Involvement of a single thiol group in the conversion of the NAD⁺-dependent activity of rat liver xanthine oxidoreductase to the O₂-dependent activity

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The effects of 2-iodosobenzoic acid, 4-chloromercuribenzoate, 5,5'-dithiobis-(2-nitrobenzoic acid) and tetraethylthioperoxydicarbonic diamide (disulphiram) on the NAD⁺-dependent activity of xanthine oxidoreductase from rat liver were investigated. Only disulphiram converted the NAD⁺-dependent activity into the O₂-dependent activity quantitatively, without changing the xanthine hydroxylation rate. The modification process was a first-order reaction with respect to time (min) and disulphiram concentration (μM). The kinetic data showed that modification of single thiol group is sufficient for loss of the enzymic activity towards NAD⁺ as electron acceptor. The complete protection afforded by NAD⁺ against the action of disulphiram suggests that the essential thiol group may be involved in binding of NAD⁺ to the xanthine oxidoreductase molecule.

Xanthine oxidoreductase occurs in mammalian tissues in the NAD⁺-dependent form (Stirpe & Della Corte, 1969; Batelli *et al.*, 1973; Francois, 1973). It is known that the conversion of the NAD⁺-dependent form to the O₂-dependent form of this enzyme can be caused by modification of the thiol groups by thiol reagents (Della Corte & Stirpe, 1972; Waud & Rajagopalan, 1976; Kamiński & Jeżewska, 1979). The dehydrogenase and oxidase forms of xanthine oxidoreductase contain about 14 and 4 thiol groups, respectively, in the proximity of each active centre of the enzyme molecule (Waud & Rajagopalan, 1976). However, it remains unknown whether modification of all 10 thiol groups of the dehydrogenase form is necessary for its conversion to the oxidase form. In most of the dehydrogenases examined so far the number of thiol groups essential for their dehydrogenase activity is only 1 or 2 (Torchinskii, 1974a).

In the present report the number of thiol groups essential for the NAD⁺-dependent activity of rat liver xanthine oxidoreductase was determined with the use of the kinetic method of Levy *et al.* (1963) and the probability method of Tsou (1962). For this purpose tetraethylthioperoxydicarbonic diamide (disulphiram) appeared to be the most suitable modifier among several thiol reagents tested. The kinetics of xanthine oxidoreductase modification by disulphiram seemed to be of special interest, because this drug has been used in the management of chronic alcoholism (Hald & Jacobsen, 1948; Haley, 1979) and the NAD⁺-dependent form of the

enzyme investigated has been postulated to play a regulatory role in purine metabolism (Kamiński & Jeżewska, 1981).

Experimental

Reagents

The sources of chemicals used were as follows: propane-1,2-diol (propylene glycol), VEB Laborchemie, Apolda, East Germany; 2-iodosobenzoic acid, Serva, Heidelberg, West Germany; 4-chloromercuribenzoic acid and 5,5'-dithiobis-(2-nitrobenzoic acid), Sigma, St. Louis, U.S.A. The remaining reagents were purchased as previously stated (Kamiński & Jeżewska, 1979). Tetraethylthioperoxydicarbonic diamide (tetraethylthiuram, disulphiram) was synthesized according to Kitson (1976), and was recrystallized five times from propan-2-ol. A saturated solution of disulphiram (approx. 650 μM) in water/propane-1,2-diol (1:1, v/v) was used as the stock solution. The exact disulphiram concentration in the stock solution was determined spectrophotometrically after dilution (20-fold) with 96% ethanol. Molar absorption coefficients of 12×10^3 and $11 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ for 250 and 282 nm, respectively, were used in the calculations (UV Atlas, 1966/1968). From the stock solution a series of diluted disulphiram solutions in water containing 10% propane-1,2-diol were prepared.

Enzyme preparation

The previously described procedure was used (Kamiński & Jeżewska, 1979, 1981). Briefly, freshly

obtained rat liver was homogenized with 5 vol. of solution containing 250 mM-sucrose, 100 mM-Hepps [4-(2-hydroxyethyl)-1-piperazinepropanesulphonic acid]/KOH buffer, pH 8.0, 15 mM-dithiothreitol and Trasylol (2500 kallikrein units/ml). The homogenate was centrifuged at 4°C for 10 min at 25 000 g and then for 60 min at 160 000 g. The fraction precipitated from the 160 000 g supernatant within the range 1.6–2.4 M-(NH₄)₂SO₄ was suspended in 250 mM-sucrose and applied to a 5'-AMP-Sepharose 4B column; xanthine oxidoreductase was eluted with 10 mM-KH₂PO₄ adjusted to pH 8.6 with KOH. Pool fractions with the highest NAD⁺-dependent activity of the enzyme were used in further experiments. The enzyme was purified 75-fold compared with the 25 000 g supernatant. The enzymic preparation exhibited mainly the NAD⁺-dependent activity of xanthine oxidoreductase, but low O₂-dependent activity was also present.

Enzymic activity measurements

Spectrophotometric measurements were performed in a Cary 118C spectrophotometer equipped with a repetitive scan, in quartz cells (light path 1 cm, volume 3 ml).

Conversion of the NAD⁺-dependent form to the O₂-dependent form by thiol reagents. Enzyme solution (0.2 ml) was preincubated with 0.2 ml of the thiol reagent solution for 5 min at 25°C. Then the incubation mixture was diluted with 50 mM-Tris/HCl buffer, pH 8.0 and (a) xanthine or (b) xanthine and NAD⁺ or (c) xanthine, NAD⁺, lactate dehydrogenase and pyruvate were added; the final volume of the mixtures was 3 ml, and xanthine and NAD⁺ were 150 μM and 350 μM, respectively. Formation of uric acid [increase of absorbance at 302 nm, A_{302} ; mixtures (a) and (c)] or formation of uric acid and NADH [increases of A_{302} and A_{340} , respectively; mixture (b)] were followed spectrophotometrically. The initial rates of these reactions were used for calculation of the activity of the individual enzyme forms: the NAD⁺-dependent form (D), NAD⁺/O₂-dependent form (D/O) and O₂-dependent form (O) as previously described (Kamiński & Jeżewska, 1979). Activities of these three forms were expressed as percentages of the total xanthine oxidoreductase activity of the enzyme preparation untreated with the thiol reagent.

Disulphiram-induced disappearance of the NAD⁺-dependent activity. The enzyme solution (0.2 ml) was preincubated with 0.2 ml of a solution of disulphiram at various concentrations, for different times, at 25°C. In some experiments preincubations of the enzyme with 15.0 μM-disulphiram alone or in the presence of NAD⁺ at various concentrations were carried out simultaneously. After preincubation, the mixtures were diluted with 50 mM-Tris/HCl buffer, pH 8.0, and xanthine and NAD⁺ were added

up to final concentrations of 150 and 350 μM, respectively; the final volume of the mixture was 3 ml. The NAD⁺-dependent activity (partly inhibited by accumulating NADH; Kamiński & Jeżewska, 1979) was measured as the absorbance increase at 340 nm. The enzymic activity was calculated taking into account the initial rates of the reaction; for short times of xanthine hydroxylation the inhibited NAD⁺-dependent activity is directly proportional to the non-inhibited one (Kamiński & Jeżewska, 1979). The enzymic activity was expressed as a percentage of the initial activity of the disulphiram-untreated enzyme preparation or as a fraction of the remaining activity (Tsou, 1962).

Results and discussion

Effects of some thiol reagents on xanthine oxidoreductase activity

In these experiments the total activity, i.e. the sum of activities of the NAD⁺-, NAD⁺/O₂-, and O₂-dependent forms, and the activity of each of these forms separately were determined before and after preincubation of the enzyme preparation with the thiol reagent as described in the Experimental section.

Preincubation of the enzyme preparation with 2 μM-2-iodosobenzoic acid (a reagent specifically oxidizing the protein thiol groups with the formation of disulphide bonds; Webb, 1966; Torchinskii, 1974b) resulted in 50% inhibition of the NAD⁺-dependent activity. This inhibition was caused by the inactivation of the enzyme, not by the conversion of the NAD⁺-dependent activity to the O₂-dependent one. The thiol reagent used at 2.5 μM concentration inactivates also xanthine oxidase from milk (Harris & Hellerman, 1956). Possibly this reagent reacts with the thiol group of cysteine in the proximity of the molybdenum centre involved in hydroxylation of purine substrate (Coughlan, 1977; Coughlan *et al.*, 1980).

On the other hand, 4-chloromercuribenzoate at concentrations up to 5 μM increased the total activity of the enzyme preparation. Higher concentrations (10–50 μM) of this reagent decreased the NAD⁺-dependent activity with simultaneous inactivation of the enzyme. It is known that 4-chloromercuribenzoate increases the activities of many enzymes and that it acts on all thiol groups, including the cysteinyl residues connected with non-haem iron, not only on those on the surface of the protein molecule (Webb, 1966).

Ellman's reagent, 5,5'-dithiobis-(2-nitrobenzoic acid), (being very specific for —SH groups; Torchinskii, 1974b), converted the NAD⁺-dependent activity to the O₂-dependent activity, without any effect on the total activity of the enzyme preparation. However, the conversion of our enzymic preparation

occurred very slowly, even at $500\mu\text{M}$ concentration of the reagent. Moreover, the results obtained by Waud & Rajagopalan (1976) indicated that this reagent also modifies thiol groups in the oxidase form of the enzyme and that thiol groups essential for dehydrogenase activity are modified only after oxidation of several non-essential ones to yield disulphide bonds. Similarly, Kitson (1978) has found that cytoplasmic aldehyde dehydrogenase was not inactivated in the presence of Ellman's reagent, although several thiol groups in the enzyme molecule became modified.

The conversion of the NAD^+ -dependent activity to the O_2 -dependent one by Cu^{2+} has been previously described (Kamiński & Jezewska, 1979). Although this conversion occurred quantitatively, without any change in the total activity, and is sufficiently fast at low Cu^{2+} concentrations, the plot of the disappearance of the NAD^+ -dependent activity against the modifier concentration was not linear and great amounts of the intermediate NAD^+/O_2 -dependent form of the enzyme were formed. Moreover, Cu^{2+} ions catalyse the formation of disulphide bonds between cysteine thiol groups in the protein molecule (Takagi & Isemura, 1964; Cavallini *et al.*, 1969), and the mechanism of this reaction is rather complex (Friedman, 1973).

Thus all the above reagents seemed to be unsuitable for the determination of the number of thiol groups essential for the NAD^+ -dependent activity of xanthine oxidoreductase.

Conversion of the NAD^+ -dependent activity to the O_2 -dependent one by disulphiram

Tetraethyldithiocarbonic diamide, disulphiram, is known to inactivate many enzymes containing thiol groups. This reagent is regarded as entirely specific for these groups (Neims *et al.*, 1966) and modifies covalently a single cysteine thiol group by forming a mixed disulphide bond between this group and its own diethyldithiocarbamyl residue (Kitson, 1978). It has been found that 1mM -disulphiram transforms the dehydrogenase activity of rat liver xanthine oxidoreductase into the oxidase activity (Della Corte & Stirpe, 1972).

In the present experiments the enzyme preparation was preincubated with disulphiram at various micromolar concentrations. Within the whole range of concentrations used, the total xanthine oxidoreductase activity of the enzyme preparation remained constant (Fig. 1, curve *a*). The decrease in the NAD^+ -dependent form of xanthine oxidoreductase (Fig. 1, curve *b*) was directly proportional up to $25\mu\text{M}$ -disulphiram. Apart from the O_2 -dependent form only small amounts of the NAD^+/O_2 -dependent form appeared, at low disulphiram concentrations (Fig. 1, curves *c* and *d*, respectively). At higher disulphiram concentrations, the NAD^+ -dependent

Fig. 1. Conversion of the NAD^+ -dependent form of xanthine oxidoreductase to the O_2 -dependent form by disulphiram

Experimental conditions and measurements of the enzymic activities are as described in the Experimental section. Preincubation (5 min) of the enzyme preparation was with disulphiram at concentrations indicated. Xanthine oxidoreductase activity: (a) total, measured in the presence of lactate dehydrogenase and pyruvate; (b) of the NAD^+ -dependent form; (c) of the O_2 -dependent form; (d) of the intermediate NAD^+/O_2 -dependent form (all expressed as a percentage of the total activity of the disulphiram-untreated enzyme).

form was transformed into the O_2 -dependent form without formation of measurable quantities of the intermediate NAD^+/O_2 -dependent form. The difference in the yield of the intermediate form between Cu^{2+} (Kamiński & Jezewska, 1979) and disulphiram may result from the difference in the least number of thiol groups modified by these two reagents (two and one, respectively). However, it cannot be excluded that the difference results from the higher rate of conversion by disulphiram. Because disulphiram at micromolar concentrations converts quantitatively the NAD^+ -dependent activity to the O_2 -dependent activity of the enzyme, without other effects on the enzyme activity, and because the time of modification was reasonably short for kinetic analysis of the modification process, this thiol reagent was used in further study. Moreover, disulphiram, having no u.v. absorbance in the region 290–350 nm did not interfere with the spectrophotometric measurements.

Determination of the number of the thiol groups essential for the NAD^+ -dependent activity of xanthine oxidoreductase

Relationships between the decrease in the NAD^+ -dependent activity of xanthine oxidoreductase, modi-

fication time and concentration of the modifying reagent were investigated.

The decrease in NAD^+ -dependent activity as a function of the modification time was examined with the use of several disulphiram concentrations, following Levy *et al.* (1963). All plots obtained (Fig. 2, note the logarithmic scale of the ordinates) were linear, in the case of the three higher disulphiram concentrations with up to an 80% loss of the initial NAD^+ -dependent activity (Fig. 2, curves *c*, *d* and *e*). This linearity shows that the modification process approximated pseudo-first-order kinetics. The rate constant of this process was calculated from the equation:

$$k = \ln(2/t_{0.5}) = 2.303 \log(2/t_{0.5})$$

where $t_{0.5}$ is the time required for the reduction of the NAD^+ -dependent activity by 50% and the constant k depends on the concentration of the modifying reagent (Levy *et al.*, 1963). The relationship between $\ln(\text{first-order constant } k)$ and $\ln(\text{disulphiram concentration})$ appeared to be linear (Fig. 3). The slope, n , of the plot, which determines the number of thiol groups essential for the NAD^+ -dependent activity, was calculated by the least squares method

Fig. 2. NAD^+ -dependent activity of xanthine oxidoreductase as a function of the time of enzyme modification by disulphiram

Experimental conditions and measurements of the NAD^+ -dependent activity are as in the Experimental section. Disulphiram concentrations (μM) used: (a) 6.4; (b) 12.9; (c) 19.3; (d) 32.2; (e) 48.3. Time of the enzyme preincubation with disulphiram is plotted against the NAD^+ -dependent activity after preincubation with disulphiram as a percentage of the total activity of the disulphiram-untreated enzyme (logarithmic scale).

Fig. 3. Dependence of the pseudo-first-order rate constant (k) of the enzyme modification on the concentration of modifying reagent (disulphiram)

Constants k were calculated from the equation:

$$k = \ln(2/t_{0.5})$$

times $t_{0.5}$, those required for reduction of the NAD^+ -dependent activity by 50% at the applied disulphiram concentration, were taken from Fig. 2. The slope ($n = 1.01$) of the plot representing $\ln(k)$ as a function of $\ln[\text{disulphiram}]$ was calculated by the method of least squares.

to be 1.10. This indicated that, on average, one molecule of disulphiram is involved in the disappearance of the NAD^+ -dependent activity of xanthine oxidoreductase.

The above conclusion was verified by the method of Tsou (1962). This method involves plotting the number of groups modified against $a^{1/i}$, where a is the fractional remaining activity of the enzyme and i is the number of groups essential for this activity. The value of i is obtained by trial and error as that which gives the best straight line. The number of groups modified can be replaced by the concentration of the modifying reagent (Kitson, 1978), if its concentration exceeds by far the enzyme concentration. Therefore, the relationship between the decrease in the NAD^+ -dependent activity of xanthine oxidoreductase and the concentration of disulphiram was examined. Three sets of experiments were performed for three fixed times of modification (6, 9 and 15 min). All plots of $a^{1/i}$ as a function of disulphiram concentration for $i = 1$, $i = 2$ and $i = 3$ were approximately linear up to $19.3 \mu\text{M}$ concentration of the modifier. The linear negative correlation coefficients $|R|$ were calculated and the following total residues $1 - |R|$ (deviation from straight line) were obtained: 0.0030, 0.0089 and 0.0118 (6 min

of modification); 0.000027, 0.0029 and 0.0049 (9 min); and 0.0018, 0.0092 and 0.0142 (15 min) for $i = 1$, $i = 2$ and $i = 3$, respectively. Therefore, the best linear fit appeared to be for $i = 1$. This result confirmed that, for the conversion of the xanthine oxidoreductase NAD^+ -dependent activity to the O_2 -dependent activity, only one thiol group in the enzyme active centre has to be modified. Probably a molecule of this enzyme, having two active centres, contains two such thiol groups.

The plots obtained for a to the 1th power against disulphiram concentration (Fig. 4) showed a bending with the increase in the modifier concentration over $19.4 \mu\text{M}$. Such change in the shape of the plot has been attributed (Tsou, 1962; Horiike & McCormick, 1979) to modification of the non-essential groups localized in the proximity of the essential one. This interpretation is consistent with the data obtained by Waud & Rajagopalan (1976), suggesting that all thiol groups in the dehydrogenase form of xanthine oxidoreductase, modified by Ellman's reagent, are located near to each other.

NAD^+ as a factor protecting the NAD^+ -dependent xanthine oxidoreductase activity against the disulphiram action

The ability of NAD^+ to protect xanthine oxidoreductase from a loss of its NAD^+ -dependent acti-

vity due to disulphiram action is shown in Fig. 5. Enzyme modification by disulphiram is much slower in the presence of $456 \mu\text{M-NAD}^+$ (physiological concentration of NAD^+ in rat liver cytosol; Tischler *et al.*, 1977) and is completely prevented by $2100 \mu\text{M-NAD}^+$. The protective effect of NAD^+ suggests that the single thiol group essential for the NAD^+ -dependent activity is involved in the binding of NAD^+ with the active centre. In contrast with the modification in the absence of NAD^+ , the modification in the presence of lower concentration of NAD^+ (Fig. 5, curves *c* and *b*, respectively) did not follow pseudo-first-order kinetics. At present there is no explanation for the reproducible linearity of plots obtained in the presence of NAD^+ .

As has been discussed earlier (Kamiński & Jezewska, 1981) the conversion of the NAD^+ -dependent activity of xanthine oxidoreductase to the O_2 -dependent activity could result in a decrease in the hypoxanthine salvage pathway, enhancement of purine biosynthesis *de novo*, and, as a consequence, formation of excessive quantities of uric acid. When used as a drug, disulphiram appears at micromolar concentrations in human tissues (Fairman *et al.*, 1978); therefore it cannot be ex-

Fig. 4. NAD^+ -dependent activity of xanthine oxidoreductase as a function of the disulphiram concentration (Tsou plot)

Experimental conditions and measurements of the NAD^+ -dependent activity were as in the Experimental section. Disulphiram concentrations are plotted against the NAD^+ -dependent activity after modification, expressed as a fraction (a) of remaining activity, and plotted as a^1 . Enzyme modification proceeded for: (a) 6 min; (b) 9 min; (c) 15 min.

Fig. 5. Protective effect of NAD^+ on the NAD^+ -dependent activity of the disulphiram-treated xanthine oxidoreductase preparation

Experimental conditions and measurements of the NAD^+ -dependent activity are as described in the Experimental section. The time of enzyme modification by $15.0 \mu\text{M}$ -disulphiram is plotted against the NAD^+ -dependent activity after modification, expressed as a fraction (a) of the remaining activity. The enzyme preparation was preincubated with disulphiram: (a) in the presence of $2100 \mu\text{M-NAD}^+$; (b) in the presence of $456 \mu\text{M-NAD}^+$; (c) without NAD^+ .

cluded that this modifier could affect the NAD^{+} -dependent activity of xanthine oxidoreductase *in vivo*. The above possibility and its consequences on purine metabolism remains to be investigated.

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