Concentration of NADH–Cytochrome b_5 Reductase in Erythrocytes of Normal and Methemoglobinemic Individuals Measured with a Quantitative Radioimmunoblotting Assay

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Abstract

The activity of NADH–cytochrome b_5 reductase (NADH-methemoglobin reductase) is generally reduced in red cells of patients with recessive hereditary methemoglobinemia. To determine whether this lower activity is due to reduced concentration of an enzyme with normal catalytic properties or to reduced activity of an enzyme present at normal concentration, we measured erythrocyte reductase concentrations with a quantitative radioimmunoblotting method, using affinity-purified polyclonal antibodies against rat liver microsomal reductase as probe. In five patients with the "mild" form of recessive hereditary methemoglobinemia, in which the activity of erythrocyte reductase was 4–13% of controls, concentrations of the enzyme, measured as antigen, were also reduced to 7–20% of the control values. The concentration of membrane-bound reductase antigen, measured in the ghost fraction, was similarly reduced. Thus, in these patients, the reductase deficit is caused mainly by a reduction in NADH–cytochrome b_5 reductase concentration, although altered catalytic properties of the enzyme may also contribute to the reduced enzyme activity.

Introduction

The principal enzymatic system of the red cell for the reduction of methemoglobin (MetHb)[1] consists of a soluble NADH–cytochrome b_5 (cyt b_5) reductase (1) and an intermediate electron carrier, soluble cyt b_5 (2). Soluble erythrocyte reductase is very similar enzymatically (1) and immunologically (3, 4) to a membrane-bound form of the enzyme, present on endoplasmic reticulum and outer mitochondrial membranes of a variety of tissues. Sequencing studies of the human erythrocyte (5) and steer liver microsomal (6) reductases have confirmed the high degree of homology between the two forms of the enzyme, showing also that the soluble form lacks a hydrophobic segment at the NH_2-terminus, present in the microsomal reductase.

In hereditary recessive methemoglobinemia, the activity of erythrocyte cyt b_5 reductase is generally reduced (7–9, but also see 10). In a rare form of the disease, associated with mental retardation, both the erythrocyte soluble protein and the membrane-bound forms of other tissues are lacking (11); therefore, it is thought that the two forms of the enzyme are products of the same gene (see 12 for a review). In most cases of hereditary enzymatic methemoglobinemia, however, the levels of reductase are selectively depressed in erythrocytes and are normal, or close to normal (11, 13), in other cell types; in these cases, the disease is mild and not associated with neurological disorders. Recently, a second type of mild disease has been reported in which the enzyme is deficient in both red and white blood cells and presumably present at normal levels in other tissues (14). Destruction of an altered enzyme by proteolytic enzymes present in blood cells and not in other tissues could explain this selective deficit of the reductase in the erythrocytes (and in some cases leukocytes) of patients with the mild form of the disease (15). However, until the present, only measurements of the enzyme activity of methemoglobinemic patients have been done, whereas data on the levels of reductase protein are not available. Thus, it is possible that methemoglobinemic individuals have normal levels of an enzyme with reduced activity. In this study, we have undertaken to directly measure levels of cyt b_5 reductase in erythrocytes of methemoglobinemic patients and normal subjects with a quantitative radioimmunoblotting assay, using an affinity-purified polyclonal antibody against the rat liver microsomal enzyme as probe.

Methods

Materials. In addition to materials listed in previous publications (16, 17), the following chemicals were purchased from the indicated sources: α-cellulose and Sigmacell type 50, Sigma Chemical Co., St. Louis, MO; 5'AMP–Sepharose 4B, Biotechnology International AB, Uppsala, Sweden; hemoglobin assay kit, Boehringer Mannheim GmbH, Mannheim, FRG.; 50% glutaraldehyde, Fluka AG, Buchs, Switzerland.

Purification of proteins and preparation of antibodies. The purification of the water-soluble fragment of rat liver microsomal NADH–cyt b_5 reductase, the production of antisera in rabbits, and the purification of antireductase antibodies by affinity chromatography have been described in previous publications (16, 18). Briefly, the Cathepsin D–solubilized form of cyt b_5 reductase, purified by standard procedures and conjugated to keyhole limpet hemocyanin, was used as antigen. Antisera were passed over a column containing Sepharose B conjugated to the same reductase preparation as the one used as antigen. Antireductase antibodies were eluted with a pH 2.2 buffer. The resulting affinity-purified antibodies recognize only one band on immunoblots of rat liver microsomes and human erythrocytes (see Fig. 2), and immunoprecipitate a polypeptide that yields two-dimensional peptide maps indistinguishable from those obtained with the enzyme purified by standard procedures (18).

A partially purified preparation of human erythrocyte soluble cyt b_5 reductase was obtained from 225 ml of packed red blood cells. Cells were lysed with 36 vol of hypotonic buffer (1 mM EDTA, 1 mM

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1. *Abbreviations used in this paper:* cyt b_5, cytochrome b_5; MetHb, methemoglobin; MetHb.FeCN, MetHb-ferrocyanide complex.

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phenylmethylsulfonyl fluoride (PMSF), and 5 mM Na^+ phosphate buffer, pH 7), and centrifuged at 25,000 g_{max} for 30 min (Sorvall GSA rotor; DuPont de Nemours & Co., Inc., Sorvall Instruments Div., Newton, CT). Soluble reductase was recovered in the resulting supernate, which was supplemented with solid $(NH_4)_2SO_4$ to a final concentration of 64.5% of saturation. After allowing the $(NH_4)_2SO_4$ to dissolve, the suspension was left for 2 h at 4°C and then centrifuged at 25,000 g_{max} for 10 min (Sorvall GSA rotor; DuPont de Nemours & Co., Inc.). The supernate was discarded, and the precipitate was taken up with phosphate buffered saline (PBS) containing 1 mM EDTA plus 1 mM PMSF to a volume of 150 ml. This solution was concentrated to 25 ml by ultrafiltration (Amicon PM-10 filter; Amicon Corp., Danvers, MA), and dialysed extensively against PBS plus 1 mM EDTA plus 1 mM PMSF. After addition of Triton X-100 and EDTA to final concentrations of 2% and 5 mM, respectively, the solution was clarified by ultracentrifugation and then loaded slowly (~ 1 ml/min) onto a 5'AMP–Sepharose 4B column (2 × 4 cm). The effluent was passed through the column again two times. The column was washed with 100 ml PBS containing 1 mM EDTA and 0.2% Triton X-100. Reductase was then eluted with PBS containing 1 mM EDTA and 0.2 mM NADH. The reductase-containing fractions were concentrated to 1.25 ml by ultrafiltration and loaded onto a Sephadex G-100 column (1 × 35 cm) equilibrated in 0.1 mM EDTA, 5 mM Tris-HCl, pH 8. The reductase-containing fractions eluting from this column were pooled, divided into aliquots, and liophylized. Before use, a liophylized aliquot was dissolved in a small volume of 0.015% Triton X-100, and the protein concentration was determined. After appropriate dilution in bovine serum albumin solution (0.1 mg/ml), the reductase solution was used as a standard in quantitative radioimmunoblotting experiments.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and radioimmunoblotting. SDS-PAGE on 8 or 10% polyacrylamide gels 1.5 mm thick was done as previously described (16). After electrophoresis, gels were either stained with silver (19) or electroblotted as previously described (17, 20).

For quantitative radioimmunoblotting of a large number of samples, only the portion of the gel containing the reductase was used. The appropriate region of the gel was identified by visualizing molecular weight markers in a lane exposed to 0.25 M KCl for 5 min, a treatment which results in the precipitation of the SDS contained in the polypeptide bands (21). A strip of gel centered around the expected position of the reductase was excised and electroblotted onto a nitrocellulose filter in a methanol-containing buffer (20) for 90 min with an applied voltage gradient of 20 V/cm. The plexiglass blotting box was kept immersed in a water bath held at 15°C by circulating tap water. After electrophoretic transfer, the blot was stained by sequential incubation with affinity-purified antibodies and ^{125}I–protein A. After exposing the blot to an x-ray film, the autoradiogram was used as a guide to excise the reductase bands from the nitrocellulose filter. An area comprising both the membrane-bound and the soluble form of the reductase was excised. The radioactivity contained in the bands was quantitated in a gamma counter (Beckman 4000; Beckman Instruments, Inc., Fullerton, CA). A background of ~ 1,000 cpm, corresponding to the radioactivity contained in an area of equal size excised from a region of the blot devoid of bands, was subtracted. Samples were always run in duplicate or triplicate and the values obtained were averaged. Details of this procedure for quantitative radioimmunoblotting are given in a recent publication (17).

Preparation and fractionation of erythrocytes. Samples of heparinized whole blood of methemoglobinemic patients of normal controls were passed through microcrystalline cellulose–α-cellulose columns to remove leukocytes (22). 6 ml of blood were passed through 10 ml columns packed in 50 ml disposable syringes. The effluent was centrifuged (150 g_{max}), and the packed red blood cells were washed twice with PBS. No leukocytes could be detected in these preparations, which were diluted in 5% acetic acid–0.25% methylene blue (Türk's solution). Whole blood collected from decapitated male Sprague–Dawley rats was treated in the same way as the human blood. 96% of the leukocytes were removed from the rat blood by passage through the microcrystalline cellulose–α-cellulose column.

Washed erythrocytes were lysed, and the lysate fractionated into a ghost and soluble fraction, as previously described (16), but the last wash of the ghost pellet was with PBS. Before SDS-PAGE, the soluble fraction was concentrated by liophylization followed by resuspension in one-tenth the original volume of PBS. In one experiment the soluble reductase was concentrated by $(NH_4)_2SO_4$ precipitation (16).

Biochemical assays. Reductase activity in whole blood or erythrocyte preparations was determined by the rate of reduction of the ferrihemoglobin-ferrocyanide complex (Methb.FeCN), as described by Hegesh et al. (23) with slight modification. The hemoglobin (Hb) substrate preparation was centrifuged before use (40,000 g_{max} for 30 min [Sorvall SA600 rotor; DuPont de Nemours & Co., Inc.]), and additional centrifugation after addition of the sample was found to be unnecessary. The amount of Hb substrate solution used was varied, so that the final Hb concentration (substrate plus sample) was constant (2.7 mg/ml). The reaction was followed in a double-beam recording spectrophotometer (Lamda 5; Perkin-Elmer Corp., Norwalk, CT) in 1 ml cuvettes with a 1-cm light path. Values were corrected for a blank, containing an equal amount of substrate Hb and NADH, but no sample. In all assays linearity of the reaction rate with respect to sample concentration was verified.

In erythrocyte ghost fractions, enzyme activity was determined both with the Hegesh assay for NADH-MetHb.FeCN reductase as well as with the NADH-FeCN reductase assay described by Sottocasa et al. (24). In both cases, 1% Triton X-100 was included in the assay (25). Samples were preincubated for 5 min in the detergent containing assay mixture before starting the reaction by addition of NADH.

Hb was determined with a test combination kit (Boehringer Mannheim, GmbH), and protein was measured by the method of Lowry et al. (26), using bovine serum albumin as standard.

Patients. Five methemoglobinemic patients from three different families were referred to us by northern Italian hospitals. On the basis of their low levels of erythrocyte NADH-MetHb.FeCN reductase activity and of the observation of normal intellectual development and absence of neurological disorders, they were diagnosed as being affected by the mild form of recessive hereditary methemoglobinemia.

Results

To measure reductase antigen levels in human erythrocytes with our anti–rat liver reductase antibodies, it was first necessary to partially purify a small amount of the human red cell reductase, to be used as a standard in our assays. Fig. 1 illustrates the quality of our preparation, analysed by SDS-PAGE: lanes *B* and *C* show the polypeptides eluted by NADH from a 5'AMP affinity column, whereas lanes *D* and *E* show the final reductase-enriched fractions, collected from a Sephadex G-100 column. This preparation was enriched in a polypeptide running slightly more slowly than the water-soluble fragment of rat liver microsomal cyt b_5 reductase (Fig. 1, lane *F*). The molecular weight of this polypeptide (M_r of ~ 32,000) was close to that reported by others for purified erythrocyte reductase (5, 27). Moreover (Fig. 2), it was the only polypeptide of the preparation recognized by our antireductase antibodies and could thus be identified as human erythrocyte cyt b_5 reductase. Starting from 225 ml of packed red blood cells, we obtained 200 μg of protein in our final preparation. Densitometric scanning of a gel lane like the one of Fig. 1, lane *D* indicated that ~ 60% of the protein in the preparation was cyt b_5 reductase.

In the experiment shown in Fig. 2, lanes *A* and *B*, we compared the ability of our anti–rat reductase antibodies to bind to rat liver and human erythrocyte reductase. Equal

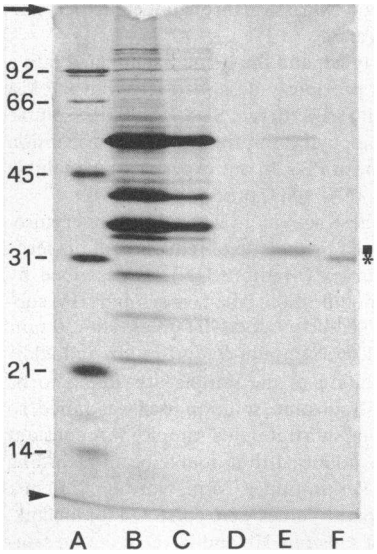

Figure 1. Purification of erythrocyte cyt b_5 reductase, analysed on a 10% SDS-polyacrylamide gel. Lanes contained: *A*, low molecular weight standards (Bio-Rad Laboratories, Richmond, CA); *B* and *C*, input to final Sephadex G-100 column (1.34 and 0.33 μl, respectively); *D* and *E*, final reductase-enriched fractions eluted from Sephadex G-100 column (50 and 100 ng of protein, respectively); and *F*, purified water-soluble fragment of rat liver microsomal cyt b_5 reductase (100 ng). The gel was stained with silver. Numbers on the left indicate molecular weights ($\times 10^{-3}$) of standards. Square and asterisk indicate erythrocyte reductase and water-soluble fragment of rat liver microsomal reductase, respectively. Arrow and arrowhead point to origin and front of the gel, respectively.

amounts of the hydrophylic fragment of rat liver microsomal reductase and human erythrocyte reductase (considered to be 60% of the partially purified preparation) were run on an SDS-polyacrylamide gel, electroblotted, and then radioimmunostained with anti-rat reductase antibodies and ^{125}I-protein A. The amounts of ^{125}I-protein A bound to the two bands were roughly the same, confirming the strong immunological similarity between the two proteins (3, 4).

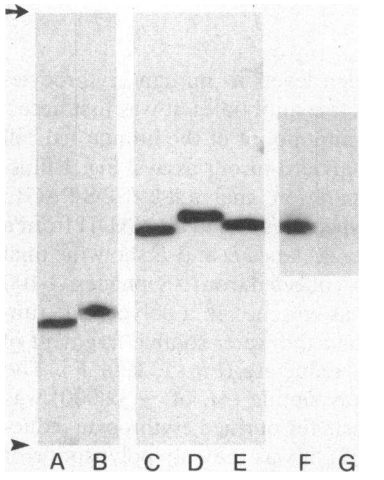

Figure 2. Radioimmunoblots of reductase-containing samples. Blotting was from 8% (lanes *A* and *B*) or from 10% (lanes *C–G*) SDS-polyacrylamide gels. Lanes contained: *A*, purified water-soluble fragment of rat liver microsomal reductase (10 ng); *B*, partially purified human erythrocyte reductase (sample shown in lanes *D* and *E* of Fig. 1, 15 ng of protein); *C*, soluble fraction of the erythrocyte of a normal individual, concentrated by $(NH_4)_2SO_4$ precipitation (50 ng of protein); *D*, erythrocyte ghost fraction of a normal individual (6.1 μg of protein); *E*, rat liver microsomes (0.5 μg of protein); *F*, erythrocytes of a normal control (300 μg of Hb); and *G*, erythrocytes of a methemoglobinemic patient (523 μg of Hb). Arrow and arrowhead point to origin and front of the gels, respectively. In the experiment shown in lanes *F* and *G*, only a limited portion of the gel, containing the reductase band, was electroblotted. Control experiments showed that in methemoglobinemic patients, no other immunoreactive bands were present on the gel. The membrane-bound and soluble forms of the enzyme are not resolved in the experiment shown in these last two lanes.

When tested against the soluble and ghost fraction of human erythrocytes, the anti-rat reductase antibodies recognized only one band in radioimmunoblotting experiments (Fig. 2, lanes *C* and *D*). The polypeptide in the ghost fraction migrated more slowly than its counterpart in the soluble fraction. The ghost reductase migrated also slightly behind the rat liver microsomal enzyme (Fig. 2, lane *E*).

Lanes *F* and *G* of Fig. 2 show the result obtained when red blood cells of a normal individual (lane *F*) and a methemoglobinemic patient (lane *G*) were analyzed by radioimmunoblotting. Although a larger sample of red blood cells was loaded for the methemoglobinemic subject, very little reductase could be detected in this patient's erythrocytes.

To assess the differences in reductase concentrations between patients and normal controls, we used the radioimmunoblotting system quantitatively, comparing the radioactivity bound to the reductase bands of red cell samples with that bound to the bands of known amounts of erythrocyte reductase standard. In preliminary experiments, we determined that it was unnecessary to electroblot for a time > 90 min since a further 90 min of electroblotting resulted in the binding of only 10% more reductase to a fresh nitrocellulose filter than the amount bound during the first 90 min of transfer, as quantitated by antibody plus ^{125}I-protein A binding (results not shown). Moreover, when a second sheet of nitrocellulose was placed behind the first one, 90% of the reductase revealed by radioimmunostaining (after 90 min of electroblotting) was found on the first sheet and only 10% on the second sheet, indicating that, with this time of electroblotting, the reductase was efficiently retained on the nitrocellulose filter (results not shown).

The typical standard curve of Fig. 3 *a* shows that the radioimmunoblotting assay was linear from 1 to 8 ng. Fig. 3 *b* shows how the amount of reductase measured in the assay increased linearly with increasing amounts of the same red blood sample loaded on the gel. Linearity of the assay was tested routinely on all samples.

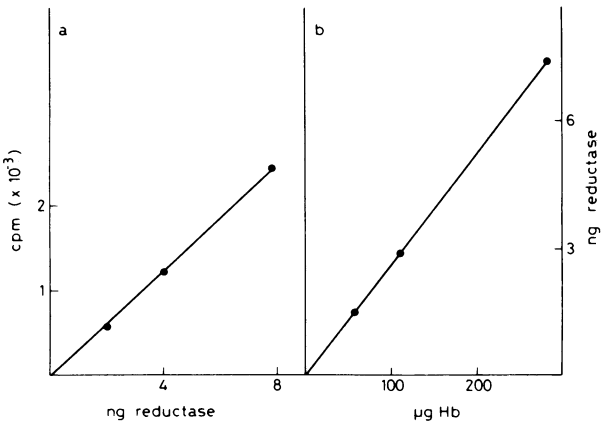

Figure 3. Quantitative radioimmunoblotting assay for erythrocyte cyt b_5 reductase. (*a*) A typical standard curve obtained with three quantities of partially purified human erythrocyte reductase. The quantities indicated on the abscissa are corrected for contaminants present in the preparation (40%). (*b*) The results of an experiment in which increasing amounts of the same red blood cell sample (*abscissa*) were assayed for reductase content (*ordinate*) with the radioimmunoblotting assay.

Table I. Distribution of Protein and Cyt b_5 Reductase in Rat and Human Erythrocyte Subfractions*

	Soluble fraction		Ghosts	
	Human§	Rat‡	Human§	Rat‡
Total protein (%)‖	99.6±0.1	98.2	0.4±0.1	1.8
Reductase antigen (%)‖	90.2±0.9	93.5	9.8±0.9	6.5
Nanogram reductase per milligram protein	22.6±3.6	19.3	574±124	117

* Erythrocytes were purified by the method of Beutler et al. (22).
‡ Values from one experiment.
§ The values for human erythrocytes are averages of two experiments ± half-range.
‖ Values are percentage of total recovered in soluble plus ghost fraction. Recoveries of protein and reductase with respect to the starting erythrocyte preparations were 93–110% and 93–130%, respectively.

With this quantitative radioimmunoblotting assay, we first compared amounts and subcellular distribution of erythrocyte reductase in the human and in the rat (Table I). As can be seen from row *2* of Table I, in both species most of the erythrocyte reductase was recovered in the soluble fraction. The concentration of reductase in the soluble fraction was similar in the two species (Table I, row *3*) and close to the value previously determined by us in rat erythrocytes with a competition radioimmunoassay, using rat liver reductase as standard (16). In contrast, the concentration of reductase in the ghost fraction was ~ 5 times higher in humans than in the rat and 25 times higher than in the soluble fraction (Table I, row *3*). The high concentration of reductase antigen in human erythrocyte ghosts is in agreement with the high values for the specific enzyme activity of human red cell membrane reductase found previously by others (25).

Table II summarizes the results obtained with methemoglobinemic patients. Patients from three different families (B.C., B.T., B.A.M., P.C., and P.R.) had NADH-MetHb.FeCN reductase activity levels 13% or less of control values (Table II, column *3*). Similarly, when cyt b_5 reductase levels were determined by quantitative radioimmunoblotting in these individuals, it was found that the levels of antigen were reduced in the patients (Table II, column *4*). However, a close inspection of the data reveals that the levels of antigen in the patients were not reduced to the same extent as the levels of enzyme activity. This is better seen by comparing the absolute specific activity values (enzyme activity per milligram reductase determined as antigen). These values were lower in the patients than in the controls (Table II, column *5*). The average value for the absolute specific activity of the enzyme of the patients was reduced to ~ 60% of the control value.

The NADH-MetHb.FeCN reductase assay carried out on total erythrocytes in the absence of detergent measures mainly the soluble enzyme (25). However, it is known that in recessive hereditary methemoglobinemia, the ghost-associated reductase is affected as well as the soluble form (25). Therefore, it seemed worthwhile to compare antigen concentration and enzyme activity in the erythrocyte ghosts of our patients. The enzyme assays on ghost fractions were carried out in the presence of detergent, which was found to activate the enzyme nearly 20-fold, in accordance with a previous report (25). Exposure of the soluble enzyme to detergent had no effect on the activity (data not shown). In agreement with previous work (25), we found that NADH-MetHb.FeCN reductase activity was reduced in erythrocyte ghosts of methemoglobinemic subjects (Table III, column *3*). The extent of reduction of the membrane-bound activity for the individual patients correlated with the decrease in enzyme activity measured in total erythrocytes (compare the data of Tables II and III). Similar results were obtained also with another enzyme assay for the reductase, the NADH-FeCN reductase assay. As expected, membrane-bound reductase, measured as antigen, was also reduced in the patients (Table III, column *2*), and the extent of this diminution was similar to that observed for total erythrocyte antigen (compare the data of Tables II and III). The data of Table III were used to calculate absolute specific activities both for NADH-MetHb.FeCN and for NADH-FeCN reductase (columns *4* and *6*). In normal individuals, the absolute specific activity for NADH-MetHb.FeCN reductase was somewhat lower than that of the enzyme measured in total erythrocytes. This could be due to incomplete activation of the mem-

Table II. Levels of Cyt b_5 Reductase Measured as Enzyme Activity or Antigen in Methemoglobinemic Patients and Normal Individuals

Subject	Relationship	Enzyme activity (NADH-MetHb.FeCn reductase)*	Reductase antigen (*μg/g Hb*)	Absolute specific activity (*IU/mg reductase*)‡
Control I		3.17	30.0	106
Control II		3.56	25.6	139
Control III		3.67	28.6	128
Average±SD		3.47±0.26	28.1±2.2	124±17
B.C.		0.22	3.68	60
B.T.	Sister of BC	0.46	6.40	72
B.A.M.		0.26	3.58	73
P.C.		0.24	2.18	110
P.R.	Brother of PC	0.14	2.02	69
Average±SD§		0.26±0.12	3.57±1.76	77±19
		(7.5)	(12.7)	(62)

* Activity is given in international units (micromole substrate reduced/minute) per gram Hb. ‡ Values in this column are obtained by dividing enzyme activity (international units per gram Hb) by antigen concentration (milligram reductase per gram Hb). § Numbers within parentheses indicate percentages of average control values.

Table III. Levels of Cyt b_5 Reductase Measured as Antigen or Enzyme in Erythrocyte Ghosts of Methemoglobinemic Patients and Normal Individuals

Subject	Reductase antigen (*μg/g protein*)	NADH-MetHb.FeCN reductase*		NADH-FeCN reductase*	
		IU/g protein‡	Absolute specific activity (*IU/mg reductase*)	IU/g protein‡	Absolute specific activity (*IU/mg reductase*)
Control I	505	41.2	82	349	702
Control II	461	35.5	77	334	724
Average±half-range	483±22	38.3±2.8	79.5±2.5	341±8	713±11
B.C.	62	1.9	31	29.1	469
B.T.	98	7.1	72	78.6	802
B.A.M.	37	1.6	43	16.0	432
Average±SD§	65.7±30.7	3.5±3.1	49±17	41.2±27.0	567±204
	(13.6)	(9.2)	(62)	(12.1)	(79)

* Enzyme assays were carried out in the presence of 1% Triton X-100. ‡ International Units are micromole substrate reduced per minute.
§ Numbers within parentheses indicate percentages of average control values.

brane enzyme by detergent. Two of the patients (B.C. and B.A.M.) had lower values than the controls with both enzyme assays. In contrast, one of the patients (B.T.) had a membrane-bound enzyme with absolute specific activity similar to that of the controls.

Discussion

In previous studies, we have used affinity-purified rabbit antibodies against the water-soluble fragment of rat liver microsomal cyt b_5 reductase to study the localization and biosynthesis of the enzyme in rat liver (17, 18, 28–30), as well as its properties and subcellular distribution in rat erythrocytes (16). In the present work, we have used these same antibodies to quantitate the enzyme in human erythrocytes and to compare its levels in red cells of normal individuals and patients affected by the mild form of recessive hereditary methemoglobinemia. The quantitative radioimmunoblotting assay we used proved to be very sensitive, in that it could detect nanogram amounts of the reductase, in samples where it was present in an extremely low proportion (< 1/100,000 of the total protein in methemoglobinemic individuals).

Using this assay, we found that, like in the rat, most of the human erythrocyte cyt b_5 reductase is soluble, but that considerable amounts are found also in the ghost fraction. The membrane-associated form was found to migrate more slowly than its soluble counterpart, in agreement with previous studies on the purified enzyme (31). The concentration of the enzyme in the ghost fraction (nanogram reductase per milligram protein) was considerably higher than that in the rat and 25 times higher than that in the soluble fraction. A high concentration of reductase in erythrocyte ghost fractions has been reported also by others on the basis of enzyme activity measurements (25, 31). On the basis of specific enzyme activity measurements of the purified ghost reductase and of a total ghost fraction, it can be calculated from the data of Kitajima et al. (31) that the membrane-associated reductase represents 1/2,500 of the total ghost protein, in very close agreement with our results. The functions of the NADH–cyt b_5 reductase system on erythrocyte membranes are at present not known.

When we used quantitative radioimmunoblotting to measure reductase concentrations in red cells of methemoglobinemic patients, we found that antigen levels were reduced to 7–20% of the values found in controls. A similar diminution was found also for the membrane-bound enzyme, measured in ghost fractions. Thus, the reductase deficit in these patients must be attributed mainly to a reduction in the levels of enzyme protein. In addition to the lower concentrations of reductase measured as antigen, our experiments also suggested a lower activity of the enzyme (~ 60% of normal) in methemoglobinemic patients. This effect was seen both in total erythrocytes (reflecting the soluble enzyme) and in the ghost fractions of two of the patients examined. In contrast, in one of the patients, the membrane-associated enzyme behaved in a similar manner to that of the controls. This result was surprising since this patient (B.T.) is the sister of B.C., whose membrane-bound reductase showed reduced absolute specific activity; moreover, the soluble reductase of this same patient (B.T.) had reduced absolute specific activity similar to that of her sister. However, reductase antigen levels of B.T. were in any case considerably higher (both in total erythrocytes and in the ghost fraction) than those in the other patients examined, including the sister. It is possible that these two sisters do not share both reductase alleles, but we do not have the necessary information to reach any conclusion on this point.

The lower levels of reductase antigen found in methemoglobinemic patients could be due either to increased degradation or to decreased synthesis of the enzyme, or to both these factors. Since it has been observed that cyt b_5 reductase decays in the erythrocytes even of normal individuals (32, 33), and, moreover, an unstable variant of the enzyme in Puerto Rican methemoglobinemic patients has been described (34), we favour the idea that a higher than normal susceptibility of an altered reductase to proteolysis in the red cell is the main cause of many of the mild forms of recessive hereditary methemoglobinemia. It is of interest that, in our patients, the membrane-bound and soluble antigen were affected to comparable degrees, suggesting that they are degraded with a similar mechanism.

In conclusion, our results show that, in the patients examined in this study, the reduced levels of NADH-MetHb reductase activity in the red cell are due mainly to reduced concentrations of enzyme and probably in small part to reduced activity of the residual enzyme. Reduced levels of immunologically reactive enzyme protein have also been reported in other hereditary enzymopathies, such as glucose-6-phosphate dehy-

drogenase (35), adenosine deaminase (36), and three types of hypoxanthine-guanine phosphoribosyltransferase deficiency (37). In contrast, one variant of the latter enzyme has drastically reduced absolute specific activity and is present in increased concentration in the erythrocytes of affected individuals (37). The assay described in this paper, which could be used to study the reductase variants described in the literature (38), as well as to measure antigen levels in other cell types of methemoglobinemic individuals, ought to be a useful tool for increasing our knowledge at a molecular level of the etiology of this disease.

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