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Structural requirements for the electron transfer between a flavoprotein and viologens

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Abstract

The covalent binding of the viologen N-methyl-N'(aminopropyl)-4,4'-bipyridinium (APMV) to the flavoprotein ferredoxin-NADP$^+$ reductase, a photosynthetic enzyme from the cyanobacterium *Anabaena* PCC 7119, facilitates the transfer of electrons between the FAD group in the enzyme and oxygen molecules present in the solution. This oxidase activity, which is completely absent in the native protein, indicates that there is an efficient electron transfer between the FAD group in the enzyme and the viologen molecule. This reaction is much more effective than that where the viologen is free in solution since, in the latter case, higher concentration of the carrier is required for similar oxidase activity. The computer graphics model of the FNR-viologen adduct indicates that the interaction between both redox groups could take place when two aromatic rings (one the FAD group in the protein and the other belonging to the bipyridinium core in the viologen) are in the same plane and also when the methyl groups at positions 7-α and 8-α in the flavin ring are pointing to the viologen molecule. These type of studies could give important information on the structural requirements for the electron transfer reaction between redox groups present in biological molecules.

Key words: Viologen; Cyanobacterium; Ferredoxin-NADP$^+$ reductase; Electron transfer; Chemical modification; Computer graphics

1. Introduction

Electron transfer reactions are essential for biological energy transformation processes such as photosynthesis, respiration, nitrogen fixation and other biosynthetic and degradative reactions. These reactions, involving either one or two electrons exchange, are remarkably fast and highly specific with respect to the proteins involved, although the electrons have to cover quite a large distance. It is generally assumed that proteins involved in electron transfer chains form tight complexes which are stabilized by several charged amino acid residues, while others could be directly involved in the electron transfer reaction. Recent reports have shown that one acidic amino acid residue and other aromatic one are essential for the intermolecular electron exchange reaction, since their replacement by another residue decreases the kinetic constant by approx. 50 000-fold [1].

The study of the parameters that control the transfer of electrons in protein-protein complexes involved in electron transfer chains is a difficult task, since the thermodynamical parameters that control the reaction are very much influenced by the interaction of the enzymic cofactors with the polypeptide chain and also by the structural features that are derived from the interaction between both proteins. On that score, it should be pointed out the change in the midpoint redox potential that is usually observed in enzymic cofactors when they are located in proteins, compared with that shown when they are free in solution [2]. The distance between the two cofactors exchanging elec-

Abbreviations: FNR, ferredoxin-NADP$^+$ reductase; EDC, *N*-ethyl-3-(3-dimethylaminopropyl) carbodiimide; MV, *N,N'*-dimethyl-4,4'-bipyridinium dichloride or methyl viologen; BV, *N,N'*-dibenzyl-4,4'-bipyridinium dichloride or benzyl viologen; PDQ, *N,N'*-trimethylene-2,2'-pyridinium dibromide; APMV, *N*-methyl-*N'*-(aminopropyl)-4,4'-bipyridinium dibromide; Fld, flavodoxin; cyt *c*, cytochrome *c*; SHE, standard hydrogen electrode.

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trons and also the geometry of the interaction are still a subject of speculation, since the information concerning the structure of electron exchanging complexes is known only in very specific cases [3,4]. Moreover, that information obtained from the crystal structure of the proteins forming a complex does not necessarily implies that it is the one present during turnover. For this reason, it seems adequate to study simpler electron transfer reactions than those involving protein–protein interactions. That is the case of the reaction between the flavoprotein ferredoxin-$NADP^+$ reductase (FNR; EC 1.18.1.2) from the cyanobacterium *Anabaena* PCC 7119 and some derivatives of 4,4′-, and 2,2′-bipyridine (viologens). The enzyme FNR participates in the reductive side of photosynthesis, mediating the transfer of electrons from reduced ferredoxin to $NADP^+$ [5]. There is a FAD group non-covalently bound to the protein which shows a midpoint redox-potential of −344 mV (NHE, pH 7) [6]. The three dimensional structure of the enzyme from *Anabaena* PCC 7119 has been determined at 1.8 Å resolution [7].

Viologens are commonly used as mediators in enzymic reactions to improve the accessibility of the prosthetic group of the enzyme by the substrate [8,9]. They have also been found to be very useful as mediators to establish the equilibrium between an electrode and the enzyme in electrochemical enzymatic processes [10–12]. The usefulness of viologens in these reactions is based on their small size which allows them to diffuse into the protein structure for effective interaction with the cofactor. They are also highly soluble in aqueous solution and operate at very low (negative) redox potentials, similar to those of redox proteins having flavin and iron-sulphur groups [13].

In the present paper, the steady-state kinetic constants for the electron exchange reaction between the enzyme FNR and viologens free in solution are compared to that obtained when the viologen is covalently bound to the protein structure. In the last case, important changes in the oxidase activity of the protein have been observed. On this subject, there is little information concerning the covalent introduction of viologen groups into proteins to act as artificial electron transfer centers, and no change in the catalytical specifity of the enzyme after modification has been reported [14].

Modelling by computer graphics of the modified enzyme permits some conclusions to be drawn concerning the geometry of the interaction between the two groups exchanging electrons.

2. Materials and methods

2.1. Materials

Ferredoxin-$NADP^+$ reductase was purified from *Anabaena* PCC 7119 as described previously [15], from cells that were grown autotrophically on nitrate. An extinction coefficient of 9.4 $mM^{-1}\,cm^{-1}$ at 459 nm was used to calculate the concentration of FNR. *N,N′*-Dibenzyl-4,4′-bipyridinium dichloride (BV) (Sigma), *N,N′*-dimethyl-4,4′-bipyridinium dichloride (MV) (Serva) and *N,N′*-trimethylene-2,2′-pyridinium dibromide (PDQ) (ICI) were used as received. *N*-Methyl-*N′*-(aminopropyl)-4,4′-bipyridinium dibromide (APMV) was a gift from A.L. de Lacey. All other reagents were purchased from commercial sources and were of analytical grade.

2.2. Enzymic assays

The FNR-dependent NADPH cytochrome *c* reductase (EC 1.6.99.3) activity was assayed as described [16], using flavodoxin instead of ferredoxin. Oxidase activity of native and modified enzyme was measured by the decrease in the absorbance at 340 nm (maximal absorbance for NADPH), using 0.25 mM NADPH and 0.3 μM native or modified FNR in 50 mM Tris-HCl buffer, at pH 8. When native FNR was assayed different amounts of oxidized viologens (between 0 and 7 mM) were used.

All the activities were performed in a Hewlett-Packard diode array spectrophotometer held at 25°C.

2.3. Anaerobic measurements of cytochrome c reductase activity

Experiments were carried out in a 1 ml necked cuvette equipped with a side arm. Anaerobic conditions were established by repeated cycles of evacuation and flushing with argon, which had been purified by passage over a heated BASF catalyst. Reaction mixtures contained, in a final volume of 1 ml, 35 nM FNR, 0.2 mM NADPH, 0.75 mgr cytochrome *c* and 19 μM flavodoxin from *Anabaena* PCC 7119 in Tris-HCl buffer (pH 8). Anaerobic conditions were established previous to the addition of $NADP^+$ and FNR. Reaction was started by adding the enzyme through a rubber septum.

2.4. Chemical modification of FNR

FNR (35 μM) was incubated, in the dark, in a reaction mixture containing 10 mM Pipes buffer, pH 6.0, 39 mM APMV and 2 mM EDC at 25°C for 90 min. After that, removal of excess reagents was carried out by washing with 25 mM phosphate buffer at pH 7 in an Amicon 10 ml ultrafitration cell provided with a YM 10 membrane. Disappearance of absorbance at 260 nm (absorbance maximum for oxidized APMV) indicated that free viologen was eliminated. No stopping buffer was used in the reaction to avoid its deleterious effect on FNR activity.

3. Results

The critical step in the mechanism by which a reduced flavoprotein can efficiently reduce dioxygen is the formation of the covalent flavin 4α-hydroperoxide. Steric restrictions or the presence of substrates can affect the formation or the stability of this species, and subsequently determine the ability of a flavoprotein to act as an oxidase [17]. Electron exchange between the substrate NADPH and molecular oxygen is not observed when native FNR from *Anabaena* PCC 7119 is assayed. However, if a small molecule, such as a viologen, is acting as electron carrier between the enzyme and the oxygen molecule, then very efficient electron transport can be measured. Fig. 1 shows the effect of the viologen concentration on the rate of NADPH oxidation by FNR assayed in the presence of several viologen molecules and aerobic conditions. Different rates are obtained depending on the structure of the viologen used. These viologens are PDQ (E'_0 −550 mV (SHE), electric charge of 2+ in the oxidized state), BV (E'_0 −359 mV (SHE), electric charge 2+ in the oxidized state), MV (E'_0 −440 mV (SHE), electric charge 2+ in the oxidized state) and APMV (E'_0 −430 mV (SHE), electric charge 3+ in the oxidized state). A more extensive study has revealed that the main parameter controlling the rate of electron transfer is the difference in redox-potential between the electron donor and acceptor, the number of charges present in the carrier molecule having a modulating effect (Bes et al., unpublished data). The information in Fig. 1 could be interpreted in the sense that the rate of electron exchange between the enzyme FNR and oxygen is regulated by the interaction between the FAD group in the enzyme and the viologen used as the electron

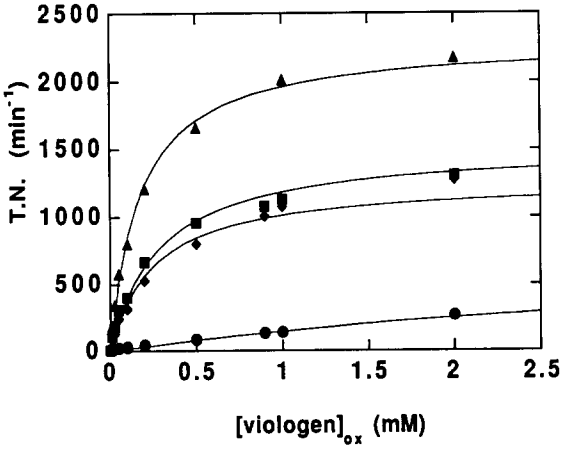

Fig. 1. Aerobic oxidation of NADPH by FNR in the presence of several viologens. Measurements were performed in a cuvette containing 0.25 mM NADPH, 0.3 μM FNR and different amounts of oxidized viologens in 50 mM Tris-HCl (pH 8.0). The disappearance of NADPH was followed by the change in absorbance at 340 nm. (▲) BV. (■) APMV. (♦) MV. (●) PDQ.

Table 1
NADPH-cytochrome *c* reductase and oxidase activities of ferredoxin-NADP$^+$ reductase and the reductase-APMV covalent adduct

Assay	Sample	Turnover number (min^{-1})	
		Aerobic	Anaerobic
NADPH → FNR → cyt *c*	FNR	40	17
	FNR + Fld	199	125
	[FNR-APMV] [a]	1128	49
	[FNR-APMV] + Fld	1124	229
NADPH → FNR → O_2	FNR	7	
	FNR + APMV	90	
	[FNR-APMV]	604	

The cytochrome *c* reductase activity assay mixture contained 0.75 mg/ml cytochrome *c* and 0.2 mM NADPH in 50 mM Tris-HCl (pH 8.0). Concentration for FNR and the adduct was 35 nM. Flavodoxin was 19 μM. The oxidase activity assay mixture contained 0.26 mM NADPH in 50 mM Tris-HCl (pH 8.0). FNR and [FNR-APMV] concentration was 0.35 μM. APMV was 12 μM.
[a] [FNR-APMV] is equivalent to FNR-APMV adduct.

carrier, this interaction being more effective as the redox potential of the carrier molecule becomes less negative.

The viologen APMV has a positive charge in the amino group located at the end of the propyl chain. In the presence of the carbodiimide EDC a covalent cross-linkage between carboxylic groups in the polypeptide chain of the protein and this amino group can be formed, producing the covalent adduct FNR-APMV. The presence of the viologen group covalently bound to the protein is demonstrated by the change in the isoelectric point of the protein and also by photochemical reduction of the adduct under anaerobic conditions (not shown). A stoichiometry of 0.5 mol of viologen/mol of enzyme is observed. This fact could indicate that the number of modified residues is limited and that the chemical modification does not affect all the protein molecules.

The modified enzyme shows now a quite high oxidase activity, in the absence of external carrier molecules, indicating that the covalently bound viologen has free access to the FAD group in the enzyme and also to the oxygen in the solution. Table 1 shows the comparison of the turnover number for the oxidase reaction of the native FNR in the presence of 12 μM APMV (90 min^{-1}) with that of the modified protein, where APMV is only 0.15 μM (604 min^{-1}). The result indicates that the effective concentration of the viologen accessible to the enzyme when it is immobilized in the polypeptide chain increases dramatically when it is covalently bound to the enzyme. Table 1 also shows that the modified enzyme presents a NADPH-cytochrome *c* activity which is not dependent on the presence of flavodoxin, since it exhibits maximal activity in the presence or absence of this electron transfer pro-

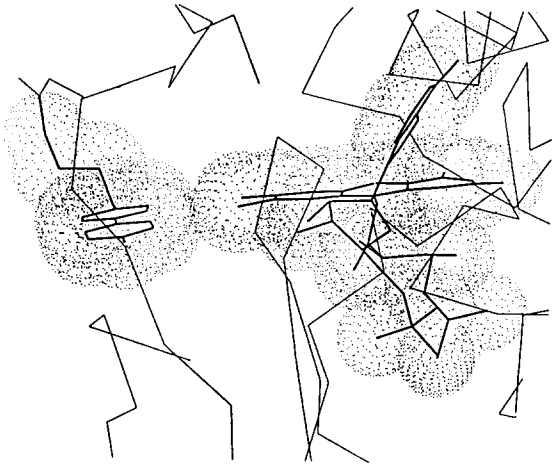

Fig. 2. Computer graphics model showing the relative positions of the residue Glu-139 in FNR and the covalent attached viologen. The *Anabaena* PCC 7119 FNR model was obtained using the coordinates provided by Dr. M. Frey (CNRS, Grenoble, France) which were used for the determination of the FNR structure described in reference [7]. The FAD and the viologen are displayed in thick lines.

tein. This is not the case for the native enzyme, which requires the presence of ferredoxin or flavodoxin (electron carrier protein synthesized by the cyanobacterium when it grows under low levels of iron) to show any cytochrome *c* reductase activity. The involvement of the oxygen in this reaction is supported by the difference in turnover numbers measured in the absence of oxygen in the reaction mixture (Table 1).

All these data indicate that an effective interaction between the FAD present in FNR and external viologen molecules occurs for the electron transfer reaction. This interaction is facilitated when the viologen molecule is covalently bound to a residue of the protein chain so that the access to the flavin group is more effective, requiring lower concentration of the electron carrier. The chemically modified molecule could be used, for this reason, as a model for the study of the structural requirements for effective electron transfer between redox groups in biological molecules. Since the three-dimensional structure of FNR from *Anabaena* has been determined at high resolution [7] the identification of the amino acid residue(s) where the viologen group is bound to the enzyme molecule could be attempted. The procedure followed for this study will be reported elsewhere (Bes et al., unpublished data) and Glu-139 was considered the most likely residue at which the viologen was bound. The position of this residue in the FNR structure indicates that it is highly exposed to the solvent and without any steric hindrance for accessing to the FAD group. The insertion of the APMV molecule in this residue with the help of the computer graphic model indicates that the Van der Waal's spheres of the viologen and FAD groups are interacting. Rotation of the molecule around the different chemical bonds in the molecule allows many different relative positions of the two redox groups (the FAD from the protein and the bipyridinium core in the viologen). Among them, the one in which the two aromatic groups are located in the same plane seems as the most favourable, since it has been reported that this is the case for many different redox structures of electron exchanging proteins [17]. Moreover, the flavin portion which is exposed to the viologen molecule is proposed to be that where the two methyl groups are at positions 7-α and 8-α. This side of the FAD molecule is the one usually exposed to the solvent and has also been proposed previously to be the site for electron exchange between flavodoxin and other electron transfer proteins [18].

We can conclude that the covalent binding of a viologen analog to the enzyme FNR produces an enzymatic species where intramolecular electron exchange takes place and where certain structural aspects of the electron transfer reaction could be studied. Forthcoming experiments in our laboratory will allow to introduce the viologen molecule in other specific amino acid residues where other geometries are observed. This will require the production of site-directed mutants to specifically bind the viologen molecule.

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