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PURIFICATION AND PROPERTIES OF GLUTATHIONE REDUCTASE OF HUMAN ERYTHROCYTES

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SUMMARY

1. Human erythrocyte glutathione reductase (NAD(P)H:oxidized glutathione oxidoreductase, EC 1.6.4.2) was purified 47 000-fold by column chromatography. The enzyme contained FAD as the prosthetic group. From the flavin content a minimum mol. wt. of 56 600 was calculated.

2. The mol. wt. was determined by gel filtration with Sephadex G-200 and was found to be 115 000 ± 4000. Blue dextran-2000 was not usable to determine the void volume of the column as it interacted with glutathione reductase.

3. The K_m value for FAD binding to the apoenzyme was temperature dependent. The Arrhenius plot of the rate of recombination was nonlinear. Restoration of full activity occurred at least through several steps.

4. Preincubation of the apoenzyme with FMN retarded the restoration of the activity with FAD. Full restoration of the activity did not guarantee that the original enzyme conformation had been acquired.

INTRODUCTION

Glutathione reductase (NAD(P)H:oxidized glutathione oxidoreductase, EC 1.6.4.2) catalyzes the reaction GSSG + NADPH + $H^+ \leftrightharpoons$ 2 GSH + $NADP^+$; the enzyme is also active although less effective with NADH. MELDRUM AND TARR[1] were the first to report the reduction of GSSG by NADPH catalyzed by erythrocyte preparations. FRANCOEUR AND DENSTEDT[2] demonstrated that both NADPH and NADH were effective as coenzymes in human hemolysates. SCOTT *et al.*[3] described a 18 000-fold purified enzyme from erythrocytes while more recently ICÉN[4] reported a 31 000-fold purification. Glutathione reductase obtained from other sources[5–8] has been reported to be a flavoprotein with FAD as the prosthetic group; the enzyme from human erythrocytes is not different in this repect[3,4].

MASSEY AND WILLIAMS[9] and MASSEY *et al.*[10] pointed out the similarities between enzymes catalyzing the reduction of S–S bridges. Recent studies in our laboratory[11]

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

have shown that the molecular weight of the apoenzyme of lipoamide dehydrogenase is half that of the holoenzyme; furthermore it was possible to modify lipoamide dehydrogenase by different treatments and to affect the catalytic properties.

In order to explain the decreased glutathione reductase activity in the erythrocytes of a patient with a hemolytic anemia[12], the normal as well as abnormal enzyme has been purified and characterized with respect to chemical and enzymatic properties. This paper deals with the enzyme obtained from normal human erythrocytes.

MATERIALS AND METHODS

Chemicals

NADPH, $NADP^+$, NADH and NAD^+ were obtained from the Sigma Chemical Co. and from Boehringer and Soehne; GSSG, GSH, FAD, FMN and riboflavin from Boehringer and Soehne. DEAE-Sephadex A-50, capacity 3.5 ± 0.5 mequiv/g, particle size 40–120 μ; CM-Sephadex C-50, capacity 4.5 ± 0.5 mequiv/g, particle size 40–120 μ; and Sephadex G-200 were purchased from Pharmacia, Uppsala. Calcium phosphate gel columns were prepared according to MASSEY[13]. Human erythrocytes were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands.

Determination of activity

The assays were carried out at 25° (with a Zeiss spectrophotometer PMQ II in combination with a Photovolt recorder model 43) by following the change in absorbance at 340 nm due to oxidation of NADPH or NADH with GSSG as an acceptor. NADPH–GSSG reductase activity was determined in 0.2 M sodium phosphate buffer (pH 6.8) and 5 mM EDTA in a final volume of 2.5 ml containing 1 mM GSSG, 120 μM NADPH and enzyme. NADH–GSSG reductase activity was determined in 0.2 M sodium phosphate buffer (pH 6.3) and 5 mM EDTA in a final volume of 2.5 ml containing 1 mM GSSG, 150 μM NADH and enzyme. A unit of activity is defined as the amount of enzyme required to oxidize 1 μmole of NAD(P)H per min under the conditions mentioned above. The specific activity is defined as units/mg protein. The protein content was determined by the method of Lowry and Folin[18] with crystalline bovine serum albumin as a standard.

Molecular weight determination

The molecular weight was estimated according to ANDREWS[19]. The reference proteins used for calibration of the column are given in Table I.

TABLE I

REFERENCE PROTEINS USED IN THE ESTIMATION OF THE MOLECULAR WEIGHT OF GLUTATHIONE REDUCTASE

Protein	*Manufacturer*	*Mol. wt.*	*Ref. No.*
Pyruvate kinase	Boehringer	237 000	14
Lactate dehydrogenase	Boehringer	135 000	15
Serum albumin (human)	Gift of Dr. R. Ballieux (Utrecht)	69 000	16
Chymotrypsin	Nutr. Biochem. Corp.	25 000	17

RESULTS

Isolation of glutathione reductase

(1) Approx. 1 l of erythrocytes was mixed with an equal volume of water containing 50 ml of chloroform. After standing in the cold for a few h, the hemolysate was collected by centrifugation (10 000 × *g* for 30 min); about 2 l of hemolysate were obtained.

(2) To the hemolysate were added 2 l of DEAE-Sephadex suspension prepared according to the instructions of the manufacturers, equilibrated with 0.01 M phosphate buffer (pH 7.2) and stirred 1 h. The Sephadex was repeatedly washed with 0.01 M phosphate buffer (pH 7.2) until the supernatant remained colorless. This procedure removed most of the hemoglobin while glutathione reductase remained bound to the Sephadex. The enzyme was eluted with 4 l of 0.1 M sodium phosphate buffer (pH 7.2) containing 0.5 M KCl.

(3) The enzyme solution obtained in the previous step was dialyzed overnight against 0.01 M phosphate buffer (pH 6.6). The enzyme from 1 l of solution was absorbed on a CM-Sephadex column (3.5 cm × 25 cm) equilibrated with 0.01 M phosphate buffer (pH 6.6). The column was washed with 0.01 M phosphate buffer (pH 6.6) until the eluate was colorless. The enzyme was eluted with 300 ml of 0.01 M phosphate buffer (pH 8.0).

(4) To 1200 ml of the enzyme solution an equal volume of DEAE-Sephadex suspension equilibrated with 0.01 M phosphate buffer (pH 7.2) was added. Washing of the Sephadex and elution of the enzyme occurred in the same way as described under (2).

(5) 2100 ml of the enzyme solution were obtained and $(NH_4)_2SO_4$ (350 g/l) was added. The precipitate was collected by centrifuging (10 000 × *g* for 10 min) and was suspended in 200 ml of 0.1 M phosphate buffer (pH 7.2). Any insoluble material was removed by centrifugation. The enzyme fraction was heated 30 min at 60°, and the precipitate was removed by centrifuging 15 min at 10 000 × *g*. This procedure removed most of the red material while glutathione reductase was not inactivated (see *Stability of the enzyme*).

(6) The enzyme fraction obtained in the previous step was dialyzed overnight against 0.01 M phosphate buffer (pH 7.3) and was absorbed on a DEAE-Sephadex column (2 cm × 20 cm) equilibrated with 0.01 M phosphate buffer (pH 7.3). The enzyme was eluted with 300 ml of 0.05 M phosphate buffer (pH 7.3). About 20–30% of the enzyme remained bound to the column.

(7) The enzyme was dialyzed against 0.01 M phosphate buffer (pH 7.5) and absorbed on a calcium phosphate gel column, equilibrated with 0.01 M phosphate buffer (pH 7.5). The enzyme was eluted with 550 ml of 0.03 M phosphate buffer (pH 7.5).

(8) After dialyzing against 0.01 M phosphate buffer (pH 7.5), the enzyme fraction was absorbed on a calcium phosphate gel column (1.5 cm × 15 cm) equilibrated with 0.01 M phosphate buffer (pH 7.5). The enzyme was eluted with 110 ml of 0.03 M phosphate buffer (pH 7.5).

(9) The enzyme fraction obtained in the previous step was dialyzed against 0.01 M phosphate buffer (pH 7.5) and was absorbed on a calcium phosphate gel column (1 cm × 7.5 cm), followed by elution with 25 ml of 0.03 M phosphate buffer (pH 7.5).

The whole purification procedure is summarized in Table II.

TABLE II

Steps of purification	*Total volume (ml)*	*Total protein (mg)*	*Total activity (units)*	*Specific activity (units/mg protein)*	*Yield (%)*	*Accumulative purification (-fold)*
1	2000	$300 \cdot 10^3$	1060	$3.5 \cdot 10^{-3}$	100	1
2	4000	$12 \cdot 10^3$	960	$8 \cdot 10^{-2}$	90	22
3	1200	$1.32 \cdot 10^3$	432	$36 \cdot 10^{-2}$	40	102
4	2100	$8.82 \cdot 10^2$	386	$44 \cdot 10^{-2}$	36	125
5	200	$3.6 \cdot 10^2$	385	1.1	35	310
6	300	78	204	2.6	19	742
7	550	11	132	12	12	3 420
8	110	1.1	82	65	7	18 500
9	25	0.3	53	165	5	47 000

Properties of the enzyme

The purest concentrated enzyme solution is yellow and has an absorption spectrum characteristic for a flavoprotein. Maxima occur at 462, 372 and 278 nm, while there are minima at 325 and 405 nm; near 485 nm a shoulder is present. The absorbance ratios for the different maxima are $A_{280\ nm}/A_{462\ nm} = 9.0$ and $A_{372\ nm}/A_{462\ nm} = 1.1$. In Table III the values obtained by MASSEY AND WILLIAMS[9] and MASSEY *et al.*[10] are given for the yeast enzyme and by SCOTT *et al.*[3] and ICÉN[4] for the human erythrocyte enzyme. From this data it can be concluded that our values are in good agreement with those. FAD is the prosthetic group.

TABLE III

REFERENCE VALUES OF THE ABSORBANCY SPECTRUM AND THE MOLECULAR WEIGHT OF GLUTATHIONE REDUCTASE

Source	λ_{max} *(nm)*	$\frac{A_{280\ m}}{A_{462\ nm}}$	*Mol. wt. (min.)*	*Mol. wt.*	*Ref. No.*
Yeast	462; 372	8.9:9.3	56 500	118 000	9
	462; 372			118 000	20
Erythrocyte	462; 372			132 000	3
	460–462	{8.8	{65 500	115 000	4
	365–375			125 000	4
	462; 372	9.0	56 600	115 000	This publication

On the basis of the flavin content of the protein as determined spectrophotometrically and on the assumption that the molar extinction coefficient of the bound flavin is identical with that of free FAD *cf.* $11.3 \cdot 10^3\ M^{-1} \cdot cm^{-1}$, for two purified enzyme preparations, a minimum mol. wt. of 56 600 g/mole FAD could be calculated. This value agrees with those given by MASSEY AND WILLIAMS[9] for the yeast enzyme and by ICÉN[4] but differs from the value reported by SCOTT *et al.*[3] (Table III).

From Fig. 1 a mol. wt. of 115 000 ($\pm$ 4000) was calculated using the method of ANDREWS[19]. COLMAN AND BLACK[20] reported on the basis of sedimentation–equilibrium studies a value of 118 000 for the mol. wt. of the yeast enzyme. More recently MAVIS

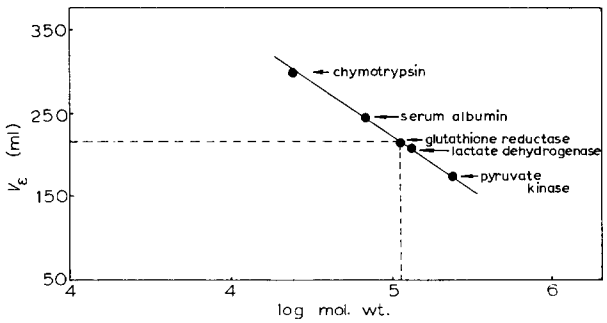


Fig. 1. Estimation of the molecular weight by gel filtration on Sephadex G-200. Column size 3.5 cm × 40 cm; buffer: 0.05 M phosphate buffer (pH 7.4) containing 0.5 M KCl. For the reference proteins see Table I.

AND STELLWAGEN[21] showed that the yeast enzyme consists of two subunits with a mol. wt. of 56 000. ICÉN[4] calculated 115 000–125 000 for the human erythrocyte enzyme as determined by gel filtration. Preliminary experiments to determine the molecular weight of the apoenzyme using the gel filtration method do not indicate that the molecular weight of the apoenzyme differs from that of the holoenzyme. This is in contrast with the molecular weight of lipoamide dehydrogenase apoprotein, which is half that of the holoenzyme (*cf.* ref. 11).

In the course of these studies an interesting observation was made. If blue dextran-2000 was used with a mixture of reference proteins and either glutathione reductase holoenzyme or apoenzyme to estimate the void volume of the Sephadex G-200 column, the glutathione reductase activity was found mainly in the void volume. In the absence of blue dextran, the enzyme appeared in the fraction as indicated in Fig. 1. The reason for this phenomenon is not known-probably it is due to absorption of the enzyme in the dye rather than with an association of the protein. This indicates a strong interaction between this enzyme and the blue dextran polymer and suggests that blue dextran-2000 is not as inert as it is generally believed to be.

Stability of the enzyme

Glutathione reductase is very heat stable even at low concentrations. Heating 1 h at 60° in a 0.1 M sodium phosphate buffer (pH 7.2) does not lead to loss of activity. However, temperatures higher than 70° lead to denaturation of the protein and result in a complete loss of activity within 10 min. Addition of $(NH_4)_2SO_4$ largely protects the enzyme against heating at 72° for 5 min.

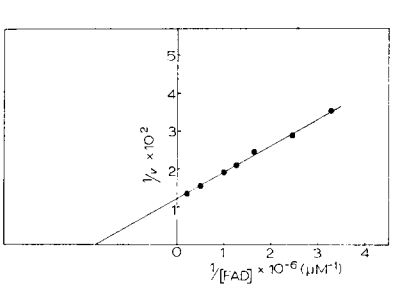
Oxidized glutathione reductase (0.1 mg/ml) was incubated 24 h at 0° and 25° in the presence of 2, 4, 6 and 8 M urea. After 24 h there was no loss of activity at 2, 4 and 6 M urea at both temperatures which contrasts with the results obtained with diluted (<0.1 mg/ml) lipoamide dehydrogenase[23]. However, no activity could be demonstrated when the enzyme was incubated with 8 M urea under the conditions mentioned above; no difference was observed in the rate of inactivation at both temperatures. In agreement with MASSEY AND WILLIAMS[9] and ICÉN[4], it has been found that incubation of the enzyme in the presence of 4 M urea and NADPH leads to complete inactivation within approx. 30 min.

Glutathione reductase has a low activity with 2,6-dichlorophenolindophenol (DCIP), as is the case with lipoamide dehydrogenase. When diluted glutathione reductase is dialyzed against 0.01 M phosphate buffer (pH 7.2) to remove EDTA and $(NH_4)_2SO_4$, it undergoes no change in activity with both GSSG and DCIP after freezing 24 h at −15°. This is in contrast with lipoamide dehydrogenase[24] in which the DCIP activity is strongly enhanced while the lipoate activity is diminished.

Preparation of the apoenzyme and recombination with FAD

The apoenzyme can be prepared according to WARBURG (*cf.* ref. 3); the method of STRITTMATTER[25] gives the same results. The enzyme solution was 55% saturated with $(NH_4)_2SO_4$ and was brought to pH 3.1 with 1 M HCl. After standing 10 min in an ice bath, the apoenzyme was collected by centrifugation and was dissolved in 0.2 M phosphate buffer (pH 6.8) containing 1 mM EDTA. This solution had only 8% of its original activity.

FMN and riboflavin cannot restore the enzymatic activity and only FAD is active. Fig. 2 shows the effect of the FAD concentration on the reactivation of the apoenzyme. From these data an apparent K_m for FAD of $5.5 \cdot 10^{-7}$ M and K_{ass} for FAD of $1.8 \cdot 10^6$ M^{-1} can be calculated (a value found in three independent experiments).



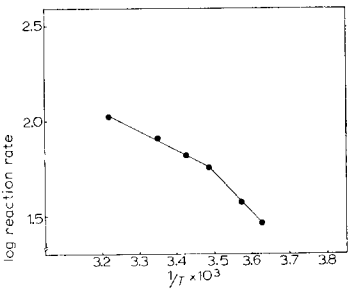


Fig. 2. Effect of FAD concentration on the reactivation of the apoenzyme by FAD. The apoenzyme was prepared as described in the text. After a 5-min incubation of 7 μg apoenzyme with the concentration FAD indicated, samples were withdrawn and activities measured. v is expressed as μmoles NADPH oxidized per min per mg protein.

Fig. 3. Arrhenius plot of the rate of restoration of glutathione reductase activity with FAD. Incubation mixtures (0.25 ml) contained 5 μM FAD, 6 μg apoenzyme and 0.2 M phosphate buffer (pH 6.8). Activities were measured under standard conditions at 25° after a 5-min incubation at the given temperatures. The reaction rate is expressed as μmoles NADPH oxidized per min per mg protein.

Fig. 3 shows that the binding of FAD to the apoenzyme is a temperature-dependent process. The Arrhenius plot of the reactivation rate of the apoenzyme by FAD binding is nonlinear and shows a break at 13°. The activition energy for this process in the temperature range from 3 to 13° is 10 500 cal · $mole^{-1}$ and 5200 cal · $mole^{-1}$ in the temperature range from 13 to 37°.

Though FMN is not capable of restoring the catalytic activity upon incubation with the apoenzyme, it is not excluded that the protein has affinity for this flavin compound. To demonstrate such interactions in flavoproteins which strongly interact with their specific flavin compound, preincubation of the apoenzyme with the aspecific

flavin is more useful than adding both flavins. This method has been followed by VISSER AND VEEGER[23] in the case of lipoamide dehydrogenase. In Fig. 4 the restoration of GSSG activity is shown upon FAD addition to an apoenzyme which was either preincubated at 0° with a large amount of FMN or was not preincubated. The restoration of the activity with FAD alone is complete within 1 h after incubation on ice. Preincubation with FMN retards the restoration by FAD extensively, but ultimately the same activity level is reached. Adding FMN after the full restoration of activity by FAD leads to a decline in the activity, as shown in Fig. 4. If the same amount of FMN is added to the holoenzyme, no loss in activity is demonstrated. The insert in Fig. 4 shows that the rate of recombination (with and without preincubation with FMN) is a first-order reaction.

In Fig. 5 the restoration of GSSG activity is shown upon addition of different FAD concentrations to an apoenzyme either with or without preincubation at 0° with a large amount of FMN. In the control experiment with FAD, the same K_m values were obtained after 10, 20, 40 and 60 min of recombination, but the v_{max} increased with the incubation time. However, after preincubation with FMN, the K_m value for FAD altered with time and approached the K_m value of the control.

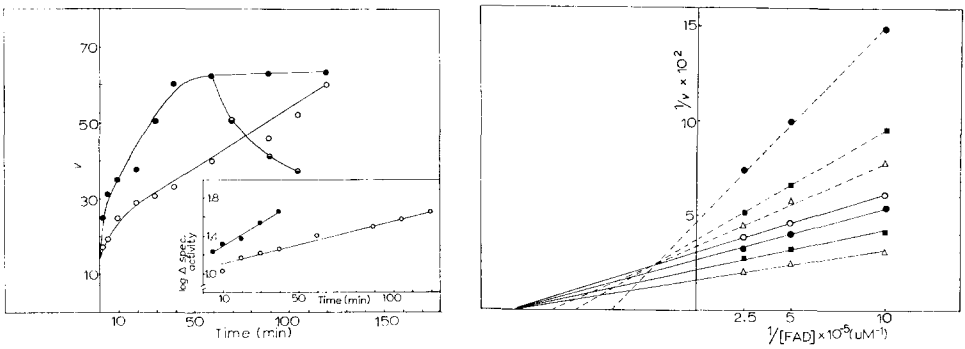


Fig. 4. Influence of FMN on the restoration of GSSG activity. 8 μM FAD were added to the apoenzyme (0.05 mg/ml) at 0°. ○—○, preincubation at 0° during 30 min with $5 \cdot 10^{-5}$ M FMN; ●—●, control, not preincubated with FMN; ◒—◒, $5 \cdot 10^{-4}$ M FMN added after full restoration of activity by FAD. Samples were withdrawn at different times and the activities measured at 25°. The activities are expressed as μmoles NADPH oxidized per min per mg protein. Buffer: 0.2 M sodium phosphate (pH 6.8). Insert: first-order return of activity calculated from the experimental data. ●—●, without preincubation with FMN; ○—○, preincubated with FMN.

Fig. 5. Influence of FMN on the restoration of GSSG activity of the apoenzyme in the presence of different FAD concentrations at 0°. Activities were measured after 10 (○), 20 (●), 40 (■) and 60 (△) min of incubation with the FAD concentrations given in the figure. — — —, preincubated with $5 \cdot 10^{-5}$ M FMN; ———, control without FMN. v is expressed as μmoles NADPH oxidized per min per mg protein. Buffer: 0.2 M sodium phosphate (pH 6.8).

DISCUSSION

The human erythrocyte glutathione reductase purified by this procedure had a specific activity of 165 at 25°, corresponding to a 47 000-fold purification of the original hemolysate. SCOTT *et al.*[3] obtained a value of 61 and ICÉN[4] of 145 at 30°. The ratio of the absorbances at 280 and 460 nm (9.0) is similar to the ratio 8.8 reported by ICÉN[4] for the human erythrocyte enzyme and to the ratios 8.9 – 9.3 reported by MASSEY

AND WILLIAMS[9] for the yeast enzyme. Our purest enzyme preparation is comparable in purity with the yeast enzyme and the enzyme preparation described by ICÉN[4].

A minimum mol. wt. of 56 000 per mole FAD could be calculated from the flavin content. This value is in good agreement with that obtained by MASSEY AND WILLIAMS[9] for the yeast enzyme (56 500) but differs to some extent from that reported by ICÉN[4] (65 500). It is clear that the value of SCOTT *et al.*[3] (130 000) is due to the low purity of the enzyme. Recently MAVIS AND STELLWAGEN[21] using 5 M guanidine hydrochloride proved that the yeast enzyme contains two subunits, having a mol. wt. of 51 500. In combination with the mol. wt. of 115 000 for the human erythrocyte glutathione reductase as determined by the method of ANDREWS[19], it can be concluded that the native enzyme contains 2 molecules of FAD and is built up by 2 polypeptide chains. This seems to be the general case for many flavoproteins, *cf.* lipoamide dehydrogenase[23], D-amino-acid oxidase[26], L-amino-acid oxidase[27] and luciferase[28].

Though lipoamide dehydrogenase and glutathione reductase seem to have many common[29] properties, there are some striking differences (*cf.* ref. 31). Up to now we could not obtain evidence that the apoenzyme of glutathione reductase is a monomer, as is the case with lipoamide dehydrogenase (*cf.* ref. 11); upon recombination of the apoenzyme with FAD, no stimulation of the DCIP activity was found as is the case with the latter enzyme[24]. Furthermore the results obtained from the incubation experiments with urea and the freezing and thawing experiments, do not favor the idea that the holoenzyme might dissociate into its monomers. This is in contrast with lipoamide dehydrogenase. At concentrations less than 0.1 mg/ml the dimer dissociates with the latter enzyme which leads to an increased sensitivity toward urea at low protein concentrations[23].

The recombination is a temperature-dependent process as shown in Fig. 3. A possible explanation could be that the apoenzyme exists in different conformations in temperature-dependent equilibrium (*cf.* ref. 30) with the form existing at lower temperatures having a lower association constant for FAD than the one present at higher temperatures ($> 13°$). However, it cannot be concluded from these results that the part of the Arrhenius plot below 13° represents the transition between the two forms (*cf.* ref. 30). An apparent difference between the Arrhenius plots of the rates of recombination process with FAD of glutathione reductase apoenzyme and that of lipoamide dehydrogenase is the linearity of it in the latter case. This is probably due to the completely different processes involved, *e.g.* FAD binding to a dimer and temperature-dependent restoration of activity in the case of glutathione reductase compared with binding of FAD to a monomer followed by temperature-dependent dimerization of the FAD containing monomer of lipoamide dehydrogenase (*cf.* ref. 11).

Though FMN cannot function as a prosthetic group it has affinity for the apoenzyme (Fig. 4). The recombination with and without preincubation with FMN proceeds in a first-order reaction which indicates that the formation of an inactive or partially active FAD–apoenzyme complex preceeds the return of a fully activated enzyme. The restoration process includes conformational changes, occuring over at least one h. FMN influences the activity of the fully restored system, while it does not influence the activity of the native holoenzyme. The formation of the holoenzyme from apoenzyme and FAD can be summarized as follows:

			?	
apo + FAD	$\rightleftharpoons$	apo_I–FAD	$\rightarrow$ apo_{II}–FAD	$\rightarrow$ holoenzyme
$\Downarrow$ + FMN		inactive or	100% active,	as isolated,
apo_I–FMN		partially	FMN	100% active,
inactive		active	sensitive	not FMN sensitive

Via an inactive intermediate (apo_I–FAD) the fully active apo_{II}–FAD conformation is formed. FMN can retard the formation of the apo_{II}–FAD conformation but the reaction is almost irreversible. Therefore only a high concentration of FMN can shift the equilibrium towards apo_I-FAD, apoenzyme and inactive apo_I–FMN. The observation that high concentrations of FMN have no influence on the activity of the isolated enzyme indicates that an almost irreversible conformational change may occur from apo_{II}–FAD. Although the present experiments do not include information concerning the conversion of the FMN-sensitive apo_{II}–FAD complex into the FMN-insensitive enzyme as isolated, such a process cannot be excluded, since it has been observed with lipoamide dehydrogenase[23]. The FMN-sensitive enzyme formed from lipoamide dehydrogenase apoenzyme and FAD, which is fully active with lipoate, is slowly converted into a FMN-insensitive enzyme by incubation at 25° overnight. During this period no change in activity is observed. Thus a similar conversion may occur with glutathione reductase. About the nature of these conformational changes one can only speculate, but work is in progress on the nature of these interesting phenomena.

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