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THE SULPHATASE OF OX LIVER

XIII THE ACTION OF CARBONYL REAGENTS ON SULPHATASE A

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SUMMARY

The inhibition of sulphatase A by certain carbonyl reagents and by ascorbate has been shown to require the presence of metal ions in the system The inhibition is prevented by EDTA which can also reverse any prior inhibition by these reagents A metal-free preparation of sulphatase A was not inhibited by phenylhydrazine Copper seems to be the metal primarily involved and it is suggested that these reagents inhibit sulphatase A either by forming an enzymically inactive sulphatase–copper–modifier complex or by reducing Cu^{2+} to Cu^{+}, the latter being the true inhibitor of the enzyme Of these two possibilities the latter seems the more probable but direct proof has not been possible

INTRODUCTION

The inhibition of the arylsulphatase (aryl-sulphate sulphohydrolase, EC 3 1 6 1) of Takadiastase (takasulphatase) by carbonyl reagents was first shown by ROSENFELD AND RUCHELMAN[1] and was later confirmed by other workers[2,3] One feature of these early results was their variability for example the concentration of CN^- required to give a 50% inhibition of takasulphatase was variously reported as 0 03 mM (ref 2) and as 2 mM (ref 3) Even taking into account the different techniques used in the assay of the enzyme these discrepancies seem very large More recently it has been shown that the arylsulphatase of *Alcaligenes metalcaligenes*[4] and the sulphatase A of ox liver (unpublished observations) are also inhibited by these reagents

In no case has the chemistry of this inhibition been clarified and although it was proposed[1] that takasulphatase must contain carbonyl groups no direct evidence for this postulate has been forthcoming Two recent observations suggested that a reinvestigation of the action of carbonyl reagents on the sulphatases would be useful First, the discovery[5,6] of aminoaldehydes in collagen suggested that these might be the hydroxylamine- or phenylhydrazine-reactive groups in the arylsulphatases Second, the observation that the carboxylesterases of pig and ox liver are inactivated

by the hydroxylating action of hydroxylamine and traces of Cu^{2+} present in the enzyme preparations suggested that sulphatase A might be similarly inhibited by the hydroxylation of the tyrosyl residue(s) involved in the action of this enzyme[8]

METHODS

Enzyme

Sulphatase A (specific activity 150 units/mg) was prepared from ox liver as previously described[9] Several preparations were used during the work

Modifiers

These were commercial preparations except for *N*-phenylhydroxylamine which was synthesized by a standard method and for dehydroascorbic acid which was prepared immediately before use by the oxidation of ascorbic acid with KIO_3 (ref 10)

The modifiers were used as freshly prepared aqueous solutions adjusted to pH 5 6 with NaOH or HCl

Assay of enzymic activity

Sulphatase A was assayed in a pH-stat at 37° and at pH 5 6 with 3 mM nitrocatechol sulphate as substrate[9] The volume of the reaction mixture was 10 ml

The effect of modifiers on the activity was studied as follows to 20 μl of a solution of sulphatase A (0 2 mg/ml, 2 μM) in acetate buffer (I = 0 1, pH 5 6) were added 20-μl portions of the required reagents, also at pH 5 6, to give a total volume of 60 μl After standing for 1 h at room temperature (25–30°) a sample (50 μl) of the modified enzyme was taken for assay Unless specified below, the substrate used in these assays contained the modifier in the same concentration as did the treated enzyme

Reaction under metal-free conditions

Sulphatase A was dialysed for 3 days against repeated changes of 100 vol of 0 01 M EDTA (pH 7 4) and the latter subsequently removed by exhaustive dialysis against 0 01 M Tris–HCl (pH 7 4) The dialysis tubing and apparatus used in this, and subsequent, procedures had been rendered metal-free by soaking in 3% acetic acid followed by rinsing with glass-distilled water All the reagents were purified as described in ref 11 with the additional precaution of carrying out the first crystallizations in the presence of EDTA

RESULTS AND DISCUSSION

Carbonyl reagents and sulphatase A

The results given in Table I show that certain carbonyl reagents are powerful inhibitors of sulphatase A With 0 5 mM hydroxylamine as modifier the mean inhibition in 9 experiments was 41% (range 23–71%) and with 0 5 mM phenylhydrazine (6 experiments) the corresponding figures were 89 and 82–100% Not all carbonyl reagents inhibited the enzyme neither dimedone (0 5 mM), CN^- (5 mM) nor β-aminopropionitrile (5 mM) showed any inhibitory effects when tested under the above conditions

TABLE I

THE CONCENTRATIONS OF CERTAIN CARBONYL REAGENTS REQUIRED TO GIVE A 50% INHIBITION OF SULPHATASE A WHEN ALLOWED TO REACT WITH THE ENZYME FOR 1 h AT pH 5.6 AND AT 25°

The reaction mixture used in the assay of the modified enzyme contained the carbonyl reagent at the same concentration as did the enzyme solution

Reagent	*Concn (mM)*
Semicarbazide	10
Hydrazine	5
Hydroxylamine	1
Phenylhydrazine	0.05
N-Phenylhydroxylamine	0.03

Treatment of sulphatase A with *N*-methylbenzthiazolinone hydrazone at pH 5.0 and 40° for 2 h caused only a 7–14% inhibition of the enzyme (three different preparations). It was not possible to detect significant numbers of azine groups in the modified enzyme although these are formed under such conditions with simple carbonyl compounds[12] and with tropocollagen (which contains aminoaldehydes)[5]. Certainly if any azine were present in the treated sulphatase A, it occurred in amounts of less than 1 mole per mole of enzyme.

The inhibition of sulphatase A by hydroxylamine or phenylhydrazine is not of a simple reversible type because it develops only slowly over several hours, as shown in Fig. 1. Nevertheless the inhibition is partly reversible by dialysis for 24 h or by omitting the carbonyl reagent from the substrate solution so that the reagent is diluted 200-fold on commencing the assay.

Sedimentation of the modified enzyme

When a solution of sulphatase A in acetate buffer, (pH 5.0, $I = 0.1$) containing 0.01 M phenylhydrazine was examined by ultracentrifugation[9] the material showed a single symmetrical boundary with an $s_{20,w}$ (at a concentration of 0.4 g per 100 ml) of 13.7 S, indistinguishable from that of 13.6 S for the untreated enzyme at the same concentration. Modification of sulphatase A with phenylhydrazine is therefore not accompanied by any drastic change in the size or shape of the enzyme.

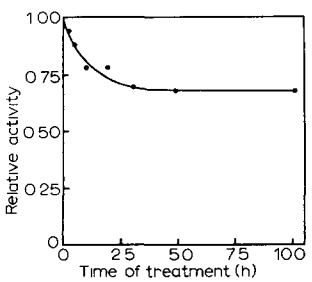

Fig. 1. The inhibition of sulphatase A by treatment with 0.05 mM phenylhydrazine at pH 5.0 and at 25°. The enzyme activity was determined in the presence of 0.05 mM phenylhydrazine and is expressed relative to that of the untreated enzyme.

TABLE II

THE EFFECT OF EDTA AND METAL IONS ON THE INHIBITION OF SULPHATASE A BY HYDROXYLAMINE, PHENYLHYDRAZINE AND ASCORBATE AT pH 5.6 AND AT 25°

In these assays the substrate did not contain the modifier so that the latter was diluted 200-fold at the start of the assay. The concentrations in the table are those pertaining during the modification of the enzyme. The activity of the modified enzyme is expressed relative to that of an untreated control. In the experiment marked with an asterisk the EDTA was added to the modified enzyme immediately before its assay.

	Hydroxylamine	*Phenylhydrazine*	*Ascorbate*
	5 mM	*0.5 mM*	*0.5 mM*
EDTA (50 mM)		0.96	
Modifier	0.65	0.20	0.40
EDTA + modifier	0.87	0.95	0.83
*Modifier + EDTA	0.88	0.90	0.90
	5 mM	*0.1 mM*	*0.1 mM*
Modifier	0.64	0.70	0.51
Modifier + Cu^{2+} (0.01 mM)	0.27	0.29	0.09
Modifier + Fe^{2+} (0.1 mM)	0.71	0.74	0.86
Modifier + Co^{2+} (0.1 mM)	0.97	0.75	0.73
Modifier + Zn^{2+} (0.1 mM)	1.01	0.74	0.66

Inhibition by ascorbate

The carbonyl reagents listed in Table I all show reducing properties which could be the reason for their being inhibitors. The action of ascorbate, a powerful reducing agent which is without action on carbonyl groups, was therefore investigated and, as shown in Table II, this compound was a powerful inhibitor of sulphatase A.

Role of metal ions in the inhibition

The results in Table II show that neither hydroxylamine, phenylhydrazine nor ascorbate inhibited sulphatase A when EDTA was present in the reaction mixture and, further, that the inhibition caused by these reagents in the absence of EDTA was completely reversed by the subsequent addition of the latter to the modified enzyme. Metal ions must therefore be involved in the reaction between these modifiers and the enzyme which excludes the possibility of these reagents reacting directly with carbonyl groups. Further, the fact that the inhibition can be reversed by EDTA shows that it cannot have been caused by the hydroxylation of tyrosyl residues[7,13] because this would not be reversible.

A metal-free preparation of sulphatase A was scarcely inhibited by phenylhydrazine alone yet it was completely inhibited by the simultaneous addition of the reagent and 0.01 mM $CuSO_4$ (Table III). The metal-free enzyme had a specific activity of 147 units/mg compared with that of 150 units/mg for the untreated enzyme showing that the metal ions involved in the reaction with the modifiers did not alter the catalytic properties of sulphatase A.

Further information on the role of metal ions in these reactions is given in Table II. It is clear that the inhibitory action of the modifiers is increased by the addition of Cu^{2+} but not by the addition of Fe^{2+}, Co^{2+} or Zn^{2+} which can in fact provide a certain degree of protection to the enzyme. In the absence of any modifier

TABLE III

THE EFFECT OF PHENYLHYDRAZINE ON A METAL-FREE PREPARATION OF SULPHATASE A UNDER METAL-FREE CONDITIONS

Details of the procedure are given in the text and the general conditions are the same as those specified in Table II

Treatment	*Relative activity*
Control	1 0
$CuSO_4$, 0 01 mM	0 89
Phenylhydrazine, 0 5 mM	0 91
Phenylhydrazine + $CuSO_4$	0 00

these metal ions were themselves without significant action on the enzyme, the maximum effect being caused by Zn^{2+} which gave a 5% inhibition at 0 1 mM

Mechanism of the inhibition

Although the results given above clearly implicate metal ions, in particular copper ions, in the inhibition of sulphatase A by 'carbonyl' reagents and by ascorbate it is difficult to specify the exact mechanism of this inhibition There appear to be two possibilities First, that the enzyme reacts with Cu^{2+} (endogenous or exogenous) and the modifier to give a ternary complex which is enzymically inactive examples of such ternary complexes were considered by GURD AND WILCOX[14] Second, that the modifiers act as reducing agents which convert Cu^{2+} into Cu^{+}, the latter being the true inhibitor of the enzyme such a situation apparently exists with β-glucuronidase[15] The second mechanism could be further complicated by the formation of a ternary complex of enzyme, Cu^{+} and reducing agent but for practical purposes this can be disregarded because the essential feature would still be the reduction

It is difficult to design experiments to unambiguously differentiate between these two possibilities If the first mechanism, ternary complex formation with Cu^{2+}, be the correct one then suitable metal-binding agents should be inhibitors even if they are not reducing agents However, neither NH_4^+ (5 mM), dehydroascorbic acid (1 mM), histidine (3 mM) nor gluconolactone (5 mM) showed any inhibitory action although the first two components should have comparable metal-binding properties to hydrazine and ascorbate respectively This therefore suggests that the inhibition by the reagents listed in Tables I and II cannot be due to their ability to form metal complexes but must be dependent upon their reducing properties, as in the second mechanism The interpretation of experiments using metal-binding reagents is, however, complicated because a failure to cause inhibition could be due to the metal-binding properties being either too weak, when complex formation would not occur, or too strong, when metal ions would be stripped from the enzyme as by EDTA

Direct evidence for the occurrence of the second mechanism has not been obtained because it has not been possible to devise a system which will reduce Cu^{2+} to Cu^{+} at pH 5 6 without the possibility of complex formation The direct addition of CuCl to the enzyme caused no inhibition but this is not unexpected because Cu^{+} cannot exist in solution and the reagent commonly used to prepare CuCl, sulphite[16], cannot be used in the present instance because it itself is a powerful competitive

inhibitor of sulphatase A with a K_i of approx $2\,\mu M$ (ref 17) Attempts to use inorganic reducing agents have not been successful neither CN^-, $Fe(CN)_6^{4-}$, $S_2O_4^{2-}$ nor $NaBH_4$ caused any inhibition when added together with Cu^{2+} Once again these results cannot be interpreted unambiguously because reduction may not have occurred at pH 5 6 or because the products could have been complex For example, the reduction of Cu^{2+} by $S_2O_4^{2-}$ is known to produce, among other compounds, CuS (ref 18) and reduction by $NaBH_4$ may produce metal hydrides[19] In both cases only vanishingly small concentrations of Cu^+ need have been present

The results therefore suggest that 'carbonyl' reagents and ascorbate inhibit sulphatase A by reducing traces of copper in the enzyme preparation to Cu^+ which is the true inhibitor and presumably combines with the enzyme in the same way as Ag^+, perhaps through an essential histidyl residue[8] The possibility of the inhibition being due to the formation of a ternary enzyme–Cu^{2+}–modifier complex cannot, however, be excluded Both types of inhibition would be prevented, or reversed, by EDTA

It cannot be assumed that the inhibition of other sulphatases by 'carbonyl' reagents must be of the same type and until these enzymes are available in a purified form a decision will not be possible In the case of the inhibition of the arylsulphatase of *A metalcaligenes* by CN^-, binding of the inhibitor to the enzyme has apparently been shown[20] but the participation of metal ions has not been demonstrated Preliminary experiments (unpublished work) with takasulphatase have likewise given no evidence for the participation of metal ions in the inhibition of this enzyme by carbonyl reagents

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