Vol. 85, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 14, 1978 Pages 402-407

ACTIVATION OF CHICKEN LIVER DIHYDROFOLATE REDUCTASE BY TETRATHIONATE

Elizabeth K. Barbehenn and Bernard T. Kaufman

National Institute of Arthritis, Metabolism, and Digestive Diseases,

National Institutes of Health, Bethesda, Maryland 20014

Received September 25,1978

SUMMARY

Dihydrofolate reductase from chicken liver (5,6,7,8-tetrahydrofolate:$NADP^+$ oxidoreductase, EC 1.5.1.3) is activated approximately six-fold by tetrathionate. A 30-fold excess is required for full activation within a 24 hour period. The activation is accompanied by stoichiometric binding of a sulfur-containing moiety of tetrathionate, presumably thiosulfate, to the single sulfhydryl group of the enzyme. The effect can be completely reversed with β-mercaptoethanol even after several days in the activated state. The activated enzyme is stable for at least a week at 0°.

INTRODUCTION

The activity of chicken liver dihydrofolate reductase is markedly influenced by treatment with certain reagents which can interact with protein sulfhydryl groups. These compounds include organic mercurials (1), iodine (2), DTNB[1,2], and NEM[2]. Organic mercurials and iodine cause a marked stimulation of enzyme activity ranging between 5 and 10 fold whereas DTNB and NEM are both inhibitors. Tetrathionate is a less commonly used sulfhydryl reagent and has, in general, been found to inactivate those enzymes with which it has been tested (3-6). However, our experiments indicate that not only does tetrathionate activate chicken liver dihydrofolate reductase, but it can be shown to bind mole/mole with the single sulfhydryl group present.

MATERIALS AND METHODS

[^{35}S] Sodium thiosulfate, specific activity 25 mCi/mmole, was obtained from Amersham. Sodium tetrathionate was from either K & K laboratories, Plainview, N.Y. or Pierce Chemical Company. All other chemicals were the purest grade available commercially.

(1) Abbreviations used: DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); NEM, N-ethylmaleimide.
(2) Unpublished results.

0006-291X/78/0851-0402$01.00/0

[^{35}S] $S_4O_6^{2-}$ was synthesized from [^{35}S] sodium thiosulfate by the method of Gilman et al (7). It had a specific activity of 8,000 cpm/nmole.

Chicken liver dihydrofolate reductase was purified according to Kaufman and Kemerer (8) and had a specific activity of 16 units/mg. It was assayed as previously described (8). Nanomoles of enzyme used were calculated on the basis of 0.34 units/nmole.

Gel filtration was performed in a Sephadex G-25 (fine) column (0.9 X 25 cm; Pharmacia) with 0.1 M potassium phosphate buffer pH 7.0 at a flow rate of 18 ml/hour. Fractions of 0.65 ml were collected and aliquots of 0.5 ml counted using the full ^{14}C channel. Three ul aliquots were assayed for activity.

Incubations of enzyme and tetrathionate were routinely carried out in a total volume of 200 ul in 12 x 75 mm tubes in ice. The enzyme, unless stated otherwise, was present at 2.6 units/ml in 0.05 M Tris-HCl pH 8.2.

RESULTS

In order to have the reaction of tetrathionate and chicken liver dihydrofolate reductase proceed to completion over a period of 24 hours or less, an excess of tetrathionate is required. Fig. 1 shows the rate of this reaction as a function of the ratio of tetrathionate to enzyme. Although the same final maximum of 6.5-fold stimulation is reached at both 30- and 60-fold excess, that maximum is reached more quickly with the higher tetrathionate level. The half-times at 0° are 2.5 and 4.4 hours for the 60- and 30-fold excesses of tetrathionate, respectively. In contrast, when enzyme was incubated with up to a 600-fold excess of sodium thiosulfate, no change in enzyme activity was observed (Fig. 1).

It has been postulated (3) that the initial reaction of enzyme and tetrathionate occurs as follows:

$$\text{Enz-SH} + S_4O_6^{2-} \rightleftharpoons \text{Enz-S-}S_2O_3^{-} + S_2O_3^{2-} + H^+ \qquad (1)$$

To test that this reaction was indeed occurring in our case, [^{35}S] $S_4O_6^{2-}$ was synthesized (see Methods). It was determined to be tetrathionate by incubating it with the enzyme under the conditions of 60-fold excess and finding that the increase in dihydrofolate activity followed the curve shown in Fig. 1 precisely (date not shown).

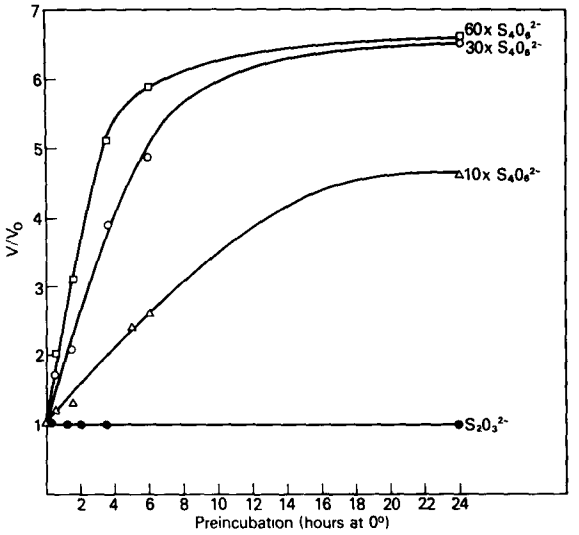

Fig. 1 Effect of tetrathionate on chicken liver dihydrofolate reductase activity. Enzyme was incubated with either a 10-fold (Δ), 30-fold (○), or 60-fold (□) excess of $S_4O_6^{2-}$ or a 600-fold (●) excess of thiosulfate as described in Methods and aliquots removed for assay at the times indicated.

The enzyme was then incubated with a 30-fold excess of [^{35}S] $S_4O_6^{2-}$ overnight and applied to a G-25 column (see Methods). Fig. 2 shows the coincidence of the peaks of both label and enzyme activity in the void volume of the column. From the known specific activity of the $S_4O_6^{2-}$, it can be calculated that 10.8 nmoles S_2O_3 are bound to 10.6 nmoles of enzyme, one mole per mole sulfhydryl group.

A summation of the enzyme activity shown in Fig. 2 shows a 6-fold increase over the activity that had been in the original incubation mixture, in agreement with previous data (Fig. 1). This activated enzyme preparation, devoid of excess tetrathionate, was stored at 0° and its activity tested over a period of 9 days. At the end of this time, it still retained 90% of the original 6-fold increase.

The increase in activity, while stable at 0°, can be reversed by incubation with β-mercaptoethanol at 28°. Enzyme was incubated as described in Methods with a 60-fold excess of tetrathionate. At the times indicated, an

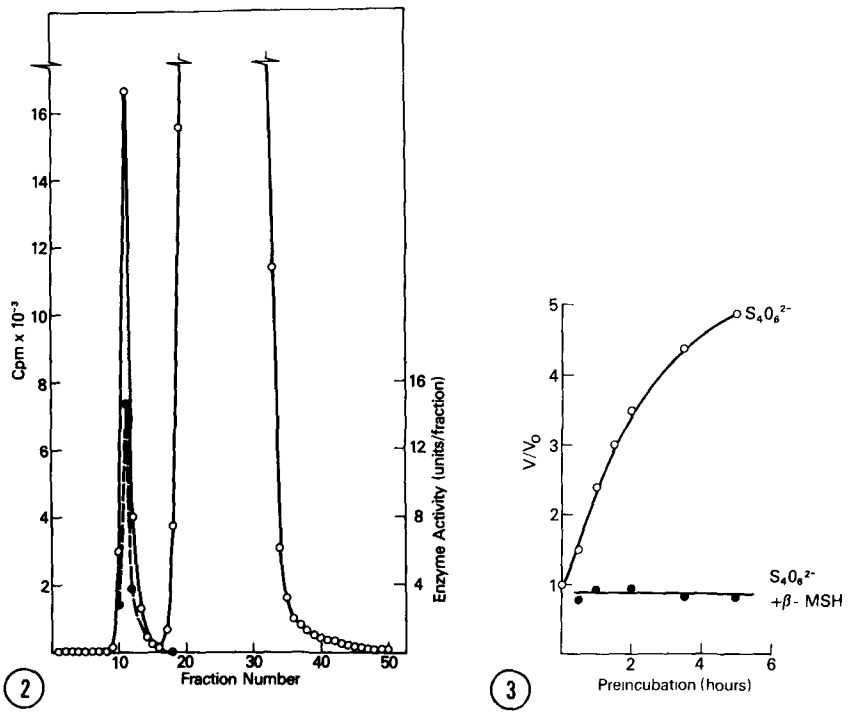

Fig. 2 Binding of [^{35}S] $S_4O_6^{2-}$ to chicken liver dihydrofolate reductase. Ten nmoles of enzyme and 300 nmoles of [^{35}S] $S_4O_6^{2-}$ were incubated in pH 8.2 buffer at 0° for 22 hours. The material was applied to a G-25 column which was run as described in Methods. Separate aliquots were assayed for enzyme activity (●) and radioactivity (○).

Fig. 3 Reversibility of tetrathionate activation. Enzyme was incubated with a 60-fold excess of tetrathionate (see Methods). At the time indicated, 3 ul aliquots were removed and either assayed directly (○) or given a 2 min incubation with 0.3 mM β-mercaptoethanol in a cuvette at 28° before adding substrates (●).

aliquot was removed and incubated with β-mercaptoethanol (Fig. 3). This treatment caused an immediate return of the activity to values slightly below those seen with untreated enzyme.

DISCUSSION

Chicken liver dihydrofolate reductase contains a single sulfhydryl group(8). The data presented here show that the six-fold activation of the enzyme be tetrathionate is due to a 1:1 binding of a sulfur-containing moiety of tetrathionate to this single sulfhydryl group. This reaction is presumed to occur as shown in

equation 1 forming an enzyme-sulfenyl thiosulfate derivative. This is the first time that an increase in enzyme activity has been found to result from a reaction with tetrathionate.

In the enzymes examined to date for their ability to react with tetrathionate (3-5,9), disulfide formation appears to be the usual product although the intermediate sulfenyl thiosulfate can be observed with glyceraldehyde 3-phosphate dehydrogenase if the reaction is kept at 0^{o} (3,4). Higher temperatures, however, lead to intramolecular cross-linking (4):

$$R\begin{matrix}S\\SH\end{matrix} \; - \; S_2O_3^- \longrightarrow R\begin{matrix}S\\|\\S\end{matrix} \; + \; S_2O_3^{2-} \; + \; H^+ \qquad (2)$$

Chicken liver dihydrofolate reductase has only one sulfhydryl group with which the sulfenyl thiosulfate derivative forms a stable activated enzyme. The disulfide which might be formed from the linking of two molecules of enzyme would result in a loss of radioactivity and this is not observed.

In marked contrast to the apparently instantaneous activation by stoichiometric amounts of the mercurials and iodine (1,2), stimulation of activity by tetrathionate requires preincubation of the enzyme with relatively high levels of reagent. This may reflect the difficulty in the approach of the negatively charged tetrathionate into a hydrophobic region of the molecule. That this is likely the case is shown by the increasing ability to react as one goes from IAA (no reaction)[2] to NEM (slow reaction)[2] to pHMB (instantaneous reaction) (1) in a direct correlation with increasing hydrophobicity.

Thus,tetrathionate may be included in the group of reagents along with organic mercurials (1) and iodine (2) which uniquely activate chicken liver dihydrofolate reductase. From these preliminary studies, the tetrathionate-activated enzyme appears to be quite similar to that resulting from activation by organic mercurials and iodine. Sequence studies indicate that the single sulfhydryl group is located on the eleventh amino acid residue from the N-terminal valine[2]. Binding to this residue via organic mercurials to form a mercaptide, iodine to form a sulfenyl iodide and, in this study, tetrathio-

nate to form a sulfenyl thiosulfate, yields a form of the enzyme with markedly enhanced catalytic activity. In view of the observation (10) that a similar enhancement of dihydrofolate reductase activity occurs in the presence of 4 M urea, it is assumed that the formation of these various sulfhydryl derivatives of the enzyme result in a specific conformational change in the protein which is characterized by increased enzyme activity.

REFERENCES

1. Kaufman, B.T. (1964) J. Biol. Chem. 239, PC 669.
2. Kaufman, B.T. (1966) Proc. Natl. Acad. Sci. 56, 695-700.
3. Pihl, A. and Lange, R. (1962) J. Biol. Chem. 237, 1356-1362.
4. Parker, D.J. and Allison, W.S. (1969) J. Biol. Chem. 244, 180-189.
5. Chung, S.I. and Folk, J.E. (1970) J. Biol. Chem. 245, 681-689.
6. Allison, W.S. and Kaplan, N.O. (1964) Biochem. 3, 1792-1800.
7. Gilman, A., Philips, F.S., Koelle, S.S., Allen, R.P., and St. John, E. (1946) Am. J. Physiol. 147, 115-126.
8. Kaufman, B.T. and Kemerer, V.F. (1977) Arch. Biochem. Biophys. 179, 420-431.
9. Degani, Y., Veronese, F.M., and Smith, E.L. (1974) J. Biol. Chem. 249, 7929-7935.
10. Kaufman, B.T. (1968) J. Biol. Chem. 243, 6001-6008.