

Local Anesthetic Binding to Thylakoid Membranes. Relation to Inhibition of Light-Induced Membrane Energization and Photophosphorylation

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Z. Naturforsch. **46c**, 79–86 (1991); received September 21, 1990

Chloroplast, Local Anesthetic, Dibucaine, Uncoupling, Photophosphorylation, Amine Binding

The association of the lipophilic tertiary amine and local anesthetic dibucaine with osmotically shocked chloroplasts of *Spinacia oleracea* L. cv. Monatol was investigated. Dibucaine, known as an effective inhibitor of thylakoid membrane energization and ATP synthesis, exhibited three distinct binding classes with chloroplasts: partitioning in the lipid phase of the membranes, electrostatic screening of negative electrical charges on the thylakoid surface and light-induced association of an as yet unknown nature. Evidence is presented that the mechanism of inhibition of the transthylakoid pH gradient, ΔpH , by dibucaine is distinct from 'classical' amine-type uncoupling. The inhibitory effect of dibucaine on ΔpH was independent of the initial strength of ΔpH . Light-induced dibucaine binding was independent of the volume of the intrathylakoid space and of the strength of ΔpH as varied by medium pH. Judged from a comparison of the data on dibucaine binding and on inhibition of ΔpH and photophosphorylation, dibucaine bound *via* partitioning in the membrane lipid phase is responsible for the uncoupler-like effects of the local anesthetic. A mechanism for the inhibition of thylakoid energization by local anesthetic amines is discussed.

Introduction

The investigation of local anesthetic effects has long been extended to problems beyond the question of how anesthesia may block the excitation of nerve membranes. Local anesthetics, *e.g.* dibucaine and tetracaine, were shown to alter the lipid composition of plant membranes [1] and to be effective inhibitors of photosynthesis [2, 3]. A special focus of investigation was the inhibition by local anesthetics of energy transduction at ATP synthesizing membranes in mitochondria [4, 5] and chloroplasts [6, 7]. In chloroplasts, dibucaine inhibited the light-induced transthylakoid pH gradient, ΔpH , and photophosphorylation [6]. However, this inhibition was unlike that exerted by 'classical'

uncouplers, since the pH-dependent photosynthetic control of electron transport at the cytochrome-b/f-complex [8] and at PS II [9] persisted in the presence of dibucaine despite a decreased ΔpH [10]. Further effects of dibucaine on primary reactions of photosynthesis are summarized in [7].

In the present study, we characterize the association of the lipophilic tertiary amine dibucaine with chloroplast membranes. Dibucaine was regarded as a model compound for local anesthetics and was chosen for binding studies since the protonated (monocation) form exhibited strong fluorescence at room temperature which may be used for a determination of dibucaine concentration [11]. Amine binding to chloroplast membranes was intensively studied with 'classical' amine-type uncouplers, *e.g.* ammonia or methylamine [12, 13] and with fluorescent probes of light-induced thylakoid energization, *e.g.* acridines [14, 15], the latter being uncouplers themselves. Several of these studies evinced an involvement of membrane-bound protons in energy transduction at the thylakoid membrane [14, 16, 17]. As suggested previously, the existence of localized proton domains at the thylakoid membrane may also be indicated by the effects of local anesthetics on thylakoid energization [7].

Abbreviations: A_b , amount of dibucaine bound to chloroplast membranes; A_f , concentration of free dibucaine in the medium; 9-AA, 9-aminoacridine; Chl, chlorophyll; ΔpH , transthylakoid pH gradient; Mes, 2-(N-morpholino)ethanesulfonic acid; PS, photosystem; Taps, 3-((tris-(hydroxymethyl)methyl)amino)-propanesulfonic acid; Tris, Tris-(hydroxymethyl)-methylamine; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; PAR, photosynthetically active radiation.

Reprint requests to H. Laasch.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0939–5075/91/0100–0079 \$ 01.30/0

With this paper we tried to characterize different types of association of dibucaine with chloroplast membranes and to relate these to the effects of dibucaine on transmembrane ΔpH and ATP synthesis. Some characteristics of 'classical' amine-type uncoupling by ammonia were set against those of 'selective' uncoupling by dibucaine.

Materials and Methods

Preparation of chloroplasts

Intact chloroplasts were isolated from six-week-old leaves of glass-house grown *Spinacia oleracea* L. cv. Monatol. The isolation procedure was described by [7]. Chloroplasts were stored at 0 °C in the dark until use. The integrity of chloroplast envelopes was 85 to 95%. The chloroplasts were osmotically shocked immediately before use.

Binding of dibucaine

In order to simplify terminology, any type of amine association with chloroplast membranes was termed binding. The concentration of dibucaine in binding assays was measured fluorometrically with excitation and emission wavelengths of 325 and 403 nm, respectively, with a halfband width of 2 or 10 nm, using a spectrofluorometer (F-2000, Hitachi). The pH of fluorescence assays was always adjusted to 8. Dibucaine was dissolved in methanol, the final solvent concentration in binding assays was < 1%. The standard reaction medium contained 20 mM Hepes/KOH, pH 8, 20 mM KCl, 1 mM MgCl_2 , 0.3 M sorbitol, and 200 U catalase (EC 1.11.1.6.). Unless stated otherwise, PS I electron flow was allowed by additions of 50 μM dichlorophenolindophenol, 2 mM Na-ascorbate and 5 μM methylviologen. When the pH dependency of amine binding was studied, the chloroplast medium was composed as described for photophosphorylation experiments. When the salt concentration in the reaction medium was varied, isosmolarity of the medium was maintained by addition of sorbitol. Effects of salt concentration on dibucaine fluorescence were corrected. Measurements of dibucaine fluorescence were either carried out in the presence of chloroplasts or after chloroplast sedimentation by centrifugation.

Method a): Fluorescence after sedimentation of chloroplasts. A suspension of chloroplasts in a

translucent reaction vial was supplied with dibucaine in the dark and placed into a centrifuge (Minifuge E, Beckman) with a translucent cover. The samples were either illuminated with white light of $2500 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, PAR, or kept in the dark for 90 s. Chloroplasts were then sedimented at $13,000 \times g$ for 20 s. The fluorescence in the supernatant was measured. For a calculation of binding data, the fluorescence intensity was calibrated against amine concentration. The amount of amine bound was calculated from amine found in the supernatant and total amine added.

Method b): Fluorescence in the presence of chloroplasts: A chloroplast suspension equivalent to $25 \mu\text{g Chl} \cdot \text{ml}^{-1}$ was supplied with dibucaine in a stirred fluorescence cuvette. The time course of fluorescence emission was followed during a dark/light ($2000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, PAR) cycle.

Determination of thylakoid osmotic space

The osmotic space was determined by incubation of osmotically shocked chloroplasts in a medium containing 20 mM Hepes/KOH, pH 8, 2 mM MgCl_2 , 10 mM KCl and sorbitol, varying between zero and 0.5 M. 20 kBq [^3H]- H_2O and 9 kBq [^{14}C]sorbitol (Amersham Buchler, Braunschweig) were added. Subsequently, the chloroplasts were centrifuged for 20 s at $13,000 \times g$ through a layer of silicon oil mixture, AR 20 and AP 150 (Wacker, München), with 65 to 83% AR 20 depending on the sorbitol concentration applied, into a compartment containing 3 M HClO_4 . The radioactivity in the HClO_4 compartment was determined by dual-label scintillation counting.

Displacement of divalent cations

In a reaction medium containing 10 mM Hepes/KOH, pH 8, 5 mM KCl, < 0.1 mM MgCl_2 and 75 mM sorbitol, chloroplasts were incubated for 30 s in the dark. Then the sorbitol concentration was raised to 0.3 M without changes of buffer and KCl. After 2 min in the dark or light, in the absence or presence of dibucaine, the samples were centrifuged for 20 s at $13,000 \times g$. The concentration of divalent cations in the supernatant was determined by addition of 50 μM Eriochrome Blue SE and 1 ml Tris/HCl, pH 9, to 0.4 ml of chlorophyll free supernatant and subsequent measurement of the Eriochrome Blue SE/cation fluores-

cence with excitation and emission wavelengths of 515 and 594 nm, respectively, at a half-band width of 10 nm. Eriochrome Blue SE without bound divalent cations was not fluorescing at pH 9.

Photophosphorylation

ATP formation in the light was determined enzymatically, using hexokinase (EC 2.7.1.1.) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49.). The reaction medium contained 0.3 M sorbitol, 30 mM KCl, 20 mM Hepes/KOH, pH 8, 1 mM EDTA, 1 mM MnCl_2 , 2 mM KH_2PO_4 , 10 μM diadenosinepentaphosphate and 0.5 mM ADP. ATP synthesis was allowed for 2 min during illumination ($2000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, PAR). PS II + I electron flow was mediated by 20 μM methylviologen.

Determination of light-induced ΔpH

5 μM 9-AA was added to osmotically shocked chloroplasts equivalent to 20 μg $\text{Chl} \cdot \text{ml}^{-1}$, in the presence of 10 μM methylviologen. The chloroplasts were illuminated with red light of $2000 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, PAR. Dibucaine was added after 9-AA and prior to illumination. 9-AA fluorescence was excited by light of 400 nm wavelength and was measured at 465 nm. The strength of ΔpH was calculated, using the equation $\Delta\text{pH} = \log(\Delta F \cdot F_R^{-1} \cdot V_E \cdot V_I^{-1})$ (ΔF = light-induced fluorescence quenching; F_R = fluorescence remaining in the light; V_E = external volume). An intrathylakoid volume, V_I , of 10 $\mu\text{l} \cdot \text{mg}^{-1}$ Chl was assumed.

Results

Comparison of methods for binding assay

Binding of dibucaine to osmotically shocked chloroplasts was assayed by fluorometric determination of dibucaine concentrations after light or dark treatment and following sedimentation of the membranes (method a). For verification that neither dibucaine desorption from membranes during centrifugation nor dibucaine sorption to surfaces of reaction vials influenced the binding data obtained, an alternative approach was carried out. Dibucaine fluorescence in the presence of chloroplast membranes in the dark and light was measured (method b). Dibucaine binding was quantified from fluorescence data for method a) by the

equation $A_n/A_f = (F_D - F_L)/F_L$ (A_n , A_f : concentrations of bound and free dibucaine; F_D , F_L : fluorescence in dark and light-treated samples after removal of chloroplasts). For method b), analogously, binding was quantified by the equation $A_n/A_f = F_q/F_R$ (F_q , F_R : fluorescence quenching and remaining fluorescence in the light in the presence of chloroplasts). In Fig. 1, the quotients A_n/A_f determined by both methods are shown for dibucaine concentrations up to 100 μM . The data fitted well to a straightline with a slope of one, suggesting that both methods reveal equivalent data. Furthermore, the data may indicate that dibucaine bound to chloroplast membranes is non-fluorescent. In the following, dibucaine binding was determined by method a).

Dibucaine binding in light and dark

The equilibration of dibucaine binding in the light or dark was generally completed after at most 60 s (data not shown). Therefore, a minimum incubation time of shocked chloroplasts with amine of 90 s was chosen. In Fig. 2 dibucaine bound to chloroplasts in dark and light, A_b , is shown in dependence of the free amine concentration present. In light-treated samples, PS I driven electron

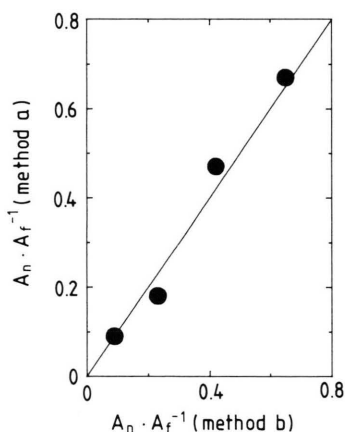


Fig. 1. Comparison of methods for determination of dibucaine binding. Dibucaine fluorescence was measured at pH 8 after sedimentation (method a) and in the presence (method b) of osmotically shocked, light and dark treated chloroplasts. A_n and A_f are the concentrations of bound and free amine, respectively. The Chl concentration was 25 $\mu\text{g} \cdot \text{ml}^{-1}$, the dibucaine concentration was varied between 20 and 100 μM .

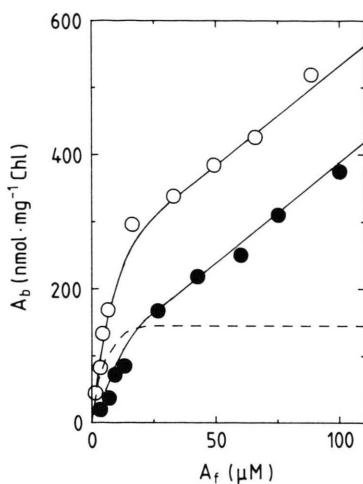


Fig. 2. Binding of dibucaine to osmotically shocked thylakoid membranes in the light (O) and dark (●) at pH 8. The dashed line indicates 'light-induced' binding, as calculated by a subtraction of the amounts of amine bound, A_b , under light and dark conditions. A_b is shown in dependence on the free amine concentration, A_f . The Chl concentration was $100 \mu\text{g} \cdot \text{ml}^{-1}$.

transport was operated since the latter, in contrast to PS II dependent electron flow, was unaffected by dibucaine up to concentrations of 0.5 mM . Two major binding classes appear in the dark: in a concentration range of $A_f < 30 \mu\text{M}$ a curvilinear dependence of bound amine, A_b , on A_f appeared. Between $A_f = 30$ and $300 \mu\text{M}$, a linear relation of A_b and A_f was observed (not shown).

In the light, dibucaine binding exceeded the dark level. Light-dependent binding, estimated by subtraction of A_b values in light and dark, was saturated at about $20 \mu\text{M}$ of free dibucaine (Fig. 2). The slopes of the linear branches of binding curves in light and dark coincided, suggesting that the underlying processes are not influenced by light. A partitioning of lipophilic dibucaine molecules [18] between membrane lipids and aqueous medium was assumed. Previously bound dibucaine re-emerged in the medium when chloroplast membranes were resuspended in the absence of free dibucaine (not shown). Hence, dibucaine binding was largely reversible.

Effects of metal cations on dibucaine binding

When dibucaine was added to osmotically shocked chloroplasts in the dark in the presence of

either high or low concentrations of salts, binding in high salt was lower than that in low salt media (Fig. 3). Under high-salt condition the curvilinear shape of the binding curve disappeared. The slopes of the linear branches of the curves, however, were unaffected. This indicates two binding classes in the dark: first, binding which is apparently saturated at free dibucaine concentrations of about $30 \mu\text{M}$ and may interact with salts and second, binding which predominates at $A_f > 30 \mu\text{M}$ and is unaffected by salts. When the data obtained by subtraction of the curves in Fig. 3, were redrawn in a double-reciprocal plot, a straightline could be fitted to the data, suggesting a hyperbolic relationship between A_b and A_f values of salt dependent binding (not shown). Apparent values for the 'concentration' of salt-sensitive binding sites and the dissociation constant of amine/membrane complexes were obtained with $280 \text{ nmol mg}^{-1} \text{ Chl}$ and $37 \mu\text{M}$, respectively.

The type of cations added was decisive for the strength of salt effects. Divalent were more effective in dibucaine displacement from chloroplasts than monovalent cations and the more tightly binding Ca^{2+} [19] was more effective than Mg^{2+} (Fig. 4). The anion used was always Cl^- .

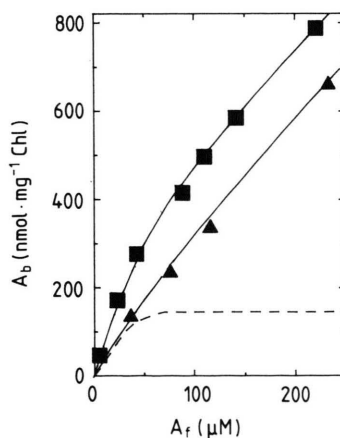


Fig. 3. Influence of salt concentrations in the medium on dibucaine binding to osmotically shocked chloroplasts in the dark. For high salt condition (\blacktriangle), 50 mM MgCl_2 and 50 mM KCl were added, for low salt conditions (\blacksquare) 10 mM KCl . The dashed line shows 'pure' salt-dependent binding, as calculated by subtraction of the related A_b values. The Chl concentration was $100 \mu\text{g ml}^{-1}$.

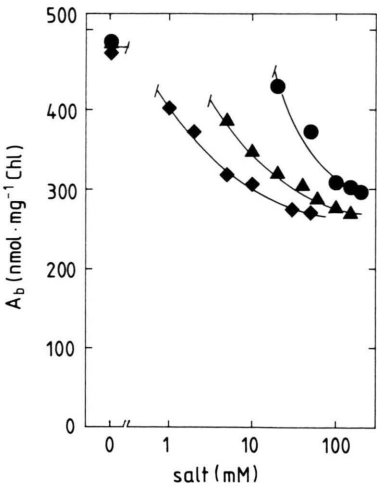


Fig. 4. Displacement of membrane-bound dibucaine by increasing concentrations of KCl (●), MgCl₂ (▲) and CaCl₂ (◆) in the dark. The total concentration of dibucaine present was 100 μM, the Chl concentration 100 μg · ml⁻¹ and the initial salt concentration 10 mM KCl. 3 min equilibration in the presence of amine and salts were allowed before sedimentation of the chloroplasts.

In Fig. 5 we show that, in reverse to the displacement of amine pictured in Fig. 4, dibucaine may displace divalent cations associated with chloroplast membranes. The concentration of divalent cations was monitored by fluorescence emission of

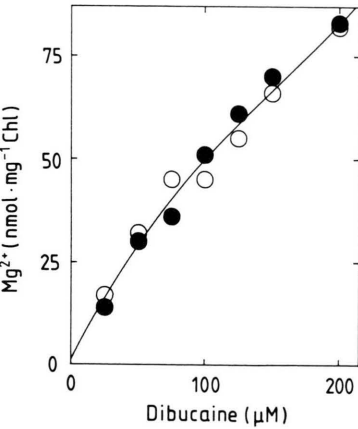


Fig. 5. Displacement of Mg²⁺ from chloroplasts under the influence of dibucaine in the dark (●) and under white light of 2500 μE · m⁻² · s⁻¹, PAR (○). The ordinate values were obtained by subtraction of Mg²⁺ efflux in the absence of dibucaine from efflux in its presence. Chl concentration was 100 μg · ml⁻¹.

Eriochrome-Blue SE. Eriochrome Blue SE exhibited a 20-fold higher affinity for Mg²⁺ than for Ca²⁺. Since the concentration of Mg²⁺ in chloroplasts in addition is higher than that of Ca²⁺, it appears that the data in Fig. 5 largely represent a displacement of Mg²⁺. When 200 μM dibucaine was added to intact chloroplasts, only little Mg²⁺ (< 20 nmol mg⁻¹ Chl) appeared in the suspension medium, although dibucaine easily penetrates the chloroplast envelope [10]. This indicates that the amine did not cause a leakiness for Mg²⁺ of the chloroplast envelope. A treatment of chloroplasts in a slightly hypotonic medium, in the absence of dibucaine, induced a release of about 30 nmol Mg²⁺ per mg Chl. When dibucaine was added to chloroplasts treated in this way, a Mg²⁺ efflux occurred which increased together with dibucaine concentration and was similar in dark and light (Fig. 5).

On the nature of light-dependent binding

Upon illumination of chloroplasts the binding of dibucaine was enhanced (Fig. 2). After a light-dark transition, light-induced binding disappeared. Also an addition of nigericin to chloroplasts in the light inhibited light-dependent binding (not shown). This suggested that light-induced binding may be related to proton accumulation in the thylakoid lumen. Having in view the effect of ‘classical’ amine-type uncouplers, e.g. methylamine or ammonia [12, 13], an accumulation of protonated dibucaine in the thylakoid lumen in the light may be assumed. However, a ΔpH induced accumulation of dibucaine is challenged by the data shown in Table I. While an increase of the

Table I. Dependence of light-induced dibucaine binding on the volume of the intrathylakoid space (V₁). The light-induced binding of dibucaine to osmotically shocked chloroplasts was measured in the presence of 50 μM dibucaine, a Chl concentration of 80 μg · ml⁻¹ and sorbitol concentrations as indicated. The volume of the intrathylakoid space, V₁, was determined under comparable conditions in the presence of dibucaine.

Concentration of sorbitol [M]	V ₁ [μl · mg ⁻¹ Chl]	Amine bound [nmol · mg ⁻¹ Chl]
0	18	160
0.1	15	160
0.2	11	145
0.3	7	140
0.5	4	130

sorbitol concentration from zero to 0.5 M reduced the intrathylakoid volume, V_i , by a factor of four, the amount of dibucaine bound by a light-induced mechanism only decreased by 20% (Table I).

With increasing medium pH the strength of the light-induced ΔpH increases ([12]; see also Fig. 7). Assuming a ΔpH dependent accumulation of dibucaine to underly light-induced binding, the latter should increase with medium pH as well. When light-induced dibucaine binding was studied in dependence of medium pH, no variation was found over the range of pH considered (Fig. 6). Binding of amine in the dark, however, increased with the external pH. Since the latter is assumed to be largely produced by partitioning, an increase of the concentration of unprotonated (uncharged) amine species in the medium with rising pH may be responsible for increased dark binding.

Evidence against a dibucaine induced proton shuttle across the thylakoid membrane also appears from a comparison of the inhibitory effect of ammonia and dibucaine on ΔpH in dependence on medium pH (Fig. 7). While the inhibitory effect on ΔpH of ammonia, a classical amine-type uncoupler, increased with external pH as expected, the effect of dibucaine on ΔpH was constant over the range of pH considered. The pK_a of ammonium of 9.3 is comparable to $pK_a = 9.0$ of dibucaine [20].

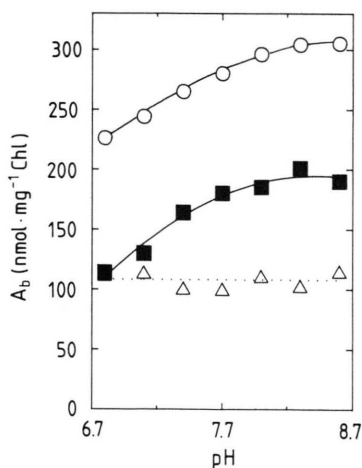


Fig. 6. Dependence of dibucaine binding in the dark (■) and under white light of $2500 \mu E \cdot m^{-2} \cdot s^{-1}$, PAR (○) on medium pH. 'Pure' light-dependent binding (△) was obtained as described in Fig. 2. The Chl concentration was $80 \mu g \cdot ml^{-1}$. PS II+I electron flow was mediated by $10 \mu M$ methylviologen. The dibucaine concentration was $30 \mu M$.

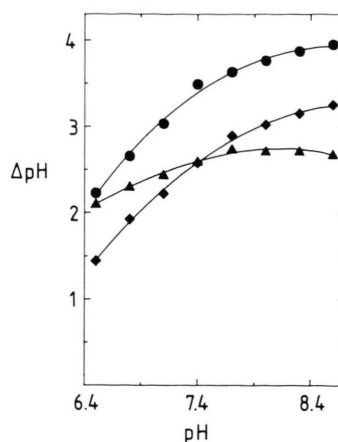


Fig. 7. Inhibition of light-induced transthylakoid ΔpH , in the absence of ADP and P_i , by 3 mM ammonia (▲) and $35 \mu M$ dibucaine (◆) in dependence on medium pH. ΔpH in the absence of uncoupler is shown by (●). ΔpH was calculated from 9-AA fluorescence quenching.

Hence, the different effects of medium pH on ammonium and dibucaine may not be explained by different degrees of protonation at the pH values tested.

Photophosphorylation by thylakoid membranes increases with external pH (Fig. 8). This is largely

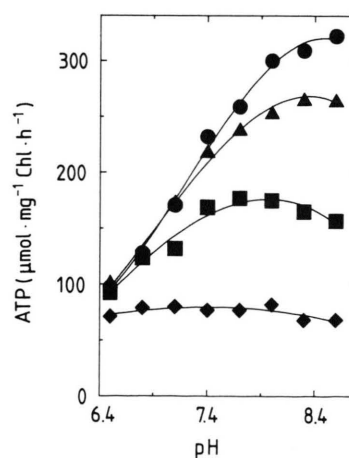


Fig. 8. Influence of medium pH on the inhibition of photophosphorylation by $15 \mu M$ (▲), $25 \mu M$ (■) and $35 \mu M$ (◆) dibucaine. Controls (absence of amine) are shown by (●). The Chl concentration was $25 \mu g \cdot ml^{-1}$. Dibucaine was added 60 s before illumination of chloroplast membranes.

due to a rising ΔpH (Fig. 7, [12]) and enhanced catalytic activity of the coupling factor [21]. In the presence of rather low dibucaine concentration, *e.g.* 15 to 25 μM , almost no inhibition occurred at low external pH (Fig. 8), although considerable dibucaine binding occurred under this condition (Fig. 6). At higher dibucaine concentration, the degree of inhibition of ATP synthesis increased with medium pH.

Discussion

Characterization of amine binding classes

In the experiments shown, we demonstrated the existence of three major binding classes of dibucaine (Fig. 2, 3). Besides a partitioning of amine between the aqueous medium and the lipid phase of the membranes, a salt-dependent and a light-dependent binding class were indicated.

There were several lines of evidence that the linear parts of the binding curves in Fig. 2, 3 reflect a partitioning of amine. First, no indication of binding saturation appeared between $A_f = 30$ and 300 μM . The total amount of amine bound per mg Chl could be raised beyond 1000 nmol mg^{-1} Chl, indicating a 1:1 stoichiometry of bound amine and Chl. This value greatly outnumbers the amount of potential specific binding sites, such as components of electron transport chains, ATPase complexes [22], or membrane surface charges [23]. Second, estimating the volume of the lipid phase of thylakoid membranes with 3 $\mu\text{l} \cdot \text{mg}^{-1}$ Chl [24], a partition coefficient, p , of about 1000 was calculated from the slope of the linear phase of binding in Fig. 2 and the ratio of external (medium) volume and volume of the lipid phase. This is in the same order of magnitude as the octanol water partition coefficient of dibucaine, determined previously to be 2500 at pH 8 [20]. Third, the type of binding considered was influenced neither by illumination, nor by additions of salts and increased with medium pH, *i.e.* with the concentration of uncharged dibucaine (Fig. 6).

The salt-dependent binding of dibucaine in the dark showed saturation characteristics, indicating a limited concentration of binding sites. Salt-dependent binding is apparently due to an electrostatic screening of negative surface charges by the monovalent cationic (protonated) amine, since divalent cations proved to be more effective in dis-

placement of dibucaine than monovalent cations (Fig. 4). The 'concentration' of binding sites is comparable to the 'concentration' of negative surface charges which is about 210 $\text{nmol} \cdot \text{mg}^{-1}$ Chl. The latter was calculated from the estimated thylakoid surface of 1.67 $\text{m}^2 \cdot \text{mg}^{-1}$ Chl [23] and a thylakoid surface charge density of $-12 \text{ mC} \cdot \text{m}^{-2}$ at pH 8 [25]. A screening of negative surface charges may also occur after a polarization of net uncharged dibucaine molecules [26, 27].

Upon illumination of broken chloroplasts, dibucaine binding increased (Fig. 2, 6). Light-dependent binding was saturated already at A_f of about 20 μM (Fig. 2). Interpreting light-induced binding as a Langmuir isotherm, the 'concentration' of binding sites of this binding class was estimated at 150 nmol mg^{-1} Chl. Hereby, light-induced dibucaine binding is distinct from binding of other amines, *e.g.* methylamine or 9-AA. Following ref. [28], 9-AA binding is dominated by an accumulation of 9-AA in the thylakoid lumen after acidification of this compartment. Thus, over a wide range of amine concentration the ratio of bound over free 9-AA concentrations should be equal to that of proton concentration inside and outside the thylakoid vesicles. This was in fact observed with 9-AA [29] and methylamine [12], but not with dibucaine (Fig. 2).

Inhibition of thylakoid energization by dibucaine

Searching for the mechanism of 'selective' uncoupling, *i.e.* the mechanism by which dibucaine inhibits the energization of thylakoid membranes and ATP synthesis, a 'classical' amine-type mechanism might be assumed: in this case protonated amine would accumulate in the thylakoid lumen upon acidification of the compartment and promote a proton shuttle over the membranes when a threshold, determined by membrane permeability and transmembrane concentration difference of protonated amine, is exceeded.

However, such a mechanism is obviously not operative in the presence of dibucaine. A drastic increase of V_i did hardly affect light-dependent dibucaine binding (Table I). A variation of the medium pH was without effect on light-dependent dibucaine binding although ΔpH drastically increased with medium pH (Fig. 6, 7; [12]). The inhibitory effects of dibucaine on ΔpH , in contrast to the effects of ammonium, did not increase with

medium pH, although the ratio of proton concentrations inside and outside of the thylakoid membrane increased by a factor of about 40 in controls without amine added (Fig. 7). Evidence against a proton shuttle across the thylakoid membrane by dibucaine also comes from the observation of spontaneous deprotonation of monovalent cationic, protonated dibucaine upon contact of the amine with a hydrophobic environment [26, 27].

A comparison of the pH dependences of binding (Fig. 6) and inhibition of ATP synthesis (Fig. 8) revealed that inhibition of photophosphorylation increased with external pH in line with dibucaine binding *via* partitioning. Yet, it cannot be excluded that partitioning is not a homogenous class, *i.e.*, that more specific binding sites may be hidden in the bulk phase of amine partitioning.

Inhibition of thylakoid energization

By excluding a 'classical' amine-type mechanism for the effects of dibucaine on ΔpH , the question

for the mechanism of 'selective' uncoupling returns to the focus of interest. Previously we presented evidence that energy transfer inhibition on the level of the ATP synthase complex and electron transport inhibition are not the reasons for the decline of photophosphorylation under influence of local anesthetics [7]. In that study and in Fig. 5, however, we showed a strong interference of dibucaine with cation binding to thylakoid surfaces. Therefore, a mechanism for inhibition of ΔpH may be assumed which is based on the interaction of dibucaine and the strong ion concentration difference across thylakoid membranes [30, 31].

Acknowledgements

Skillful technical assistance was provided by Gabriele Luhn and Regina Glosch. The financial support of this study by the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

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