Biochem. J. (1972) **126**, 187–191
Printed in Great Britain

The Enzymic Degradation of L-Serine *O*-Sulphate by a Specific System from Pig Liver

STUDIES ON THE MECHANISM OF THE REACTION

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(*Received* 23 *July* 1971)

1. By using [^{18}O]water it was demonstrated that L-serine *O*-sulphate undergoes C–O cleavage during enzyme-catalysed degradations. 2. C–O cleavage of substrate also occurs under the agency of alkali and pyridoxal phosphate, but, as expected, acid-catalysed degradation involves S–O scission. 3. Aminoacrylate was identified as an intermediate in the enzyme-catalysed degradation of L-serine *O*-sulphate.

An enzyme specific for the degradation of *N*-terminal L-serine *O*-sulphate residues has now been obtained from pig liver in a homogeneous form. The enzyme is separable by isoelectric focusing into two immunologically distinct isoenzymes with molecular weights approximating to 52000 (Tudball *et al.*, 1971).

The products of enzyme action with L-serine *O*-sulphate as the substrate have been shown to be pyruvate, SO_4^{2-} and NH_4^+, which are liberated in equimolar amounts (Thomas & Tudball, 1967). It was further suggested that the most likely mechanism for their production was an $\alpha\beta$-elimination. Subsequent studies showed that the enzyme did not require the participation of pyridoxal phosphate as a cofactor (Tudball *et al.*, 1969), and that carbonyl-specific reagents did not have a marked effect on enzyme activity (Thomas & Tudball, 1967). It thus seems unlikely that the role normally assumed by pyridoxal phosphate in enzyme-catalysed $\alpha\beta$-eliminations can be assigned to some other carbonyl function, thus indicating a novel mechanism for the abstraction of the α-hydrogen atom. Both aspartate aminotransferase (John & Fasella, 1969) and alanine aminotransferase (see Tudball *et al.*, 1969) are also able to degrade L-serine *O*-sulphate to yield pyruvate, NH_4^+ and SO_4^{2-}, but in view of their requirement for pyridoxal phosphate the details of the mechanism must differ even though the overall effect is the same as that observed for the above system.

To detail the mechanism of the enzymic degradation of L-serine *O*-sulphate it is necessary to possess information on the position of cleavage of the sulphate ester grouping. This knowledge would enable us to differentiate between the suggested $\alpha\beta$-elimination mechanism and a hydrolytic cleavage, which is the mode of action normally associated with the degradation of sulphate esters by the sulphatase group of enzymes (Spencer, 1958). It is possible to distinguish between these alternatives by using [^{18}O]water as the aqueous medium of the reaction mixture. Cleavage of the S–O bond would result in one of the oxygen atoms of the sulphate ion having its origin in the oxygen of the aqueous medium so that the species of ion formed would be $S^{18}O^{16}O_3^{2-}$. If scission of the C–O bond occurred then ^{18}O would not be present in the liberated SO_4^{2-}.

This paper deals with the mode of sulphate removal from the substrate under various experimental conditions and also considers the involvement of L-serine and aminoacrylate as intermediates in the reaction and the possibility of effecting the reversal of the reaction.

Materials and Methods

Chemicals

Water containing 42.1 atoms % excess of ^{18}O was obtained from the Weizmann Institute of Science, Rehovoth, Israel. L-[3-^{14}C]Serine and sodium [U-^{14}C]pyruvate were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Pyridoxal 5′-phosphate, $NaBH_4$, L-alanine, D-alanine, DL-alanine, lactate dehydrogenase and NADH were obtained from Sigma (London) Chemical Co., London S.W.6, U.K. Triton X-100 and catalase were obtained from British Drug Houses, Poole, Dorset, U.K., and 2,5-diphenyloxazole was from Beckman Instruments, Fullerton, Calif., U.S.A.

^{14}C-labelled and unlabelled potassium L-serine *O*-sulphate were prepared by the method of Tudball (1962) and potassium *p*-nitrophenyl sulphate was prepared as described by Burkhardt & Lapworth (1926).

The purified L-serine *O*-sulphate-degrading enzyme was obtained from pig liver by the method of Tudball *et al.* (1971). Both α- and β-enzymes were employed as enzyme sources for the subsequent experiments.

D-Amino acid oxidase and L-alanine aminotransferase were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Assay of enzyme activities

L-Serine *O*-sulphate-degrading activity was assayed by the procedure of Tudball *et al.* (1971). Alanine aminotransferase activity was assayed by the method of Tonhazy *et al.* (1950). D-Amino acid oxidase was assayed in the following manner. The substrate (1 mg), 0.1 mg of NADH, 10 μl of lactate dehydrogenase (10 mg/ml), and 5 μl of catalase (1000 units) were made up to 0.9 ml with 0.1 M-tris–HCl buffer, pH 8.0, that had previously been saturated with air. The reaction was initiated by adding D-amino acid oxidase (100 μl of a 1 mg/ml solution of the enzyme in the above tris buffer) and the reaction followed by observing the decrease in E_{340} at 25°C with a Unicam SP. 800 recording spectrophotometer fitted with a scale-expansion accessory.

Preparation of barium sulphate for infrared analysis

Total incubation mixtures were washed into a 10 ml tapered centrifuge tube with water (approx. 2 ml) and inorganic sulphate was precipitated by the addition of 2 M-HCl (1 ml) followed by 10% (w/v) $BaCl_2$ (1 ml). The precipitated $BaSO_4$ was removed by centrifuging and washed with 3 × 3 ml of 2 M-HCl, 5 × 3 ml of water, 2 × 3 ml of ethanol and 3 × 3 ml of ether. The washed precipitate was finally dried at 110°C for 24 h and stored *in vacuo* over P_2O_5.

Infrared analysis of barium sulphate

The method employed for analysing $BaSO_4$ was a modification of that of Spencer (1959). Samples of $BaSO_4$ (7.5 mg) were mixed with infrared-grade KBr (250 mg) and dried at 110°C for 24 h. The mixture was pressed into a disc at 10^8 N/m^2 (7 tons/in^2) and then examined with a Perkin–Elmer 257 grating infrared spectrophotometer. The ^{18}O content in the $BaSO_4$ was determined by comparing the peaks at 981 and 961 cm^{-1} as described by Spencer (1959).

Experimental and Results

Preparation of standard $BaS^{16}O_3{}^{18}O$

Spencer (1958) has established that 100% splitting of the O–S bond occurs during the acid hydrolysis of *p*-nitrophenyl sulphate. Precipitated $BaSO_4$ prepared after the hydrolysis of the ester in media containing various concentrations of ^{18}O was accordingly used as standard samples of $BaS^{16}O_3{}^{18}O$ for infrared analysis.

Potassium *p*-nitrophenyl sulphate (60 mg) was dissolved in [^{18}O]water (150 μl), and 50 μl of 8 M-HCl was added. The final concentrations of ^{18}O employed were 31.5, 21.0, 15.75 and 10.5 atoms % excess of ^{18}O. No correction was made for the % difference of water content introduced by adding 8 M-HCl. Hydrolyses were performed at 110°C for 12 h in Pyrex tubes sealed under N_2. A control experiment was similarly performed by using K_2SO_4 (50 mg) and 421 atoms % excess of ^{18}O. $BaSO_4$ was prepared from the hydrolysates for infrared analysis as described above.

No peak at 961 cm^{-1} due to the S–^{18}O vibration was detected in the control sample. The ratios of the peaks at 981 cm^{-1} and 961 cm^{-1} for the $BaSO_4$ preparations obtained after the hydrolysis of *p*-nitrophenyl sulphate in 31.5, 21.0, 15.75 and 10.5 atoms % excess of ^{18}O were taken as representative of $BaS^{16}O_3{}^{18}O$ containing 7.87, 5.25, 3.94 and 2.62 atoms % excess of ^{18}O respectively.

Mode of removal of β-substituent in the enzymic and non-enzymic degradation of L-serine O-sulphate

The enzymic degradation of L-serine *O*-sulphate was performed by incubating the substrate (50 mg) and tris (2.4 mg) with enzyme in ^{18}O-enriched water (200 μl), the mixture being adjusted to pH 7.0 with 2 M-HCl. The final concentration of ^{18}O in the reaction mixture was either 31.5 or 21.0 atoms % excess of ^{18}O. Incubations were performed at 37°C for 3 h. Under these conditions over 80% of the substrate underwent degradation. Reactions were stopped by immersing reaction vessels in a boiling-water bath for 60 s, and $BaSO_4$ was prepared for infrared examination as described above.

The non-enzymic degradation was examined by subjecting 60 mg of the ester to treatment with either 2 M-HCl, 2 M-KOH or a pyridoxal phosphate and $CuCl_2$ mixture (final concns. 10 and 2 mM respectively) made up in 0.1 M-tris–HCl buffer, pH 8.0. The reaction mixtures were sealed under N_2 in Pyrex tubes and incubated at 110°C for 12 h. $BaSO_4$ was again prepared for infrared analysis as described above.

No traces of ^{18}O were detected in the $BaSO_4$ obtained from the enzyme-catalysed breakdown of L-serine *O*-sulphate. Similarly degradation of the ester by pyridoxal phosphate and alkali failed to incorporate ^{18}O into the precipitated $BaSO_4$. As expected, however, the $BaSO_4$ obtained after acid hydrolysis did contain ^{18}O to the extent of 5.25 atoms % excess of ^{18}O, which represents the theoretical value for 100% splitting of the O–S bond of the ester.

Non-involvement of L-serine as an intermediate

The scission of the C–O–S linkage between the C and O atoms in the enzymic degradation of L-serine

O-sulphate should mean that it occurs via a mechanism, e.g. an $\alpha\beta$-elimination, that would not involve the participation of L-serine as an intermediate. A hydrolytic cleavage, on the other hand, would indicate that L-serine was an intermediate. To amplify the results obtained from the investigations with ^{18}O it was decided to examine the possible participation of L-serine as an intermediate. If L-serine were an intermediate in the enzyme-catalysed reaction, then the incorporation of L-[3-^{14}C]serine into an incubation mixture of enzyme and substrate could result in an exchange of label between the amino acid and the substrate.

L-[3-^{14}C]Serine (100μl of 0.04M-L-serine O-sulphate), and 100μl of L-serine and 100μl of K_2SO_4 were used; the L-serine and K_2SO_4 were employed at two different equimolar concentrations (namely 0.4M and 0.04M). All solutions were made up in 0.1M-tris–HCl buffer, pH7.0. The reaction was started by the addition of enzyme (100μl, containing 30μg of protein) and proceeded at 10°C for 30min before being stopped by the addition of 25% (w/v) trichloroacetic acid (100μl). Controls with heat-denatured enzyme were run simultaneously. Precipitated protein was removed by centrifuging and the resulting clear supernatants were fractionated on columns (0.5cm×1cm) of Dowex 50 ion-exchange resin (200–400 mesh; H^+ form). L-Serine O-sulphate, SO_4^{2-} ion and pyruvate were removed by washing with water (10ml). The columns were then washed with 2M-NH_3 (10ml) to remove L-serine. Samples (1ml) of the water and alkali washings were added to scintillation fluid (15ml) prepared by mixing 2,5-diphenyloxazole (5g), toluene (1 litre) and Triton X-100 (500ml), before measurement of their ^{14}C content in a Beckman LS-100 liquid-scintillation counter.

Not more than 0.015% of the total radioactivity was detected in the water washings, the remainder being found in the ammonia wash, indicating that no exchange between L-[3-^{14}C]serine and L-serine O-sulphate had occurred.

Identification of aminoacrylate as an intermediate in the enzyme-catalysed degradation of L-serine O-sulphate

The demonstration that no exchange of label occurred between the substrate and serine in the above experiments cannot be regarded as unequivocal proof that serine is not an intermediate in the enzyme-catalysed degradation of L-serine O-sulphate. However, this result might have been expected from the observation that the β-substituent is liberated after the cleavage of the C–O bond. Under these circumstances aminoacrylate presents itself as a much more plausible intermediate. If aminoacrylate were an intermediate in the enzyme-catalysed reaction then the presence of $NaBH_4$ in the reaction mixture should give rise to alanine. The following experimental procedure was adopted to test this possibility.

Preliminary experiments indicated that the enzyme was not inhibited by $NaBH_4$ at any of the concentrations employed in subsequent experiments. L-[3-^{14}C]-Serine O-sulphate (final concn. 0.05M; radioactivity corresponding to 200000c.p.m./mg) and 0.5mg of a mixture of the α- and β-enzymes contained in 0.1M-tris–HCl buffer, pH7.0 (300μl), were incubated at 37°C for 1h. $NaBH_4$ was added to the incubation mixture in portions (approx. 1.5mg) every 10min (total amount 10mg). Control experiments were run alongside, from which either the enzyme or the $NaBH_4$ was omitted. At the end of the incubation period 25% trichloroacetic acid (30μl) was added to stop enzyme activity. Precipitated protein was removed by centrifuging and portions (10μl) of the clear supernatants were subjected to paper chromatography and paper electrophoresis. Standard preparations of L-[3-^{14}C]serine O-sulphate, L-[3-^{14}C]-serine, [U-^{14}C]pyruvate and DL-alanine were run concurrently for comparison. Descending chromatography was performed on Whatman no. 1 paper with the following solvent systems: I, butan-1-ol–acetic acid–water (50:12:25, by vol.); II, 2-methylpropan-2-ol–formic acid–water (8:3:4, by vol.); III, ethanol–water (7:3, v/v). Paper electrophoresis was carried out on Whatman 3MM paper in 0.1M-ammonium formate–formic acid buffer, pH6.4, for 2h at a potential of 12V/cm. Ninhydrin-positive material was located on the dried papers by spraying with a 0.1% solution of ninhydrin in acetone, followed by heating at 85°C for 5min. The radioactive zones were located by using the Packard model 7201 radiochromatogram scanner.

In the test samples a ninhydrin-positive radioactive zone was observed that had the same mobility as that of alanine on both paper chromatography and paper electrophoresis. This material was absent from the appropriate controls. Elution of this material from untreated chromatograms and co-chromatography with authentic DL-alanine resulted in the appearance of a single radioactively labelled ninhydrin-positive zone under all the above chromatographic conditions. This material, which accounted for 8% of the total radioactivity, was presumed to be alanine. Further confirmation of this claim was provided by enzyme analysis.

An unlabelled preparation of the above material was performed on a large scale. L-Serine O-sulphate (500mg) and 2mg of enzyme in 0.1M-tris–HCl buffer, pH7.0 (2ml), were incubated at 37°C for 3h. $NaBH_4$ (5mg portions) was added at 20min intervals. The reaction was stopped by immersing the reaction vessel in a boiling-water bath for 2min and coagulated protein removed by centrifuging. The resulting clear

supernatant was passed through a column (10cm × 1cm) of Dowex 50 ion-exchange resin (200–400 mesh; H^+ form) and washed with 50ml of water. Further washing of the column was carried out with 2M-NH_3 (25ml) and the resulting eluate subjected to freeze-drying. The resulting material was taken up in water (10ml) and again freeze-dried. This procedure was repeated a further five times. The resulting white powder was dissolved in water (0.1ml) and precipitated by the dropwise addition of ethanol. After separation by centrifugation the resulting material was recrystallized twice with 70% (v/v) ethanol, then washed with ethanol followed by ether and finally dried *in vacuo* over P_2O_5 at room temperature (yield, 9.5mg).

The resulting material was chromatographically identical with authentic DL-alanine in the above solvent systems. An infrared spectrum of the sample analysed as a KBr disc was identical with that of an authentic DL-alanine sample. When used as a substrate for either alanine aminotransferase or D-amino acid oxidase, activities were obtained that were identical with those obtained with authentic DL-alanine as assay substrate.

Irreversibility of the enzyme-catalysed reaction

It is generally assumed that the hydrolysis of aminoacrylate is a spontaneous reaction that does not require the participation of an enzyme. Under these circumstances the above enzyme-catalysed reaction must be presumed to be irreversible. The reversibility or otherwise of the reaction was thus examined by attempting to trap any aminoacrylate formed via the back reaction by reduction with $NaBH_4$.

The reaction mixture was prepared by mixing 100μl of 0.15M-sodium [U-^{14}C]pyruvate (100μCi), 100μl of 0.15M-NH_4Cl and 100μl of enzyme (0.2mg). All reagents were made up in 0.1M-tris–HCl buffer, pH7.0. Incubations were performed at 37°C for 3h. Portions (1mg) of solid $NaBH_4$ were added at 15min intervals during the incubation. The reaction was stopped by placing the reaction tubes in a boiling-water bath for 2min, after which time the precipitated protein was removed by centrifuging. Control experiments were run alongside, with heat-denatured enzyme. The clear supernatants were analysed by descending paper chromatography on Whatman no. 1 paper as described above. Alternatively the whole supernatant was applied to a column (0.5cm × 5cm) of Dowex 50 ion-exchange resin (200–400 mesh; H^+ form), and washed with 10ml of water, then with 2M-NH_3 (10ml). The alkali washings were freeze-dried, taken up in 1ml of water and added to 15ml of scintillation mixture before determination of the amount of ^{14}C present as described above. No evidence was obtained with either analytical procedure to indicate the presence of [^{14}C]alanine in the test system.

Discussion

The results obtained in the present investigation bring into focus some of the events in the enzymic degradation of L-serine *O*-sulphate. It is now certain that release of sulphate occurs as a result of the scission of the C–O bond. In this respect the enzyme resembles pyridoxal phosphate and NaOH in its mode of action, but differs from the typical acid hydrolysis, which as expected leads to S–O-bond cleavage. The liberation of the β-substituent by C–O-bond cleavage would clearly be expected to give rise to aminoacrylate as an intermediate, and not serine. Such an involvement is well substantiated by the isolation of alanine after the incorporation of $NaBH_4$ in the incubation mixture. This amino acid could only have arisen by the reduction of an unsaturated compound whose structure corresponded to that of aminoacrylate. Since the amino acid was liberated in the DL-form it seems likely that reduction of aminoacrylate did not take place when it was enzyme-bound, implying that the subsequent hydrolysis to pyruvate and NH_4^+ occurs non-enzymically. This conclusion receives support but not absolute confirmation by the demonstration that it is not possible to carry out the reverse enzyme-catalysed formation of aminoacrylate from pyruvate and NH_4^+. The apparent irreversibility of the reaction indicates that the enzyme would be incapable of acting as a transferase of either SO_4^{2-} or serine and one can only presume that the enzyme functions in a purely degradative capacity.

The enzymic degradation of L-serine *O*-sulphate by the specific liver system is similar to that catalysed non-enzymically by pyridoxal phosphate. The reaction is also probably similar to that proposed by John & Fasella (1969) for the $\alpha\beta$-elimination of L-serine *O*-sulphate by aspartate aminotransferase and for the $\alpha\beta$-elimination of β-chloro-L-alanine by aspartate β-decarboxylase (Tate *et al.*, 1969). Clearly the mechanism concerned with the latter reactions can be accommodated by the general reaction scheme proposed by Metzler *et al.* (1954) for $\alpha\beta$-eliminations. With the enzyme-catalysed breakdown of L-serine *O*-sulphate by the pig liver system the general mechanism probably still applies, though in this instance a function other than pyridoxal phosphate is involved in an as yet undefined manner.

P. T. is grateful to the Science Research Council for a studentship.

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