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THE SULPHATASE OF OX LIVER

XXIII. THE NATURE OF SUBSTRATE-MODIFIED SULPHATASE A

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Summary

An improved method is described for the preparation of milligram quantities of substrate-modified sulphatase A. The latter has the same molecular weight and the same ability to form a tetramer as has native sulphatase A. It has been shown that the modified enzyme prepared with nitrocatechol [^{35}S]sulphate as substrate contains 1 mol $^{35}SO_4^{2-}$ per mol enzyme and that any treatment which causes reversion of the modified enzyme to native enzyme is accompanied by the loss of the bound SO_4^{2-}. Dialysis of the ^{35}S-modified enzyme against a solution containing SO_4^{2-} causes a loss of $^{35}SO_4^{2-}$ with no change in the amount of modified enzyme in the preparation.

It has been shown that the activation of the substrate-modified enzyme by SO_4^{2-} does not lead to the formation of a third stable form of sulphatase A.

Introduction

The 'anomalous' kinetics of sulphatase A (aryl-sulphate sulphohydrolase, EC 3.1.6.1) were first described by Roy [1] for the sulphatase A of ox liver and were investigated in considerable detail by Baum et al [2] using the corresponding enzyme from human liver. The latter authors showed [3] that the 'anomalous' kinetics were the result of the enzyme being inactivated during its catalytic cycle and subsequently being reactivated by the reaction products, particularly by SO_4^{2-}. Further evidence for this explanation was provided by

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Abbreviation: PPO, 2,5-diphenyloxazole.

Nicholls and Roy [4]. Although it is now clear that the production of a substrate-modified enzyme is typical of the behaviour of sulphatase A functioning as an arylsulphatase, but not as a cerebroside sulphatase [5], there has been no indication of the nature of the change undergone by the enzyme in the presence of substrate. It appears to have been assumed to be conformational [6] because it is freely reversible at 37°C in the absence of substrate [4,6]. Recent work in this laboratory [7] has, however, shown that the substrate-modified sulphatase of ox liver contains 1 mol SO_4^{2-} per mol enzyme. A similar finding has been reported by Waheed and Van Etten [8] with the sulphatase A of rabbit liver, although in this case there are 2 mol SO_4^{2-} bound per mol substrate-modified enzyme.

The present paper describes some of the physical and chemical properties of the substrate-modified sulphatase A of ox liver.

Experimental

Preparation of sulphatase A. Sulphatase A was prepared from ox liver by a slight modification of the method of Nichol and Roy [9]. Several different preparations were used; their specific activities ranged from 203 to 248 μmol · min^{-1} · mg^{-1}. These values are higher than those previously reported because they are based on computed values of the initial velocities determined in 0.1 M KCl (see below).

In the following, the molar concentration of sulphatase A is always expressed in terms of its monomer (M_r 107 000), whatever be the polymeric form of the enzyme dominant under particular experimental conditions.

Determination of sulphatase A activity. All determinations were made in a pH-stat (assembly PHM26-TT11-SBR2-ABU12, Radiometer Ltd., Copenhagen). Routine assays were carried out with 3 mM nitrocatechol sulphate as substrate in 0.1 M KCl, pH 5.6, at 37°C. The volume of the reaction mixture was 10 ml.

Initial velocities were computed by fitting readings from the pH-stat recordings, at 8 s intervals over the first 3 min of the reaction, to the exponential function described by Roy [10].

The substrate-modified enzyme was activated by adding to the above reaction mixture 50 μl 0.6 M K_2SO_4. The velocity, 20—30 min after the addition of K_2SO_4, was obtained from the slope of the pH-stat recording, determined by linear regression of readings at 1-min intervals.

The fraction of substrate-modified sulphatase A in any preparation was calculated in either of two ways. In the first, the specific activity of the preparation, measured from v_0, expressed as a fraction of that of the corresponding native enzyme gave directly the amount of the latter remaining in the preparation. In the second, the ratio of v_0 to the velocity measured after the addition of SO_4^{2-} was used [4] to give the extent of the modification. Both methods assume [4] that the substrate-modified enzyme is devoid of activity in the absence of SO_4^{2-} and they give similar results. The first method was preferred because the enzyme concentrations were such that the reasonably high values of v_0 could be precisely determined. This was not so with the second method which was useful when only small amounts of enzyme were available.

Preparation of substrate-modified sulphatase A. Modified enzyme was

prepared by incubating sulphatase A (25 μg/ml) in 0.05 M nitrocatechol sulphate/0.1 M Tris-HCl, pH 7.5, at 37°C for 30 min. In routine preparations, 50 ml reaction mixture containing 1.25 mg sulphatase A were used. After incubation the mixture was cooled to 4°C and applied to a Sephadex G-25 column (25 × 3.5 cm) in 0.01 M Tris-HCl/0.15 M NaCl (pH 7.4) and eluted with the same buffer. In this and all subsequent steps the temperature was kept at 4°C. The portion of the eluate containing the protein, determined by prior calibration of the column with bovine serum albumin, was then applied to a DEAE-Sephadex A-50 column (7 × 1 cm) equilibrated with the above buffer. After thorough washing with the same buffer, the enzyme was eluted with 0.05 M Tris-HCl/0.5 M NaCl, pH 7.4. 0.5-ml fractions were collected and the enzyme was located by the method of Baum et al. [11] which depends upon the activation of the substrate-modified enzyme by pyrophosphate. The fractions containing the modified sulphatase A were combined, the protein concentration determined spectrophotometrically ($A^{1\%}_{280nm}$ = 7.0; see below), and then stored at −4°C.

Fluorimetric determination of protein. When the concentrations of preparations of substrate-modified sulphatase A were too low to be determined spectrophotometrically, they were obtained by the method of Benson and Hare [12]. To 0.5 ml of a solution of sulphatase A (15—20 μg/ml) in 1 M NaCl were added 0.5 ml *o*-phthaladehyde reagent [12] and 1 min after mixing the resulting fluorescence was measured in an Aminco-Bowman spectrofluorimeter (λ_{ex} = 340 nm; λ_{em} = 455 nm). A standard emission value for native sulphatase A compared to bovine serum albumin was determined, and secondary standards of the latter were run simultaneously with each set of determinations. It was assumed that native and substrate-modified sulphatase A gave derivatives having the same fluorescence.

Synthesis of nitrocatechol [^{35}S]sulphate. Nitrocatechol [^{35}S]sulphate was prepared by the method of Flynn et al. [13]. To 1.5 g 4-nitrocatechol in 3.2 ml CS_2 and 3.7 ml *N,N*-dimethylaniline were added 5 mCi chloro[^{35}S]sulphonic acid (Radiochemical Centre, Amersham) mixed with 0.7 ml of redistilled chlorosulphonic acid. The reaction mixture was worked up in the usual way [13] to give approx. 0.7 g nitrocatechol [^{35}S]sulphate with a specific activity of about 0.1 Ci/mol. The purity of the product was verified spectrophotometrically.

Measurement of radioactivity. Radioactivity (^{35}S) was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer, model 3255, using either Bray's scintillation fluid or a mixture of xylene and Triton X-114 (8.3 : 5, v/v) containing 0.5% 2,5-diphenyloxazole (PPO).

Equilibrium ultracentrifugation. This was carried out by the method of Yphantis [14], as previously described [15], in a Spinco Model E ultracentrifuge using an An-D rotor. The conditions were as follows. At pH 7.5 and 5°C, 18 000 rev./min for 27 h; at pH 5.0 and 5°C, 10 000 rev./min for 33 h; at pH 5.0 and 20°C, 10 000 rev./min for 23 h. Molecular weights were computed by the standard method, assuming that the $\bar{v}$ of substrate-modified sulphatase A was the same as that determined for native sulphatase A, 0.715 [15].

Results

Isolation of substrate-modified sulphatase A

The method described above allowed the isolation of substrate-modified sulphatase A in mg quantities, the usual yield being about 80% in terms of protein and the final solution having a concentration of about 0.7 mg/ml. Typically, such preparations contained about 90% of substrate-modified sulphatase A, similar to those previously described [4].

When such a preparation was incubated for a second time with nitrocatechol sulphate, as described above, and the enzyme again isolated then the amount of substrate-modified sulphatase in the latter rose to about 97%. As little was to be gained by the use of such highly modified preparations, they were not routinely prepared and samples containing about 90% of substrate-modified sulphatase A were used in the experiments described below.

Stability of substrate-modified sulphatase A

The modified enzyme was apparently stable indefinitely when stored frozen or at temperatures close to 0°C. At 37°C and pH 7.4, it was much less stable and reverted to what was apparently native sulphatase A in a reaction having a $t_{½}$ of about 5 h. At 20°C and pH 7.4, the $t_{½}$ was about 60 h, and at pH 5.0 and 37°C it was about 30 h. From the rates of the reaction at 20 and 37°C, the activation energy for the reversion of substrate-modified sulphatase A to native sulphatase A at pH 7.4, was calculated to be about 6 kJ · mol^{-1}.

At pH 5.6, the optimum pH for the arylsulphatase reaction, the addition of 3 mM K_2SO_4 made little difference to the rate of reversion to the native enzyme but the addition of 3 mM 4-nitrocatechol did increase the rate, as shown in Table I. This increase did not occur when both K_2SO_4 and 4-nitrocatechol were present.

Activation of substrate-modified sulphatase A

Substrate-modified sulphatase A is activated by SO_4^{2-} in the presence of nitrocatechol sulphate [2—4,6] but nothing is known of the mechanism of this reaction, or of the nature of the activated enzyme. That the latter is not yet a third stable form of sulphatase A was shown as follows.

TABLE I

REVERSION OF SUBSTRATE-MODIFIED TO NATIVE SULPHATASE A

The substrate-modified enzyme (0.1 mg/ml) was incubated in 0.125 mM sodium acetate 0.125 mM NaCl, pH 5.6, at 37°C with the addition of K_2SO_4 or 4-nitrocatechol as required. At the appropriate time, the amount of native enzyme was determined by measuring v_0, as described in the text. The substrate-modified enzyme initially contained 13% of native enzyme.

Addition	Native enzyme (%)	
	3 h	6 h
None	13	15
K_2SO_4 (3 mM)	17	20
4-Nitrocatechol (3 mM)	24	36
K_2SO_4 + nitrocatechol	18	18

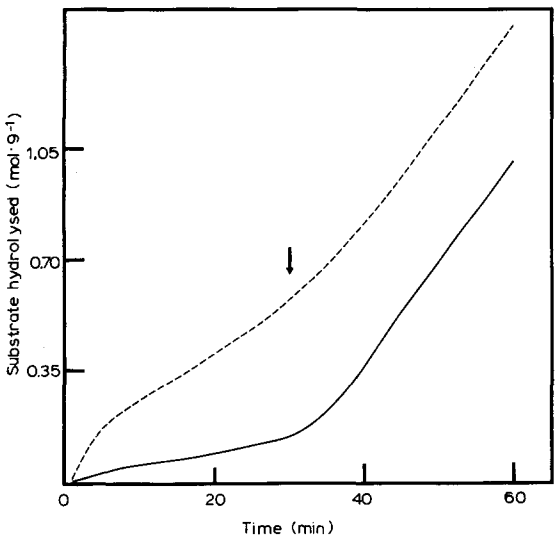

Fig. 1. Progress curves for the hydrolysis of 3 mM nitrocatechol sulphate by a standard preparation of substrate-modified sulphatase A (———) and by the same sample which had been activated by SO_4^{2-} and reisolated (- - - - -). In both cases 30 μmol SO_4^{2-} were added to the reaction mixtures at 30 min.

50 ml of a reaction mixture containing 25 μg of substrate-modified sulphatase A in 3 mM nitrocatechol sulphate and 0.1 M KCl were incubated in the pH-stat for 30 min at pH 5.6 and then made 3 mM in K_2SO_4 before incubating for a further 30 min. At the end of this time the enzyme was isolated in the usual way by chromatography on Sephadex G-25 and DEAE-Sephadex. The concentration of protein, determined fluorimetrically, showed a recovery of 18 μg (72%) sulphatase A. Progress curves for the hydrolysis of nitrocatechol sulphate by the recovered and original preparations of substrate-modified sulphatase A are shown in Fig. 1. It is clear that the progress curve with the recovered enzyme is similar to that with the original preparation of substrate-modified sulphatase A and that there is no indication that the activation by SO_4^{2-} has led to the formation of a stable form of sulphatase A different from the latter. If such a stable form had been produced the progress curve of the recovered enzyme would have been expected to be linear from the beginning of the reaction and the velocity to be equal to that attained after the addition of SO_4^{2-} prior to the reisolation of the enzyme. Also, no further activation would have been expected following the addition of SO_4^{2-} to the reaction mixture containing the recovered enzyme. It is, however, also clear from Fig. 1 that there has been, as expected [4], some regeneration of native sulphatase A during the reaction resulting in a increase in the initial velocity associated with the recovered enzyme.

Physical properties of substrate-modified sulphatase A

Ultraviolet absorption. The concentration of a sample of substrate-modified sulphatase A in 0.1 M Tris-HCl buffer, pH 7.5, was determined by differential refractometry, assuming a specific refractive increment of 0.184 ml · g^{-1}. Mea-

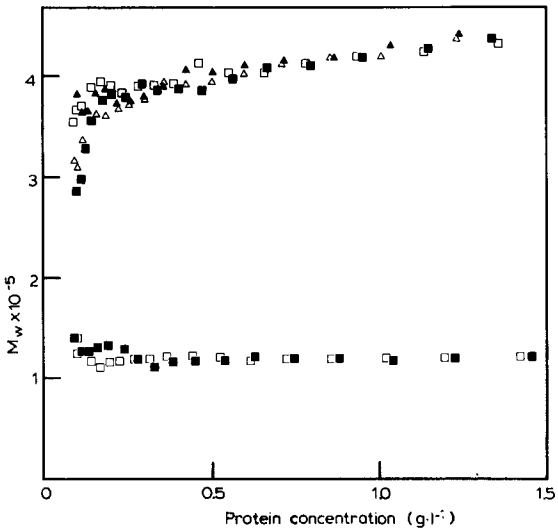

Fig. 2. Point-average values of the weight-average molecular weights of native and substrate-modified sulphatase A as a function of the total protein concentration during equilibrium ultracentrifugation at 5°C, $I = 0.1$. □,■; upper curve, substrate-modified sulphatase A after centrifugation for 31 and 33 h, respectively, at pH 5.0; lower curve, substrate-modified sulphatase A after 25 and 27 h centrifugation at pH 7.5. △,▲; native sulphatase A after centrifugation at pH 5.0 for 31 and 33 h.

surements in a Zeiss PMQ3 Spectrophotometer gave a value of 7.0 for $A^{1\%}_{280nm}$, the same as that for native sulphatase A [15].

There was no obvious difference between the ultraviolet absorption spectra of the native and substrate-modified forms of sulphatase A.

Molecular weight. In diethylbarbiturate buffer, pH 7.5, $\mu = 0.1$, at 5°C, substrate-modified sulphatase A had a molecular weight, determined by the Yphantis method [14], of 119 000 (range 116 000—121 000 in 4 measurements). This is within the range of values previously reported for native sulphatase A [15].

At high protein concentrations in acetate buffer, pH 5.0, $\mu = 0.1$, and at 5°C, substrate-modified sulphatase A existed predominantly as a tetramer but, as shown in Fig. 2, the tetramer dissociated at low protein concentrations. Native sulphatase A showed identical behaviour when examined under the same conditions (Fig. 2). In previous studies [15], no dissociation of the tetramer of native sulphatase A was noted during ultracentrifugation at pH 5.0 and 20°C. The latter experiments were therefore repeated, using the same sample of sulphatase A as was used to obtain the data in Fig. 2. No dissociation of the tetramer of sulphatase A occurred under these conditions, confirming its greater stability at higher temperatures. Its molecular weight was 448 000 which, as can be seen from Fig. 2, is the same as those of the tetrameric forms of both native and modified sulphatase A at pH 5.0 and 5°C.

^{35}S-Substrate-modified sulphatase A

Modification with nitrocatechol [^{35}S]sulphate. In these experiments, the substrate-modified enzyme was isolated by exactly the same procedure as

TABLE II

$^{35}SO_4^{2-}$ IN SUBSTRATE-MODIFIED SULPHATASE A

The value in the final column is obtained by assuming a nonspecific binding of 0.16 mol SO_4^{2-} per mol of total enzyme (see text).

Preparation	Fraction modified	$^{35}SO_4^{2-}$ per mol of		
		Total enzyme	Modified enzyme	Corrected
1	0.84	0.95	1.13	0.94
2	0.87	1.15	1.32	1.14
3	0.91	1.31	1.44	1.26
4	0.94	0.89	0.98	0.78

before, except that the substrate was nitrocatechol [^{35}S]sulphate. The protein concentration of the preparation was determined spectrophotometrically, the fraction of modified enzyme was obtained from measurements of v_0, as described above, and the amount of $^{35}SO_4^{2-}$ present was measured. The results, with four independent preparations of the substrate-modified enzyme, are shown in Table II. It is clear that approximately 1 mol of $^{35}SO_4^{2-}$ is associated with each mol of substrate-modified sulphatase A.

In control experiments, sulphatase A was incubated with $^{35}SO_4^{2-}$ under the conditions used for the preparation of the substrate-modified enzyme, except that no substrate was present. The concentration of SO_4^{2-} was 0.02 M, approximately that produced by the hydrolysis of nitrocatechol sulphate during the preparation of the substrate-modified enzyme. After incubation with $^{35}SO_4^{2-}$, the enzyme was recovered and examined as before. In two separate experiments the amounts of SO_4^{2-} bound to the native enzyme were 0.12 and 0.21 mol/mol, respectively.

Properties of ^{35}S-substrate-modified sulphatase A. A sample of ^{35}S-substrate-modified sulphatase A was activated by SO_4^{2-} during its reaction with nitrocatechol sulphate as has already been described for the unlabelled enzyme. As before, this treatment gave some reversion to the native enzyme and this was

TABLE III

$^{35}SO_4^{2-}$ IN SUBSTRATE-MODIFIED SULPHATASE A AFTER VARIOUS TREATMENTS

Details of the various treatments are given in the text. In each case, values are given for two independent experiments and are not corrected for any nonspecific binding of $^{35}SO_4^{2-}$.

Treatment	Fraction modified	$^{35}SO_4^{2-}$ per mol modified enzyme
Activated by SO_4^{2-}	0.61	1.10
	0.77	1.54
Incubated at 37°C	0.57	0.99
	0.52	1.07
Dialysed against SO_4^{2-}	0.83	0.0
	0.86	0.0

accompanied by a loss of $^{35}SO_4^{2-}$ so that the reisolated enzyme still contained 1 mol $^{35}SO_4^{2-}$ per mol of substrate-modified enzyme, as shown in Table III.

A further sample of the ^{35}S-substrate-modified enzyme was incubated at pH 7.5 and 37°C for 5 h and the enzyme recovered by chromatography on Sephadex G-25. This caused a considerable reversion to the native enzyme which was accompanied by a loss of $^{35}SO_4^{2-}$ so that the amount in the residual modified enzyme remained at 1 mol/mol (Table III).

A sample of [^{35}S]substrate-modified sulphatase A was dialysed for 3 days at 5°C against several changes of 0.1 M Tris-HCl, pH 7.4, containing 0.01 M K_2SO_4 and then against repeated changes of the buffer alone to remove K_2SO_4. Such treatment caused little change in the amount of substrate-modified enzyme present but there was a complete loss of $^{35}SO_4^{2-}$, indicating a facile exchange between SO_4^{2-} in solution and $^{35}SO_4^{2-}$ bound to the substrate-modified enzyme.

Discussion

The method for preparing substrate-modified sulphatase A requires little comment. It is similar to that previously described [4] except for the second chromatographic step, adsorption on DEAE-Sephadex, which allows an easy concentration of the eluate from the Sephadex G-25 column so that the present method is more easily adapted for the preparation of large amounts of the substrate-modified enzyme. The yield is about 80% and the preparations contain about 90% of substrate-modified enzyme. In accord with the demonstration [4] that there is an equilibrium between the native and substrate-modified forms of sulphatase A when these coexist with the substrate and the reaction products, it has been shown that the amount of residual native enzyme in the standard preparation of substrate-modified enzyme can be reduced to 2—3% by repeating the preparative procedure. Presumably even more highly-modified preparations could be obtained by further repetitions.

Attempts have been made to remove SO_4^{2-} from the reaction mixture, and so drive the equilibrium in favour of the modified enzyme [4], by having Ba^{2+} present. Such experiments have not been satisfactory because the very powerful adsorption of the enzyme to the precipitated $BaSO_4$ meant that the recovery of the modified enzyme was vanishingly small.

The reconversion of substrate-modified sulphatase A to native sulphatase A which occurs on incubation of the former at temperatures much above zero is not only of theoretical interest but also of practical importance. It means that investigations of the physical properties of the modified enzyme must be carried out at temperatures close to zero to prevent changes in the proportions of native and modified forms during the measurements. As has been shown in Table I, SO_4^{2-} has no effect on the reversion to native enzyme while 4-nitrocatechol stimulates it slightly.

The physical properties of the substrate-modified enzyme so far examined are not different from those of the native enzyme. Modification is not accompanied by any change in the molecular weight, the ability to form a tetramer at pH 5 or in the ultraviolet spectrum.

The dissociation of the tetrameric forms of both native and substrate-

modified sulphatase A which was obvious (Fig. 2) during equilibrium ultracentrifugation at pH 5 and 5°C was unexpected and would not have been predicted from the apparent association constant for tetramerisation of the native enzyme, $3 \cdot 10^9\ l^3 \cdot g^{-3}$, found for this system by chromatographic methods [16]. The apparent association constant pertaining to the system in the ultracentrifuge was computed by the method of Milthorpe et al. [17] to be $0.5 \cdot 10^6\ l^3 \cdot g^{-3}$, much less than that previously reported [16]. This value gives a curve which fits the experimental points in Fig. 2 despite the fact that the method is not well suited to the investigation of the sulphatase A system where the degree of dissociation of the tetramer is slight and the extrapolation to $\Omega_0(r)$ is hazardous [17]. The reason for the discrepancy between the present results from equilibrium ultracentrifugation and the previous ones from chromatography [16] is not obvious but may lie in the very different concentration ranges required for the two techniques, 0.1—1 $g \cdot l^{-1}$ in the ultracentrifuge and 0.5—50 $mg \cdot l^{-1}$ in chromatography. The apparent association constant for the latter was computed on the assumption that the system was of a monomer and tetramer; this simplification [15,16] may become increasingly invalid at higher protein concentrations and unfortunately the method of Milthorpe et al. [17], which does not require such a simplification, cannot properly handle the present system. More detailed studies are required, but for the present purpose the conclusion that there is no obvious difference between the association behaviour of native and substrate-modified sulphatase A is justified.

The most important finding in the present work is that [^{35}S]substrate-modified sulphatase A contains 1 mol $^{35}SO_4^{2-}$ per mol enzyme. Strictly speaking, all that has been shown is that the enzyme contains one atom ^{35}S per mol enzyme, but it is most unlikely that the very stable sulphate group could have been altered under the experimental conditions used for the preparation of the modified enzyme. Although the enzyme-$^{35}SO_4^{2-}$ link is stable during the preparation, the bound $^{35}SO_4^{2-}$ is lost when the modified enzyme reverts to the native enzyme, and also by dialysis of the modified enzyme against SO_4^{2-}.

Nothing is known of the nature of the bound sulphate in the modified enzyme. Its most likely form would be a sulphate ester or related compound, for example, a sulphate ester ($R \cdot OSO_3^-$) of tyrosine, serine or threonine; a sulphamate ($R \cdot NH \cdot SO_3^-$) of an amino group or a ring nitrogen in histidine; or a thiosulphate ($R \cdot SSO_3^-$) of cysteine. Perhaps pertinent is the observation that the Arrhenius activation energy for the reversion of substrate-modified sulphatase A to native sulphatase A, that is, for the removal of the bound SO_4^{2-}, is approximately 6 $kJ \cdot mol^{-1}$. A similar value was given by Lee and Van Etten [6] for the reversion of the substrate-modified sulphatase A of rabbit liver, and it is therefore interesting that the activation energies for the acid-catalysed hydrolysis of sulphate esters range from 5.7—6.5 $kJ \cdot mol^{-1}$ [18]. On the other hand, attempts to prepare 'substrate-modified' sulphatase A by sulphation of the native enzyme, in aqueous solution, with pyridine-sulphur trioxide have not been successful; such treatment inactivates sulphatase A, but there is nothing to suggest that this is due to the formation of a 'substrate-modified' enzyme because it is not reactivated by SO_4^{2-}.

Further information on the nature of the bound sulphate will be difficult to obtain because the modified enzyme must be kept at temperatures close to

zero to prevent its reversion to native sulphatase A. This requirement effectively prevents the use of most of the chemical techniques available for the investigation of protein structure.

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