View metadata, citation and similar papers at core.ac.uk
brought to you by CORE
provided by Archivio istituzionale della ricerca - Università dell'Insubria

Ferredoxin–ferredoxin NADP reductase interaction

Catalytic differences between the soluble and thylakoid-bound complex

Giorgio Forti and Marcella Bracale

Centro CNR per lo Studio della Biologia Molecolare delle Piante, Dipartimento di Biologia, Università degli Studi, Via Celoria 26, 20133 Milano, Italy

Received 10 October 1983; revised version received 23 November 1983

Ferredoxin-NADP reductase (FNR) and ferredoxin form a complex when the former is membrane-bound as they do when both components are in solution, with the same dissociation constant. The rate constant of NADP photoreduction, first order with respect to the complex, is more than 20-times higher when FNR is membrane-bound than when the enzyme is in solution. The Arrhenius activation energy is identical in both conditions. These observations are interpreted in terms of 'entropic catalysis' of NADP reduction by the thylakoid-bound FNR.

Photosynthesis NADP reduction Ferredoxin Ferredoxin-NADP reductase Catalysis Membrane

1. INTRODUCTION

We have previously shown that the rate of NADP photoreduction by thylakoids is severely reduced whenever ferredoxin-NADP reductase (FNR) is present in solution instead of bound to the membranes as it is in nature [1]. Other reactions catalyzed by FNR, such as the diaphorase reaction, are unaffected by the solubilization of the enzyme [1].

We report here the results of further investigations of the influence of the FNR–membrane interactions on the formation of the complex of FNR with ferredoxin (Fd) [2] and the thermodynamic aspect of catalysis of NADP reduction.

2. MATERIALS AND METHODS

Chloroplasts were isolated from freshly harvested spinach leaves as in [1], except that 5 mM $MgCl_2$ was present at all stages of the preparation. NADP photoreduction was measured in a buffer containing 15 mM Tricine–NaOH (pH 8.0), 5 mM NaCl, 2.5 mM $MgCl_2$, 1 mM NADP, 2.5 μM gramicidin, and Fd and FNR as indicated; chlorophyll was 10–12 μg/ml. Illumination, at saturating intensity, was provided by a projector equipped with a 500 W lamp. Light was filtered through 5 cm of water, a heat filter (Balzer) and a Corning 4-96 filter. The sample was illuminated for 2 min (the reaction rate was constant during this time); illumination was then discontinued and oxidized glutathione was added at a final concentration of 2.5 mM together with an excess of glutathione reductase (Boehringer). After 1 min, HCl (25 μl) was added to bring the pH below 2. The samples were then centrifuged, and an appropriate fraction was placed in a spectrophotometer cuvette together with Tris buffer, to bring the pH to 8.5, and 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The increase in absorbance at 412 nm upon addition of this last reagent was used to calculate the concentration of reduced glutathione (Ellman's reaction) from which the concentration of NADP in the reaction mixture was calculated. The method is about 4-times more sensitive than direct estimation of NADPH at 340 nm, since the molar extinction coefficient of

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00 © 1984 Federation of European Biochemical Societies

reduced DTNB is 13600, and two molecules of reduced glutathione are formed per NADPH. The small absorbance of non-illuminated samples treated according to the same procedure was subtracted. The inactivation of thylakoid-bound FNR by its specific antibody was performed by incubation of the membranes (about 300 μg chlorophyll/ml) with the required amount of antibody, which was established by titration of the NADP photoreduction activity. The control thylakoids were similarly incubated for 5 min at room temperature without antibody. The antibody against FNR was prepared and its purity established as in [1,3]. The FNR bound to thylakoids was determined from the FAD content of the membranes and its NADPH diaphorase activity. FAD was extracted with HCl at room temperature for 3 min and estimated fluorimetrically in the neutralized extract (excitation at 450 nm, emission at 535 nm). The increase in fluorescence upon addition of phosphodiesterase was measured, and addition of known amounts of authentic FAD was used for calibration. FNR was purified, and its diaphorase activity was measured as in [4]. Fd was prepared as in [5].

3. RESULTS

Fd and FNR are known to form a 1:1 complex when mixed in solution [2], with a dissociation constant of 7×10^{-8} M, at pH 7, extrapolated to zero ionic strength [2], which increases with increasing ionic strength [2,6]. There is evidence that the formation of this complex is required for NADP photoreduction by isolated chloroplasts [7]. However, the differential spectroscopy method used to estimate the formation of the complex from the pure components in solution cannot be used with membrane-bound FNR because of the high absorbance of the membranes at the relevant wavelengths. We have therefore measured the formation of the complex between added soluble Fd and membrane-bound FNR by estimating the amount of Fd left in solution after incubation with thylakoids containing measured amounts of FNR, under controlled temperature and ionic strength. The equilibrium concentrations of FNR and of the complex were calculated from the measured equilibrium concentration of Fd and the measured initial concentrations of FNR and Fd, on the assumption that the complex formed has the same 1:1 composition as observed with the soluble components [2]. The dissociation constant, K_d, was calculated according to the formula

$$\frac{[\mathrm{Fd}]_{\mathrm{eq.}} \cdot [\mathrm{FNR}]_{\mathrm{eq.}}}{[\mathrm{FNR} \cdot \mathrm{Fd}]_{\mathrm{eq.}}} = K_d \qquad (1)$$

The results (table 1) indicate that the dissociation constant is 5.3×10^{-6} at an ionic strength of 0.046, and 9.65×10^{-6} when the ionic strength is raised to 0.092. Both the value of K_d and its

Table 1

Dissociation constant of the complex of ferredoxin and membrane-bound FNR in thylakoids

Exp. no.	Chlorophyll (mg/ml)	Ferredoxin		FNR		Fd–FNR complex at equilibrium[e]	K_d ($\times 10^{-6}$)
		Initial[a]	Final[b]	Initial[c]	Final[d]		
1	1.66	1.70	1.292	1.719	1.311	0.408	4.15
2	1.71	1.70	1.396	1.773	1.469	0.304	6.74
3	2.98	1.70	1.052	3.36	2.712	0.648	4.40
3	1.49	1.70	1.385	1.68	1.365	0.315	6.00
							mean 5.32
4	1.99	1.70	1.458	2.115	1.873	0.242	11.30
4	0.996	1.70	1.528	1.058	0.886	0.172	8.00
							mean 9.65

All concentrations are in μM. The medium was 15 mM Tricine buffer (pH 8), containing 2.5 mM $MgCl_2$ and 5 mM NaCl (ionic strength 0.046; in exp.4 the ionic strength was raised to 0.092 with NaCl). Temperature, 20°C. The data under a, b, and c are measured concentrations; d was calculated as c minus e; e was calculated as a minus b

dependence on the ionic strength are very close to those reported for the complex formation of the soluble, purified proteins [2,6]. The value of the dissociation constant is only slightly affected by temperature in the range 4–30°C (not shown), as reported for the pure proteins in solution [2].

One can therefore calculate the concentration of the FNR–Fd complex in a thylakoid suspension, provided the concentrations of FNR and Fd are estimated. We measured the rate of NADP photoreduction at different temperatures, under two conditions: (a) washed thylakoids, where FNR is bound to the membranes [1], and (b) thylakoids where the membrane-bound enzyme was titrated with the specific antibody and activity was restored by the addition of known concentrations of FNR in solution. The reaction was measured under conditions where it was first order with respect to Fd (Fd, 1 μM) and with respect to the FNR–Fd complex calculated as above. The first order rate constant, $k = v/[\text{FNR–Fd}]$, was measured as 8, 16, 24 and 30°C in the two conditions mentioned (FNR bound to the membranes or in solution, and thus the FNR–Fd complex). The values obtained were used to construct an Arrhenius plot of $\ln k$ *vs* $1/T$. The results are shown in fig.1 (average of 3 experiments, run in duplicate). It is shown that the straight lines obtained have the same slope, i.e., the same apparent activation energy, whereas the rate constants are more than 20-times higher in the native system, where FNR is membrane-bound, than in the reconstituted system, where FNR is soluble. The situation would be identical if the rate constants were calculated on the basis of the concentration of Fd rather than that of the complex. The Arrhenius apparent activation energy was 6.57 and 6.04 kcal/mol of NADPH in the control and reconstituted systems, respectively. It should be emphasized that in these experiments with uncoupled thylakoids the concentration of Fd, and therefore of the FNR–Fd complex was the rate-limiting factor, and therefore the observed activation energy is that dictated by the Fd–FNR-catalyzed NADP reduction step. Different (higher) values of the activation energy are observed when Fd is added in saturating concentrations, and some other step in the electron transport chain becomes rate-limiting.

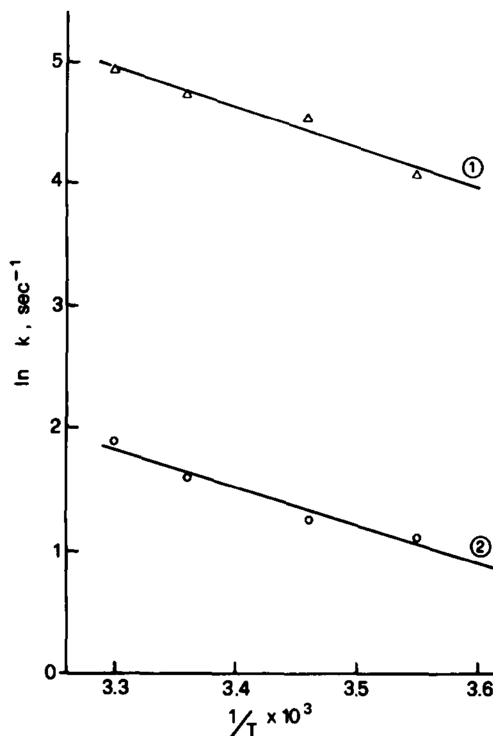

Fig.1. Arrhenius plot of NADP photoreduction by isolated thylakoids. (1) Control, (2) reconstituted system with FNR in solution. Conditions, see section 2. The reaction was performed in a cell jacketed with thermostated water, and the temperature was measured in the cell.

4. DISCUSSION

We demonstrate here that the complex between FNR and Fd forms with the thylakoid-bound FNR with the same dissociation constant previously reported when the two proteins are in solution [2,6]. The fact that the value of the constant of 5.3×10^{-6} (table 1) is almost identical to the value of 4.5×10^{-6} reported for the K_m for Fd [1,8] of NADP photoreduction under similar conditions of pH and ionic strength also provides independent evidence that the FNR–Fd complex is an intermediate in NADP reduction, as reported in [7] on the basis of the chemical modification of Fd experiments. The two constants also have the same dependence on ionic strength. Our results also show that the catalytic efficiency of NADP reduction by thylakoids is influenced at the terminal step

catalyzed by the Fd–FNR system by the binding of FNR to the membrane. The rate of the reaction is much higher when the enzyme is membrane-bound (as it is in nature) rather than in solution, even though the concentration of the FNR–Fd complex, an intermediate in the reaction [7], is the same in both conditions.

Catalysis may accelerate a process by influencing the enthalpic or the entropic factor, or both, of the energy of activation. If we assume that pressure and volume of our system are identical in the two conditions (when FNR is membrane-bound or in solution), i.e., if we assume that the pressure in the microenvironment at the membrane surface is not significantly different from that in the bulk solution, to a first approximation the rate constant depends on the free energy of activation according to the equation

$$k = \frac{k_B \cdot T}{h} \cdot e^{-\Delta G^*/RT} = \frac{k_B \cdot T}{h} \cdot e^{-\Delta H^*/RT} \cdot e^{\Delta S^*/R} \quad (2)$$

where ΔG^*, ΔH^* and ΔS^* are respectively the free energy, enthalpy, and entropy of activation, k_B Boltzmann's constant, and h Planck's constant. In the Arrhenius formulation, the energy of activation, E_a, is linked to the above formulation by the relation

$$\Delta H^* = E_a - RT \cong E_a \quad (3)$$

(if RT is small relative to E_a, as is usually the case). We can therefore take the estimation of E_a from an Arrhenius plot as an estimation of ΔH^* of eq.2. In our case, the value of ΔH^* is the same in the two conditions mentioned. The suggestion is therefore made that the large difference observed in the rate constants can be explained as due to a higher entropy of activation of the reaction when catalyzed by membrane-bound FNR. The terminal step of NADP reduction by thylakoids in natural conditions might then be an example of 'entropic catalysis', a concept recently discussed as regards the catalysis of protein synthesis by the elongation factors [9].

Our observations on the influence of binding to the membrane on the catalytic properties of FNR must have a counterpart in changes imposed on the conformation of the enzyme by its interaction with the membrane. Though these are not known in detail, it has been reported that conformational changes of FNR are caused by different factors affecting the membrane itself [10].

REFERENCES

[1] Forti, G., Cappelletti, A., Nobili, R.L., Garlaschi, F.M., Gerola, P.D. and Jennings, R.C. (1983) Arch. Biochem. Biophys. 221, 507–513.

[2] Foust, G.P., Mayhew, S.G. and Massey, V. (1969) J. Biol. Chem. 244, 964–970.

[3] Jennings, R.C., Garlaschi, F.M., Gerola, P.D. and Forti, G. (1979) Biochim. Biophys. Acta 546, 207–219.

[4] Forti, G. and Sturani, E. (1968) Eur. J. Biochem. 3, 461–472.

[5] Yasunobu, K.T. and Tanaka, M. (1980) Methods Enzymol. 69, 228–238.

[6] Batie, C.J. and Kamin, H. (1981) J. Biol. Chem. 256, 7756–7763.

[7] Davis, D.J. and San Pietro, A. (1977) Biochem. Biophys. Res. Commun. 74, 33–40.

[8] Carrillo, N., Lucero, H.A. and Vallejos, R.H. (1981) J. Biol. Chem. 256, 1058–1059.

[9] Chetverin, A.B. and Spirin, A.S. (1982) Biochim. Biophys. Acta 683, 153–179.

[10] Carillo, N. and Vallejos, R.H. (1983) Trends Biochem. Sci. 8, 52–56.