Volume 278, number 2, 151–154 FEBS 09143 January 1991
[991 Federation of European Biochemical Societies 0014-5793/91/$3.50
4DONIS 0014579391000103

Identification of sulfurtransferase enzymes in *Azotobacter vinelandii*

S. Pagani[1], E. Franchi[1], R. Colnaghi[1] and C. Kennedy[2]

[1] *Dipartimento di Scienze Molecolari Agroalimentari and Centro Interuniversitario per lo Studio delle Macromolecole Informazionali, University of Milan, Italy* and [2] *AFRC Institute of Plant Science Research, Nitrogen Fixation Laboratory, Brighton, BN1 9PQ, UK*

Received 22 November 1990

Rhodanese and 3-mercaptopyruvate sulphurtransferase have been identified in *A. vinelandii*. Two distinct active fractions of the two sulphurtransferases were obtained after FPLC ion exchange chromatography of material partially purified from crude extracts. Rhodanese has been purified to homogeneity, and it consists of one polypeptide chain of M_r ca 25000. A partial purification of 3-mercaptopyruvate sulphurtransferase was obtained.

Azotobacter vinelandii, Sulphurtransferase, Rhodanese, 3-Mercaptopyruvate sulphurtransferase

1 INTRODUCTION

The sulphurtransferase rhodanese (Rd) (thiosulphate-cyanide sulphurtransferase EC 2.8.1.1) and 3-mercaptopyruvate sulphurtransferase (3-MST) (EC 2.8.1.2) catalyze the transfer of a sulphane sulphur atom from a donor molecule to a thiophilic acceptor substrate [1–4]. Although widely distributed, the physiological role of these enzymes has not been established [1,5]. Previous work demonstrated that mammalian Rd is effective in the in vitro synthesis of heterometallic clusters [6–9]. In addition, inactive Fe-protein of the nitrogenase from *Klebsiella pneumoniae*, lacking the full complement of iron and sulphide, could be reactivated for acetylene reduction after incubation with Rd and its substrates [10].

Low levels of Rd and 3-MST were detected in cultures of *K. pneumoniae* [11]. In preliminary experiments, activities in *Azotobacter vinelandii*, another nitrogen fixing organism, were more stable and ten times higher for Rd. The aim of the work presented here was to verify whether the sulphurtransferase activities detected in *A. vinelandii* extracts corresponded to specific endogenous enzymes. Sulphurtransferase enzymes generally show low substrate specificity [5], and recently Aird et al. purified from the prokaryote *Acinetobacter calcoaceticus lwoffi* a sulphurtransferase which utilized a wide range of acceptor substrates [12]. We report here the identification and purification of Rd and 3-MST from cultures of *A. vinelandii*.

Correspondence address: S. Pagani, Dipartimento di Scienze Molecolari Agroalimentari, Via Celoria 2, 20133 Milano, Italy

2 MATERIALS AND METHODS

Azotobacter vinelandii strain UW136 (from NFL collection) was grown in Burk's sucrose medium [13] in a 200 litre New Brunswick stainless steel fermentor. Aeration rate was 50 l min^{-1}. Cells were harvested in a Westphalia centrifuge when the culture reached $OD_{600} = 2.0$.

Extracts were produced by thawing 50 g of frozen cells in 75 ml of 50 mM phosphate buffer containing 3.5 mM thiosulphate (pH 7.0). The suspension was passed twice through a Cell Fractionator at 28 psi of argon. Cell debris was removed by centrifugation at 13 000 × *g* for 60 min, the supernatant was used for the purification of sulphurtransferases.

Proteins were estimated either by the biuret method [14] or in the case of partially purified material, from the absorbance at 280 nm by using the extinction coefficient of 1.50 for a solution containing 1.0 mg/ml.

Rhodanese activity was determined by the colorimetric assay based on the absorption at 460 nm of the ferric thiocyanate complex formed from the reaction product thiocyanate and ferric nitrate [15]. The activity figures were corrected for the absorbance at 460 nm of a parallel assay in which the sample was added after the addition of HCHO in the cubation mixture. The 3-mercaptopyruvate sulphurtransferase assay is based on the detection of the product pyruvate [4,16]. The absorbance figures were corrected by subtracting the value obtained when the enzyme preparation was added after $CdDl_2$. One unit of Rd and 3-MST activity is defined as the amount of enzyme that produce 1 μmol of thiocyanate or pyruvate per min at 37°C.

2.1 *Purification of the sulphurtransferases*

2.1.1 Step 1. Ammonium sulphate precipitation

The extract was made 65% saturated with ammonium sulphate. After centrifugation at 13 000 × *g* for 30 min, the precipitate was redissolved in 50 mM phosphate buffer, pH 7.0, containing 3.5 mM thiosulphate (buffer A). Unless otherwise stated, all procedures were carried out at 4°C.

2.1.2 Step 2. Sephadex G50 fractionation and ion-exchange chromatography

The redissolved proteins from Step 1 (50 ml) were fractionated on a column of Sephadex G50 Fine (Pharmacia, 7.5 × 40 cm), equilibrated with buffer A, at a flow rate of 200 ml/h. The fractions with high sulphurtransferase activities were pooled and passed through a

column of DEAE Sephacel (Whatman, 5 × 7 cm) equilibrated in buffer A. Sulphurtransferase activities were recovered in the non bound fraction. The active material eluted from the DEAE Sephacel column was pooled and concentrated by precipitation with ammonium sulphate (65%) at pH 7.0. After centrifugation the precipitate was redissolved in 5 ml of 10 mM phosphate buffer pH 7.0, containing 3.5 mM thiosulphate (buffer B).

2.1.3. Step 3. Desalting and FPLC ion exchange chromatography

The material from Step 2 was desalted on Sephadex G25 equilibrated with buffer B. The eluate was divided in aliquots of 2–2.5 mg protein which were separately loaded on a TSK-DEAE 5PW column (LKB, 0.8 × 7.5 cm) equilibrated in buffer B in a FPLC apparatus (LKB) at room temperature. Elution of bound proteins was achieved by a linear gradient of buffer B to buffer A in 25 min (Fig. 1). This step essentially separated Rd and 3-MST activities.

2.1.4. Step 4. Gel filtration by FPLC

The combined material with Rd activity from three FPLC-DEAE elutions (peak B of Fig. 1) was concentrated about 50-fold by using a Centricon 10 (Amicon) device, and loaded on a column of Superose 12 10/30 HR (LKB, 1 × 30 cm) equilibrated in 50 mM phosphate buffer containing 0.1 M NaCl (pH 7.5) in FPLC apparatus. Rd was eluted with the same buffer at a flow rate of 0.5 ml/min. The same procedure was followed for the peak A of Fig. 1 containing essentially 3-MST.

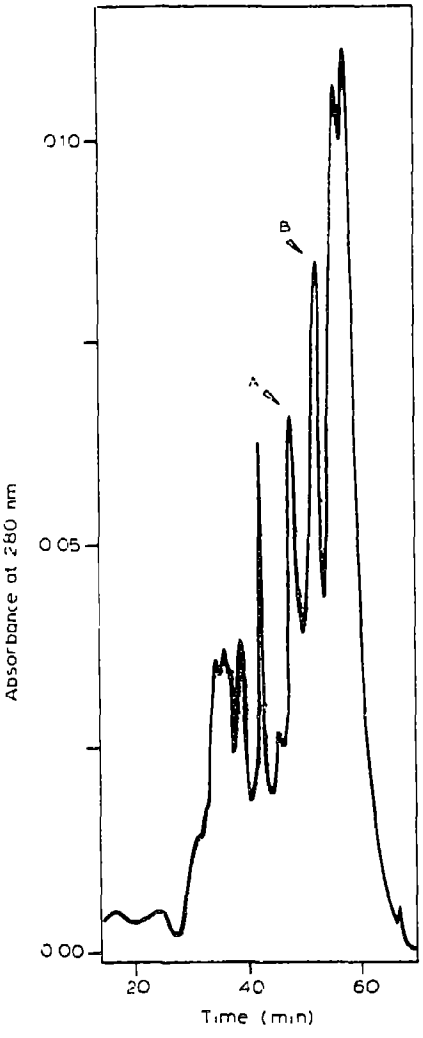

Fig. 1. Chromatographic separation of Rd and 3-MST. A typical chromatogram obtained with aliquots of material from Sephadex G25 passed through the TSK-DEAE 5PW FPLC column is shown. A and B are peaks of 3-MST and Rd activities, respectively.

3. RESULTS AND DISCUSSION

Rhodanese and 3-mercaptopyruvate sulphurtransferase activities were found in crude extracts of *A. vinelandii* grown with either 15 mM ammonium acetate (spec. act. of 0.142 and 0.183, respectively, for Rd and 3-MST) or dinitrogen (spec. act. of 0.300 and 0.410, respectively) as nitrogen source. The activities were only slightly higher in the dinitrogen-grown cells, suggesting that neither was a product of a nitrogen fixation-specific gene. Since sulphurtransferase activities in the presence of different donor molecules are not necessarily specific for the identification of two separate enzymes [5,12], we attempted the purification of the Rd and 3-MST activities.

3.1. *Purification of the enzymes from N_2-grown cells*

The scheme for purification is shown in Table I. During the early stages, both activities were recovered in the same fractions. In order to achieve separation on FPLC ion-exchange chromatography, we found it necessary to first remove bulky contaminating proteins on a Sephadex G50 column, although this led to significant loss of material. After FPLC chromatography, two distinct peaks were recovered (Fig. 1). The fraction corresponding to 3-MST contained 3.3% of residual Rd activity. The peak containing Rd activity eluted just behind that of 3-MST, in the Rd fraction the contaminating 3-MST accounted for 5% of the total sulphurtransferase activities. Further purification of the two enzymes was achieved by FPLC gel chromatography on Superose 12HR (Table I). Both enzymes were, at this final stage, purified more than 1000-fold compared to the crude extract specific activities and no cross-contaminating activities were found (Table I).

3.2. *Homogeneity and molecular weight of the purified enzymes*

When the 3-MST fraction from FPLC-DEAE was chromatographed on Superose, two major and several minor peaks were obtained (not shown). The 3-MST activity was found only in one peak which eluted at a position corresponding to an apparent M_r or 25–26 kDa. This peak gave two major polypeptide bands (Fig. 2) when analyzed by SDS-PAGE that accounted for 61% and 33% of the total proteins loaded. Their apparent M_rs correspond to 38 and 29 kDa, respectively. Since we did not succeed in the purification of 3-MST to homogeneity, the definition of its molecular weight requires further investigation.

Table 1

Purification of sulphur transferases from *A. vinelandii*

Step	Total protein (mg)	Rd				3-MST			
		Total units	Specific act.	Purification (x fold)	Yield (%)	Total units	Specific act.	Purification (x fold)	Yield (%)
Crude extract	2275	780	0.340	1	100	643	0.280	1	100
Sephadex G50 fine	555	310	0.560	1.6	40	247	0.450	1.6	38
DEAE Sephacel	63	250	3.975	11.6	32	200	3.170	11.2	31
FPLC chromatography DEAE peak A	0.3	2.2	7.500	-	0.3	65	218	770	10
3-MST from Superose	0.2	0.0	0.0	-		61	283	1010	9.5
DEAE peak B	0.5	119	244	715	15	6.1	12.5	-	0.2
Rd from Superose	0.27	110	350	1030	14	0.0	-		

After chromatography on Superose of the Rd fraction from FPLC-DEAE one single peak with an apparent M_r of 24 kDa was found (not shown) SDS-PAGE of the Rd eluted from Superose further demonstrated the presence of a single polypeptide species (Fig. 2) with an apparent M_r of 29 kDa.

Molecular weights of the same order of magnitude as that of mammalian Rd (33 kDa) [17] and also of less than 20 kDa have been reported for Rd's isolated from prokaryotic organisms. Thiosulphate-utilising bacteria contain Rd's with sizes ranging from 34 to 39 kDa [18,19]. Rd from *Escherichia coli*, on the other hand, had an M_r close to 14 kDa [20] Rd from the soil bacterium *Acinetobacter calcoaceticus* showed a M_r of around 35 kDa [21]; also recently Aird et al. [12] purified from the same bacterium a sulphane sulphurtransferase with an apparent M_r of 17 kDa. Thus the rhodanese purified here from *A. vinelandii* falls somewhat midway in size between the two extremes of molecular sizes reported for this enzyme.

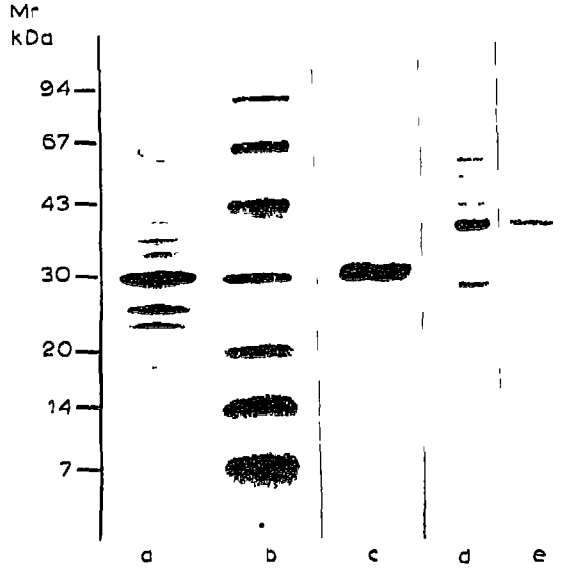

Fig. 2 Electrophoretic analysis of the final enzyme preparation SDS-PAGE was performed according to Laemmli [25] Samples were denatured in the presence of 1.25% 2-mercaptoethanol and 2% SDS (Lane a) Rd active peak from the TSK-DEAE 5PW column (15 μg), (lane b) protein markers (15 μg total of a mixture containing phosphorylase *b*, bovine serum albumin, egg albumin, carbonic anhydrase, trypsin inhibitor, α-lactalbumin, and aprotinin) (Lane c) Rd from the Superose 12HR column (10 μg), (lane d) 3-MST active peak from the TSK-DEAE 5PW column (20 μg), (lane e) 3-MST active peak from the Superose 12HR column (10 μg)

Enzymes belonging to the class of sulphur transferases utilize or produce sulphane compounds [1,5,22] The physiological function of sulphane sulphur, however, has not been well defined [23]. It could be the source of the inorganic sulphur used for synthesis of iron-sulphur centres. Though the enzymic system based on rhodanese has been extensively utilized in the in vitro synthesis of metal-sulphur structures [6–9], it remains unclear whether this enzyme can be regarded as the in vivo catalyst of reactions involved in the synthesis of metal-sulphur structures [24]. The identification of two different sulphurtransferases in *A. vinelandii*, as well as the purification of rhodanese to homogeneity, represent an important first step to investigate the role of these enzymes by combined biochemical-genetic approaches

Acknowledgements We wish to thank Dr R R Eady for helpful discussion, F Sessa for technical assistance, R Humphrey for growing large scale cultures, and Prof B E Smith for constructive comments on the manuscript The present investigation was supported by grants from CNR (Italy) C T 88 02275 06 115 06073 and C T 89 04895 06 115 06073

REFERENCES

[1] Westley, J (1977) in Bioorganic Chemistry, vol 1 (Van Tamelen, E E , ed) pp 371–390, Academic Press, Orlando
[2] Villarejo, M and Westley, J (1963) J Biol Chem 238, 4016–4020

[3] Volini, M. and Westley, J. (1966) J. Biol. Chem. 241, 5168–5176
[4] Jabarak, R. and Westley, J. (1978) Arch. Biochem. Biophys. 185, 458–465
[5] Westley, J. (1980) in: Enzymatic Basis of Detoxification, vol. 2 (Jacoby, W.B. ed.) pp. 245–261
[6] Pagani, S., Bonomi, F. and Cerletti, P. (1984) Eur. J. Biochem. 142, 361–366
[7] Bonomi, F., Pagani, S. and Kurtz, D.M. (1985) Eur. J. Biochem. 148, 67–73
[8] Bonomi, F., Werth, M.T. and Kurtz, D.M. (1985) Inorg. Chem. 24, 4331–4335
[9] Bonomi, F., Iametti, S., Kurtz, D.M. and Pagani, S. (1988) in: Nitrogen Fixation: Hundred Years After (Bothe, H., de Bruijn, F.J., Newton, W.E. eds) p. 63
[10] Pagani, S., Eldridge, M. and Eady, R.R. (1987) Biochem. J. 244, 485–488
[11] Pagani, S., Bonomi, F. and Kennedy, C. (1988) Biochem. (Life Sci. Adv.) 7, 275–281
[12] Aird, B.A., Heinrikson, R.L. and Westley, J. (1987) J. Biol. Chem. 262, 17327–17335
[13] Newton, J.W., Wilson, P.W. and Burris, R.H. (1953) J. Biol. Chem. 204, 445–451
[14] Iizaki, R.I. Gill, O.M. (1964) Anal. Biochem. 9, 401–410
[15] Sorbo, L. (1953) Acta Chem. Scand. 7, 1137–1145
[16] Vachek, H. and Wood, J.L. (1972) Biochim. Biophys. Acta 258, 133–146
[17] Ploegman, J.H., Drent, G., Kalk, K.H., Hol, W.G.J., Heinrikson, R.L., Deim, P., Weng, L. and Russell, J. (1978) Nature 273, 124–129
[18] Truper, H.G. (1981) in: Biology of Inorganic Nitrogen and Sulphur (Bothe, H. and Trebst, A. eds.) pp. 199–211, Springer, Berlin
[19] Stemmetz, M.A. and Fisher, U. (1985) Arch. Microbiol. 142, 253–258
[20] Alexander, K., Volini, M. (1987) J. Biol. Chem. 262, 6595–6604
[21] Vandenbergh, P.A. and Berk, R.S. (1980) Can. J. Microbiol. 26, 281–286
[22] Roy, A.B. and Trudinger, R.V. (1970) in: The Biochemistry of Inorganic Compounds of Sulphur, Cambridge Univ. Press, Cambridge UK
[23] Schneider, J.F. and Westley, J. (1969) J. Biol. Chem. 244, 5735–5740
[24] Cerletti, P. (1986) Trends Biochem. Sci. 11, 369–372
[25] Laemmli, U.K. (1970) Nature 227, 680–685