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Bioelectrochemistry 63 (2004) 61–65
Bioelectrochemistry
www.elsevier.com/locate/bioelechem

Analysis of the interaction of a hybrid system consisting of bovine adrenodoxin reductase and flavodoxin from the cyanobacterium *Anabaena* PCC 7119

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Received 23 June 2003; received in revised form 1 October 2003; accepted 10 October 2003

Abstract

The mitochondrial steroid-hydroxylating system in vertebrates and the NADPH producing electron transfer chain in photosynthetic organisms contain structurally and functionally similar components. Examination of a potential hybrid reconstitution of the electron transfer chain between different components of both systems could help to improve our knowledge on protein–protein interaction and subsequent electron transfer. Here we analyzed the interaction between bovine adrenodoxin reductase and flavodoxin from the cyanobacterium *Anabaena* PCC 7119. Optical biosensor as well as steady state and fast kinetic experiments showed their ability to form distinct productive complexes. Compared with the corresponding physiological systems the electron transfer is rather slow, probably due to the lack of specificity at the interaction surface.

Keywords: *Anabaena*; Bovine adrenodoxin reductase; Flavodoxin

1. Introduction

In the adrenal steroid-hydroxylating system adrenodoxin reductase (AdR), a NADPH-dependent FAD-containing enzyme and adrenodoxin (Adx), a [2Fe–2S] vertebrate-type ferredoxin function as electron carrier proteins to the mitochondrial cytochrome *P*450 [1]. The binding between AdR (50.3 kDa) and Adx (14 kDa) is mainly based on electrostatic interactions. AdR displays a highly asymmetric charge distribution rendering a cleft between the FAD and NADPH domains almost completely surrounded by basic residues [2]. This basic cleft of AdR is considered to be the main binding domain of the highly negatively charged Adx [3]. Site-directed mutagenesis studies and the recently solved crystal structure of a 1:1 Adx/AdR complex have provided a detailed insight into the electrostatic binding mechanism between these proteins pointing to essential residues in the basic cleft region of AdR (Arg 211, Arg 240 and Arg 244) [4].

From the functional point of view, AdR is closely related to the ferredoxin-$NADP^+$-reductase (FNR) of plants and cyanobacteria. Both enzymes contain a FAD cofactor, are NADPH-dependent, and use a [2Fe–2S] ferredoxin as single-electron carrier. In algae and cyanobacteria flavodoxin (Fld), a low-potential FMN-containing flavoprotein (~ 20 kDa) functionally replaces the plant-type ferredoxin (Fd) under iron-deficient conditions [5]. Like the Adx/AdR complex, the complex formed between FNR and Fld displays a 1:1 stoichiometry, presumably with a participation of electrostatic interactions [5,6]. Analysis of the Fld structure clearly shows the dipole character of the protein with the negative end located around the FMN cofactor [7]. Studies on the interaction between bovine Adx and FNR from the cyanobacterium *Anabaena* PCC 7119 showed that both proteins are able to form complexes and exchange electrons, suggesting that other proteins of these different systems might also be exchangeable [8].

Despite the low homology between Fld and Fd both electron carrier proteins are proposed to bind to the same interaction surface on the reductase [6], suggesting the

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doi:10.1016/j.bioelechem.2003.10.014

existence of a general recognition pattern which might be also common in several other electron transfer (ET) proteins such as Adx and AdR. In the present work we analyzed the interaction and ET between bovine AdR and Fld from the cyanobacterium *Anabaena* PCC 7119. Analysis of this hybrid system could render new insights into the nature of protein–protein interactions as well as electron transfer reactions between two flavoproteins.

2. Experimental procedures

All samples were prepared in a 10 mM potassium phosphate buffer, pH 7.4.

2.1. Biological material

Fld from *Anabaena* sp. PCC 7119 was overexpressed in *Escherichia coli* and purified as described previously [9]. Bovine AdR was expressed and purified as described elsewhere [10].

2.2. Cross-linking experiments

Cross-linking reactions were performed at 25 °C using 30 μM of each protein and 2 mM 1-Ethyl-3-(3-dimethylamino propyl) carbodiimide (EDC). The reaction was stopped after 1 h by Laemmli-buffer addition, [11], followed by a separation of all proteins on a SDS-polyacrylamide gel.

2.3. Optical biosensor measurements

Analysis of the binding behavior between AdR and Fld was accomplished by using a BIAcore2000 system. Throughout the measurement a continuous flow of BIAcore HEPES buffer including salt (HBS-EP) was maintained over the sensor surface. Immobilization of either Fld or Adx to a CM5 sensor chip was performed according to previously described principles [12,13]. About 400 RU (resonance units) of either Fld or Adx were coupled via free amino groups to the activated carboxyl groups by injecting a 30 μM solution of the respective protein until that value was reached. After blocking different analyte concentrations in the range from 500 nM to 5 μM were injected and the refractive index was measured. Determination of K_d values was achieved by using the BIAcore evaluation software 3.1.

2.4. Steady state and fast kinetic measurements

The reaction between NADPH-reduced AdR (4 μM) and different Fld_{ox} concentrations in the range from 1 to 60 μM was followed under steady state (Shimadzu MultiSpec-1501 spectrophotometer) and anaerobic conditions (N_2/H_2 (95%:5%) atmosphere). The continuous reduction of AdR was achieved by addition of NADPH to a final concentration of 200 μM ($k_{obs,max} \sim 13\ s^{-1}$, unpublished data). The reduction of Fld was followed at 465 nm in periods of 1 s for 1200 s at 20 °C. The observed kinetic traces fitted to a monoexponential reaction process providing observed rate constants (k_{obs}), which were plotted against the corresponding Fld concentration (Fig. 2). The reaction between 5-deazariboflavin photoreduced Fld [6] and AdR_{ox} was followed at 465 nm and 20 °C under pre-steady state conditions as described previously [14].

3. Results and discussion

The study of hybrid systems, consisting of proteins involved in similar ET chains, such as the reductase of the mammalian mitochondrial steroid-hydroxylating system, AdR, and an electron carrier protein from the photosynthetic ET chain of photosystem I in the cyanobacterium *Anabaena*, Fld, can be a suitable tool for the improvement of our knowledge on the molecular bases of protein–protein interactions and ET.

Analysis of cross-linked complexes (Fig. 1A) between AdR and Fld showed that both proteins are able to form complexes with higher stoichiometries than 1:1, suggesting different interaction modes between these proteins. Chemical modification and site-directed mutagenesis studies have implicated two negatively charged regions in Fld important for proper complex formation with FNR and for efficient ET [15–17]. Each of these regions contains a group of three carboxylate residues (Asp123, Asp126, Asp129 and Asp144, Glu145, Asp146) [15] and could be involved in complex formation with AdR. Nevertheless, a 2:1 complex formation between AdR_{ox} and Fld_{ox} seems to be sterically and thermodynamically unfavored. The association rate (k_{on} value) for such a complex, obtained from optical biosensor experiments, was extremely slow ($4.9 \times 10^{-6}\ M^{-1}\ s^{-1}$), indicating an unfavored 2:1 complex formation. The K_d value for a 1:1 AdR_{ox}/Fld_{ox} interaction (Fig. 1B) was 26- and 42-fold increased compared to the reported K_d values for the corresponding physiological systems (Table 1). Taken together, this data indicates that the interaction between AdR and Fld is not as specific as in the corresponding physiological systems.

Kinetic analysis of the reaction between AdR_{red} and Fld_{ox} (Fig. 2A and B) rendered reproducible k_{obs} values, which were considerably slower than those reported under similar conditions for the reduction of Adx by AdR_{red} (~ 250-fold) [13] and Fld by FNR_{red} (~ 60-fold) [18,19] (Table 1). Since the reduction of AdR by NADPH is ~ 4000 times faster, this reaction will not influence the reduction of Fld_{ox} by AdR_{red}. Upon increasing the Fld/AdR concentration ratio, the k_{obs} value for this monoexponential reaction decreased ($V_{i,max} = 0.046\ s^{-1}$; $K_{i,obs} = 1.34$ μM, see Fig. 2B), probably due to a Fld concentration-dependent inhibition. Taking all present data into account, the rate-limiting step for this interaction seems to be the formation of a productive complex in which ET is possible. Since

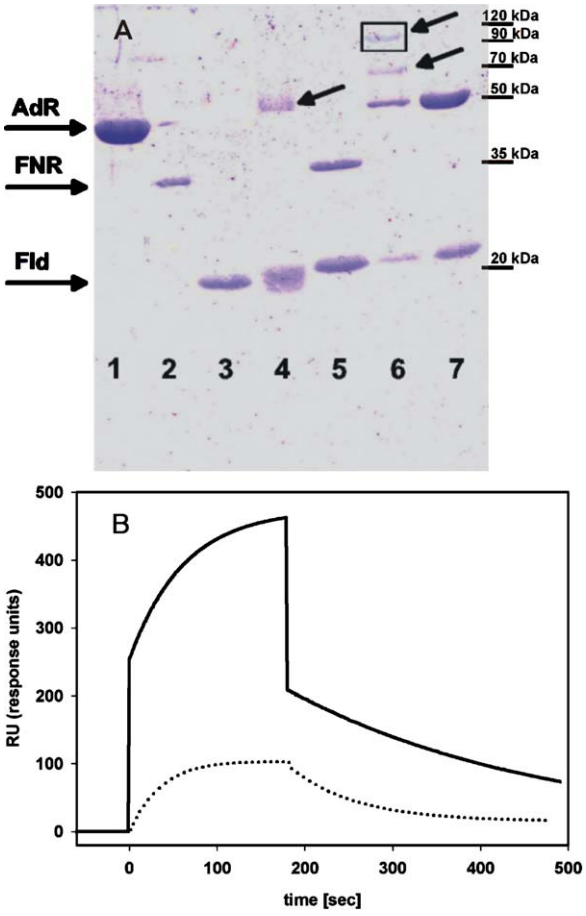
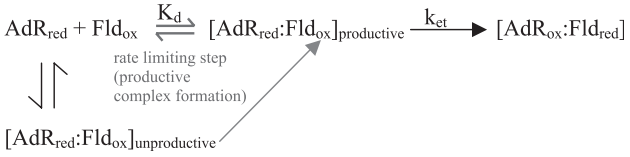


Fig. 1. Analysis of the interaction between AdR_{ox} and Fld_{ox}. (A) SDS/PAGE of the complexes after EDC treatment. (1) AdR; (2) FNR; (3) Fld; cross-linking between Fld and both FNR (4) and AdR (6); mixture of Fld and FNR (5) or AdR (7) without EDC. Complexes are indicated by an arrow. The higher AdR/Fld complex band has been electronically enforced and framed. (B) Binding kinetics (BIAcore2000 system) for the interaction of 1 μM AdR_{ox} and both Fld_{ox} (dotted line) or Adx_{ox} (solid line). K_d values were determined by analyzing different AdR concentrations (500 nM–5 μM) after immobilization of ~400 RU Adx_{ox} or Fld_{ox}.

complex formation between AdR_{ox} and Fld_{ox} takes place within a similar time scale as the Fld reduction, the observed slow k_{obs} values could mainly depend on the slow formation of a productive complex. Therefore we propose the following reaction mechanism:



Kinetic analysis of the reverse reaction rendered k_{obs} values with very high standard deviations (data not shown), suggesting the formation of different productive complexes that lead to the reduction of AdR. The k_{obs} value obtained for the most productive complex in this orientation is considerably slower (~3000-fold) compared with the value reported for the corresponding physiological system (Table 1) [16].

Thermodynamically, the reaction between Fld_{red} (−436 mV) [20] and AdR_{ox} (−295 mV) [1] is favored over the opposite direction (Fld_{ox}: −212 mV [20]; AdR_{red}: −295 mV). This could explain our observation that the reaction between Fld_{red} and AdR_{ox} is faster compared with the reverse reaction.

Compared to the recently published analysis of a hybrid system consisting of FNR and wild type Adx (WT-Adx) [8], the results obtained here show that the interaction between Fld and AdR is even less specific (~1.7-fold increased K_d value; see Table 1). In contrast to this, the reduction rate between FNR_{red} and Adx_{ox} was 10-fold decreased compared to the reduction of Fld by AdR. Moreover, here we were able to detect a reaction between the reduced electron carrier and the reductase (Fld_{red} + AdR_{ox}), whereas a reaction between reduced Adx and FNR was not detectable (see Table 1). These findings show that even if the complex formation between AdR and Fld is unfavored over the FNR/Adx complex formation electron transfer in this AdR/Fld system is significantly faster, probably due to a close distance between both prosthetic groups in the formed complex, which could allow a faster electron transfer.

Concluding, our results indicate that AdR and Fld are able to form productive interactions which lead to a subsequent ET. However, the observed low interaction and binding specificity between these proteins suggests that mainly weak electrostatic interactions must be involved in the complex formation. The fact that ET is achieved in the AdR/Fld system supports the idea that the interaction between each reductase and the ET protein cannot only take place through a highly specific complementarity of the

Table 1
Thermodynamic and kinetic parameters for the AdR-Fld interaction

Reductase/electron carrier system	K_d[a] (μM)	k_{obs} (s^{-1})	k_{obs} (s^{-1})
AdR/Fld	AdR_{ox}/Fld_{ox}[b] 41.8	$AdR_{red} + Fld_{ox}$[c] 0.03 ± 0.008	$Fld_{red} + AdR_{ox}$[d] 0.6–1.8
FNR/Fld	FNR_{ox}/Fld_{ox} 1.6	$FNR_{red} + Fld_{ox}$[e] 1.8	$Fld_{red} + FNR_{ox}$[f] 5300 ± 200
AdR/Adx	AdR_{ox}/Adx_{ox} 1.0	$AdR_{red} + Adx_{ox}$[g] 7.3	$Adx_{red} + AdR_{ox}$ no reaction
FNR/Adx	FNR_{ox}/Adx_{ox}[h] 25	$FNR_{red} + Adx_{ox}$[h] 0.003	$Adx_{red} + FNR_{ox}$[h] not detectable

[a] Standard deviation for all shown K_d values is ± 40%.
[b] Dissociation constant of a 1:1 complex.
[c] Maximal determined k_{obs} value.
[d] Averaged k_{obs} value determined from Fld_{red}/AdR_{ox} (ratio of 1:4 and 10:1). The standard deviation is ± 90%.
[e] [19].
[f] [16].
[g] [13].
[h] [8].

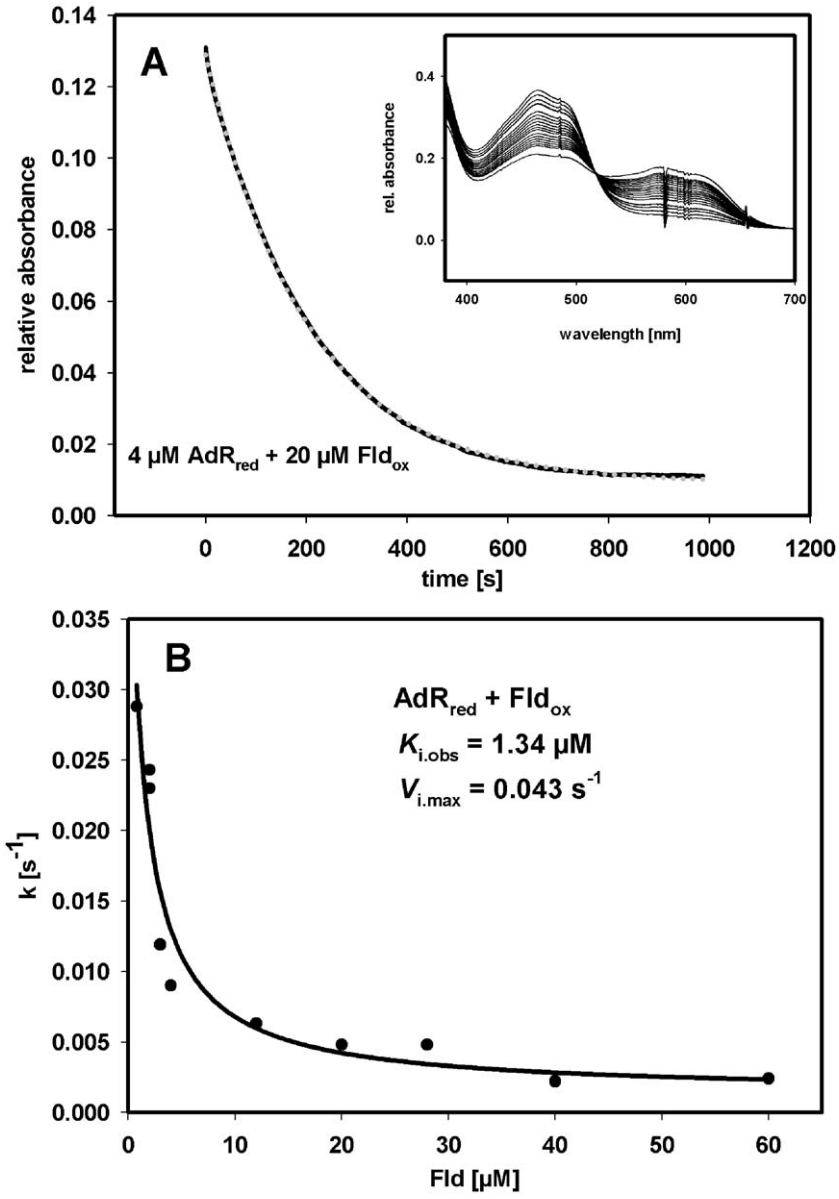


Fig. 2. Steady state kinetical analysis (AdR_{red} + Fld_{ox}). (A) Typical time trace and the corresponding monoexponentiell fit (dotted) for the reduction of Fld_{ox} by AdR_{red} followed at 465 nm. The insert shows the spectral changes observed for the anaerobic reaction between AdR_{red} and Fld_{ox}. (B) The Fld concentration dependence of the k_{obs} values was calculated from transients at 465 nm by fitting them monoexponentially, as shown in Fig. 2A. The $K_{i,obs}$ and $V_{i,max}$ values for the inhibition reaction were determined by fitting the k_{obs} vs. Fld concentration plot hyperbolically. $V_{i,max}$ represents the maximal velocity of this Fld concentration dependent inhibition whereas $K_{i,max}$ corresponds to the Fld concentration leading to $V_{i,max} \times 0.5$.

protein surfaces, but seems to proceed also as a consequence of various weak interactions.

Acknowledgement

Volkswagen Stiftung for financial support.

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