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A negative cooperativity between NADPH and adrenodoxin on binding to NADPH:adrenodoxin reductase

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Adrenodoxin stimulated the oxidation of NADPH by 1,4-benzoquinone, catalyzed by NADPH:adrenodoxin reductase. It prevented the enzyme inhibition by NADPH and formed an additional pathway of benzoquinone reduction presumably via reduced adrenodoxin. In the presence of 100–400 μM $NADP^+$, which increased the K_m of NADPH, adrenodoxin acted as a partial competitive inhibitor for NADPH decreasing its TN/K_m by a limiting factor of 3. K_i of adrenodoxin decreased on the $NADP^+$ concentration decrease and was estimated to be about 10^{-8} M in the absence of $NADP^+$.

NADPH:adrenodoxin reductase; Adrenodoxin; Kinetics, steady-state; Substrate binding

1. INTRODUCTION

Flavoprotein NADPH:adrenodoxin reductase (E.C. 1.18.1.2) functions in the mitochondrial electron transport chain for cytochrome P-450-dependent steroid hydroxylation. It donates the electrons to Fe_2S_2-protein adrenodoxin which further transfers them to cytochromes $P\text{-}450_{scc}$ or $P\text{-}450_{11\beta}$ [1]. Adrenodoxin reductase (ADR) and adrenodoxin (ADX) form a stable stoichiometric complex, the stability of which depends on the ionic strength, medium composition and oxidation state of proteins [2–5]. Cysteine, histidine and arginine residues are essential for NADP(H) binding by ADR [6,7], and a lysine residue is located at the ADX-binding site [8]. It is assumed that the reduced ADR-$NADP^+$ complex is the form of the enzyme transferring an electron to ADX [5]. The evidence available indicates that the ADX-binding domain is distinct from the pyridine nucleotide binding site [1].

The formation of a ternary $NADP^+$-flavoprotein-ferredoxin complex was demonstrated for ferredoxin:$NADP^+$ reductase [9,10], which possesses a catalytic mechanism similar to that of ADR. However, a negative cooperativity between NADP(H) and ferredoxin binding was assumed on the basis of kinetic and difference spectroscopy data [9,10].

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Abbreviations: ADX, adrenodoxin; ADR, NADPH:adrenodoxin reductase; BQ, 1,4-benzoquinone; *TN*, turnover number; TN/K_m, bimolecular rate constant

In this paper we present the evidence from the steady-state kinetic data on the existence of the negative cooperativity between NADPH and ADX binding to NADPH:adrenodoxin reductase.

2. MATERIALS AND METHODS

Adrenodoxin ($A_{414}/A_{280} = 0.83$) and adrenodoxin reductase ($A_{271}/A_{450} = 7.7$) were prepared from bovine adrenal mitochondria [11]. The concentrations of ADX and ADR were determined spectrophotometrically with the use of $\epsilon_{414} = 10\ mM^{-1}cm^{-1}$ and $\epsilon_{450} = 11\ mM^{-1}cm^{-1}$, respectively. NADPH, $NADP^+$, cytochrome *c* (Serva) were used without further purification. 1,4-Benzoquinone (Reakhim, USSR) was purified by sublimation in vacuum. The reaction rate was determined spectrophotometrically using a Hitachi-557 spectrophotometer at 25 ± 0.1°C to the NADPH absorption decrease ($\Delta\epsilon_{340} = 6.2\ mM^{-1}cm^{-1}$). The rate of nonenzymatic NADPH oxidation by BQ never exceeded 3% of the total reaction rate and was subtracted from it. The ADX-linked reduction of cytochrome *c* was monitored using $\Delta\epsilon_{550} = 20\ mM^{-1}cm^{-1}$. Cytochrome *c* concentration was 40 μM. 0.1 M K-phosphate solution (pH 7.0) containing 1 mM EDTA was used. *TN*, with an exception of cytochrome *c* reduction, corresponds to a number of NADPH consumed by FAD per 1 s.

3. RESULTS AND DISCUSSION

The data on the initial rates of ADR-catalyzed NADPH oxidation by benzoquinone linearize the Lineweaver-Burke coordinates (fig.1). *TN* of the reaction makes up 22–25 s^{-1} when 10–30 μM of NADPH are used. The data are in accordance with the reported low values of the K_m of NADPH (1.8–2.5 μM [12,13]) and *TN* is close to 28 s^{-1} corresponding to the maximal rate of enzyme reduction by NADPH [3]. TN/K_m for BQ diminishes on the NADPH concentration increase. NADPH is supposed to be a competitive inhibitor for

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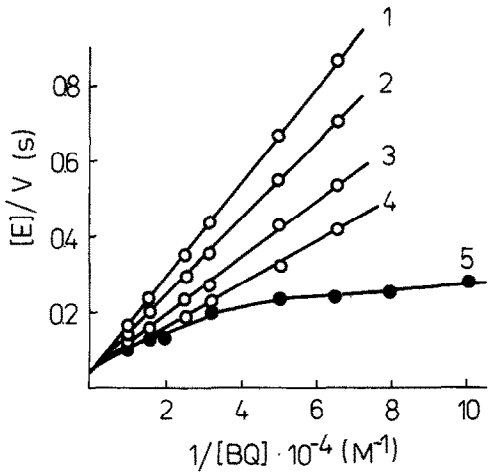

Fig.1. The dependence of the initial rate of ADR-catalyzed oxidation of NADPH by benzoquinone on the substrate concentrations. NADPH concentrations: 28 μM (1), 22 μM (2), 16.5 μM (3), 9.7 μM (4), and 22 μM NADPH in the presence of 0.5 μM adrenodoxin (5).

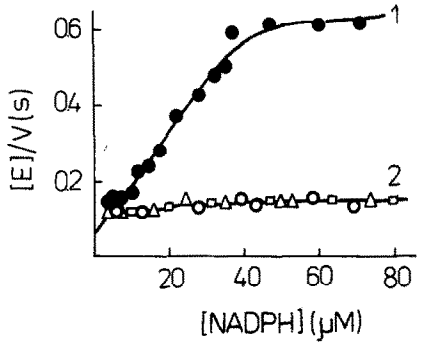

Fig.2. The rate of enzymatic oxidation of NADPH by 50 μM benzoquinone in the absence (1) and presence (2) of adrenodoxin. ADX concentrations: 0.1 μM (○), 0.3 μM (△), 0.6 μM (□).

quinoidal electron acceptors [14], and the K_i of NADPH, estimated from the dependence of the slopes of the Lineweaver-Burke plots (fig.1) on the NADPH concentrations is close to 5 μM. TN/K_m for BQ, obtained by extrapolation to zero concentrations of NADPH, makes up $6.5 \cdot 10^5$ $M^{-1}s^{-1}$. One must note, that the inhibition is incomplete and the reaction rate drops to a constant value at NADPH concentrations exceeding 50 μM. These results explain previously reported low diaphorase activities of ADR [2,3]. However, our data do not allow to conclude, whether the inhibition reflects the displacement of $NADP^+$ from its complex with reduced ADR [3] or the presence of a second $NADP^+$/NADPH binding site [13].

Addition of ADX increases the reaction rate. TN is close to 20 s^{-1} (fig.1), and the rate does not depend on the NADPH concentration (10–30 μM, data not shown). At low BQ concentrations the break is observed in the Lineweaver-Burke plot, and TN of the second 'slow' phase in the range 3.5–5 s^{-1} may be estimated (fig.1). This biphasicity may be caused by the BQ reduction via adrenodoxin, since at 0.5 μM of ADX the rate of ADX-linked cytochrome *c* reduction by ADR is 8 s^{-1} (on a one-electron basis). Analogous breaks are also characteristic of yeast flavocytochrome b_2, where quinoidal acceptors oxidize the heme at a higher rate constant than $FMNH_2$, but the electron transfer from $FMNH_2$ to heme is slower than the FMN reduction by lactate [15]. Thus, ADX prevents the reductase inhibition by NADPH and probably takes part in the reduction of quinone.

Further experiments were performed at relatively high BQ concentrations where the influence of possible side reactions might be less. The data of fig.2 indicate that 0.1–0.6 μM of ADX give almost the same activating effect. It is not possible to conclude whether ADX affects the affinity of ADR for NADPH in this case, since the K_m of NADPH is too low. For this reason the large concentrations of $NADP^+$ which increased the K_m of NADPH were used. The data of fig.3 indicate that in the presence of 400 μM $NADP^+$ TN/K_m for NADPH is close to $3 \cdot 10^5$ $M^{-1}s^{-1}$. Since $NADP^+$ is known to be a competitive inhibitor for NADPH possessing a K_i close to 10 μM [13] it corresponds to about a 40-fold decrease of TN/K_m. In the presence of $NADP^+$ adrenodoxin decreased TN/K_m for NADPH and to some extent raised TN (fig.3). However, the almost constant values of TN/K_m were reached at sufficiently high ADX concentrations (fig.4). This is valid for all concentrations of $NADP^+$ tested. K_i for ADX, estimated from fig.4, was close to 0.4 μM at 400 μM of $NADP^+$. It is evident that K_i decreased on the $NADP^+$ concentration decrease and diminished about 4-fold when 100 μM of $NADP^+$ were used.

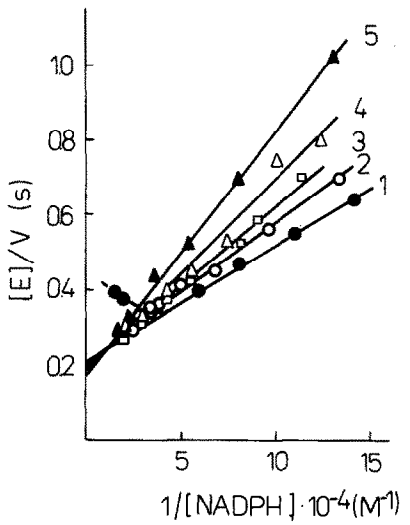

Fig.3. The effect of adrenodoxin on the rate of ADR-catalyzed NADPH oxidation by 50 μM benzoquinone in the presence of 400 μM $NADP^+$. Adrenodoxin concentrations: 0 (1), 0.1 μM (2), 0.2 μM (3), 0.4 μM (4) and 6 μM (5).

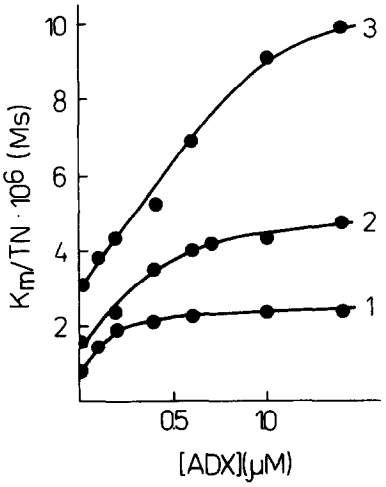

Fig.4. The effect of adrenodoxin on TN/K_m for NADPH in the presence of 100 μM (1), 200 μM (2) and 400 μM $NADP^+$ (3).

The data of figs 3 and 4 demonstrate a negative cooperativity between the ADX and NADPH binding and indicate that ADX decreases the affinity of ADR for NADPH by a limiting factor of 3. This value is several-fold less than that reported for ferredoxin:$NADP^+$ reductase [9]. Since ADX does not change the standard potential of reductase [5] the change in affinity is probably due to induced sterical hindrances or a partial overlapping of binding domains. The increase of *TN* at fixed BQ concentrations on adrenodoxin binding (fig.3) evidences a rather complex influence of ADX on catalytic properties of reductase.

It is evident from fig.4 that $NADP^+$ decreases the ADR affinity for ADX. One may assume that the K_i of ADX, obtained from these data, corresponds to the K_d of the ADR-oxidized ADX complex at the given $NADP^+$ concentration. However, it seems that the limiting value of K_d is not reached at these $NADP^+$ concentrations (fig.4). Again, assuming that the K_d of the oxidized ADR-$NADP^+$ complex, which corresponds to the competitive K_i for NADPH, is close to 10^{-5} M [3,13,16] one may suggest that the K_d of the ADR-ADX complex is increased about 40-fold at 400 μM of $NADP^+$. Thus, K_d of this complex must be close to 10^{-8} M. It is consistent with the value of 10^{-9} M, estimated from the ADX-stimulated diaphorase reaction of ADR at a significantly lower ionic strength [2].

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