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ELECTROPHORETIC VARIANTS OF XANTHINE DEHYDROGENASE IN *DROSOPHILA MELANOGASTER*

II. ENZYME KINETICS

TERENCE T. T. YEN* AND EDWARD GLASSMAN

Department of Biochemistry and The Genetics Curriculum, University of North Carolina, Chapel Hill, N.C. (U.S.A.)

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SUMMARY

1. The activity of xanthine dehydrogenase in *Drosophila melanogaster* is affected by mutations at three loci: maroon-like *(ma-l)* on the X chromosome, and rosy *(ry)* and the low xanthine dehydrogenase *(lxd)* on the third chromosome. Previous studies on four types of naturally-occurring electrophoretic variants of xanthine dehydrogenase have shown that *ry* is a structural gene for xanthine dehydrogenase.

2. In the present study, kinetic analyses were carried out on these four electrophoretic variants. These include the K_m's of 2-amino-4-hydroxpyteridine, xanthine, methylene blue, and nicotinamide–adenine dinucleotide, and the K_i's of 2-amino-4-hydroxypteridine-6-carboxyaldehyde, ammeline, and 8-azaguanine. The data have shown no significant differences in the kinetic parameters of the xanthine dehydrogenase from the electrophoretic variants or from *lxd*.

3. The experiments of this study also indicate the possible presence of two active sites on xanthine dehydrogenase, one for purines and one for pteridines, and that pyridoxal can bind both active sites.

4. The biological significance of the electrophoretic variation is discussed in view of the present data.

Abbreviations: *AHP*, 2-amino-4-hydroxypteridine; *AHPC*, 2-amino-4-hydroxypteridine-6-carboxyaldehyde; *by*, blistery (3-48 ±: third chromosome, locus 48 ±), a recessive wing mutant; F-type, fast type electrophoretic variant of xanthine dehydrogenase; I-type, intermediate type electrophoretic variant of xanthine dehydrogenase; Ins CXF, an inversion on the third chromosome; *lxd*, low xanthine dehydrogenase (3-33 ±), recessive; OR, Oregon-R wild-type fly; PAC, Pacific wild-type fly; *Pm*, Plum, a dominant eye-color mutant marking an inversion of the second chromosome; *ru*, roughoid (3-0), a recessive eye mutant; S-type, slow type electrophoretic variant of xanthine dehydrogenase; *Sb*, Stuble (3-58.2), a dominant bristle mutant; SI-type, slow-intermediate type electrophoretic variant of xanthine dehydrogenase whose mobility is between those of S-type and I-type; Tris–albumin: 0.05 M Tris (pH 8) containing 1 mg bovine albumin per ml; Tris–EDTA: 0.05 M Tris (pH 8) containing 10^{-3} M EDTA; *W*: wrinkled (3-46.0), a dominant wing mutant; *Xa*: Xasta, a dominant wing mutant marking a translocation involving the second and the third chromosomes.

* Present address: Biological Research Division, Eli Lilly and Company, Indianapolis, Indiana, 46206, U.S.A.

INTRODUCTION

The activity of xanthine dehydrogenase in *Drosophila melanogaster* is affected by mutations at three loci: maroon-like eye color *(ma-l)* at 64 ± on the X chromosome, rosy eye color *(ry)* and low xanthine dehydrogenase *(lxd)* at 52 ± and 33 ±, respectively, on the third chromosome. When homozygous, *ma-l* or *ry* flies have only traces of xanthine dehydrogenase activity, while *lxd* flies have only 20 to 25% enzyme activity of the wild-type flies. Interactions of the products of these three loci are discussed elsewhere[1].

Four types of naturally-occurring variants with xanthine dehydrogenase activity have been demonstrated with vertical polyacrylamide gel electrophoresis[2,3]. They have relatively slow (S), slow intermediate (SI), intermediate (I), and fast (F) mobilities toward the anode at pH 8.9. Genetic analyses have shown that all four variants are due to alterations at the *ry* locus, which is the structural gene for xanthine dehydrogenase. These four *ry* alleles are named according to the mobility of their products; namely, $ry^{el\text{-}S}$, $ry^{el\text{-}SI}$, $ry^{el\text{-}I}$, and $ry^{el\text{-}F}$, where *el* stands for an electrophoretic variant. The existence of these variants in *D. melanogaster* provides an opportunity to examine the biological significance of electrophoretic variation. This report presents data which show no change in kinetic parameters among these xanthine dehydrogenase variants.

MATERIALS AND METHODS

Chemicals

The sources of the chemicals were: 2-amino-6-oxy-8-azaguanine and methylene blue (California Corp. for Biochemical Research); $(NH_4)_2SO_4$ and xanthine (Mann Research Lab.); bovine plasma albumin and protein standard solution (Armour Pharmaceutical Co.); EDTA and Folin phenol reagent (Fisher Scientific Co.); NAD^+ (P–L Biochemicals, Inc.); Sephadex G-200 (Pharmacia Fine Chemicals, Inc.); 2-amino-4-hydroxypteridine-6-carboxyaldehyde (AHPC) (a gift from Dr. B. JACOBSON); 2-amino-4-hydroxypteridine (AHP) (a gift from Dr. H. S. FORREST); milk xanthine oxidase (Worthington Biochemical Corp., a gift from Dr. I. FRIDOVICH); ammeline (American Cyanamid Co., a gift from Dr. I. FRIDOVICH).

Drosophila stocks and media

Flies were grown at 25 ± 1° on a medium containing 82.2% water, 0.4% agar, 4.9% dextrose, 2.4% sucrose, 8.0% cornmeal, 1.5% dried yeast, 0.08% phosphoric acid, 0.4% propionic acid, and 0.06% benzyl benzoate.

Five strains of *D. melanogaster* were used. They were Oregon-R wild type (OR), which has F-type xanthine dehydrogenase; Pacific wild type (PAC) and *ru lxd by*, which have I-type xanthine dehydrogenase; *W Sb/Ins CXF*, which has SI-type xanthine dehydrogenase.

Purification of xanthine dehydrogenase

Xanthine dehydrogenase was isolated and purified according to a method of KARAM[4]. All steps were carried out at 6° or below. The phenotype of all flies was checked under the microscope. The electrophoretic mobility of the purified xanthine

dehydrogenase was determined using the method of electrophoresis described previously[3]. Each extract contained only one band of xanthine dehydrogenase of the correct type; the band, however, tends to lose crispness after purication. This is due to the formation of a second molecular form of xanthine dehydrogenase (SHINODA AND GLASSMAN, in preparation).

Adult flies were homogenized in two volumes (v/w) of Tris–EDTA (pH 8) and the homogenate was centrifuged at 30 000 × *g* for 20 min. The resulting supernatant solution was adjusted to pH 5.2 with 1 M acetic acid, centrifuged immediately at 30 000 × *g* for 5 min, and the supernatant solution adjusted to pH 8 with 1 M KOH. Xanthine dehydrogenase was precipitated from this supernatant solution with $(NH_4)_2SO_4$ between 35 to 45% satn. The precipitate was dissolved in Tris–EDTA and chromatographed on a Sephadex G-200 column. The column was eluted with Tris–EDTA, and the fractions containing xanthine dehydrogenase were pooled for kinetic studies. This method achieved a purification of about 40 fold.

A modification of the method of LOWRY *et al.*[5] was used to determine the amount of protein.

Assays of xanthine dehydrogenase

Two assays of xanthine dehydrogenase were used.

AHP assay[6]. The reaction mixture contained 7 μM AHP, 20 μM methylene blue (NAD^+ was substituted for methylene blue when the K_m of NAD^+ was determined), an appropriate amount of xanthine dehydrogenase, and Tris–albumin (pH 8) to make the final volume 1 ml. The ingredients were added to a fluorometer cuvette which was kept in a dry bath (Thermoline) maintained at 30°. The enzyme was added last. Readings were taken at 1- to 2-min intervals for 10 min in a fluorometer (Photovolt No. 540) equipped with a 347-mμ primary filter (Photovolt) and a 405-mμ secondary filter (Turner No. 110-812). The sensitivity of the fluorometer was adjusted so that a solution of 1.6 μM quinine in 0.1 M H_2SO_4 read 100 on the scale. The high blank of the reaction mixture was reduced by zero suppression. The cuvette was always returned to the dry bath between readings. One unit of xanthine dehydrogenase is defined as that amount of enzyme which converts 1 $\mu\mu$mole of AHP to isoxanthopterin per min. A change of 1 unit on the fluorometer scale corresponds to approx. 2 xanthine dehydrogenase units.

Xanthine assay. The standard reaction mixture contained 50 μM xanthine, 500 μM NAD^+, an appropriate amount of xanthine dehydrogenase and Tris–albumin (pH 8) to make the final volume 1 ml. This mixture was pipetted into a fluorometer cuvette which was kept in a dry bath (Thermoline) maintained at 30°. The enzyme was always added last, and readings were taken at 1-min intervals for 5 min in a fluorometer (Photovolt No. 540) equipped with a 340-mμ primary filter (Photovolt) and a 449-mμ secondary filter (Photovolt). The sensitivity of the fluorometer was adjusted so that a solution of 0.5 μM quinine in 0.1 M H_2SO_4 read 80 on the fluorometer scale. The cuvette was always returned to the dry bath between readings. With this assay, one unit of xanthine dehydrogenase is defined as that amount of enzyme which causes the production of 1 mμmole of NADH due to the conversion of xanthine to uric acid. A change of one unit on the fluorometer scale corresponds to approx. one-fourth of a xanthine dehydrogenase unit.

The reaction rate was linear for at least 10 min at 30° (Fig. 1A) and was

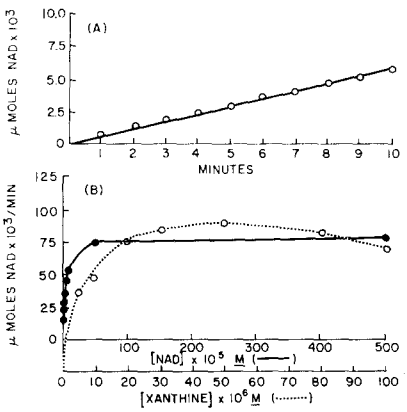


Fig. 1. (A) The time course of xanthine assay of xanthine dehydrogenase. The reaction mixture contained 0.01 ml xanthine dehydrogenase, 100 μM NAD^+, 50 μM xanthine, and Tris–albumin (pH 8) to 1 ml. (B) Substrate inhibition by xanthine (- - - -). The reaction mixture contained 0.02 ml xanthine dehydrogenase, 500 μM NAD^+, 1 to 100 μM xanthine, and Tris–albumin (pH 8) to 1 ml. Saturation curve with NAD^+ in xanthine assay (——). The reaction mixture contained 0.02 ml xanthine dehydrogenase, 0.01 to 5 mM NAD^+, 50 μM xanthine, and Tris–albumin (pH 8) to 1 ml.

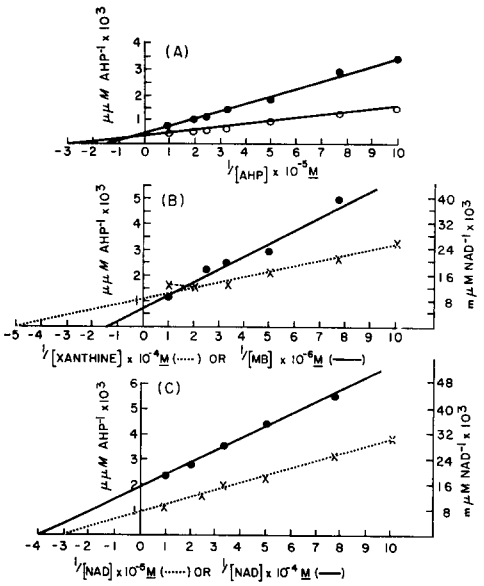
dependent upon xanthine and xanthine dehydrogenase. Occasionally endogenous activity was observed without substrate; a cuvette without substrate was also included as a control. The activity observed in this cuvette, if any, was subtracted from that of other reaction mixtures. Substrate inhibition by xanthine was observed at concentrations higher than 50 μM, but no inhibition was found with excess NAD^+ (Fig. 1B).

RESULTS

Kinetics of I-type xanthine dehydrogenase

Xanthine dehydrogenase was purified from PAC which contained the I-type variant, and various kinetic properties were studied. The K_m of AHP with methylene blue as the electron acceptor was 7.1 μM at pH 8 and 4.0 μM at pH 7 (Fig. 2A). That of methylene blue was 0.83 μM with AHP as the electron donor (Fig. 2B). Using NAD^+ as the electron acceptor, the K_m of xanthine was 18 μM (Fig. 2B). The K_m of NAD^+ using AHP as the substrate was 3.3 μM (Fig. 2C), but with xanthine it was 25 μM (Fig. 2C). The K_m of AHP remained constant at 30° and 50° (Fig. 3A), but the K_m of xanthine changed from 18 μM at 30° to 40 μM at 50° (Fig. 3B). Similar results were obtained with milk xanthine oxidase: the K_m of xanthine also changed with temperature[7], but the K_m of AHP did not (Fig. 3C).

AHPC, a pteridine, competitively inhibited the oxidation of AHP (Fig. 4A), while ammeline and 8-azaguanine, a triazine and a purine, respectively, inhibited the oxidation of xanthine in a competitive manner (Fig. 4B). The K_i's were 0.25 μM for AHPC, 37 μM for 8-azaguanine, and 21 μM for ammeline. When xanthine oxidation was inhibited by AHPC (Fig. 4C), and when AHP oxidation was inhibited by ammeline and 8-azaguanine (Fig. 4D), the inhibition was of the noncompetitive type, which suggested the presence of two active sites on the enzyme, one site for



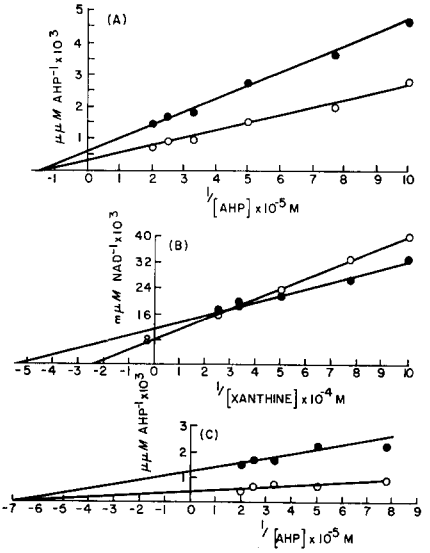


Fig. 2. Lineweaver–Burk plot determining the K_m of: (A) AHP at pH 7 (○) and at pH 8 (●). The reaction mixture contained 0.01 ml xanthine dehydrogenase (PAC), 20 μM methylene blue, 1 to 10 μM AHP, and Tris–albumin, pH 7 or 8, to 1 ml. (B) Xanthine (- - - -), the reaction mixture contained 0.02 ml xanthine dehydrogenase (PAC), 500 μM NAD^+, 10 to 100 μM xanthine, and Tris–albumin (pH 8) to 1 ml. Substrate inhibition is indicated by the dashed line. Methylene blue (MB) (—), the reaction mixture contained 0.01 ml xanthine dehydrogenase (PAC), 10 μM AHP, 0.1 to 1 μM methylene blue, and Tris–albumin (pH 8) to 1 ml. (C) NAD^+ with AHP (- - - -), the reaction mixture contained 0.01 ml xanthine dehydrogenase (PAC), 10 μM AHP, 1 to 10 μM NAD^+, and Tris–albumin (pH 8) to 1 ml. NAD^+ with xanthine (—), the reaction mixture contained 0.02 ml xanthine dehydrogenase (PAC), 50 μM xanthine, 10 to 100 μM NAD^+, and Tris–albumin (pH 8) to 1 ml.

Fig. 3. Lineweaver–Burk plot determining the K_m at 30° (○) and 50° (●) of: (A) AHP with xanthine dehydrogenase. The reaction mixture contained 0.02 ml xanthine dehydrogenase, 20 μM methylene blue, 1 to 5 μM AHP, and Tris–albumin (pH 8) to 1 ml. (B) Xanthine with xanthine dehydrogenase. The reaction mixture contained 0.02 ml xanthine dehydrogenase, 500 μM NAD^+, 10 to 100 μM xanthine, and Tris–albumin (pH 8) to 1 ml. (C) AHP with milk xanthine oxidase. The reaction mixture contained 0.01 ml milk xanthine oxidase, 1 to 5 μM AHP, and Tris–albnmui (pH 8) to 1 ml.

purines and the other for pteridines. The respective K_i's of AHPC, ammeline, and 8-azaguanine in these experiments were 0.51 μM, 45 μM, and 71 μM.

In contrast, experiments using milk xanthine oxidase showed that AHPC, ammeline, and 8-azaguanine inhibited the oxidation of AHP in a competitive manner (Fig. 5). The K_m of AHP with milk xanthine oxidase was 1 μM; the K_i's were 16 μM for ammeline, 1.2 μM for 8-azaguanine, and 0.016 μM for AHPC. This result agreed with the report that 2-amino-4-hydroxy-6-pteridylaldehyde inhibited the oxidation of AHP, xanthopterin, and xanthine competitively[8].

Pyridoxal oxidase is believed to be related to xanthine dehydrogenase since it is present in wild type and *ry* flies but not in *ma-l* and *lxd* flies[9,10]. It has also been observed that pyridoxal prevents the loss of xanthine dehydrogenase activity at 50° due to hypoxanthine[11]. For this reason, the effect of pyridoxal on the oxidation of AHP (Fig. 6) and xanthine (Fig. 7) was tested. It was found that the oxidation

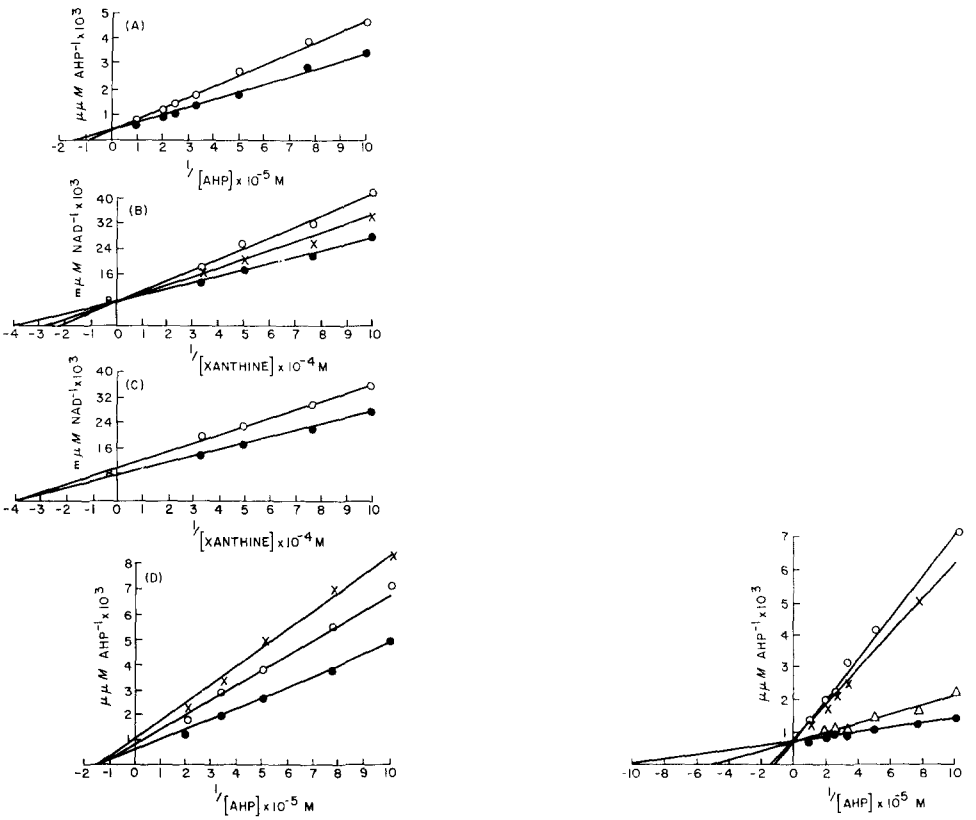


Fig. 4. Inhibition of the oxidation of: (A) AHP by AHPC. The reaction mixture contained 0.01 ml xanthine dehydrogenase (PAC), 20 μM methylene blue, 1 to 10 μM AHP, with (○) or without (●) 0.1 μM AHPC, and Tris–albumin (pH 8) to 1 ml. (B) Xanthine by ammeline or 8-azaguanine. The reaction mixture contained 0.02 ml xanthine dehydrogenase (PAC), 500 μM NAD^+, 10 to 100 μM xanthine, with 100 μM ammeline (×), or with 30 μM 8-azaguanine (○), or without both (●), and Tris–albumin (pH 8) to 1 ml. (C) Xanthine by AHPC. The reaction mixture contained 0.02 ml xanthine dehydrogenase (PAC), 500 μM NAD^+, 10 to 100 μM xanthine, with (○) or without (●) 0.2 μM AHPC, and Tris–albumin (pH 8) to 1 ml. (D) AHP by ammeline or 8-azaguanine. The reaction mixture contained 0.01 ml xanthine dehydrogenase (PAC), 20 μM methylene blue, 1 to 10 μM AHP, with 30 μM ammeline (×) or 8-azaguanine (○), or without both (●), and Tris–albumin (pH 8) to 1 ml.

Fig. 5. Inhibition by ammeline, 8-azaguanine, or AHPC of the oxidation of AHP by milk xanthine oxidase. The reaction mixture contained 0.01 ml xanthine oxidase, 1 to 10 μM AHP, with 0.1 μM AHPC (×), or with 10 μM 8-azaguanine (○), or with 30 μM ammeline (△), or without inhibitor (●), and Tris–albumin (pH 8) to 1 ml.

of both substrates was inhibited in a competitive pattern. At 30° and 50° the K_m of AHP was increased form 7.1 μM in the abscence of pyridoxal to 11 μM in the presence of pyridoxal. The K_i of pyridoxal at both temperatures was 89 μM with AHP and methylene blue as substrates. When xanthine and NAD^+ were substrates, the K_m of xanthine increased from 18 μM in the absence of pyridoxal to 36 μM in its presence at 30°. At 50° the K_m of xanthine changed from 40 μM to 59 μM when pyridoxal is present. The K_i of pyridoxal was 50 μM at 30° and 110 μM at 50°.

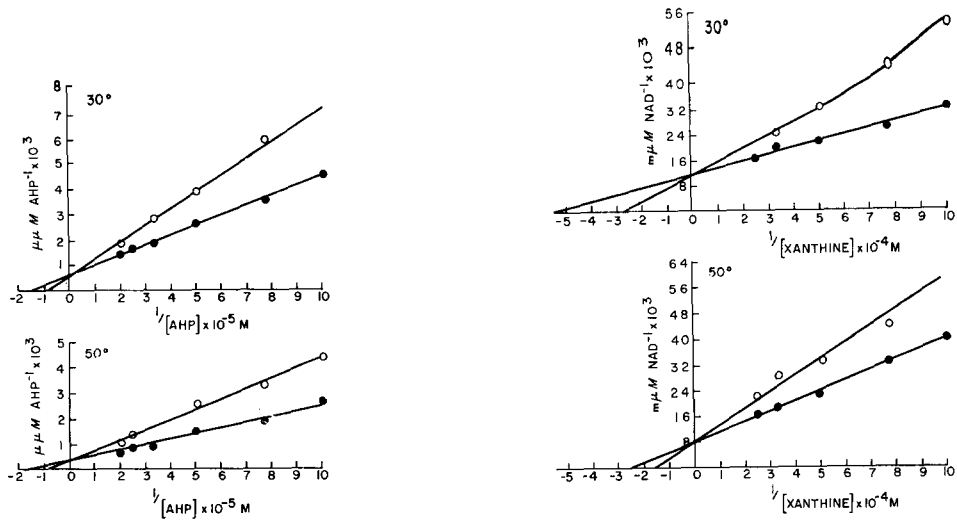


Fig. 6. Lineweaver–Burk plot determining the K_m of AHP at 30° (top) and 50° (bottom) in the presence of pyridoxal. The reaction mixture contained 0.02 ml xanthine dehydrogenase, 20 μM methylene blue, 1 to 5 μM AHP, with (○) or without (●) 50 μM pyridoxal, and Tris–albumin (pH 8) to 1 ml.

Fig. 7. Lineweaver–Burk plot determining the K_m of xanthine at 30° (top) and 50° (bottom) in the presence of pyridoxal. The reaction mixture contained 0.02 ml xanthine dehydrogenase, 500 μM NAD^+, 100 to 10 μM xanthine, with (○) or without (●) 50 μM pyridoxal, and Tris–albumin (pH 8) to 1 ml.

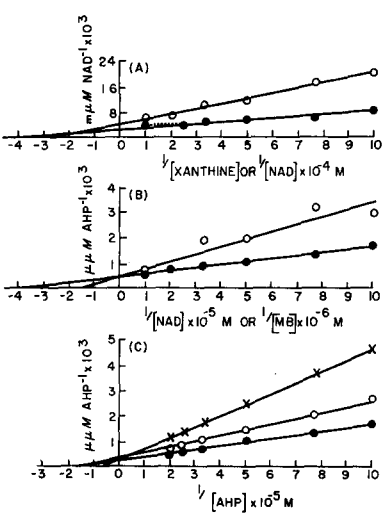


Fig. 8. Kinetic properties of xanthine dehydrogenase from *lxd.* (A), K_m of xanthine (●) and K_m of NAD^+ with xanthine (○). (B), K_m of NAD^+ (●) and methylene blue (MB) (○) with AHP. (C), K_m of AHP (●) and K_i of AHPC (×, using 0.5 μM AHPC) and ammeline (○, using 30 μM ammeline) with AHP and methylene blue as substrates.

TABLE I

SUMMARY OF KINETIC PROPERTIES OF XANTHINE DEHYDROGENASE FROM VARIANTS AND FROM *lxd* FLIES

Kinetic constants	*Variants*				
	S-type	*SI-type*	*I-type*	*F-type*	*lxd I-type*
I. Substrates: AHP and MB					
K_m, AHP, pH 7 ($\times 10^{-6}$ M)	5.0	5.6	4.0	5.9	
K_m, AHP, pH 8 ($\times 10^{-6}$ M)	7.1	6.3	7.1	6.7	6.7
K_m, methylene blue ($\times 10^{-7}$ M)	6.7	10	8.3	5.0	5.6
K_i, AHPC ($\times 10^{-7}$ M)	2.5	1.3	2.5	1.5	2.5
K_i, ammeline ($\times 10^{-5}$ M)	3.3	2.3	4.5	3.0	4.0
K_i, 8-azaguanine ($\times 10^{-5}$ M)	5.0	7.5	7.1	5.4	
II. Substrates: AHP and NAD+					
K_m, NAD+ ($\times 10^{-6}$ M)	2.5	2.2	3.3	2.0	2.5
III. Substrates: xanthine and NAD+					
K_m, xanthine ($\times 10^{-5}$ M)	1.8	1.9	1.8	2.4	2.5
K_m, NAD+ ($\times 10^{-5}$ M)	2.7	2.5	2.5	3.7	4.0
K_i, AHPC ($\times 10^{-7}$ M)	5.3	5.4	5.1	3.3	
K_i, ammeline ($\times 10^{-5}$ M)	4.8	3.0	2.1	3.0	
K_i, 8-azaguanine ($\times 10^{-5}$ M)	4.8	3.8	3.7	3.0	

Kinetics of other xanthine dehydrogenase variants and of lxd

Xanthine dehydrogenase was also purified from the other three variants and its kinetic characteristics examined. The results are summarized in Table I. No significant differences are observed between these 12 kinetic parameters among these electrophoretic variants.

Since *lxd* flies have only a quarter of xanthine dehydrogenase activity of the wild type[12], it was of interest to see if the xanthine dehydrogenase isolated from *lxd* had the same affinities toward these substrates and inhibitors. The data are summarized in Fig. 8 and Table I. The limited data obtained indicate that xanthine dehydrogenase of *lxd* is not different from that of the wild type.

DISCUSSION

The kinetic properties of Drosophila xanthine dehydrogenase have been extensively studied in this investigation. The results generally agree with the limited data reported previously on this enzyme. Thus, using NAD+ as the electron acceptor, the K_m of xanthine has been reported as 25 μM by GLASSMAN AND MITCHELL[14] and 23.6 μM by PARZEN AND FOX[15]. It averaged 21 μM in our study (Table I). Also, the K_m of AHP has been reported to be 6.7 μM by GLASSMAN AND MITCHELL at pH 8 (ref. 14), which is the same value as we obtained in these experiments. These K_m's are higher than the K_m's for the milk xanthine oxidase, which are 1.7 μM for xanthine[13] and 1 μM for AHP (ref. 8). Not only the K_m's differ; our studies also indicate that the kinetics of the fly xanthine dehydrogenase differ from that of the milk enzyme.

Using AHPC (a pteridine), 8-azaguanine (a purine), and ammeline (a triazine),

inhibition studies have given some insight into the mechanism of the enzymatic function of Drosophila xanthine dehydrogenase. When both the substrate and the inhibitor are pteridines or purines (Fig. 4), the inhibition is of the competitive type, which indicates that the inhibitor and the substrate are competing for the same site on the enzyme. Ammeline, a triazine, behaves as a purine (Fig. 4), as reported by FRIDOVICH[16]. The result, however, is different when a purine is the substrate and a pteridine is the inhibitor, or *vice versa* (Fig. 4); the inhibition in both cases is of the noncompetitive type. This implies the presence of two active sites on the enzyme, one site for purines and the other for pteridines. The binding of one type of substance at one site might cause what may be an allosteric transition of the enzyme molecule, which impairs the binding of another type of substance at another site. Xanthine oxidase from milk apparently has only one active site for both purines and pteridines: purines inhibit the oxidation of pteridines in a competitive manner, and *vice versa* (Fig. 5). The evolutionary significance of this type of allosterism must await further studies on the enzyme from other organisms using both types of substrates.

The molecular relationship between xanthine dehydrogenase and pyridoxal oxidase of Drosophila is not clearly defined. Previous experiments demonstrated no pyridoxal oxidase activity in *ma-l* and *lxd* flies[9,10] and suggested that these enzymes might have subunits or a cofactor in common[1]. The fact that pyridoxal protects xanthine dehydrogenase activity at 50° from destruction in the presence of hypoxanthine suggests that pyridoxal has an affinity for xanthine dehydrogenase[11]. Our present data show that pyridoxal inhibits the oxidation of both AHP (Fig. 6) and xanthine (Fig. 7) in a competitive manner. This indicates that pyridoxal, which is not a substrate of xanthine dehydrogenase, can bind with both active sites on Drosophila xanthine dehydrogenase, and it seems reasonable to conclude that the protection by pyridoxal[11] could be due to the competition between pyridoxal and substrate for a common site on the enzyme molecule.

The discovery of electrophoretic variations of proteins and enzymes in general brings up the question of the biological significance of their existence. This study was designed to answer the question of whether these four types of electrophoretic variants of xanthine dehydrogenase differ in their kinetic properties so that one type has some adaptive advantage functionally over another type. The present data indicate no significant differences in the kinetic parameters studied among these electrophoretic variants. This implies that, if there is any adaptive value related to electrophoretic variation, the difference among variants is not reflected in their kinetic characteristics, which generally represent the biological significance of an enzyme. The present data do not rule out the possibility that the adaptive advantage resides in some other properties of the enzyme molecule which were not measured in this study. These properties could include other kinetic parameters: the conformation of the molecule; the stability of its enzymatic activity; the freedom of movement of the molecule in the cell and through the membranes; the susceptibility of the molecule to regulatory mechanisms; and many others. On the other hand, the present data demonstrate that the alteration of amino acid composition in xanthine dehydrogenase, as reflected by various electrophoretic mobilities, does not change the protein molecule so as to affect its catalytic functions. It may well be that this type of variation, like many other types of variations we encounter throughout the

biological world, could be the result of spontaneous mutations which do not have adaptive significance.

ACKNOWLEDGMENTS

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