Rhodanese from *Thiobacillus* A2: Determination of Activity by Proton Nuclear Magnetic Resonance Spectroscopy

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SUMMARY

Rhodanese from *Thiobacillus* A2 was shown by proton nuclear magnetic resonance (NMR) spectroscopy to use dihydrolipoate or dihydrolipoamide as acceptor of the sulphane moiety of thiosulphate with the formation of α -lipoate or lipoamide respectively. Correlation is shown between assays of the enzyme activity by NMR spectroscopy and by ultraviolet spectrophotometry.

INTRODUCTION

Rhodanese from *Thiobacillus* A2 uses dihydrolipoate or dihydrolipoamide as the acceptor of the sulphane moiety from thiosulphate with the production of α -lipoate and lipoamide respectively; the persulphides of these compounds are transitory intermediates in this reaction (Silver & Kelly, 1976). We now present confirmatory evidence obtained by proton nuclear magnetic resonance (NMR) spectroscopy for this reaction. This technique is particularly suitable for studying enzymic reactions which result in either major chemical change or deuteration of the substrate, as it detects changes in the chemical environment of all ^1H nuclei present in the reaction mixture.

METHODS

Rhodanese was isolated from *Thiobacillus* A2 as described previously (Silver & Kelly, 1976). The enzyme for the NMR assay was precipitated by dialysis for 3 h against 1000 vols saturated $(\text{NH}_4)_2\text{SO}_4$ (pH 5.0), containing 10^{-3} M- $\text{Na}_2\text{S}_2\text{O}_3$, recovered by centrifuging at 13000 g, and redissolved in a quantity of D_2O equal to half the original volume. This resulted in the loss of 60 to 70 % of the enzyme activity. $\text{Na}_2\text{S}_2\text{O}_3$ and dihydrolipoate were also dissolved in D_2O ; dihydrolipoamide was dissolved in 50 % (v/v) CD_3OD in D_2O . In all cases, solutions were adjusted to pD 10.2 by the addition of solid Na_2CO_3 .

The activity of rhodanese was determined colorimetrically by the formation of thiocyanate or spectrophotometrically by the formation of α -lipoate or lipoamide as described previously (Silver & Kelly, 1976) with sufficient solid Na_2CO_3 added in place of Tris to adjust the pD to 10.2. The NMR reaction mixture contained: 5 μmol dihydrolipoate or dihydrolipoamide; enzyme; and 5 μmol thiosulphate, with which the reaction was routinely initiated. The volume of the reaction mixture was adjusted to 0.5 ml by adding D_2O . A rhodanese preparation containing 24 μg protein was used; this quantity of enzyme catalysed the formation of

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Fig. 1

Fig. 2

Fig. 1. Partial ^1H -NMR spectra of (a) dihydrolipoate and (b) α -lipoate.

Fig. 2. Partial ^1H -NMR spectra of rhodanese reaction mixture containing dihydrolipoate after incubation times (min) of (a) 0, (b) 60, (c) 120 and (d) 180.

0.22 μmol thiocyanate in 10 min when assayed by the colorimetric method. The reaction was monitored by an automated observation of the NMR spectrum (Couch, Howarth & Moore, 1975) every 15 or 20 min at 28 $^\circ\text{C}$ in a Bruker spectrometer operating at 90 MHz. A delay of 4 to 8 min was experienced between the initiation of the reaction and the midpoint of the first spectrum recorded. For clarity, therefore, this first spectrum was taken arbitrarily as the initial point in calculation of the enzyme activity. Standard spectra of α -lipoate and dihydrolipoate in D_2O , adjusted to pD 11.0 with solid Na_2CO_3 , and lipoamide and dihydrolipoamide in CDCl_3 , were recorded independently. Dihydrolipoate and dihydrolipoamide always contained 5 to 15 % α -lipoate and lipoamide respectively as contaminants.

D_2O , CD_3OD and CDCl_3 (minimum isotope purity, 99 atom % D) were obtained from Merck, Sharp and Dohm Canada Ltd, Montreal. The sources of all other materials have been described previously (Silver & Kelly, 1976).

Fig. 3. Correlation of NMR assay and spectrophotometric assay of rhodanese in D_2O or H_2O . NMR assay carried out in D_2O with dihydrolipoate (■) or dihydrolipoamide (□) as substrate; spectrophotometric assay carried out in H_2O with dihydrolipoate (▲) or dihydrolipoamide (△), or in D_2O with dihydrolipoate (●) or dihydrolipoamide (○).

RESULTS AND DISCUSSION

The multiplets centred at $\delta = 2.68$ and 3.00 p.p.m. in the NMR spectrum of dihydrolipoate and at $\delta = 3.22$ and 3.71 p.p.m. in the α -lipoate spectrum (Fig. 1) arose from the CH_2-S and $CH-S$ protons respectively, which are well separated in chemical shift. The spectra of lipoamide and dihydrolipoamide were very similar in this region.

When NMR spectra were recorded at 20 min intervals during the reaction of rhodanese with dihydrolipoate, the formation of α -lipoate could be seen (Fig. 2). The ratio of the heights of the α -lipoate and dihydrolipoate multiplets equals the ratio of concentrations of the two species, as it is independent of any minor line-broadening in the spectrum, and is also independent of the observed pD decrease of between 0.4 and 0.5 units during the course of the reaction.

Correlation between the formation of α -lipoate from dihydrolipoate and lipoamide from dihydrolipoamide when assayed by NMR spectroscopy or by u.v. spectrophotometry in either D_2O or H_2O (Fig. 3) shows that, as reported previously (Silver & Kelly, 1976), the reaction was faster with dihydrolipoamide than with dihydrolipoate. In this case, the reaction measured by NMR spectroscopy was 38 % faster with dihydrolipoamide than with dihydrolipoate. Similar differences were noted when the reactions were measured spectrophotometrically; little difference was noted in the rates of reaction in the presence of D_2O or H_2O . The lack of a linear relationship of the enzyme activity with respect to time may be due either to the instability of the enzyme or to the aforementioned increase in acidity during the reaction. The former reason seems more probable, as the difference between the activity at pH 10.2 and that at pH 9.7 is only approximately 10 %, whereas the enzyme preparation used in the previous study (Silver & Kelly, 1976), which was more stable than that used in this study, lost 50 % of its activity in 4 h at 0 to 4 °C.

We conclude that NMR spectroscopy is a further method for the study of rhodanese activity and has provided additional evidence for the production of α -lipoate and lipoamide from the reaction of thiosulphate with dihydrolipoate and dihydrolipoamide catalysed by the *Thiobacillus* A2 enzyme.

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