

The Regulation of Rat Liver Xanthine Oxidase

INVOLVEMENT OF THIOL GROUPS IN THE CONVERSION OF THE ENZYME ACTIVITY FROM DEHYDROGENASE (TYPE D) INTO OXIDASE (TYPE O) AND PURIFICATION OF THE ENZYME

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1. The 'xanthine oxidase' activity of rat liver supernatant, most of which behaves as an NAD^+ -dependent dehydrogenase (type D) can be rapidly converted into an oxidase (type O) by thiol reagents such as tetraethylthiuram disulphide, copper sulphate, 5,5'-dithiobis-(2-nitrobenzoic acid), *N*-ethylmaleimide and *p*-hydroxymercuribenzoate. Treatment with copper sulphate, if prolonged, leads to almost complete inactivation of the enzyme. The effect of these reagents is prevented by dithioerythritol, and in all cases but that of *N*-ethylmaleimide is reversed by the same thiol. 2. Dithioerythritol prevents and reverses the conversion of xanthine oxidase from type D into type O brought about by storage of rat liver supernatant at -20°C , preincubation under anaerobic conditions, treatment with carbon or with diethyl ether, and reverses, but does not prevent, the conversion obtained by preincubation of the whole liver homogenate. 3. Conversion of the enzyme from type D into type O is effected by preincubation of rat liver supernatant with the sedimentable fraction from rat liver but not from chick or pigeon liver. The xanthine dehydrogenase activity of chick liver supernatant is not changed into an oxidase by preincubation with the sedimentable fraction from rat liver. 4. The enzyme activity of rat liver supernatant is converted from type D into type O during purification of the enzyme: the purified enzyme can be reconverted into type D by dithioerythritol. 5. The enzyme appears as an oxidase in the supernatant of rat heart, intestine, spleen, pancreas, lung and kidney. The enzyme of all organs but intestine can be converted into a dehydrogenase by dithioerythritol.

It has been reported that most of the activity of the xanthine oxidase (xanthine-oxygen oxidoreductase, EC 1.2.3.2) of rat liver supernatant appears as a NAD^+ -dependent dehydrogenase (called type D) if the supernatant is prepared with a minimum of manipulation and is assayed promptly. The activity is converted into an oxidase (type O) by a variety of treatments of the supernatant, such as storage at -20°C (Della Corte & Stirpe, 1968*a*), treatment with proteolytic enzymes (Della Corte & Stirpe, 1968*b*), preincubation before assay with particulate sub-cellular fractions or under anaerobic conditions, treatment with organic solvents or with carbon (Stirpe & Della Corte, 1969). Only the changes after preincubation under anaerobic conditions could be reversed by keeping the supernatant at 37°C in air. Similar changes were observed with the xanthine oxidase of human liver supernatant (Della Corte *et al.*, 1969), whereas in all other rat organs examined the enzyme activity appeared entirely as an oxidase (Stirpe & Della Corte, 1969).

These two activities were interpreted as being due to two interconvertible forms of xanthine oxidase. The experiments reported in the present paper were performed (i) to clarify the nature of the conversion of xanthine oxidase, (ii) to purify the enzyme in its D

form, and (iii) to ascertain the possible role of the two forms *in vivo*. The results demonstrate that the conversion of xanthine oxidase from type D into type O may be obtained with thiol reagents and may be reversed by thiols. The use of thiols allows the purification of xanthine oxidase in the D form.

Experimental

Chemicals

Xanthine (monosodium salt), NAD^+ , *p*-hydroxymercuribenzoate, dithioerythritol, *N*-ethylmaleimide, 5,5'-dithiobis-(2-nitrobenzoic acid) and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.; lactate dehydrogenase (from rabbit muscle) was from Boehringer und Soehne G.m.b.H., Mannheim, Germany; tetraethylthiuram disulphide (disulfiram) was from Fluka, Buchs, Switzerland.

Animals

Rats of both sexes (2–3 months old) were of the Wistar–Glaxo strain, bred in our Laboratory. Adult pigeons and day-old chicks were obtained commercially, and used after at least a week.

Table 1. *Modifications of xanthine oxidase by reagents of thiol groups, and the effect of EDTA and of dithioerythritol*

The enzyme activity was measured as described by Stirpe & Della Corte (1969). The reaction mixture contained, in a final volume of 3 ml: 0.1M-tris-HCl buffer, pH 8.1, 60 μ M-xanthine, 0.67 mM-NAD⁺ (when present) and 0.2 ml of rat liver supernatant. (Different preparations were used with each reagent). Xanthine was omitted from reference cuvettes. Formation of uric acid at 25°C was measured at 292 nm at 1 min intervals. Inhibitors, EDTA and dithioerythritol were mixed with supernatant at the concentration indicated; an equal amount of water was added to control samples. Disulfiram was dissolved in propylene glycol. Propylene glycol, EDTA and dithioerythritol had no effect on xanthine oxidase.

Reagent ...	Xanthine oxidase activity (milliunits/100mg of liver)											
	Disulfiram (1 mM)		CuSO ₄ (1 mM)		Dithiobisnitrobenzoic acid (1 mM)		N-Ethylmaleimide (1 mM)		p-Hydroxymercuribenzoate (1 mM)			
	O ₂	NAD ⁺ +O ₂	O ₂	NAD ⁺ +O ₂	O ₂	NAD ⁺ +O ₂	O ₂	NAD ⁺ +O ₂	O ₂	NAD ⁺ +O ₂	O ₂	NAD ⁺ +O ₂
None	7.4	33.7	7.4	36.0			2.9	23.4	8.2	30.5		
Reagent	33.1	29.4	39.3	32.1			7.8	22.9	7.8	20.7		
Assayed immediately	30.3	33.7	6.1	6.0			22.9*	27.8*	36.8*	39.3*		
After 30 min at 37°C												
EDTA (10 mM) then reagent												
Assayed immediately			8.6	38.7			8.2	24.0	7.4	33.8		
After 30 min at 37°C			10.5	38.7			20.0*	28.9*	30.3*	43.6*		
Reagent, then 10 mM-EDTA												
Assayed immediately			38.0	37.0								
After 30 min at 37°C	27.8	43.0	35.2	46.9								
Reagent for 30 min at 37°C then 10 mM-EDTA												
Assayed after 30 min at 37°C	22.9	29.9	29.0*	32.7			23.3**†	31.1**†	41.7**†	44.7**†		
None‡			6.6	22.3	6.1	30.5						
Reagent												
Assayed immediately			29.0	20.7	40.9	32.2						
After 30 min at 37°C			4.1	3.8	41.2	45.8						

Table 2. Effect of EDTA and of dithioerythritol on the modifications of xanthine oxidase activity caused by various treatments

Experimental conditions were as described in Table 1. Treatments were as follows: for trypsin the supernatant was preincubated at 37°C for 90 min with trypsin (100 µg/ml of supernatant) and 1 mM-xanthine, then it was dialysed and assayed; for preincubation of the whole homogenate the whole homogenate was preincubated at 37°C for 5 min, then the supernatant was prepared and assayed as described in the Experimental section, for carbon, supernatant was shaken for 5 min at 0°C with carbon (50 mg/ml of supernatant), then was filtered and assayed; for diethyl ether, supernatant was shaken with an equal volume of diethyl ether, which was separated by centrifugation and removed.

Treatment .. Additions and treatment	Xanthine oxidase activity (milliunits/100mg of liver)														
	Storage at -20°C for 24h			1 mM-Xanthine and trypsin		Anaerobiosis for 90 min at 37°C		Preincubation of the whole homogenate for 5 min at 37°C				Carbon		Diethyl ether	
	O ₂	NAD ⁺ +O ₂		O ₂	NAD ⁺ +O ₂	O ₂	NAD ⁺ +O ₂	O ₂	NAD ⁺ +O ₂	O ₂	NAD ⁺ +O ₂	O ₂	NAD ⁺ +O ₂	O ₂	NAD ⁺ +O ₂
None	8.2	39.2		8.2	39.2	8.2	39.2	5.7	32.6	8.2	26.2	8.2	26.2	8.2	26.2
Treated	46.6	41.4		30.3	39.3	35.9	42.5	45.0	43.5	22.9	28.3	31.1	33.8	31.1	33.8
EDTA (10 mM) before treatment	42.5	42.5		40.0	41.4	6.9	38.1	38.0	38.1			13.1	43.6	13.1	43.6
Treated, then 30 min at 37°C with 10 mM-EDTA	38.4	44.7		31.9	40.3	4.9	37.1	42.9*	43.3*	7.4†	29.4†	32.7†	38.2†	32.7†	38.2†
Treated, then 30 min anaerobiosis at 37°C with 10 mM-EDTA						6.9	37.1								
None†	8.3	45.0		8.3	45.0										
Treated	44.1	46.6		39.3	40.9										
Dithioerythritol (10 mM) before treatment	7.4	45.8		40.1	41.7	5.7	37.1	10.6	30.5			4.5	24.0		
Treated, then 10 min at 37°C with 10 mM-dithioerythritol	6.9	43.3		35.9	40.5	4.5	39.3	5.2*	31.0*	3.3‡	28.3‡	6.5†	30.5†	6.5†	30.5†

* Supernatant alone was kept 30 min at 37°C.

† 15 min instead of 30.

‡ When no values are given, the experiments were performed together and with the same enzyme as those of the series above.

fully active in the presence of O₂, and NAD⁺, when present, was not reduced). The effect of disulfiram was both prevented and reversed by 10mM-dithioerythritol but was not reversed by EDTA.

This unexpected effect of disulfiram and its reversibility by a thiol led us to suppose that the disulphide might act by reacting reversibly with a thiol group (or groups) of xanthine oxidase, as it has been observed with D-amino acid oxidase (Neims *et al.*, 1966). This possibility was investigated further by using other thiol reagents, some of which are known to inhibit milk xanthine oxidase (Bergel & Bray, 1956; Green & O'Brien, 1967) or chick liver xanthine dehydrogenase (Doisy *et al.*, 1955).

Copper sulphate, dithiobisnitrobenzoic acid, *N*-ethylmaleimide and *p*-hydroxymercuribenzoate brought about conversion of xanthine oxidase from type D into type O (Table 1) immediately (in the case of CuSO₄ and dithiobisnitrobenzoic acid) or after some time at 37°C (with the other reagents). Keeping the supernatant at 37°C in the presence of 1 mM-CuSO₄ caused an almost complete inhibition of enzyme activity. EDTA prevented, but did not reverse, the conversion caused by treatment with copper, but did not influence the effect of other treatments (it was not tested on dithiobisnitrobenzoic acid). EDTA reversed also the inhibition after preincubation of supernatant with copper sulphate, and the enzyme activity reappeared as type O. Dithioerythritol prevented the effects of the various reagents, and reversed them, except the conversion effected by *N*-ethylmaleimide. It also relieved the inhibition by copper sulphate, but in this case the enzyme activity reappeared as type D.

It should be mentioned that immediately upon the addition of some reagents (invariably with disulfiram, copper sulphate and dithiobisnitrobenzoic acid; sometimes with *p*-hydroxymercuribenzoate) the xanthine oxidase activity appeared to be converted into type O, as judged from the increased activity with O₂ alone; however, some NAD⁺, when present, was still reduced, so that the amount of uric acid formed in the presence of NAD⁺ did not stoichiometrically correspond to the sum of the amount of uric acid formed in the presence of O₂, plus the amount of NAD⁺ reduced. Very little NADH was formed, and the stoichiometry was within the limits of experimental error if the supernatant was preincubated at 37°C with the reagents. For instance, in the experiments with disulfiram (Table 1) the amount of NADH formed during the reaction was 15.4 milliunits/100 mg of liver immediately upon addition of the reagent, and 3.9 after 30 min at 37°C.

Effect of EDTA and of dithioerythritol on the conversion of xanthine oxidase from type D into type O

The effect of treatment with dithioerythritol upon the conversion of xanthine oxidase from type D into

Table 3. Purification of xanthine oxidase, and conversion of the purified enzyme from type O into type D

Step Assayed with ...	Volume (ml)	Enzyme concentration (milliunits/ml)			Total enzyme activity (milliunits)			Protein (mg/ml)	Specific enzyme activity (milliunits/mg of protein)			Yield* (%)	Relative purification*
		O ₂	NAD ⁺	O ₂ + NAD ⁺	O ₂	NAD ⁺	O ₂ + NAD ⁺		O ₂	NAD ⁺	O ₂ + NAD ⁺		
Supernatant	200	9.5	49	1900	40000	23	0.41	2.13	100	1			
First (NH ₄) ₂ SO ₄ fraction	25	229	371	5725	9275	44	5.2	8.43	23	3.9			
Hydroxyapatite eluate	23	180	191	4140	4393	2.1	85.7	90.9	11	42.7			
Second (NH ₄) ₂ SO ₄ fraction	6	665	560	3930	3360	4	163.7	140.0	8.4	65.7			
DEAE-cellulose eluate, pooled	25	53	45	1325	1125	0.076	697	592	2.8	278			
DEAE-cellulose eluate, most active fraction	5	33	33	165	165	0.029	1138	1138	534				
Preincubated with 10 mM-dithioerythritol for 20 min at 37°C													
DEAE-cellulose eluate, pooled		9.8	36				129	473.6					
DEAE-cellulose eluate, most active fraction		5.5	23.2				189.6	800					

*Calculated from the assays with NAD⁺ + O₂.

type O obtained with various treatments (Stirpe & Della Corte, 1969) was investigated, and the results are reported in Table 2. The conversion obtained by storage at -20°C was prevented and could be reversed by dithioerythritol, but was unaffected by EDTA. Both reagents prevented and could reverse the conversion from type D into type O obtained on preincubation of the supernatant under anaerobic conditions, but neither of these reagents influenced the conversion caused by trypsin. The conversion effected by preincubation of the whole homogenate was not affected by EDTA, but was partially prevented by and was completely reversed by dithioerythritol (Table 2). The xanthine oxidase activity of all organs examined except liver appeared to be entirely as an oxidase (Stirpe & Della Corte, 1969). This was confirmed, but it was observed that the activity of the supernatants from all organs but intestine could be converted into a dehydrogenase upon preincubation at 37°C with dithioerythritol. In almost all organs examined the addition of pyruvate and lactate dehydrogenase stimulated the dehydrogenase activity, thus indicating that the NADH formed inhibits the enzyme of these organs, as it does with the enzyme from the liver (Della Corte & Stirpe, 1970).

Experiments with liver from birds

The sedimentable fraction from rat liver homogenate contains a heat-labile factor that converts xanthine oxidase from type D into type O, and this conversion is reversed by thiols (Stirpe & Della Corte, 1970). This converting capacity is not present in the sediment prepared from chick, which contains xanthine dehydrogenase but not oxidase, or of pigeon liver, which is devoid of xanthine oxidase or dehydrogenase (Richert & Westerfeld, 1951). The xanthine dehydrogenase of chick liver supernatant was not converted into an oxidase by preincubation with the sedimentable fraction prepared from rat liver.

Purification of xanthine oxidase and conversion of the purified enzyme

The results described above were used to devise a procedure for the purification of the xanthine oxidase in its D form. Previous attempts to do this had failed, because of the rapid conversion of the enzyme into the O form at the very early stages of the purification. Rather than purify the enzyme in the presence of a thiol throughout the procedure to keep it in the D form, the purification was performed by allowing the xanthine oxidase to be converted into the O form, and then reconvert the purified enzyme into the D form. A summary of the purification is given in Table 3. The purified enzyme was entirely an oxidase, but

could be reconverted almost completely into a dehydrogenase by treatment at 37°C with 10mM-dithioerythritol, although at the expense of some loss of activity.

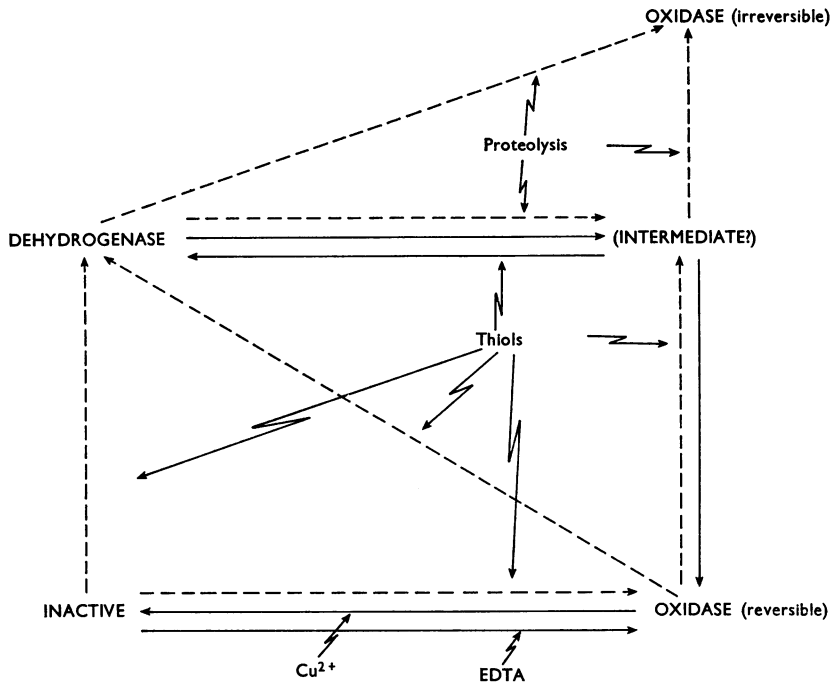
Discussion

On the basis of the effect of thiol and of reagents that react with thiol groups, the treatments converting xanthine oxidase from the D into the O form may be divided into two categories. The effects of treatments of category I are not affected by thiols. The only treatment of this type is incubation with trypsin (and presumably with other proteolytic enzymes). In category II are treatments with reagents containing thiol groups and all other treatments the effects of which are prevented and/or reversed by thiols. Thus, the mechanisms whereby a variety of apparently unrelated treatments convert xanthine oxidase into xanthine dehydrogenase may be restricted to two types, (I) proteolysis and (II) modification(s) of thiol groups. Some uncertainty may remain about the conversion after preincubation under anaerobic conditions which is reversed by shaking the supernatant in air. In other cases (treatment with carbon or diethyl ether) the presence of traces of metal seems necessary, since EDTA prevents the effect of these treatments.

The existence of two distinct O forms of xanthine oxidase is suggested also by the observation that after treatment with trypsin the enzyme cannot react with NAD^+ under anaerobic conditions, whereas this occurs when the conversion $\text{D} \rightarrow \text{O}$ is effected in other ways (Stirpe & Della Corte, 1969). The change from the O form to the inactive form seems to be different from the other ones, in that it may be reversed by EDTA, and this is in agreement with the results obtained by Bergel & Bray (1956) with milk xanthine oxidase.

Further, in some cases the first effect of the treatment seems to be the conversion of the xanthine oxidase from the D form into a form, presumably intermediate, that reacts with O_2 in the absence of NAD^+ but that is still capable of reacting with NAD^+ if this is present in the reaction mixture. These results, together with the effects of trypsin, are summarized in Scheme 1.

The nature of the changes remains unknown. It may be assumed as a working hypothesis that the conversions are due to conformational changes, or alternatively to inactivation of functionally important thiol group(s), with activation of other functional group(s) reacting with O_2 , but other possibilities should be considered. Massey & Edmondson (1970) have demonstrated that milk xanthine oxidase is reversibly inactivated by cyanide, which acts by extracting sulphur from the enzyme: it cannot be excluded that some of the thiol reagents act in the



Scheme 1. *Interconversion of xanthine oxidase among its various possible forms*

Broken arrows indicate uncertain or alternative pathways.

same way. It is also possible that the oxidation and reduction of thiol groups are accompanied respectively by disaggregation and aggregation of subunits, as has been demonstrated by Maley & Maley (1968) with deoxycytidylate deaminase.

The conversion D \rightarrow O is also induced by a thermolabile factor present in the sedimentable fraction from rat liver, the effect of which is reversed by dithioerythritol. It was supposed that this factor could be an enzyme, possibly unspecific, i.e. capable of acting on other proteins (Stirpe & Della Corte, 1970). It seems noteworthy that this factor is absent from the liver of birds, whose xanthine dehydrogenase, when present, cannot be converted into an oxidase.

In rat organs other than liver the xanthine oxidase appears in the O form, and in all cases but intestine it may be converted into a dehydrogenase by dithioerythritol. Thus the intestinal enzyme seems different from that of other organs, and this is consistent with the different electrophoretic mobilities of the liver and intestinal xanthine oxidases (Sackler, 1966). It is difficult, at the moment, to ascertain the possible physiological role of the two forms of xanthine oxidase, and whether the conversion of the enzyme from one form into another may occur *in vivo*.

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