglycerol; PC, phosphatidylcholine.

liposomes when the latter are added to chlorophyllide dispersed in an aqueous medium. This means that chlorophyllide formation can be monitored by means of its fluorescence [2].

The experimental results described below suggest that the lipids which are contained in isolated chlorophyllase play a role in the regulation of the enzyme activity in that they rapidly remove the reaction product chlorophyllide from the active center of the enzyme.

Materials and Methods

On the whole, the materials and methods are the same as described in the preceding article [1].

Chlorophyllin (sodium-copper salt) was purchased from Sigma.

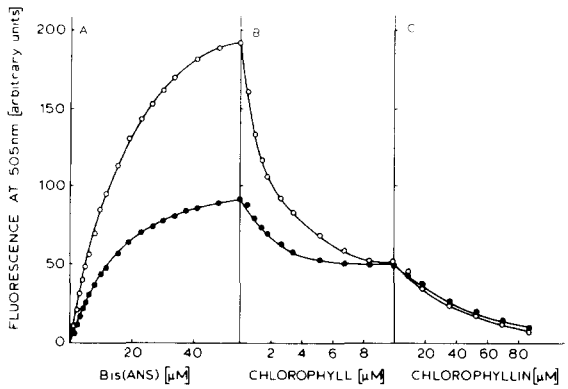

Fig. 1. (A) Fluorescence course of bis(ANS) upon its addition to chlorophyllase, measured in an aqueous medium in the presence or absence of $MgCl_2$. 66 μg chlorophyllase in 200 μl 20 mM Tris-HCl (pH 8.0) with (○) or without (●) 10 mM $MgCl_2$, were titrated with a $4 \cdot 10^{-4}$ M or $8 \cdot 10^{-4}$ M bis(ANS) solution in the same medium. The final volume was 220 μl. Data were corrected for chlorophyllase dilution. Excitation was at 365 nm. (B) Fluorescence decline of the chlorophyllase-bis(ANS) complex upon the addition of chlorophyll, measured in the presence (○) or absence (●) of $MgCl_2$. Chlorophyllase-bound bis(ANS), obtained in the experiments part (A) was titrated with 2 mM chlorophyll in acetone. The final acetone concentration was under 0.5%. The final volume was 240 μl. Data were corrected for chlorophyllase dilution. Chlorophyll(ide) fluorescence at 505 nm was negligible. (C) Influence of chlorophyllin addition on 505 nm fluorescence of chlorophyllase-bis(ANS)-chlorophyllide complex, measured in the presence (○) or absence (●) of $MgCl_2$. Chlorophyllin (2 mM in 20 mM Tris-HCl buffer (pH 8.0)) was added to the chlorophyllase-bis(ANS)-chlorophyllide complexes, obtained in the experiments of part (B). The final volume was 250 μl. Data were corrected for chlorophyllase dilution.

Chlorophyll is added to the reaction mixtures as a freshly prepared acetone extract of spinach chloroplasts: 1–2 g chloroplasts (wet weight) were extracted at about 4°C with 5 ml acetone. The chlorophyll concentration (about 2 μg/μl) was determined according to the method of Bruinsma [3]. The acetone extract also contains carotenoids and other lipids [4]. This unpurified chlorophyll solution was used because lipids are required for chlorophyllase-catalyzed chlorophyll hydrolysis [4] (experiment of Fig. 4).

Chlorophyllide was prepared by incubation of 0.4 nmol chlorophyll with 2 μg chlorophyllase for 15 min at room temperature. In this case, too, unpurified chlorophyll was used as a substrate. The amount of lipids or chlorophyllase present in the chlorophyllide preparation was too small to interfere with the fluorescence experiments (Figs. 1–3).

The fluorescence of lipid-bound chlorophyllide was excited at 365 nm and measured either at 678 nm or in the wavelength range from 650 to 700 nm. Generally the absorbance of protein or chromophore in the cuvette was kept below 0.1 at 365 nm to ensure linearity of concentration and fluorescence. In the experiment shown in Fig. 1c, the final absorbance rose to 0.2. Even then the chromophore concentration and its fluorescence were reasonably linear.

Results and conclusions

Interaction of chlorophyllide and chlorophyllin with chlorophyllase-bis(ANS) complex

Bis(ANS) exhibits very low fluorescence in an aqueous medium, but fluoresces strongly upon its binding to chlorophyllase [1]. Fig. 1A shows the course of the fluorescence intensity both in the presence and absence of Mg^{2+}, when chlorophyllase is titrated with bis(ANS) until near-saturation values are reached. When at this point chlorophyll is added to the chlorophyllase-bis(ANS) complex, a strong quenching of the bis(ANS) fluorescence is observed (Fig. 1B). Despite the presence of bis(ANS), the chlorophyllase concentration used in these experiments is so high that chlorophyll is instantaneously hydrolyzed; therefore, the decrease in the bis(ANS) fluorescence is actually caused by chlorophyllide and not by chlorophyll.

The fluorescence of chlorophyllase-bound bis(ANS) does not disappear entirely upon the addition of excess chlorophyll(ide), but reaches a steady value. This residual fluorescence intensity is independent of the presence or the absence of Mg^{2+}. Results reported in the previous article [1] show that with saturating amounts of bis(ANS) the number of 'protein binding sites' of chlorophyllase is unaffected by Mg^{2+}. Moreover, the florescence intensity of the maximum amount of bis(ANS) molecules bound to the protein binding site per mg chlorophyllase, n_1 in Ref. 1, agrees well with the residual fluorescence intensity observed in the present experiment (Fig. 1B). It is concluded that this residual fluorescence originates from bis(ANS) bound to the protein binding site of chlorophyllase. Thus, the decrease in fluorescence of chlorophyllase-bis(ANS) complex upon the addition of chlorophyll (Fig. 1B) is caused by the interaction between chlorophyllide and bis(ANS) bound to the lipid component of the enzyme (see also the following section).

In the preceding paper [1] it was concluded that bis(ANS) and chlorophyll compete for the active site of chlorophyllase. As the fluorescence of bis(ANS) bound to the protein binding site of the enzyme is not significantly influenced by the presence of chlorophyllide (Fig. 1B), it follows that chlorophyllide is rapidly removed from the active site. In Fig. 1C it is shown that chlorophyllin (a substrate analogue that resembles chlorophyll, but lacks the two esterified alcohols) is able to quench the bis(ANS) fluorescence that is left when excess chlorophyllide has been added. Chlorophyllin, like bis(ANS), inhibits chlorophyllase, presumably by binding to the catalytic centre of chlorophyllase. The fluorescence of the protein-bound bis(ANS) may be quenched either through the displacement by chlorophyllin or through the transfer of excitation energy to chlorophyllin. Since chlorophyllin does not exhibit any detectable fluorescence, it has not been possible to determine which explanation is the correct one. In any case, these results provide additional evidence that the protein binding site for bis(ANS) is at or near the catalytic site of chlorophyllase.

Energy transfer from bis(ANS) to chlorophyllide

It was shown in the previous section that the quenching of the bis(ANS) fluorescence upon addition of chlorophyll (Fig. 1B) is a result of the interaction between chlorophyllide and those bis(ANS) molecules that are bound to the lipid site of chlorophyllase. The number of lipid-bound bis(ANS) molecules increases upon the addition of Mg^{2+} [1]. Fluorescence quenching by chlorophyllide is at its maximum when excess chlorophyllide is present. Again, this fluorescence decrease can be caused either by displacement of the lipid-bound bis(ANS) by chlorophyllide or by excitation energy transfer from bis(ANS) to chlorophyllide. In this case we can discover which process is involved by measuring the fluorescence of bis(ANS) and of chlorophyllide, excited at 365 nm, in two different wavelength regions: 470–570 nm (maximum bis(ANS) fluorescence) and 650–700 nm (maximum chlorophyllide fluorescence). Chlorophyllide in an aqueous medium is virtually non-fluorescent. It was observed that chlorophyllide bound to chlorophyllase fluoresces at 678 nm (Fig. 2A; solid lines). This fluorescence can be measured in the absence of added lipids when large amounts of chlorophyllase are used: this observation can serve as an additional argument for the presence of a

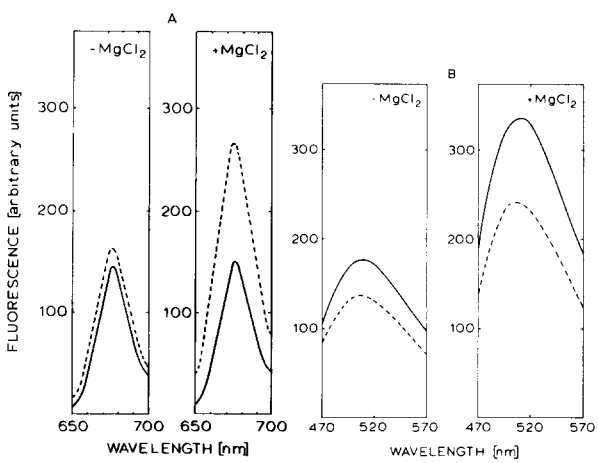

Fig. 2. (A) Effect of bis(ANS) (- - - -) on the fluorescence (678 nm) of chlorophyllase-bound chlorophyllide, measured in the absence or in the presence of $MgCl_2$. (B) Effect of chlorophyllide (----) on the fluorescence (505 nm) of chlorophyllase-bound bis(ANS), measured in the absence or in the presence of $MgCl_2$. Reaction mixtures: 100 μg chlorophyllase; 40 μM Bis(ANS); no or 10 mM $MgCl_2$; 2 μM chlorophyllide; 20 mM Tris-HCl (pH 8.0). Total aqueous volume, 200 μl. Fluorescence excitation was at 365 nm.

lipid component in chlorophyllase (cf. Ref. 2). Addition of bis(ANS) enhances the 678 nm fluorescence of a chlorophyllase-chlorophyllide complex (Fig. 2A; dotted lines). The observed enhancement is larger in the presence of Mg^{2+}, due to an increased number of lipid-bound bis(ANS) molecules. We checked that the chlorophyllide fluorescence is unaffected by Mg^{2+} in the absence of bis(ANS) (Fig. 2A; solid lines). Fluorescence of bound bis(ANS), on the other hand, is decreased by chlorophyllide (Fig. 2B; dotted lines). From all these results it is concluded that the lipid-bound bis(ANS) and the lipid-bound chlorophyllide are in such proximity that excitation energy transfer occurs from bis(ANS) to chlorophyllide. However, the possibility that some displacement of bis(ANS) by chlorophyllide may still occur cannot be ruled out.

Further evidence for energy transfer between adjacent bis(ANS) and chlorophyllide molecules, both bound to the lipid site of chlorophyllase, is derived from the results of the experiments depicted in Fig. 3. Here DGDG liposomes were used instead of chlorophyllase. Essentially the same picture emerges: bis(ANS) enhances chlorophyllide fluorescence (Fig. 3A) and, in turn, bis(ANS) fluorescence is quenched by chlorophyllide (Fig. 3B). PC liposomes gave similar results. These experiments provide further evidence that energy transfer observed between bis(ANS) and chlorophyllide in the presence of chlorophyllase occurs at a lipid site on the chlorophyllase molecule.

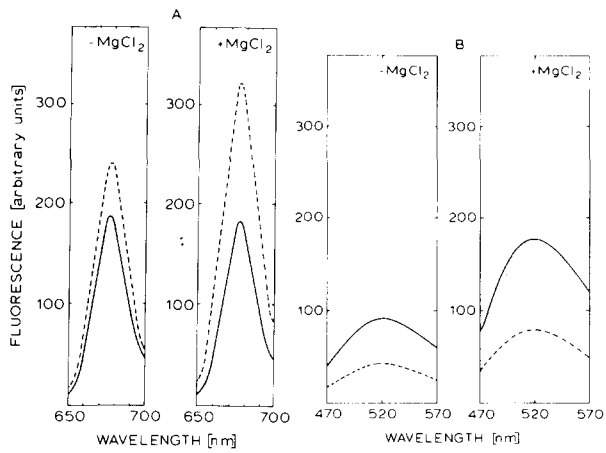

Fig. 3. (A) Effect of bis(ANS) (----) on the fluorescence (678 nm) of DGDG-bound chlorophyllide, measured in the absence or in the presence of $MgCl_2$. (B) Effect of chlorophyllide (----) on the fluorescence (505 nm) of DGDG-bound bis(ANS), measured in the absence or in the presence of $MgCl_2$. Reaction mixtures: 40 μg DGDG (liposomes); 40 μM bis(ANS); no or 10 mM $MgCl_2$; 2 μM chlorophyllide; 20 mM Tris-HCl (pH 8.0). Total aqueous volume, 200 μl. Fluorescence excitation was at 365 nm.

Chlorophyllide fluorescence in the absence of bis(ANS)

From the results of the two previous sections one can say that as soon as chlorophyllide is formed after chlorophyll hydrolysis, it migrates from the active center of chlorophyllase to a lipid component that forms a part of the enzyme. The results of the experiments presented in Fig. 4 show that chlorophyllide thereafter can enter the aqueous phase surrounding the chlorophyllase molecule. Fig. 4, curve a, shows the increase of chlorophyllide fluorescence at 678 nm, when chlorophyll is added to a small amount of chlorophyllase in the presence of an excess of lipids (DGDG). Since chlorophyllide dispersed in an aqueous environment is virtually non-fluorescent, it is assumed that the measured fluorescence is due to lipid-bound chlorophyllide (cf. ref. 2). When, in the absence of added lipids, chlorophyll is added to a large amount of chlorophyllase (Fig. 4, curve b), an immediate increase of chlorophyllide fluorescence is observed; this fluorescence is ascribed to chlorophyllide bound to chlorophyllase-associated lipids. However, the observed fluorescence is lower than in Fig. 4, curve a, although, with the high enzyme concentration used, it can be assumed that chlorophyll has been completely converted into chlorophyllide. This suggests that part of the chlorophyllide is not bound to the limited amount of intrinsic lipids of chlorophyllase but is present in the aqueous phase in a non-fluorescent form. This hypothesis was verified by the observation that addition of excess lipids (Fig. 4, arrow curve b) shifts the binding equilibrium completely to the lipid phase and thus maximum fluorescence is observed. The results shown in Fig. 4, curve c, once more confirm our hypothesis. In these experiments the conditions were the same as those for curve a in Fig. 4, but no external lipids were added. Only a small fluorescence increase is observed and this is ascribed to the presence of a

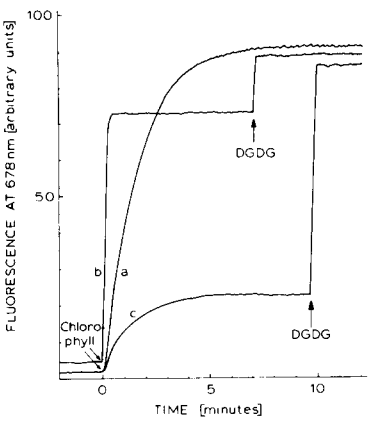

Fig. 4. Chlorophyllase-catalyzed chlorophyll hydrolysis, measured from the increase of the chlorophyllide fluorescence at 678 nm. Reaction mixtures: (a) 2 μg chlorophyllase; 40 μg DGDG (liposomes); 20 mM Tris-HCl (pH 8.0); 10 mM dithiothreitol; total volume, 200 μl. 0.16 nmol chlorophyll was added at zero time; (b) 100 μg chlorophyllase; 20 mM Tris-HCl (pH 8.0); 10 mM dithiothreitol; total volume, 180 μl. 0.16 nmol chlorophyll was added at zero time and 40 μg DGDG (liposomes) in 20 μl 20 mM Tris-HCl (pH 8.0) were added after 7 min; (c) the same as under (a), but now DGDG was added after 10 min incubation with chlorophyll. Temperature, 21 ± 1 °C.

small amount of lipid present in the chlorophyll extract [4]. Upon addition of excess lipids (Fig. 4, arrow curve c) maximum fluorescence again shows up. This shows that chlorophyll had been completely hydrolyzed and that most of the chlorophyllide was present in the aqueous phase.

Discussion

Results of previous experiments indicated that bis(ANS) binds to the catalytic site of chlorophyllase [1]. This hypothesis is supported by the observation, made in the present experiments, that the substrate analogue, chlorophyllin, is able to quench the fluorescence of bis(ANS) bound to this site (Fig. 1C).

Whereas the binding site mentioned above is located in the protein part of the enzyme, fluorescence spectra of chlorophyllase-bound bis(ANS) suggested that a second binding site for bis(ANS) consists of enzyme-associated lipids [1]. Our present results provide additional evidence for this hypothesis. With DGDG liposomes, containing both bis(ANS) and chlorophyllide, it was shown that lipid-bound chlorophyllide quenches the fluorescence of lipid-bound bis(ANS) through excitation energy transfer (Fig. 3). Since the same phenomenon was observed when chlorophyllide and bis(ANS) were bound to chlorophyllase, it is concluded that (i) chlorophyllide and bis(ANS) are both bound to the lipid component of the enzyme and (ii) the two molecules are so close to each other within this lipid component that energy transfer is possible (Fig. 2). Only part of the total bis(ANS) fluorescence is quenched upon addition of chlorophyll to a chlorophyllase-bis(ANS) complex (Fig. 1B). The residual fluorescence is ascribed to bis(ANS) bound to the active site. On a time-scale of minutes this fluorescence is not influenced by chlorophyllide, indicating that energy transfer from this bis(ANS) to chlorophyllide does not occur. On a time scale of seconds quenching of active site-bound bis(ANS) fluorescence upon chlorophyll addition is expected to occur through displacement of bis(ANS) by chlorophyll. Since relatively high concentrations of chlorophyllase are used in these fluorescence experiments (Fig. 1), the conversion of chlorophyll into chlorophyllide is almost instantaneous. This may explain why a decrease in active site-bound bis(ANS) fluorescence is not observed, even when all lipid-bound bis(ANS) fluorescence is quenched. The results indicate that once chlorophyllide is formed at the active site it can be readily removed and enters a lipid site which is part of the enzyme (Fig. 2). Earlier, evidence was found that the active center of chlorophyllase is near the carbohydrate part of the molecule and is exposed to the aqueous medium [2]. Our present results suggest that this active centre is located on the boundary between the hydrophylic and lipophylic part of the enzyme.

Although other membrane proteins have been shown to contain lipids after their purification [5–11], it has been shown only in a few cases that these endogenous lipids are directly involved in the functioning of the membrane protein, e.g., light-harvesting chlorophyll-protein complex [12], NADH:ubiquinone reductase [13], cytochrome oxidase [14,15], Ca^{2+}-ATPase [16,17].

Chlorophyllide is not restricted to the lipid phase; it has been shown (Fig. 4) that it can also enter the aqueous phase. Apparently chlorophyll-

ide prefers a lipid environment, since the addition of relatively small amounts of lipids shifts the partitioning equilibrium to the lipid phase.

The combined results presented in this and the preceding paper lead us to the following tentative model for chlorophyllase action: the substrate chlorophyll is attached to the active site of the enzyme. The accessibility of this site, which is situated close to the hydrophobic carbohydrate part of the molecule, may be regulated by Mg^{2+}. After the chlorophyll is hydrolyzed, the reaction product – chlorophyllide – is immediately removed to a site on the enzyme which consists of protein-associated lipids. The headgroups of these lipids can again be influenced by Mg^{2+}. Finally, the chlorophyllide can migrate from this lipid site to the aqueous medium surrounding the (isolated) enzyme.

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References

1 Lambers, J.W.J., Terpstra, W. and Levine, Y.K. (1984) Biochim. Biophys. Acta 789, 188–196
2 Terpstra, W. (1982) Biochim. Biophys. Acta 681, 233–241
3 Bruinsma, J. (1961) Biochim. Biophys. Acta 52, 576–578
4 Terpstra, W. and Lambers, J.W.J. (1983) Biochim. Biophys. Acta 746, 23–31
5 Lemaire, M., Møller, J.V. and Tanford, C. (1976) Biochemistry 15, 2336–2342
6 Rawyler, A., Henry, L.E.A. and Siegenthaler, P-A. (1980) Carlsberg Res. Commun. 45, 443–451
7 Trémolières, A., Dubacq, J-P., Ambard-Bretteville, F. and Rémy, R. (1981) FEBS Lett. 130, 27–31
8 Peters, W.H.M., Fleuren-Jakobs, A.M.M., De Pont, J.J.H.H.M. and Bonting, S.L. (1981) Biochim. Biophys. Acta 649, 541–549
9 Heinz, E. and Siefermann-Harms, D. (1981) FEBS Lett. 124, 105–111
10 Peters, W.H.M., Fleuren-Jakobs, A.M.M., Schrijen, J.J., De Pont J.J.H.H.M. and Bonting, S.L. (1982) Biochim. Biophys. Acta 690, 251–260
11 Gounaris, K., Whitford, D. and Barber, J. (1983) FEBS Lett. 163, 230–234
12 Rémy, R., Trémolières, A., Duval, J.C., Ambard-Bretteville, F. and Dubacq, J.P. (1982) FEBS Lett. 137, 271–275
13 Ragan, C.I. (1976) Biochim. Biophys. Acta 456, 249–290
14 Yu, C., Yu, L. and King, T.E. (1975) J. Biol. Chem. 250, 1383–1392
15 Robinson, N.C. and Capaldi, R.A. (1977) Biochemistry 116, 375–381
16 Fiehn, W. and Hasselbach, W. (1970) Eur. J. Biochem. 13, 510–518
17 Warren, G.B., Toon, P.A., Birdsall, N.J.M., Lee, A.G. and Metcalfe, J.C. (1974) Biochemistry 1, 5501–5507