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Bioelectrochemistry and Bioenergetics 47 (1998) 179–183

BIOELECTROCHEMISTRY AND BIOENERGETICS

Short communication

Investigation of the diaphorase reaction of ferredoxin-NADP$^+$ reductase by electrochemical methods

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Received 30 July 1998; accepted 9 September 1998

Abstract

The enzyme ferredoxin-NADP$^+$ reductase (FNR) can be used in amperometric enzyme electrodes for NADP$^+$ detection. In order to evaluate the performance of such electrodes, a critical step is the comparison of the catalytic rate constants of immobilized FNR enzyme to the FNR in solution. In this work, electrochemical techniques such as cyclic voltammetry have been applied to investigate the catalysis of NADPH oxidation by ferredoxin-NADP$^+$ reductase using one-electron redox mediators such as ferrocenemethanol. The activity of the enzyme in solution mediated by ferrocenemethanol is comparable to other one-electron redox mediators previously measured.

Keywords: Ferredoxin-NADP$^+$ reductase; NADPH; Diaphorase activity; Cyclic voltammetry; Ferrocenemethanol; Gold electrode

1. Introduction

The enzyme Ferredoxin-NADP$^+$ reductase (FNR, DE 1.18.1.2) mediates the transfer of electrons from reduced ferredoxin to NADP$^+$ in the reductive site of photosynthesis [1]. This enzyme can also use NADPH as electron donor in a diaphorase activity with natural or artificial acceptors [2]. The enzyme isolated from the cyanobacterium *Anabaena* PCC 7119 contains a noncovalently bound FAD group that participates in the redox catalytic cycle of the enzyme. The three-dimensional structure of the enzyme from *Anabaena* has been determined to 1.8 Å resolution [3]. An amperometric enzyme electrode for NADP$^+$ based on FNR and viologen-modified glassy carbon electrode has been described. Glutaraldehyde cross-linking in the presence of bovine serum albumin was used for immobilization [4]. A critical evaluation of the performance of the electrode would require a comparison between the catalytic rate constants of the immobilized FNR enzyme to the FNR in solution. Furthermore, the enzyme FNR has been considered the prototype of a family of enzymes which includes others such as nitric oxide synthase, cytochrome P450 reductase, sulfite reductase, nitrate reductase, phthalate dioxygenase reductase, among others [5]. The results presented in this paper could be extended to those systems in which these enzymes participate.

Construction of electrodes using the enzyme FNR could find many useful applications since they could be used as biosensors for the detection of NADP$^+$ or NADPH present in biological mixtures. They could also be used for the electrochemical removal of NADP$^+$ being produced in the enzymatic reaction which could be taking place in a bioreactor. Finally, the enzyme FNR immobilized on an electrode could be used for the regeneration of NADPH to be used in reactions in which this cofactor is required as substrate.

For enzymes such as FNR the reaction proceeds by way of a series of binary complexes with an intermediate form of the enzyme carrying the hydride to be transferred. Such a system has been termed two-step transfer or ping-pong mechanism and guidelines for the determination of K_m for both substrates with soluble enzyme are well established [6]. However it is a formidable task to measure the K_m of the immobilized enzyme using the classical approach of enzyme kinetics based on the measurements of initial rates when varying the concentrations of the substrates. Besides, the experimental values of K_m thus obtained are complex function of several rate constants corresponding to different elementary steps [6]. Therefore suitable procedures for

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0302-4598/98/$ - see front matter
PII: S0302-4598(98)00175-5

a detailed kinetic analysis of this type of enzyme electrodes are needed.

In a very elegant work Bourdillon et al. [7] have recently described the utilization of cyclic voltammetry, a transient electrochemical technique, for the investigation of the catalysis of glucose oxidation by the soluble flavoprotein glucose oxidase. Moreover, the same group has demonstrated that cyclic voltammetry can also be efficiently used for the investigation of the glucose oxidase activity when immobilized onto an electrode [8]. These illuminating works open to us the possibility to investigate the effect of the immobilization procedure on the catalytic performance of the redox enzyme FNR. In the present work we present the kinetic analysis by electrochemical techniques of the oxidative reaction of NADPH with the oxidized form of ferrocenemethanol catalyzed by native FNR in solution.

2. Materials and methods

Freshly prepared solutions of ferrocenemethanol (Aldrich) and NADPH (Sigma) were used all along this work. Recombinant enzyme ferredoxin $NADP^+$ reductase from *Anabaena* PCC 7119 was purified as previously described [9].

Polycrystalline gold wires electrodes were cleaned, polished and their areas were determined as previously described [10]. All electrochemical experiments were carried out at 25°C in an electrochemical cell with an electrolyte volume of 1 ml. A platinum auxiliary electrode and an Ag/AgCl, 3 M NaCl, reference electrode connected to the cell by a salt bridge filled with the electrolyte solution were used, as well as a BAS analyzer, model CV-50 W. Supporting electrolyte was 50 mM phosphate buffer, KCl 150 mM, pH 7.5.

3. Results

Cyclic voltammetry was used to investigate the kinetic behaviour of the enzyme FNR in solution following the methodology developed by Bourdillon et al. [7] for the analysis of the catalytic parameters of oxidases. Here the diaphorase activity of FNR has been determined using NADPH as the reductive substrate and oxidized ferrocenemetanol, a one-electron exchanging mediator, (E_0 = 0.19 V), as the oxidized substrate. The catalytic process consists of the following sequence of reactions:

$$\mathrm{FNR} + \mathrm{NADPH} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} \mathrm{FNR\text{-}NADPH} \quad (1)$$

$$\mathrm{FNR\text{-}NADPH} \overset{k_2}{\rightarrow} \mathrm{FNRH_2} + \mathrm{NADP^+} \quad (2)$$

$$\mathrm{FNRH_2} + 2\mathrm{FM_{ox}} \overset{k_3}{\rightarrow} \mathrm{FNR} + 2\mathrm{FM_{red}} \quad (3)$$

The first step consists of the reversible binding of the enzyme to the substrate NADPH forming the transient complex FNR-NADPH (Eq. (1)) which allows the transfer of the hydride to the prosthetic group of the enzyme, the FAD (Eq. (2)). Finally the reduced enzyme transfers the electrons to the ferriciniummethanol (the oxidized form of the mediator, generated at the surface of the electrode during the anodic scan) of which two molecules are required to regenerate the initial oxidized form of FNR.

The aim of this work was the determination of the catalytic parameters (k_1, k_1, k_2, and k_3) of this reaction catalyzed by soluble FNR. Electrochemical techniques can be used for this purpose provided that the methodology developed by Saveant and cols. for the study of oxidases [7] is used. Accordingly, cyclic voltammograms of ferrocenemethanol are recorded before and after the addition of NADPH to the electrolyte solution containing a known amount of the enzyme FNR (Fig. 1). A noticeable increase of the anodic signal that reaches a plateau can be observed at the same time that the reduction peak disappears. The trace of the cathodic scan almost superimposes the anodic one indicating that the catalytic transformation of FM_{ox} is faster than the diffusion of FM to the electrode and that the consumption of NADPH in the diffusional layer is negligible.

Experiments in which the concentration of both substrates were modified while maintaining the pH constant were performed. The mediator concentration C_{FM} ranged from 1.25×10^{-6} to 10^{-4} M while that of NADPH, C_{NADPH} ranged from 5×10^{-4} to 5×10^{-3} M. The pH and enzyme concentration were maintained unchanged

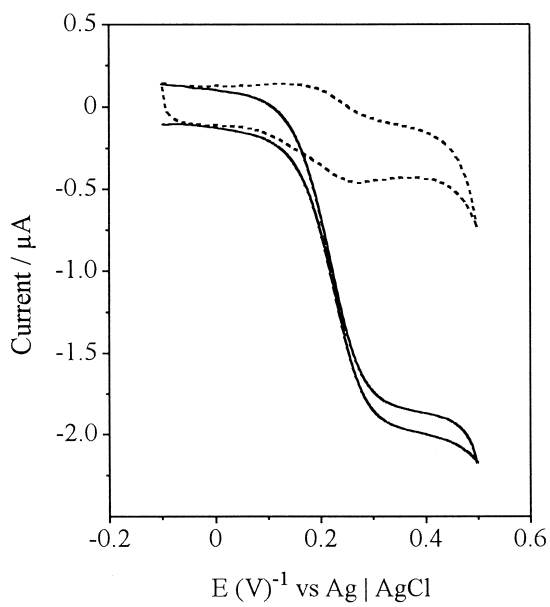

Fig. 1. Cyclic voltammograms of ferrocenemethanol in supporting electrolyte containing soluble Ferredoxin-$NADP^+$ reductase (10^{-7} M), before (solid line), and after addition of 1 mM NADPH to the solution (dashed line). FM was 10 μM; scan rate 0.05 V s^{-1}; temperature 25°C. Phosphate buffer 50 mM, KCl 0.15 M, pH 7.5.

throughout all the experiments being 7.5 the first, and $C_{FNR} = 10^{-7}$ M. Under these experimental conditions it was assured that the mediator concentration was always over 50-fold higher than that of the enzyme in order to ensure the applicability of the steady-sate approximation. Moreover, NADPH concentration is always at least 50-fold higher than that of the mediators to ensure that there was not significant change of the concentration of the reduced substrate on the surface of the electrode. Under these experimental conditions it has been established [7] that the time and space distribution of the oxidized form of the mediator ferrocenemethanol is described by the following equation:

$$\frac{\delta[FM_{ox}]}{\delta t} = D\frac{\delta^2[FM_{ox}]}{\delta x^2} - \frac{2k_3C^0_{FNR}[FM_{ox}]}{1 + 2k_3\left(\frac{1}{k_2} + \frac{k_{-1}+k_2}{k_1k_2[NADPH]}\right)[FM_{ox}]} \quad (4)$$

Eq. (4) holds in those conditions where the plateau current is observed and also when the catalysis is weak and a peak of current appears.

According to Bourdillon et al. [7], in these experimental conditions, the ratio between the catalytic current at the plateau, i_p, and the reversible wave peak current corresponding to the mediator in the absence on NADPH, i^0_p, is given by the dimensionless Eq. (5):

$$\frac{i_p}{i^0_p} = \frac{\lambda^{1/2}}{0.446}\left\{\frac{2}{\sigma}\left[1 - \frac{1}{\sigma}\ln(1+\sigma)\right]\right\}^{1/2} \quad (5)$$

Were λ and σ are, respectively:

$$\lambda = \frac{2k_3C^0_{FNR}}{v}\frac{RT}{F} \quad (6)$$

$$\sigma = \frac{k_3C^0_{FM}}{k_2}\left(1 + \frac{k_{-1}+k_2}{k_1C^0_{NADPH^+}}\right) \quad (7)$$

The relationships between the ratio i_p/i^0_p and the dimensional parameters λ and σ can be found in Fig. 2 of Ref. [7]. For each value of σ, the ratio i_p/i^0_p tends toward unity as $\lambda \rightarrow 0$ (see Fig. 2).

It must be considered that first-order conditions correspond to those in which $\sigma \rightarrow 0$. This can be experimentally achieved by lowering the concentration of ferrocenemethanol in solution until it reaches a value such that a further decrease in the concentration does not produce any change in the catalytic current. In our case to get into these limits it was necessary to decrease C_{FM} to values below 2.5 μM. For each concentration of mediator, experiments were performed at different scan rates, at fixed concentrations of enzyme and NADPH, and the experimental i_p/i^0_p values were plotted versus the ratio

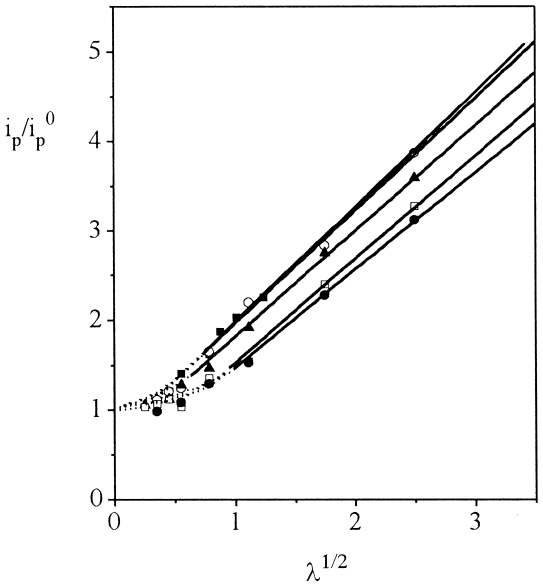

Fig. 2. Variation of the anodic currents of electrocatalytic NADPH oxidation by FNR and ferrocenemethanol with the scan rate. The concentration of ferrocenemethanol are (μM): 1,5 (■); 2,5 (○); 5 (▲); 10 (□) and 20 (●). The experimental data (points in the figure) were fitted to the working curves (solid lines) by adjusting the experimental abscissa axis $(((2C^0_{FNR})/v)((RT)/F))^{1/2}$ to the theoretical abscissa axis ($\lambda^{1/2}$) according to Eq. (6) in the text in order to obtain the value of k_3.

$(C^0_{FNR}/v)^{1/2}$, as is indicated in Fig. 2. Notice that, in order to fit the experimental values to the theoretical curves (Fig. 2 of Ref. [7]), a value of k_3 that best approaches the experimental $(C^0_{FNR}/v)^{1/2}$ to the corresponding theoretical $\lambda^{1/2}$ was chosen and used to establish the values of the experimental abscissa axis. The best fit was obtained with a value of 2.15×10^6 M^{-1} s^{-1} for k_3 (Table 1).

In order to determine the k_1, k_{-1} and k_2 values it was necessary to move away from the experimental conditions described above in which first-order kinetics are obtained. This can be achieved by increasing the mediator concentration and lowering that of NADPH (while keeping a ratio NADPH/FM above 50) since in these conditions the σ value increases, according to Eq. (7). These conditions were used in the determination of the kinetic constants of the reductive half-reaction. Similar plots to those of Fig. 2 were obtained when representing the i_p/i^0_p values versus $(C^0_{FNR}/v)^{1/2}$, obtained with NADPH concentrations in the range 2.0 to 5.0 mM while keeping the mediator concen-

Table 1
Characteristic rates constants of the enzyme Ferredoxin $NADP^+$ Reductase (FNR)[a]

k_3	$(2.15 \pm 0.14) \times 10^6$ M^{-1} s^{-1}
k_2	(332 ± 35) s^{-1}
k_{red}	$(1.63 \pm 0.17) \times 10^4$ M^{-1} s^{-1}

[a] Diaphorase reaction with the oxidized form of ferrocenemethanol as electron acceptor in 50 mM phosphate buffer, 150 mM KCl, pH 7.5, 25°C. Values are the average of 5 independent measurements.

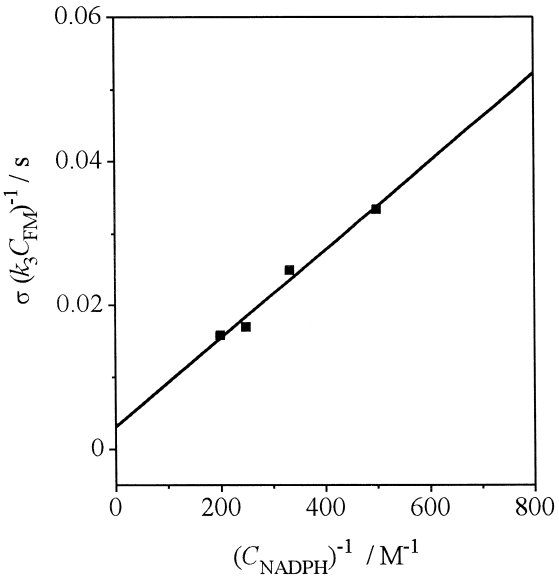

Fig. 3. Variation of the parameter $\sigma / k_3 C_{FM}$ with NADPH concentration. Other conditions as in Fig. 1.

tration C_{FM} at 10 μM (not shown). Again, the fitting of the experimental data values to the theoretical curves was obtained by adjusting the abscissa axis $(C^0_{FNR}/\upsilon)^{1/2}$, to the theoretical $\lambda^{1/2}$ axis (Eq. (6)) by using the previously determined k_3 value. New σ values were calculated for each set of results. The ensuing variation of the parameter $\sigma/k_3 C^0_{FM}$ with the inverse of the NADPH concentration is shown in Fig. 3. The intercepts with the vertical axis provide the value of $1/k_2$ and the slope gives that of $(k_{-1} + k_2)/k_1 k_2$, which is the inverse of k_{red}. The experimental values obtained with native FNR at pH 7.5 and ferrocenemethanol are summarized in Table 1.

The rate k_3 is, in fact, an overall rate constant for the oxidative half-reaction involving several elementary steps during which two electrons are exchanged between the reduced form of the enzyme and two molecules of oxidized ferrocenemethanol. A comparison of the value for k_3 obtained in this work and the k_{cat}/K_M measured for the single-electron reduction of several quinones by ferredoxin-$NADP^+$ reductase by colorimetric methods [11] shows that the value of k_3 here obtained is in the same order than the rate constants obtained for these quinone mediators. Bimolecular rate constants of reduction of these quinones range from 6.7×10^3 to 2.0×10^6 (M^{-1} s^{-1}) and are assigned in the mentioned paper to the oxidation of semiquinone form of FNR. It is thus comparable to the value of 2.15×10^6 (M^{-1} s^{-1}) for k_3 obtained here with ferrocenemethanol as mediator. Similarly, previous enzymatic assays of the diaphorase activity of ferredoxin-$NADP^+$ reductase with ferricyanide as electron acceptor [12] show a similar value of k_{cat}/K_M for the ferricyanide of 1.3×10^6 (M^{-1} s^{-1}). Considering the differences among the chemical and electrochemical characteristics of these electron acceptors, the almost identical values experimentally found of k_3 suggest that the electron transfer from NADPH to the flavin is the limiting step during the catalysis by FNR.

4. Discussion

Kinetics parameters of diaphorase activity of FNR here measured by electrochemical methods are comparable to those measured by conventional colorimetric methods with other single-electron mediators as described in Ref. [11]. This supports the proposal that electrochemical methods are appropriate for the determination of the kinetic parameters of flavoenzymes when free in solution. To this respect it is interesting to note that colorimetric methods do not allow to measure the reactivity of FNR with compounds which, like ferrocenemethanol, are isolated and are stable only in their reduced form; the oxidized active form of the substrate is being generated at the surface of the electrode during the anodic scan and its concentration can be precisely known at each value of the applied potential from the Nerst's equation [13].

Nevertheless, the main advantage of using electrochemical methods for the kinetics characterization of redox enzymes is that the same technique can be used for the investigation of the enzyme in the soluble form and when immobilized onto the electrode surface, under identical experimental conditions [8]. Rate constants for elementary steps of the oxidation of NADPH by the soluble Ferredoxin $NADP^+$ reductase with a monoelectronic electron acceptor have been measured, as well as the k_{red} constant which is related to the affinity of the enzyme for NADPH. The kinetics analysis of this enzyme immobilized onto solid electrodes is in progress. These results will allows us to ascertain the effect of the immobilization procedure onto the enzymatic activity and to evaluate the number of enzyme molecules that remain active after immobilization. This is being pursued in our laboratory in the research on well controlled methods for oriented immobilization of enzymes. A previous account of that has been recently published [10].

Acknowledgements

We thank Dr. A.L. De Lacey for his useful comments during the experimental work. This work was supported by collaborative grants BIO97-0912-C01 to CGM and C02 to VMF from the *Comision Interministerial de Ciencia y Tecnologia*, Spain.

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