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CATALYTIC AND IMMUNOCHEMICAL PROPERTIES OF ARYLSULPHATASE A FROM URINE, MODIFIED BY POTASSIUM FERRATE

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Arylsulphatase A (EC 3.1.6.1.) from urine was inactivated with potassium ferrate, a strong oxidizing agent. The inhibition could be prevented by competitive inhibitors, tetraborate and orthophosphate. Tetraborate which was shown to be a powerful competitive inhibitor (determined $K_I = 4 \times 10^{-5}$ M) gave more efficient protection. The partially inactivated enzyme exhibited a K_m value similar to that of the unmodified arylsulphatase A, and its V_{max} decreased in proportion to the loss of enzymatic activity. The partially modified enzyme did not lose its ability to catalyse hydrolysis of *p*-nitrocatechol sulphate according to the "anomalous kinetics" exhibited towards this substrate and characteristic for arylsulphatase A. The immunochemical properties of arylsulphatase A either fully or partially inactivated were similar to those of the native enzyme. The results allow to conclude that ferrate reacts with arylsulphatase A in its active site. Thus ferrate seems to be a very sensitive probe for amino acid residues essential for catalytic activity of arylsulphatase A.

Lee and Van Etten [1] have shown that a histidine residue is essential for catalytic activity of arylsulphatase A from rabbit liver and that competitive inhibitor, orthophosphate, partially preserved the enzyme against modification of its histidine residue by diethyl pyrocarbonate [2].

Potassium ferrate, a potent oxidizing agent, a structural analogue of orthophosphate which modifies irreversibly, among other residues, also histidine was often used to identify amino acids present in active sites of enzymes which interact with phosphate compounds as competitive inhibitors and/or substrates [3, 4].

Theretore, to find a tool for further determination of amino acid residues present in the active site of arylsulphatase A and/or involved in its catalytic activity we attempted to modify the enzyme with potassium ferrate, and compare catalytic and immunochemical properties of the modified and native enzyme. The studies were carried out on arylsulphatase A from urine which was isolated and characterized as described recently [5].

MATERIALS AND METHODS

Materials. Arylsulphatase A was isolated as described previously [5]. *p*-Nitrocatechol sulphate was purchased from Sigma Chemical Company and diethyl pyrocarbonate from Fluka AG. Potassium ferrate was synthesized according to the method of Thompson *et al.* [6]. All other chemicals were reagent grade.

Enzyme assays. Enzymatic activity of arylsulphatase A towards *p*-nitrocatechol sulphate was determined at 37°C in 0.5 M sodium acetate buffer, pH 5.5, as follows: 50 µl of enzyme solution was added to 250 µl of 5 mM substrate solution in the buffer and the mixture was left for 5 min in a water bath. The reaction was stopped by adding 1 cm^3 of 1 M NaOH. The amount of *p*-nitrocatechol liberated was measured spectrophotometrically at 515 nm and enzyme activity was calculated using molar absorbance value $\varepsilon^{515}_{p\text{-NC}} = 12\,600\ M^{-1}$.

Modification of arylsulphatase A with potassium ferrate (K_2FeO_4) was carried out as described by Steczko *et al.* [7]. Increasing known amounts of potassium ferrate were added to the samples of known amounts of enzyme (10 - 45 µg/cm^3) previously dialysed against the buffer of pH 5.5 or 7.0. The mixtures of ferrate and the enzyme were left for 5 min at room temperature and then the enzymatic activity of arylsulphatase A was determined in every sample. Potassium ferrate solutions were always prepared immediately before the experiment and were kept in an icebath until use.

Catalytic properties of arylsulphatase A, inhibition of its enzymatic activity by tetraborate ($B_4O_7^{2-}$) and the effect of ferrate modification on kinetic parameters K_m and V_{max} were determined by the methods generally used in studies of enzyme kinetics. The "anomalous kinetic" behaviour of arylsulphatase A during hydrolysis of *p*-nitrocatechol sulphate was observed both for the ferrate-modified and unmodified enzyme by the method of Waheed & Van Etten [8].

Double immunodiffusion of ferrate-modified and native arylsulphatase A was carried out in 1% agar gel [9].

Labelling of arylsulphatase A with $Na^{125}I$ and radioimmunological analysis of native arylsulphatase A and the enzyme modified under different conditions with

ferrate were based on the radioimmunoassay test developed by Laidler *et al.* [10] and Laidler *et al.* (in press).

Modification of arylsulphatase A with diethyl pyrocarbonate was carried out by the generally used methods [2, 11].

RESULTS AND DISCUSSION

Ferrate ion (FeO_4^{2-}), a structural analogue of orthophosphate and a potent oxidizing agent was used to inactivate arylsulphatase A (EC 3.1.6.1) from urine. The inhibition was pH-dependent: the inactivation was more efficient at pH 7.0 than at pH 5.5, e.g. a 50% loss of enzymatic activity at pH 7.0 was observed for about ten times lower concentration of ferrate than at pH 5.5 (Fig. 1). Inhibition

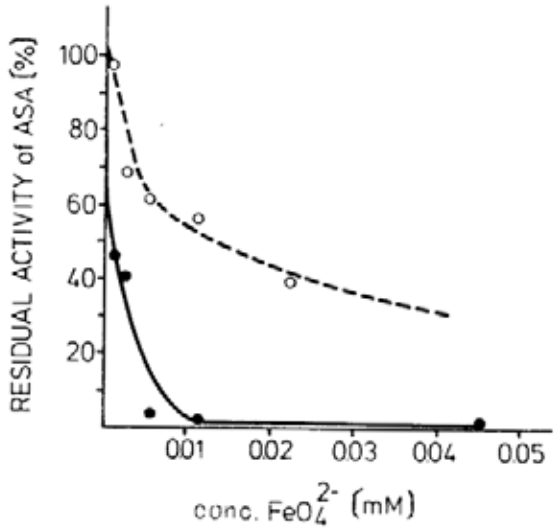

Fig. 1. The effect of ferrate on enzymatic activity of arylsulphatase A. pH 5.5 (○); pH 7.0 (●); enzyme concentration 8.3×10^{-2} μM.

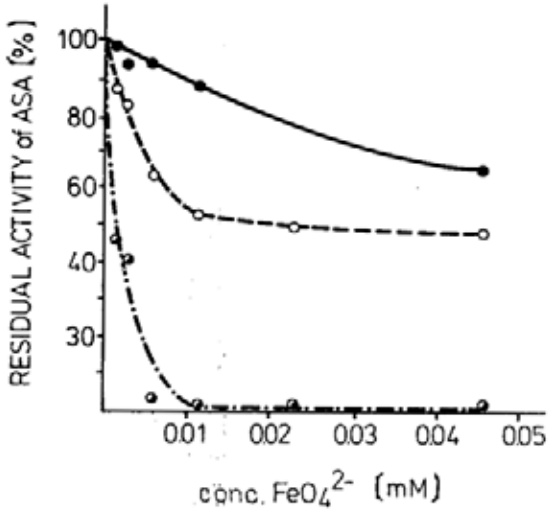

Fig. 2. Effect of competitive inhibitors of arylsulphatase A on inactivation of the enzyme by ferrate; without inhibitors (◑); 10 mM orthophosphate (○); 1 mM tetraborate (●); enzyme concentration 8.3×10^{-2} μM.

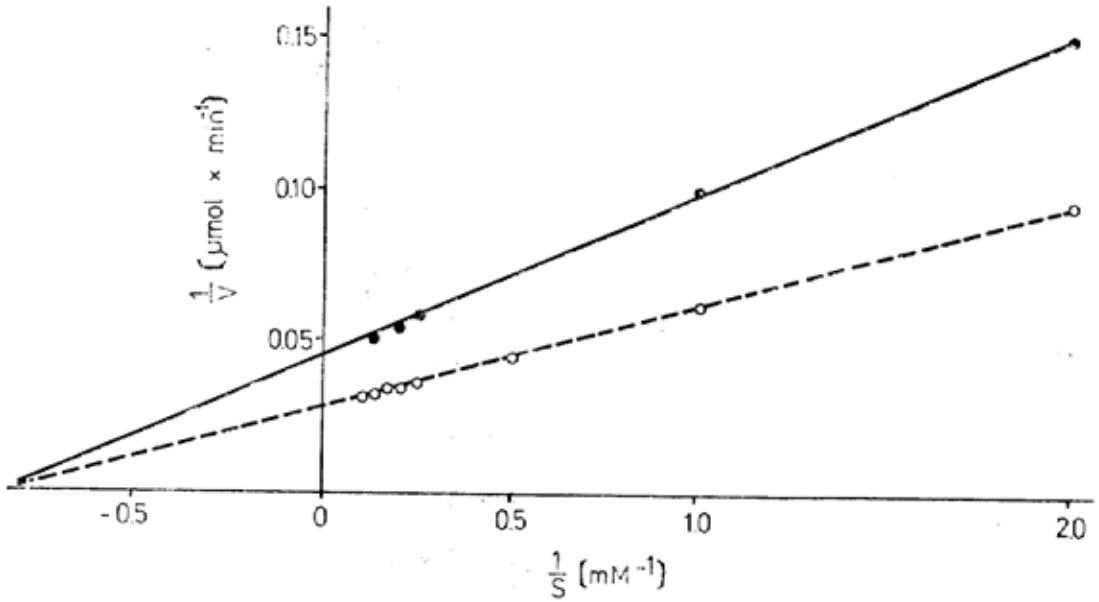

Fig. 3. Effect of ferrate-modification on K_m and V_{max} of arylsulphatase A; native enzyme, pH 5.5, enzyme concn. 6.6×10^{-3} μM, $K_m=1.20$ mM, $V_{max}=36.3$ μmol×min^{-1} (○); ferrate-modified enzyme pH 5.5, 8.5×10^{-3} μM, $K_m=1.24$ mM, $V_{max}=23.4$ μmol×min^{-1} (●) Modification of arylsulphatase A was carried out so as to get 50% inactivation of its enzymatic activity. Such a modified enzyme, 8.5×10^{-3} μM, exhibited $V_{max}=23.4$ μmol×min^{-1} which corresponds to $V_{max}=46.8$ for the native enzyme of the same concentration.

of arylsulphatase A by ferrate was prevented by competitive inhibitors which indicates that the action of ferrate is site-specific. At pH 7.0 the protective effect of orthophosphate was weaker than that of tetraborate-$B_4O_7^{2-}$ (Fig. 2). These results were supported by the finding that tetraborate is a fairly strong competitive inhibitor of arylsulphatase A. The calculated $K_i = 4 \times 10^{-5}$ M for tetraborate is in fact much lower than for orthophosphate: $K_i = 3 \times 10^{-4}$ M.

The partially inactivated enzyme (50%, pH 7.0) exhibited a K_m similar to that of the native enzyme and its V_{max} decreased with the loss of the enzymatic activity (Fig. 3). At the same time the enzyme partially modified by ferrate did not lose its ability to catalyse hydrolysis of *p*-nitrocatechol sulphate according to "anomalous kinetics" characteristic with this substrate (Fig. 4). These results suggest that those molecules of arylsulphatase A which had reacted with ferrate ion lost entirely their catalytic properties.

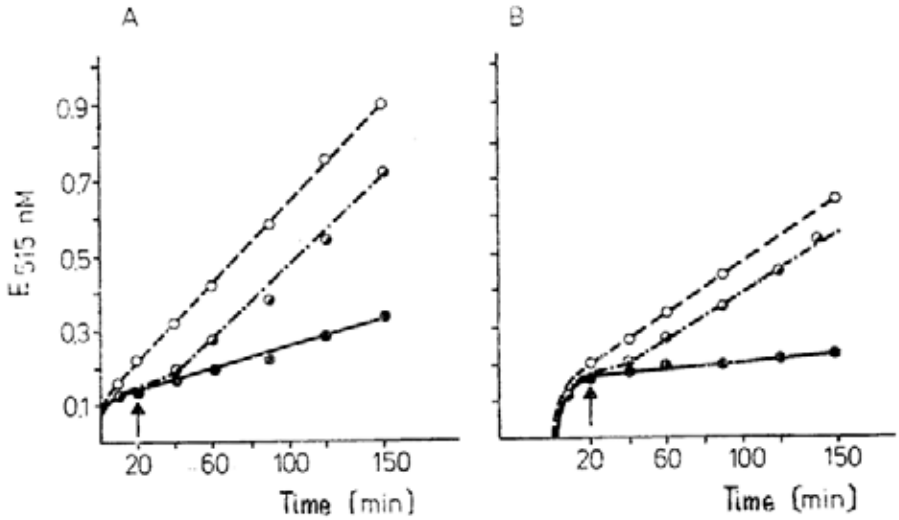

Fig. 4. Progress curves for the hydrolysis of 4 mM *p*-nitrocatechol sulphate by native and ferrate-modified arylsulphatase A. Reactions were carried out at 37°C in 0.5 M sodium acetate, pH 5.5, as follows: enzyme+substrate (●); enzyme+substrate+5 mM SO_4^{2-} added after 20 min of incubation (◑); enzyme+substrate+5 mM SO_4^{2-} present in the mixture since the beginning of incubation (○); A. native enzyme, 3×10^{-3} μM; B. ferrate-modified enzyme (50% inactivation), 8×10^{-3} μM.

Neither partial nor total inactivation of arylsulphatase A by ferrate at pH 5.5 or 7.0 led to observable changes in antigenic properties of the enzyme. This was demonstrated for arylsulphatase A modified under different conditions when compared with native enzyme on double immunodiffusion in 1% agar gel (Plate 1). The same results were obtained when the radioimmunoassay, a test more sensitive for detection of slight antigenic differences, was used (Fig. 5).

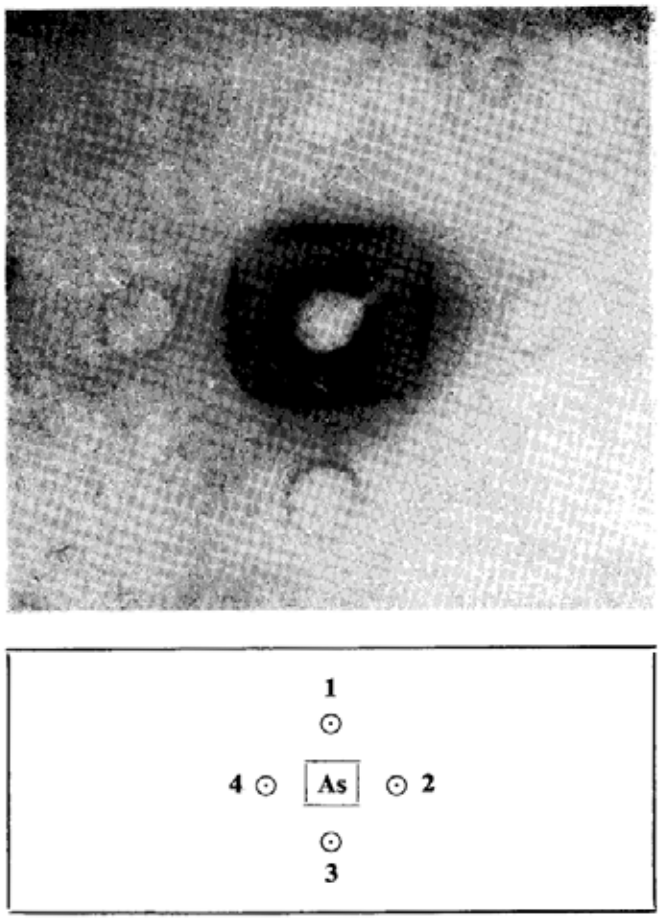

Plate 1. Immunodiffusion of arylsulphatase A from urine. As - rabbit antiserum against arylsulphatase A. Wells 1 and 3 contained each 0.7 μg of the enzyme modified by ferrate at pH 7.0 (96% inactivation). Wells 2 and 4 contained each 0.7 μg of native enzyme.

The lack of appreciable changes in immunochemical properties of arylsulphatase A despite full inhibition of its enzymatic activity even at pH 5.5 where a rather high concentration of potassium ferrate is necessary for total inactivation of the enzyme, supports the idea that ferrate reacts specifically with arylsulphatase A in its active site. It may be particularly the case at pH 7.0 where fairly low concentration of ferrate is used.

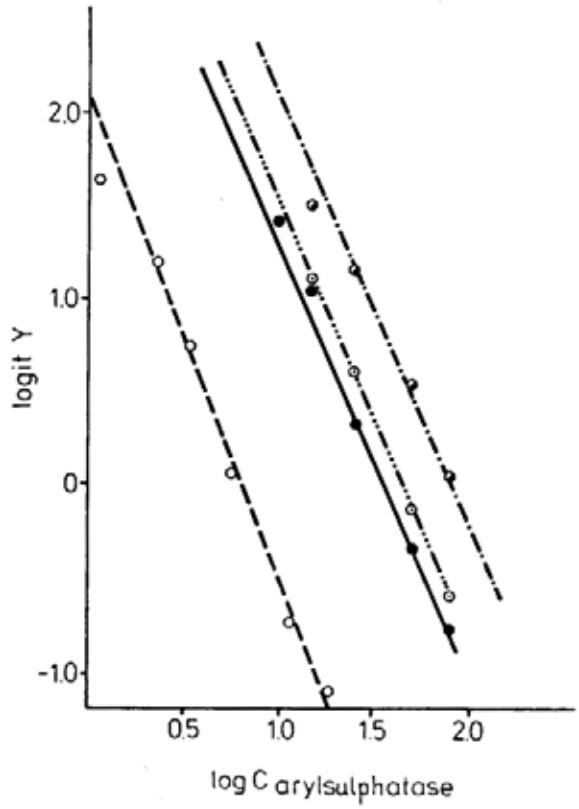

Fig. 5. Radioimmunological comparison of antigenic properties of native and ferrate-modified arylsulphatase A; native arylsulphatase A treated as standard (○); logit $Y = -2.43 \times \log$ enzyme concn. $+1.91$, $r = 0.995$; ferrate-modified arylsulphatase A, pH 5.5 (○), (50% inactivation); logit $Y = -2.34 \times \log$ enzyme concn. $+3.87$, $r = 0.9996$; ferrate-modified arylsulphatase A at pH 5.5 (95% inactivation) (●); logit $Y = -2.02 \times \log$ enzyme concn. $+3.93$, $r = 0.996$; ferrate-modified arylsulphatase A at pH 7.0 (93% inactivation) (◑); logit $Y = -2.50 \times \log$ enzyme concn. $+3.92$, $r = 0.998$. The slopes of these curves did not differ significantly from each other as tested by Student's t - test for parallelism.

Histidine is one of the amino acid residues which are able to react with ferrate [7], Steczko (in press). In the case of arylsulphatase A the involvement of histidine seems to be likely enough in the light of data of Lee and Van Etten [2] for arylsul-

phatase A from rabbit liver and of the observed loss of enzymatic activity of arylsulphatase A from urine following its reaction with diethyl pyrocarbonate (Fig. 6).

Thus arylsulphatase A from urine was found to be quite sensitive to the action of ferrate. The enzyme was almost entirely inactivated by ferrate at pH 7.0 at the ferrate-to-enzyme molar ratio of about 70:1. This ratio was not as low as in the case of some phosphatases but still much lower than for e.g. triose phosphate isomerase [12] or muscle adenylate kinase [13] where site-specific action of ferrate is still observed.

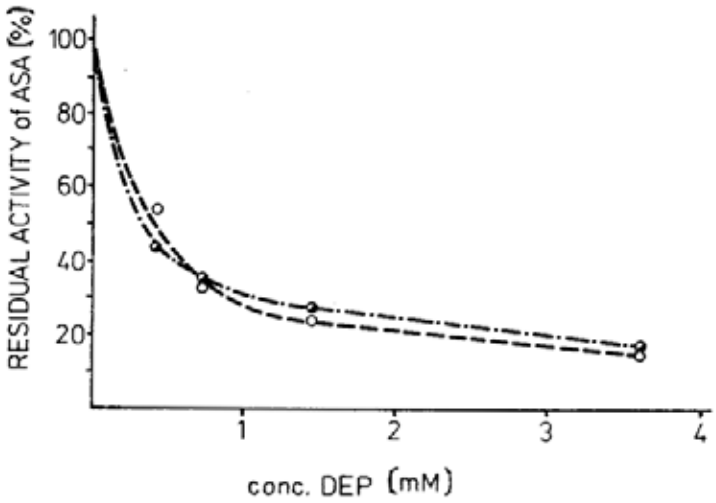

Fig. 6. The inactivation of arylsulphatase A with diethyl pyrocarbonate (DEP). The reactions were carried out for 15 min at room temperature, enzyme concn. 7.5×10^{-2} μM; pH 5.5 (◑); pH 7.0 (○).

Arylsulphatase A sensitivity to ferrate, enzyme protection against ferrate by competitive inhibitors, the effect of ferrate modification on K_m and V_{max} of arylsulphatase together with the absence of any detectable changes in the enzyme immunochemical properties indicate unequivocally that, at least at pH 7.0, ferrate is an active-site specific agent.

This allows to undertake more detailed studies to identify the amino acid residues present in the active site of arylsulphatase A and possibly involved in catalytic activity of this enzyme.

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