Interaction of Milk Xanthine Oxidase with Folic Acid

INHIBITION OF MILK XANTHINE OXIDASE BY FOLIC ACID AND SEPARATION OF THE ENZYME INTO TWO FRACTIONS ON SEPHAROSE 4B/FOLATE GEL*

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Inhibition of xanthine oxidase by folic acid was reexamined after complete removal of the contaminant which was responsible for time-dependent inactivation (Lewis, A. S., Murphy, L., Meailla, C., Pfeary, M., and Thereill, S. (1984) J. Biol. Chem. 259, 10784–10786). From turnover experiments using stopped flow equipment with a limited amount of xanthine and excess oxygen, and from kinetic analyses with an oxygen electrode, folic acid was found to be an inhibitor of xanthine oxidase. The inhibition was competitive with xanthine with a $K_i$ value of $4.2 \times 10^{-5}$ M. From the behavior of the enzyme in affinity chromatography using a Sepharose 4B/folate column, folic acid was also confirmed to be a competitive inhibitor of xanthine oxidase. When enzyme which had been pretreated with oxipurinol was applied to the affinity column, two fractions of xanthine oxidase were separated. The first fraction was found to contain the fully active form (double-active dimers) from the analyses of spectral changes on addition of xanthine, oxipurinol titration, and ESR slow signal, whereas the second fraction was assumed to contain mixed dimers and double-inactive dimers. The ratio of the content of the first fraction to that of the second fraction supports the hypothesis that there are three enzyme species and that there is no interaction either in catalytic activity or in sulfuration or desulfuration reactions between the two subunits.

Inhibition of xanthine oxidase by folic acid was first reported by Kalcker et al. (1), but subsequent investigation by himself demonstrated that this inhibition was due to pterine-6-aldehyde (2-NH$_2$-4-OH-pteridine-6-aldehyde), a photolytic breakdown product of folic acid (2). The recent report (3) that folic acid was a potent inactivator of xanthine oxidase was also refuted promptly for the same reason (4). It is well established that xanthine oxidase and related enzymes contain an inactive form (5). An inactive form is known to be caused by treatment with cyanide, which results in the release of an essential sulfur atom as thiocyanate (6). The recent evidence showed that the sulfur atom existed as a terminal sulfide of molybdenum (Mo=S) in the active form (7, 8). The inactive form usually exists to some extent in enzyme samples prepared by normal procedures. Resolution of active and inactive forms was reported by Edmondson et al. (9) who used an allopurinol analogue as an affinity ligand. We reported previously a much easier method for preparation of a highly active enzyme by using affinity chromatography on Sepharose 4B/folate gel (10). In this chromatography the enzyme behaved as though it had an affinity for folate, suggesting that folate was actually an inhibitor of the enzyme. Although the highly active enzyme sample was prepared by affinity chromatography on the folate column, the content of the inactive form in the enzyme preparation has not been analyzed in detail previously.

In this paper we re-examined whether folic acid was an inhibitor and also analyzed in detail the content of the inactive form in the enzyme preparation purified by the affinity chromatography.

MATERIALS AND METHODS

Xanthine oxidase was purified by the method of Bell (11), Massey et al., (12), or Hart et al. (13). Sepharose 4B/folate gel was prepared as described previously (10). The folate column was kept in the cold room in the dark and was repeatedly used by washing successively with 10 volumes each of 0.1 M pyrophosphate buffer, pH 8.5, and distilled water. The following buffer mixtures were used for affinity chromatography: A; 20% 0.1 M pyrophosphate buffer, pH 8.5, containing 0.2 mM EDTA and 80% 0.05 M Tris-HCl, pH 7.8, containing 0.2 mM EDTA, B; 30% 0.1 M pyrophosphate buffer, pH 8.5, containing 0.2 mM EDTA and 70% 0.05 M Tris-HCl, pH 7.8, containing 0.2 mM EDTA. Xanthine and hypoxanthine were obtained from Sigma. Folic acid was from Sigma or Wako Chemicals. Folic acid was purified by DEAE-cellulose column chromatography according to the method of Shiota et al. (14). Then a trace amount of contaminant was removed by the following procedure. 10 mM folic acid solution in 0.1 M pyrophosphate buffer, pH 8.5, was mixed with an equal volume of 0.1 M pyrophosphate buffer, pH 8.5, and was kept at 4 °C in the dark overnight. After filtration in the dark with a Millipore Moulcut filter (type SJGC), the filtrate was used for experiments. Absorption spectra were recorded on a Hitachi U-320 recording spectrophotometer. Xanthine oxidase activity was measured spectrophotometrically by following the absorption at 295 nm in 0.1 M pyrophosphate, pH 8.5, at 25 °C.

Specific activity was expressed as AFR (16). Kinetic analyses by an oxygen electrode were performed in a 3.0-mL reaction mixture containing 0.1 M pyrophosphate buffer, pH 8.5, with various concentrations of xanthine, oxygen, and folic acid. An oxygen electrode used was from Yellow Springs Corp. Absorption spectra were recorded on

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1 The abbreviation used is: AFR, activity to flavin ratio (enzyme activity defined as the absorbance change/min at 295 nm, monitoring conversion of xanthine to uric acid, divided by the enzyme absorbance at 450 nm in the standard assay condition).
an Amino-Chance DW-2a spectrophotometer or a Hitachi 557 spectrophotometer. Turnover experiments were performed with a Hitachi 557 spectrophotometer equipped with a stopped flow apparatus. The ESR slow signal was obtained by reduction of the sample for 20 min with dithionite at a final concentration of about 4 mM (17). Spectra were recorded on a Varian E9 spectrophotometer linked to a computer (18). Simulation of probability was performed using a personal computer NEC Model PC 8001.

RESULTS

We confirmed the previous reports (2, 4) that progressive inactivation of xanthine oxidase by folic acid was due to an impurity in commercially obtained reagent. However, we found that folic acid, from which the contaminant responsible for time-dependent inactivation was removed completely, was a competitive inhibitor of xanthine oxidase. This trace amount of contaminant could be removed by incubation of folic acid with enough xanthine oxidase to trap the contaminant as an enzyme-inhibitor complex followed by filtration with a Millipore Mol-cut membrane filter. The time course of the oxidation of xanthine was linear in the presence of the purified folic acid as shown in Fig. 1, whereas the time course declined progressively in the presence of commercially obtained folic acid in agreement with previous reports (1, 3, 4). The turnover experiment using a stopped flow spectrophotometer was performed by following the absorbance at 450 nm of xanthine oxidase with a limited amount of xanthine and excess oxygen in the presence of the purified folic acid. As shown in Fig. 2 purified folic acid was clearly found to be an inhibitor of xanthine oxidase. A slower reduction rate of the enzyme and delayed consumption of xanthine was seen in the presence of folic acid. On analysis by the method of Gibson et al. (19) these data yielded the Lineweaver-Burk plots as shown in Fig. 3. The inhibition was competitive with xanthine with $K_i = 5 \times 10^{-5} \text{ M}$. Further analysis by monitoring oxygen consumption by an oxygen electrode showed that folic acid was a competitive inhibitor of xanthine oxidase with $K_i = 4.2 \times 10^{-5} \text{ M}$ (Fig. 4).

Separation of Xanthine Oxidase into Two Fractions by Affinity Chromatography on Sepharose 4B/Folate Gel—When enzyme purified up to the second ammonium sulfate step by a modification of Ball’s (11) method was applied to the folate column, all the enzyme was absorbed on the column and most was eluted with buffer containing hypoxanthine. This is consistent with the result that folic acid is a competitive inhibitor of xanthine oxidase. In this affinity chromatography neither separation of enzyme into fractions nor improvement of the AFR value was observed. However, when the enzyme pre-treated with oxipurinol was applied to the folate affinity column, it separated into two fractions (top section in Fig. 5): the first fraction of the enzyme passed through the column and the second fraction was eluted with buffer containing hypoxanthine. It should be noted that the two fractions were not interconvertible. Both fractions were eluted mostly at the original positions when each fraction was subjected to rechromatography after re-treatment with oxipurinol (middle and bottom sections in Fig. 5). In rechromatography, the recoveries of the enzymes of each fraction were about 70% of original fraction. As both fractions bound oxipurinol tightly, neither fraction had xanthine-O$_2$ activity soon after elution from the column. It is well established that only the reduced form of

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**Fig. 1.** Time course of oxidation of xanthine by xanthine oxidase in the presence of folic acid. Reaction was followed at 295 nm in 3 ml of mixture containing 0.1 mM xanthine, 0.1 M pyrophosphate buffer, pH 8.5, and 5 μg of xanthine oxidase in the presence of purified (●) or original Sigma’s (○) 10 μM folic acid at 25°C.

**Fig. 2.** Changes in absorbance at 450 nm when xanthine oxidase was mixed with a limited amount of xanthine and excess oxygen. Enzyme (A$_{295}$ = 0.419) in 0.1 M pyrophosphate buffer, pH 8.5, saturated with argon was mixed at 25°C with an equal volume of 20 μM xanthine and 0 or 0.56 mM folic acid in 0.1 M pyrophosphate buffer, pH 8.5, saturated with air.

**Fig. 3.** Lineweaver-Burk plots of the xanthine oxidase-catalyzed oxidation of xanthine with O$_2$ as electron acceptor. The lines were obtained by analysis (19) of the data of Fig. 2.

**Fig. 4.** Analysis of the inhibition of xanthine oxidase by folic acid using an oxygen electrode. The solution was initially saturated with air and the xanthine concentration was changed systematically at a series of fixed concentration of folic acid. The folic acid concentrations used were (from bottom to top): 0, 35, 70, and 140 μM. The $K_i$ value was obtained from secondary plots of apparent $K_m$ versus the folic acid concentration.

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The properties required for fully active enzyme were the two phases, the fast bleaching followed by a very much slower bleaching. The active enzyme can bind oxipurinol tightly and can be reactivated by re-oxidation of the reduced molybdenum by ferricyanide. This suggested that the first fraction contained mostly fully active enzyme since the theoretical value of fully active enzyme was expected to be 210 under these conditions (9). This partial activity is not due to contamination of fully active enzyme of the first fraction, but rather is due to active monomer in dimeric molecules, because rechromatography did not give the fraction containing the fully active enzyme.

Analysis of the Enzyme Purified by Affinity Chromatography—The properties required for fully active enzyme were described by Edmondson et al. (9). In order to know whether the first fraction with high AFR value was really fully active form, the properties of the first fraction were examined applying several criteria.

Morell (21) showed that the reduction of xanthine oxidase by xanthine under anaerobic conditions proceeded in two phases, the fast bleaching followed by a very much slower one. He suggested that the two phases of reduction were due to the presence of active and inactive enzymes. Edmondson et al. (9) demonstrated subsequently that the extent of rapid bleaching was linearly correlated with the specific activity of the enzyme, confirming the conclusion by Morell. On addition of xanthine to the enzyme which was obtained from the first fraction in the affinity chromatography and was reactivated with ferricyanide up to AFR 195, a large rapid decrease in the intensity of the visible spectrum was observed. The subsequent slower decrease was less than 5% of the total bleaching (Fig. 6). This indicates that the sample obtained by affinity chromatography is mostly the active form. This also confirms the conclusion by Morell that the biphasic reduction is due to the presence of the active and inactive enzymes.

Another piece of evidence for the existence of inactive form was put forward by Massey et al. (20) based on titration of the enzyme with oxipurinol. From titration experiments, they obtained the result that the ratio of oxipurinol to enzyme FAD required for complete inactivation was about 0.7 instead of 1.0, suggesting the existence of an inactive form, which could not bind oxipurinol, in this enzyme preparation. They also demonstrated the linear correlation between the specific activity and the amount of oxipurinol bound to enzyme FAD (9, 20). Essentially the same experiment was performed in the present work with the sample purified by affinity chromatography. As shown in Fig. 7, oxipurinol bound to the purified (AFR 190, 90% activity) enzyme with the ratio of 0.93, whereas it bound to the original enzyme (AFR 130, 62% activity) with the ratio of 0.6. The increase of the ratio of bound oxipurinol to enzyme FAD indicates that inactive...
enzyme was essentially eliminated from the sample by affinity chromatography.

Desulfo form of the enzyme has been known to show a distinctive ESR slow signal (17). For information on the desulfo form in the enzyme sample purified by affinity chromatography, it was subjected to ESR. ESR showed a definite slow signal, but the integration corresponding to only 1.8% of desulfo form in the sample, as shown in Table I.

**The Ratio of Active and Inactive Forms in the Preparation—**
In order to know the content of fully active form in the enzyme preparation, the preparation was applied to the affinity column and the ratio of the content of first fraction to second fraction was calculated from absorbance at 450 nm of each pooled fractions. When we applied the sample of highest AFR (149, 70% active) obtained by a modification of Ball's method to the affinity column, 59% of the total eluted sample was in the first fraction whereas 41% was in the second fraction. When we applied the samples of AFR 130 (62% active), which was the specific activity usually obtained in routine preparations, about 40% of the eluted sample was in the first fraction in the average of 10 experiments. Thus the amount of active form eluted in the first fraction was not linearly proportional to the percent activity of the original enzyme. As the subunit structure of the enzyme was known to be dimeric (22, 23), three species of enzyme molecules could be predicted: double-active dimer, mixed dimer, and double-inactive dimer. The existence of three such molecular species had been suggested previously (5, 9). The first fraction was considered to contain fully active enzyme (double-active dimer) whereas the second fraction was considered to contain mixed dimer and double-inactive dimer. This was also supported by the fact that the second fraction possessed partial activity, but the elution profile of rechromatography of the second fraction in the same condition as above was not changed as described above.

If two subunits are identical and there are no interactions between them either in catalytic activity or in their sulfuration of desulfuration reactions, then a random distribution of sulfur atoms to the three molecular species can be expected. Based on this model the populations of molecular species in the enzyme preparation with particular specific activity can be calculated from simulation of probability. 100 random trials were actually performed at every 1% of specific activity using a computer and assuming the number of enzyme molecules to be 100 (200 subunits). Fig. 8 shows the result of 100 simulations. The experimental data of the affinity chromatography shown above seem to fit in well with the estimated result in Fig. 8 (curve 1). This supports that there are three species of enzyme molecules and that no interactions occur between the two subunits either in catalytic activity or in their sulfuration and desulfuration reactions.

**DISCUSSION**

It is clear from the present study that folic acid is a competitive inhibitor of xanthine oxidase. We confirmed the previous reports that time-dependent inactivation of xanthine oxidase by folic acid was due to contamination by an impurity usually contained in the commercial reagent. It was suggested that the impurity was pterine-6-aldehyde, a photolytic breakdown product of folic acid (2, 4). In the present study a trace amount of impurity could be removed easily and effectively by incubation of folic acid with enough xanthine oxidase to bind the impurity followed by filtration with a Millipore Molicut filter. Time-dependent inactivation of xanthine oxidase was abolished after purification of folic acid, and purified folic acid was found to be a competitive inhibitor. The inhibition of xanthine oxidase by folic acid is not strong enough to permit analysis of the inhibition by normal spectrophotometric assay at 295 nm without being disturbed by the high absorbance of folic acid itself at this wavelength. From the kinetic analyses of oxygen consumption by oxygen electrode, the $K_a$ value was found to be $4.2 \times 10^{-9}$. It is not surprising that folic acid acts as a competitive inhibitor, because many purines, pterines, and other heterocyclic compounds are known to be inhibitors (24). Administration of folic acid reportedly had no effects on serum uric acid concentration (25). This might be due to the weak inhibitory effect of folic acid. However, a weak inhibitory effect of folic acid rather made it possible to use it as an affinity ligand for purification of xanthine oxidase. Too tight binding may make it difficult to elute the sample from an affinity column. The fact that the enzyme could be eluted from the folate column by hypoxanthine confirmed the inhibition by folic acid as being the competitive type. The possibility of binding of the enzyme to pterine-6-aldehyde as a ligand of the affinity gel can be ruled out, because pterine-6-aldehyde cannot couple to the AH-Sepharose with the carbodiimide reaction.

We have reported previously that highly active xanthine oxidase was obtained by using affinity chromatography on Sepharose 4B/folate gel (10). As we expected previously, the enzyme in the first fraction of the affinity chromatography was found to be very close to fully active from the analyses of spectral changes on addition of xanthine to the enzyme, the titration of the enzyme with oxipurinol, and the observation of the ESR slow signal. However, a few percent of inactive form was found to exist in the purified enzyme. This small amount of inactive form might be due to inactivation during the reactivation procedure, because further purification by rechromatography did not decrease the content of the inactive form. On the other hand, the second fraction is considered to contain two other molecular species.

Recovery from the affinity column was usually about 70% under our experimental conditions, but it depended on the size of the column and the amount of the sample applied.
Generally we got better recovery by applying a larger amount of the enzyme sample and using a smaller size of the column. In order to get good separation and recovery it is important to test the column at first with a large amount of enzyme without prior oxipurinol treatment (first affinity chromatography as described previously) (10). From this first affinity chromatography, we can estimate the amount of sample which should be applied to the column for best results. If any lack of success occurs in performing the affinity chromatography using the folate column (26), it might be due to inappropriate conditions in performing the chromatography. The different enzyme preparations made by the methods of Massey et al. (12) and Hart et al. (13) did not affect the behavior in affinity chromatography (data not shown). Loss of the sample to some extent (about 30%) in our affinity chromatography might be due to nonspecific binding to the column. However, most of this can be washed out from the column by the normal washing procedure with 0.1 M pyrophosphate buffer, pH 8.5. As the recoveries of both fractions in chromatography were almost the same, we estimated the ratio of the content of fully active form to those of half-active and inactive forms. The ratio obtained seems to fit in well with the value estimated from the simulation of probability based on the hypothesis that there are three enzyme species (molecular forms) and that the two halves of the molecule are identical and independent. No interaction in catalytic activity between two subunits is consistent with the result that the binding of oxipurinol/enzyme FAD is linearly proportional to the specific activity of the enzyme (20). As the content of fully active form was not linearly proportional to the specific activity of the original sample (curve 1 in Fig. 8), it is important for getting fully active enzyme in good quantity to use enzyme preparations with higher specific activity. Only 25% of fully active enzyme exists in the 50% active enzyme, whereas 56% of fully active enzyme exists in 75% active enzyme.

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