THE TITRATION OF RHODANESE WITH SUBSTRATES

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1. Introduction

The transfer of sulfur catalyzed by rhodanese (EC 2.8.1.1.) is thought to proceed through a double displacement mechanism where the enzyme is first charged with the donor sulfur and then discharged by the acceptor [1]. Earlier workers have reported that the enzyme accepts from 1 to 1.9 atoms of sulfur per mole of enzyme of mol. wt. of 37 000 (2–4). The large difference being mainly due to the procedure adopted for the purification of the enzyme and to the method used for the determination of transferable sulfur. As summarized in a recent review [5] the form of sulfur bound to rhodanese has been debated for a long time without receiving a satisfactory answer. In recent investigations carried out in our laboratory [6] it has been found that rhodanese, crystallized from beef kidney, exhibits a definite absorbancy in the form of a shoulder in the area of 335 nm which disappears on the addition of cyanide. This absorbancy, which has been ascribed to the presence of a persulfide group (R-SSH) in the active site of rhodanese, provides a new technical means for establishing the chemical form of transferable sulfur and for studying the catalytic mechanism of this enzyme. The present note describes the titration of rhodanese with cyanide and with thiosulfate, using the absorbancy at 335 nm as a guide, aimed at establishing the correlation between this absorbancy and the amount of sulfur loosely bound to the enzyme.

2. Materials and methods

Crystalline rhodanese has been prepared from beef kidney as reported earlier [7]. The titrations have been made by using a Beckman DU2 spectrophotometer equiped with a temperature control. Readings were taken at 7°C in stoppered silica cuvettes 1 cm light path. The crystalline enzyme after centrifugation was dissolved in 0.05 M sodium acetate and dialyzed overnight against 3 liters of the same solution. The final solution of the enzyme had a concentration in the order of 24 mg/ml, which was estimated by reading at 280 nm a scaled down aliquot of the solution and using the value $E^{1\%}_{1\,cm} = 17.5$ [7]. The potassium cyanide used for the titration was obtained from Erba C. Milano, it had a purity of 98% as determined by argentometric titration. The cyanide solution was brought to pH 9 by the careful addition of the required amount of acetic acid, under the control of a pH-meter.
Sodium thiosulfate was a Merck product used as such. Spectrophotometric curves in the area of the specific absorbancy around 335 nm were registered by a DK2 Beckman spectrophotometer. The reactants were added to the enzyme solution by using Pedersen pipettes. The reported values have been corrected, when necessary, for dilution.

3. Results and discussion

The titration of the crystallized and dialyzed enzyme by cyanide is shown on the left of fig. 1. The decrease of the 335 nm absorbancy at any addition of cyanide is apparently immediate and stops when an amount of cyanide equivalent to 1.35 atoms (a mean value of three titrations) of sulfur per mole of enzyme (mol. wt.= 37 000) has been added. The spectral curves in this area before and after the titration with cyanide are shown in fig. 2 (curves A and B). On the right of fig. 1 it is reported the back titration of rhodanese with thiosulfate soon after the titration with cyanide

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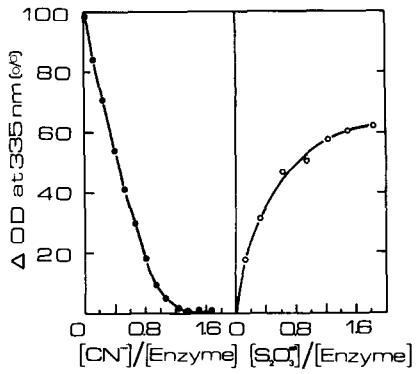

Fig.1. Titration of rhodanese with substrates. To 0.65 mM rhodanese in 0.05 M sodium acetate successive amounts of KCN (•—•—•) were added following the absorbancy at 335 nm. At the end of the titration with KCN the reverse experiment was performed by adding successive amounts of $Na_2S_2O_3$ (o—o—o). The data are plotted as the percentage variation in optical density at 335 nm against the molar ratio between substrate and enzyme.

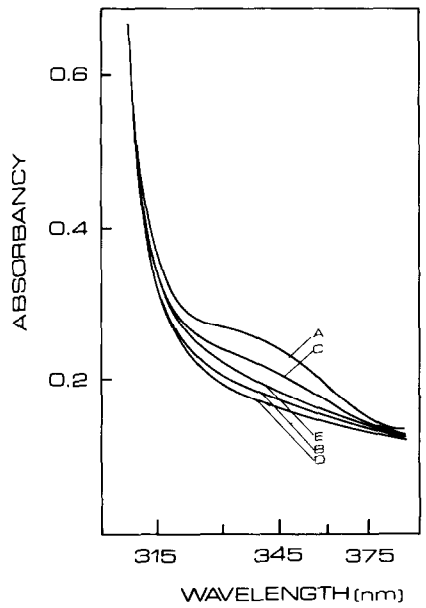

Fig.2. Absorbancy of rhodanese in the area of 335 nm. 0.65 mM rhodanese in 0.05 M sodium acetate: before titration with cyanide (A); after titration with cyanide (B); after titration with cyanide and back titration with thiosulfate (C). The enzyme at the end of the experiment reported in fig. 3 gives the following spectral curves: after recrystallization in the presence of mM thiosulfate (A); after precipitation with excess ammonium sulfate (D); after precipitation with excess ammonium sulfate in the presence of 8 mM thiosulfate (E).

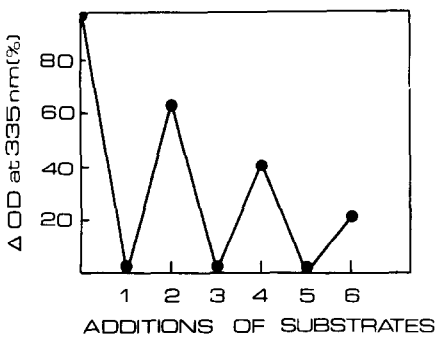

Fig.3. Percentage variation of optical density at 335 nm of rhodanese after successive additions of total dosages of cyanide and thiosulfate. The experimental conditions were the same of the experiment reported in fig. 1 with the difference that for each dosage the molar ratio between substrates and enzyme was 20% in excess. 1, 3, 5, additions of cyanide. 2, 4, 6, additions of thiosulfate.

has been ended. It is evident an increase of the absorbancy at 335 nm, which is immediate after any addition of thiosulfate. The addition of thiosulfate however does not restore the initial absorbancy exhibited by rhodanese before the addition of cyanide, but stops at a level of about 60% of that expected for a complete recharge of rhodanese with sulfur. Further increases of thiosulfate produce only a slight increase of the absorbancy. The spectral curve at the end of titration is therefore intermediate between that of the initial enzyme and that of the enzyme titrated with cyanide (fig. 2 curve C). When an amount of cyanide and thiosulfate, able, respectively, to discharge the enzyme, is added successively to the enzyme, as it is reported in fig. 3, the increase of absorbancy by thiosulfate is in any case lower than that expected by the previous decrease caused by cyanide. The enzyme recrystallized at the end of an experiment as that reported in fig. 3, in the presence of 8 mM thiosulfate, gives a spectral curve identical to that of the original enzyme. If not recrystallized but precipitated by excess of ammonium sulfate in the presence of 1 mM thiosulfate gives the curve E in fig. 3, while if precipitated by ammonium sulfate in the absence of thiosulfate gives the curve D in the same fig. 3.

As a conclusion of the experiments described above it is confirmed that the absorbancy exhibited by rhodanese in the area of 335 nm has to be related to the

presence of transferable sulfur bound to the enzyme possibly in the form of a persulfide group. The initial titration with cyanide indicates that the enzyme, as it is usually crystallized is not fully saturated with transferable sulfur. The average of 1.35 atoms of sulfur per mole of enzyme is in fact less than 2 atoms of sulfur expected by the dimeric form of the enzyme and by the possible occurrence of two active sites (1, 2). Moreover it is evident that the sulfur responsible for the absorbancy at 335 nm does not represent a definite structural feature of rhodanese, its content being strongly dependent upon the actual state of the enzyme, whether freshly crystallized or precipitated or treated with one or the other substrate.

Acknowledgements

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