Biliverdin-IXβ reductase in human erythrocytes is a multifunctional enzyme

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Biliverdin-IX β reductase in human erythrocytes has been studied as NADPH-methemoglobin reductase at first, and then as NADPH-flavin reductase, or green-heme binding protein. At last, it was identified to be same with biliverdin-IX β reductase based on the amino acid sequence and base sequence of the cDNA. However, for the patients of hereditary methemoglobinemia, in which the main methemoglobin reductasing enzyme, NADH-cytochrome b_5 reductase is deficient, the NADPH-flavin reductase activity is indispensable for their lives. This paper summarized the history of the studies on the enzyme.

Hemoglobin in human erythrocytes binds oxygen and transports oxygen to various tissues, but always some part of hemoglobin is oxidized to non-functional hemoglobin, methemoglobin. In erythrocytes, there are two methemoglobin reducing enzyme systems, NADH- and NADPH-methemoglobin reductases to reduce back the methemoglobin produced to functional (oxy)hemoglobin. The former NADH-methemoglobin reductase is the major enzyme, and the latter one is minor in function. In the case of deficiency of the major enzyme, the patients have the hereditary methemoglobinemia, and suffered from cyanosis in mild cases, or mental retardation and neurological impairments in severe cases. In those cases, the minor enzyme is considered to save the patient life functioning as the salvage enzyme to reduce the methemoglobin. However, the NADPH-methemoglobin reductase is not always sufficient to support the patient life.

Gibson (1948) first described the deficiency of methemoglobin reducing enzyme in human erythrocytes as one of the responsible enzyme to cause human disease, and since then studies on NADH- and NADPH-methemoglobin reductases have been done by many researchers. However, properties of the enzymes were not clarified for a long time until the major methemoglobin reducing enzyme system was identified clearly by Hultquist and Passon (1971) to be the electron transfer system of the soluble forms of NADH-cytochrome b_5 reductase and cytochrome b_5 in erythrocytes. They showed that NADH-cytochrome b_5 reductase reduces cytochrome b_5, and then the enzymatically reduced cytochrome b_5 reduces non-enzymatically methemoglobin rapidly to functional oxyhemoglobin.

In normal human erythrocytes, methemoglobin concentration is maintained to be less than 1% of total hemoglobin mainly by NADH-cytochrome b_5 and cytochrome b_5. Function of the minor methemoglobin reducing enzyme, NADPH-methemoglobin reductase was also not unclear for long time, because in the patient erythrocytes with hereditary methemoglobinemia, in which NADH-cytochrome b_5 reductase is deficient, methemoglobin is often accumulated up to around 30%, even though NADPH-methemoglobin-

reducing enzyme exists sufficiently in the cells. NADPH-methemoglobin reductase, in fact, cannot reduce methemoglobin directly as studied by *in vitro* experiments. To characterize the physiological function of the NADPH-methemoglobin-reducing enzyme, many research groups tried to purify the enzyme from human erythrocytes, but those were almost hampered with contamination of hemoglobin, and were unsuccessful.

We have succeeded in purifying the enzyme from human erythrocytes, and found that flavins are effective electron carriers among the physiological substances so far tested (FMN, FAD, riboflavin, hemin, GSSG) (Yubisui *et al.*, 1976) to reduce methemoglobin, and named the enzyme NADPH-flavin reductase (Yubisui *et al.*, 1976; Yubisui *et al.*, 1979). In the *in vitro* experiments, the purified enzyme reduced FMN or FAD, and then the reduced form of flavins transferred electron to methemoglobin nonenzymatically to reduce to oxyhemoglobin (Yubisui *et al.*, 1979), and the reaction was analyzed to be very fast by the stopped-flow experiments (Yubisui *et al.*, 1980). The rate constant for the reduction of methemoglobin by reduced FMN was determined to be $3.3 \times 10^8 \ M^{-1}min^{-1}$ at 25°C. This value is extremely high than the reduction rate of methemoglobin with ascorbic acid ($1.8 \times 10^{-1} \ M^{-1}min^{-1}$) (Yubisui *et al.*, 1980). As described above, the purified enzyme did not reduce methemoglobin directly, but can reduce effectively via flavin. Based on these results, we confirmed that riboflavin added to intact human erythrocytes, reduced effectively methemoglobin to oxyhemoglobin by *in vitro* experiments (Matsuki *et al.*,1978).

Then, oral administration of riboflavin to patients with hereditary methemoglobinemia was tried to reduce methemoglobin in their erythrocytes, and the treatment was found to be effective (Matsuki *et al.*,1978, Kaplan and Chirouze, 1978), although the reduction of methemoglobin in patient erythrocytes was not so fast as compared with methylene blue (Goluboff and Wheaton, 1961). Methylene blue has long been used to reduce patient's methemoglobin in emergency, but the injection of the blue dye was unpleasant for patients, as their urine becomes blue. Contrary to this, flavins are the physiological substances and do not give any side effect to patients. Thus, NADPH-methemoglobin-reducing enzyme was called NADPH-flavin reductase, and the oral administration of flavin to patients was accepted as the general and effective therapeutics.

DeFilippi and Hultquist (1978) purified previously a green-heme binding protein from bovine erythrocytes. The structure and function of the green-heme, named by them was unidentified to any known heme. Later, in the process to purify the green-heme binding protein, they tried to reduce the bound heme with NADPH-flavin reductase of bovine erythrocytes, as the heme bound to the protein was unstable in the oxidized form (1991). Following the purification procedure of Yubisui *et al.* (1976, 1979) to purify the NADPH-flavin reductase from bovine erythrocytes, they found that the behaviors of the enzyme during purification are very similar to their green-heme binding protein (Quandt *et al.*, 1991). Then, they analyzed the primary structures of both the purified green-heme binding protein and NADPH-flavin reductase, and found that both of them are very similar to each other (Quandt *et al.*, 1991) as shown in Fig. 1.

After their report (Quandt *et al.*, 1991), we cloned a cDNA for human reticulocyte NADPH-flavin reductase (Chikuba *et al.*, 1994). The cDNA we cloned from a human reticulocyte cDNA library was consisted of about 800 bp with 621 bp coding sequence, which encodes 206 amino acid residues, and a polyadenylation signal in the 3'-noncoding region. The amino acid sequence deduced from the base sequence of the cDNA matched well with the partial amino acid sequences of peptides prepared from the flavin reductase purified from human erythrocytes (Chikuba *et al.*, 1994).

On the other hand, Yamaguchi *et al.* have been studied on the bilirubin metabolism in human tissues. They purified biliverdin-IXβ reductase from human liver and analyzed the primary structure of the enzyme (Yamaguchi *et al.*, 1993). Their amino acid sequence determined was almost identical with that of our NADPH-flavin reductase except that the liver enzyme lacks the N-terminal methionine and the C-terminal glutamine (Fig. 1).

We subsequently expressed the recombinant human NADPH-flavin reductase in *E. coli* using the cloned cDNA to analyze whether our enzyme has the biliverdin-IXβ reductase activity (Chikuba *et al.*, 1999). The

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HumFR    MAVKKIAIFGATGQTGLTTLAQAVQAGYEVTVLVRDSSRLPSEGPRPAHVVVGDVLQAAD
HumBR    -AVKKIAIFGATGQTGLTTLAQAVQAGYEVTVLVRDSSRLPSEGPRPAHVVVGDVLQAAD
BovFR    MVVKKIALFGATGNTGLTTLAQAVQAGYEVTVLVRDFSRLPSEGPQPAHVVVGDVRQPAD
         .*****;*****;************************.********;*********  *.**

HumFR    VDKTVAGQDAVIVLLGTRNDLSPTTVMSEGARNIVAAMKAHGVDKVVACTSAFLLWDPTK
HumBR    VDKTVAGQDAVIVLLGTRNDLSPTTVMSEGARNIVAAMKAHGVDKVVACTSAFLLWDPTK
BovFR    VDKTVAGQDAVIVLLGTRNDLSPTTVMSEGAQNIVAAMKAHGVDKVVACTSAFLLWDPSK
         ******************************;*****************************;*

HumFR    VPPRLQAVTDDHIRMHKVLRESGLKYVAVMPPHIGDQPLTGAYTVTLDGRGPSRVISKHD
HumBR    VPPRLQAVTDDHIRMHKVLRESGLKYVAVMPPHIGDQPLTGAYTVTLDGRGPSRVISKHD
BovFR    VPPRLQDVTDDHIRMHKVLQQSGLKYVAVMPPHIGDHPLTGAYTVTLQDGRGPSRVISKHD
         ******  ************;;***************;**********************

HumFR    LGHFMLRCLTTDEYDGHSTYPSHQYQ
HumBR    LGHFMLRCLTTDEYDGHSTYPSHQY-
BovFR    LGHFMLHCLTTDKYDGHTTYPSHVYE
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Figure 1. The alignment of amino acid sequences of flavin reductases and biliverdin-IXβ reductase.

The upper sequence is the amino acid sequence of human erythrocyte flavin reductase (HumFR), the middle one is that of human liver biliverdin-IXβ reductase (HumBR), and the bottom one is that of bovine erythrocyte flavin reductase (BovFR). Amino acid sequence data was obtained from the database, HumFR and HumBR (P30043), and BovFR (P52556).

recombinant human NADPH-flavin reductase showed significant biliverdin-IXβ reductase activity with apparent Km values of 0.4 μM for NADPH and 0.3 μM for biliverdin-IXβ, respectively, and the enzyme did not reduce biliverdin-IXα isomer (Chikuba et al., 1999) as shown in Table 1. The Km value determined for biliverdin-IXβ is a quite low value against the high Km value of 50 μM for FMN (Yubisui et al., 1976; Yubisui et al., 1979). Therefore, the biliverdin-IXβ reductase activity is more plausible and physiological activity of the enzyme in human erythrocytes rather than that of the flavin reductase activity. The cellular concentration of the free flavin in erythrocytes is quite low (less than 1 μM); therefore, the enzyme cannot play a role practically as the flavin reductase in erythrocytes without externally added flavin. In the case of oral administration of riboflavin to patients with hereditary methemoglobinemia, however, the flavin incorporated in the patient erythrocytes activates the reduction of methemoglobin to save the patient life (Matsuki et al., 1978; Kaplan and Chirouze, 1978).

Table 1. Enzyme activities of human flavin reductase and kinetic properties.

Electron acceptors	Concentrations (μM)	Enzyme activity (nmol/min/mg)		Km (μM)
		Recombinant FIR	FIR[a]	
FMN	50	95	72	50
FAD	50	39	33	-
Riboflavin	50	70	53	-
Methylene blue	50	272	118	-
(NADPH)	-	-	-	0.4[b]
Biliverdin-IXβ	0.6	nd[c]	-	-
Biliverdin-IXα	0.6	190	-	0.3
(NADPH)	-	-	-	0.4[d]

a. Purified from human erythrocytes.
b. Km value for NADPH of the FMN reductase activity.
c. Not detectable.
d. Km value for NADPH of the biliverdin-IXβ reductase activity.

A part of hemoglobin in erythrocytes is always oxidized to methemoglobin by loosing one electron from the heme in hemoglobin by chance accompanying the release of oxygen from the heme. The released oxygen with one electron becomes superoxide, and this superoxide may cleave the heme methen-bridge to biliverdin, the tetrapyrole compound. As another case to cause methemoglobin, some drugs we took also

oxidize hemoglobin, and some parts of the oxidized hemoglobin (methemoglobin) are degraded to bilirubin. NADPH-flavin reductase (biliverdin IXβ reductase) may function to reduce biliverdin-IXβ isomer. Usually in human liver cells, the α-methen-bridge of free heme is cleaved by heme oxygenase to produce biliverdin-IXα, but in human erythrocytes the major biliverdin-IX isomer was analyzed to be the β-form (94%), and the α-form (6%) was rather a minor one by Yamaguchi et al. (1994). At present we have no evidence to show the presence of heme oxygenase in erythrocytes to produce biliverdin. However, superoxide anion as described above, and also other unknown mechanism may cleave the heme, and in fact, the analyzed data (Yamaguchi et al., 1994) shows that the biliverdin-IXβ is predominant (94%) in human erythrocytes.

Crystal structures of biliverdin-IXβ and -α reductases to reduce these isomers were already analyzed, and those structures are clearly different from each other (Pereira et al., 2001; Whitby et al., 2002). The structure of the biliverdin-IXβ reductase revealed that it binds the β-isomer, and discriminates against the α-isomer through steric hindrance at the binding pocket (Pereira et al., 2001).

This review summarized briefly the interesting history on the studies of biliverdin-IXβ reductase in human erythrocytes. The enzyme was first called NADPH-methemoglobin reductase, but was later identified as NADPH-flavin reductase. The enzyme works well as NADPH-flavin reductase to reduce methemoglobin in patient erythrocytes to save their lives with oral administration of flavin. The identical enzyme in bovine erythoryctes, was studied as the green-heme binding protein (Quandt et al., 1991), but was later identified as flavin reductase. Xu et al. (1993) reported that pyrroloquinoline quinone acts with flavin reductase to reduce ferryl myoglobin in vitro and protects isolated heart from re-oxygenation injury, but the physiological concentration of pyrroloquinoline quinone in cells is very low to function.

Based on the kinetic studies on the recombinant flavin reductase, the most reasonable physiological function of the enzyme in human erythrocytes was concluded at last to be biliverdin-IXβ reductase activity. However, the function of the NADPH-flavin reductase as a methemoglobin-reducing enzyme in erythrocytes is an indispensable activity for the patients with hereditary methemoglobinemia to maintain their lives. Thus, this enzyme can acts multifunctionally depending on the physiological conditions.

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