Biochimica et Biophysica Acta, 973 (1989) 7–12
Elsevier

BBA 42920

The effect of azide on regulation of the chloroplast H^+-ATPase by ADP and phosphate

Ignat B. Minkov and Heinrich Strotmann

Institut für Biochemie der Pflanzen, Universität Düsseldorf, Düsseldorf (F.R.G.)

(Received 12 July 1988)

Key words: Chloroplast; ATPase, H^+-; Enzyme regulation; Azide inhibition; Enzyme kinetics

The light + dithiothreitol-induced chloroplast ATPase is rapidly deactivated by tight binding of ADP to CF_1 whereas ADP binding and ADP-dependent inactivation, respectively, are decelerated by inorganic phosphate. Sodium azide specifically prevents the effect of phosphate on ATPase activity but does not change tight ADP binding significantly. Azide is concluded to interact as a competitor to phosphate with an intermediate enzyme form containing a loosely bound ADP. The ATPase · azide · ADP complex, in contrast to the ATPase · phosphate · ADP complex, forms an inactive enzyme species.

Introduction

In bacteria, mitochondria and chloroplasts, synthesis of ATP from ADP and phosphate by utilization of energy from a transmembrane electrochemical gradient is catalyzed by proton-translocating ATPase complexes (F_0-F_1). Although many details are known about the structure and the functional properties of the ATPases, fundamental questions of energy conversion within the complexes and their regulation are still open.

ATPase inhibitors are useful tools in the study of partial steps of the reaction chain. For example, the covalent reaction of the carbodiimide, *N,N'*-dicyclohexylcarbodiimide (DCCD), with the proteolipid subunit of the F_0 moiety elucidated the significance of this subunit in proton translocation [1–3]. On the other hand, a variety of compounds are known to react with the F_1 moiety, such as phlorizin [4], Dio 9 [5] or various anthraquinone dyes [6], which are inhibitors of the chloroplast ATPase. Among others, azide was found to interact with the F_0-F_1-type ATPase of mitochondria and bacteria. Interestingly, the ATP-hydrolyzing activity and the ATP-synthesizing activity of mitochondria [7,8] and *E. coli* [9] showed different sensitivities to azide. This result was taken as evidence for different conformations or catalytic sites being involved in the forward and backward reactions, respectively. In chloroplasts, neither ATP synthesis nor ATP hydrolysis is affected by azide. There is, however, a very particular effect of azide on regulation of the ATPase, as will be shown in this communication.

Multiple regulatory mechanisms are responsible for control of the H^+-ATPase of chloroplasts, the main regulatory factors being the transmembrane proton gradient, thiols and nucleotides. Thiols like dithiothreitol (DTT) or the natural thiol donor protein thioredoxin are capable of reducing a specific disulfide bond in γ-subunit of CF_1 [10,11]. While the oxidized enzyme is catalytically active only above a certain threshold $\Delta\tilde{\mu}_{H^+}$ [12], the reduced form may retain activity over several minutes, even in a fully deenergized membrane, and therefore is capable of hydrolyzing added ATP [13,14]. However, the addition of ADP at micromolar concentrations before the addition of substrate ATP triggers deactivation within a few seconds [15]. Inactivation is caused by 'tight' binding of ADP to CF_1 [16,17]. Binding of ADP as well as inactivation is largely abolished by inorganic phosphate [15,17,18]. The results shown in this paper demonstrate that azide interferes with the regulatory action of ADP and phosphate on the chloroplast ATPase.

Methods

Thylakoids from spinach leaves were isolated as in Ref. 16. Photosynthetic electron transport was measured by oxygen consumption using an oxygen elec-

Abbreviations: DCCD, *N,N'*-dicyclohexylcarbodiimide; DTT, dithiotheritol; PMS, phenazine methosulfate; Chl, chloride.

Correspondence: H. Strotmann, Institut für Biochemie der Pflanzen, Universität Düsseldorf, Universitätsstrasse 1, D-4000 Düsseldorf, F.R.G.

TABLE I

Effect of azide on photosynthetic electron transport

Thylakoids (50 μg Chl/ml) were incubated in assay medium (see Methods) with the indicated supplements. NaN_3 (5 mM) was added 15 s before the start of the reaction by light.

Additions	Rate of electron transport (μmol O_2/ml Chl per h)	
	control	+ azide
–	34.0	34.7
5 mM P_i	45.1	45.1
20 μM ADP	20.8	25.8
5 mM P_i + 1 mM ADP	70.5	72.8
0.5 μM Nigericin	149.4	148.0

trode in a system employing methylviologen as electron acceptor. The medium contained 25 mM Tricine buffer (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 1 mM KCN and 0.5 mM methylviologen. Further additions are indicated in the legend of Table I. The final volume was 2 ml, the temperature was 20 °C and the light intensity (red light, RG 630 filter, Schott) was 300 $W \cdot m^{-2}$.

For measurement of light-triggered ATP hydrolysis, thylakoids were preilluminated for 2 min (red light, 300 $W \cdot m^{-2}$) in a medium containing 25 mM tricine buffer (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 10 mM DTT and 50 μM phenazine methosulfate (PMS), before [γ-^{32}P]ATP was added as substrate in the dark. Further experimental details are given in the legends. Release of [^{32}P]P_i was analyzed as phosphomolybdate complex extracted by isobutanol/toluene as in Ref. 19.

Transmembrane ΔpH formation was registered by the fluorescence of 9-aminoacridine [20] as described in Ref. 21. The medium was identical with the one used for the assay of ATP hydrolysis. Binding of [^{14}C]ADP was measured as in Ref. 29.

Results

The effect of 5 mM NaN_3 on photosynthetic electron transport from water to methylviologen was studied in an experiment shown in Table I. The results reveal no significant alteration of either uncoupled, coupled or basal electron transport (measured in the absence of ADP), indicating that azide acts neither as an electron transport inhibitor, nor as an ATPase inhibitor, nor as an uncoupler. This is corroborated by the lack of inhibition of photophosphorylation (not shown). A slight increase of basal electron transport in the presence of ADP (without P_i) or ATP (not shown) is observed.

The stability of the activated state of the ATPase established by preillumination of the thylakoids in the presence of DTT is strongly affected by ADP and phosphate (see Introduction). Table II shows rates of light-triggered ATP hydrolysis following 15 s reaction in

TABLE II

Rates of light-triggered ATP hydrolysis as affected by preincubation with phosphate, ADP and azide

Thylakoids (25 μg Chl/ml) were preilluminated for 2 min as described in Methods. After 15 s in the dark, the indicated additions were made and after a further 15 s, [^{32}P]ATP (0.2 mM) was injected. Kinetics of [^{32}P]P_i formation were followed by taking samples after 10, 20, 30 and 60 s.

Additions (15 s before [^{32}P]ATP)	Rate of ATP hydrolysis	
	(μmol [^{32}P]P_i/mg Chl per h)	(%)
1 mM P_i	87.8	100
1 mM P_i + 2.5 mM NaN_3	87.8	100
5 μM ADP	14.7	17
5 μM ADP + 2.5 mM NaN_3	0.2	0
5 μM ADP + 1 mM P_i	81.8	93
5 μM ADP + 1 mM P_i + 2.5 mM NaN_3	35.6	41

the dark with 1 mM P_i or 5 μM ADP or both compounds together, respectively. In parallel samples, 2.5 mM NaN_3 was added together with P_i and ADP under the same conditions. No effect of azide is observed in the control sample (P_i present). Preincubation with ADP causes about 80% deactivation of the ATPase, but full inhibition is observed when azide is supplied in addition to ADP. The enzyme is almost completely protected from ADP-induced inactivation by simultaneous addition of P_i, but protection is largely abolished in the presence of azide.

Hydrolysis of ATP is coupled with the translocation of protons from the medium into the thylakoid lumen [22,23]. The formation of ATP-induced ΔpH under conditions similar to those employed in Table I was measured by 9-aminoacridine fluorescence (Fig. 1). The fluorescence traces indicate complete prevention of ΔpH

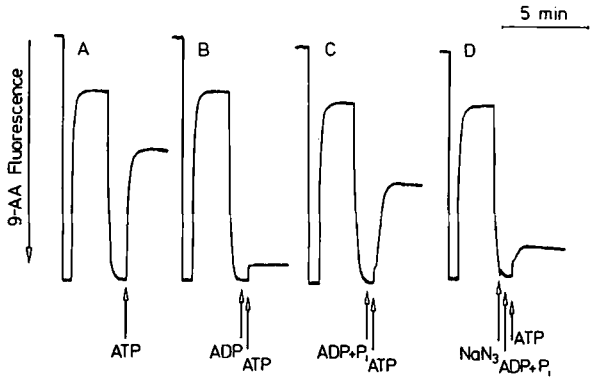


Fig. 1. Effect of 20 s preincubation of thylakoids with ADP, ADP + P_i or ADP + P_i + NaN_3 on ATP-induced formation of ΔpH as measured by 9-aminoacridine fluorescence quenching. Thylakoids (24 μg Chl/ml) were preilluminated for 2 min in reaction medium (see Methods) containing 5 μM 9-aminoacridine. At the arrows, the indicated compounds were added to yield the following final concentrations: 0.2 mM ATP, 5 μM ADP, 0.7 mM P_i and 0.5 mM NaN_3.

TABLE III

Effect of azide on ATP-induced ΔpH in the presence of ADP and phosphate

The indicated compounds were added after 2 min preillumination at the following final concentrations: 0.2 mM ATP, 1 mM P_i, 5 μM ADP, 1 mM NaN_3. ΔpH values were calculated from traces of 9-aminoacridine fluorescence according to Ref. 20 on the basis of an internal thylakoid volume of 30 μl/mg chl, which was determined as in Ref. 21.

Additions			ΔpH	%	$[H^+]_i$ (μM)	%
Time (s) after preillumination: 15	25	55				
–	–	ADP, P_i, ATP	3.03	100	10.7	100
NaN_3	–	ADP, P_i, ATP	2.90	96	7.9	74
–	–	ADP, P_i, ATP, NaN_3	2.89	95	7.8	72
–	P_i	ADP, ATP	3.01	100	10.2	100
NaN_3	P_i	ADP, ATP	2.87	95	7.4	72
–	ADP, P_i	ATP	2.96	100	9.1	100
NaN_3	ADP, P_i	ATP	2.20	74	1.6	17
NaN_3 (5 mM)	ADP, P_i	ATP	1.72	58	0.5	6
–	ADP, P_i	ATP, NaN_3	2.62	89	4.2	46

formation by previous reaction for 30 s with 5 μM ADP (the instantaneous small decrease of fluorescence is due to artificial quenching caused by the addition of ATP) and the protective effect by simultaneous addition of P_i. As in Table II, this protection is largely annulled by azide.

Table III shows ΔpH values obtained by ATP hydrolysis when ADP, P_i and azide were added together with or before the addition of substrate ATP. When ADP and P_i are supplied together with ATP, a rather low inhibitory effect of azide is observed, irrespective of whether the compound is added simultaneously or before addition of substrates. Likewise, inhibition is weak when azide and phosphate are allowed to react before the simultaneous addition of ADP and ATP or when azide is introduced together with ATP after preincubation with ADP and phosphate. However, significant inhibition of ΔpH formation is observed when azide is present during the pretreatment with ADP and P_i. The inhibitory effect is even more pronounced when internal proton concentrations (calculated from the ΔpH values) are considered. These results permit the conclusion that azide prevents phosphate-dependent protection of the ATPase from ADP-induced deactivation and that the catalytic reaction itself is not sensitive to azide. The latter conclusion is confirmed by a result which demonstrates that azide is ineffective as an inhibitor of ATP hydrolysis in the presence of a pyruvate kinase system which provides continuous regeneration of the emerging ADP to ATP (not shown).

A possible explanation of the azide effect might be competition of the azide anion with the phosphate anion at the site where phosphate displays its regulatory function. In order to scrutinize this working hypothesis, the abolition of ADP-dependent inactivation was analyzed as a function of phosphate concentration in the absence and presence of 0.5 mM NaN_3. The magnitude of ΔpH generated by subsequent addition of ATP was evaluated in Fig. 2A. Evidently, the effect of azide can be overcome by increasing the concentration of phosphate. At a phosphate concentration of 0.3 mM, for example, almost maximal ATPase activity is retained in the controls, but virtually no activity is detected in the presence of azide. On the other hand, at 1.5 mM

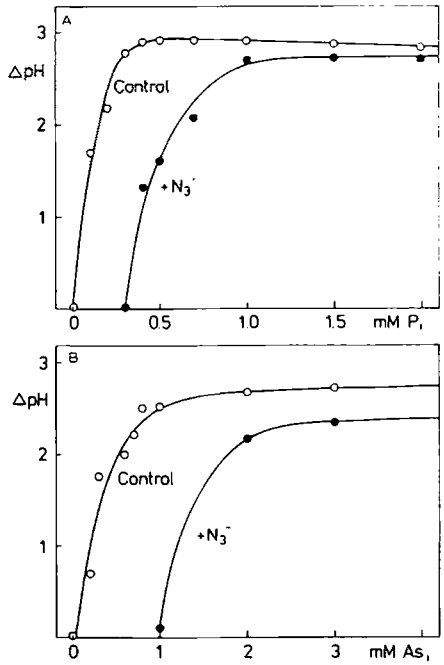


Fig. 2. Protection of ADP-dependent deactivation of the ATPase by phosphate (A) and arsenate (B) in the absence and presence of 0.5 mM NaN_3. As a measure of activity, the amplitude of ATP-induced ΔpH formation (measrued by 9-aminoacridine fluorescence) was evaluated. Thylakoids were preilluminated as in Fig. 1. After 30 s in the dark, NaN_3 was added where indicated. 10 s later, 5 μM ADP plus phosphate or arsenate, respectively, at the indicated concentration were injected. The ATPase reaction was started by the addition of 0.2 mM ATP after a further 30 s. ΔpH values were calculated from the 9-aminoacridine signals, as in Table III.

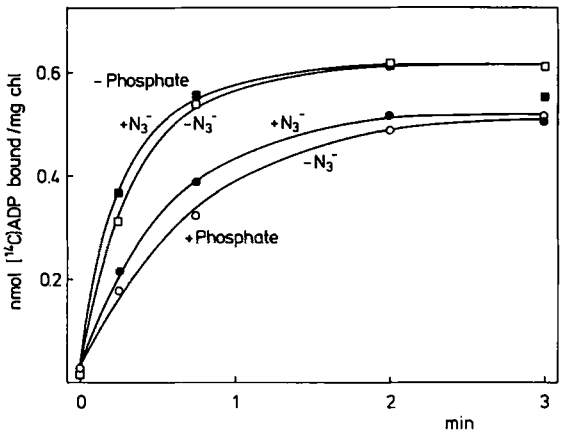


Fig. 3. Effect of 0.5 mM NaN_3 on tight binding of [^{14}C]ADP in the absence and presence of 0.3 mM phosphate. Thylakoids (100 μg chl/ml) were preilluminated in reaction medium (see Methods) for 2 min. After 30 s in the dark, 0.5 mM NaN_3 was added (filled symbols). 10 s later, 5 μM [^{14}C]ADP without (squares) or with (circles) 0.3 mM phosphate was injected. The open symbols show control curves (absence of NaN_3). At the indicated times, the samples were quenched and further treated as in Ref. 29. Total [^{14}C]ADP was about 50 mol/mol CF_1, the maximal binding of [^{14}C]ADP (measured after 15 min) was about 0.9 mol/mol CF_1.

phosphate, the ΔpH values were almost identical in the azide-containing and in the control samples. As expected in a competitive system, the curve is shifted towards higher phosphate concentrations when NaN_3 is raised from 0.5 to 1 mM (cf. Table III). Fig. 2B shows similar results when arsenate is employed instead of phosphate. The protective arsenate concentrations are, however, higher by a factor of 2–3 than the phosphate concentrations.

As phosphate decelerates tight binding of ADP and azide apparently acts competitively with phosphate, it was investigated whether NaN_3 is able to release the inhibitory effect of phosphate on ADP binding. Fig. 3 shows the corresponding experiment which was conducted at 0.3 mM P_i and 0.5 mM NaN_3 in order to obtain the maximal azide effect according to Fig. 2A. Actually azide results in some stimulation of ADP binding which was suppressed by phosphate, but this effect is much lower than expected from the results obtained in Fig. 2.

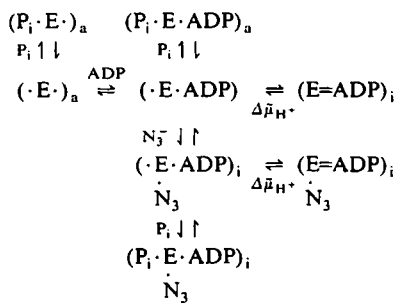
Discussion

Effects of azide on ATPases from different sources have been reported in the literature. ATPase activity of membrane-bound and isolated F_1 from *E. coli* was found to be non-competitively inhibited by azide. While ATP hydrolysis was strongly affected even at low concentrations ($K_i = 39$ μM), inhibition of ATP synthesis and ATP–P_i exchange required much higher azide concentrations [9]. More recently, it was reported that azide inhibited steady-state ATPase activity of *E. coli* F_1 by lowering catalytic site cooperativity [30].

Non-competitive inhibition by azide of a membrane-bound DCCD-sensitive ATPase from the photosynthetic bacterium *Chlorobium thiosulfatophilum* has been reported by Burns and Midgley [24]. Reconstitution of isolated subunits of F_1 from the thermophilic bacterium PS 3 yielded ATP-hydrolyzing enzymes when the complexes either contained $\alpha + \beta + \delta$ or $\beta + \gamma$. Only when subunit γ was present, the complex showed sensitivity against azide, like the native ATPase [25]. Pederson [7] found about 70% inhibition of the isolated ATPase F_1 from rat liver mitochondria by 0.1 mM NaN_3. He could not detect any effect of azide on tight binding of ADP. Some insight into the mechanism of azide inhibition permitted the study by Moyle and Mitchell [26]. Similar to the results reported in this paper, they found that phosphate and phosphate analogues like arsenate, sulfate, etc. increased whereas azide and some other univalent anions decreased the equilibrium between active and inactive ATPases of submitochondrial particles. The ATPase of submitochondrial particles or isolated F_1 is inactivated by preincubation with low concentrations of ADP in the presence of Mg^{2+} [8]. Reactivation, which can be effected by phospho*enol*-pyruvate/pyruvate kinase, is blocked by NaN_3, suggesting that the inhibitory effect may be due to stabilization of an inactive enzyme-ADP complex [8].

Lack of inhibition of both ATP synthesis and ATP hydrolysis may be the reason for the low interest in azide in studies of the chloroplast ATPase. There is, however, a very specific effect of azide in nucleotide- and phosphate-dependent regulation of this ATPase, as shown by the preceding experiments. In deenergized membranes, the thiol-modified ATPase is deactivated by tight binding of ADP and this reaction is decelerated by phosphate. Regulatory binding of ADP is described by two consecutive steps, the reversible binding of the nucleotide molecule to form a 'loose' enzyme-ADP complex followed by the transfer to a 'tight' complex, which includes a conformational change of the binding site [16]. Since the latter step needs membrane energization for reversal, the overall reaction is practically unidirectional under deenergized conditions and is terminated when every ATPase molecule has incorporated one ADP molecule [17]. The initial rate of tight ADP binding in deenergized membranes was found to be inhibited by phosphate in a non-competitive manner [27]. Provided that the formation of the tight enzyme-ADP complex is the rate-limiting reaction, the non-competitive type of inhibition may be interpreted as an interaction of phosphate with the nucleotide-free enzyme form as well as the loose enzyme-ADP complex. Seemingly azide obviates the protective effect of phosphate in ADP-dependent deactivation without changing the kinetics of ADP binding significantly. Since higher concentrations of phosphate in part can overcome the effect of azide, we may conclude competition between

phospahte and azide. The fact that the action of azide requires the simultaneous presence of ADP indicates competition of phosphate and azide for the intermediate loose enzyme-ADP complex. The resulting ATPase · azide · ADP complex may be concluded to be inactive and further react to inactive tight ATPase · azide · ADP complex or to a ternary loose complex by subsequent binding of phosphate, which is also inactive. Reaction of phosphate with the loose enzyme-ADP complex on the other hand forms an active ATPase · phosphate · ADP complex [27]. The context between regulatory binding of ADP, phosphate, and azide may be summarized in the following reaction scheme:



Scheme I. The indices a and i mean active and inactive forms, respectively. Loosely bound ADP is marked by a dot, tightly bound ADP by a double bar.

The model explains competition between phosphate and azide with regard to ADP-dependent inactivation as well as the synergistic effects of ADP and azide in deactivation of the ATPase. Uncouplers stimulate the rate of ATP hydrolysis by preactivated thylakoids due to release of feed-back control by the proton gradient. In the course of the reaction, however, the rate of uncoupled ATP hydrolysis progressively decreases [28]. This fact can be explained by the necessity of $\Delta\tilde{\mu}_{H^+}$ for continuous reactivation of the ATPase, which is inactivated by tight binding of ADP accumulating during ATP hydrolysis [23,27]. The above model predicts acceleration of deactivation of the uncoupled ATPase by azide after enough ADP has been generated by ATP hydrolysis, because an additional pathway of inactivation is opened by the interaction of azide with the loose enzyme-ADP complex. Actually this prediction is verified experimentally. Fig. 4 shows no effect of azide on the initial rate of ATP hydrolysis either in the absence or in the presence of the uncoupler nigericin. After 1 min reaction time, however, complete inhibition of the uncoupled ATPase is found, whereas coupled ATP hydrolysis is much less affected by azide (after this paper was submitted for publication, a similar result was published by Wei, J., Howlett, B. and Jagendorf, A.T. (1988) in Biochim. Biophys. Acta 934, 72–79). Lack of inhibition of photophosphorylation by azide may be explained by reversal of the inactive enzyme · azide · ADP complex to loose enzyme-ADP complex which is forced by the light-induced proton gradient. In addition, the presence of the substrate of photophosphorylation, P_i, provides protection from azide inactivation because of its competitive effect.

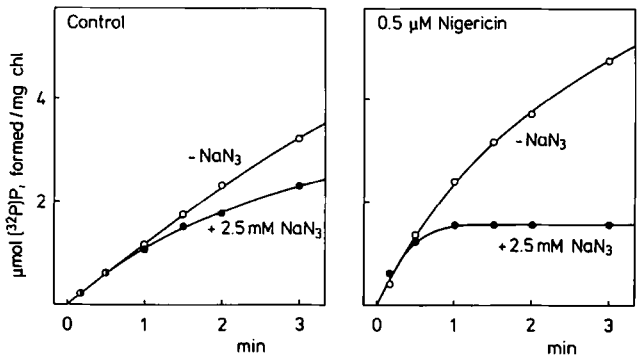


Fig. 4. Effect of NaN_3 on the kinetics of ATP hydrolysis by activated thylakoids in the absence and presence of the uncoupler nigericin. Thylakoids (25 µg Chl/ml) were preilluminated in reaction medium as in Table II. After 15 s in the dark, the indicated concentrations of nigericin and NaN_3 were added together with substrate [^{32}P]ATP (0.2 mM).

Acknowledgements

This work was supported by a grant from Deutsche Forschungsgemeinschaft to H.S. I.B.M. (Department of Animal and Human Physiology, Sofia University, Bulgaria) was recipient of a scholarship from Deutsche Forschungsgemeinschaft. The authors thank Mrs. Mechthild Gokus for competent technical assistance and Dr. Jürgen Schumann for valuable discussion.

References

1 Beechey, R.B., Holloway, C.T., Knight, I.G. and Roberton, A.M. (1958) Biochem. Biophys. Res. Commun. 23, 75–80.
2 McCarty, R.E. and Racker, E. (1967) J. Biol. Chem. 242, 3435–3439.
3 Sebald, W., Hoppe, J. and Wachter, E. (1979) in Function and Molecular Aspects of Biomembrane Transport (Quagliariello, E., Palmieri, F., Papa, S. and Klingenberg, M., eds.), pp. 69–74, Elsevier, Amsterdam.
4 Izawa, S., Winget, G.D. and Good, N.E. (1966) Biochem. Biophys. Res. Commun. 22, 223–226.
5 McCarty, R.E., Guillory, R.J. and Racker, E. (1965) J. Biol. Chem. 240, 4822–4823.
6 Strotmann, H., Brendel, K., Boos, K.S. and Schlimme, E. (1982) FEBS Lett. 145, 11–15.
7 Pedersen, P. (1975) Biochem. Biophys. Res. Commun. 64, 610–616.
8 Vasilyeva, E.A., Minkov, I.B., Fitin, A.F. and Vinogradov, A.D. (1982) Biochem. J. 202, 15–23.
9 Kobayashi, H., Maeda, M. and Anraku, Y. (1977) J. Biochem. 81, 1071–1077.
10 Moroney, J.K., Fullmer, C.S. and McCarty, R.E. (1984) J. Biol. Chem. 259, 7281–7285.
11 Ketcham, S.R., Davenport, J.W., Warncke, K. and McCarty, R.E. (1984) J. Biol. Chem. 259, 7286–7293.

12 Junesch, U. and Gräber, P. (1985) Biochim. Biophys. Acta 809, 429–434.
13 McCarty, R.E. and Racker, E. (1968) J. Biol. Chem. 243, 129–137.
14 Petrack, B., Craston, A., Sheppy, F. and Farron, F. (1965) J. Biol. Chem. 240, 906–914.
15 Carmeli, C. and Lifshitz, Y. (1972) Biochim. Biophys. Acta 267, 86–95.
16 Strotmann, H. and Bickel-Sandkötter, S. (1977) Biochim. Biophys. Acta 460, 126–135.
17 Schumann, J. and Strotmann, H. (1981) in Photosynthesis II. Photosynthetic Electron Transport and Photophosphorylation (Akoyunoglou, G., ed.), pp. 881–892. Balaban, Philadelphia.
18 Dunham, K. and Selman, B.R. (1981) J. Biol. Chem. 256, 10044–10049.
19 Shigalowa, T., Lehmann, U., Krevet, M. and Strotmann, H. (1985) Biochim. Biophys. Acta 809, 57–65.
20 Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64–70.
21 Strotmann, H. and Lohse, D. (1988) FEBS Lett. 229, 308–312.
22 Carmeli, C. (1970) FEBS Lett. 7, 297–300.
23 Strotmann, H., Kleefeld, S. and Lohse, D. (1987) FEBS Lett. 221, 265–269.
24 Burns, D.D. and Midgley, M. (1966) Eur. J. Biochem. 67, 323–333.
25 Yoshida, M., Sone, N., Hirata, H. and Kagawa, Y. (1977) J. Biol. Chem. 252, 3480–3485.
26 Moyle, J. and Mitchell, P. (1975) FEBS Lett. 56, 55–61.
27 Strotmann, H., Niggemeyer, S. and Mansy, A.R. (1987) in Progress in Photosynthesis Research (Biggins, J., ed.), Vol. III, pp. 29–36, Martinus Nijhoff Publishers, Dordrecht.
28 Bakker-Grunwald, T. and van Dam, K. (1973) Biochim. Biophys. Acta 292, 808–814.
29 Strotmann, H., Bickel-Sandkötter, S. and Shoshan, V. (1969) FEBS Lett. 101, 316–320.
30 Noumi, T., Maeda, M. and Futai, M. (1987) FEBS Lett. 213, 381–384.