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SUBSTRATE SPECIFICITY OF THE L-SERINE *O*-SULPHATE DEGRADING ACTIVITIES OF *PSEUDOMONAS* FR

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Summary

1. Two enzyme systems obtained from *Pseudomonas* FR capable of catalysing the $\alpha\beta$-elimination of L-serine *O*-sulphate exhibit a wide range of substrate specificity. Greatest activity was exhibited towards β-substituted serine and cysteine derivatives. Enzyme A shows a marked preference for the L-isomeric form and enzyme B shows a preference for D-isomers.

2. The alternative activities were shown to be properties of the same enzyme by inhibition properties and heat denaturation experiments.

3. The assay of enzyme A by a number of alternative substrates at various stages during its purification confirmed the multi-substrate specificity of the system.

4. Growth of *Pseudomonas* FR on *S*-methyl-L-cysteine as the sole carbon source also resulted in the induction of enzyme B. Growth patterns and levels of induced enzyme were similar to those obtained when L-serine *O*-sulphate was employed in comparable circumstances.

Introduction

It has recently been demonstrated that *Pseudomonas* FR, an organism isolated from soil and capable of growth on L-serine *O*-sulphate as the sole carbon source, possesses two distinct systems capable of degrading the ester [1]. One of these was inducible and present only when the organism was grown on the ester sulphate as the sole source of carbon. The other system was constitutive and present when growth occurred on a variety of media. Unlike the corresponding mammalian system [2], the bacterial systems required pyridoxal phosphate as a cofactor.

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Since L-serine *O*-sulphate has not been shown to occur naturally, it has been suggested [3] that activity towards this substrate represents an alternative but not the normal function of the system in question. Other mammalian enzymes are known to be capable of utilizing the ester sulphate as a 'quasi' substrate, namely alanine [2] and aspartate [4] aminotransferases and cystathionine β-synthase[5]. The aminotransferases are clearly not responsible for the bacterial L-serine *O*-sulphate degrading activities [1]. Nonetheless there exist in Nature many activities capable of performing αβ-elimination reactions that superficially closely resemble the degradation of L-serine *O*-sulphate. In order to make unequivocal statements about the exact nature of the systems associated with *Pseudomonas* FR which degrade the ester sulphate it was necessary to obtain comprehensive information on the nature of compounds capable of acting as substrates. This communication deals with the results of such investigations and attempts to draw meaningful conclusions about the normal functions of the L-serine *O*-sulphate degrading systems of *Pseudomonas* FR.

Material and Methods

Chemicals

The potassium salts of the *O*-sulphate esters of L-homoserine, α-methyl L-serine, L-threonine, ethanolamine and L-serine were prepared essentially as described by Tudball [6]. D-Glyceric acid-3-sulphate was prepared according to Fitzgerald et al., [7], 3-cyano-L-alanine by the method of Ressler and Ratzkin [8], 3-chloro-L-alanine by the method of Fischer and Raske [9], *O*-succinyl-L-serine by the method of Guggenheim and Flavin [10] and *O*-succinyl-L-homoserine by the method of Flavin and Slaughter [11]. *S*-Methyl-L-cysteine, DL-lanthionine, *O*-benzyl-L-serine, *S*-benzyl-L-cysteine, L-cysteine, D-cysteine, L-cystine, D-cystine, *O*-phospho-D-serine, L-homoserine, DL-alanine, L-cysteine sulphinic acid, L-cysteic acid, NADH and lactate dehydrogenase were obtained from Sigma (London) Ltd., London, S.W.6, (U.K.). L-Methionine, L-cystathionine, D-cystathionine, L-serine, D-serine, L-threonine and β-ureido α-aminopropionic acid were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England. *O*-Methyl-DL-serine was obtained from Mann Research Laboratories Inc., New York, (U.S.A.), α-methyl-L-serine from Calbiochem, Los Angeles, (U.S.A.), diazoacetyl-L-serine from Parke-Davis, Hounslow, London, (U.K.), *O*-acetyl-L-serine from Miles Laboratories Inc., Rehovoth, (Israel) and *S*-carbamyl-L-cysteine and *S*-ethyl-L-cysteine from Fluka AG, Buchs SG, (Switzerland). All other chemicals were purchased from British Drug Houses Ltd., Poole, Dorset, (England).

Enzyme preparations

Preparations of both L-serine *O*-sulphate degrading enzymes (A and B) were prepared as previously described [1]. Both systems yielded one major and one minor protein-containing band on analytical polyacrylamide gel electrophoresis. Only the major band in each case was associated with ester sulphate degrading activity.

Enzyme assays

In those experiments employing sulphate esters as substrates, liberated SO_4^{2-}

ions were measured using the method of Dodgson [12]. In order to check for either the $\alpha\beta$ or $\alpha\gamma$-elimination of the substrates under test and L-serine *O*-sulphate degrading activity, keto acid production was followed using a coupled assay procedure employing lactate dehydrogenase [3]. Except where otherwise stated enzymes A and B were assayed employing optimum conditions recorded for L-serine *O*-sulphate as substrate. L-Aspartate and L-glutamate decarboxylase activities were determined according to the method of Najjar [13].

Microbiological procedures

These were performed as described by Tudball and O'Neill [1].

Results

Substrate specificity of enzymes A and B from Pseudomonas FR

The optimum conditions for the assay of the A and B enzymes when L-serine *O*-sulphate was used as substrate were generally employed for the initial examination of other potential substrates. The low solubilities of some of the compounds examined or alternatively their limited availability necessitated in some instances the use of lower substrate concentrations.

TABLE I

SUBSTRATE SPECIFICITY OF ENZYMES A AND B OF *PSEUDOMONAS* FR

Substrate	Conc. (mM)	Relative enzyme activity (%)	
		Enzyme A	Enzyme B
L-Serine *O*-sulphate	25	100	100
O-Benzyl DL-serine	1	60	40
O-Methyl DL-serine	50	58	47
S-Methyl L-cysteine	25	28	18
L-Cystathionine	10	29	20
DL-Lanthionine	2.5	7	20
D-Serine *O*-sulphate	25	3	173
D-Serine	25	0.3	30
DL-Cystine	10	0.5	12
D-Cysteine	1	5.0	110
D-Cystathionine	10	5.0	89
O-Benzyl-L-serine	1	82	0.2
O-Acetyl-L-serine	25	30	1.7
O-Diazoacetyl-L-serine	10	25	0.2
O-Succinyl-L-serine	25	42	2.0
S-Ethyl-L-cysteine	20	43	0.9
S-Benzyl-L-cysteine	1	54	0.1
L-Cystine	1	50	3.0
3-Chloro-L-alanine	25	14	0.9
L-Serine	25	2.1	0.2
L-Cysteine	10	1.8	0.8
S-Carbamyl-L-cysteine	10	3.3	0.3
S-Sulpho-L-cysteine	25	3.7	0.6
3-Cyano-L-alanine	25	2.0	0.5
β-Ureido-α-aminopropionic acid	10	0.5	0.1
L-Threonine *O*-sulphate	25	0.3	0.1

Table I records the list of those compounds that were capable of acting as substrates for both enzymes. Both systems were found to be totally inactive towards all other compounds examined. With the exception of 3-chloro-L-alanine, 'good' substrates (those exhibiting more than 10% the activity shown towards L-serine *O*-sulphate) for either system were serine and cysteine derivatives. Both enzyme systems exhibited a wide substrate specificity, with the enzyme A having a preferential activity for L-isomers and enzyme B for D-isomers.

In order to obtain additional information about the suitability of compounds other than L-serine *O*-sulphate to act as substrates, the K_m and V values when some of these compounds acted as substrates were obtained (Table II). It can be seen that for either enzyme system some of the alternative substrates can be considered to be better substrates than L-serine *O*-sulphate.

Since enzymes A and B were not homogeneous entities with respect to protein the alternative activities exhibited by these systems may have been associated with a system which was not that catalysing the degradation of L-serine *O*-sulphate. Evidence for or against this possibility was therefore sought.

Inhibition of enzyme activities

A series of competitive inhibitors for the mammalian L-serine *O*-sulphate degrading system have been described that are conformationally similar to the ester sulphate [14]. One of these, isophthallic acid, also proved to be an efficient competitive inhibitor of enzyme A and enzyme B. The relative efficiency of isophthallic acid to act as an inhibitor of both systems employing a number of alternative substrates is shown in Table III. The values for K_i were computed by the method of Dixon [15]. In all cases the inhibition proved to be competitive. Whereas the K_i values were quite distinct for enzyme A and B, the respective values changed little in response to alteration in substrate, sug-

TABLE II

SOME KINETIC PROPERTIES OF ENZYMES A AND B USING ALTERNATIVE SUBSTRATES

Substrate	Enzyme A		Enzyme B	
	K_m (mM)	Relative V	K_m (mM)	Relative V
L-Serine *O*-sulphate	7.9	1.11	5.4	2.40
O-Acetyl-L-serine	20.0	0.49	—	—
D-Serine *O*-sulphate	—	—	0.14	4.60
O-Benzyl-L-serine	1.3	1.35	—	—
D-Serine	—	—	8.3	1.10
S-Methyl-L-cysteine	17.8	0.49	56	3.33
O-Benzyl-DL-serine	—	—	2.0*	2.85*
S-Ethyl-L-cysteine	38	0.54	—	—
L-Cystathionine	9.1	0.67	8.5	1.67
S-Benzyl-L-cysteine	1.1	0.88	—	—
D-Cystathionine	—	—	2.2	2.23
L-Cystine	2.0	1.24	—	—
D-Cystine	—	—	0.17	2.44

* Calculated on the basis of the non-reactivity of the L-isomer.

TABLE III

INHIBITION OF ENZYMES A AND B BY ISOPHTHALLIC ACID IN THE PRESENCE OF DIFFERENT SUBSTRATES

Substrate	Enzyme A K_i (mM)	Enzyme B K_i (mM)
L-Serine *O*-sulphate	6.3	1.1
D-Serine *O*-sulphate	6.8	—
D-Serine	7.1	—
O-Acetyl-L-serine	—	0.9
O-Benzyl-L-serine	—	1.1
O-Benzyl-DL-serine	7.6*	—
S-Methyl-L-cysteine	7.9	1.2
S-Ethyl-L-cysteine	—	0.9
S-Benzyl-L-cysteine	—	1.2
L-Cystathionine	8.1	1.4

* Calculated on the basis of the non-reactivity of the L-isomer.

gesting the various activities associated with A and B respectively were properties of a single enzyme.

Heat inactivation

It was known that the activities of enzymes A and B towards L-serine *O*-sulphate were sensitive to heat. The effect of heat on the activities towards other substrates was thus examined.

Enzymes A and B in 0.1 M Tris buffer adjusted to pH 8.3 and pH 7.8 respectively, were maintained at 50°C. After varying intervals of time 100 µl of enzyme solution was taken and the change in activity monitored using L-serine *O*-sulphate, *O*-benzyl-DL-serine and L-cystathionine. Similar enzyme solutions maintained at 1°C were used as controls. The rates and degree of inactivation of enzymes A and B differed in response to this treatment but the decline in activity of the respective activities was similar using any of the above substrates. After 15 min enzyme B exhibited 30, 32 and 38% of the starting activity towards L-serine *O*-sulphate, *O*-benzyl-DL-serine and L-cystathionine respectively, whereas the corresponding activities of enzyme A were 71, 75 and 71%.

Specificity of enzyme A during purification

Further evidence for the multi-substrate activities of enzyme A was sought by assaying the enzyme during normal purification procedures using a variety of substrates. This type of experiment was not meaningful when applied to the purification of enzyme B, since enzyme A was also present during the early stages of the purification [1]. The results of these experiments are presented in Table IV, from which it can be seen that there is a very close relationship between the four activities examined at each stage of the purification.

Growth of Pseudomonas FR on S-methyl-L-cysteine

Previous attempts to induce the L-serine *O*-sulphate degrading system in *Pseudomonas* FR by growing the organism on media containing compounds

TABLE IV

RELATIVE ACTIVITIES OF ENZYME A AT DIFFERENT STAGES OF A NORMAL PURIFICATION PROCEDURE USING ALTERNATIVE SUBSTRATES

Purification procedure	Vol. (ml)	Protein conc. (mg/ml)	Total* units	Specific* activity	Ratio L-Serine *O*-sulphate to L-cystathionine	Ratio L-Serine *O*-sulphate to *O*-benzyl L-serine	Ratio L-serine *O*-sulphate to *S*-methyl L-cysteine	Yield*
1. High speed supernatant	210	13.5	83.8	0.029	2.03	1.48	3.70	100
2. Protamine sulphate supernatant	215	8.5	80.4	0.044	2.00	1.69	3.44	96
3. Acetone precipitate (45—55%)	37	9.3	48.5	0.141	2.13	1.61	3.22	49
4. DEAE-cellulose chromatography	80	0.33	26.8	1.012	2.03	1.53	3.84	32
5. Calcium phosphate gel supernatant	80	0.16	24.1	1.88	2.03	1.63	3.57	30
6. Sephadex G-200 chromatography	18	0.04	10.9	14.9	2.08	1.58	3.57	13

* Figures based on assay using L-serine *O*-sulphate.

other than the ester sulphate as the sole carbon source proved unsuccessful [1]. Since it has now been established that enzymes A and B are capable of degrading alternative substrates in vitro, the response of the organism to the presence of these alternative substrates in the culture medium was contemplated. Within limits, results similar to those observed with the ester sulphate would be obtained, if the substrates were as readily available to the organism as the ester sulphate. Many of the alternative substrates could not be used as growth media, partly owing to their limited solubility but *S*-methyl-L-cysteine was a suitable substrate. Growth media containing this compound as sole carbon source were prepared and inoculated with *Pseudomonas* FR as previously described [1]. The organism exhibited a growth pattern similar to that observed with L-serine *O*-sulphate when maintained on *S*-methyl-L-cysteine. Washed preparations of cells grown on *S*-methyl-L-cysteine were able to oxidize the ester sulphate and furthermore the level of the L-serine *O*-sulphate degrading activity of these cells corresponded closely to the level observed after growth on the ester itself [1]. These observations offer further substantiative evidence for the multi-substrate activity of enzyme B.

Discussion

It now seems reasonably certain that enzymes A and B isolated from *Pseudomonas* FR and originally identified as a result of their ability to degrade L-serine *O*-sulphate exhibit the property of multi-substrate specificity. Detailed mechanistic studies have not been performed but it is probable that the alternative activities exhibited by these systems correspond to $\alpha\beta$-elimination reactions. Most of the reactions catalysed by these enzymes involve the scission of C-O and C-S linkages. Only very feeble activity was observed towards com-

pounds containing C-N, C-C or C-Cl linked β-substituents.

In non-enzymic αβ-eliminations mediated by pyridoxal an increased electronegativity of the β-substituent normally leads to a more rapid expulsion of the substituent [16]. Similar conclusions cannot be drawn for the substrate specificities exhibited by enzymes A and B. Thus *O*-methyl-DL-serine is a better substrate for enzyme A than is 3-chloro-L-alanine. A similar observation has also been reported for tryptophan synthetase of *Eschericia coli* which also utilizes these substrates [17]. Since the affinity of enzyme A for *S*-methyl-L-cysteine is greater than that exhibited towards *S*-ethyl-L-cysteine this suggests that stereochemical factors play an important role in determining the rates of reaction. At first sight, the relatively high affinity of the enzyme for *S*-benzyl L-cysteine would seem to negate this argument, but the electron-delocalizing effect of the benzene ring would undoubtedly assist in the expulsion of the hydrogen attached to the α-carbon atom, a necessary prerequisite for β-elimination and could outweigh the disadvantage of a bulky β-substituent. A similar argument could be proposed for the activities of enzyme B.

In spite of the fact that enzyme B was induced in the presence of L-serine *O*-sulphate, the enzyme shows a preference for D-isomers as substrates. Functionally D-serine *O*-sulphate is a shorter molecule than the L-isomer and it is to be expected on this basis that enzymes A and B would behave differently towards the inhibitor isophthallic acid. Isophtallic acid resembles the conformation of L-serine *O*-sulphate very closely in its distribution of functional groups [14] and should thus be a more efficient inhibitor towards systems preferentially utilizing the L-isomer. Such an explanation does receive some degree of experimental support.

Enzymes A and B of *Pseudomonas* FR are probably not normally associated with the degradation of L-serine *O*-sulphate, since they are clearly capable of utilizing many naturally occurring compounds. A review of the literature reveals that a number of apparently distinct systems are capable of catalysing many of the alternative reactions exhibited by enzymes A and B, particularly those utilizing cystathionine, cystine and *S*-methyl-cysteine. *S*-Alkyl-cysteine lyase from *Pseudomonas cruciviae* primarily functions in the degradation of *S*-methyl-L-cysteine; in this case neither cystathionine nor cystine have been reported as substrates [18]. However, a similar enzyme from *Albizzia lophanta* was able to utilize DL-cystathionine and L-cystine as substrates [19]. This C-S lyase was quite distinct from a β-cystathionase isolated from spinach [20], which was unable to degrade *S*-methyl-L-cysteine. *Bacillus subtilis* possesses two distinct β-cystathionase systems [21], one repressed, the other unaffected by methionine. The cystathionase unaffected by methionine is believed to be a general C-S lyase and has similar substrate specificities to that exhibited in the present study by enzyme A. Apart from the finding that enzyme B was induced in the presence of *S*-methyl-L-cysteine and was capable of affecting some of the reactions catalysed by C-S lyases, it cannot be stated with any surety that the two systems are similar due to the preference for D-isomers shown by enzyme B. However, little information is available in the literature about the ability of C-S lyases to utilize the D-forms of substrates and further comparison is best deferred until such information becomes available.

The conclusion that L-serine *O*-sulphate degrading activities of *Pseudomonas*

FR normally have little direct association with the ester sulphate can probably also be extended to all other systems previously shown to be active towards this substrate. The results of the present investigations suggest that it would be profitable to explore further the possibility that β-substituted cysteine derivatives are physiological substrates for these enzymes.

Many enzymes that appear superficially to be quite distinct and unrelated entities are now known to be capable of catalysing essentially similar reactions with the same substrates. In some cases specifity is conferred only by the lack of activity towards a particular substrate. It has been suggested [5] that a number of C_3-specific β-replacing lyases are probably variants of a specific enzyme activity or are closely related members of the same group or family. We propose a similar explanation to account for the numerous activities observed towards L-serine *O*-sulphate. In bacteria this could provide the necessary framework in which mutations may occur to yield novel enzyme activities. Studies of the primary sturctures of such related enzymes would undoubtedly provide relevant information in this context and could contribute much to our knowledge of the processes of evolution and induction of novel enzyme activities in microorganisms.

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