The Effect of Substrate Concentration and pH on the Enzymic Sulphation of L-Tyrosyl Derivatives

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1. The kinetics of the enzymic transfer of sulphate from adenosine 3'-phosphate 5'[³⁵S]-sulphatophosphate to derivatives of L-tyrosine were investigated with a partially purified enzyme preparation from rat liver. 2. At pH 7.5 and 37°C the K_m values for L-tyrosine methyl ester and adenosine 3'-phosphate 5'[³⁵S]-sulphatophosphate are 0.3 mM and 8 nM respectively. The K_m value for either substrate is independent of the concentration of the other. The available data are consistent with the sulphation reaction proceeding according to a rapid-equilibrium random Bi Bi mechanism. 3. From the effect of pH on the K_m and V_{max} values for L-tyrosine methyl ester, tyramine and *N*-acetyl-L-tyrosine ethyl ester it is concluded that the enzyme is specific for substrate molecules with a free and unprotonated amino group and an un-ionized hydroxyl group. 4. The only ionizing group that can be positively attributed to the enzyme appears to influence the binding of adenosine 3'-phosphate 5'[³⁵S]-sulphatophosphate and has an apparent pK value of approx. 9.5. It is suggested that this group may be an essential thiol. 5. The enzyme is inhibited by iodoacetamide at pH 7.5 and 30°C and this inhibition is prevented by the presence of adenosine 3'-phosphate 5'[³⁵S]-sulphatophosphate but not by L-tyrosine methyl ester.

The preceding paper (Mattock & Jones, 1970) contrasted some of the properties of the enzymes responsible for the sulphation of L-tyrosine methyl ester and tyramine on the one hand and *p*-nitrophenol on the other. From the evidence quoted, it appears that the two enzymes are distinct from one another. Hence it is not likely that the appearance of L-tyrosine *O*-sulphate in mammalian urines (John, Rose, Wusteman & Dodgson, 1966) is an artifact of a detoxication type of reaction. However, whether the enzyme that catalyses the sulphation of simple derivatives of L-tyrosine is responsible for the appearance of sulphated tyrosine residues in proteins is unknown, and an investigation of the substrate requirements of this enzyme may throw light on its possible metabolic function. In this connexion, Segal & Mologne (1959) have shown that, with a rat liver enzyme preparation, the rate of transfer of sulphate from *p*-nitrophenyl sulphate via adenosine 3',5'-diphosphate to carboxyl-substituted derivatives of L-tyrosine was higher at pH 9.3 than at pH 7.8. These authors suggested that these observations reflected the ionization of the amino group of the substrates in

addition to ionizations of groups on the enzyme. The present paper describes a detailed investigation of the effect of pH on the rate of enzymic sulphation of derivatives of L-tyrosine and confirms the view that the variation in enzyme activity with pH reflects ionizations of one of the substrates and of the enzyme itself. Some evidence is also presented suggesting that the enzyme-catalysed transfer of sulphate from adenosine 3'-phosphate 5'-sulphatophosphate to L-tyrosine methyl ester is a rapid-equilibrium random Bi Bi reaction.

MATERIAL AND METHODS

Preparation of the enzyme. The enzyme was prepared from female rat livers as explained by Mattock & Jones (1970). Enzyme from stage 4 of that preparative procedure was employed, as it was stable and free from other enzymes that might interfere with the assay procedure by modifying the substrates or products of the enzyme under investigation.

Preparation and assay of adenosine 3'-phosphate 5'[³⁵S]-sulphatophosphate. This was as described by Mattock & Jones (1970).

Determination of sulphotransferase activity. The general conditions of assay were incubation of enzyme (10 μ l) with substrates in a medium (vol. 160 μ l), containing 3 mM-dithiothreitol (Calbiochem Ltd., Basingstoke,

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Hants., U.K.) for 10 min at 37°C, during which time the reaction followed zero-order kinetics. The type, concentration and pH of the buffer and the concentration of substrates were as described for each experiment. The reaction was stopped by the addition of 0.4 ml of 0.05M-H₂SO₄ and the amount of ³⁵S-labelled ester produced was then determined by the slight modification of the method of Wengle (1964) described by Mattock & Jones (1970).

EXPERIMENTAL AND RESULTS

Kinetics of enzymic sulphation of L-tyrosine methyl ester. The rate of transfer of [³⁵S]sulphate from adenosine 3'-phosphate 5' [³⁵S]-sulphatophosphate to L-tyrosine methyl ester (Ralph N. Emanuel Ltd., London S.E.1, U.K.) was measured at each of four concentrations of the nucleotide (4 nM, 8 nM, 16 nM and 32 nM) and at each of four concentrations of the sulphate acceptor (0.25 mM, 0.5 mM, 1.0 mM and 2.0 mM). The completed assay mixtures also contained 0.48 μmol of dithiothreitol, 100 μl of 0.1 M-tris-HCl buffer, pH 7.5, and approx. 20 μg of protein. The amount of radioactive ester produced in 10 min at 37°C was determined as described above. The results were expressed as double-

reciprocal plots (Lineweaver & Burk, 1934) and the Michaelis constant for each substrate was determined by extrapolating lines to cut the abscissa. Fig. 1 shows that all reciprocal plots were linear and the *K_m* for one substrate is independent of the concentration of the second substrate. The *K_m* values for L-tyrosine methyl ester and adenosine 3'-phosphate 5' [³⁵S]-sulphatophosphate were 0.3 mM and 8 nM respectively. From these reciprocal plots, secondary plots of intercept on the ordinate and slopes against the reciprocal of the concentration of the second substrate were made. These secondary plots were also linear and from them values of the kinetic constants were derived by the procedure of Dalziel (1957) for a rate equation of the form:

$$\frac{1}{V_0} = \phi_0 + \frac{\phi_1}{[S_1]} + \frac{\phi_2}{[S_2]} + \frac{\phi_{12}}{[S_1][S_2]}$$

The values of these kinetic constants were found to be: ϕ_0 , 223 min nmol⁻¹; ϕ_1 , 18 min; ϕ_2 , 57 min; ϕ_{12} , 4.56 min nmol. These kinetic coefficients exhibit the following relationship:

$$\frac{\phi_1\phi_2}{\phi_{12}} = 225 = \phi_0$$

The kinetic constants quoted by Dalziel (1957) are true constants and independent of the concentration of the enzyme. However, in the case quoted here, the concentration of the enzyme was unknown and is therefore included in the values for all the kinetic constants. Accordingly these values will vary from experiment to experiment and the dimensions of the various ϕ values are different from those quoted by Dalziel (1957).

Effect of pH on the enzymic sulphation of tyrosyl derivatives. (a) At a constant concentration of adenosine 3'-phosphate 5' [³⁵S]-sulphatophosphate and various concentrations of L-tyrosine methyl ester or tyramine. The rate of transfer of [³⁵S]sulphate from 0.06 mM-adenosine 3'-phosphate 5' [³⁵S]-sulphatophosphate to L-tyrosine methyl ester (concentration range 0.13 mM–2.0 mM) and tyramine (Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.) (concentration range 0.1 mM–5 mM) was measured at various pH values over the range pH 6.5–10.5. Michaelis barbital-sodium acetate buffer and Sørensen glycine buffer were used to cover the pH ranges 6.5–9.0 and 9.5–10.5 respectively (see Documenta Geigy Scientific Tables, 1968, p. 314). Each incubation mixture contained 100 μl of the appropriate buffer, which was used as a vehicle for the sulphate acceptor and the dithiothreitol. Preliminary experiments showed that the same results were obtained with 100 μl of 0.1 M-tris-hydrochloric acid buffer, pH 9.0. Further, during the time of assay, the rate of production of sulphate ester was linear at all pH values, indicating that

Fig. 1. Effect of varying the concentration of the second substrate on the reciprocal plots for L-tyrosine methyl ester and adenosine 3'-phosphate 5' [³⁵S]-sulphatophosphate at pH 7.5. (a) For L-tyrosine methyl ester at different concentrations of adenosine 3'-phosphate 5' [³⁵S]-sulphatophosphate: ○, 4 μM; △, 8 μM; ●, 16 μM; □, 32 μM. (b) For adenosine 3'-phosphate 5' [³⁵S]-sulphatophosphate at different concentrations of L-tyrosine methyl ester: ○, 0.25 mM; △, 0.5 mM; ●, 1.0 mM; □, 2.0 mM. See the text for details. *v* = nmol of ³⁵S transferred/min.

Fig. 2. pH-dependence of K_m and V_{max} values for (a) L-tyrosine methyl ester and (b) tyramine at a constant concentration of adenosine 3'-phosphate 5' [^{35}S]sulphatophosphate (0.06 mM). Values were obtained in Michaelis barbital-sodium acetate buffer (○) and Sørensen glycine buffer (●). See the text for details. V_{max} = nmol of ^{35}S transferred/min.

there was no appreciable loss of enzyme activity. From the experimental results, the K_m and V_{max} values for both acceptors were calculated from double-reciprocal plots as described above. Fig. 2 shows how K_m and V_{max} , expressed as pK_m ($-\log K_m$) and $\log V_{max}$, vary as a function of pH. (b) At a constant concentration of L-tyrosine methyl ester and various concentrations of adenosine 3'-phosphate 5' [^{35}S]sulphatophosphate. The rate of transfer of [^{35}S]sulphate from adenosine 3'-phosphate 5' [^{35}S]sulphatophosphate (concentration range 4 nM to 0.06 mM) to 4 mM-L-tyrosine methyl ester was measured over the range pH 6.5–10.5 as described in the previous experiment. The variation in pK_m for the nucleotide with pH is shown in Fig. 3. The V_{max} values agreed closely with those obtained from the previous experiment when the concentration of L-tyrosine methyl ester was varied and with a constant and high concentration of adenosine 3'-phosphate 5' [^{35}S]sulphatophosphate.

Kinetic constants for the enzyme-catalysed reaction between adenosine 3'-phosphate 5' [^{35}S]sulphatophosphate and N-acetyl-L-tyrosine ethyl ester or L-tyrosine ethyl ester. N-Acetyl-L-tyrosine ethyl ester (BDH Ltd., Poole, Dorset, U.K.) was found to be a substrate for this enzyme preparation and the rate of transfer of sulphate to this compound was directly compared with the rate of transfer to the unsubstituted molecule, L-tyrosine ethyl ester

Fig. 3. pH-dependence of K_m for adenosine 3'-phosphate 5' [^{35}S]sulphatophosphate at a constant concentration of L-tyrosine methyl ester (4 mM). Values were obtained in Michaelis barbital-sodium acetate buffer (○) and Sørensen glycine buffer (●). See the text for details.

(BDH Ltd.). Accordingly adenosine 3'-phosphate 5' [^{35}S]sulphatophosphate (0.06 mM) was incubated under the usual assay conditions with either L-tyrosine ethyl ester or N-acetyl-L-tyrosine ethyl ester (concentration range 0.39 mM–6.25 mM) with 0.1 M-tris-hydrochloric acid buffer, pH 7.5 (100 μl). Sulphotransferase activity was assayed in the usual way and the results, expressed as double-reciprocal plots, are shown in Fig. 4. The calculated K_m values were 15 mM and 0.7 mM for N-acetyl-L-tyrosine ethyl ester and L-tyrosine ethyl ester respectively. The corresponding V_{max} values were 0.14 nmol/min and 0.35 nmol/min. The K_m and V_{max} values for N-acetyl-L-tyrosine ethyl ester

(concentration range 0.8 mM–6.3 mM) were found to be the same at pH 7.1, 7.5 and 8.2 under the usual assay conditions with 0.1 M-tris-hydrochloric acid buffers.

Effect of iodoacetamide on the enzymic sulphation of L-tyrosine methyl ester. A sample (0.8 ml) of the enzyme preparation was activated by mixing with 100 μ l of a solution of 0.2 M-dithiothreitol in 0.1 M-tris-hydrochloric acid buffer, pH 7.5. After standing for 2 h at 0°C the mixture was dialysed against 2 litres of 0.05 M-tris-hydrochloric acid buffer, pH 7.5, for 2 h to remove excess of dithiothreitol. Samples (10 μ l) of the activated enzyme were then incubated at 30°C in a total volume of 110 μ l with iodoacetamide [1.1 μ mol; Sigma (London) Chemical Co.] alone and with iodoacetamide in the presence of either L-tyrosine methyl ester (1 μ mol) or adenosine 3'-phosphate 5' [35 S]-sulphatophosphate (0.015 μ mol). Each reagent was dissolved in 100 μ l of 0.1 M-tris-hydrochloric acid buffer and the pH adjusted to 7.5 with M-sodium hydroxide. Control experiments were performed in an identical manner except that iodoacetamide was omitted from the incubation mixtures. After 30 min, substrates (L-tyrosine methyl ester, 1 μ mol, or adenosine 3'-phosphate 5' [35 S]-sulphatophosphate, 0.015 μ mol, or both substrates simultaneously) were added to the appropriate tubes to make a complete solution suitable for the assay of sulphotransferase activity. Again the substrates were dissolved in 100 μ l of 0.1 M-tris-hydrochloric acid buffer, pH 7.5. The completed assay solutions were incubated for

5 min at 30°C and the sulphotransferase activity remaining in each tube was then measured. The results (Table 1) show that the enzyme is inactivated by incubation with 10 mM-iodoacetamide and is protected from this inactivation by the presence of 0.14 mM-adenosine 3'-phosphate 5' [35 S]-sulphatophosphate but not by 9 mM-L-tyrosine methyl ester.

Titration curves of L-tyrosine methyl ester and tyramine. Approximate values for the macroscopic ionization constants for L-tyrosine methyl ester and tyramine were obtained by titration in aqueous solution. Samples (10 ml of 4 mM solutions) of L-tyrosine methyl ester and tyramine hydrochloride were placed in the titration compartment, maintained at 37°C by water circulation, of an automatic titrator (Radiometer, Copenhagen, Denmark) and titrated with 0.5 ml of 0.1 M-sodium hydroxide. All solutions were kept under nitrogen throughout. The macroscopic constants for the two compounds were calculated from the titration curves by standard procedures (see Edsall & Wyman, 1958).

The calculated apparent pK values were: pK_1 7.14 and pK_2 9.88 for L-tyrosine methyl ester; pK_1 9.25 and pK_2 10.9 for tyramine. These values agree reasonably well with other values quoted for tyramine and esters of L-tyrosine at 25°C (Martin, Edsall, Wetlaufer & Hollingworth, 1958; Ogston, 1936; Yamazaki, Fujiki & Murata, 1965).

DISCUSSION

The kinetics of the enzymic transfer of sulphate from adenosine 3'-phosphate 5' [35 S]-sulphatophosphate to L-tyrosine methyl ester show that the K_m values for the two substrates are independent of one another. Similar observations have been made for androstenedione sulphotransferase (Banerjee & Roy, 1967), choline sulphotransferase

Fig. 4. Reciprocal plots for the effect of substrate concentration on the enzymic sulphation of L-tyrosine ethyl ester (●) and N-acetyl-L-tyrosine ethyl ester (○) at pH 7.5. The concentration of adenosine 3'-phosphate 5' [35 S]-sulphatophosphate was maintained constant at 0.06 mM. v = nmol of 35 S transferred/min.

Table 1. *Effect of iodoacetamide on the enzymic sulphation of L-tyrosine methyl ester*

The enzyme was incubated with the various reagents for 30 min at pH 7.5 and 30°C before assay of sulphotransferase activity towards L-tyrosine methyl ester.

Incubation mixture	Enzyme activity remaining after 30 min (arbitrary units)
Enzyme alone	100
Enzyme+iodoacetamide (10 mM)	52
Enzyme+iodoacetamide (10 mM)+adenosine 3'-phosphate 5' [35 S]-sulphatophosphate (0.14 mM)	84
Enzyme+iodoacetamide (10 mM)+L-tyrosine methyl ester (9 mM)	47

(Orsi & Spencer, 1964) and phenol sulphotransferase (Banerjee & Roy, 1968). Dalziel (1957) considered a number of possible mechanisms for two-substrate enzymic reactions and drew up characteristic relationships between the measured kinetic constants for each mechanism considered. A mechanism involving the formation of two binary and one ternary enzyme-substrate complexes, which are in rapid equilibrium with the substrates, exhibits the following diagnostic relationship between the kinetic coefficients:

$$\phi_0 = \phi_1\phi_2/\phi_{12}$$

The results recorded here are in good agreement with these requirements and it is therefore suggested that the enzymic sulphation of L-tyrosine methyl ester is a rapid-equilibrium random Bi Bi reaction (see Cleland, 1963). It must be emphasized that the results presented here, although being compatible with the proposed mechanism, cannot exclude all others. However, similar results to those quoted here have been reported for adenosine 3'-phosphate 5'-sulphatophosphate-phenol sulphotransferase (EC 2.8.2.1) from guinea-pig liver (Banerjee & Roy, 1968), and the mechanism has been confirmed, by employing product inhibition studies (Cleland, 1963), as that of a rapid-equilibrium random Bi Bi reaction.

The variation, with pH, of K_m and V_{max} , for an enzymic reaction may be interpreted in terms of ionizations of groups in the enzyme, substrate or enzyme-substrate complexes (see Dixon & Webb, 1964).

The graph of pK_m versus pH for L-tyrosine methyl ester (Fig. 2) shows two negative changes of slope corresponding to apparent pK values of 7.6 and 9.5, which are attributable to either the free enzyme or the substrate. These pK values compare favourably with those obtained, by titration, for the ionization of L-tyrosine methyl ester and hence may correspond to the ionization of the amino and hydroxyl groups of the substrate. However, it is possible that the groups involved are on the enzyme and the presence of groups in the substrate with similar pK values is coincidental. To investigate this point further the K_m value for tyramine has been determined over the same pH range. The results (Fig. 2) show only one negative change of slope, at pH 9.0, which corresponds approximately to the pK value of the first ionization of tyramine, presumably the amino group. The ionization of the hydroxyl group of tyramine (pK approx. 11) would not be expected to affect significantly the pK_m value for this substrate over the pH range studied. Therefore it is possible to conclude that there are no groups on the enzyme that ionize within the pH range studied that affect the affinity of the enzyme for L-tyrosine methyl ester or tyramine. However,

the enzyme appears to be specific for substrate molecules with their amino and hydroxyl groups in the base and acid form respectively. The requirement for a free and unprotonated amino group is further supported by the pH-independent and low rate of transfer of sulphate groups to *N*-acetyl-L-tyrosine ethyl ester by this enzyme preparation between pH 7.0 and 8.5.

The graph of pK_m versus pH for L-tyrosine methyl ester also shows two ionizing groups in the enzyme-substrate complex. The proximity of the pK values of these groups (pK approx. 7.0 and 10.0) to those attributed to the free substrate may be interpreted (Dixon & Webb, 1964) as showing that these groups are not directly involved in the binding of substrate to the enzyme, but this combination enhances the ionization of the amino group and suppresses the ionization of the hydroxyl group. This is in keeping with the hypothesis that the enzyme binds the substrate when the amino and hydroxyl groups are in their base and acid forms respectively. A similar argument may be applied to the ionization of the amino group of tyramine (Fig. 2). However, in this instance the suppression of the ionization of this group on combination with the enzyme is far more pronounced than with L-tyrosine methyl ester. This observation is perhaps not surprising in view of the fact that, with the ester, the ionization of the amino group is already influenced by the presence of a carbonyl group on the α -carbon atom, which decreases considerably the basic character of the amino group.

The variation of V_{max} , with pH for L-tyrosine methyl ester (Fig. 2) shows the presence of two groups in the enzyme-substrate complex having the same apparent pK values as those groups in the enzyme-substrate complex that affect the K_m value for this substrate. A similar observation may be made in the case where tyramine was used as substrate (Fig. 2). It is therefore possible to conclude that the groups that affect the reactivity of the enzyme-substrate complex are the same as those that affect the binding of the substrates to the enzyme, namely the amino and hydroxyl groups of the substrates.

The graph of pK_m versus pH for adenosine 3'-phosphate 5' [^{35}S] sulphatophosphate at high concentration of L-tyrosine methyl ester shows the presence of two ionizing groups in the free enzyme or substrate with apparent pK values of 6.9 and 9.5. The more acidic group is again affected by combination of substrate and enzyme when its pK value is raised to 7.2. With the available evidence it is not possible to decide whether this group should be assigned to the enzyme or to one of the phosphate groups of the substrate. The second ionizing group (pK approx. 9.5) may be attributed to the enzyme,

as adenosine 3'-phosphate 5' [^{35}S]-sulphatophosphate would not be expected to ionize within this range. This group could conceivably be an essential thiol, which may be expected to ionize within this pH range if associated with a carboxyl group (Cecil & McPhee, 1959). Support for this hypothesis comes from the fact that the enzyme is inhibited by a number of reagents known to react with thiol groups (Mattock & Jones, 1970). Further, the inhibition of the enzyme by iodoacetamide is prevented by the presence of adenosine 3'-phosphate 5' [^{35}S]-sulphatophosphate but not by the presence of L-tyrosine methyl ester. Therefore it is reasonable to draw the tentative conclusion that this enzyme possesses a functional thiol group in its nucleotide-binding site.

The results reported here have yielded valuable information about the binding of substrates to an enzyme that catalyses the sulphation of compounds which may be considered as derivatives of the amino acid L-tyrosine. Hence the enzyme appears to possess two independent substrate-binding sites: one for adenosine 3'-phosphate 5'-sulphatophosphate, which probably contains a functional thiol group, and one for the sulphate acceptor molecule. This latter site appears to be specific for substrate molecules with a free and unprotonated amino group and an un-ionized hydroxyl group. These observations may throw valuable light on the physiological function of this enzyme. It was argued previously (Mattock & Jones, 1970) that the incorporation of sulphate groups into tyrosine residues of certain proteins must occur either at the tyrosyl-tRNA stage of protein biosynthesis or after incorporation of the amino acid into the protein or a polypeptide precursor. If the enzyme described here is involved in this process, it does not seem

likely that peptide-bound tyrosine residues would be suitable substrates. However, a tyrosine residue, linked through its carboxyl group to a tRNA molecule, may be an ideal substrate for this enzyme.

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