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CHARACTERIZATION OF GLUTATHIONE REDUCTASE FROM PORCINE ERYTHROCYTES

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Summary

Glutathione reductase (NAD(P)H : oxidized-glutathione oxidoreductase, EC 1.6.4.2) was purified to homogeneity from porcine erythrocytes by use of affinity chromatography on 2',5'-ADP-Sepharose 4-B. Analytical ultracentrifugation experiments were analysed to give the following physical parameters for the enzyme: $s_{20,w} = 5.7$ S, $D_{20,w} = 50\ \mu m^2/s$, and $M_w = 103\ 000$ (protein concentration, 0.5 mg/ml). The frictional ratio was 1.37 and the Stokes radius was 4.3 nm. The enzyme molecule is a dimer composed of subunits of equal size each containing a FAD molecule. The amino acid compositions and circular dichroism spectra of the porcine and human enzymes indicated extensive structural similarities. The isoelectric point was at pH 6.85 (at 4°C). The absorption spectrum of the oxidized enzyme had maxima at 377 and 462 nm. In vivo the enzyme appears to be partially reduced. At a physiological concentration of reduced glutathione the apparent Michaelis constants for glutathione disulfide and NADPH were higher than in the absence of reduced glutathione. At 0.15 M ionic strength the catalytic activity obtained with NADPH as reductant was optimal at pH 7 and more than 200 times higher than that obtained with NADH. *S*-sulfoglutathione and some mixed disulfides of glutathione were poor substrates with the exception of the mixed disulfide of coenzyme A and reduced glutathione. The purified enzyme displayed low transhydrogenase activity with oxidized pyridine nucleotide analogs and diaphorase activity with 2,6-dichlorophenolindophenol as acceptor substrates; both NADPH and NADH served as donors.

Introduction

Glutathione reductase (NAD(P)H : oxidized-glutathione oxidoreductase, EC 1.6.4.2) has a key role in the maintenance of cellular glutathione in its reduced

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form. The importance of the enzyme is due to the absolute requirement of reduced glutathione for the normal functioning of the cell. Reduced glutathione contributes to the stability of erythrocytes by counteracting oxidative events and protecting sulfhydryl groups of intracellular proteins. The NADPH required for reduction of glutathione disulfide in the erythrocyte is provided by the initial steps of the hexose monophosphate pathway involving glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase [1]. Although the enzyme is most active with NADPH as a reductant, it has also some activity with NADH. The earlier suggestion that the erythrocyte has two distinct glutathione reductases, one dependent on NADPH and one on NADH for activity, has been refuted [2]. The enzyme also lacks absolute specificity towards disulfides [3]. In combination with thioltransferase [4] it will effect the reduction of naturally occurring low-molecular-weight disulfides and thiosulfate esters [3,5,6] as well as mixed disulfides of reduced glutathione and proteins [7].

Glutathione reductase has been purified from a wide variety of sources. The successful application of the affinity matrix 2′,5′-ADP-Sepharose 4-B for the purification of erythrocyte glutathione reductase [8] has made possible the preparation of homogeneous enzyme from porcine blood, which is a source available in large quantities. The present paper reports the characterization of the purified porcine enzyme. Some of the results have been presented [9].

Materials and Methods

Chemicals. The mixed disulfides of coenzyme A and reduced glutathione [10] and L-cysteine and reduced glutathione [11] as well as *S*-sulfoglutathione [12] were synthesized as earlier described. The mixed disulfide of cysteamine and reduced glutathione was prepared by treatment of reduced glutathione with an excess of cystamine; the product was purified by ion-exchange chromatography. The purity of the above glutathione sulfenyl derivatives was verified by paper electrophoresis. The mixed disulfide of egg-white lysozyme and reduced glutathione was synthesized as described by Axelsson and Mannervik [13]. All other chemicals used were standard commercial products of high purity.

Assay of enzymatic activities

The rate of oxidation of NADPH by oxidized glutathione at 30°C was used as the standard assay of glutathione reductase activity. The composition of the reaction system was that described by Worthington and Rosemeyer [14]. Transhydrogenase activity was measured at 30°C in 0.1 M potassium phosphate buffer (pH 7.0), 1 mM EDTA, by observing the formation of reduced 3-acetylpyridine adenine dinucleotide phosphate at 375 nm [15] and reduced thio-$NADP^+$ at 395 nm [16], respectively. The nucleotides (donors as well as acceptors) were normally present at 0.1 mM.

Estimation of molecular weight by gel filtration

Estimation of molecular weight of glutathione reductase was carried out by gel filtration on a Sephadex G-150 (Superfine) column (1.5 × 95 cm). A sample (2% of the bed volume) containing porcine erythrocyte glutathione reductase,

horse liver alcohol dehydrogenase (80 000 daltons), rabbit muscle lactate dehydrogenase (140 000 daltons), pig heart malate dehydrogenase (70 000 daltons), carbonic anhydrase (30 000 daltons) was applied to the column. The column was developed with 10 mM Tris-HCl buffer (pH 7.5), 0.1 M KCl, 1 mM EDTA, 1 mM dithioerythritol. The reference proteins were identified in the eluate by use of standard assay methods.

Analytical ultracentrifugation

Ultracentrifugation was carried out on a Beckman model E analytical centrifuge by Dr. H. Pertoft, University of Uppsala. The rotor speeds were 52 000 and 14 000 rev./min for determination of sedimentation coefficient and molecular weight, respectively. The molecular weight was determined by the method of long-column meniscus depletion using interference optics. The concentration of enzyme used was 0.5 mg/ml. The partial specific volume of the enzyme was 0.73 ml/g as calculated from the amino acid composition.

Determination of isoelectric point. Isoelectric focusing was performed at 4°C in a column (model 8101, LKB Produkter) according to the instruction of the manufacturer. Ampholytes of 1% concentration (pH 5—8) were used to create the pH-gradient. Measurements of pH were carried out at 4°C with a pH-meter calibrated against standard buffer solutions at this temperature.

Amino acid analysis. Amino acid analysis was carried out on a Durrum D-500 amino acid analyzer by Dr. D. Eaker, University of Uppsala. 0.1-mg samples were hydrolyzed for 24 and 72 h. Norleucine was used as an internal standard.

Preparation of apoenzyme and restoration of enzyme activity. Apoenzyme was prepared according to the procedure of Icén [17]. Restoration of activity was tried at different FAD and FMN concentrations at 0° C and 30° C.

Recording of spectra. Spectra were recorded on a Beckman model 25 spectrophotometer under aerobic conditions. Circular dichroic spectra were recorded on a Jasco J-41A spectropolarimeter by Dr. T. Sjödin, University of Uppsala. The instrument was calibrated with D-10-camphorsulfonic acid.

Kinetic analysis. An analysis of the steady state kinetics of the purified glutathione reductase was carried out on initial velocities which were determined on an Aminco DW-2 dual wavelength spectrophotometer. Fitting of rate equations to the experimental data was done with a Gauss-Newton nonlinear regression program (cf. ref. 18). Criteria for discrimination between alternative rate equations and for judgement of goodness-of-fit have been given earlier [19,20].

Results and Discussion

Physical properties of the purified porcine glutathione reductase

Glutathione reductase from porcine erythrocytes was obtained in homogeneous form with a specific activity of 230 units/mg. A crucial technique in the purification procedure (Boggaram, V., Larson, K. and Mannervik, B., unpublished data) was affinity chromatography on 2′,5′-ADP-Sepharose 4-B (cf. ref. 8), which largely solved the difficult problem of separating the enzyme from hemoglobin.

The molecular weight of the enzyme was estimated by gel filtration to be 116 800 ± 800 (± S.D., $n = 3$). Sodium dodecyl sulfate polyacrylamide gel electrophoresis demonstrated a band corresponding to a molecular weight of 59 000 ± 600 (± S.D., $n = 3$), indicating a dimeric structure of glutathione reductase composed of two similar or identical subunits. A peptide map of a tryptic digest of yeast glutathione reductase indicated that the two subunits of this enzyme are identical [21].

Analytical ultracentrifugation showed that the enzyme was homogeneous within the limitations of analysis by the ultraviolet and Schlieren optics. The $s_{20,w}$ was 5.7 S at a protein concentration of 0.5 mg/ml in 5 mM sodium phosphate (pH 7.0) containing 0.1 mM dithiothreitol and 0.1 mM EDTA. The molecular weight, determined at the same concentration, was $M_w = 103\,000$ (υ calculated to be 0.73 ml/g). A plot of log c vs. r^2 was linear (correlation coefficient 0.999). The frictional ratio f/f_0 was calculated to be 1.37, corresponding to an axial ratio of about 7 assuming no hydration. The diffusion coefficient was estimated as 50 $\mu m^2/s$. The Stokes radius was calculated as 4.3 nm. The asymmetry expressed in the frictional ratio is consistent with the structure determined by X-ray crystallography [22]. The nonspherical (prolate) shape of the enzyme molecule is evidently also the explanation of a somewhat too high value of the molecular weight obtained by gel filtration. The molecular weights of the human erythrocyte enzyme and its subunits have been reported as 100 000 and 50 000, respectively [23], and it thus appears as if the porcine enzyme has a physical structure which is very similar to that of the human enzyme.

The isoelectric point of glutathione reductase from porcine erythrocytes was at pH 6.85 (at 4°C) as determined by isoelectric focusing. This value is very close to the isoelectric point of hemoglobin and explains the great difficulties experienced in separating porcine glutathione reductase and hemoglobin by ion-exchange chromatography and other techniques, which are based on electrochemical properties. The isoelectric point of rat liver glutathione reductase is also somewhat below pH 7 [6], but a notable difference is found in the isoelectric point of the enzyme from yeast (*Saccharomyces cerevisiae*) which is at pH 4.9 (4°C) [24].

The amino acid composition of glutathione reductase from porcine erythrocytes is given in Table I. The data for the human enzyme are given for comparison. It is evident that the amino acid compositions are similar although notable discrepancies appear for serine and methionine. The difference index of Metzger et al. [26] was 4.7 for human vs. porcine glutathione reductase indicating a high degree of sequence similarity.

Spectral properties

The spectrum of the purified enzyme was very similar to spectra previously recorded (see refs. 14 and 27). Absorbance maxima were obtained at 377 and 462 nm; minima at 325 and 405 nm. The absorbance ratio A_{280nm}/A_{462nm} was 6.5 (uncorrected for light scattering). These data refer to enzyme with flavin in its oxidized form. However, in the living cell the enzyme encounters reductants which may affect the redox state of the flavin. Both reduced glutathione and NADPH have been found to reduce the enzyme [28,29]. The

TABLE I

AMINO-ACID COMPOSITIONS OF GLUTATHIONE REDUCTASE FROM PORCINE AND HUMAN ERYTHROCYTES

The values for the porcine enzyme are means ± S.D. of 5 separate preparations, and are calculated for a molecular weight of 103 000. Those of the human enzyme [23] are for a molecular weight of 100 000.

Amino acid	Enzyme	
	Porcine	Human
Asx	77.2 ± 1.4	74
Thr	60.4 ± 2.9 *	56
Ser	78.8 ± 11.8 *	54
Glx	95.5 ± 7.0	86
Pro	40.9 ± 3.9	42
Gly	97.3 ± 7.2	88
Ala	77.3 ± 4.5	80
Cys	24.1 ± 2.7 **	20
Val	86.8 ± 1.6	88
Met	15.9 ± 3.2	32
Ile	55.2 ± 3.1	56
Leu	72.9 ± 6.1	66
Tyr	24.4 ± 1.4	24
Phe	27.7 ± 3.1	26
His	31.6 ± 3.7	30
Lys	65.7 ± 1.8	66
Arg	37.6 ± 3.9	30
Trp	5.5 ***	8

* Determined by extrapolation to zero time.

** Determined as cysteic acid after performic acid oxidation ($n = 2$).

*** Determined separately according to [25] ($n = 1$).

concentrations of reduced glutathione and NADPH in human erythrocytes are reported to be about 2 mM [30] and 0.03 mM [31], respectively. The effect on the spectrum of adding physiological concentrations of these reductants to the enzyme under aerobic conditions was investigated. Reduced glutathione caused a partial reduction of the flavin which was somewhat augmented by subsequent addition of NADPH. A physiological concentration of $NADP^+$ ($\leqslant 10\,\mu M$) had no significant influence on this reduced-flavin spectrum. Since the concentration of glutathione disulfide in erythrocytes is very low [30] and insufficient to reoxidize the enzyme in the presence of NADPH, the results indicate very strongly that in vivo glutathione reductase is maintained in a partially reduced form. This conclusion is not limited to erythrocytes, but is also applicable to other cells. The participation of partially reduced enzyme as an intermediate in the catalytic mechanism has been discussed (cf. ref. 32), but the identification of a reduced form as the most abundant species under steady-state conditions in vivo has to our knowledge not previously been made.

Flavin content

The flavin of the enzyme was FAD as judged from reactivation studies on the apoenzyme. The apoenzyme had a residual activity of 3.8% of the holoenzyme and the activity rose to 72% after treatment with 5 μM FAD at 30°C. FMN had no stimulatory effect on the apoenzyme.

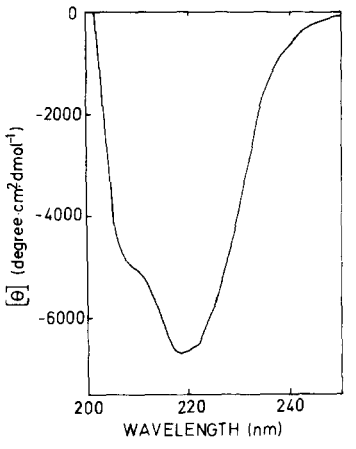

Fig. 1. Circular dichroism spectrum of glutathione reductase from porcine erythrocytes. The mean residue ellipticity, [θ], was calculated on the basis of an enzyme concentration determined from an assumed optical absorbance of $A^{0.1\%}_{280nm}$ = 1.35 (per cm) [14,23].

On the basis of an extinction coefficient of 11.3 $mM^{-1} \cdot cm^{-1}$ at 462 nm [29] for the flavin and determination of protein concentration by the absorbance at 280 nm ($A^{0.1\%}_{280nm}$ = 1.35 [14,23]) a value of 2 mol FAD per mol enzyme (103 000 daltons) was determined.

Circular dichroic spectrum

The circular dichroic spectrum of glutathione reductase purified from porcine erythrocytes was recorded in the far-ultraviolet region (Fig. 1). The corresponding spectrum of the human erythrocyte enzyme was also obtained and appeared to be very similar. Thus, a far-reaching degree of similarity between the secondary structures of the porcine and human enzymes may be predicted. The fractions of helical structure, β-structure, and unordered ('random') structure of the porcine enzyme were estimated to be 15, 15 and 70%, respectively, as calculated according to [33]. However, only the data in the range of 215—240 nm were used because inclusion of the values at shorter wavelengths destroyed the fit. Calculations based on a larger part of the spectrum gave somewhat lower values (about 10%) of helix and β-structure. These estimates are apparently very unreliable, because a recent X-ray diffraction analysis of the human enzyme showed that 31% and 28% of the amino acid residues are in helices and β-sheets, respectively [34].

Steady-state kinetics

Initial velocities were determined with variable concentrations of glutathione disulfide and NADPH. In a v vs. v/[S] plot the lines corresponding to different fixed concentrations of the nonvaried substrate converged in a point on the v/[S]-axis (Fig. 2A). This result is expected for a ping-pong mechanism [35] and corresponds to the kinetic pattern previously obtained with glutathione reductase from other sources [27,29,36,37]. However, at high oxidized glutathione concentrations deviations from this simple pattern appears, which in combination with product-inhibition studies indicate a branching mechanism

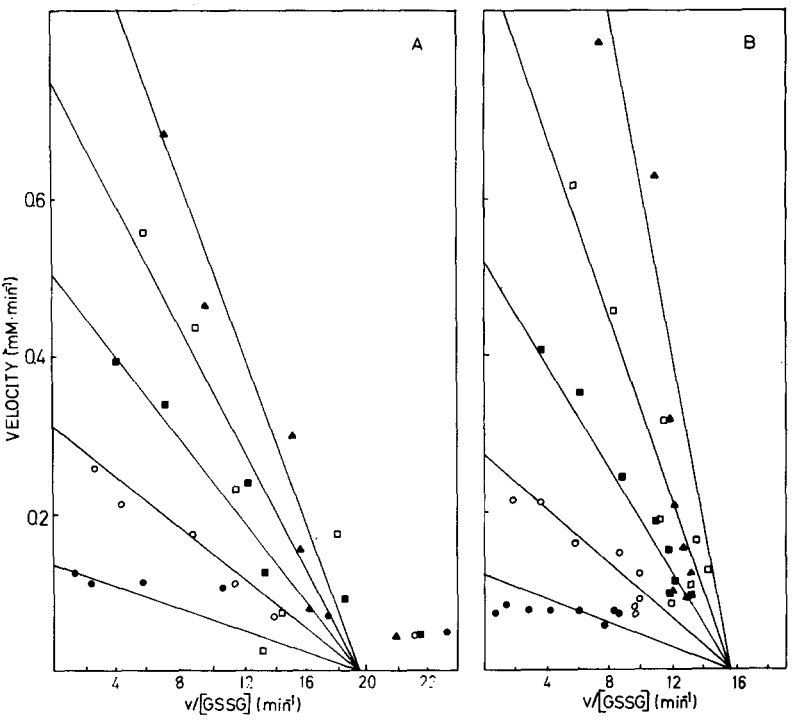

Fig. 2. Dependence of initial velocity on substrate concentration in the absence (A) and presence (B) of reduced glutathione. The straight lines were computed under the assumption that the rate equation of a simple ping-pong mechanism is applicable. The concentrations used were (A) [NADPH]: 2.2 (●); 5.4 (○); 10.9 (■); 21.9 (□); 54.7 μM (▲); and [reduced glutathione]: zero; [glutathione reductase]: 17.1 nM (34.2 nM flavin). (B) [NADPH]: 2.4 (●); 5.9 (○); 11.9 (■); 23.7 (□); 59.3 μM (▲); and [reduced glutathione]: 2.15 mM; [glutathione reductase]: 30.5 nM (60.9 nM flavin). GSSG = glutathione disulfide.

composed of a ping-pong and a sequential pathway as first proposed for the yeast enzyme [38—40]. When the glutathione disulfide concentration is low the rate law of the branching mechanism may degenerate to that of a simple ping-pong mechanism:

$$v = \frac{V[\mathrm{GSSG}][\mathrm{NADPH}]}{K_m^{\mathrm{GSSG}}[\mathrm{NADPH}] + K_m^{\mathrm{NADPH}}[\mathrm{GSSG}] + [\mathrm{GSSG}][\mathrm{NADPH}]}$$

This equation is probably applicable for the conditions in vivo, in which the GSSG level is low, and provides a reasonable approximation of the data presented in Fig. 2. The parameters obtained by fitting the above equation to data in the substrate-concentration domain bounded by [NADPH]: 2—60 μM and [GSSG]: 2—100 μM were:

V = 21 700 ± 1400 μmol/min per μmol flavin;

K_m^{GSSG} = 76 ± 8 μM;

K_m^{NADPH} = 21 ± 2 μM.

However, in the intact erythrocytes reduced glutathione will affect the kinetics and the same experiment and analysis were consequently carried out in the

presence of 2 mM reduced glutathione to simulate in vivo conditions (Fig. 2B). The apparent constants obtained were:

$V = 29\,800 \pm 4100$ μmol/min per μmol flavin;

$K_m^{GSSG} = 231 \pm 37$ μM;

$K_m^{NADPH} = 71 \pm 11$ μM.

Thus, the effective concentrations of oxidized glutathione and NADPH required to reach half-maximal velocity are increased in the presence of a physiological concentration of reduced glutathione.

pH-dependence and specificity towards reduced pyridine nucleotides

The specificity towards NADPH and NADH was tested as a function of pH at constant glutathione disulfide concentration and physiological ionic strength of 0.15 M (Fig. 3). In the physiological pH range NADPH gave more than 200 times the activity obtained with NADH; the optimal activity was at pH 7. The activity obtained with NADH was optimal at about pH 5. No indication for a separate NADH-dependent glutathione reductase has been obtained in the present investigation.

Activity with mixed disulfides of glutathione and with S-sulfoglutathione

Experiments on the activity of purified porcine glutathione reductase with glutathione *S*-sulfenyl derivatives included *S*-sulfoglutathione and mixed disulfides of coenzyme A and reduced glutathione, cysteine and reduced glutathione, cysteamine and reduced glutathione, and lysozyme and reduced glutathione. Previously, the purified rat liver enzyme has been shown to be active with some of these compounds [27] and the results obtained with the porcine enzyme were not significantly different from those obtained with the rat liver enzyme. The mixed disulfide of coenzyme A and reduced glutathione was next to glutathione disulfide the best substrate. Like glutathione reductase

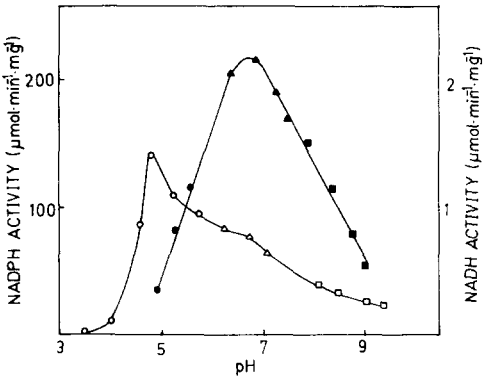

Fig. 3. pH vs. activity profiles of glutathione reductase from porcine erythrocytes. The activity was measured with 1 mM glutathione disulfide and 0.1 mM NADPH (filled symbols) or NADH (open symbols). The following buffers were used in 20 mM concentration: sodium acetate/acetic acid (●,○); Na_2HPO_4/NaH_2PO_4 (▲,△); Tris-HCl (■,□). The ionic strength in the assay system was maintained at 0.15 M by addition of appropriate amounts of KCl.

TABLE II

TRANSHYDROGENASE ACTIVITY OF PURIFIED GLUTATHIONE REDUCTASE FROM PORCINE ERYTHROCYTES

The nucleotides were used at a concentration of 0.1 mM. The activity is expressed as % of the activity obtained with glutathione disulfide and NADPH as substrates in the standard assay system.

Reaction	Activity (%)
Thio-$NADP^+$ + NADPH → thio-NADPH + $NADP^+$	0.33
Thio-$NADP^+$ + NADH → thio-NADPH + NAD^+	0.068
3-Acetylpyridine-ADP^+ + NADPH → 3-acetylpyridine-ADPH + $NADP^+$	0.12
3-Acetylpyridine-ADP^+ + NADH → 3-acetylpyridine-ADPH + NAD^+	0.23
3-Acetylpyridine-AD^+ + NADPH → 3-acetylpyridine-ADH + $NADP^+$	<0.001
3-Acetylpyridine-AD^+ + NADH → 3-acetylpyridine-ADH + NAD^+	<0.003

from yeast and rat liver [13] the porcine enzyme had no detectable activity with the mixed disulfide of lysozyme and reduced glutathione. As in the case of yeast and rat liver [3,6,24], no evidence for a separate 'CoASSG reductase' in erythrocytes has been obtained.

Transhydrogenase activity

Purified glutathione reductase from porcine erythrocytes was assayed with three oxidized pyridine-nucleotide analogs for transhydrogenase activity with NADPH and NADH as reductants (Table II). The oxidation of reduced pyridine nucleotides in the absence of added electron acceptor was negligible in comparison with the transhydrogenase or diaphorase activities. The $NADP^+$ analogs gave low transhydrogenase activities (<1% of the activity with glutathione disulfide and NADPH as substrates) whereas the NAD^+ analog was essentially inactive under the conditions investigated. NADPH, but not NADH, displayed a pronounced substrate inhibition. Use of 10 μM NADPH instead of 0.1 mM, which was used in the measurements reported in Table II, doubled the initial velocity with acetylpyridine adenine dinucleotide phosphate as acceptor substrate and gave a 4.3-fold increase in the case of thio-$NADP^+$. Transhydrogenase activity has previously been demonstrated with thio-$NADP^+$ and glutathione reductase from yeast [41], rat liver and calf liver (Carlberg, I. and Mannervik, B., unpublished data). The transhydrogenase activity of glutathione reductase could be predicted on the basis of the branching reaction scheme for which separate binding sites for glutathione disulfide and pyridine nucleotide were postulated [38]. The structure determined by X-ray diffraction analysis corroborates the assumption of topographically distinct sites for glutathione disulfide and NADPH, and establishes their locations on separate sides of a subunit of the enzyme [22,34]. The demonstration of transhydrogenase activity shows that the pyridine-nucleotide-binding cavity of the enzyme has catalytic activity per se. It is suggested that the transfer of reducing equivalents between the pyridine nucleotides in the transhydrogenase reaction is mediated by the redox-active FAD of the enzyme. By analogy it is postulated that also lipoamide dehydrogenase, which has a corresponding transhydrogenase activity [42], has a catalytically active pyridine-nucleotide-binding site, which is distinct and remote from the lipoamide binding site. A corresponding postulate is also made for the related thioredoxin reductase.

Diaphorase activity

Diaphorase activity of the purified enzyme was measured with 2,6-dichlorophenolindophenol as electron acceptor [43]. The activities with 0.1 mM NADPH and NADH were 0.60 and 0.18%, respectively, of the activity obtained with glutathione disulfide and NADPH in the standard assay system. In distinction from DT-diaphorase [43] glutathione reductase was not inhibited by dicoumarol. Human erythrocyte glutathione reductase has previously been found to have diaphorase activity [36,44], but judging from the specific activities reported the enzyme had not been purified to homogeneity in the earlier studies. The diaphorase activity demonstrated with the homogeneous porcine glutathione reductase in the present investigation, thus, proves that the diaphorase activity is an endogenous property of glutathione reductase and not the result of contamination by extraneous material.

Apart from the characterization of the porcine enzyme, which in many properties resembles glutathione reductase from other sources, the present investigation has approached the physiological conditions more closely than previous studies. Thus, it has been found that the enzyme most probably exists in a partially reduced state in vivo. The effective kinetic constants of the steady-state rate law have been determined at a physiological reduced glutathione concentration, and the specificity for pyridine nucleotides, previously found to be highly dependent on the salt concentration [17,36], has been studied at a physiological ionic strength. By extending such studies the properties relevant in vivo can be delineated step by step and a more complete picture of the biological role of glutathione reductase will emerge.

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