The Essential SH-Groups in Folate Reductase

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(Received October 13, 1967)

SUMMARY

The number of essential SH-groups per amethopterin binding site has been determined in several preparations of folate reductase derived from a subline of S-180 cells grown *in vitro*. The content of folate reductase in terms of amethopterin binding sites varied in the different preparations between 15 and 20 mμmoles per milligram of protein and was based on titrations with amethopterin. In every preparation two SH-groups per binding site were essential for enzymatic activity and amethopterin binding. These two were protected against 5,5′-dithiobis-(2-nitrobenzoic acid) by TPNH, folate, and amethopterin and by low temperatures. The remainder of the SH-groups, which varied in different preparations, could not be protected and reacted with SH-reagent without interfering with the enymatic activity or amethopterin binding capacity. Folate reductase was found to be sensitive to inactivation by urea, which also accelerated the reaction with the SH-reagent.

INTRODUCTION

An enzyme preparation less than 100% pure rarely lends itself for a quantitative estimation of essential structural units. Folate reductase, also termed dihydrofolate reductase or tetrahydrofolate dehydrogenase, is exceptional in that it can be accurately estimated in terms of amethopterin binding sites by titration with this drug. Moreover, the intactness of the amethopterin binding site is essential for activity and vice versa (1). This enzyme from different sources has been shown to be either inhibited or stimulated by, or insensitive to, SH-reagents [for review see Hakala and Suolinna (1)]. Folate reductase of S-180 cells grown *in vitro* is one which is inactivated by SH-reagents with a parallel loss of amethopterin binding capacity. The purpose of the present study was to determine the number of essential SH-groups per amethopterin binding site. The means to distinguish the essential from the nonessential SH-groups in these preparations was provided by the fact that substrates, coenzymes, and competitive inhibitors protect this enzyme against SH-reagents (1).

MATERIALS AND METHODS

Enzyme preparation. The amethopterin-resistant subline of S-180 cells, AT/3000, the growth media, and the methods used for collecting and storage of these cells have been previously described (1). Packed cells (20–22 g), were homogenized in a 2-fold volume of folate solution (100 μg/ml, pH adjusted to 7.0 with ammonia). The high speed supernatant fluid (1 hr at 105 000 *g*) was applied to a column (9 cm × 35 cm) of Sephadex G-75 (new bead form) that had been equilibrated with the folate solution, which also was used for elution. Fractions were collected and analyzed as described (1, 2). The fractions containing folate reductase were combined (600–700 ml) and lyophilized. This gave 170–190 mg of a yellow powder which was then suspended in 7–8 ml of 0.05 M sodium citrate, pH 8.0. To remove folate, these suspensions were dialyzed in a cold room for 3 days against 8 × 900 ml of 0.05 M

sodium citrate. The dialyzed preparation was centrifuged and divided into 1-ml portions, which were stored at —75°. The average purity of three of such preparations was 37% (2), the yield about 60%, and the concentration of folate reductase in the solution about 0.1 mM (see below). The only exception to the above was encountered when the folate solution used throughout was supplemented with 1 mM mercaptoethanol. The yield then dropped to one-fourth of the usual, and the purity to 24%.

Folate reductase activity. Folate reductase activity was measured by two methods. In most cases the reaction product, tetrahydrofolate, was estimated by diazotization as has been described (1). However, since some compounds, especially urea, interfered with diazotization the spectrophotometric assay at 340 mμ was used also (3). This was done by recording the decrease of absorbance at 340 mμ at 37° in a Zeiss PMQ II spectrophotometer equipped with a Haake thermostat. The reaction mixture (1 ml) contained 0.1 M sodium citrate pH 6.0, 0.04 mM folic acid, 0.1 mM TPNH, and the enzyme. Reaction was initiated with TPNH; the blank was devoid of folate. A decrease of absorbance at 340 mμ of about 0.15–0.2 in 10 min was caused by 0.1 mμmole of enzyme.

Titration of folate reductase with amethopterin. The method has been described in detail elsewhere (1). This titration permitted estimation of folate reductase in terms of molar equivalents of amethopterin binding sites. It served as the basis for the calculation of the number of SH-groups per mole of folate reductase, assuming one amethopterin binding site per molecule.

Titration of SH-groups. The initial trials to determine SH-groups by using a spectrophotometric assay with *p*-chloromercuribenzoate (4) failed because heavy precipitates were formed. Ellman's reagent, 5,5′-dithiobis-(2-nitrobenzoic acid), DTNBA, purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin (5), proved to be a useful tool for these titrations after some important modifications had been adopted. Most of these titrations (exceptions will be pointed out) were performed as follows: an aliquot of a folate reductase preparation, whose content in terms of molar equivalents of amethopterin binding sites was accurately known, was diluted to 1 ml with 0.05 M sodium citrate, pH 8.0, to give about 2×10^{-5} M titratable SH-groups, and then mixed in a test tube with 0.1 ml of 0.01 M DTNBA in 0.2 M Tris, pH 8.0. The mixture was then centrifuged for 5–10 min at 3000 rpm and the supernatant fluid poured into a 1 cm microcuvette, kept at 37° in the spectrophotometer compartment. The increase in absorbance at 412 mμ was recorded against a reagent blank. If precipitate was formed in the sample during measurement it was removed by centrifugation. The rate of change in absorbance at 37° was 0.035–0.075 per 10 min, and was linear with time for the first 20 min. Thereafter it tapered off so that a plateau was reached in about an hour, which was followed by a decline of the absorbance. The presence of protecting substances influenced mainly the final extent of the reaction, but also slowed it down slightly. Protectors also prevented the formation of precipitates which otherwise plagued these titrations. In the presence of 4 M urea no precipitates were formed, and the initial rate of the reaction was increased by 5- to 10-fold. The absorbance of the enzyme without DTNBA at 412 mμ was also determined and varied from 0.010 to 0.040, depending on the dilution used. The number of SH-groups was calculated from the maximum absorption (minus the absorbance of the corresponding enzyme blank) using reduced glutathione, Schwarz BioResearch, as the standard ($A_{412} = 10700\ M^{-1}\ cm^{-1}$). The control (free of DTNBA) was incubated under identical conditions and lost no activity.

Protein determination. Protein was assayed using the Folin reagent (750 mμ) (6). Since Kaufman has reported that dihydrofolate reductase of chicken liver has a high content of aromatic amino acids (7), the possibility that albumin may not be a proper standard was considered. Therefore, the protein of a 37% pure folate reductase preparation was also estimated using Ell-

man's microbiuret assay (8). No differences between the results obtained by the two types of assays were noted and, therefore, the Folin method, because of its greater sensitivity, was used throughout this study.

RESULTS

Effect of Temperature, Folic Acid, or Amethopterin on the Reaction with DTNBA

The reaction of DTNBA with glutathione is very rapid even at room temperature, and is complete in a few minutes. In contrast, even though DTNBA at room temperature reacted rapidly with some of the SH-groups in samples of folate reductase, the inactivation of the enzyme was only slight. Thus, with one particular preparation it was found that approximately one SH per amethopterin binding site reacted within an hour. However, the absorbance at 412 mμ continued to increase so that a plateau had not been reached even after 7 hours at room temperature. At that time 1.6 SH-groups had reacted and resulted in 23% inactivation of the enzyme. One of the lines in Fig. 1 demonstrates the relationship of enzymatic activity (and amethopterin binding capacity) to the number of SH-groups reacted with DTNBA at 25°, 35°, and 37°. Even at 37° folate reductase was not totally inactivated (60–80%) by reaction with DTNBA. In view of our previous findings (1) it was to be expected that the presence of amethopterin would influence the number of SH-groups that could react with DTNBA. It was found that three molar equivalents of amethopterin protected the SH-groups to the same degree as 10 or 50 molar equivalents. Figure 1 shows that in the presence of amethopterin (points corresponding to 100% activity) only about one SH-group per binding site could react with DTNBA in these particular preparations. As will be shown later, this number varied in different preparations and was in one case more than two SH-groups per binding site (Fig. 3). Figure 1 also presents the SH-titration of another enzyme preparation performed in the presence or absence of 0.3 mM folate at

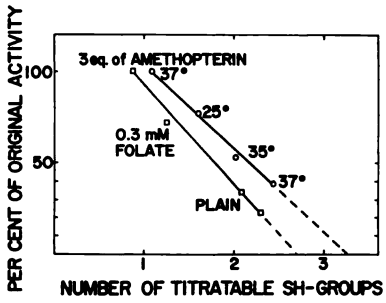

FIG. 1. *Relationship of the number of SH-groups per amethopterin binding site which have reacted with DTNBA and the remaining folate reductase activity in two different enzyme preparations*

Where indicated, folate or amethopterin were present during the DTNBA reaction. After completion of the SH-titrations the samples were analyzed for folate reductase activity and amethopterin binding capacity; activity values shown are based on these determinations. ○, SH-titrations performed at varied temperatures as indicated; □, SH-titrations performed at 37° in the presence or absence of protectors as indicated. Each point represents a mean of two or more separate experiments.

37°. Extrapolation of the two lines to the abscissa indicates a total of 2.7 and 3.3 SH groups per mole of folate reductase. It appears that from these SH-groups 1.8 or 2.2 are essential for activity.

Titration of SH-Groups in the Presence of Urea

It was hoped that urea by its unfolding effect on proteins, in general, would make the last inaccessible fraction of SH-groups in folate reductase available for reaction with DTNBA. Kaufman has reported a 5-fold stimulation of dihydrofolate reductase activity of chicken liver by 4 M urea (9). Figure 2 illustrates that 1–2 M urea, when present during enzyme assay, caused only a slight stimulation of folate reductase of S-180 cells while 3–4 M urea was quite inhibitory.

To determine the effect of SH-group titrations on enzyme activity the samples must be incubated for about 60 min at pH 8.0 and 37°, and the enzymatic activity at pH 6.0 is determined only after that. As

is seen in Fig. 2, such preincubation in 1 M urea (without DTNBA) caused some loss of activity (10–15%), and in 2 M urea extensive inactivation occurred (40–55%). On the other hand, if DTNBA and urea were present together during the SH-titration, complete inactivation occurred already in 1 M urea. On account of this strong urea effect, SH-titrations with DTNBA were made in the presence of urea only to determine the total number of SH-groups

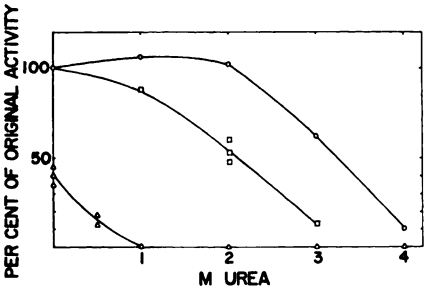

FIG. 2. *Effect of urea with or without DTNBA on folate reductase activity*

○, Urea present only during the enzymatic assay at pH 6.0 at the concentrations indicated. △, Urea present during SH-titrations. For the subsequent enzyme activity measurement, urea was diluted down to insignificant concentrations. □, Control for SH-titration: incubation with urea in the absence of DTNBA. For details see Materials and Methods.

in these preparations, and not to correlate number with activity. Indeed, 4 M urea was ideal for this purpose since it prevented the formation of precipitates which otherwise complicated the SH-titrations whenever enzyme protectors were missing. Moreover, 4 M urea speeded up the reaction with DTNBA so that at 37° it was complete in 20 min, instead of 60 min as usual. It was gratifying to find, as is shown in Fig. 3, that the total number of SH-groups which reacted in 2–4 M urea was only slightly larger than in the absence of urea and was identical with the extrapolated value for the total number of SH-groups.

SH-Titration in the Presence of TPNH

It was shown previously that not only folic acid and its analogs but also TPNH protected the activity and amethopterin binding capacity of folate reductase against *p*-chloromercuribenzoate and iodoacetamide (1). Depending on the concentration of TPNH used, the number of SH-groups which could be titrated with DTNBA varied (Fig. 3). Thus, 8.1 mM TPNH pro-

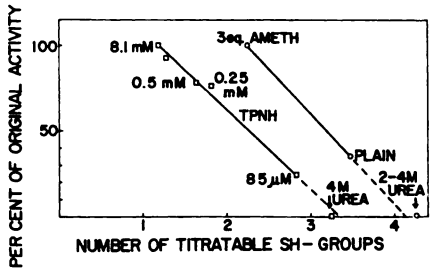

FIG. 3. *Relationship of the number of SH-groups per amethopterin binding site which have reacted with DTNBA and the remaining folate reductase activity in two different enzyme preparations*

After SH-titrations at 37°, the samples were analyzed for activity and amethopterin binding capacity. ○, SH-titrations performed either in the presence or absence of 3 equivalents of amethopterin or 2 and 4 M urea. Each point is an average of 2–6 separate experiments. □, SH-titrations performed in the presence of 0.085 mM to 8.1 mM TPNH or 4 M urea.

vided complete protection of enzyme activity and amethopterin binding capacity while it permitted more than one SH-group to react with DTNBA. In contrast, 85 μM TPNH protected neither activity nor SH-groups, as compared with the TPNH-free samples. In the same figure the analysis of another preparation is shown in the presence and absence of 3 equivalents of amethopterin, or in 2–4 M urea. Both of these analyses again suggest that 2 SH-groups per mole of folate reductase must be protected to retain full activity.

Figure 4 summarizes all the available data in a form that allows one to focus attention on those SH-groups that are essential for activity, unencumbered by the differences between different preparation with respect to the number of unessential SH-groups. This figure includes not only data presented in Figs. 1 and 3, but also those from several additional experiments.

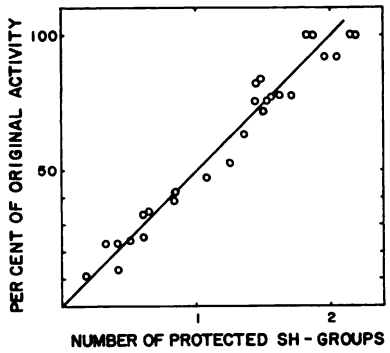

FIG. 4. *Relationship of folate reductase activity to the number of protected SH-groups*

This is composed of data on seven different folate reductase preparations analyzed as presented in Figs. 1 and 3 using TPNH, folate, amethopterin, or varied temperatures to permit partial inactivation by DTNBA. The number of protected SH-groups was calculated by deducting the number actually titrated in the varied conditions from the total number of SH-groups found in the particular preparation. The total number was determined either in the presence of urea as in Fig. 3 or by extrapolation as in Fig. 1. The values corresponding to 100% activity include also those that were determined in the presence of 3 or more equivalents of amethopterin.

It brings out more clearly the result of the present studies—i.e., that two SH-groups in folate reductase are essential for enzyme activity and amethopterin binding.

DISCUSSION

The subline of Sarcoma-180 cells grown *in vitro* which was used as the source of folate reductase for these studies performs the major part of enzyme "purification" within the cells. It produces a supernatant fluid containing 2 mμmoles of folate reductase per milligram of protein, a purity equal to a 400- to 500-fold purified chicken liver dihydrofolate reductase. For the present study the S-180 enzyme was purified 10 times further. This provided material with a relatively low total SH-content, 2.7 to 4.2 SH-groups per amethopterin binding site in different preparations. From these always two SH-groups were essential for enzymatic activity and for binding of amethopterin.

This study reveals that the essential SH-groups in folate reductase are not readily accessible for reaction with DTNBA. This is in agreement with our previous studies, which showed that relatively high concentrations of SH-reagents, and long incubations were necessary for inactivation of the enzyme. It probably explains the generally observed high stability of folate reductase during storage. It might also explain the reports which claim that dihydrofolate reductases of sheep and chicken liver are insensitive to SH-reagents (10, 11). Recent investigations have indeed revealed 1 or 2 cysteine residues in the latter enzyme (12).

This type of study is unable to establish the exact location of the SH-groups in the enzyme molecule. All we have shown is that two SH groups in folate reductase are vital and are so situated that the presence of a substrate, competitive inhibitor, or the coenzyme makes these groups inaccessible for DTNBA and to other SH-reagents (1) and by so doing protects the enzymatic activity as well as the amethopterin binding capacity. Whether this protection is a result of direct shielding or of conformational change is debatable. However, the fact that increase in temperature as well as the presence of urea increased the accessibility of the SH-groups suggests that conformational changes might well be involved here.

ACKNOWLEDGMENTS

The author wishes to express her appreciation to Mrs. L. Kenny and Miss E.-M. Suolinna for assistance in this study.

This investigation was supported in part by research grant CA-04175 from the National Cancer Institute of the U.S. Public Health Service.

REFERENCES

1. M. T. Hakala and E.-M. Suolinna, *Mol. Pharmacol.* **2**, 465 (1966).
2. S. F. Zakrzewski, M. T. Hakala and C. A. Nichol, *Mol. Pharmacol.* **2**, 369 (1966).
3. M. J. Osborn and F. M. Huennekens, *J. Biol. Chem.* **233**, 969 (1958).
4. R. Benesch and R. E. Benesch, *Methods Biochem. Anal.* **10**, 43 (1962).
5. G. L. Ellman, *Arch. Biochem. Biophys.* **82**, 70 (1959).
6. O. H. Lowry, N. J. Rosebrough, A. L. Farr

and R. J. Randall, *J. Biol. Chem.* **193,** 265 (1951).
7. B. T. Kaufman and R. C. Gardiner, *J. Biol. Chem.* **241,** 1319 (1966).
8. G. L. Ellman, *Anal. Biochem.* **3,** 40 (1962).
9. B. T. Kaufman, *Biochem. Biophys. Res. Commun.* **10,** 449 (1963).
10. J. M. Peters and D. M. Greenberg, *J. Am. Chem. Soc.* **80,** 6679 (1958).
11. C. K. Mathews and F. M. Huennekens, *J. Biol. Chem.* **238,** 3436 (1963).
12. G. P. Mell, G. H. Schroeder and F. M. Huennekens, *Federation Proc.* **25,** 277 (1966).