BBA 65647

THE ENZYMATIC REDUCTION OF FERRIHEMOGLOBIN

II. PURIFICATION OF A FERRIHEMOGLOBIN REDUCTASE FROM HUMAN ERYTHROCYTES

EMANUEL HEGESH AND MORDHAY AVRON

Biochemistry Section, Weizmann Institute of Science and the Philip Handel Research Institute, Kaplan Hospital, Rehovoth (Israel)

(Received April 4th, 1967)

SUMMARY

1. An enzyme was purified from human erythrocytes which catalyses the reduction of ferrihemoglobin by NADH in the presence of ferrocyanide. About 1000-fold purification of the enzyme was achieved.

2. The enzyme was free of NADPH-dependent 'ferrihemoglobin reductase'. Its pH optimum was 5.2. The apparent K_m of the enzyme for ferrihemoglobin was 8 μM. The enzyme was specific toward NADH as the electron donor for ferrihemoglobin reduction.

3. The purest enzyme preparation obtained was also active in the reduction of dichlorophenolindophenol, methylene blue, cytochrome *c* and myoglobin. NADPH could also serve as a donor in some of these systems.

4. The substrate of the enzyme, ferrihemoglobin, was purified and freed of enzymes involved in its reduction. Crystalline hemoglobin preparations obtained commercially or prepared by accepted procedures were shown to contain large amounts of the enzyme.

INTRODUCTION

Several enzyme preparations capable of promoting ferrihemoglobin reduction have been isolated from human erythrocytes[1–4]. All exhibited high diaphorase activity but were comparatively poor ferrihemoglobin reductases. Addition of an electron carrier, such as methylene blue, was necessary to demonstrate high rates of ferrihemoglobin reduction.

In this report, an enzyme which promotes ferrihemoglobin reduction in the absence of an added electron carrier was purified and characterized. A procedure resulting in the purification of hemoglobin from enzymes involved in ferrihemoglobin reduction is also presented.

EXPERIMENTAL

Materials

NADH (grade III), NADPH (type II), phenazine methosulfate, FAD (grade III), FMN and cytochrome *c* (horse heart, type III) were purchased from the Sigma Chemical Comp. Methylene blue and dichlorophenolindophenol were preparations of Eastman, Organic Chemicals Department. Dowex AG 1-X10 (analytical grade anion-exchange resin chloride form, 200–400 mesh) was a product of the Bio-Rad Corp. for Biochemical Research. DEAE-cellulose (DE II) and carboxymethyl-cellulose (CM II) were supplied by Whatman Column Chromedia. Myoglobin (whale skeletal muscle, lyophilized) was a preparation of Seravac Laboratories, Capetown, South Africa. Diaphorase (type II, from *Clostridium kluyveri*) was bought from Sigma Chemicals, St. Louis, U.S.A. NADPH-diaphorase was prepared as previously described[5,6].

All other chemicals used were of analytical grade.

Preparations

'Active hemolysate' was prepared by water and toluol treatment of washed human erythrocytes[7]. Hemolysate free of ferrihemoglobin-reducing enzymes ('inactive hemolysate') was made by an adaptation of the method of HENNESSEY *et al.*[7] as described under RESULTS. Hemolysate containing ferrihemoglobin was prepared by oxidizing active or inactive hemolysate with ferricyanide using 1 mole ferricyanide per mole hemoglobin iron. In this reaction ferricyanide is quantitatively reduced to ferrocyanide and as such bound to ferrihemoglobin[8]. Active or inactive 'Dowex hemolysate' was obtained by passing the appropriate ferricyanide-treated hemolysate through a Dowex 1 anion-exchange resin column to free it of ferrocyanide[8].

Assay of ferrihemoglobin-reducing activity (enzyme assay)

The reaction mixture contained: 0.075 μmoles ferrihemoglobin added as inactive Dowex hemolysate, 1.5 μmole EDTA, 9.0 μmoles citrate buffer (pH 4.7), enzyme, 0.3 μmoles potassium ferrocyanide and 0.3 μmoles NADH (or NADPH) in a vol. of 3 ml. NADH (or NADPH) was added at zero time and the change in absorbance was measured at 575 mμ at 30-sec intervals against a blank containing all additions, except NADH (or NADPH). The final pH of the reaction mixture was 5.2. A $\Delta\varepsilon$ of $42.0 \cdot 10^3$ (see ref. 9) for the difference in absorption between ferri- and ferrohemoglobin was used to calculate the rate of reduction. In experiments where hemolysates containing ferrohemoglobin were used, ferricyanide replaced ferrocyanide.

Several other variations of this procedure were employed. They are indicated in the appropriate legends.

Other methods

In hemolysates, total hemoglobin was determined by the method of CANNAN[10]. Protein concentration was measured by the method of LOWRY *et al.*[11] with bovine serum albumin as a standard. Flavins were determined fluorimetrically by the method of BURCH, BESSEY AND LOWRY[12] using an Aminco-Bowman spectrofluorimeter and chromatographically by the method of CRAMMER[13]. Ultracentrifugations were performed in a Beckman-Spinco analytical ultracentrifuge (Model E). Spectrophoto-

metric measurements were performed with a Beckman DU spectrophotometer or a Cary recording spectrophotometer, Model 14.

RESULTS

Purification of hemoglobin

Dialysis and crystallization are generally used in the purification of hemoglobin. One of the most commonly used methods was described by DRABKIN[14]. However, the big octahedral crystals of hemoglobin obtained by this method were found to contain NADH-diaphorase in amounts which were only slightly lower than those found in hemolysates (Table I). Ferrihemoglobin-reduction activity, as measured by our enzyme assay, diminished to about half of the activity in the original hemolysate. Therefore, DRABKIN's purification method did not result in a separation of hemoglobin from its reducing enzymes. Similarly, several commercial preparations of crystalline hemoglobin were found to be heavily contaminated with diaphorase and ferrihemoglobin reductase.

TABLE I

FERRIHEMOGLOBIN REDUCTION AND DIAPHORASE ACTIVITY IN SOLUTIONS OF HEMOGLOBIN PURIFIED BY DIFFERENT METHODS

NADH-diaphorase was determined by the method of SCOTT[15]. Preparation of active and inactive hemolysates and the estimation of ferrihemoglobin reduction are described under EXPERIMENTAL. The addition of enzyme to the reaction mixtures was omitted. Data are expressed in μmoles/min per mg total protein $\times 10^3$.

Hemoglobin preparation	*Ferrihemoglobin reduction by NADH*	*Dichlorophenol-indophenol reduction (NADH-diaphorase)*
Active hemolysate	3.00	0.40
Solution of ferrohemoglobin crystals prepared by DRABKIN's method	1.72	0.38
Inactive hemolysate	0	0

The purification of hemoglobin from the system responsible for ferrihemoglobin reduction in erythrocytes was achieved by a method based upon the procedure of HENNESSEY *et al.* for the isolation of certain enzymes from erythrocytes[7]. A convenient amount of active hemolysate (120 ml) was divided equally among 6 tubes. The following procedure represents the treatment of one of the 20-ml samples carried out at 4° without transfer of the material from the tube. To 20 ml of hemolysate, 20 ml of purified DEAE-cellulose suspension[7] was added and the mixture allowed to stand at 4° for 20 min with manual stirring. Hemoglobin which remained unadsorbed in the supernatant fraction was separated by centrifugation for 15 min at 2000 $\times$ *g*. By repeating the adsorption procedure 2–3 times, each time using fresh DEAE-cellulose, hemoglobin preparations were obtained, which were free of enzymes activating ferrihemoglobin reduction. The purification of hemoglobin from its reducing system could be shown in the ferrocyanide–activated ferrihemoglobin-reduction system, and in the diaphorase assay described by SCOTT (Table I).

The purified hemoglobin preparation was stable for at least a week if kept refrigerated; lyophilized and deep frozen, it could be used after months.

Purification of an enzyme promoting ferrihemoglobin reduction

Step 1, adsorption of enzyme on DEAE-cellulose. The first part of the procedure used in the purification of hemoglobin constituted also Step 1 in our enzyme purification method. The enzyme protein remained adsorbed on the DEAE-cellulose. A single adsorption was sufficient. Residual traces of hemoglobin were removed by washing the combined adsorbent (from 6 tubes) 3 times with a total of 90 ml of 1 mM phosphate buffer (pH 7.0) and 3 times with the same amount of 0.3 mM phosphate buffer (pH 7.0). These treatments did not remove significant amounts of enzyme from the DEAE-cellulose.

Step 2, elution of enzyme protein from DEAE-cellulose. The enzyme was eluted by a solution containing 60 mM KCl, 50 mM citrate buffer (pH 5.2) and 50 μM EDTA. 1 ml eluent was added for each ml of hemolysate used in Step 1. The DEAE-cellulose suspension was stirred for 1 h in an ice-bath. The supernatant fluid was collected by centrifugation at 2000 $\times$ g for 15 min and constituted the 'crude enzyme preparation'.

Step 3, fractionation of the crude enzyme preparation on a CM-cellulose column. Preparation of the column. CM-cellulose was washed several times with a solution containing: 3.75 mM citrate buffer (pH 5.2), 15 mM KCl and 5 μM EDTA. Small particles were discarded. A chromatographic column (2 cm $\times$ 24 cm) was prepared and washed with 2500 ml of the above solution at 4°.

Fractionation. 100 ml of the crude enzyme preparation were mixed with an equal amount of 0.01 M citrate buffer (pH 5.2), containing 0.1 mM EDTA. The prepa-

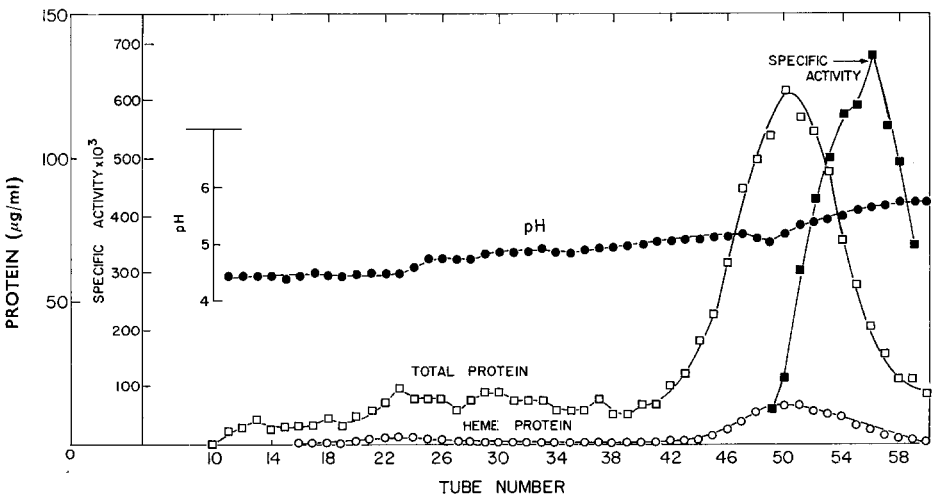

Fig. 1. Profile of the chromatographic separation of ferrihemoglobin-reducing enzyme on a CM-cellulose column. pH was determined with a Metrohm E-388 compensator; total protein was estimated by the method of LOWRY *et al.*[11]. Heme protein content was calculated from the absorption at 407 mμ and based on a molar extinction coefficient of $169 \cdot 10^3$ where 1 M represents 1 gram-atom of iron[31]. The collection of fractions was started with the introduction of the crude enzyme preparation on the top of the column. Specific activity was determined by our enzyme assay.

ration was centrifuged at 10 000 × *g* for 10 min. The clear supernatant was passed through the CM-cellulose column. Elution was performed with the same buffer solution used in the preparation of the column. The flow rate was kept at 2.0 ml per min. Fractions were collected and stored in ice. Fig. 1 demonstrates the profile of a typical chromatographic run.

While the crude enzyme extract with a pH of 6.8 passed through the column, a change in pH from about 4.8 to 5.6 could be measured in the eluted fractions. Two colored bands were evident on the column: a lower brown band and an upper orange-red band. Ferrihemoglobin-reducing activity appeared immediately following the brown band. All active fractions contained small amounts of the tail of the brown band and therefore were slightly yellowish. The color was due to ferrihemoglobin, as could be demonstrated by its typical absorption spectrum. The concentration of heme in the different fractions was determined by measuring absorption at 407 mμ. The orange-red band remained attached to the top of the column throughout the fractionation procedure. This band could be eluted with 0.01 M phosphate buffer (pH 7.0). According to spectral characteristics, its color was due to oxyhemoglobin.

Strict experimental conditions had to be maintained during the fractionation procedure; when the enzyme was exposed to lower pH values (pH < 4.5), it was denatured and remained adsorbed on top of the column. At higher pH values (pH > 6.5) most of the protein passed the column as a single band. The purification of the enzyme seemed to be mainly dependent on the slight pH gradient, which was achieved (probably by the buffer action of the proteins in the preparation).

Many attempts were made to improve the column fractionation procedure. However, three major difficulties prevented the achievement of a better separation: (a) The enzyme would adsorb to the column only at very low ionic strengths (below 0.05 M KCl), making it impossible to use strong buffer solutions to maintain a stable pH gradient. (b) The fractionation occurred within a very small pH range (within about 0.5 of a pH unit). (c) The enzyme was inactivated when exposed to very low salt concentration (below 0.01 M).

Step 4, lyophilization of enzyme protein. The fractions with the highest specific activities were combined and lyophilized. In this way, concentration of the enzyme protein was achieved without significant losses of activity.

No better or further purification of the enzyme was achieved. Precipitations with $(NH_4)_2SO_4$, $Ca_3(PO_4)_2$-gel adsorption, and chromatography on DEAE-cellulose columns were tried without success. Repetition of the CM-cellulose fractionation resulted in higher specific activities, but yields were unsatisfactory. Table II summarizes the purification and recovery data at the various stages for a typical preparation. At the final stage, the enzyme was recovered in a 15–20% overall yield. Final specific activities achieved varied between 700 and 2500 mμmoles ferrihemoglobin reduced per min per mg enzyme protein or a purification of 300–1000-fold.

The crude enzyme preparation

The crude enzyme preparation was a clear, slightly yellowish fluid. It was tested for diaphorase activities using NADH and NADPH in SCOTT's assay system[15]. It was also assayed for the ferrihemoglobin reductase activity in HUENNEKENS' system[2] and for ferrihemoglobin reduction in our ferrocyanide-activated system[8].

TABLE II

PURIFICATION AND RECOVERY

Ferrihemoglobin reduction was measured as described under EXPERIMENTAL. Where active hemolysate was used, the addition of enzyme was omitted.

Fraction	*Vol. (ml)*	*Total protein (mg)*	*Ferrihemoglobin reduction (spec. act.; μmoles/min per mg protein × 10³)*	*Recovery (%)*
Active hemolysate	100	8921*	2.6	100
Crude enzyme preparation	100	36	235	36.5
Eluate from column (best fractions)	20	1.9	2260	18.5

* Based upon hemoglobin determination.

The enzyme preparation catalyzed reduction in all three systems. The ratio of NADH/NADPH activity was different for each of the 3 assays employed. Therefore, it was possible that the crude enzyme preparation contained different enzymes, each of which was measured specifically by one of the 3 assays or one enzyme whose

TABLE III

SPECIFICITY OF ENZYME PREPARATION

Expt. 1 was done by the method of SCOTT[15]; Expt. 2 by our enzyme assay. In Expts. 3–11, the assay was modified. Inactive Dowex hemolysate (containing ferrihemoglobin as the final electron acceptor of the system) was replaced by ferricytochrome *c* (Expts. 5–7), ferrimyoglobin (Expts. 8–10) and ferricyanide (Expt. 11). The final concns. of ferricytochrome *c* and ferrimyoglobin in the reaction mixtures were 0.05 μmoles/ml while ferricyanide was present at a concn. of 0.1 μmoles/ml. Where indicated, methylene blue (0.01 μmoles/ml) replaced ferrocyanide. In all experiments 2.5 μg purified enzyme (specific activity $1000 \cdot 10^{-3}$) was added per ml of reaction mixture. Reduction of cytochrome *c* and myoglobin, and oxidation of pyridine nucleotides (in Expt. 11), were followed at 550 mμ, 575 mμ and 340 mμ using the following molar extinction coefficients: $18.5 \cdot 10^{-3}$ (see ref. 20), $10.5 \cdot 10^{-3}$ and $6.22 \cdot 10^{-3}$ respectively.

Expt. No.	*Procedure*	*Final electron acceptor*	*Activator*	*Activity**	
				With NADH	*With NADPH*
1	Diaphorase	Dichlorophenol-indophenol	None	676	42
2	Enzyme assay	Ferrihemoglobin	Ferrocyanide	4040	0
3	Modified enzyme assay	Ferrihemoglobin	None	0	0
4	Modified enzyme assay	Ferrihemoglobin	Methylene blue	772	272
5	Modified enzyme assay	Ferricytochrome *c*	Ferrocyanide	18 000	0
6	Modified enzyme assay	Ferricytochrome *c*	None	938	470
7	Modified enzyme assay	Ferricytochrom *c*	Methylene blue	2650	2950
8	Modified enzyme assay	Ferrimyoglobin	Ferrocyanide	80	40
9	Modified enzyme assay	Ferrimyoglobin	None	0	40
10	Modified enzyme assay	Ferrimyoglobin	Methylene blue	40	105
11	Modified enzyme assay	Ferricyanide	None	2350	880

* μmoles electrons transported/min per mg protein × 10³; μmoles electrons were obtained by multiplying μmoles acceptor reduced per mg enzyme protein per min × 10³ values by 2, 4, 1, 1, 1, for dichlorophenolindophenol, ferrihemoglobin, cytochrome *c*, myoglobin and ferricyanide respectively.

pyridine nucleotide specificity changed with the assay system used. The crude enzyme preparation, when kept at 4°, was stable for about a week. At −20° there was no loss of activity even during a year. In the absence of ferrocyanide, ferrihemoglobin reduction by NADH was not catalyzed by the crude enzyme preparation.

Properties of the purified enzyme preparation

The following experiments were done on lyophilized enzyme preparations, kept for 1–10 months at −20°. Before use, the protein, whose color varied from a very slight to a deeper yellow, was dissolved in 0.9% NaCl to achieve a concentration of 0.2–1.0 mg/ml. Only preparations with a specific activity of more than $800 \cdot 10^{-3}$ were employed.

Stability. No decrease in ferrihemoglobin reduction activity occurred in lyophilized enzyme preparations kept at −20° for periods up to 1 year. Frozen solutions of the enzyme kept under similar conditions were stable for several months. The presence of salt helped in stabilizing the enzyme. Prolonged dialysis against distilled water caused the complete loss of activity.

Specificity. The enzyme was absolutely specific for NADH, when measured as a ferrihemoglobin reductase (Table III, 2).

When tested with other electron acceptors, the specificity of the enzyme preparation changed with respect to the two pyridine nucleotides. In the dichlorophenol-indophenol–diaphorase assay[15], the enzyme was relatively specific for NADH: with NADPH it had only 6.7% of the activity (Table III, 1). Ferrihemoglobin was not reduced in the absence of ferrocyanide (Table III, 3). Addition of methylene blue in place of ferrocyanide initiated ferrihemoglobin reduction. However, in this case both NADH and NADPH were active. The NADPH/NADH ratio was 0.35 (Table III, 4). If cytochrome *c* was used as the final electron acceptor in place of ferrihemoglobin, other activity ratios were obtained: the enzyme preparation reduced cytochrome *c* in the absence of activators, the NADPH/NADH ratio was 0.5 (Table III, 6). In the presence of methylene blue the ratio changed to 1.1 (Table III, 7). Potassium ferrocyanide caused a marked activation of cytochrome *c* reduction which was then NADH specific (Table III, 5). With ferrimyoglobin as an electron acceptor, direct reduction was observed only with NADPH (Table III, 9). Addition of methylene blue to the latter system rendered it less NADPH specific (Table III, 10), while in the presence of ferrocyanide it had an NADH/NADPH ratio of 2 (Table III, 8).

The enzyme could catalyse the reduction of potassium ferricyanide at high rates by either pyridine nucleotide (Table III, 11).

Affinity. The affinity of the enzyme for the ferrihemoglobin–ferrocyanide complex was rather high. The enzyme was completely saturated at about 55 μM. The concentration giving half-maximal velocity, or apparent K_m, was approximately 8 μM.

Purity. Different batches of purified enzyme were examined for their content of other enzymatic activities and compared with the crude enzyme preparation. Table IV summarizes the results.

The comparative study demonstrated a parallelism between the enzyme activity measured by our enzyme assay and the NADH-diaphorase activity in SCOTT's system. This fact suggested that one enzyme was probably responsible for both activities. A decrease in the NADPH-diaphorase activity with purification was also observed.

While this activity was not detectable in the purest enzyme preparations using HUENNEKENS' assay system, it could still be demonstrated by the more sensitive method of SCOTT. It seems therefore that our method of purification resulted in the loss of most of the NADPH-dependent enzyme.

The enzyme was run in an analytical ultracentrifuge at a protein concentration of 0.2%. A homogeneous symmetrical peak was obtained with a sedimentation constant of $s_{20,w}$, $9.2 \cdot 10^{-13}$. Traces of non-homogenous contaminants of smaller molecular weights accompanied even the purest preparations obtained.

Flavin content. Flavin analyses were made only on one of the purest enzyme preparation achieved (spec. act. $2200 \cdot 10^{-3}$). By paper chromatography according to the method of CRAMMER[13], flavin could not be detected. 200 μl of a trichloroacetic acid extract containing 230 μg of the enzyme protein were applied as a single spot to the filter paper. Standards were run in parallel. The limit of detectability was 0.3 μg for FAD and FMN and 0.5 μg for riboflavin. By the fluorimetric method of BURCH, BESSEY AND LOWRY[12], fluorescence at 530 mμ was obtained, which corresponded to a total flavin content of no more than 50 μg (as riboflavin) for 100 mg of enzyme protein. These data indicate that the preparation contains not more than 1 mole riboflavin per 727 000 g of enzyme, if at all.

Heme content. The concentration of ferrihemoglobin in different preparations of pure enzyme protein varied from 4 to 20%. The amount of the pigment, measured as heme, was found to be in no correlation to the specific activity of the preparation or its protein content. The absence of parallelism between activity and heme content was also evident from analyses run during the purification procedure. Samples, taken at different points along the purification procedure demonstrated the appearance of activity where heme concentration declined (see Fig. 1). These data may be taken as evidence that the pigment responsible for the light absorption at 407 mμ plays no role in the functioning of the enzyme.

pH optimum. The pH optimum for the reduction of ferrihemoglobin by pure

TABLE IV

ENZYME ACTIVITIES IN DIFFERENT BATCHES OF PURIFIED ENZYME

Ferrihemoglobin reduction was determined by our enzyme assay, diaphorase by the method of SCOTT[15]. NADPH-dependent ferrihemoglobin reductase was measured according to HUENNEKENS *et al.*[2].

Fraction	*Ferrihemoglobin reduction**		*Diaphorase***		HUENNEKENS' *reductase****	
	NADH	*NADPH*	*NADH*	*NADPH*	*NADH*	*NADPH*
Crude enzyme	207	0	75	13	10	20
Purified enzyme Batch a	710	0	172	19	13	20
Purified enzyme Batch b	1000	0	338	21	0	3
Purified enzyme batch c	1100	0	326	12	0	0

* Data expressed in μmoles ferrihemoglobin/min per mg enzyme $\times 10^3$.
** Data expressed in μmoles dichlorophenolindophenol/min per mg enzyme $\times 10^3$.
*** Data expressed in μmoles pyridine nucleotide/min per mg enzyme $\times 10^3$.

enzyme in the ferrocyanide-activated system was around 5.2. The shape of the pH-activity curve did not differ from that found for ferricyanide hemolysates[8].

Inhibitors. The action of possible inhibitors, such as *N*-ethylmaleimide, atebrin, *p*-chloromercuribenzoate, bathophenanthroline, carbonylcyanide *m*-chlorophenylhydrazone, on the activity of the purified enzyme was tested. Among these substances and at a concentration of 100 μM, only *p*-chloromercuribenzoate was found to be a potent inhibitor of the enzyme.

Comparison with other diaphorases. NADH diaphorase from *Cl. kluyveri*, NADPH-dependent diaphorase from chloroplasts and our purified enzyme preparation were tested for their activity in reducing ferrihemoglobin as compared with their activity as diaphorases. The catalytic effect of each enzyme in the reduction of ferricyanide by the respective pyridine nucleotide was taken in these experiments as a measure of diaphorase activity. Table V summarizes the results.

TABLE V

THE ACTIVITY OF DIFFERENT DIAPHORASES AS FERRIHEMOGLOBIN–FERROCYANIDE REDUCTASES

Ferrihemoglobin reduction was mesaured by our enzyme assay. Diaphorase activity was determined by modifying the same assay: hemolysate was omitted from the reaction mixture and ferrocyanide replaced by ferricyanide. NADH or NADPH oxidation was measured at 340 mμ. The amounts of enzymes used were rate limiting in all cases.

Enzyme	*Electron donor*	*Diaphorase activity**	*Ferrihemoglobin reduction** (A)*	*B/A ratio* $\times 10^3$ *(B)*
1. NADPH-diaphorase from chloroplasts	NADPH	13 000	330	25
2. NADH-diaphorase from *Cl. kluyveri*	NADH	915	55	60
3. Purified enzyme	NADH	2350	1680	720

* Data expressed in μmoles reduced pyridine nucleotide oxidized/min per mg enzyme protein $\times 10^3$.

** Data expressed in μmoles ferrihemoglobin reduced per min per mg enzyme protein $\times 10^3$.

As could be shown, other diaphorases acted also as ferrihemoglobin reductases. However, among the diaphorases examined, our purified enzyme was by far the best ferrihemoglobin reductase (see B/A ratio). The data in Table V are not considered to be due to an effect of the low pH of 5.2. Thus, at a higher pH (7–9) the NADPH-diaphorase isolated from chloroplasts is known to have a much higher diaphorase activity than at pH 5.2 (ref. 5), while its ferrihemoglobin–ferrocyanide reductase activity is lower under these conditions. It follows that measurements at an higher pH would emphasize the conclusions drawn even more.

The fact that the B/A ratios differ for the three enzymes is an additional argument that potassium ferrocyanide does not act as a mere carrier as was shown earlier[8].

DISCUSSION

It seems fairly well established that in human erythrocytes NADH is the major reductant for ferrihemoglobin. The most important evidence supporting this view

is the accumulation of ferrihemoglobin in red blood cells deficient in an NADH-dependent enzyme having diaphorase activity[15]. As to the participation of NADPH in the reduction of ferrihemoglobin, the information available to date is confusing. There is convincing evidence on the presence of an NADPH-dependent enzyme in human erythrocytes[1,2]. However, direct reduction of ferrihemoglobin by NADPH could be demonstrated in hemolysates only at extremely low rates[3]. Moreover, the fact that ferrihemoglobin does not accumulate in red blood cells deficient in glucose-6-phosphate dehydrogenase[16], and therefore in NADPH, is definite evidence that an alternative route of reduction exists. This route must be sufficiently effective to keep practically all the hemoglobin in its reduced state. The role of other reduction mechanisms in human erythrocytes seems to be minimal[3,17,18].

The present information on enzyme systems capable of promoting ferrihemoglobin reduction in human erythrocytes can be summarized as follows: (a) The methemoglobin reductase purified by HUENNEKENS *et al.*[2], was found to be NADPH specific. Thus it was suggested by the author that ferrihemoglobin reduction in the intact cell is brought about by the reduced form of this pyridine nucleotide produced in the glucose-6-phosphate dehydrogenase shunt. However, in order to activate ferrihemoglobin reduction, the purified enzyme had an absolute requirement for methylene blue. Since reduced methylene blue non-enzymatically reduces ferrihemoglobin, HUENNEKENS' enzyme cannot reasonably be considered as more than a NADPH-diaphorase. The same methylene blue dependency was observed also with the methemoglobin reductase purified by KIESE, SCHNEIDER AND WALLER[1], except that with crude preparations these investigators could measure some ferrihemoglobin reduction induced by NADPH. These findings can be interpreted as indicating that either ferrihemoglobin is reduced by other enzymes, different from those of HUENNEKENS and KIESE, or that an unknown factor is lost during the purification procedures used.

(b) The "NADH dehydrogenase" of SCOTT, DUNCAN AND EKSTRAND[3] is the only enzyme for which good evidence exists to support its participation in ferrihemoglobin reduction *in vivo*[15]. It is also the only enzyme purified from human erythrocytes which seems to catalyze the reduction of ferrihemoglobin by NADH directly[3]. However, its efficiency as a ferrihemoglobin reductase is rather poor. As was shown by SCOTT himself, the activity of this enzyme in reducing ferrihemoglobin was only $1 \cdot 10^{-4}$ of its diaphorase activity[4].

The properties of the enzyme purified by us show some similarities with the NADH dehydrogenase 1 (NADH-diaphorase) isolated by SCOTT, DUNCAN AND EKSTRAND[3]: (1) Specific activities measured at different purification steps by our enzyme assay paralleled specific activities obtained in SCOTT's assay system (see Table IV). (2) The pyridine nucleotide specificity (NADPH/NADH activity ratio), when measured by the dichlorophenolindophenol reduction assay, was similar to that given by SCOTT[15]. Our procedure resulted generally in a 1000-fold purification of the enzyme. The protein seemed fairly homogeneous according to ultracentrifugal experiments. However, the presence of heme and traces of other proteins observed in the ultracentrifugal run indicates that at least 10% of the protein was a contaminant.

No conclusive evidence is available regarding the prosthetic group of the enzyme. It was suggested by GIBSON[19] that the NADH-dependent enzyme involved in ferrihemoglobin reduction is a flavoprotein. This hypothesis was based on the

observation that Straub's diaphorase activated ferrihemoglobin reduction in hemolysates from a patient with congenital ferrihemoglobinemia of the enzyme deficiency type. SCOTT AND MCCRAW[4] supported this view by demonstrating that the FAD content increased with purification of their NADH-diaphorase. KIESE, SCHNEIDER AND WALLER[1] presented evidence that the NADPH-dependent enzyme was a flavoprotein, while HUENNEKENS *et al.*[2] thought that he had sufficient information to prove that the enzyme purified by him was a heme protein. The data presented in this study suggest that the heme content of the enzyme was an impurity (found generally in ferrihemoglobin reductases isolated from erythrocytes). On the other hand, no reliable data could be obtained on flavins as being part of the purified enzyme. The lack of inhibition of the ferrihemoglobin reduction by EDTA suggests that no common metal is involved in the action of the enzyme.

The relatively low pH optimum of the enzyme is surprising. It may be of interest to note that it is of general practice to store blood for longer periods at a pH lower than physiological. This may be related to the fact that under these conditions ferrihemoglobin formed will be reduced rapidly to hemoglobin.

The enzyme seems to be absolutely specific for NADH when assayed with ferrihemoglobin–ferrocyanide (or with cytochrome *c*–ferrocyanide). When assayed with other electron acceptors (dichlorophenolindophenol, methylene blue(O_2), ferricyanide), activity could be measured also with NADPH. This could be interpreted as indicating: (1) The purified preparation contained two enzymes both active as diaphorases, but only the NADH specific one reacting with ferrihemoglobin–ferrocyanide. (2) Only one diaphorase exists, but it is a pyridine nucleotide–enzyme complex which is the active reducing agent. Only the NADH–enzyme complex can interact with ferrihemoglobin while both can interact with dichlorophenolindophenol, methylene blue(O_2) or ferricyanide. If we assume that our enzyme and SCOTT's NADH–diaphorase are identical, then the fact that the ratio of activity with NADPH/NADH changed with purification would favor the first alternative.

The purified enzyme catalyzed the reduction of cytochrome *c* directly (without the addition of an activator) at a considerable rate. Therefore, it is best defined as a cytochrome-*c* reductase.

If the enzyme is indeed responsible for the reduction of ferrihemoglobin *in vivo* it must be assumed that an activator (or a second active site on the enzyme) is lost during purification. The fact that the NADH diaphorase isolated by SCOTT has ferrihemoglobin reductase activity may be due to the presence of some activator in the system used by this investigator.

It is obvious from the foregoing that our enzyme, as all others described to date, is not capable of reducing ferrihemoglobin directly at significant rates. However, since potassium ferrocyanide activation cannot, in our view, be considered as an electron-carrier effect (like methylene blue activation)[8], this seems to be the only enzyme described which can rapidly reduce ferrihemoglobin, although only when the pigment is in a slightly modified form. The potassium ferrocyanide requirement for such reduction is most reasonably explained in terms of a change in the ferrihemoglobin structure, which permits the enzyme to approach the site of reduction much more freely.

One of the main problems in the investigation of ferrihemoglobin reduction results from the fact that the final electron acceptor (ferrihemoglobin) and the enzyme

system catalyzing its reduction are present together in hemolysates. In order to be able to study intermediates involved in the electron transfer, the separation of hemoglobin from the reducing system appears necessary.

It was demonstrated that purification of hemoglobin by selective adsorption on DEAE-cellulose resulted in a preparation which was free of NADH- and NADPH-dependent diaphorase activities, as measured by the method of SCOTT, by methylene blue reduction and by the ferrocyanide-activated ferrihemoglobin reduction assay.

It is considered, therefore, that the hemoglobin thus produced is a suitable substrate in the investigation of enzymatic ferrihemoglobin reduction.

REFERENCES

1 M. KIESE, C. SCHNEIDER AND H. D. WALLER, *Arch. Exptl. Pathol. Pharmakol.*, 231 (1957) 158.
2 F. M. HUENNEKENS, R. W. CAFFREY, R. E. BASFORD AND B. W. GABRIO, *J. Biol. Chem.*, 227 (1957) 261.
3 E. M. SCOTT, J. W. DUNCAN AND V. EKSTRAND, *J. Biol. Chem.*, 240 (1965) 481.
4 E. M. SCOTT AND J. C. MCGRAW, *J. Biol. Chem.*, 237 (1962) 249.
5 M. AVRON AND A. T. JAGENDORF, *Arch. Biochem. Biophys.*, 65 (1965) 475.
6 M. AVRON AND A. T. JAGENDORF, *Arch. Biochem. Biophys.*, 72 (1957) 17.
7 M. A. HENNESSEY, A. M. WALTERSDORPH, F. M. HUENNEKENS AND B. W. GABRIO, *J. Clin. Invest.*, 41 (1962) 1257.
8 E. HEGESH AND M. AVRON, *Biochim. Biophys. Acta*, 146 (1967) 91.
9 L. HEILMEYER, in *Spectrophotometry in Medicine*, Adam Hilger, London, 1943, p. 97.
10 R. K. CANNAN, *Am. J. Clin. Pathol.*, 30 (1958) 211.
11 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
12 H. B. BURCH, O. A. BESSEY AND O. H. LOWRY, *J. Biol. Chem.*, 175 (1948) 457.
13 J. C. CRAMMER, *Nature*, 161 (1948) 349.
14 D. L. DRABKIN, *J. Biol. Chem.*, 185 (1950) 231.
15 E. M. SCOTT, *J. Clin. Invest.*, 39 (1960) 1176.
16 E. BEUTLER, M. ROBSON AND E. BUTTENWEISER, *J. Clin. Invest.*, 37 (1957) 617.
17 C. A. FINCH, *New Engl. J. Med.*, 239 (1948) 470.
18 W. THAL AND L. LACHHEIN, *Klin. Wochschr.*, 39 (1961) 1022.
19 Q. H. GIBSON, *Biochem. J.*, 42 (1948) 13.
20 E. MARGOLIASH, *Biochem. J.*, 56 (1954) 535.
21 D. L. DRABKIN, in J. E. FALK, R. LEMBERG AND R. K. MORTON, *Haematin Enzymes*, Pergamon, Oxford, 1961, p. 142.