A POSSIBLE REGULATION OF ACTIVITY OF FERREDOXIN–NADP REDUCTASE AND FERREDOXIN SYSTEM BY IONIC STRENGTH: CATALYTIC SIGNIFICANCE OF THE ONE TO ONE COMPLEX

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1. Introduction

It has been demonstrated by difference spectrum and sedimentation experiments [1–4] that spinach ferredoxin–NADP reductase (FNR) and ferredoxin (Fd) form, at low ionic strength, a tightly bound 1:1 complex, which can be completely dissociated into its components by increasing ionic strength [3, 4]. This tightly bound 1:1 complex was thought as important for the enzymic activities of FNR, such as Fd-linked cytochrome *c* reductase activity [3,4].

Contradictory to this implication, however, Fd was found to be a potent inhibitor of FNR-catalyzed reactions at low ionic strength [5]. The FNR-Fd system was more effective in initiating the sulfite oxidation at a high ionic strength than at a low ionic strength [6]. Cammack et al. [7] showed that a 'difference' circular dichroism spectrum, ascribed to complex formation, still remained at an ionic strength (0.3 M NaCl) which was sufficiently high to cause the dissociation of the complex [3, 4].

In the present investigation, a more comprehensive interpretation of these contradictory facts is reported, i.e. there are at least three states of interaction between FNR and Fd, depending on the ionic strength of the environment: 1) a very tightly bound complex is present at near zero ionic strength where Fd is inhibitory toward FNR-catalyzed reactions; 2) a comparatively loosely bound but catalytically effective complex is seen at around 0.1 M NaCl concentration; and 3) a complex of decreased activity when FNR is inactivated by high ionic strength is observed.

It is likely that the intracellular increment of salt concentration plays a regulatory role on the enzymic activities by changing the degree of dissociation of the complex into its components and reversible inactivation of the flavoprotein itself.

2. Materials and methods

Fd and FNR were purified from spinach by the methods of Tagawa and Arnon [8] and Shin et al. [9], respectively. The concentrations of these proteins were estimated by measuring the absorbances on the basis of molar extinction coefficients [10, 11]. NADPH was purchased from Sigma and its concentration was determined spectrophotometrically. Diaphorase activities were measured spectrophotometrically with 2,6-dichlorophenolindophenol (DCPIP) as an electron acceptor. Spectrophotometers used were Hitachi-Perkin Elmer (Model 124 and Model 139).

3. Results and discussion

Fig. 1 shows the effect of NaCl on FNR-catalyzed diaphorase activities in the absence and presence of Fd. When Fd was not added, the diaphorase activity decreased gradually with increase of ionic

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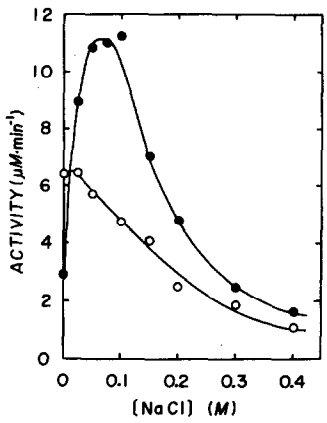

Fig. 1. Effect of NaCl concentration on FNR-catalyzed diaphorase activities in the absence (○) and presence (●) of Fd. Reactions were measured spectrophotometrically at 600 nm in 0.01 M tris-HCl buffer, pH 7.4, at 25°. Concentrations (M): FNR 6.3×10^{-9}; Fd (when added) 2.6×10^{-7}; NADPH 2.4×10^{-5}; DCPIP 1.2×10^{-5}.

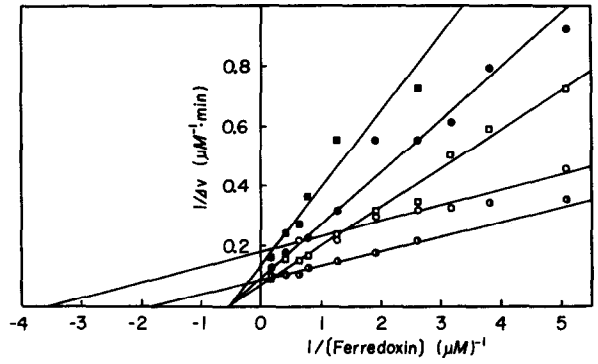

Fig. 2. The plot of $1/\Delta\nu$ vs. 1/[Fd]. Activity was measured spectrophotometrically at 600 nm in 0.01 M tris-HCl buffer, pH 7.4, at 25°. Concentrations (M): FNR 7.1×10^{-9}; NADPH 5.2×10^{-5}; DCPIP 2.6×10^{-5}. Concentrations of NaCl (M): 0.05 (●), 0.10 (○), 0.15 (□), 0.20 (●), 0.30 (■).

strength. This is due to an inactivation of FNR by the high ionic strength environment. This inactivation was reversible, since the activity was fully restored upon dilution with buffer of the enzyme, which had been pre-incubated with a 0.3 M NaCl solution for 10 min.

Fd was found to have two opposite effects on the diaphorase activity depending on ionic strength. In the absence of NaCl, it was fairly inhibitory in agreement with Nelson and Neumann [5]; on the other hand, it stimulated the activity when NaCl was added (over 0.025 M NaCl). A maximal activity was attained at about 0.1 M NaCl, and then activity decreased with increase of salt concentration. The decrease in activity in this portion of the graph parallels that of the diaphorase activity *without* Fd: the ratios of the activity at 0.15 M NaCl to that at 0.30 M NaCl ($A_{0.15}/A_{0.30}$) were 2.3 for the diaphorase activity *without* Fd and 2.2 for the activity *with* Fd, respectively. Therefore, the activity decrease at high ionic strength (over 0.15 M NaCl) could also be caused by FNR-inactivation due to the high ionic strength, but not due to the dissociation of the complex into components as previously considered [3, 4]. On the other hand, the activity increase at lower ionic strength (below 0.10 M NaCl) could be due to changes in the affinity between FNR and Fd, resulting in a more effective state of interaction between these two proteins, and also due to a recovery from the Fd-inhibition by increasing the accessibility of NADPH to the reductase.

Stimulation by Fd at a fairly high ionic strength was studied. It was found that the reaction rate increased with increasing Fd when other conditions were kept constant. It therefore seems reasonable to assume that some effective state of interaction, probably a loosely bound complex, between FNR and Fd was present at high ionic strength. On the assumption that the increase in the reaction rate on addition of Fd, $\Delta\nu$, is directly proportional to the amount of this species, the following relationship can be derived [12]:

$$\frac{1}{\Delta\nu} = \frac{1}{\Delta V_{max}} + \frac{K_{FF}}{\Delta V_{max}} \frac{1}{[Fd]} \qquad (1)$$

where K_{FF} is the apparent interaction constant between FNR and Fd. The experimental results obtained under various ionic strengths are shown in fig. 2. A satisfactory straight line was obtained in each case. The values of the constant, K_{FF}, calculated from the intercepts on the abscissa are listed

Table 1
Apparent interaction constant, K_{FF}, between FNR and Fd in high ionic strength media.

NaCl concn. (M)	K_{FF}* (M)
0.05	2.9×10^{-7}
0.10	6.1×10^{-7}
0.15	2.0×10^{-6}
0.20	2.0×10^{-6}
0.30	2.0×10^{-6}

* Obtained from equation (1).

in table 1. It is important to note that the value increased abruptly with increase of ionic strength up to 0.15 M NaCl, but remained constant above 0.15 M, indicating that FNR and Fd are still in a complexed state at high ionic strength but have quite a decreased activity because of the inactivation of FNR. The above facts also well account for the observation of Cammack et al. [7] that the decrease in the Cotton effects indicates the existence of the complex in the presence of even 0.3 M NaCl, where Fd-linked cytochrome *c* reductase activity is seriously damaged [3]. Also, the absorbance increment in the flavin region upon complexing does not entirely reflect in events leading to the formation of the complex, but reflect a sort of spectral perturbation of the flavin moiety.

In this context, the interpretation by Foust et al. [3] and Nelson and Neumann [4] must be somewhat modified. The effects of ionic strength on the FNR–Fd system were revealed to have a dual function: (1) the dissociation of the complex into the components, at a rather low ionic strength (below 0.10 M NaCl); (2) the reversible inactivation of FNR itself, which becomes pronounced at a higher ionic strength (over 0.15 M NaCl). The latter effect has been so far overlooked by other investigators.

A demonstration of the existence of the FNR–Fd complex by ultracentrifugation is unclear, due to the small difference in sedimentation velocities of the complex, free FNR (M.W. 40,000) and Fd (11,000).

Taking into account the relatively high ion content (above 0.1 M) in an intact chloroplast [13], the physiological significance of the FNR–Fd complex at near zero ionic strength is questionable. Of far greater physiological significance is the fact that the ionic environment of the flavin and iron-sulfur proteins *in vivo* can regulate the expression of enzymic activities through the degree of dissociation of the complex and inactivation of the flavoprotein. In view of the close similarity of the adrenal steroid hydroxylase system to the electron transport system of chloroplast photosynthetic pyridine nucleotide reductase, the proposed regulatory mechanism is probably also valid in the former system. This finding is supported by a previous observation that the conversion of cholesterol to pregnenolone was greatly stimulated by inorganic phosphate (optimal concentration about 10 mM) but higher concentrations of inorganic phosphate were inhibitory [14]. This mode of regulation of enzymic activity by ionic strength could be a general phenomenon for multisubunit enzymes.

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