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ENZYMIC N-DEMETHYLATION REACTION CATALYSED BY RED BLOOD CELL CYTOSOL

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Abstract—Red blood cell cytosol promotes enzymic N-demethylation reactions which display typical Michaelis–Menten kinetics with respect to *N*-methylaniline as substrate. The demethylase activity is linked with hemoglobin (Hb) and is enhanced in the presence of NADH and the NADH–methemoglobin reductase system. It has been adduced that Hb in its oxygenated form is involved in the reaction.

The metabolic activity of red blood cells has attracted increasing interest in recent years [1]. Although mature red cells do not contain an effective P450 system, various experiments suggest that erythrocytes might be a site of xenobiotic metabolism and that hemoglobin is the key molecule involved in the reactions. This has been indicated by *in vitro* mutagenicity tests in which red blood cells were used as a metabolic activation system for several prematugens [2, 3]. Other studies have shown that the red blood cell can perform some typical reactions normally catalysed by the cytochrome P450 system. In this respect, it is noteworthy that hemoglobin (Hb‡) has been implicated as a catalyst of various reactions including decarboxylation of 3,4-dihydroxyphenylalanine [4], hydroxylation of aniline [5, 6] and oxidation of styrene [7]. Aniline hydroxylation has been studied widely using intact red blood cells [8] or reconstituted systems containing purified Hb, cytochrome P450 reductase and NADPH [5]. In most studies, Hb appears to catalyse aniline hydroxylation in a manner typical of the hepatic microsomal cytochrome P450 [8], but it has also been postulated that hydroxylations could occur as intermediate reactions in peroxidations catalysed by Hb [9].

Although oxidative N-demethylation of substrates catalysed by liver microsomal cytochrome P450 requiring O_2 and NADPH is considered a characteristic monooxygenation reaction of this enzymic system, several studies have shown that in the presence of cumene hydroperoxide a peroxidase mechanism is also active [10]. Other hemoproteins reported to catalyse the peroxide-mediated N-demethylation of substrates include horseradish peroxidase and myoglobin [11, 12]. In the presence of peroxide, Methb is also able to catalyse dealkylation reactions [10, 11]. However, the oxygen-dependent demethylation reaction catalysed by oxyhemoglobin has been investigated to a lesser extent.

The studies of Starke *et al.* [13] and Tyce *et al.* [14] demonstrated that hemoglobin could catalyse demethylation reactions. However, these authors reached contradictory conclusions concerning the requirement of reduced pyridine nucleotides and the nature of the one-carbon product.

In the present work, we studied the *N*-demethylase activity of a red cell supernatant containing oxyhemoglobin. Optimal conditions for the incubation were determined and kinetic parameters were calculated. In an attempt to characterize the mechanism involved in the reaction, the influence of several inhibitors and cofactors was examined.

MATERIALS AND METHODS

Chemicals. NMA and KCN were purchased from Janssen Chimica (Beerse, Belgium). Metyrapone was obtained from Aldrich (Beerse, Belgium). NADH and catalase from beef liver were obtained from Boehringer Mannheim (Germany). Rat Hb and human Hp were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Carboxymethyl-cellulose (CMC) was purchased from Serva (Heidelberg, Germany).

Red blood cell supernatant preparation. Whole blood was obtained by cardiac puncture from male Wistar rats. The red cells were purified by filtration of the blood through a Sepacell leucocyte removal filter (Fenwall Laboratory, Deerfield, U.S.A.). The preparation and biochemical characterization of the hemoglobin-containing supernatant obtained after membranes removal according to Dodge *et al.* [15] is described elsewhere [16].

Dosages. The protein content was determined by the method of Lowry *et al.* [17] and hydroxyl radicals were measured as described by Richmond *et al.* [18]. Methb content was measured according to Bauer [19].

Spectroscopic measurements. Spectroscopic measurements were carried out in a Jasco double beam UV/vis spectrophotometer model 7800. Spectral analysis of the Hb-containing supernatant was performed at pH 6.9 or 7.5.

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‡ Abbreviations: CMC, carboxymethyl-cellulose; Hb, hemoglobin; Hp, haptoglobin; Methb, methemoglobin; NMA, *N*-methylaniline.

Kinetics of demethylation. NMA demethylase activity was measured on the basis of formaldehyde production according to Nash [20]. The incubation was carried out in a phosphate buffer (60 mM) unsupplemented with cofactors. Appropriate blanks were run for each assay. The reaction was started by addition of substrate and stopped with 0.5 mL of 40% trichloroacetic acid. Apparent V_{max}, K_m and K_i values were obtained by non-linear regression analysis [21].

CMC chromatography. The supernatant was separated into Hb and non-Hb proteins fractions by chromatography on a CMC column. The supernatant was eluted on CMC as proposed by Riggs [22] for Hb preparation with the following modifications. The supernatant containing about 1.5% Hb was dialysed overnight against 0.01 M phosphate buffer pH 6.7 and applied to the column. The non-Hb proteins were eluted with this phosphate buffer. Hb adsorbed on the column was then eluted with 0.01 M sodium phosphate buffer pH 6.9. Each 2-mL fraction collected was analysed by spectroscopy. Hb and non-Hb proteins were detected on the basis of absorbance at 400–420 and 280 nm, respectively. NADH-reductase activity was measured using 2,6-dichloroindophenol as described by Jaffe [23]. Fractions 1–30 were devoid of Hb and pooled: this preparation is referred to as the non-Hb protein fraction. Aliquots of the Hb and non-Hb protein fractions were tested for demethylase activity in a phosphate buffer containing 0.2 mM NADH.

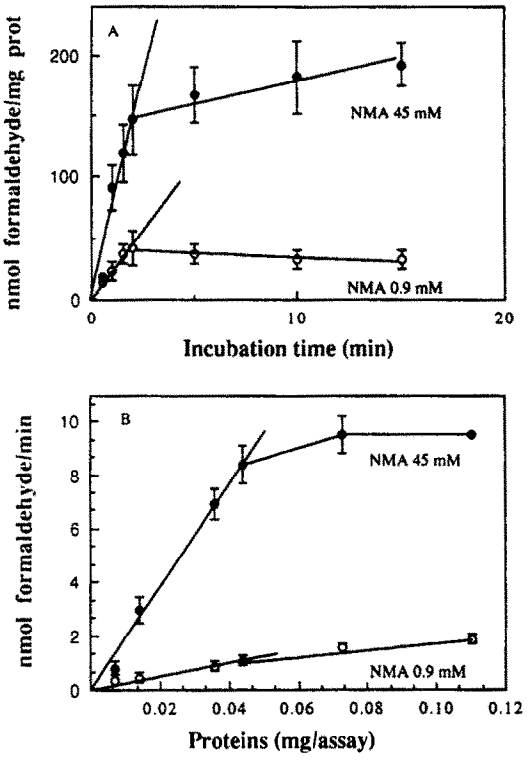

Fig. 1. Demethylation of NMA as a function of time (A) and protein content (B).

RESULTS

Kinetics of NMA dealkylation

NMA demethylation was carried out in phosphate buffer not supplemented with cofactors in the presence of the supernatant fraction of red blood cells, and displayed optimal pH and temperature at pH 7.5 and 40°. The measurement of light absorption spectra of the preparations showed that Hb exhibited a typical oxyhemoglobin absorption spectrum with maxima at 413–414, 540–541 and 575–576 nm, and minima at 509–511 and 559–561 nm. This spectrum remained unchanged during the 1.5-min incubation with substrate. No Methb could be detected in the medium. Figure 1A and B shows that the reaction is linear for up to 2 min and for protein content up to 0.04 mg/assay. An incubation time of 1.5 min and a protein concentration of 0.035 mg/assay (corresponding to 0.3 μM Hb) were chosen in the linear region of the graph. The kinetics displayed a substrate saturation curve typical of Michaelis–Menten kinetics (Fig. 2) with a V_{max} of 154.8 ± 6.3 nmol/min/mg protein and K_m of 6.9 ± 1.1 mM.

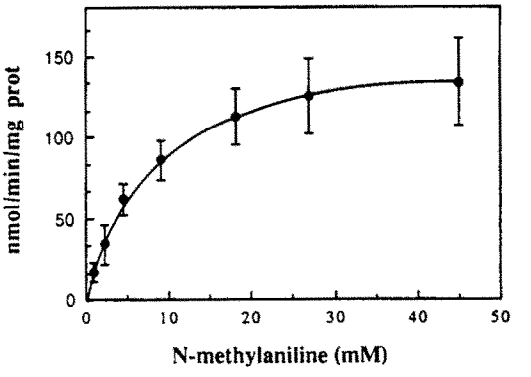

Fig. 2. Dependence of the rate of demethylation on NMA concentration. Each point represents the average (± SE) of at least six determinations.

Is a Fenton mechanism involved in the reaction?

In order to determine whether a Fenton reaction involving ferrous iron and hydrogen peroxide to generate hydroxyl radicals is involved in the reaction, typical inhibitors were tested. However, iron chelators (10 mM EDTA, 0.5 mM diethylenetriamine pentaacetic acid or 1 mM desferrioxamine) and typical radicals scavengers (1 mM thiourea, 85 mM ethanol, 1 mM dimethyl sulfoxide or 50 mM arginine) did not inhibit the reaction nor could OH· radicals be detected by the salicylate hydroxylation method (data not shown). Moreover, catalase (650 U/assay) which would reduce the hydrogen peroxide necessary for the Fenton reaction did not affect the demethylase activity (Table 1).

In contrast, boiled supernatant almost completely inhibited the reaction (Table 1).

Inhibition studies

In order to confirm the role of Hb as catalyst of

Table 1. Influence of modifying factors on the demethylase activity of a red cell supernatant

Incubation mixture	% Activity
Control	100
+ CO	25
+ Hp 0.08 mg/assay	60
+ Catalase 650 U/assay	94
Heat-denatured supernatant	5

100% activity = 134 ± 26 nmol/min/mg protein.
NMA, 45 mM.

Table 2. Kinetic parameters of the demethylase activity of a red cell supernatant: effect of inhibitors

Reaction mixture	V_{max} (nmol/min/mg protein)	K_m (mM)
Control	154.8 ± 6.3	6.9 ± 1.1 (6)
KCN 0.1 mM	118.8 ± 9.5*	8.1 ± 2.0 (4)
KCN 0.5 mM	68.0 ± 8.7*	8.7 ± 3.6 (2)
Metyrapone 1.0 mM	118.0 ± 4.9*	9.5 ± 1.3 (2)
Metyrapone 1.5 mM	109.2 ± 12.0*	7.8 ± 3.4 (2)

* Significantly different from control ($P < 0.01$).
( ) Number of repetitive experiments.

the reaction, the influence of Hp was examined. Hp is a serum glycoprotein which binds Hb in an irreversible manner. The formation of the hemoprotein–antihemoprotein antibody complex is highly specific *in vitro* and *in vivo* [24, 25]. As shown in Table 1, the addition of Hp to the incubation medium inhibited the demethylase activity of the supernatant.

The addition of metyrapone to the reaction mixture decreased the maximal velocity of the reaction ($K_i = 2.84 \pm 0.32$ mM) and only 25% of the activity was recovered in the presence of CO (Tables 1 and 2).

Cyanide which acts as a ligand to the iron in the ferric form of hemoproteins was an effective inhibitor of the reaction ($K_i = 0.33 \pm 0.04$ mM) suggesting the involvement of a ferric form of the protein (Table 2). However, since spectral analysis and Methb measurement did not reveal any Methb formation during the incubation period, the form $HbFe^{3+}$ is likely to exist only as an intermediate in the reaction.

Importance of NADH and the reductase system in the reaction

We investigated whether the catalytic activity could be enhanced by supplying reducing equivalents to the system (Fig. 3). The addition of 0.2 mM NADH to the medium significantly increased the V_{max} of the reaction ($V_{max} = 338.0 \pm 14.8$ nmol/min/mg protein). This result suggests that NADH might stimulate the reductase system present in the red cell preparation.

The role of the reductase was examined further in the following experiment. When Hb was separated

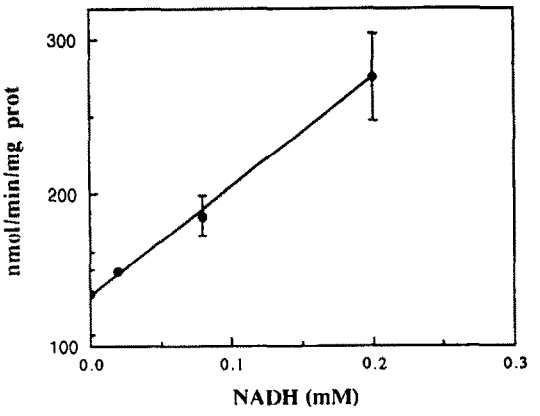

Fig. 3. Influence of increasing concentrations of NADH on the demethylase activity of the red cell supernatant. NMA, 45 mM.

from the red cell supernatant by CMC chromatography, NADH–reductase activity could be detected in fractions 7–10 of the non-Hb protein eluate. Table 3 shows that the non-Hb protein fraction *per se* did not have any demethylating activity but enhanced the activity associated with the Hb fraction. In addition, the reaction was investigated using commercial purified Methb. This oxidized form of hemoglobin did not exert any activity *per se*, the latter being recovered after addition of the reductase fraction.

DISCUSSION

It is known that ferrous iron and hydrogen peroxide react to produce the reactive OH· radical [26]. This has been designated as the "Fenton reaction". Reduced Hb can replace ferrous iron in this reaction [27]. It seemed, therefore, important to elucidate whether a Fenton type mechanism was implied in the demethylase activity observed. The failure of several inhibitors to reduce the activity in addition to the incapability of boiled supernatant to mediate the reaction indicates an enzymic reaction mechanism rather than a chemical one of the type of the Fenton reaction.

The inhibition observed with Hp indicates that Hb is involved in the reaction. In contrast to the enhancement of the peroxidatic activity of Hb generally observed with Hp–Hb complexes, the demethylase activity was strongly inhibited by Hp suggesting that, under our conditions, the well known peroxidatic activity of Hb was not involved. This conclusion was corroborated by the failure of catalase to affect the reaction and by the inhibitory effect of CO which would not inhibit peroxidases. Indeed, the influence of metyrapone and CO suggest that the oxygenated form of Hb participates in the reaction.

The mature red blood cell does not possess any cytochrome P450 but contains most of the elements of an electron transport system which in other tissues forms part of a drug-metabolizing system. Red blood

Table 3. Demethylase activity of different supernatant fractions isolated by ion exchange chromatography

Fraction	Formaldehyde (nmol/min/mg protein)
Non-Hb fraction	ND
Hb fraction*	32.0 ± 5.2
Hb fraction* + non-Hb fraction	76.6 ± 15.0
Purified Methb†	ND
Purified Methb† + non-Hb fraction	30.5 ± 6.3

* OxyHb isolated from the supernatant.

† Purified Methb from Sigma. The reaction mixture was supplemented with 0.2 mM NADH and contained an amount of protein similar to that added when the whole supernatant was used.

Substrate concentration, 9 mM. ND, not detectable.

cells are equipped with two electron transport systems that maintain Hb in the ferrous oxidized state. The primary pathway involves a NADH-dependent reductase associated with cytochrome *b*5 [28]; the secondary NADPH-dependent pathway is of minor importance in normal conditions and can be recruited by the administration of Methylene blue as exogenous electron carrier [29].

We observed an enhancing effect of NADH and of the fraction containing the NADH–reductase system on the demethylation: these results underline the importance of maintaining Hb in its oxygenated form to obtain maximal activity. Similar findings have been reported for styrene oxidation [7] and aniline hydroxylation [8] catalysed by Hb.

In conclusion, the present experiments indicate that, in addition to its widely studied hydroxylase activity, Hb in its oxygenated form exhibits demethylase activity. In contrast to the studies reported by Tyce *et al.* [14], formaldehyde rather than methanol was detected as the one-carbon product and the reaction was shown to be enhanced by the presence of NADH. Formaldehyde was also detected as the product of the Hb-mediated demethylation of various substrates by Starke *et al.* [13]. But in addition to most studies on Hb-catalysed reactions where an exogenous cytochrome P450–reductase system was added [5, 6, 13], the Hb-dependent catalytic activity was found to be enhanced by its own NADH-dependent reductase system.

These results indicate that the red blood cell which lacks cytochrome P450 can participate in typical reactions involved in xenobiotic metabolism. Extended studies in this field would be of considerable interest in order to investigate the possible use of human red cell fractions as an alternative exogenous metabolic activating system in *in vitro* toxicology tests.

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