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Role of Lys-110 of human NADH-cytochrome b_5 reductase in NADH binding as probed by site-directed mutagenesis

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Lys-110 of human NADH-cytochrome b_5 reductase was replaced by Ala, Met, or Arg by site-directed mutagenesis to evaluate the role of the residue. K_m values of purified Lys-110 → Ala and Lys-110 → Met mutants for NADH were approximately 200-fold and 1,100-fold higher than that of the wild-type, respectively, while the value of the Arg mutant was almost the same as that of the wild-type. These results indicate that the positive charge at position 110 is important for NADH binding. The k_{cat} value of Lys-110 → Ala was not affected, indicating that the residue only participates in the binding process in the reaction by forming an ionic interaction with phosphoryl group of NADH.

NADH-cytochrome b_5 reductase; NADH binding; Site-directed mutagenesis

1. INTRODUCTION

NADH-cytochrome b_5 reductase (b_5R; EC 1.6.2.2) is an FAD-containing oxidoreductase which transfers two electrons from NADH to two molecules of cytochrome b_5 [1,2]. The enzyme participates in methemoglobin reduction in erythrocytes [3], while in other tissues it plays a role in elongation and desaturation of fatty acids [4,5]. P-450-mediated drug metabolism [6], and cholesterol biosynthesis [7] as a part of the microsomal electron transfer system.

Porter et al. [8] first noted homology in the FAD and NAD(P)H binding sites of b_5R, ferredoxin-NADP$^+$ reductase (FNR) and NADPH-cytochrome P-450 reductase. It has now become evident that b_5R is a member of a family that consists of FNR, sulfite reductase, NADPH-cytochrome P-450 reductase, nitrate reductase, and nitric oxide synthetase, because important residues in FAD and NAD(P)H binding determined by the atomic structure of FNR at 2.6 Å [9], are conserved in all of these enzymes. These enzymes do not have the GxGxx(G/A) sequence motif which is widely found in the canonical dinucleotide binding site. The enzymes in this novel family are, therefore, likely to share a new aspect in NADH binding.

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Abbreviations: b_5R, NADH-cytochrome b_5 reductase; FNR, ferredoxin-NADP$^+$ reductase; K110A, the mutant enzyme with a Lys residue at position 110 (Lys-110) replaced by Ala; K110M, the mutant with Lys-110 replaced by Met; K110R, the mutant with Lys-110 replaced by Arg.

Recently Hackett et al. [10] reported that specific acetylation of Lys-110 of the enzyme elevates K_m values for NADH by 300-fold and causes a significant decrease in the activity. Because conclusions deduced from chemical modification experiments in many cases cannot exclude the possibility of steric hindrance, we have examined in the present study the role of the Lys residue of the enzyme on the basis of site-directed mutagenesis. During the preparation of this manuscript, Strittmatter et al. [11] reported a site-directed mutagenesis study of Lys-110 of steer b_5R. They converted Lys-110 to Gln or His and observed significant elevation of K_m values in both mutants, and significant reduction of k_{cat} values in the Lys-110 → Gln mutant. From these results they concluded that Lys-110 might play roles both in binding of NADH and in further processing, e.g. orientation of the bound substrate. In contrast, our results from a K110A mutant indicates that this residue participates only in the binding process, because k_{cat} values of the mutant were retained at almost the same level as that of the wild-type enzyme.

2. MATERIALS AND METHODS

2.1. *Materials*

Construction of the expression plasmid for the wild-type enzyme and *E. coli* RB791 used for expression were described previously [12]. Restriction enzymes and T4 polynucleotide kinase were obtained from Takara Shuzo (Kyoto, Japan) and the in vitro mutagenesis system was the product of Amersham (UK). Sequenase used for dideoxy chain-termination [13] was purchased from US Biochemicals (Cleveland, OH, USA). Oligonucleotides for mutagenesis were synthesized with a DNA synthesizer (model 8600; Milligen Biosearch Inc., San Rafael, USA). DEAE-Toyopearl and 5′-AMP-Sepharose 4B were the products of Tosoh (Tokyo, Japan) and Pharmacia LKB Biotechnology (Uppsala, Sweden), respectively. Bovine α-thrombin was purchased

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from Sigma (St. Louis, MO, USA). Other reagents were obtained commercially.

2.2. *Site-directed mutagenesis and construction of expression plasmid*

Mutagenesis was carried out by the method of Taylor et al. [14] as described previously [15]. Antisense primers used to isolate mutants were 5′-AAGTAAACCGCGATGACCAGG-3′ for K110A and 5′-AAGTAAACC(A or C)TGATGACCAGG-3′ for K110M and K110R (the changed nucleotides are underlined). Construction of the expression plasmid was essentially the same as described [16]. Human erythrocyte cytochrome b_5, which was used as an electron acceptor in the enzyme assay, was purified as described previously [16].

2.3. *Purification of the wild-type and mutant enzymes*

The wild-type and mutant enzymes were purified to homogeneity as described [12] with some modifications, as described below, from *E. coli* RB791 harboring the respective mutant plasmids. The activity of the K110M and K110A mutants was assayed as described [16] except that 1 mM NADH was used instead of 100 μM because of higher K_m value for NADH of these mutant enzymes. Affinity chromatography with 5′-AMP Sepharose 4B was unsuccessful in the cases of K110A and K110M mutants. Therefore, gel filtration chromatography using Sephacryl S-200 (3.2 × 85 cm) was performed. Yields of activity of the mutant enzymes were 40–50% and 15–50 mg of enzymes were obtained from 1.6 l of culture. No significant decrease in enzyme activity was observed during α-thrombin cleavage to release the authentic soluble form of the enzyme from the fusion protein with β-galactosidase [12] for all the mutant enzymes. Purity of the mutant b_5Rs was judged by polyacrylamide gel (12.5%) electrophoresis in the presence of sodium dodecyl sulfate [17].

2.4. *Steady-state kinetics*

Enzyme activity was assayed and kinetic properties were obtained as described previously [18].

3. RESULTS AND DISCUSSION

3.1. *Purification of the mutant enzymes*

We have constructed three expression plasmids which overproduce K110M, K110A, and K110R mutant enzymes as described in section 2 to clarify the role of Lys-110 which was formerly suggested to be involved in NADH binding by the chemical modification experiments of Hackett et al. [10]. Since the K_m values of K110M and K110A mutants for NADH were increased, the purification procedure for these mutants was modified as described in section 2.

3.2. *Kinetic properties of the mutant enzymes*

The kinetic properties of prepared mutant and the wild-type enzymes are presented in Table I. K_m values of purified Lys-110 → Ala and Lys-110 → Met mutants for NADH were 202-fold and 1,120-fold higher than that of the wild-type, respectively, while the value of the Arg mutant was almost the same as that of the wild-type. The higher K_m value of the Met mutant for NADH might be due to a conformational change caused by replacement of Lys-110 by a more hydrophobic residue, Met, as expected from the elevated K_m for cytochrome b_5 and reduced k_{cat} value of the mutant. In contrast to K110M, K110A showed a milder change in both K_m and k_{cat} values, suggesting that K110A has little conformational change; this replacement is therefore more suitable to evaluate the role of the residue. According to the study of Bordo and Argos [19], the replacement of Lys with Met seems not to be appropriate to evaluate the role of the Lys residue, although this has been performed in many site-directed mutagenesis studies probably because of the similar length and bulkiness of the side chains of Lys and Met. Their statistic study of the amino acid sequences of evolutionarily related proteins showed that a Lys to Met substitution in the course of evolution is rarely found either in buried and exposed residues [19]. Ala and Thr are the most suitable residues to substitute for Lys to evaluate the functional role of amino group of the residue [19]. K110A was shown to have 200-fold elevated K_m value and 85% of the k_{cat} value, compared with the values of the wild-type. The kinetic properties of K110R were essentially the same as those of the wild-type. From these results we concluded that a positive charge at position 110 only participates in the NADH binding process in the reaction by making an ionic interaction with the phosphoryl group of NADH. Apparent binding energy (ΔG_a) of the original side chain was −3.2 kcal/mol according to the equation, $\Delta G_a = RT \ln(k_{cat}/K_m)_{mutant}/(k_{cat}/K_m)_{wild\text{-}type}$ [20], when calculated with the values of the Ala mutant. This value may correspond well to the deletion of one charged interaction in the binding process [20].

In contrast to our results presented above, Strittmatter et al. [11] performed site-directed mutation of Lys-110 → Gln of steer b_5R and observed a 130-fold reduction of the k_{cat} value, and concluded that Lys-110 participates in both binding of NADH and orientation of the bound substrate. Replacement of Lys by Gln, which is a substitution less frequently found during evolution [19], might cause a significant conformational distortion leading to a reduction of the k_{cat} value. In this respect, the higher k_{cat} values of K110A obtained in the present study might be a better reflection of the effect of the loss of charge at position 110.

The role of Lys-116 at the homologous position in FNR, which was shown to belong to the same family as b_5R [9], was reported by Aliverti et al. [21]. They also changed the Lys to Gln and observed a significant reduction of the k_{cat} value. This may be also caused by

Table I

Kinetic properties of mutant NADH-cytochrome b_5 reductases

Enzymes	K_m (μM)		k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)	
	NADH	cyt. b_5		NADH	cyt. b_5
Wild-type[a]	0.6	6.6	872	1,453 × 10^6	13.2 × 10^7
K110A	121	14.2	742	6.2 × 10^6	5.2 × 10^7
K110M	672	28.5	563	0.84 × 10^6	2.0 × 10^7
K110R	2.3	16.7	877	381 × 10^6	5.3 × 10^7

[a] Data are from Shirabe et al. [18].

conformational distortion as discussed above. In both cases, however, the role of the positive residue in NAD(P)H binding was demonstrated, indicating that, in this family of enzymes, a positive residue seems to play an important role in substrate–dinucleotide binding. In addition, the α-helix dipole might be also important for substrate binding in the enzymes of the FNR family as implicated by close contact of the phosphate group of the substrate analog, 2′-phospho-AMP, to the NH_2-terminus of the α-helix in the $NADP^+$ binding site, as shown by X-ray analysis of FNR [9]. This family of enzymes has the consensus sequence, GxGxxP, at the NH_2-terminal portion of the α-helix instead of GxGxx(G/A), which is widely found in dinucleotide binding sites [22], and the dihedral angles of the first two Gly residues of FNR are different from those of the GxGxx(G/A) motif [9,22]. Furthermore, participation of the positively charged residue has been shown in only a few cases of classical dinucleotide binding proteins [22], in contrast to the FNR family demonstrated in the present study. Therefore, the enzymes of the FNR family are distinct from others in that both their positively charged residue and dipole moment of the α-helix, with novel structural motif, might be important in orientating the dinucleotide at the proper position for the reaction.

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