Preliminary Communications

Activation of Dihydrofolic Reductase by Organic Mercurials

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(Received for publication, October 23, 1963)

Dihydrofolic reductase catalyzes the reduced triphosphopyridine nucleotide-dependent reduction of dihydrofolate to tetrahydrofolate. In addition, this enzyme is believed to cause, although much less rapidly, the reduction of folic acid to the tetrahydro level. Recent studies from this laboratory (1) have shown that the activity of this enzyme is markedly stimulated under conditions which suggest that activation may be the result of a change in configuration or dissociation of the protein molecule. A possible mechanism to account for this effect might involve the exposure of functional groups at the active site which would facilitate the reaction. As an initial attempt to explore this hypothesis, the participation of sulfhydryl groups in the catalytic activity of dihydrofolic reductase was investigated. It seemed particularly appropriate to examine the sulfhydryl properties of this enzyme, since one recent report (2) concluded that dihydrofolic reductase from several sources appeared to be insensitive to the usual sulfhydryl inhibitors, whereas other investigators (3, 4) have reported an inhibitory effect with p-chloromercuribenzoate. In this communication, it is shown that very low concentrations of organic mercurials do not inhibit the dihydrofolic reductase derived from chicken liver but rather cause a marked stimulation of enzyme activity similar to that previously reported for 4 M urea and other activating agents (1).

Dihydrofolic reductase was purified approximately 50- to 100-fold from a high speed supernatant extract of a chicken liver homogenate as previously described (1). Fig. 1 illustrates the rather unexpected observation with respect to the effects of three organic mercurials on the rate of reduction of dihydrofolic acid. The most striking stimulation is seen with methyl mercuric bromide or hydroxide. Stimulation of activity from 6- to 12-fold has been consistently observed under these conditions. Increasing levels of the mercurial result in progressive loss of the stimulated rate, and at an approximately 10-fold increase in concentration above the level for maximal stimulation, the residual activity is again reduced to approximately the unstimulated rate. Further addition of the mercurial to the reaction mixture results in progressive inhibition below the original activity. Similar results are obtained with the more complex mercurials, p-chlormercureibenzoate, phenylmercuric acetate, and mesalyl (salicyl-(γ-hydroxymercuri-β-methoxypropyl)-amido-O-acetate). The stimulating effects of the mercurials on the reaction rates occurred without any detectable time lag. No preliminary incubation of enzyme with mercurial was required to obtain the maximal effect. Inorganic mercury derivatives could not be tested in this system since mercury in either oxidation state apparently reacts with TPNH (6). Other sulfhydryl reagents, such as iodoacetate, iodoacetamide, o-iodosobenzene, and N-ethylmaleimide, neither inhibited nor stimulated the enzyme activity in either the presence or absence of 4 M urea.

Definitive evidence that the stimulation of the reduction of dihydrofolate acid is due to an activation of the enzyme is illustrated by the preparation of the activated enzyme-mercurial complex. A small volume of the partially purified enzyme (i.e., 1 to 2 ml containing 10 mg of protein) was titrated directly with successive increments (0.01 ml) of 10 mM methylmercuric hydroxide. After each addition of the mercurial, the activity of an aliquot was determined in the standard reaction mixture without additional mercurial. At the point of maximal stimulation (0.052 μmole of methylmercuric hydroxide per mg of protein), the solution was freed of excess mercurial by passage through a Sephadex G-25 column and then fractionated with ammonium sulfate. The material precipitated between 60 to 80% saturation was resuspended in a minimal volume of buffer (0.5 m Tris, pH 7.4) and desalted by another passage through a Sephadex G-25 column. Table I summarizes the preparation and properties of the activated enzyme. The enzyme-methylmercuric complex appears to be relatively stable as evidenced by the maintenance of the activated rate throughout these manipulations involving dilution and concentration as well as repeated freezing and thawing. At each stage of the above procedure, the activation is abolished and the characteristic initial activity recovered on removal of the mercurial group by the addition of mercaptoethanol, cysteine, or glutathione. On the other hand, a large excess of EDTA had no significant effect on the stimulated reaction. The p-chlormercureibenzoate enzyme was prepared in a similar manner and also proved to be quite stable. The initial rate of the reaction was linear with respect to protein concentration over a 15-fold concentration range for the enzyme-mercurial complex as well as for the untreated enzyme. These observations, and particularly the reversal of the activation and recovery of the original activity at each step in the purification procedure, make rather improbable any explanation of the activation in terms of a release of inhibition by removal of an inactivator combined with the enzyme protein or of some impurity in reaction mixtures.

Fig. 2 illustrates the rate of reduction of dihydrofolate catalyzed by the enzyme-methylmercuric complex and the original enzyme as a function of pH. This change in the pH-activity curve is essentially identical with that observed during the urea activation of the enzyme (1). However, in contrast to the urea effect, the activity of mercurial-enzyme complex is still equal to or higher than the untreated enzyme at pH values less than 5. Consequently, it proved feasible to examine the effect of the mercurials on the reduction of folic acid in the region of its pH optimum, i.e., 4 to 5. Similar experiments to those described above with dihydrofolate were repeated with folate and the methylmercurial-enzyme complex. The mercurial enzyme also exhibited the enhanced catalysis with folate as
The specific activation of chicken liver dihydrofolic reductase by the organic mercurials suggests that this enzyme possesses two sites concerned with catalytic activity. The active site which binds the substrate or antifolate appears to be relatively insensitive to the organic mercurials and completely insensitive to other commonly used sulphydryl reagents. The other site, which is apparently concerned with the activation or control of the enzyme, is specifically affected by the mercurials and from this standpoint might be considered to be sulphydryl in nature. The similarity of these observations and those described for glutamic dehydrogenase (7, 8), phosphoribosyl-ATP-pyrophosphorylase (9), and particularly aspartate transcarbamylase (10), suggests that the activation site in dihydrofolic reductase may be similar to the allosteric site (11) involved in feedback phenomena. However, to date, no inhibitor of dihydrofolic reductase has been found which could be called a physiological feedback inhibitor. Compounds examined have included DNA, RNA, nucleoside mono-, di-, and triphosphates, amino acids, steroids, and various folate derivatives. The similarity between the activation of the enzyme by 4 mM urea and the organic mercurials suggests that the activation is a result of a change in configuration or dissociation of the protein molecule. Consequently, an attempt was made to examine the sedimentation properties of the mercurial-enzyme complex with respect to the untreated enzyme by means of the sucrose gradient technique (12). No significant difference in the two forms of the enzyme.

FIG. 1. The effect of three organic mercurials on the activity of dihydrofolic reductase. The reaction mixture, in a volume of 1 ml, contained: 50 mM potassium phosphate buffer, pH 7.5; 0.2 mM TPNH; 0.05 mM dihydrofolate; and enzyme preparation (equivalent to 0.1 mg of protein). Suitable aliquots of a 1 mM solution of the mercurial were added to yield the indicated final concentrations in the 1-ml reaction mixture. The reaction was initiated by the addition of the enzyme, and the initial rate was determined from the decrease in absorbance (5), automatically recorded. The control consisted of the same reaction mixture minus dihydrofolate. PCMB, p-chloromercuribenzoate.

TABLE I

| Purification and properties of enzyme-methylmercuric complex |
|-----------------|-----------------|
|                 | \( \Delta A_{260}/\text{min/mg of protein}^* \) |
|                 | No addition     | + Mercaptoethanol† |
| Original enzyme | 0.214           | 0.214              |
| Enzyme-mercurial complex | 1.360 | 0.200              |
| Sephadex C-25 eluate | 1.360 | 0.105              |
| Ammonium sulfate fraction, 60-85% saturated | 4.100 | 0.600              |
| Sephadex G-25 eluate | 3.950 | 0.585              |

* Assay as described in Fig. 1, omitting the mercurial.
† Mercaptoethanol, 20 mM.

substrate; however, a maximal activation of only about 2- to 3-fold was observed at pH 4.5; at higher pH values, no significant activity could be observed with either form of the enzyme.

Because the inhibition of dihydrofolic reductase by the antifolic drugs (aminopterin, amethopterin, etc.) has been described as the basis for the unique pharmacological action of these compounds (2), it was of interest to compare the effects of aminopterin on the enzyme-mercurial complex and on the untreated enzyme. On a percentage basis, the degree of inhibition was identical over the inhibitor concentration range when dihydrofolate was utilized as the substrate.
could be detected by this method. However, the sedimentation studies did show that chicken liver dihydrofolic reductase is a relatively small protein with a molecular weight in the range of 15,000 to 20,000. Thus, it would appear that activation may involve a reversible alteration of the molecular structure of the protein rather than any dissociation of the protein into more active subunits. It is conceivable, therefore, that urea, ionic strength, and hydrogen ion, as well as the organic mercurials, result in the same activated state; however, the mercurials yield the activated enzyme as the stabilized structural modification resulting from a direct interaction with a specific site or sites.

The generality of this phenomenon is difficult to assess at present since preliminary examination of the crude enzyme derived from rat liver exhibits only inhibition in the presence of the various mercurials, despite the fact that the rat liver enzyme shows the urea and salt activation. This does not appear to be dependent on the degree of purification since the chicken liver enzyme responds to mercurial activation at all stages of purification.

Current studies now in progress are directed toward the elucidation of the kinetic parameters of the activated enzyme with respect to the original enzyme, analysis of the proposed structural modification of the protein by physical techniques, and the possible physiological significance of this effect.

REFERENCES

Inactivation of Chymotrypsin by Cyanate

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(Received for publication, November 15, 1963)

The studies to be reported are an outgrowth of attempts to stabilize trypsin by reaction with cyanate under mild conditions.

* Supported in part by a grant from the National Institutes of Health, United States Public Health Service.
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It was anticipated that covering of the most available NH₂ groups would cause little inactivation since previous investigations (see Sri Ram, Bier, and Maurer (1) for references) have shown that NH₂ groups of trypsin can be acetylated without loss of activity. We find, however, that in the reaction with cyanate (cf. Stark et al. (2) and Stark and Smyth (3)), carboxymethylation of less than half of the ε-amino groups of lysine residues is accompanied by inactivation of the enzyme. Moreover, there is no correlation between loss of activity and substitution of α- or ε-NH₂ groups. When we observed that both chymotrypsin and subtilisin are similarly inactivated by cyanate, it seemed possible that cyanate might react with some other group, and the most likely possibility was the reactive hydroxyl group of the serine residue at the active center of each of these enzymes. Because chymotrypsin is relatively stable and is the best characterized enzyme of the three, it was selected for more detailed study.

The rate of inactivation of α-chymotrypsin (Worthington Biochemical Corporation, Lot CDC47) by cyanate under different conditions is shown in Fig. 1. Activity is lost far more rapidly at pH 6.5 than at pH 8.5. The enzyme is protected from inactivation at pH 6.5 by the presence of the competitive
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