Studies on the one- and two-electron FAD-mediated reactions by ferredoxin-NADP$^+$ oxidoreductase from *Anabaena*

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The NADPH-diaphorase activity of the flavoprotein ferredoxin-NADP$^+$ oxidoreductase from the cyanobacterium *Anabaena* sp. strain 7119 shows a very alkaline optimum pH value, about 9.5–10.0, when assayed with two-electron acceptor dyes, such as dichlorophenolindophenol or iodonitrotetrazolium. In contrast, the enzyme reactions mediated by the physiological one-electron carrier ferredoxin exhibit an almost neutral optimum pH value. EPR spectra and pH titration of the NADPH-reduced enzyme indicate that whereas the one-electron reduced form (semiquinone) of the flavin coenzyme is formed at neutral pH values, the fully reduced form (hydroquinone) arises at the alkaline range above pH 9.0. These findings are also in agreement with data obtained from redox titrations of the enzyme FAD by using an NADPH-regenerating system and are interpreted on the basis of the hydroquinone and semiquinone forms of the flavin coenzyme being, respectively, intermediates in the diaphorase and ferredoxin-mediated catalytic activities of the reductase.

Ferredoxin-NADP$^+$ oxidoreductase *NADPH-diaphorase* *Ferredoxin-dependent reductase*
Flavin semiquinone *Flavin hydroquinone* *(Cyanobacterium)*

1. INTRODUCTION

The flavoprotein ferredoxin-NADP$^+$ oxidoreductase (FNR) (EC 1.18.1.2) is a component of the photosynthetic electron transport chain which catalyzes the reduction of NADP$^+$ by Photosystem I-reduced ferredoxin (Fd) [1]. Besides its physiological role as a NADPH-generating system, FNR catalyzes a number of other enzymatic activities, either dependent on or independent of Fd as an electron mediator, such as NADPH-diaphorase, transhydrogenase, and NADPH-hemoprotein reductase [2,3]. Recently, two Fd-dependent assays for FNR, namely, oxidation by NADP$^+$ of dithionite-reduced Fd and reduction by NADPH of cytochrome *c*, have been claimed as valid models for the in vivo reduction of NADP$^+$ in comparison to the artificial diaphorase assay [4,5].

Here, a comparative study of both enzymatic activities is presented using a purified cyanobacterial FNR. The pH profiles for the Fd-dependent and diaphorase assays using two-electron acceptor dyes, such as dichlorophenolindophenol (DPIP) [6] or 2-(*p*-iodophenyl)-3-nitrophenyl-5-phenyltetrazolium chloride (INT) [7], exhibit, respectively, a pronounced decrease and increase in activity around pH 8.5–9.0. These changes show a noteworthy parallelism with the pH-dependent redox behavior of the FNR flavin-cofactor reduced by NADPH, suggesting that each kind of FNR activity is related to either the semiquinone or hydroquinone reduced form of the enzyme FAD.

2. EXPERIMENTAL

FNR [8] and Fd [9] were purified as described from photoautotrophically grown cells of the cyanobacterium *Anabaena* sp. strain 7119.

The Fd-dependent NADPH-cytochrome *c* reductase activity was assayed as in [2]. The oxidation by NADP$^+$ of dithionite-reduced Fd was determined as in [4]. NADPH-diaphorase activity using DPIP or INT as electron acceptor was determined as in [10] and [7], respectively, but using 0.5 mM NADPH to start the reaction. The reaction mixtures for activity measurements were in all

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cases buffered with a 60 mM equimolar mixture of 4-morpholinepropanesulfonic acid (Mops), Tris and glycine adjusted with NaOH to the indicated pH values.

EPR spectra of the purified FNR were performed using a Bruker Spectroscopin instrument equipped with a low temperature accessory. The temperature was 77 K, the frequency 9.3 GHz, and the modulation amplitude 2 G. The enzyme (19 μM) was reduced by the addition of solid NADPH to reach a final concentration of 5 mM in the presence of either 50 mM Tris–HCl buffer (pH 7.5), or 50 mM glycine–KOH (pH 10.5). The solutions were frozen in liquid nitrogen before performing the spectra.

pH-Titration of the NADPH-reduced enzyme was performed at 22°C under argon in an anaerobic cuvette containing 2.0 ml of 20 mM Tris–HCl buffer (pH 6.0), FNR (8 μM) and NADPH (2 mM) by adding stepwise aliquots of 2 N NaOH with a Hamilton gas-tight microsyringe and monitoring the pH by means of a BioRad Combination pH Microelectrode. The absorbance spectra in the range between 350 and 700 nm were simultaneously recorded with an Aminco-Chance DW-2a spectrophotometer.

Redox titration of the enzyme FAD was carried out at several pH values by using an NADPH-regenerating system (isocitrate/isocitrate dehydrogenase/$NADP^+$) as in [11], but utilizing as buffer a 0.1 M equimolar mixture of Mops, Tris and glycine adjusted with NaOH to the indicated pH values. The amounts of NADPH and oxidized FNR were determined at time intervals during the reduction process from the absorbances at 340 nm (ϵ = 6.2 $mM^{-1} \cdot cm^{-1}$) and 456 nm (ϵ = 10.75 $mM^{-1} \cdot cm^{-1}$), respectively.

Mops, Tris, glycine, sodium isocitrate, heart horse cytochrome *c* and isocitrate dehydrogenase were purchased from Sigma (St. Louis, MO); sodium dithionite and DPIP from Merck (Darmstadt); NADPH and $NADP^+$ from Boehringer (Mannheim); and INT from Serva (Heidelberg).

3. RESULTS AND DISCUSSION

3.1. *Comparison of pH profiles for diaphorase and Fd-dependent reductase activities*

The pH profiles for diaphorase assay, using the two-electron acceptor dyes DPIP and INT, as well as those for Fd-mediated reactions, namely, dithionite-reduced Fd-$NADP^+$ reductase and NADPH-cytochrome *c* reductase, are shown in fig.1. The pH profiles for diaphorase exhibit a marked increase in activity when the pH is shifted towards the alkaline side in pH range 7–10, with maxima at pH values between 9.5–10.0, and resemble titration curves for pH-dependent transitions between two enzyme states with apparent pK_a values for DPIP and INT of 9.1 and 8.7, respectively (cf. [6]). On the other hand, Fd-dependent activities show rather different pH profiles in comparison with diaphorase assays, the optimum pH value being close to neutrality, about 7.5. Above this pH Fd-dependent activities decrease with pH, showing apparent pK_a values of 8.8 (NADPH-cytochrome *c* reductase) and 8.6 (dithionite-reduced Fd-$NADP^+$ reductase). Both kinds of pH profiles are therefore not only different, but, in addition, they manifest a marked transition in opposite directions at about the same pH value.

3.2. *Effect of pH on the redox properties of the FNR flavin-cofactor*

We have previously reported [12] that *Anabaena* FNR contains 1 FAD prosthetic group per mole-

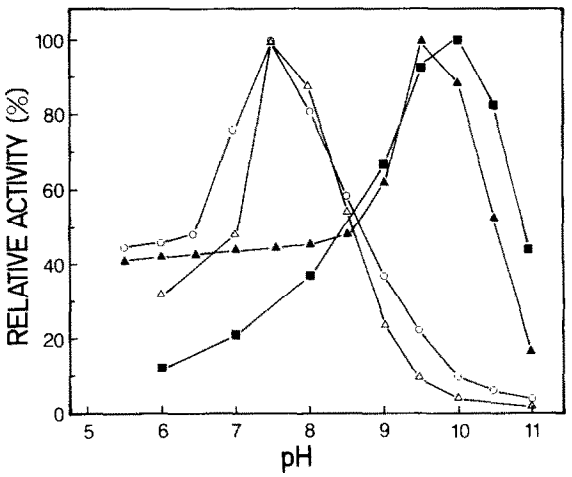

Fig.1. pH profiles for one- and two-electron FAD-mediated enzymatic activities of FNR from the cyanobacterium *Anabaena*. Data are expressed as relative values with respect to activity at optimum pH. (○) NADPH-cytochrome *c* reductase; (△) dithionite-reduced Fd-$NADP^+$ reductase; (▲) DPIP diaphorase; (■) INT diaphorase. Assay conditions are described in section 2.

cule. At neutral pH values and in the presence of NADPH this flavin-cofactor undergoes partial reduction to the semiquinone form, as demonstrated spectrophotometrically and confirmed by EPR data.

Fig.2 shows the EPR spectra at pH 7.5 of both the oxidized and the NADPH-reduced FNR. It can be seen that only the reduced enzyme exhibits a clear EPR signal with no hyperfine structure at a g value of 2.005, the peak-to-peak linewidth being about 20 G. Both values are characteristic of neutral (blue) flavoprotein radicals, in which the flavin-cofactor is partially reduced to the semiquinone form [13]. In contrast, neither the oxidized nor the NADPH-reduced enzyme exhibited EPR signal when the spectra were performed at pH 10.5.

Fig.3 shows in detail the pH-effect on the redox state of NADPH-reduced FNR along the pH range between 6 and 11. The spectrophotometric data obtained from this pH-titration of the NADPH-reduced enzyme reveal the appearance at neutral pH values of two absorbance peaks at 525 and 602 nm, which are associated with the formation of the blue semiquinone form. However, as the pH rises to more alkaline values, absorbance beyond 500 nm disappears and reaches a minimum above pH 10, the spectrum of NADPH-reduced FNR being then very similar to that of the dithionite-reduced enzyme, which, at any pH, presents fully

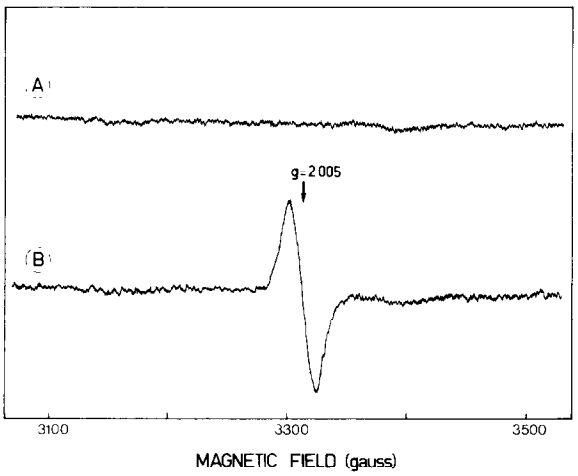

Fig.2. EPR spectra at pH 7.5 under different redox conditions of purified FNR from *Anabaena* sp. (A) Oxidized enzyme; (B) NADPH-reduced enzyme. Other conditions are described in section 2.

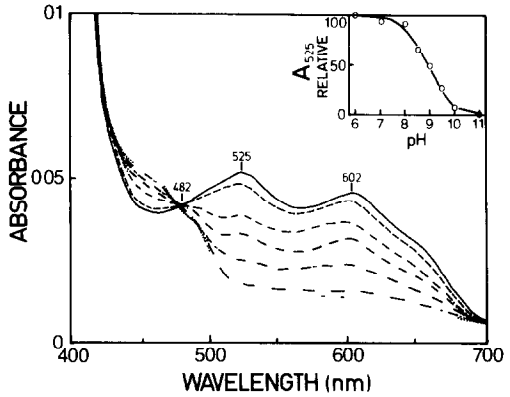

Fig.3. pH-Titration of NADPH-reduced FNR from *Anabaena*. Spectra were performed at pH values of 6 (——), 8 (– – –), 8.5 (– · –), 9 (– · · –), 9.5 (– · · · –), 10 (– · · · · –) and 11 (· · ·). The inset shows the pH-dependent absorbance changes at 525 nm. Other conditions are described in section 2.

reduced its FAD (hydroquinone form) (cf. [11]). The inset of fig.3 clearly reveals that the absorbance at 525 nm – a distinctive feature of semiquinone formation – of NADPH-reduced FNR exhibits a pH-dependent transition with an apparent pK_a of about 8.9. Similar results have been obtained with NADPH-reduced FNR using as buffer Mops–Tris–glycine (total concentration, 0.1 M) adjusted to different pH values. In this regard it should be mentioned that incubation of the cyanobacterial enzyme with NADPH in the absence of any electron acceptor induces, at pH values above 10, a time-dependent inactivation that, in contrast with that observed around neutral pHs, is total and irreversible leading to splitting of the FAD cofactor [14].

The oxido-reduction behavior of *Anabaena* FNR has also been investigated from spectra obtained during the course of the redox process by using an NADPH-regenerating system. The midpoint redox potentials and the electrons involved in the corresponding redox reactions were estimated, as described in section 2, at several pHs by using the couple NADPH/$NADP^+$ as a redox indicator (table 1). At pH values near neutrality the enzyme FAD undergoes a one-electron reduction to its semiquinone form (cf. [11]), whereas at pH 10 a two-electron process results that gives rise to the fully reduced hydroquinone form, the data thus being in agreement with those obtained from both EPR spectra and pH-titration.

Table 1

Midpoint redox potential for enzyme FAD and number of electrons involved in the reduction by NADPH of *Anabaena* FNR at different pH values

pH	E_m for enzyme FAD (mV)	Electron number
6	−256 ± 13	1.2 ± 0.08
8	−359 ± 8	1.3 ± 0.07
10	−438 ± 11	2.1 ± 0.10

The experiments were carried out under argon at 22°C using an anaerobic cuvette containing in a volume of 2.5 ml: Mops–Tris–glycine buffer (total concentration, 0.1 M) adjusted with NaOH to the indicated pH values; 8 μM FNR; 30 μM $NADP^+$; 3 mM $MgCl_2$; 4 mM DL-isocitrate, and 25 μg of isocitrate dehydrogenase. The E_m values and the number of electrons involved were calculated, respectively, from two independent experiments, from the *y*-axis intercept and the slope of the plots of ambient redox potentials – estimated from the $[NADP^+]/[NADPH]$ ratio – '*vs*' log $[FNR_{ox}]/[FNR_{red}]$. Other conditions are described in section 2

In summary, a close parallelism seems to exist at the alkaline pH range between the decrease in the Fd-dependent FNR activities and the decline in the proportion of the enzyme FAD semiquinone form generated by reduction with NADPH. Accordingly, high semiquinone levels and optimum enzyme activity for the reactions mediated by the one-electron carrier Fd appear tightly related, thus suggesting participation of the semiquinone form in such reactions. In this regard it is worthwhile to point out that the semiquinone form of the FNR flavin-cofactor has been reported to be a functional intermediary of the photosynthetic electron-transport chain in intact *Chlorella* cells [15]. On the other hand, FNR diaphorase activity assayed with two-electron acceptor dyes exhibits an optimal pH at about 10, a pH value at which the enzyme FAD undergoes a two-electron reduction by NADPH to its hydroquinone form. It seems therefore that at the alkaline pH range the enzyme FAD may shuttle between its fully oxidized and reduced forms, thus behaving as a more effective electron-carrier for two-electron acceptors than the neutral semiquinone form. This interpretation is not incompatible with the explanation proposed in [6] about the involvement of -SH groups in activity transitions. Actually, both thiol groups [16] and flavin semiquinone radicals [17] have a similar pK_a value, about 8.5, where the FNR activity transitions are observed.

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REFERENCES

[1] Böger, P. (1979) in: Encyclopedia of Plant Physiology, New Series (Gibbs, M. and Latzlo, E. eds) vol.6, pp.401–404, Springer, Berlin.
[2] Shin, M. (1971) in: Methods in Enzymology (San Pietro, A. ed) vol.23, pp.440–447, Academic Press, New York.
[3] Zanetti, G. and Curti, B. (1980) in: Methods in Enzymology (San Pietro, ed) vol.69, pp.250–255, Academic Press, New York.
[4] Masaki, R., Yoshibara, S. and Matsubara, H. (1982) Biochim. Biophys. Acta 700, 101–109.
[5] Zanetti, G. and Curti, B. (1982) in: Flavins and Flavoproteins (Massey and Williams, C.H. eds) pp.667–671, Elsevier, Amsterdam, New York.
[6] Davis, D.J. and San Pietro, A. (1977) Arch. Biochem. Biophys. 184, 572–577.
[7] Zanetti, G. (1981) Plant Sci. Lett. 23, 55–61.
[8] Serrano, A. and Rivas, J. (1982) Anal. Biochem. 126, 109–115.
[9] Mitsui, A. and Arnon, D.I. (1971) Physiol. Plant. 25, 135–140.
[10] Riov, J. and Brown, G. (1976) Physiol. Plant. 38, 147–152.
[11] Keirns, J.J. and Wang, J.H. (1972) J. Biol. Chem. 247, 7374–7382.
[12] Serrano, A., Rivas, J. and Losada, M. (1982) Abstr. Spec. FEBS Meet. on Cell Function and Differentiation, Athens, p.256.
[13] Palmer, G., Müller, F. and Massey, V. (1971) in: Flavins and Flavoproteins (Kamin, H. ed) pp.123–140, University Park Press, Baltimore.
[14] Serrano, A., Rivas, J. and Losada, M. (1982) Abstr. 10th SEB Meet., Santander, abstr.no.53.
[15] Bouges-Bocquet, B. (1978) FEBS Lett. 94, 95–99.
[16] Jocelyn, P.C. (1972) in: Biochemistry of SH Group, pp.51–55, Academic Press, London, New York.
[17] Massey, V. and Hemmerich, P. (1980) Biochem. Soc. Trans. 8, 246–257.