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STUDIES ON THE ACTION MECHANISM OF THE MEMBRANE ENZYME CHLOROPHYLLASE

I. BINDING OF THE FLUORESCENCE PROBE 4,4′-BIS(1-ANILINO-8-NAPHTHALENE SULFONATE) [BIS(ANS)]; INFLUENCE OF Mg^{2+} AND INTRINSIC LIPIDS

JOHANNES J.W. 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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Isolated chlorophyllase (chlorophyll chlorophyllido-hydrolase, EC 3.1.1.14) interacts with the probe molecule 4,4′-bis(1-anilino-8-naphthalene sulfonate) (bis(ANS)), resulting in a strongly fluorescent chlorophyllase-bis(ANS) complex. Analyses of the fluorescence spectra and of bis(ANS)-binding kinetics reveal that isolated chlorophyllase contains two different kinds of binding site for bis(ANS): a high-affinity and a low-affinity one. The high-affinity site is at or near the active site of chlorophyllase and therefore is located in the protein part of the enzyme. Bis(ANS) competes with chlorophyll for this active site. The low-affinity site is located in tightly chlorophyllase-bound lipids. Mg^{2+} affects the binding of bis(ANS) to the two kinds of binding site on chlorophyllase. In the case of the high-affinity (protein) site Mg^{2+} causes a large increase in the affinity of the enzyme for bis(ANS), but the binding capacity remains the same. In the case of the low-affinity (lipid) site Mg^{2+} causes only a small increase in the affinity for bis(ANS), but the binding capacity of the enzyme for bis(ANS) is enlarged 2–3-fold. The relation between these observations and the effects that Mg^{2+} has on chlorophyllase activity is discussed.

Introduction

The hydrolysis of chlorophyll into chlorophyllide and phytol is usually considered to be the first step in the degradation of chlorophyll in plant organisms. This hydrolysis is catalyzed by the enzyme chlorophyllase (chlorophyll chlorophyllido-hydrolase, EC 3.1.1.14). The enzyme is an intrinsic membrane glycoprotein, localized in thylakoid membranes [1–4]. The main goal of our research is to gain insight into the structure and function of the isolated chlorophyllase and to determine the factors that are involved in the regulation of enzyme activity; one of these factors, for instance, is the presence or absence of divalent cations: Mg^{2+} activates the chlorophyllase-catalyzed chlorophyll hydrolysis [5]. For this activation the presence of certain chloroplast lipids is required [6]. Since various interactions between chlorophyllase, chlorophyll(ide), lipids and cations occur, the interpretation of experimental results is complicated [6,7]. In order to elucidate the mechanism by which chlorophyllase functions, it is therefore necessary

Abbreviations: MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; PG, phosphatidylglycerol; SQDG, sulphoquinovosyldiacylglycerol; PC, phosphatidylcholine; ANS, 1-anilino-8-naphthalene sulfonate; bis(ANS), 4,4′-bis(1-anilino-8-naphthalene sulfonate).

to separate the factors that ultimately determine the enzyme activity. This approach has enabled us to determine which plant lipids are directly involved in the interaction with the enzyme. It was found that chlorophyllase could be reconstituted with a mixture of galactolipid (MGDG or DGDG) and PG and that this reconstitution effectively prevents the enzyme from heat denaturation [8].

To study the interaction of the substrate, chlorophyll, and the product, chlorophyllide, with isolated chlorophyllase, we combined the fluorescence probe bis(ANS) with the enzyme. In this paper we describe the way in which bis(ANS) binds to chlorophyllase and its interaction with chlorophyll; in a second, accompanying paper [9] we discuss the interaction of chlorophyllase-bound bis(ANS) with chlorophyllide.

Bis(ANS) has essentially the same fluorescence characteristics as the monomer ANS [10,11]. Like ANS, bis(ANS) is virtually non-fluorescent in aqueous solutions, but becomes strongly fluorescent in non-aqueous solvents or when it is bound to hydrophobic sites in proteins [10] or several lipids (see this paper). Unlike ANS, however, bis(ANS) binds readily to chlorophyllase. Several other investigators have found that bis(ANS) is strongly absorbed to certain proteins, whereas the monomer shows hardly any interaction [12–19]. The results presented here show that bis(ANS) binds to two different kinds of binding site on the isolated chlorophyllase: a 'protein binding site' with relatively high affinity and a 'lipid binding site' with relatively low affinity for bis(ANS). Binding of bis(ANS) to the protein binding site occurs at or near the active center of chlorophyllase, resulting in a competitive inhibition of enzyme activity.

Mg^{2+}, which activates the enzyme reaction, induces an enhancement of the fluorescence of the chlorophyllase-bis(ANS) complex. The reasons for this enhancement are analyzed and the implications for the functioning of the enzyme are discussed.

Materials and Methods

PC (egg lecithin) and PG were purchased from Sigma and MGDG, DGDG and SQDG from Lipid Products, Nutfield Nurseries, Surrey, U.K. Part of the bis(ANS) (dipotassium salt) was a gift from Dr. D. Jameson, University of Illinois, Urbana; the rest was purchased from Molecular Probes. All other chemicals used were of analytical quality.

Chlorophyllase was isolated from *Phaeodactylum tricornutum*, according to the method described in Ref. 6. The concentration of bis(ANS) was determined spectroscopically, using a molar absorption coefficient of $16.8 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ at 385 nm [11]. A stock solution of $1 \cdot 10^{-3}$ M bis(ANS) in 20 mM Tris-HCl buffer (pH 8.0) was used for the experiments. Lipids were extracted from isolated chlorophyllase according to the method of Bligh and Dyer [20]. The lipid components were separated by one-dimensional thin layer chromatography on silica plates (HPTLC, silica-gel 60, Merck). The solvent system used was $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v). Protein content was estimated either from 280 nm absorption, assuming an $E_{1\ cm}^{0.1\%}$ of 1.0 [6], or by the Bio-Rad microassay [21]. Lipid suspensions ('liposomes') were prepared as follows: lipids in hexane or chloroform were evaporated to dryness in an N_2 current, 20 mM Tris-HCl buffer (pH 8.0) was added to the residue, and the suspension was sonicated under N_2 for 30 min in a Bransonic bath.

Two different methods were used to determine the chlorophyllase activity:

(a) The 'chemical assay'; with this method, which was described in Ref. 6, chlorophyll in an acetone extract of spinach chloroplasts and chlorophyllase were incubated together for 17 h at 23°C; thereafter, chlorophyll and chlorophyllide were separated by partitioning between aqueous acetone and petroleum spirit.

(b) The 'fluorescence assay'; with this method chlorophyllide formation was monitored directly by its fluorescence in added lipids [22]. Fluorescence excitation was at 546 nm. Enzyme activity was determined from the initial slope of the curve of chlorophyllide fluorescence (678 nm) versus time. Details of the reaction mixtures are given with the figures.

Fluorescence was measured with an apparatus essentially the same as that designed by Goedheer [23]. A 125 W mercury lamp (Philips) was used as a light source. Excitation light of 365 and 546 nm

(see the individual experiments) was selected with interference filters. The sample cuvette was placed in this light beam at an angle of 45°. The emitted fluorescence light passed through a Bausch and Lomb monochromator (7.2 nm bandwidth; 500 mm focal length) and was detected with an RCA 4832 photomultiplier. The signal was fed into a lock-in amplifier and then recorded. Samples (200 µl) were placed in cuvettes with an optical path of 1 mm. A standard of 5 µM quinine sulfate in 0.1 M H_2SO_4 was used throughout all the experiments to permit direct comparison of the results.

Chlorophyllase was titrated with bis(ANS) by adding small aliquots of bis(ANS) solutions ($4 \cdot 10^{-4}$ M or $8 \cdot 10^{-4}$ M) to a chlorophyllase preparation. Details of the titration procedure are given with the figures. Fluorescence of unbound bis(ANS) is negligible. In all instances the absorbance of protein or chromophore in the cuvette at the wavelength of the incident light did not exceed 0.1. This ensures linearity of fluorescence with chromophore concentration. As the maximum of ANS fluorescence lies at a wavelength where absorbance is negligible, reabsorption of fluorescence can be neglected. In the conversion of fluorescence data to binding data, the following equation was used:

$$\frac{[\text{bis(ANS)}]_b}{[\text{bis(ANS)}]_{total}} = \frac{F_{obsd}}{F_\infty}$$

where $[\text{bis(ANS)}]_{total}$ is the total amount of bis(ANS) (nmol per mg enzyme), $[\text{bis(ANS)}]_b$ the chlorophyllase-bound bis(ANS) (nmol per mg enzyme), F_{obsd} the observed fluorescence at 505 nm of the chlorophyllase-bis(ANS) complex and F_∞ the calculated fluorescence at 505 nm when all the bis(ANS) present is bound to the enzyme. This fluorescence value of totally bound bis(ANS) is obtained by extrapolation to infinite protein concentration, using plots of $1/F_{obsd}$ vs. $1/P_0$ (P_0 = protein concentration) at a fixed bis(ANS) concentration [24]. The binding data obtained were plotted according to the method of Scatchard [25]:

$$\frac{[\text{bis(ANS)}]_b}{[\text{bis(ANS)}]_f} = \frac{1}{K_d}(n\text{-bis(ANS)}_b)$$

where $[\text{bis(ANS)}]_b$ is chlorophyllase-bound bis(ANS) (nmol per mg enzyme); $[\text{bis(ANS)}]_f$, unbound bis(ANS) (mol/l); n, binding capacity, i.e., maximum amount of chlorophyllase-bound bis(ANS) (nmol per mg enzyme); K_d, dissociation constant of the chlorophyllase-bis(ANS) complex (mol/l).

The binding data were fitted, using a non-linear least-squares method, with a Langmuir-type equation:

$$[\text{bis(ANS)}]_b = \frac{n_1 \cdot [\text{bis(ANS)}]_f}{K_{d1} + [\text{bis(ANS)}]_f} + \frac{n_2 \cdot [\text{bis(ANS)}]_f}{K_{d2} + [\text{bis(ANS)}]_f} + \dots$$

Here n_1, n_2 ... are the respective maximum amounts of bound bis(ANS) and K_{d1}, K_{d2} ... the respective dissociation constants of chlorophyllase-bis(ANS) of the different kinds of binding site. Quantum yields were determined by comparison with bovine serum albumin, assuming an absolute quantum yield of bovine serum albumin-bis(ANS) complex of 0.7 [10].

Results

Fluorescence enhancement of bis(ANS) upon its interaction with chlorophyllase

Bis(ANS) in aqueous solution shows very little fluorescence and has an emission maximum at 555 nm (Fig. 1, curve b). When chlorophyllase is added to a bis(ANS) solution, the bis(ANS) fluorescence intensity is considerably enhanced and there is a concomitant blue shift of the emission maxima (Fig. 1, curves c–e). In the absence of bis(ANS) chlorophyllase does not exhibit any measurable fluorescence in the wavelength range studied (Fig. 1, curve a). These results show that bis(ANS) interacts with chlorophyllase. Closer examination of the chlorophyllase-bis(ANS) interaction reveals that at relatively low bis(ANS) concentrations (Fig. 1, curve c) the fluorescence maximum lies at 500 nm, but with increasing bis(ANS) concentrations the fluorescence maximum gradually shifts towards longer wavelengths (508–510 nm) (Fig. 1, curves d and e). In the literature no information could be found about bis(ANS) binding to biomembranes. However, with ANS, similar shifts of the fluorescence maximum towards longer wavelengths have been shown to occur upon its binding to biomembranes [26–29]. In these cases ANS is assumed to bind to both proteins and

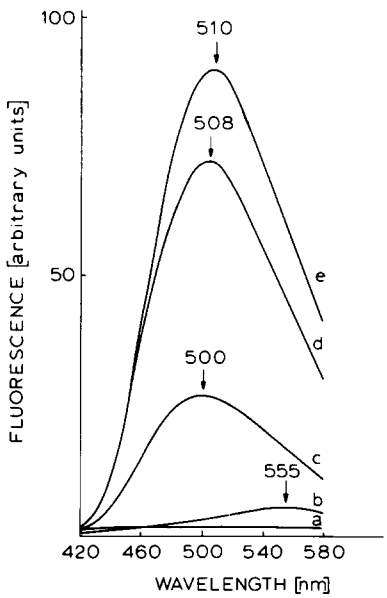

Fig. 1. Fluorescence emission spectra of chlorophyllase, bis(ANS) and chlorophyllase-bis(ANS) complexes in an aqueous medium. (a) 0.5 mg/ml chlorophyllase; (b) 60 μM bis(ANS); (c) 0.5 mg/ml chlorophyllase + 5 μM bis(ANS); (d) 0.5 mg/ml chlorophyllase + 30 μM bis(ANS); (e) 0.5 mg/ml chlorophyllase + 60 μM bis(ANS). Aqueous medium: 20 mM Tris-HCl (pH 8.0). Fluorescence excitation at 365 nm.

lipids present in the membrane (see also the following sections). Since chlorophyllase is a membrane enzyme, the observed fluorescence shifts (Fig. 1, curves b–e) could well be caused by the presence of two different kinds of binding site for bis(ANS) on the molecule, presumably a protein site and a lipid site. Extraction of an isolated chlorophyllase preparation with methanol/chloroform and analysis of the extract by TLC on silica-gel plates (see Materials and Methods), did indeed show the presence of lipids. MGDG, PG and carotenoids were most prominent, but traces of other typical plant lipids, such as DGDG, SQDG and PC, could be detected as well (data not shown). No quantitative analysis of the lipids was made, but at a rough estimate 100–200 μg lipid per mg enzyme was present. Further evidence for the presence of lipids and their involvement in the binding of bis(ANS) is presented in the following sections.

Competitive inhibition of chlorophyllase activity by bis(ANS)

Chlorophyllase-catalyzed chlorophyll hydrolysis is inhibited by bis(ANS). 'Chemical' as well as 'fluorescence' assays of chlorophyllase activity (see Materials and Methods) yielded similar results in this respect. Fig. 2 shows the effect that bis(ANS) at various concentrations has on the rate of chlorophyll hydrolysis. The results, presented in a double-reciprocal plot (Lineweaver-Burk plot), indicate that bis(ANS) acts as a competitive inhibitor; i.e., the rate of chlorophyll hydrolysis decreases with the addition of increasing amounts of bis(ANS), but in the presence of a sufficient amount of the substrate (chlorophyll) the same maximum enzyme activity can be reached at all bis(ANS) concentrations (Fig. 2; intercept with ordinate equals $1/V_{max}$). This strongly suggests that bis(ANS) competes with chlorophyll for the active site of chlorophyllase. As can be derived from Fig. 2, the maximum enzyme activity *under the specified conditions* and in the absence of bis(ANS) is 19 nmol chlorophyll/min per mg enzyme and the K_m is 0.5 μM.

A plot of the strength of inhibition (i.e., the slopes of the lines in Fig. 2) versus various inhibitor (bis(ANS)) concentrations does not yield a straight line (insert Fig. 2); the inhibition increases to a specific level and then increases no further. This type of inhibition is referred to as partially competitive and indicates that, contrary to what occurs with fully competitive inhibition, the binding of bis(ANS) does not entirely prevent the binding of chlorophyll to the active site (and vice versa). This type of inhibition indicates that a chlorophyllase-chlorophyll-bis(ANS) complex may exist [30]. For the reasons mentioned above, it is not possible to determine an exact value for the

TABLE I

BINDING PARAMETERS OF CHLOROPHYLLASE-BIS(ANS)

K_d, dissociation constant of chlorophyllase-bis(ANS) complex. n, binding capacity, i.e., maximum amount of chlorophyllase-bound bis(ANS) (nmol per mg enzyme). The data were derived from the results of experiments shown in Fig. 4B and C.

	$-Mg^{2+}$	$+Mg^{2+}$
K_{d1}	$6.2 \cdot 10^{-6}$ M	$8.6 \cdot 10^{-7}$ M
K_{d2}	$2.0 \cdot 10^{-5}$ M	$1.2 \cdot 10^{-5}$ M
n_1	13.5	13.7
n_2	14.4	37.8

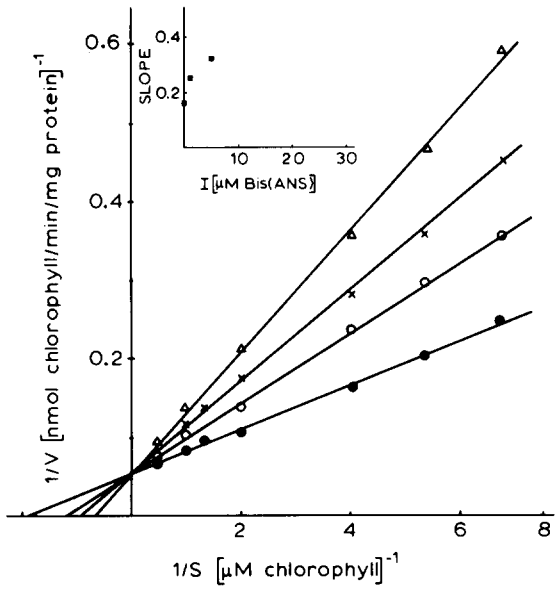

Fig. 2. Competitive inhibition of chlorophyllase activity by bis(ANS), as shown in double-reciprocal (Lineweaver-Burk) plots of enzyme activity (V) versus substrate concentration (S). Enzyme activity was determined by the 'fluorescence assay' (see Materials and Methods). Reaction mixtures: 0.4 μg chlorophyllase, 20 μg PG ('liposomes') 10 mM dithiothreitol, 10 mM $MgCl_2$, bis(ANS) (various concentrations), 20 mM Tris-HCl (pH 8.0). The final aqueous volume was 200 μl. At zero time, chlorophyll in a spinach chloroplast extract in acetone (2 mM solution) was added to the reaction mixtures. Maximum acetone concentration 0.1%. [Bis(ANS)] (μM): ●, none; ○, 1; ×, 5; △, 25. Inset: Plot of the slopes of the lines from the Lineweaver-Burk plot versus various bis(ANS) concentrations (I).

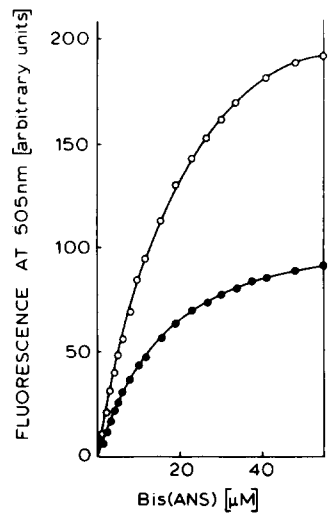

Fig. 3. Fluorescence course of bis(ANS) upon its addition to chlorophyllase, measured in an aqueous medium in the presence (○) or absence (●) of $MgCl_2$. Procedure: 66 μg chlorophyllase in 200 μl 20 mM Tris-HCl (pH 8.0) with or without 10 mM $MgCl_2$ was titrated with a 4×10^{-4} M or 8×10^{-4} M bis(ANS) solution in the same medium. The final volume was 220 μl. Data were corrected for chlorophyllase dilution and represent the average of three determinations. The standard error did not exceed 5% of the measured value.

dissociation constant (K_i) of the chlorophyllase-bis(ANS) complex by linear extrapolation.

Analysis of the binding of bis(ANS) to chlorophyllase; effect of Mg^{2+}

The binding of bis(ANS) to chlorophyllase was studied by fluorimetric titration of the enzyme with bis(ANS). The experiments were performed both in the presence and absence of Mg^{2+}. Typical titration curves are shown in Fig. 3. The addition of 10 mM Mg^{2+} (cf. Ref. 5) to the reaction mixture resulted in a considerable fluorescence enhancement of the chlorophyllase-bis(ANS)complex. The data were analyzed with Scatchard plots [25] (Fig. 4A). The curves obtained are non-linear, indicating that there is more than one kind of binding site on the chlorophyllase molecule. On using non-linear least-squares methods (see Materials and Methods), the best fit of these data is obtained by assuming two different kinds of binding site of chlorophyllase for bis(ANS). The binding parameters, determined after fitting, are shown in Table I. Before analyzing the curves of Fig. 4A, we made two assumptions: first, all binding sites of each kind are equal and independent, and secondly, the fluorescence quantum yield of all bound bis(ANS) molecules for both kinds of site is the same. At least the last condition is satisfactorily fulfilled, since double-reciprocal plots of enzyme concentration versus fluorescence intensity at a fixed bis(ANS) concentration [24] yield straight lines. In addition, extrapolation results in identical maximum fluorescence values, irrespective of the presence or absence of Mg^{2+} (Fig. 5).

The data above corroborate the hypothesis, derived from experiments described in the first section of this chapter, that in chlorophyllase there are two kinds of binding site for bis(ANS). These sites can be characterized in the following way:
a 'protein-binding site' with relative high affinity

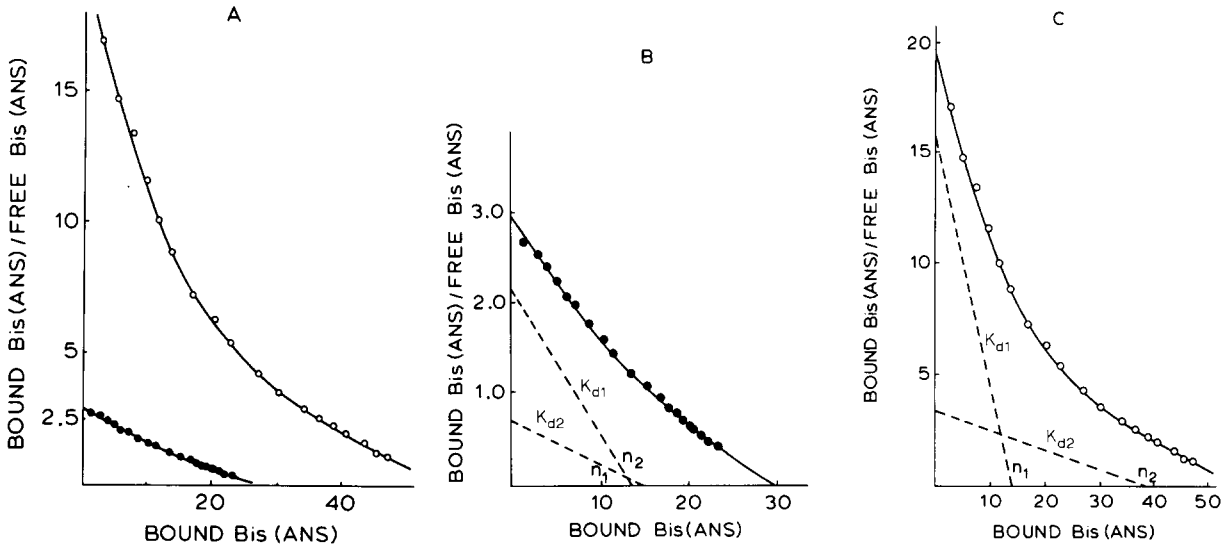

Fig. 4. (A) Scatchard plots for the binding of bis(ANS) to chlorophyllase in the absence (●) and presence (○) of $MgCl_2$. Bis(ANS) fluorescence at 505 nm was determined during the titration of chlorophyllase (66 μg in 200 μl 20 mM Tris-HCl buffer (pH 8.0) with or without $MgCl_2$) with a $4 \cdot 10^{-4}$ M and $8 \cdot 10^{-4}$ M bis(ANS) solution, respectively. The temperature was 21 ± 1°C. Corrections were made for dilutions. Bound bis(ANS) is expressed in nmol/mg enzyme, free bis(ANS) in mol/l. Excitation was at 365 nm. (B,C) Analysis of the curves from (A): (B) $-MgCl_2$; (C) $+MgCl_2$. Fitting was done by the non-linear least-squares method. The best fit was obtained with two different kinds of bis(ANS)-binding site per chlorophyllase molecule. (B) $K_{d1} = 6.2 \cdot 10^{-6}$ M; $K_{d2} = 2.0 \cdot 10^{-5}$ M. (C) $K_{d1} = 8.6 \cdot 10^{-7}$ M; $K_{d2} = 1.2 \cdot 10^{-5}$ M.

TABLE II

SOME FLUORESCENCE PARAMETERS OF BIS(ANS) BOUND TO VARIOUS LIPIDS AND PROTEINS

Lipids and proteins, 0.2 mg/ml 20 mM Tris-HCl (pH 8.0); 10 μM bis(ANS), 10 mM $MgCl_2$, n.d., not determined.

	Spectral maximum (nm)	Fluorescence intensity at maximum (arbitrary units)	Fluorescence intensity after addition of Mg^{2+}	Quantum yield [a]
Lipids (liposomes)				
PG	514	4	32	0.23 [b]
MGDG	516	25	55	n.d. [c]
DGDG	514	45	71	0.27
PC	512	180	220 [d]	0.57 [e]
Proteins				
Chlorophyllase	502 [f]	25	52	0.29
Bovine serum albumin	499	178	180	0.70 [g]
Chymotrypsin	504	6	7	n.d. [h]

[a] See methods for determination.
[b] Determined in the presence of Mg^{2+}.
[c] Too high turbidity of MGDG in its hexagonal phase made it impossible to determine the quantum yield.
[d] With the [bis(ANS)]/[PC] ratio used here, the lipid is almost saturated with bis(ANS). At lower [PC] or higher [bis(ANS)] the Mg^{2+} effect is much larger.
[e] For ANS the quantum yield is lower: approx 0.30 (see Ref. 28).
[f] cf. Fig. 1.
[g] Determined by Ref. 10.
[h] No saturation of binding is possible and quantum yield determination is therefore difficult. No literature values are available.

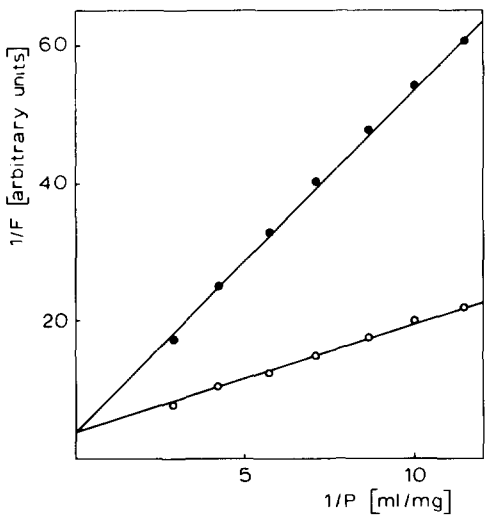

Fig. 5. Double-reciprocal plot of bis(ANS) fluorescence at 505 nm versus chlorophyllase concentration. Experimental conditions: 66 μg chlorophyllase in 200 μl 20 mM Tris-HCl (pH 8.0) with (○) or without (●) 10 mM $MgCl_2$, 10 μM bis(ANS). Chlorophyllase was diluted by adding 10 μM bis(ANS), dissolved in the same aqueous medium, in discrete steps.

for bis(ANS) (binding parameters K_{d1} and n_1), and a 'lipid-binding site' with relative low affinity for bis(ANS) (binding parameters K_{d2} and n_2).

In the absence of $MgCl_2$, the binding capacities of the protein and lipid sites are equal (Fig. 4B; Table I). The effect that Mg^{2+} has on the binding characteristics of the protein and lipid sites differs: At the 'protein binding site', the addition of Mg^{2+} does not change the maximum amount of bound bis(ANS) molecules (the value of n_1 remains virtually the same), but the binding affinity increases by a factor of 7 (K_{d1} 7-times smaller) (Figs. 4B, C; Table I).

At the 'lipid binding site', the addition of Mg^{2+} increases both the number of bound bis(ANS) molecules (by a factor 2.6; n_2 in Figs. 4B, C; Table I) and the binding affinity (by a factor 1.7; $1/K_{d2}$ in Figs. 4B, C; Table I).

The influence that Mg^{2+} has on the 'lipid binding site' of chlorophyllase is similar to the influence that divalent cations have on the binding of ANS to isolated lipids and to lipids embedded in biomembranes [31–38]; in these cases, too, the investigators found an increase in the number of ANS-binding sites with a concomitant small increase of the binding affinity. The fluorescence quantum yield of lipid-bound ANS was not influenced by cations. In order to check these results with respect to bis(ANS), some preliminary experiments were performed on the fluorescence characteristics of bis(ANS) bound to some lipids and proteins. In addition, the influence of Mg^{2+} was investigated. Table II shows that the fluorescence maximum of lipid-bound bis(ANS) is at a higher wavelength than that of protein-bound bis(ANS) (cf. Ref. 28 for similar results with ANS). Whereas with proteins (bovine serum albumin and chymotrypsin) Mg^{2+} has little effect on fluorescence intensity, fluorescence is clearly enhanced upon the addition of Mg^{2+} to lipid-bound bis(ANS). At least in the case of DGDG and PC, this enhancement results from an increased number of bound bis(ANS) molecules, since it could be established that the quantum yield is not affected by Mg^{2+}.

In short, one can say that the Mg^{2+}-induced fluorescence enhancement of the chlorophyllase-bis(ANS) complex is due mainly to two different effects: (i) a large increase in the binding affinity of the 'protein binding site' for bis (ANS) (below saturation) and (ii) an increase in the number of 'lipid binding sites' for bis(ANS).

Discussion

The experiments on the binding of bis(ANS) to chlorophyllase reveal that the isolated enzyme contains two different kinds of binding site: (a) a 'protein binding site', with relatively high affinity for bis(ANS); and (b) a 'lipid binding site', with relatively low affinity for bis(ANS) (see Fig. 4).

Bis(ANS) inhibits chlorophyllase activity competitively (Fig. 2); this means that bis(ANS) binds at or near the catalytic site of the enzyme. This strongly suggests that the protein binding site, mentioned under (a), is identical to the catalytic site of the enzyme.

So far it has not been possible to determine the number of bis(ANS) molecules bound per chlorophyllase molecule on a molar base. If it is assumed that, in the absence of Mg^{2+}, there is one 'protein binding site' and one 'lipid binding site' (i.e., two or three lipid-binding sites in the presence of Mg^{2+}), the minimum molecular weight of the isolated chlorophyllase is calculated to be 70000.

Inhibitory effects of anilinonaphthalene dyes on

enzyme activities were also described for Carlsberg subtilisin [39], phospho*enol*pyruvate carboxykinase [40,41], phosphoglycerate kinase [42], myosin subfragment I [15] and RNA polymerase [16], while competitive inhibition was found for choline-acetyl transferase [43,44], UDPgalactose-4-epimerase [45], UDPglucose-4-epimerase [46] and iodothyronine deiodinase [47]. In Refs. 15, 16 and 44 bis(ANS) was used as the probe molecule.

The results of the experiments on the binding of bis(ANS) to chlorophyllase indicate that the low-affinity site of chlorophyllase for bis(ANS), mentioned under (b), consists of chlorophyllase-associated lipids. The fluorescence emission maximum of the chlorophyllase-bis(ANS) complex shifts towards longer wavelengths when the bis(ANS) concentration is increased. This phenomenon is explained by the observation that bis(ANS), bound to proteins, generally fluoresces at shorter wavelengths (between 495 and 505 nm; Table II, see also Refs. 10, 15, 16 and 19) than bis(ANS) bound to lipids (between 510 and 520 nm; Table II). As the 'protein binding site' of chlorophyllase has a higher affinity for bis(ANS) than the 'lipid binding site', characteristic protein fluorescence is observed at low bis(ANS) concentrations. At higher bis(ANS) concentrations the fluorescence of lipid-bound bis(ANS) predominates in the total fluorescence, because a greater number of lipid sites are occupied by bis(ANS) molecules. Similar changes in fluorescence characteristics have been observed when ANS binds to certain biomembranes (Refs. 26–29, see also Refs. 48–50). Additional evidence that the low-affinity site of chlorophyllase for bis(ANS) consists of chlorophyllase-associated lipids is presented in the accompanying paper [9].

We found earlier that Mg^{2+}, particularly in the presence of dithiothreitol, effectively activates the chlorophyllase-catalyzed chlorophyll hydrolysis [5]. It was suggested that Mg^{2+} induces a conformational change of the protein. Later experiments showed that Mg^{2+} also influences the enzyme reaction because it interacts with headgroups of lipids which are present in the enzyme reaction medium [6]. The results presented here are in line with both of these action mechanisms of Mg^{2+}. The addition of Mg^{2+} to chlorophyllase results in a large increase in the binding affinity (i.e., decrease of K_{d1}; Fig. 4C; Table I) of the active site of the enzyme for bis(ANS). This effect could be due to a conformational change of the enzyme, resulting in better accessibility of the active site for bis(ANS) and thus also for chlorophyll. Various other membrane proteins have been shown to undergo conformational changes induced by divalent cations [51–57]. It should be pointed out, however, that the enhanced bis(ANS) binding can also be the result of a change in the electrostatic surface potential near the catalytic site of chlorophyllase; such a change will facilitate bis(ANS) binding, because the negative charges present will become neutralized.

As is shown in Fig. 4C and Table I, the number of lipid binding sites for bis(ANS) increases 2- to 3-fold in the presence of Mg^{2+}, with a concomitant small decrease of the dissociation constant of the bis(ANS)-lipid complex. Similar observations have been reported for ANS-lipid studies [31–38]. Some investigators [58,59] argue that divalent cations have a rigidifying effect on membrane lipids, which causes conformational changes of the membrane proteins. Such a mechanism may also play a role in chlorophyllase-lipid interaction. It seems remarkable that uncharged galactolipids (DGDG and MGDG) show enhanced fluorescence in the presence of Mg^{2+}. However, some observations by other authors serve to corroborate our results. Hauser et al. [60] pointed out that the alcohol and ether groups of glycolipids may contribute considerably to the binding of cations to biomembranes; Wieslander et al. [61] showed that in the presence of cations the hydration shell of the headgroups of glycolipids is enlarged. Both findings suggest that Mg^{2+} interacts with uncharged glycolipid headgroups and induces conformational changes, allowing more bis(ANS) molecules to bind to the lipid.

Our experimental results suggest that both kinds of bis(ANS)-binding site on the chlorophyllase molecule may play a role in the regulation of enzyme activity by Mg^{2+}. Further support for this hypothesis is presented in the accompanying paper [9].

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References

1 Bacon, M.F. and Holden, M. (1970) Phytochemistry 9, 115–125
4 Schoch, S. and Vielwerth, F.X. (1983) Z. Pflanzenphysiol. 110, 390–317
5 Terpstra, W. (1977) Z. Pflanzenphysiol. 85, 139–146
6 Terpstra, W. and Lambers, J.W.J. (1983) Biochim. Biophys. Acta 746, 23–31
7 Terpstra, W. and Lambers, J.W.J. (1983) Photobiochem. Photobiophys. 6, 93–100
8 Lambers, J.W.J., Verkleij, A.J. and Terpstra, W. (1984) Biochim. Biophys. Acta 786, 1–8
9 Lambers, J.W.J., Terpstra, W. and Levine, Y.K. (1984) Biochim. Biophys. Acta 789, 197–202
10 Rosen, C-G. and Weber, G. (1969) Biochemistry 8, 3915–3920
11 Farris, F.J., Weber, G., Chiang, C.C. and Paul, I.C. (1978), J. Am. Chem. Soc. 100, 4469–4474
12 Anderson, S.R. (1971) Biochemistry 10, 4162–4168
13 Lu, F.J. and Anderson, S.R. (1973) Biochemistry 12, 3479–3482
14 Pochon, F. and Ekert, B. (1973) Eur. J. Biochem. 36, 311–316
15 Takashi, R. and Tonomura, Y. (1977) Proc. Natl. Acad. Sci. USA 74, 2334–2338
16 Wu, F.Y-H. and Wu, C-W. (1978) Biochemistry 17, 138–144
17 York, S.S., Lawson, R.C. Jr. and Worah, D.M. (1978) Biochemistry 21, 4480–4486
18 Malencik, D.A., Anderson, S.R., Bohnert, J.L. and Shalitin, Y. (1982) Biochemistry 21, 4031–4039
19 Bohnert, J.L., Malencik, D.A., Anderson, S.R., Teller, D. and Fisher, E.H. (1982) Biochemistry 21, 5570–5576
20 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911
21 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
22 Terpstra, W. (1982) Biochim. Biophys. Acta 681, 233–241
23 Goedheer, J.C. (1964) Biochim. Biophys. Acta 88, 304–317
24 Weber, G. and Young, L.B. (1964) J. Biol. Chem. 239, 1415–1423
25 Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660–672
26 Träuble, H. and Overath, P. (1973) Biochim. Biophys. Acta 307, 491–512
27 Zierler, K. and Rogus, E. (1978) Biochim. Biophys. Acta 514, 37–58
28 Slavik, J. (1982) Biochim. Biophys. Acta 694, 1–25
29 Slavik, J., Horak, J., Rihova, L. and Kotyk, A. (1982) J. Membrane Biol. 64, 175–179
30 Dixon, M. and Webb, E.C. (1964) Enzymes, pp. 315–359, Longmans, Green & Co. Ltd., London
31 Freedman, R.B. and Radda, G.K. (1969) FEBS Lett. 3, 150–152
32 Rubalcava, B., Martinez de Munoz, D. and Gitler, C. (1969) Biochemistry 8, 2742–2747
33 Vanderkooy, J. and Martonosi, A. (1969) Arch. Biochem. Biophys. 133, 153–163
34 Feinstein, M.B., Spero, L. and Helsenfield, H. (1970) FEBS Lett. 6, 245–248
35 Gomperts, B.D., Lantelme, F. and Stock, R. (1970) J. Membrane Biol. 3, 241–266
36 Haynes, D.H. and Staerk, H. (1974) J. Membrane Biol. 17, 313–340
37 Kamo, N., Aiuchi, T., Kurihara, K. and Kobatake, Y. (1978) Colloid Polymer Sci. 256, 31–36
38 Green, D.E. and Van de Zande, H. (1982), Biochem. Biophys. Res. Commun. 107, 1300–1307
39 Karasaki, Y. and Ohno, M. (1980) J. Biochem. 87, 1235–1241
40 Silverstein, R., Lin, C-C. (1980) J. Biol. Chem. 255, 1374–1379
41 Nelson, D.L. and Silverstein, R. (1982) Biochem. Biophys. Res. Commun. 108, 696–699
42 Wiksell, E.W. and Larsson-Raznikiewicz, M. (1982) J. Biol. Chem. 257, 12672–12677
43 Currier, S.F. and Mautner, H.G. (1977) Biochemistry 16, 1944–1948
44 Mautner, G.G., Merril, R.E., Currier, S.F. and Harvey, G. (1981) J. Med. Chem. 24, 1534–1537
45 Wong, S.S. and Frey, P.A. (1978) Biochemistry 17, 3551–3556
46 Samanta, A.K. and Bhaduri, A. (1982) Ind. J. Biochem. Biophys. 19, 320–323
47 Fekkes, D., Henneman, G. and Visser, T.J. (1982) Biochem. Pharmacol. 31, 1705–1710
48 Strauss, G. (1976) Photochem. Photobiol. 24, 141–153
49 Lang, M. (1976) Biochim. Biophys. Acta 455, 947–960
50 Horie, T., Sugiyama, Y., Awazu, S. and Hanano, M. (1982) J. Pharm. Dynam. 5, 73–80
51 Dupont, Y. (1976) Biochem. Biophys. Res. Commun. 71, 544–550
52 DeMeis, L. and Vianna, A.L. (1979) Annu. Rev. Biochem. 48, 275–292
53 Champeil, P., Gingold, M.P., Guillain, F. and Inesi, G. (1983) J. Biol. Chem. 258(7), 4453–4458
54 Schuurmans Stekhoven, F.M.A.H., Swarts, H.G.P., De Pont, J.J.H.H.M., and Bonting, S.L. (1983) Biochim. Biophys. Acta 732, 607–619
55 Younis, H.M., Weber, G. and Boyer, J.S. (1983) Biochemistry 22, 2505–2512
56 Arav, R., Aderem, A.A. and Berman, M.C. (1983) J. Biol. Chem. 258, 10433–10438
57 Pick, U. and Finel, M. (1983) Eur. J. Biochem. 135, 559–567
58 Ohyashiki, T. and Mohri, T. (1982) J. Biochem. 91, 1575–1581
59 Fuyu, Y., Beiqi, G. and Yuguo, H. (1983) Biochim. Biophys. Acta 724, 104–110
60 Hauser, H., Levine, B.A. and Williams, R.J.P. (1976) Trends Biochem. 1, 278–281
61 Wieslander, A., Ulmius, J., Lindblom, J. and Fontell, K. (1978) Biochim. Biophys. Acta 512, 241–253