PHOTOSYNTHETICA 39 (4): 553-556, 2001

Three substrate binding sites on spinach ferredoxin:NADP$^+$ oxidoreductase. Studies with selectively acting inhibitors

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Abstract

The effects of phenylmercuric acetate (PMA) and apoferredoxin (apoFd) on the diaphorase activity of spinach ferredoxin:NADP$^+$ oxidoreductase (FNR) in the presence of dibromothymoquinone (DBMIB) or cytochrome *c* (Cyt *c*) were studied. PMA inhibited effectively (I_{50} = < 5 μM) ferredoxin-dependent Cyt *c* reduction but did not affect evidently the enzyme activity in the presence of DBMIB as an electron acceptor. ApoFd caused also inhibition of Cyt *c* reduction but slightly stimulated, like ferredoxin, DBMIB reduction. We confirm a hypothesis according to which three binding sites for substrates [NADP(H), Fd-Cyt *c*, quinone/dichlorophenol indophenol] occur within the molecule of isolated FNR.

Additional key words: apoferredoxin; cytochrome *c*; diaphorase activity; dibromothymoquinone; ferredoxin; phenylmercuric acetate.

Introduction

Ferredoxin:NADP$^+$ oxidoreductase (EC 1.18.1.2) is a flavoprotein involved in the terminal step of the light-driven non-cyclic electron flow in chloroplasts catalysing NADP$^+$ reduction with electrons derived from reduced ferredoxin (Fd) (for review see Carrillo and Vallejos 1987, Pschorn *et al.* 1988, Arakaki *et al.* 1997). This enzyme is located at the stromal surface of the thylakoid membranes (Jennings *et al.* 1979). *In vivo* this soluble protein occurs in monomeric or aggregated form (Zanetti and Arosio 1980, Yamasaki *et al.* 1983, Shin *et al.* 1985) and may be loosely or tightly bound to the membranes (Matthijs *et al.* 1986, Yu *et al.* 1995). Presumably, 17.5 kDa polypeptide (Vallejos *et al.* 1984) or photosystem 1-E (Andersen *et al.* 1992) mediates interaction of FNR molecules with the membrane. Some data implied that FNR, particularly a tightly membrane-bound form, is also involved in the cyclic electron flow around photosystem 1 (PS1) (Nielsen *et al.* 1995) and NADP(H) binding site is not engaged in this pathway because its blocking by specific antibodies does not influence the cyclic electron transfer (Böhme 1977, Shahak *et al.* 1981). However, up to now the participation of FNR in cyclic electron transport around PS1 has not been conclusively elucidated. The mechanism of transport of the inter-FNR-molecule reducing equivalents has been also a matter of dispute. Some information on this problem has been obtained in experiments with the isolated enzyme that exhibits diaphorase activity and may catalyse the NADPH-dependent reduction of many non-physiological substrates such as Cyt *c*, ferricyanide, dichlorophenol indophenol (DCPIP), methyl viologen (for reviews see Carrillo and Vallejos 1987, Bowyer *et al.* 1988) as well as naphthoquinones and many aromatic nitrocomponents (Anusevičius *et al.* 1997). Fd is an obligatory component only for the reduction of some of these substrates, *e.g.* Cyt *c* (Lazzarini and San Pietro 1962). However, non-physiological substrate binding site(s) on the FNR molecule has(have) not been ascertained satisfactorily. Chang *et al.* (1991) argued that the site involved in the DCPIP reduction differs from that involved in the Cyt *c* reduction. We found (Bojko and Więckowski 1999) that plastoquinone and ubiquinone analogues with short isoprenoid side chains as well as DBMIB are also reduced efficiently by NADPH in the reaction catalysed by isolated

Received 19 July 2001, *accepted* 7 November 2001.
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Abbreviations: apoFd, apoferredoxin; Fd, ferredoxin; DBMIB, dibromothymoquinone: 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCPIP, 2,6-dichlorophenol indophenol; Fd, ferredoxin; PMA, phenylmercuric acetate; PS1, photosystem 1.
Acknowledgment: This work was financially supported by the State Committee for Scientific Researches (KBN), grants No. No.6 PO4A 010 16 and 6 PO4A 022 18.

FNR and we assumed that the quinone binding site corresponds to that of the DCPIP one. We have also hypothesised that in thylakoid membranes the quinone binding site on FNR molecule may be involved in the plastoquinone pool reduction in the PS1-driven cyclic electron transfer (Bojko and Więckowski 1995, 1999, Więckowski and Bojko 1996, 1997). However, this hypothesis has not been verified experimentally. Under the applied conditions the isolated enzyme did not catalyse NADPH-dependent plastoquinone-9 reduction (Bojko and Więckowski 1999). Probably the extremely low solubility of plastoquinone-9 molecules in polar solvents (Marchal *et al.* 1997) prevents interaction of their reducible heads with FNR and this hindrance may be abolished in thylakoid membranes.

In this paper we tried to confirm the existence of two different sites on FNR molecule involved in the binding of non-physiological substrates in experiments with selectively acting inhibitors (phenylmercuric acetate, apoferredoxin).

Materials and methods

Chemicals: DBMIB was purchased from *Aldrich* (Germany), Tris and PMA were obtained from *Fluga* (Switzerland), NADPH was from *Sigma* (U.S.A.), and Cyt *c* came from *Biomed* (Poland). Other chemicals of the analytical grade were from *Polskie Odczynniki Chemiczne* (Poland).

FNR and Fd were isolated from spinach (*Spinacia oleracea* L.) purchased on the local market by the procedure described in Bojko and Więckowski (1995, 1999). The concentrations of FNR and Fd were determined spectrophotometrically at 456 nm (ε_{456} = 11.3 mM^{-1} cm^{-1}) and at 420 nm (ε_{420} = 9.8 mM^{-1} cm^{-1}), respectively. The purity of isolated proteins was verified by SDS-PAGE. Apoferredoxin was obtained from Fd by the method of Pagani *et al.* (1984).

The FNR activity in the presence of Cyt *c* or DCPIP was measured spectrophotometrically (spectrophotomer *LSM/Aminco DW 2000*, U.S.A.) by monitoring absorbance changes at 550 nm (ε_{550} = 21.1 mM^{-1} cm^{-1}) or 600 nm (ε_{600} = 20 mM^{-1} cm^{-1}), respectively. Spontaneous reoxidation of enzymatically reduced DBMIB was recorded by a Clark type oxygen electrode (*Hansatech*, Great Britain) connected to the *TZ 2000* recorder (Czech Republic) as described previously (Bojko and Więckowski 1995, 1999). All measurements were performed at 25 °C and under aerobic conditions. Each experiment was repeated 4-5-fold and then mean values and standard deviations were calculated.

Results and discussion

We have postulated that DCPIP binding site on FNR molecule is also involved in the NADPH-dependent reduction of DBMIB and prenyloquinones, and this site differs from that involved in the Fd/Cyt *c* reduction (Bojko and Więckowski 1999). This assumption agrees with the results of Nielsen *et al.* (1995) who found that decyl-PQ and vitamin K_1 at the concentrations of 100 μM inhibit DCPIP reduction significantly (I_{50} = 17 μM). Comparison of K_m value for DCPIP (24 μM, Nielsen *et al.* 1995) with that for DBMIB (16 μM, Bojko and Więckowski 1995) indicates that DBMIB and DCPIP have essentially a similar affinity to FNR. The results of Anusevičius *et al.* (1997) showed that Fd-binding site on FNR is not involved in the quinone reduction. Our results (not shown) indicate that such FNR inhibitors as heparin, 1,3-disalicylidenepropanediamine, and N-ethylmaleimid decrease the rates of both Cyt *c* and DCPIP (or DBMIB) reductions. Thus these compounds do not interact with DCPIP and Fd-binding sites. In this work we found for the first time that PMA at the concentrations 0 to 100 μM exerted little effect on the diaphorase activity of FNR in the presence of DBMIB. Treatment with high concentration of PMA (100 μM) caused inhibition of about 25 or 40 % at pH 8.7 or 7.7, respectively (Fig. 1*A*). On the other hand, PMA probably blocked selectively the diaphorase activity of FNR in the presence of Fd-Cyt *c*. The activity fell down almost totally at the concentration of 10 μM with I_{50} < 5 μM. This inhibition was observed at both pH of 7.7 and 8.7 of the assay medium (Fig. 1*B*). The Cyt *c* reduction rate was slightly enhanced at pH 8.7 as compared with that at pH 7.7. Increase in concentration of PMA in the reaction mixture from 10 to 100 μM did not lower the rate of Cyt *c* reduction in the presence of Fd. Therefore the low rate of Cyt *c* reduction (typically 0.05 or 0.10 nmol s^{-1} at pH 7.7 or 8.7, respectively) in the presence of inhibitor as well as in the absence of Fd was not directly associated with the FNR activity. Under the applied conditions this may be ascribed to its spontaneous interaction with superoxide radicals that are generated in the medium containing FNR (flavoprotein) and NADPH (see McCord and Fridovich 1969). Mercurials interact with sulfhydryl groups (Tagawa and Arnon 1965) and among the six groups occurring in FNR molecule at least one is essential for catalytic activity of the enzyme in the

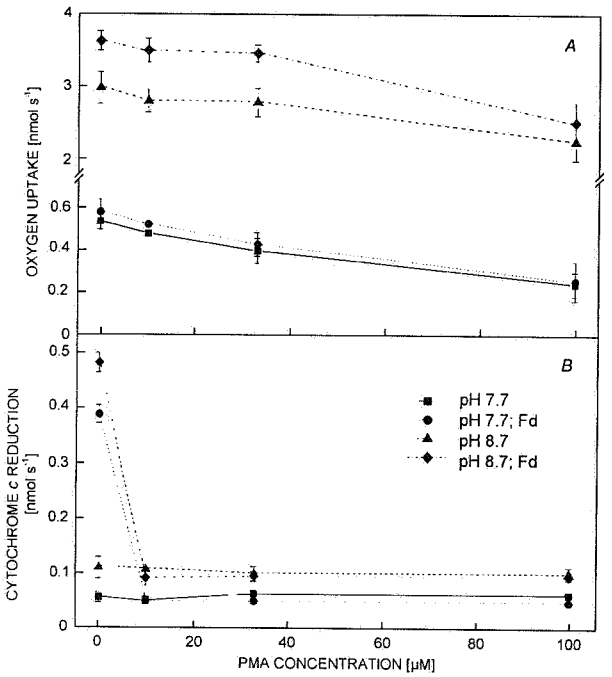

Fig. 1. Effect of phenylmercuric acetate (PMA, 0-100 μM) on the diaphorase activity of ferredoxin:NADP$^+$ oxidoreductase (FNR) in the presence of dibromothymoquinone (DBMIB) (*A*) or cytochrome *c* (*B*) as electron acceptors. Oxygen uptake was associated with re-oxidation of enzymatically reduced dibromothymoquinone. The reaction mixture consisted of 40 mM Tris-HCl buffer, 0.6 μM FNR, 1 mM NADPH, 0.6 μM Fd (where indicated), 20 μM DBMIB (*A*) or 0.6 μM cytochrome *c* (*B*), and PMA in the indicated concentrations.

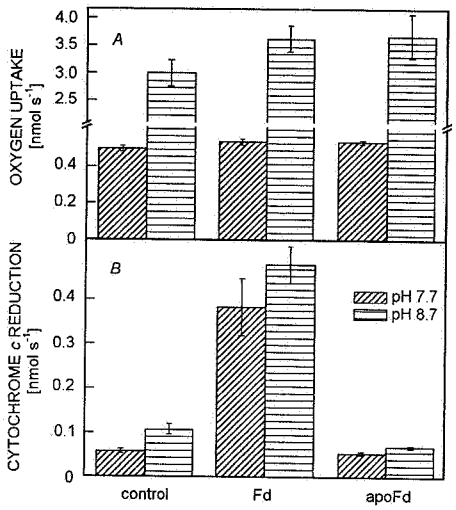

Fig. 2. Effects of ferredoxin (Fd, 0.6 μM) or apoferredoxin (apoFd, 0.6 μM) on the diaphorase activity of ferredoxin:NADP$^+$ oxidoreductase (0.6 μM) in the presence of 20 μM DBMIB (*A*) or 0.6 μM cytochrome *c* (*B*) as electron accepmtors. The reaction mixture contained also 1 mM NADPH and was buffered with 40 mM Tris-HCl.

presence of ferricyanide (Zanetti and Forti 1969). Even if Fd was also inactivated by PMA (Honeycutt and Krogmann 1972) we found that either the -SH groups were not accessible to the inhibitor (see also Zanetti and Forti 1969) or they were not essential for the diaphorase activity of FNR in the presence of DBMIB as a terminal electron acceptor. This suggests that branching pathway of electron transport occurs within the FNR molecule.

Although Fd did not participate in the DBMIB (or DCPIP) reduction, its presence in the assay medium accelerated this process (Bojko and Więckowski 1995, 1999). Pyridine nucleotide transhydrogenase activity of FNR was also stimulated by Fd (Fredricks abd Gehl 1971). Adding apoFd instead of Fd to the reaction mixture also stimulated the DBMIB reduction by the NADPH-FNR system at both pH studied (Fig. 2*A*) but inhibited Cyt *c* reduction (Fig. 2*B*). Thus, our results confirm earlier data that indicated that Fd is not involved in a pathway of quinone reduction by the FNR-NADPH system. Fd, like inactive apoFd, may cause conformational changes within FNR and thus favour also DCPIP or DBMIB reduction (see also Bojko and Więckowski 1995).

The rate of diaphorase activity of FNR, particularly in the presence of quinones, is considerably higher at pH 8.7 than at lower pH (Bojko and Więckowski 1995). The highest enzyme activity in the alkaline medium may be related not only to the fact that optimal proton concentration for FNR activity is around pH 8-9 (Batie and Kamin 1981, Carrillo *et al.* 1981), but also to the domination of anionic form(s) of the reduced quinones (Morrison *et al.* 1982). This form(s) is(are) oxidised rapidly by molecular oxygen (Sugioka *et al.* 1988, Bojko and Więckowski 1995).

Hence the presented results confirm our earlier suggestion that two different sites on FNR molecule participate in the reduction of various non-physiological substrates by reducing equivalents derived from NADPH. The importance of the prenylquinone-binding site on FNR molecule attached to the thylakoid membrane is still a matter of debate.

References

Andersen, B., Scheller, H.V., Møller, B.L.: The PSI-E subunit of photosystem I binds ferredoxin: NADP$^+$ oxidoreductase. – FEBS Lett. **311**: 169-173, 1992.

Anusevičius, Z., Martinez-Júlvez, M., Genzor, C.G., Nivinskas, H., Gomez-Moreno, C., Čenas, N.: Electron transfer reactions of *Anabaena* PCC 71119 ferredoxin: NADP$^+$ reductase with nonphysiological oxidants. – Biochim. biophys. Acta **1320**: 247-255, 1997.

Arakaki, A.K., Ceccarelli, E.A., Carrillo, N.: Plant-type ferredoxin-NADP$^+$ reductases: a basal structural framework and

a multiplicity of functions. – FASEB J. **11**: 133-140, 1997.

Batie, C.J., Kamin, H.: The relation of pH and oxidation-reduction potential to the association state of the ferredoxin-ferredoxin:NADP$^+$ reductase complex. – J. biol. Chem. **256**: 7756-7763, 1981.

Böhme, H.: On the role of ferredoxin and ferredoxin:NADP$^+$ reductase in cyclic electron transport of spinach chloroplasts. – Eur. J. Biochem. **72**: 283-289, 1977.

Bojko, M., Więckowski, S.: Diaphorase activity of ferredoxin:NADP$^+$ oxidoreductase in the presence of dibromothymoquinone. – Phytochemistry **40**: 661-665, 1995.

Bojko, M., Więckowski, S.: NADPH and ferredoxin:NADP$^+$ oxidoreductase dependent reduction of quinones and their reoxidation. – Phytochemistry **50**: 203-208, 1999.

Bowyer, J.R., O'Neill, P., Camilleri, P., Todd, C.M.: A study of the reaction between spinach ferredoxin and one-electron reduced herbicides of differing charge. – Biochim. biophys. Acta **932**: 124-129, 1988.

Carrillo, N., Lucero, H.A., Vallejos, R.H.: Light modulation of chloroplast membrane-bound ferredoxin-NADP$^+$ oxidoreductase. – J. biol. Chem. **256**: 1058-1059, 1981.

Carrillo, N., Vallejos, R.H.: Ferredoxin:NADP$^+$ oxidoreductase. – In: Barber, J. (ed.): The Light Reactions. Pp. 527-560. Elsevier, Amsterdam – New York – Oxford 1987.

Chang, K.-T., Morrow, K.J., Hirasawa, M.N., Knaff, D.B.: Monoclonal antibody studies of ferredoxin:NADP$^+$ oxidoreductase. – Arch. Biochem. Biophys. **290**: 522-527, 1991.

Fredricks, W.W., Gehl, J.M.: Stimulation of the transhydrogenase activity of spinach ferredoxin-nicotinamide adenine dinucleotide phosphate reductase by ferredoxin. – J. biol. Chem. **246**: 1201-1205, 1971.

Honeycutt, R.C., Krogmann, D.W.: Inhibition of chloroplast reactions with phenylmercuric acetate. – Plant Physiol. **49**: 376-380, 1972.

Jennings, R.C., Garlaschi, F.M., Gerola, P.D., Forti, G.: Partition zone penetration by chymotrypsin and the localization of the chloroplast flavoprotein and photosystem II. – Biochim. biophys. Acta **546**: 207-219, 1979.

Lazzarini, R.A., San Pietro, A.: The reduction of cytochrome *c* by the photosynthetic pyridine nucleotide reductase and transhydrogenase. – Biochim. biophys. Acta **62**: 417-420, 1962.

Marchal, D., Boireau, W., Laval, J.M., Moiroux, J., Bourdillon, C.: An electrochemical approach of the redox behavior of water insoluble ubiquinones or plastoquinones incorporated in supported phospholipid layers. – Biophys. J. **72**: 2679-2687, 1997.

Matthijs, H.C.P., Coughlan, S.J., Hind, G.: Removal of ferredoxin:NADP$^+$ oxidoreductase from thylakoid membranes, rebinding to depleted membranes, and identification of the binding site. – J. biol. Chem. **261**: 12154-12158, 1986.

McCord, J.M., Fridovich, J.: Superoxide dismutase. An enzymic function for erytrocupein (hemocuprein). – J. biol. Chem. **244**: 6049-6055, 1969.

Morrison, L.E., Schelhorn, J.E., Cotton, T.M., Bering, C.L., Leach, P.A.: Electrochemical and spectral properties of ubiquinone and synthetic analogs: relevance to bacterial photosynthesis. – In: Trumpower, B.L. (ed.): Function of Quinones in Energy Conserving Systems. Pp. 35-58. Academic Press, New York – London 1982.

Nielsen, H.L., Andersen, B., Scheller, H.V.: Ferredoxin:NADP$^+$ reductase bound to the PSI-E subunit of photosystem I is inefficient in NADP$^+$-reduction but catalyzes the reduction of quinones. – In: Mathis, P. (ed.): Photosynthesis: from Light to Biosphere. Vol. II. Pp. 847-850. Kluwer Academic Publ., Dordrecht – Boston – London 1995.

Pagani, S., Bonomi, F., Cerletti, P.: Enzymic synthesis of the iron-sulfur cluster of spinach ferredoxin. – Eur. J. Biochem. **142**: 361-366, 1984.

Pschorn, R., Rühle, W., Wild, A.: Structure and function of ferredoxin:NADP$^+$-oxidoreductase. – Photosynth. Res. **17**: 217-229, 1988.

Shahak, Y., Crowther, D., Hind, G.: The involvement of ferredoxin-NADP$^+$ reductase in cyclic electron transport in chloroplasts. – Biochim. biophys. Acta **636**: 234-243, 1981.

Shin, M., Ishida, H., Nozaki, Y.: A new protein factor, connectein, as a constituent of the large form of ferredoxin-NADP reductase. – Plant Cell Physiol. **26**: 559-563, 1985.

Sugioka, K., Nakano, M., Tosune-Nakano, H., Minokami, H. M., Kubata, T., Ikogami, Y.: Mechanism of $O_2^{\cdot-}$ generation in reduction and oxidation cycle of ubiquinones in a model of mitochondrial electron transport systems. – Biochim. biophys. Acta **936**: 377-385, 1988.

Tagawa, K., Arnon, D.I.: Oxidation-reduction potentials and stechiometry of electron transfer in ferredoxins. – Biochim. biophys. Acta **152**: 602-613, 1965.

Vallejos, R.H., Ceccarelli, E., Chan, R.: Evidence for the existence of a thylakoid intrinsic protein that binds ferredoxin-NADP$^+$ oxidoreductase. – J. biol. Chem. **259**: 8048-8051, 1984.

Więckowski, S., Bojko, M.: Is ferredoxin:NADP$^+$ oxidoreductase involved directly in plastoquinone reduction? – Plant Physiol. Biochem., Special Issue, p. 99, 1996.

Więckowski, S., Bojko, M.: The NADPH-dependent electron flow in chloroplasts of the higher plants. – Photosynthetica **34**: 481-496, 1997.

Yamasaki, Y., Ishida, H., Shin, M.: Reassociation of the small form of ferredoxin-NADP$^+$ reductase to the large form. – Plant Cell Physiol. **24**: 1313-1316, 1983.

Yu, S.-G., Stefánsson, H., Albertsson, P.-Å.: Molecular properties and localization of ferredoxin-NADP$^+$-oxidoreductase in the thylakoid membranes. – In: Mathis, P. (ed.): Photosynthesis: from Light to Biosphere. Vol. II. Pp. 717-720. Kluwer Academic Publ., Dordrecht – Boston – London 1995.

Zanetti, G., Arosio, P.: Solubilization from spinach thylakoids of a higher molecular weight form of ferredoxin-NADP$^+$ reductase. – FEBS Lett. **111**: 373-376, 1980.

Zanetti, G., Forti, G.: On the reactivity of the sulfhydryl groups of ferredoxin nicotinamide adenine dinucleotide phosphate reductase. – J. biol. Chem. **244**: 4757-4760, 1969.