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DIFFERENTIAL MONOOXYGENASE-LIKE ACTIVITY OF FETAL AND ADULT ERYTHROCYTES*

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SUMMARY

The monooxygenase-like activity of human erythrocytes was measured by monitoring the rate of para-hydroxylation of aniline. Erythrocytes from umbilical cord blood samples were found to be 3-5 times more active than erythrocytes from adult peripheral venous blood samples. This result may be attributed to an intrinsic difference in the reactivity of the particular form of hemoglobin which predominates in each of these erythrocyte types. Thus, the fetal hemoglobin isolated and purified from the cord blood displayed 2-6 times more activity than purified adult hemoglobin when each was tested in reconstituted aniline hydroxylation systems containing NADPH.

We and others (1-3) have shown that isolated hemoglobin can catalyze the para-hydroxylation of aniline, a reaction that is typical of the monooxygenase function of the cytochrome P450 system. Our studies demonstrated that hemoglobin could substitute equieffectively for cytochrome P450 in a reconstituted system containing the hemoprotein, NADPH, cytochrome P450-reductase and oxygen (2). The mechanism of the hemoglobin-mediated reaction implicated oxyhemoglobin as the form which interacts with substrate, and that finding prompted us to examine the hydroxylase activity of intact erythrocytes where oxyhemoglobin is the predominant species. We found that erythrocytes from various species including humans do catalyze the para-hydroxylation of aniline and related substrates, and that this reactivity was abolished if the oxyhemoglobin in the cells was oxidized to ferrihemoglobin (methemoglobin) or converted to carbonmonoxy-hemoglobin (4,5). This absolute requirement for oxyhemoglobin for the reactivity of the erythrocytes suggested that hemoglobin itself is the monooxygenase catalyst. It follows from that hypothesis

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that altered molecular forms of hemoglobin would be expected to display differential reactivity. Accordingly, this report shows that erythrocytes from human umbilical cord blood which contain predominantly fetal hemoglobin (hemoglobin F, $\alpha_2\gamma_2$ tetrameric structure (6)) are better hydroxylase catalysts than adult erythrocytes which contain predominantly adult hemoglobin (hemoglobin A, $\alpha_2\beta_2$ tetrameric structure (6)). The isolated hemoglobins displayed an analogous relationship of reactivity.

MATERIALS AND METHODS

p-Aminophenol was obtained from Aldrich Chemical Co. and recrystallized from ethanol:petroleum ether, 50:50 (v:v). Aniline and ethyl ether were from Fisher Scientific Co. Aniline was distilled and stored frozen under nitrogen until used. Ether was routinely prewashed with a $FeSO_4$ solution, then water to remove any peroxide degradation products present. NADPH and methylene blue were supplied by Sigma Chemical Co. All other chemicals were reagent grade.

Erythrocytes were prepared as described previously (4). Human umbilical cord blood was generously supplied by Dr. Jeffrey L. Blumer, Department of Pediatrics, Case Western Reserve University. Peripheral venous blood was donated by healthy adult colleagues. These studies were carried out under the approval of the human subjects committee of Case Western Reserve University.

The hydroxylation of aniline to p-aminophenol by erythrocytes was assayed by the colorimetric technique previously described (4).

Fetal hemoglobin from cord blood was separated from coexistant adult hemoglobin by treatment with dilute ammonium hydroxide followed by rapid neutralization with dilute hydrochloric acid. Further purification of the fetal hemoglobin was accomplished by the chromatographic techniques described by Eyer et al. (7). Purity was confirmed by standard clinical electrophoresis in the Special Hematology Laboratory, University Hospitals, Cleveland, Ohio. Fetal hemoglobin content was assayed by an alkaline denaturation method (8). P450 reductase from rat liver was prepared by the method of Dignam and Strobel (9). The activity of fetal hemoglobin in the reconstituted aniline hydroxylase system was investigated using the method described by Mieyal, et al. (2).

RESULTS

At the same hemoglobin concentrations, fetal erythrocytes were 3-5 times more active than adult erythrocytes in catalyzing aniline hydroxylation (Table 1A). The response of fetal erythrocytes to stimulatory substances (Table 1B) displayed a pattern similar to that described previously for adult erythrocytes (4). Thus, maximal stimulation was observed in the presence of methylene blue and glucose, again implicating NADPH as the chief cofactor

TABLE 1

ANILINE HYDROXYLASE ACTIVITY OF FETAL ERYTHROCYTES

A. Comparison with Adult Erythrocytes[1]

Erythrocyte Source	Rate of p-Aminophenol Formation, pmol/min/ml
Adult, 0.2 mM HbO_2	21.7 ± 2.8
Fetal, 0.2 mM HbO_2	73.3 ± 5.5
Adult, 1.0 mM HbO_2	96.7 ± 5.0
Fetal, 1.0 mM HbO_2	473.3 ± 51.7

B. Effect of Stimulatory Substances[2]

Additions	Relative Rate of p-Aminophenol Formation
none	1.00 ± 0.08
6 mM glucose	1.10 ± 0.03
10 mM lactate	1.12 ± 0.06
10 μM methylene blue	1.56 ± 0.21
6 mM glucose + 10 μM methylene blue	6.69 ± 0.81
10 mM lactate + 10 μM methylene blue	2.53 ± 0.27

[1] Intact erythrocytes at the designated hemoglobin concentration were incubated with 60 mM aniline at 38° C for 60 minutes, then extracted and assayed as described under "Methods". The results, expressed as pmol p-aminophenol/min/ml, are given for the mean ± standard error for at least 6 determinations.

[2] Assays were performed as described for part A, except that the substances designated were added at the concentrations shown which were previously used for adult erythrocytes (4). The hemoglobin concentration in all samples was 0.2 mM. Results are expressed relative to the experiments for which the samples contained no stimulatory agents (defined as 1.00).

for the hydroxylase activity (4).

Isolated hemoglobin F was then compared with isolated hemoglobin A in reconstituted aniline hydroxylase systems. As shown in Table 2, fetal hemoglobin was much more active than adult hemoglobin in the reaction utilizing NADPH alone. On the other hand, whereas the activity of the adult hemoglobin system was increased by the addition of P450 reductase as expected from previous work (2), the activity of the fetal hemoglobin system was decreased. One explanation for this difference might be that the cytochrome P450 reductase couples less effectively with fetal hemoglobin than with adult hemoglobin. In fact, the reductase actually may channel electrons away from the direct

interaction between NADPH and fetal hemoglobin, rather than stimulating the reaction by serving as an intermediate electron carrier.

DISCUSSION

Fetal erythrocytes display a substantially greater monooxygenase-like activity than adult erythrocytes, as shown above (Table 1). It is unlikely that this increased reactivity is due to factors other than the hemoglobin itself, because the activity of the NADPH-dependent methemoglobin reductase of fetal cells is not different from adult cells, and the activity of the NADH-dependent methemoglobin reductase is actually somewhat less (10,11). Moreover, the levels of the superoxide dismutase and glutathione peroxidase enzymes, which may serve to scavenge activated forms of oxygen and thereby possibly inhibit hydroxylase activity, also are similar to the levels in adult erythrocytes (12). Furthermore the direct comparison of the activities of the isolated fetal and adult hemoglobins confirmed that the difference was retained in the absence of all of the other cellular components (Table 2). Therefore we conclude that catalysis of aniline hydroxylation is an intrinsic property of the hemoglobin molecules themselves which apparently is dependent upon their unique molecular structures. In support of this hypoth-

TABLE 2

ANILINE HYDROXYLATION BY RECONSTITUTED SYSTEMS

	pmol/min/ml Rate of p-Aminophenol Formation
Adult Hb^{3+} + NADPH	25.3 ± 4.0
Fetal Hb^{3+} + NADPH	153.0 ± 6.7
Adult Hb^{3+} + NADPH + reductase	51.3 ± 4.7
Fetal Hb^{3+} + NADPH + reductase	96.7 ± 4.7

Assays with the reconstituted system were performed as described by Mieyal, et al. (2). The reaction mixture contained 20 µM phosphate buffer, pH 6.8, 1 µM methemoglobin, 40 mM aniline and 20 µM NADPH, and was incubated for 15 minutes. 20 µg of partially purified P450 reductase enzyme (specific activity ∿ 3.3 mmol cytochrome c reduced/min/mg) were added to each sample as designated. The results are expressed as pmol p-aminophenol/min/ml and are the mean ± standard error of at least 4 determinations.

esis, we had shown previously that this reactivity of hemoglobin is abolished by heat denaturation (2,5). Among the possible explanations for the greater hydroxylase activity of the fetal hemoglobin, several can be eliminated: (a) It cannot be ascribed to a difference in affinity for oxygen, because the greater catalytic activity was retained by the purified hemoglobin F which was "stripped" of the oxygen-affinity modifier 2,3-diphosphoglycerate. In intact erythrocytes, the hemoglobin F has a higher oxygen affinity than hemoglobin A, because its deoxy form binds the diphosphoglycerate less tightly due to the altered amino acid composition of its γ-subunits (6). The relative oxygen affinity is reversed when the two hemoglobins are isolated. (b) The differential hydroxylase activity is not related to the oxidation-reduction potential of the hemoglobins, because the redox potential of isolated hemoglobin F has been shown to be essentially equivalent to that of hemoglobin A at pH 7.4 (13). (c) Likewise intrinsic autooxidizability seems not to be the explanation. Even though one study reported that hemolysates from infant blood accumulated less methemoglobin than comparable samples from blood of older children (14), the isolated hemoglobins F and A were shown to display indistinguishable rates of autooxidation (15).

Despite the apparent lack of a difference in autooxidizability of hemoglobin F and hemoglobin A, there appears to be a greater facility of the ferrous iron atoms of hemoglobin F to donate electrons to O_2 in response to stimuli. Thus, isolated fetal oxyhemoglobin is more readily converted to methemoglobin than is adult hemoglobin by various reducing agents including nitrite (16) and ortho- and para-aminophenol (17). A reasonable mechanism for methemoglobin formation in these reactions would involve generation of peroxide from the heme-bound molecular oxygen, one electron being donated by the reducing agent and one electron from the ferrous iron atom yielding ferric iron (18). It is mechanistically analogous to suggest that the heme iron atoms of hemoglobin F would have a greater facility to transfer electrons to O_2 also in the presence of NADPH and an hydroxylatable substrate like ani-

line, the result being increased activation of oxygen and increased monooxygenase-like catalysis. It remains to be ascertained whether the greater reactivity requires the tetrameric structure of hemoglobin F or if it might be confined to the γ-subunits.

The present study illustrates an avenue for exploring the intimate molecular basis for the differential reactivity and function of hemoproteins. The relative facility of the heme iron atom to participate in electron transfer to molecular oxygen may be a key factor which distinguishes oxygen carriers from oxygen-activating enzymes. Moreover, this study highlights the caution that should be exercised in the use of drugs for pregnant women or newborns, since the oxygen-carrier function of fetal hemoglobin may be especially susceptible to alteration by drugs structurally related to aniline.

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