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Inhibition study of rhodanese by means of electrophoretically mediated microanalysis

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Abstract

A combination of the electrophoretically mediated microanalysis methodology with a partial filling technique was applied for the inhibition study of bovine liver rhodanese by 2-oxoglutarate. In this set-up, part of the capillary is filled with the best buffer for the enzymatic reaction, while the rest of the capillary is filled with the optimal background electrolyte for separation of substrates and products. The estimated value of K_I for 2-oxoglutarate was $3.62 \cdot 10^{-4} \pm 1.43 \cdot 10^{-4}$ M with respect to cyanide and $1.40 \cdot 10^{-3} \pm 1.60 \cdot 10^{-4}$ M with respect to thiosulfate. In addition, the type of inhibition was also evaluated. The findings of 2-oxoglutarate as the competitive inhibitor with respect to cyanide and as the uncompetitive inhibitor with respect to thiosulfate are in accordance with previous literature data.

Keywords: Electrophoretically mediated microanalysis; Partial filling electrophoretically mediated microanalysis; Rhodanese; Oxoglutarate; Enzymes; Enzyme inhibitors

1. Introduction

The enzyme rhodanese (thiosulfate: cyanide sulfur transferase, EC 2.8.1.1) discovered by Lang in 1933 [1] is involved in the major route of biological cyanide detoxification [2–5]. It catalyses transfer of the sulfane sulfur of thiosulfate to an acceptor, which is normally cyanide in the standard assay, and is likely to be cyanide under some physiological conditions [6,7]:

$$S_2O_3^{2-} + CN^- \rightarrow SCN^- + SO_3^{2-}.$$

Rhodaneses are widespread in the biological world: their activity has been detected in several species ranging from microorganisms through fungi, plants and animals to man [8]. Their physiological role has been debated for many years with proposals ranging from the detoxication of cyanide to the suggestion that rhodanese is important in bioenergetic oxidation of thiosulfate [9], in generating iron–sulfur protein complexes [10], in lipoate metabolism [11] and in reactivation of nitrogenase [12].

Because of the significance of rhodanese to fundamental and applied toxicology, many studies have been performed to elucidate its kinetic mechanism [13–15]. Most of them utilized spectrophotometric assays having associated limits such as consumption of relatively large amount of enzyme, no possibility of automation, etc. In order to overcome these limitations a new method based on capillary zone electrophoresis (CZE) has been recently applied to determine the kinetic parameters of rhodanese–electrophoretically mediated microanalysis (EMMA)

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[16]. In this method, substrate(s) and enzyme are introduced in the capillary as a distinct plug, the first analyte injected being that with the lower electrophoretic mobility. Upon the application of an electric field, the two zones interpenetrate due to differences in their electrophoretic mobilities. Enzymatic reaction takes place and the resultant reaction product(s) and the unreacted substrate(s) are electrophoretically transported towards the detector, where they are individually detected.

The Michaelis constants for both substrates and the effect of temperature on rhodanese reaction have been evaluated utilizing the EMMA approach. In addition the type of kinetic mechanism of enzymatic reaction has also been elucidated. The purpose of this study was to apply the EMMA methodology to the inhibition study of rhodanese by 2-oxoglutarate. To the best of the authors' knowledge it is the first application of EMMA method for an inhibition study of bi-substrate enzymatic reaction.

2. Experimental

2.1. Materials and reagents

Rhodanese from beef liver, 2-oxoglutarate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and potassium cyanide were obtained from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical reagent grade, supplied from Fluka (Buchs, Switzerland). The background electrolyte was prepared by adding hydrochloride acid to 0.1 *M* β-alanine solution up to pH 3.5. The HEPES buffer was prepared by adding 0.1 *M* sodium hydroxide to 0.1 *M* HEPES solution up to pH 8.5. All solutions were prepared with Milli-Q water (Millipore, Milford, MA, USA) and filtered through a 0.45 μm membrane filter. Enzyme, substrates and inhibitor solutions were freshly prepared in 25 m*M* HEPES buffer (pH 8.5) each day. The mixing of substrates and inhibitor was performed immediately before the measurements to prevent their reaction.

2.2. Instrumentation

A Hewlet-Packard ^{3D}CE system (Waldbronn, Germany) with diode-array UV–Vis detector was used to carry out all CZE separations. Data were collected on an HP Vectra VL5 166-MHz personal computer using the Hewlet-Packard ^{3D}CE ChemStation Software. A Polymicro Technologies (Phoenix, AZ, USA) 75 μm fused-silica capillary was used for all separations.

2.3. Monitoring of rhodanese reaction by EMMA method

A 75 μm fused-silica capillary (64.5 cm total length, 56 cm effective length) was washed with 0.1 *M* β-alanine–HCl (pH 3.5) as a background electrolyte for 3 min. The on-capillary enzymatic reaction was performed by injection of 25 m*M* HEPES buffer (pH 8.5), the enzyme solution in 25 m*M* HEPES buffer (pH 8.5), the substrates solution in 25 m*M* HEPES buffer (pH 8.5) containing 2-oxoglutarate as an inhibitor, 25 m*M* HEPES buffer (pH 8.5), and the background electrolyte all at 50 mbar for 4.0 s consecutively into the capillary. The temperature of the capillary was 25 °C. The reaction was initiated by application of −18 kV (negative polarity) separation voltage. Samples were detected at 200 nm with a bandwidth of 20 nm. The peak areas were measured using ChemStation software. Evaluation and calculation of inhibition constants were done by means of the SigmaPlot 2001 software.

3. Results and discussion

3.1. Enzyme inhibition

The inhibition of enzymatic activity by specific molecules and ions is important because it serves as a major control mechanism in biological systems. Also, many drugs and toxic agents act by inhibiting enzymes. Furthermore enzyme inhibition can provide insight into the mechanism of enzyme action.

Enzyme inhibition can be either a reversible or irreversible process. In irreversible inhibition, the inhibitor is covalently linked to the enzyme or bound so tightly that its dissociation from the enzyme is very slow. In contrast, reversible inhibition is char-

acterised by a rapid equilibrium of the enzyme and inhibitor.

Three main types of reversible inhibition — competitive, uncompetitive and non-competitive — are known.

A competitive inhibitor resembles the shape and size of an enzyme substrate. It competes for substrate binding sites on the enzyme surface and so decreases the number of bound substrate. Michaelis–Menten equation for competitive inhibition can be described as

$$v_0 = \frac{V_{max}[S]}{K_M \cdot (1 + \frac{[I]}{K_I}) + [S]} \quad (1)$$

where v_0 and V_{max} are the initial and maximum velocities, respectively, K_M is the Michaelis constant, $[S]$ is the concentration of substrate, $[I]$ is the concentration of inhibitor, and K_I is the inhibition constant. The Lineweaver–Burk (double reciprocal) plots of initial velocity on the concentrations of substrate at the different concentration of a competitive inhibitor are intersected at the point $1/V_{max}$, which is typical for competitive inhibition.

An uncompetitive inhibitor is incapable of binding to the free enzyme. It can only bind to the enzyme–substrate complex. Once the inhibitor binds, it prevents the enzyme from turning the substrate into the product. Michaelis–Menten equation can be described as

$$v_0 = \frac{V_{max}[S]}{K_M + [S] \cdot (1 + \frac{[I]}{K_I})} \quad (2)$$

The series of the Lineweaver–Burk plots at the different concentrations of an uncompetitive inhibitor form the set of parallel lines.

A non-competitive inhibitor can bind both the enzyme and the enzyme–substrate complex. It binds at a site separate from the active site and modifies the enzyme conformation to inhibit the formation of the product. Michaelis–Menten equation can be described as

$$v_0 = \frac{V_{max}[S]}{K_M \cdot (1 + \frac{[I]}{K_I}) + [S] \cdot (1 + \frac{[I]}{K_I})} \quad (3)$$

The Lineweaver–Burk plots at the different concentration of a non-competitive inhibitor are intersected at the point $1/K_M$, which is typical for non-competitive inhibition [17].

It must be emphasised that all these formulas and plots are derived for uni-substrate enzymatic reactions. For multi-substrate reactions the algebraic origins of the plots are more complex, but essentially the same patterns arise and can be analysed to yield true K_I values.

3.2. Electrophoretically mediated microanalysis (EMMA) of rhodanese

The EMMA methodology was described by Bao and Regnier in 1992 [18]. Since its discovery it has been applied in a number of biochemical systems — for assays of enzyme activities [18–24], determination of substrates [23,25,26], Michaelis constants [16,27–30], inhibitors and inhibition constants [31,32], etc. As mentioned above, EMMA utilizes the different electrophoretic mobilities of enzyme, substrate(s) and product(s) to initiate enzymatic reaction inside the separation capillary and to separate the given compounds. Its original arrangement — the same buffer used for the enzymatic reaction and the electrophoretic separation — fundamentally restricts its applicability: the electrophoretic conditions, especially the composition and pH of background electrolyte, must be favorable for both the separation of substrate(s) and product(s) of the enzymatic reaction and the enzymatic reaction itself. To solve this problem Van Dyck et al. introduced the combination of the EMMA methodology with a partial filling technique [27]. In this set-up part of the capillary is filled with the best buffer possible for the enzymatic reaction while the rest of the capillary is filled with the optimal background electrolyte for separation of substrates and products. A similar approach has been adopted for the study of kinetic parameters of rhodanese [16]. The enzymatic reaction was performed in 25 m*M* HEPES buffer (pH 8.5) [33] while the low pH background electrolyte (100 m*M* β-alanine–HCl, pH 3.5) was used for separation of substrates and products [33,34].

An identical set-up with only minor modification — the inhibitor added to the substrate solution — was used in this work. The capillary was first filled

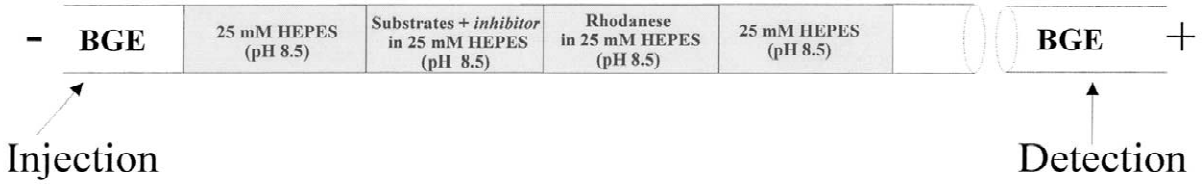

Fig. 1. Illustration of the combination of the EMMA methodology with a partial filling technique.

with the background electrolyte. Subsequently, a plug of the HEPES buffer, a plug of the enzyme solution, a plug of the substrates solution with or without the inhibitor, a plug of the HEPES buffer and finally a plug of the background electrolyte were injected hydrodynamically into the capillary (Fig. 1). The exact injections parameters are described in the Experimental section. The typical electropherogram of the on-column enzymatic reaction with the enzyme solution (0.5 mg of protein per ml) and the

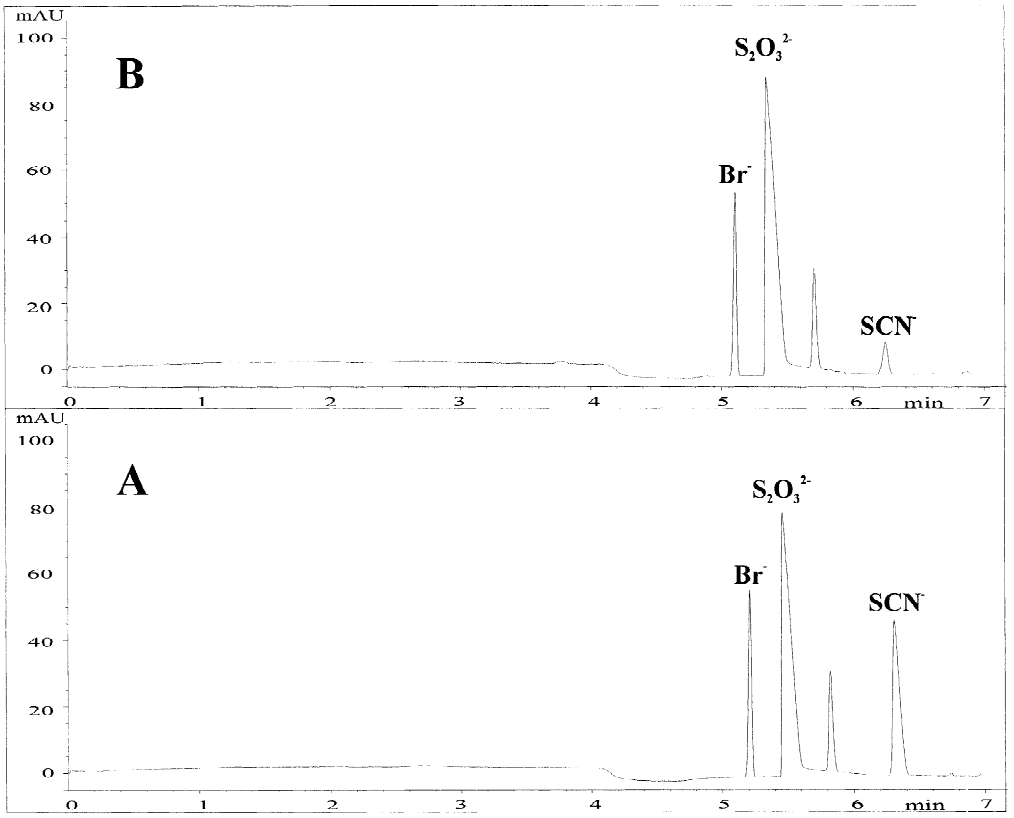

Fig. 2. Typical electropherogram of the on-column rhodanese reaction without (A) and with (B) 2-oxoglutarate as inhibitor added into the plug of substrates. The concentration of substrates was 5 m*M* thiosulfate and 5 m*M* cyanide, while the concentration of 2-oxoglutarate was 5 m*M*. The 0.4 m*M* bromide was added as an internal standard. Separation conditions: background electrolyte 0.1 *M* β-alanine–HCl (pH 3.50), separation voltage −18 kV (negative polarity), 75 μm fused-silica capillary (64.5 cm total length, 56.0 cm effective length), direct detection at 200 nm, temperature of capillary 25 °C. Injection: 25 m*M* HEPES buffer (pH 8.5), 50 mbar for 4.0 s; the enzyme solution, 50 mbar for 4.0 s; the substrates solution without or with inhibitor, 50 mbar for 4.0 s; 25 m*M* HEPES buffer (pH 8.5), 50 mbar for 4.0 s; and the background electrolyte, 50 mbar for 4.0 s, consecutively into the capillary.

substrates solution containing 5.0 m*M* sodium thiosulfate and 5.0 m*M* potassium cyanide is shown in Fig. 2A. The 0.4 m*M* bromide was added as an internal standard; the small peak between the thiosulfate and thiocyanate peaks was an unknown compound from the enzyme sample. The inhibition activity of 2-oxoglutarate on rhodanese reaction can be seen from the electropherogram measured under the same conditions but with the addition of 5.0 m*M* 2-oxoglutarate to the plug of substrates (Fig. 2B).

Since rhodanese is a bi-substrate enzyme, the inhibitory behavior of 2-oxoglutarate against each substrate was determined individually by measuring the initial velocities of the enzymatic reaction at the varying concentrations of the one substrate and the inhibitor, and at the fixed concentration of the second substrate and vice versa. Each substrate and inhibitor combination was analysed in triplicate. The initial reaction velocities were measured from the thiocyanate peak areas. As peak areas have no physical units, the reaction velocities are scaled arbitrarily.

The Michaelis–Menten plots with the potassium cyanide as varied substrate, sodium thiosulfate as fixed substrate (10.0 m*M*) and at different concentrations of 2-oxoglutarate are given in Fig. 3. The Lineweaver–Burk plots are linear intersecting the $1/v_0$ axis at the point $1/V_{max}$ and show that 2-oxoglutarate is a competitive inhibitor with respect to

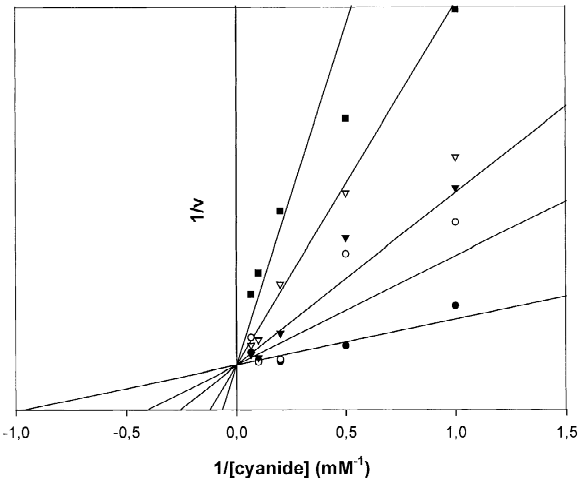

Fig. 4. The Lineweaver–Burk plots showing competitive inhibition by 2-oxoglutarate with the cyanide as the varied substrate and thiosulfate (10.0 m*M*) as the fixed substrate. The family of lines was obtained by varying the 2-oxoglutarate concentration as follows: (●) 0 m*M*; (○) 0.5 m*M*; (▼) 1.0 m*M*; (▽) 2.5 m*M*; (■) 5.0 m*M*.

cyanide (Fig. 4). The K_I value for 2-oxoglutarate with respect to cyanide, $3.62 \cdot 10^{-4} \pm 1.43 \cdot 10^{-4}$ *M*, was computed from these data.

On the other hand, Fig. 5 shows the Michaelis–Menten plots with the sodium thiosulfate as varied

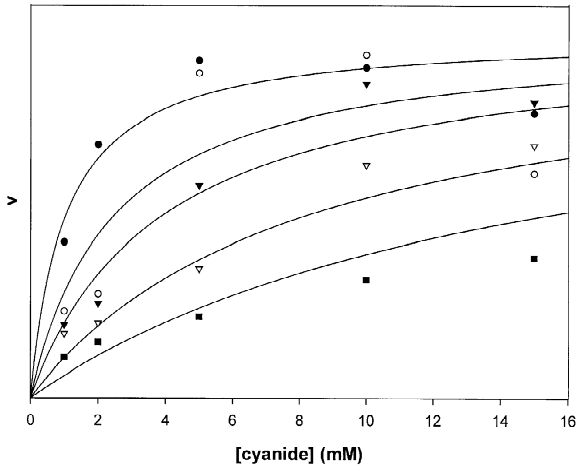

Fig. 3. The Michalis–Menten plots for the enzymatic reaction of rhodanese inhibited by: (●) 0 m*M*; (○) 0.5 m*M*; (▼) 1.0 m*M*; (▽) 2.5 m*M*; and (■) 5.0 m*M* 2-oxoglutarate with the cyanide as the varied substrate and thiosulfate (10.0 m*M*) as the fixed substrate. The separation conditions were the same as in Fig. 2.

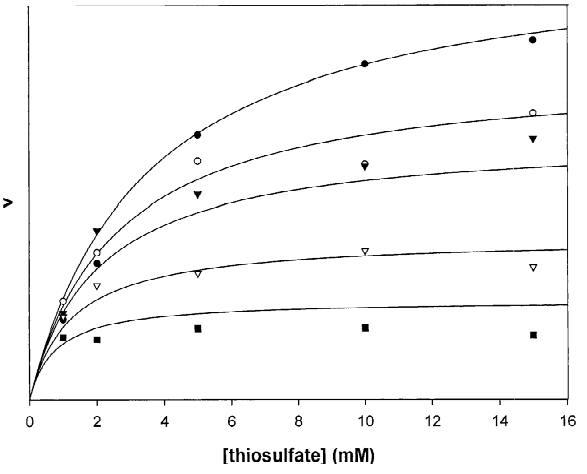

Fig. 5. The Michalis–Menten plots for the enzymatic reaction of rhodanese inhibited by: (●) 0 m*M*; (○) 0.5 m*M*; (▼) 1.0 m*M*; (▽) 2.5 m*M*; and (■) 5.0 m*M* 2-oxoglutarate with the thiosulfate as the varied substrate and cyanide (10.0 m*M*) as the fixed substrate. The separation conditions were the same as in Fig. 2.

substrate, potassium cyanide as fixed substrate (10.0 m*M*) and at different concentrations of 2-oxoglutarate. The Lineweaver–Burk plots are linear and parallel (Fig. 6) indicating 2-oxoglutarate as an uncompetitive inhibitor with respect to thiosulfate. The K_I value for 2-oxoglutarate with respect to thiosulfate, $1.40 \cdot 10^{-3} \pm 1.60 \cdot 10^{-4}$ *M*, was computed as well.

The inhibition of rhodanese activity by tricarboxylic acid cycle intermediates was first observed by Lawrence [35]. Susumu [36] later demonstrated that bovine liver rhodanese was inhibited competitively by 2-oxoglutarate with respect to cyanide and uncompetitively with respect to thiosulfate and proposed the reaction mechanism shown in Fig. 7, which includes the inhibition reaction by 2-oxoglutarate (2-OG) as well as the non-enzymatic formation of cyanohydrin (Y). All our results and conclusions are in accordance with this schematic. The inhibition study in the present paper thus provide further important evidence for elucidation of the action of rhodanese. In consequence the EMMA methodology could serve as a progressive tool of modern enzymology in the context of metabolomic research.

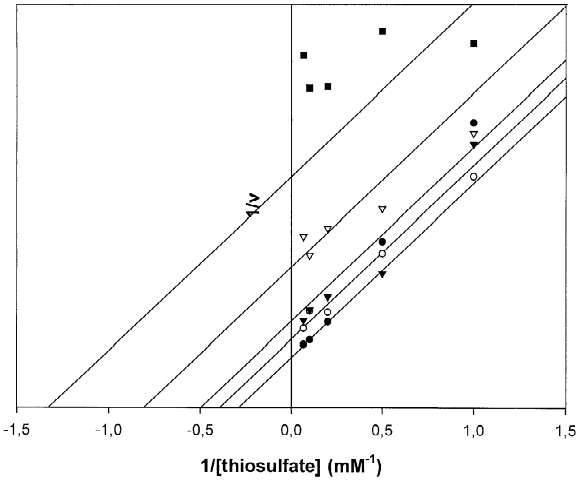

Fig. 6. The Lineweaver–Burk plots showing uncompetitive inhibition by 2-oxoglutarate with the thiosulfate as the varied substrate and cyanide (10.0 m*M*) as the fixed substrate. The family of lines was obtained by varying the 2-oxoglutarate concentration as follows: (●) 0 m*M*; (○) 0.5 m*M*; (▼) 1.0 m*M*; (▽) 2.5 m*M*; (■) 5.0 m*M*.

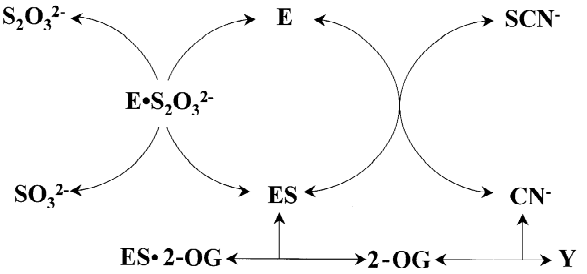

Fig. 7. Illustration of the inhibition of rhodanese by 2-oxoglutarate: 2-oxoglutarate is competitive inhibitor with respect to cyanide and uncompetitive inhibitor with respect to thiosulfate. (E, enzyme; $E \cdot S_2O_3^{2-}$, non-covalent complex between enzyme and thiosulfate; ES, covalent complex between enzyme and sulfur; 2-OG, 2-oxoglutarate; Y, cyanohydrin).

4. Conclusions

This work shows that the inhibition study of bovine liver rhodanese can be easily performed by EMMA methodology combined with partial filling technique. The method can be used not only to estimate K_I but also for the determination of the inhibition type. Compared to spectrophotometric and other discontinuous assays, the method is rapid, can be automated, and requires only small amounts of reagents, which is especially important in the case of enzymes. Consequently the method has great potential for such determinations in other enzyme-inhibitor systems.

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