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BRIEF COMMUNICATION

Effects of amino and thiol group reagents on the ferredoxin:NADP$^+$ oxidoreductase catalysed reduction of dibromothymoquinone

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Abstract

Effects of selective reagents of amino groups (fluorescamine, Fc) and thiol [5,5'-dithio-bis(2-nitrobenzoic) acid, DTNB] groups on the diaphorase activity of spinach ferredoxin:NADP$^+$ oxidoreductase (FNR, E.C 1.18.1.2) in the presence of dibromothymoquinone (DBMIB) as an electron acceptor were studied. The incubation of FNR with 250 μM Fc in the time range from 0 to 120 min led to the gradual decrease of FNR activity according to biphasic kinetics. At the initial phase the activity (defined as the rate of NADPH oxidation) decreased about 4-time faster than at the subsequent second slower phase. Incubation of FNR simultaneously with Fc and DBMIB for more than 20 min caused restoration of the activity to about 80 % of the control. The inhibitory effect of Fc on the FNR-catalysed DBMIB reduction had non-competitive character. Incubation of FNR with DTNB led also to a gradual decrease of the enzyme activity, which reached about 45 % of the control after 2 h of incubation. Thus neither amino nor thiol groups in the FNR molecule are involved directly in the DBMIB reduction. However, the presence of DBMIB in the incubation medium influenced the inhibitory pattern of Fc and DTNB, and this suggests that DBMIB modified the conformational state of the FNR molecule.

Additional key words: dichlorophenol indophenol; 5,5'-dithio-bis(2-nitrobenzoic) acid; fluorescamine; *Spinacia*.

Isolated ferredoxin:NADP$^+$ oxidoreductase (FNR) exhibits diaphorase activity catalysing electron transfer from NADPH to many exogenous non-physiological substrates such as ferricyanide, dichlorophenol indophenol (DCPIP), cytochrome *c* (Cyt *c*), some quinones, and many others (Bojko and Więckowski 2001 and cited literature). Ferredoxin (Fd) is indispensable solely for the Cyt *c* reduction. The sites at the FNR molecule engaged in the reduction of different substrates have not been established satisfactorily. The NADP$^+$ and Fd binding sites have been only relatively well defined. The first one is located in the domain at the C-terminus, whereas the second one was found in a cleft between two domains in the proximity to the FAD (Karplus *et al.* 1991, Kurisu *et al.* 2001). Chang *et al.* (1991) found that the binding site involved in Fd-Cyt *c* reduction differs from that involved in the DCPIP reduction. They isolated eleven monoclonal antibodies IgG's which specifically blocked DCPIP reduction, but had no effect on the Fd-dependent Cyt *c* reduction. Using specifically acting inhibitors we have also found that the quinone-binding site differs from that of Fd binding (Bojko and Więckowski 2001). For example, in the presence of apoferredoxin (Fd deprived of Fe-S centre) the FNR catalysed reduction of Cyt *c* was suppressed totally, whereas DBMIB reduction was not inhibited. We have also found that many prenylquinones, such as plastoquinone-1, plastoquinone-3, or plastoquinone-4, can be efficiently reduced by the NADPH-FNR system (Bojko and Więckowski 1999). Some stimulation of the quinone reduction in the presence of Fd as well as apoferredoxin was even observed. We thought that apoferredoxin, like Fd, caused conformational changes

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Abbreviations: Cyt – cytochrome; DBMIB – dibromothymoquinone, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCPIP – dichlorophenol indophenol; DTNB – 5,5'-dithio-bis(2-nitrobenzoic) acid; Fc – fluorescamine; Fd – ferredoxin; FNR – ferredoxin:NADP$^+$ oxidoreductase; PQ9 – plastoquinone 9.
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within the FNR molecule and this favoured quinone reduction.

We have postulated that in higher plants the quinone binding site at FNR molecule, anchored to the outer surface of the thylakoid membranes, may be involved in PQ9 pool reduction associated with cyclic electron flow around photosystem 1 (Bojko and Więckowski 1999). But so far we have not been able to verify experimentally this proposal. PQ9 is very inefficiently reduced in the reaction mixture containing NADPH and FNR. It is assumed that extremely high hydrophobicity of PQ9 (Marchal *et al.* 1997) prevents from interaction of its "head" with FNR molecule in hydrophilic medium. This hindrance may not exist in thylakoid membranes. We have also found (unpublished data) that the prenylquinone molecules, included PQ9, incorporated into cholate micelles are reduced more effectively by the NADPH-FNR system. All the data obtained strongly suggest that three specific sites occur at the FNR molecule, which are involved in the binding either of $NADP^+$(H), Fd, or quinones. Until now the quinone (or DCPIP) binding site has not been characterised. The mechanism of FNR-catalysed quinone reduction is also scarcely understandable.

In this communication we attempted to answer the question whether amino and thiol groups of some amino acid residues in the FNR molecule are involved in the DBMIB reduction. We used fluorescamine and 5,5'-dithio-bis(2-nitrobenzoic) acid for the modification of the amino and thiol groups, respectively. We focussed primarily to the incubation time of the enzyme with respective modifier in order to obtain some information upon the susceptibility of appropriate amino acid residues to the studied inhibitors.

FNR and Fd were isolated and purified from spinach as described in Bojko and Więckowski (1995). The activity of FNR in the presence of DBMIB and NADPH was determined spectrophotometrically (absorbance changes at 340 nm due to the oxidation of NADPH, $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$) on a *Cary UV/VIS Varian Bio 50* spectrophotometer (USA). The reaction of DTNB with thiol groups in the FNR molecules was also registered by spectrophotometer (absorbance increase at 412 nm due to the formation of yellow product of the reaction; Ellman 1959). On the other hand, the interaction of fluorescamine with free amino groups (primary amines) led to the formation of fluorescent product (Udenfriend *et al.* 1972) which was recorded by a *Perkin Elmer LS-50B* spectrofluorimeter (UK). FNR was pre-incubated with the reagent (250 μM Fc, 10 mM phenylglyoxal, or 2.5 mM DTNB) in the mixture deprived of one or both substrates (NADPH, DBMIB) for 0–120 min. Pre-incubation was performed at 20 °C. Every experiment was repeated at least three times. NADPH, DBMIB, phenylglyoxal, Fc, and DTNB were obtained from *Sigma* (USA), Tris was obtained from *Fluka Chemische Fabrik* (Switzerland) or from *Reanal* (Budapest, Hungary). Other chemicals of analytical grade came from *Polskie Odczynniki Chemiczne* (Poland).

Fc is a selective reagent for amino groups (Udenfriend *et al.* 1972). Control experiments were carried out in order to determine the extent of the Fc reaction with other than FNR components of the incubation medium. The reaction of Fc with NH_2 containing amino acid residues (N-terminal, Lys, Arg, Asn, Gln) in FNR as well as with those that occur in molecules of NADPH and Tris. In the incubation mixture NADPH occurred in a considerably higher concentration than Fc and FNR and therefore NADPH did not limit the studied reaction even if some of NADPH molecules were inactivated by Fc. The reaction of Fc with Tris did not change pH of the mixture by more than 0.1 unit.

In the first set of experiments the effects of incubation time of FNR with Fc or with Fc and substrates (NADPH or DBMIB) on the activity of the enzyme were

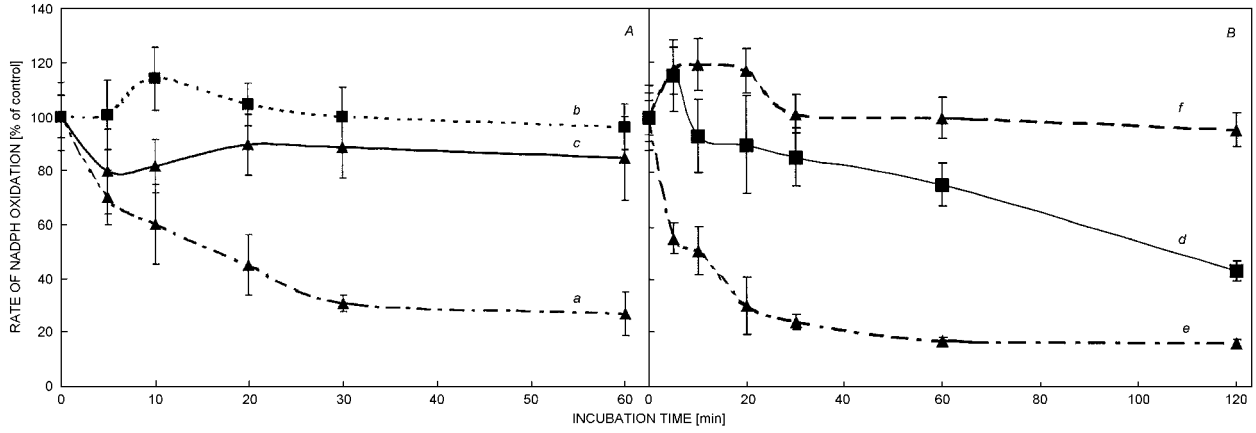

Fig. 1. Effect of incubation time of FNR (1.25 μM) with (*A*) 250 μM fluorescamine (*a*), 250 μM fluorescamine plus 2.5 mM NADPH (*b*), or 250 μM fluorescamine plus 1.5 mM DBMIB (*c*) or (*B*) with 2.5 mM DTNB (*d*), 2.5 mM DTNB plus 2.5 mM NADPH (*e*), or with 2.5 mM DTNB plus 1.5 μM DBMIB (*f*) on the enzyme activity. Incubation medium contained also 10 mM $MgCl_2$, 10 mM Na_2EDTA, and 40 mM Tris.HCl buffer (pH 8.7). Essay medium was composed of 0.05 μM FNR, 60 μM DBMIB, 100 μM NADPH, 10 mM $MgCl_2$, 10 mM Na_2EDTA, and 40 mM Tris-HCl buffer (pH 8.7). For other details see the text.

investigated. Treatment of FNR with Fc (Fig. 1*A*, curve *a*) led to a decrease of the activity in the first 30 min according to biphasic kinetics. During the first 5 min the activity declined to about 70 % of the control, and in the next 25 min it gradually decreased to about 30 % of the control. Thus, the rate of FNR inhibition was about four times faster at the first phase than at the second one. Extension of the incubation period over 30 min did not cause further decrease of the enzyme activity. Curve *b* in Fig. 1*A* shows that incubation of FNR with Fc (250 μM) and NADPH (2.5 mM) did not inhibit the activity of the enzyme. Even some stimulation was observed during the first minutes. Probably all Fc molecules interacted rapidly and efficiently with NADPH being in excess in the medium, and it is conceivable that under these conditions incubation with Fc did not influence FNR activity. Addition of DBMIB to the medium containing FNR and Fc did not influence the pattern of FNR inactivation during the first *ca*. 5 min (Fig. 1*A*, curve *c*), but the activity was restored to about 90 % of the control when incubation period was prolonged over 5 min. Thus probably the inhibitory effect of Fc on the FNR activity was a result of the interaction of Fc with amino groups in the NADP(H)- and/or DBMIB-binding sites as well as with any other free amino groups in the FNR molecule, which can impact the enzyme activity through the conformational changes.

As can be deduced from Fig. 2, the inhibition of NADPH oxidation had non-competitive character with respect to DBMIB, and this may suggest that NH_2 groups were not directly involved in DBMIB binding. The negatively charged groups in $NADP^+$ interact electrostatically with Lys 244 in the nucleotide-binding site at the FNR molecule (Carrillo and Vallejos 1983, Chain *et al.* 1985, Cidaria *et al.* 1985, Batie and Kamin 1986). Lys 116 influences the activity of this enzyme (Aliverti *et al.* 1991). Thus, the interaction of Fc with Lys 244 might exert pronounced effect, although we never observed the total inhibition of the enzyme. Since Fc interacts rapidly ($t_{1/2}$= 100–300 ms; Udenfriend *et al.* 1972) with primary amines, the two phases of the enzyme inactivation were probably associated with the different susceptibilities to the reagent of various NH_2 groups within the FNR molecule. The initial phase might be related to the modification of the Lys 244 as this residue would be more accessible to Fc, while the second phase might be associated to the modification of any other amino acid residue(s) having free NH_2 group(s), particularly those located deeply within the FNR molecule. The screening effect of DBMIB on the Fc-treated FNR is not clear. We assume that this quinone induced conformational changes within the enzyme molecule that made more difficulties for the Fc penetration into deeply located reactive groups.

Modification of the amino groups by Fc, however, did not completely inhibit the FNR activity. The residual activity (account 30 % of the control) remained in samples incubated with excess of Fc (compared with the FNR concentration) during the next 30 min.

The arginyl residues occur in nucleotide and Fd-binding sites (Zanetti *et al.* 1979, Sancho *et al.* 1990, Medina *et al.* 1992). These residues can also be modified by Fc. We found that modification of arginyl residue(s) by phenylglyoxal (Sancho *et al.* 1990) also caused time-depended lowering of the FNR activity (data not shown). The inhibition finally reached about 40 % after 40-min incubation. Thus, the data from this experiment suggest that the modification of arginyl residues in FNR probably affected indirectly the diaphorase activity of the enzyme. This might concern also other amino acid residues containing free NH_2 groups.

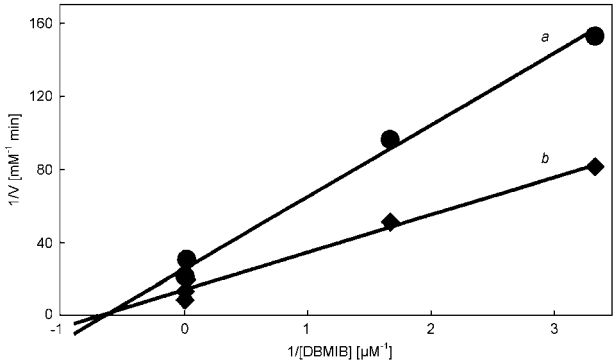

Fig. 2. The Lineweaver-Burke plot of the FNR activity (the rate of NADPH oxidation) *versus* DBMIB concentration in the sample incubated with fluorescamine (*a*) for 1.5 h and in the control (*b*). For other details see Fig. 1*A* and the text.

The last set of experiments focused on the effect of dithionitrobenzoic acid (DTNB) on the FNR activity in the presence of DBMIB as electron acceptor. We expected to obtain information about the involvement of the SH groups of FNR in the reduction of quinones. Phenylmercuric acetate, another selective reagent for SH groups, insignificantly affects the DBMIB reduction (Bojko and Więckowski 2001). It is assumed, however, that preincubation of FNR with the DTNB might enable penetration of the inhibitor into the interior part of the FNR molecule and that lead to the delay of its reaction with deeply located SH groups. Our results support this notion. In the first about 20 min of incubation with DTNB the enzyme did not significantly lose the activity (Fig. 1*B*, curve *d*), and even some transitional stimulation was observed. However, prolonged incubation caused gradual decrease in FNR activity, and after two hours it amounted to about 40 % of the initial value. Significant depression of FNR activity in the presence of NADPH (Fig. 1*B*, curve *e*) was partially coupled with the direct interaction of DTNB and NADPH (data not shown). If DBMIB was present in the incubation mixture, the inhibitory effect of DTNB was eliminated (Fig. 1*B*, curve *f*). Under these conditions the accumulation of $DTNB^{2-}$ was also not observed (data not shown). Thus, the presented results confirm our earlier data that indicated that out of the five

cysteine residues in FNR (see Protein Data Bank, structure 1FND) none is directly involved in the reduction of DBMIB. Incubation for more than 20 min caused some inhibition of the enzyme, which suggests that SH group(s) located in the interior of FNR molecule is(are) indirectly involved in the enzyme activity in the presence of DBMIB. Perhaps the interaction of DTNB with the SH group(s) led to the formation of less favoured FNR conformational state for DBMIB reduction. We think that reduced DBMIB was not involved in the DTNB reduction in any considerable extent. Thus, although NH_2 and SH groups are not engaged directly in the DBMIB binding by the FNR molecule, the quinone markedly influenced the interaction of Fc and DTNB with the enzyme. However, quinone influences also the conformational state of FNR and therefore it may interact in any way with this enzyme.

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