Two structural types of mercury reductases and possible ways of their evolution

E.S. Bogdanova and S.Z. Mindlin

Institute of Molecular Genetics, USSR Academy of Sciences, Moscow 123182, USSR

Structural differences have been revealed among mercury reductases of immunologically unrelated types from Gram-positive bacteria: enzymes of one immunological type have a molecular mass of 62–69 kDa and seem to contain an N-terminal extension of 5–15 kDa, which is easily cleaved by trypsin and chymotrypsin; enzymes of the other immunological type have a molecular mass of 52–57 kDa and are resistant to proteolysis. The existence of at least two different lines in the evolution of mercury reductases is surmised.

Mercury reductase; Limited proteolysis; Immunoblotting; Enzyme evolution; (Environmental bacteria)

1. INTRODUCTION

Mercury reductases are flavoproteins that are structurally and functionally related to glutathione reductases (GR) and lipoamide dehydrogenases (LipDH). These enzymes are believed to have had a common evolutionary precursor. In the case of mercury reductases the precursor gene fused with the DNA sequence encoding the N-terminal fragment. The same additional sequence also formed a separate gene, merP, encoding the mercury ion carrier protein [1,2].

Having studied the mercury reductases (MR) of Gram-positive bacteria, we discovered a large structural diversity among these enzymes, possibly pointing to the existence of different lines in their evolution.

2. MATERIALS AND METHODS

We obtained cell extracts, determined the MR activity and carried out immunodiffusion according to Ouchterloni as in [3].

In isolating MR from *Arthrobacter* TC28-I (to obtain im-

Correspondence address: E.S. Bogdanova, Institute of Molecular Genetics, USSR Academy of Sciences, Moscow 123182, USSR

mune rabbit serum), we used DEAE cellulose, Reactive red 120 agarose of type 3000-CL (Sigma) and Biogel A 0.5m.

To determine the molecular mass of MR and their proteolysis products, 10–100 μg of extract proteins were fractionated in SDS 10% acrylamide gel according to Laemmli [4], followed by immunoblotting.

In limited proteolysis experiments, bacterial extracts were incubated with trypsin and chymotrypsin, using protease: protein ratios from 1:1500 to 1:4 in 0.1 M sodium phosphate, pH 7.4, 0.5 mM EDTA and 10 mM mercaptoethanol for 30 min at 30°C.

3. RESULTS

From soil and water samples collected by Drs E. Kalyaeva, A. Gragerov and V. Nikiforov in Kamchatka, the Kuril islands, the Transcarpathians and Moscow region, and from the air, 71 different strains of aerobic chemoorganotrophic Gram-positive bacteria, displaying a resistance on LA medium to 10–15 μg/ml $HgCl_2$, were isolated in addition to the 24 strains described in [3]. Their MR were studied by means of antibodies against the MR of Gram-positive *Bacillus* FA8-2, *Mycobacterium* CHM19-3 [3] and newly isolated *Arthrobacter* TC28-I, denoted II, III and IV, respectively. The extracts of 25 strains exhibited no MR activity and no reaction with any of the sera upon immunodiffusion. The enzymes of 31 strains

formed precipitation bands with serum II only, 29 strains with serum III only, and the MR of one strain (*Arthrobacter* TC28-I) reacted only with homologous serum IV. Finally, the extracts of 10 strains showed an MR activity but did not form precipitation bands with any of the three sera or with the serum against Tn501 MR from Gram-negative bacteria [3]. Thus, apart from the three immunologically unrelated types, there seem to exist other, less frequently occurring, types of MR among Gram-positive bacteria.

The MR enzymes are known to consist of identical subunits [2]. It follows from the three MR gene sequences known to date, Tn501 and Tn21 from Gram-negative bacteria and pI258 from

Gram-positive *Staphylococcus aureus*, that the subunits of these enzymes consist of 561, 564 and 547 amino acid residues, respectively [5-7]. We used electrophoresis and the immunoblotting technique to determine the molecular masses of MR subunits in extracts. Subunit masses of Tn501 and pI258 MR proved to be 63.5 kDa and 62 kDa, respectively (table 1), which is close to [5,7]. Unexpectedly, the MR subunit masses of different Gram-positive bacterial strains proved to vary appreciably, ranging from 69.5 to 52 kDa (see table 1 and fig.1). The subunits of all enzymes interacting with serum III had the smallest molecular masses. It seems unlikely that the short enzyme forms are the result of proteolysis during the preparation of

Table 1

Molecular masses of mercury reductases and their proteolysis products

Immuno-logical type[a]	Bacteria	Strain[b]	Subunits' molecular mass (kDa)	Proteolysis products' molecular mass (kDa)	
				Trypsin	Chymotrypsin
I[c]	*Escherichia coli* K12 (ColE1:Tn501)	6364	63.5	56	56
II	*Bacillus sphaericus*	FA8-2	66[d]	58.5	58.5 ± 54.5
	Bacillus polymyxa	TA32-5	66[d]	58.5	ND[e]
	Bacillus sp.	TA41-4	66[d]	58.5	ND
	Rhodococcus sp.	TA32-12	66[d]	58.5	ND
	Bacillus licheniformis	FA6-12	63	54 + 53	ND
	Bacillus megaterium	TA40-8	63	ND	ND
	Bacillus sphaericus	TA41-11	62	54	ND
	Oerskovia sp.	TC38-2b	69.5	54	59 + 57 + 55 + 54
	Bacillus sphaericus	EL5-1	62	54	ND
	Oerskovia sp.	TC22-9	62	55.5	ND
	Staphyloccus saprophyticus	B4	66	58.5	ND
	Staphylococcus aureus (pI258)	8325-4	62	57	ND
	Coryneform	FA11-3	66	ND	ND
III	*Mycobacterium* sp.	CHM19-3	56	56	56
	Mycobacterium sp.	CHM22-8	57	57	57
	Mycobacterium sp.	CHM21-1	52	52	52
	Citreobacterium sp.	EL5-12	52	52	52
	Micrococcus roseus	B3	54	54	ND
	Micrococcus luteus	B2	54	54	54
IV	*Arthrobacter* sp.	TC28-1	55	54 + 52 + 43	54 + 53 + 32

[a] Enzymes of each immunological type react with their homologous serum only
[b] See [3]
[c] Strain notation indicates the area of isolation: TC, the Transcarpathian region; CHM, EL, FA, TA, different parts of the Kamchatka peninsula; B, air (Moscow)
[d] Some extracts contain an additional MR component with a molecular mass of 62-63 kDa
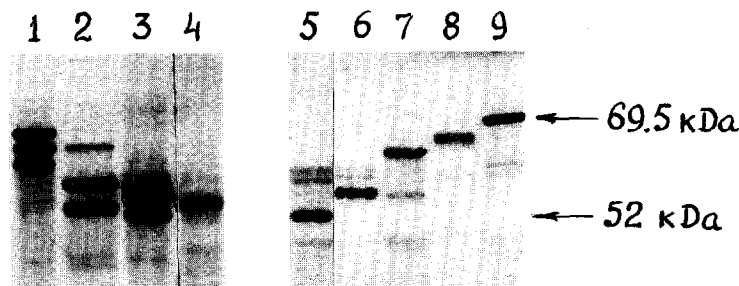[e] ND, not determined

Fig.1. Determination of the molecular masses of mercury reductase subunits by means of extract electrophoresis and immunoblotting. Lanes: 1, mixed extracts (TC38-2b, FA8-2, EL5-1 in the order of diminishing MR molecular masses); 2, the same as 1, after incubation with trypsin; 3, mixed extracts (CHM22-8, CHM19-3, CHM21-1 in the order of diminishing MR molecular masses); 4, TC28-1; 5, EL5-12; 6, CHM19-3; EL5-1; 8, FA8-2; 9, TC38-2b. The protein bands which are less intense than MR are the result of non-specific binding. This has been demonstrated by the comparison of extracts from induced and non-induced bacterial cultures.

extracts. For one thing, cells were disrupted with the maximum rapidity in the presence of a PMSF: TPCK:TLCK mixture, and the extracts were subsequently heated at 100°C in the presence of SDS. For another, when Bacillus FA8-2 cells, containing long subunits, were disrupted together with Mycobacterium CHM19-3, CHM21-I or Citreobacterium cells, carrying short subunits, there was no reduction of molecular mass or decrease of the long enzyme's share.

The N-terminal region, some 80 amino acid residues long, in Tn501 MR is either an extra-domain or forms a domain of its own and is easily hydrolyzed by trypsin and chymotrypsin. The loss of this N-terminal extension does not affect the enzyme's activity in vitro [8]. It was supposed, on the basis of the primary structure, that pI258 MR should also have such a region [9]. We examined the changing subunit mass of enzymes of different types upon the incubation of extracts with trypsin and chymotrypsin. It turned out that the enzymes reacting with serum II, including the one encoded by pI258, undergo a stepwise transformation into shorter polypeptides with a molecular mass of 54–58 kDa (see table 1, figs 1 and 2), in the manner of Tn501 MR ([8] and fig.2). Hence, the enzymes of Gram-positive bacteria may have essentially the same structure as Gram-negative Tn501 MR: a relatively proteolysis-resistant core with a molecular mass close to that of GR and LipDH from E. coli (450 and 473 amino acid residues) [10,11] and the N-terminal extension. The latter is present in all the tested enzymes of immunological type II in spite of their considerable diversity [3]. The proteolysis sensitivity of the core varies: it is the lowest

in Bacillus FA8-2 and the highest in St.saprophyticus B4.

The mercury reductase of Arthrobacter TC28-I (type IV) may contain a small N-terminal extension (table 1).

All the enzymes reacting with serum III proved to be proteolysis-resistant (table 1, fig.2). The reductases of Mycobacterium CHM19-3, CHM21-I and CHM22-8 showed some hydrolysis only at trypsin: extract protein ratios of 1:25–1:10. The resistance of these enzymes cannot be explained by the presence of protease inhibitors in the extracts, as shown by the trypsin treatment of extract mixtures with long and short enzymes: Bacillus FA8-2 and Citreobacterium or Oerskovia TC38-2b and Mycobacterium CHM22-8. The MR of this type do not seem to have the N-terminal extension.

Thus, apparently there are two structural types of MR: with and without the N-terminal extension. The length of this part of MR varies by a factor 2–3 in different enzymes: it is the smallest in pI258 and the largest in Oerskovia TC38-2b (table 1). The functional role of the N-terminal extension remains unclear. This part of MR is not required for catalysis but may be transiently involved in mercury binding [5]. The high degree of conservation of the first 23–27 N-terminal amino acid residues in Gram-positive Staphylococcus and Gram-negative MR [6,7], may mean that this region is essential to the functioning of MR of this type.

The short enzymes may belong to an independent evolutionary branch originating from a precursor shared with GR and LipDH. The short MR, GR and LipDH are close in molecular mass (table 1 and [10,11]), and the MR of this type show
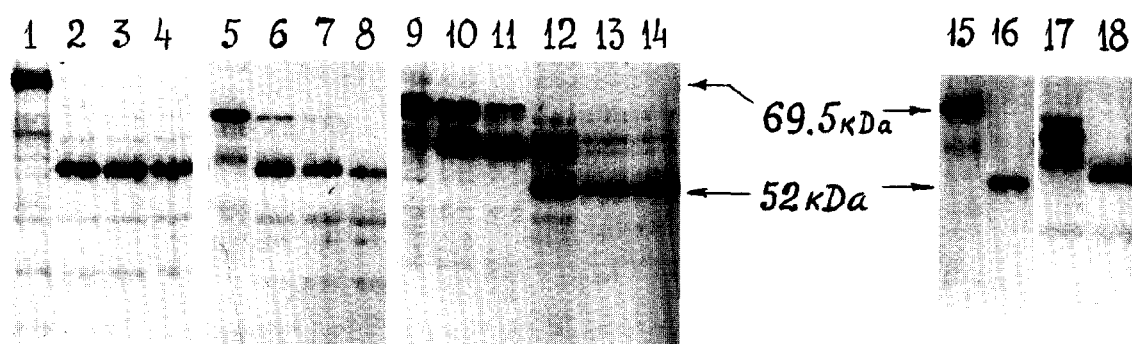
Fig.2. Limited proteolysis by trypsin of mercury reductase subunits in extracts. Lanes: 1,15, TC38-2b; 2,16, the same + trypsin 1:500; 3, the same + trypsin 1:250; 4, the same + trypsin 1:100, 5, EL5-1; 6, the same + trypsin 1:250; 7, the same + trypsin 1:100; 8, the same + trypsin 1:25; 9, FA8-2; 10, the same + trypsin 1:250; 11, the same + trypsin 1:25; 12, CHM21-1; 13, the same + trypsin 1:100, 14, the same + trypsin 1:25; 17, *E. coli* (ColE1::Tn501); 18, the same + trypsin 1:750.

a very high proteolysis resistance (see above), which is also true of LipDH [12]. However, one cannot rule out the possibility that the short enzymes might have lost, in the course of evolution, an N-terminal extension which they had had earlier.

Clearly, only the sequencing of the various types of MR and a study of their intracellular locations can help elucidate their evolitionary pathways and the function of the N-terminal structure.

REFERENCES

[1] Brown, N.L. and Goddette, D. (1984) in: Flavins and Flavoproteins, pp. 165–168, De Gruyter, Berlin.

[2] Summers, A.O. (1986) Annu. Rev. Microbiol. 40, 607–634.

[3] Bogdanova, E.S., Mindlin, S.Z., Kalyaeva, E.S. and Nikiforov, V.G. (1988) FEBS Lett. 234, 280–282.

[4] Laemmli, U.K. (1970) Nature 227, 680–685.

[5] Brown, N.L., Ford, S.J., Pridmore, R.D. and Fritzinger, D.C. (1983) Biochemistry 22, 4089–4095.

[6] Barrineau, P., Gilbert, P., Jackson, W.J., Jones, C.S., Summers, A.O. and Wisdom, S. (1984) J. Mol. Appl. Genet. 2, 601–619.

[7] Laddaga, R.A., Chu, L., Misra, T.K. and Silver, S. (1987) Proc. Natl. Acad. Sci. USA 84, 5106–5110.

[8] Fox, B.S. and Walsh, C.T. (1983) Biochemistry 22, 4082–4088.

[9] Silver, S., Rosen, B.P. and Misra, T.K. (1986) in: The Fifth International Symposium on the Genetics of Industrial Microorganisms 1986 (Alacevic, M. et al. eds) pp. 357–371.

[10] Greer, S. and Perham, N. (1986) Biochemistry 25, 2736–2742.

[11] Stephens, P.E., Lewis, H.M., Darlison, M.G. and Guest, J.R. (1983) Eur. J. Biochem. 135, 519–527.

[12] Williams, C.H., jr (1976) in: The Enzymes (Boyer, P.D. ed.) vol. 13, pp. 89–173, Academic Press, New York.