Vol. 120, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1984 Pages 28-36

# MODIFICATION OF RAT LIVER IODOTHYRONINE 5'-DEIODINASE ACTIVITY WITH DIETHYLPYROCARBONATE AND ROSE BENGAL; EVIDENCE FOR AN ACTIVE SITE HISTIDINE RESIDUE

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Received February 22, 1984

SUMMARY. Iodothyronine 5'-deiodinase activity of rat liver microsomes was rapidly and completely lost by treatment with diethylpyrocarbonate (DEP) and by photo-oxidation with Rose Bengal (RB). In both cases inactivation followed pseudo first order reaction kinetics. Inactivation by DEP was diminished in the presence of substrate or competitive inhibitors, and was reversed by hydroxylamine treatment. In addition to photo-oxidation, deiodinase activity was also inhibited by RB in the dark. This inhibition was reversible and competitive with substrate ($K_i$ 60 nM). These results suggest the location of an essential histidine residue at or near the active site of rat liver iodothyronine deiodinase.

Enzymatic 5'-deiodination of thyroxine ($T_4$) in peripheral tissues is the major pathway for the production of the biologically active form of thyroid hormone, 3,3',5-triiodothyronine ($T_3$) (1,2). The 5'-deiodinase activity of the liver and the kidneys is associated with the microsomal fractions of these tissues. It prefers 3,3',5'-triiodothyronine (reverse $T_3$, $rT_3$) as substrate, and requires the reductive equivalents of thiols, e.g. reduced glutathione, mercaptoethanol and dithiothreitol (DTT) (3-6). Deiodination is inhibited by sulfhydryl (SH) group-blocking reagents such as N-ethylmaleimide, p-chloromercuriphenylsulfonic acid and especially iodoacetate, suggesting the participation of an enzyme SH group in the catalytic process (5). This is substantiated by the findings of potent and uncompetitive inhibition of deiodination by thiouracil derivatives, compounds known to react with sulfenyl iodide groups (7,8). Supposedly, the enzyme SH group acts as an acceptor of the 5'-iodine atom.

The nucleophilicity of catalytically active enzyme SH groups is often increased by hydrogen bond formation with the imidazole group of a neighbouring histidine (His) residue (9). In this study we investi-

0006-291X/84 $1.50

gated the possible presence of an essential His residue in the deiodinase of rat liver microsomes using two His-selective reagents. At neutral or near neutral pH, limited reaction of proteins with diethylpyrocarbonate (DEP) leads to the preferential modification of His residues forming the $N^{im}$-carbethoxy derivative (10). However, especially at alkaline pH DEP may also react with lysine and cysteine residues, but only carbethoxylation of His is reversible with hydroxylamine (10-15). Another approach to modification of His residues is photo-oxidation sensitized by Rose Bengal (9,12,15). Compared with other susceptible residues reaction with His is favoured by complex formation of the anionic dye with the protonated imidazole group. The results of the present study, employing both DEP and RB, indicate the presence of an essential His residue at or near the active site of the iodothyronine deiodinase of rat liver.

MATERIALS AND METHODS. Reverse $T_3$ and 3,3'-diiodothyronine (3,3'-$T_2$) were obtained from Henning, Berlin, FRG; DEP from Aldrich; RB from Kodak-Eastman; iopanoic acid (IOP) from Sterling-Winthrop, Amsterdam, The Netherlands; and carrier-free Na $^{125}I$ from Amersham. [3',5'-$^{125}I$] $rT_3$ was prepared by radioiodination of 3,3'-$T_2$ with the chloramine-T method and purified on Sephadex LH-20 (16). All other reagents were of the highest quality commercially available. Rat liver microsomes were prepared in 0.15 M sodium phosphate (pH 7.2), 3 mM EDTA and 3 mM DTT, essentially as previously described (17). Protein was measured according to Bradford using bovine serum albumin as the standard (18,19).

Enzyme modification with DEP. DEP was dissolved and diluted to the desired concentrations in absolute ethanol. The actual DEP concentration was determined by reaction of 10 µl aliquots with 1 ml 0.1 M His in 0.1 M sodium phosphate (pH 6.0). From the increase in absorbance at 240 nm the DEP concentration was calculated using a molar extinction coefficient for $N^{im}$-carbethoxy-His of 3200 $M^{-1}.cm^{-1}$ (20). Protein modification was carried out by reaction of 10 µl DEP in ethanol with 140 µg of microsomal protein in 0.5 ml 50 mM sodium phosphate (pH 6.5 unless stated otherwise), 1 mM EDTA and 1 mM DTT, at 21C. After various times the reaction was stopped by quenching 50 µl aliquots of the mixtures in 150 µl 0.1 M His in 0.1 M sodium phosphate (pH 7.2), 3 mM EDTA and 3 mM DTT. In control incubations DEP was added to microsomes diluted in quench buffer. At the concentration of 2%, ethanol did not affect enzyme activity. Protection against carbethoxylation by substrate or competitive inhibitors was investigated by preincubation of microsomes with these substances for at least 5 min prior to addition of DEP. Reversibility of DEP inhibition was tested by incubation of carbethoxylated microsomes for 1 h at 21C with 0.5 M $NH_2OH$ in 0.1 M sodium phosphate (pH 7.2), 3 mM EDTA and 3 mM DTT.

Photo-oxidation with RB. Microsomal suspensions containing 1.4 mg protein per ml 0.15 M sodium phosphate (pH 7.2), 3 mM EDTA and 3 mM DTT, were irradiated in the presence of the indicated concentrations of RB. Irradiation was done for various times at 21C in polypropylene tubes placed at 10 cm distance from the lens of a slide projector equipped with a 300 W lamp.

To correct for reversible, competitive inhibition by RB, control incubations were carried out in the dark. Irradiation in the absence of RB did not affect enzyme activity.

Iodothyronine 5'-deiodination assay. Appropriate dilutions of the inactivation mixtures (2-5 µg microsomal protein) were incubated for 10 min at 37C with 0.1 µCi $^{125}$I-$rT_3$ and 0.5 µM unlabelled $rT_3$ in 0.25 ml 0.15 M sodium phosphate (pH 7.2), 3 mM EDTA and 3 mM DTT. The reaction was stopped by addition of 0.25 ml human serum, and the tubes were placed on ice. Serum protein-bound iodothyronines, i.e. substrate $rT_3$ and product 3,3'-$T_2$, were precipitated with 0.5 ml 10% trichloroacetic acid. Production of iodide was calculated from the radioactivity measured in 0.5 ml of the supernatant after centrifugation for 10 min at 1500 x g. The results were corrected for non-enzymatic deiodination as assessed in enzyme-free incubations, and multiplied by two to account for the equal distribution of the $^{125}$I label among the products 3,3'-$T_2$ and $I^-$. Identical results were obtained if deiodinase activity was measured by quantitation of 3,3'-$T_2$ production from unlabelled $rT_3$ with a specific radioimmunoassay (2).

## RESULTS

Reaction with DEP. Incubation of rat liver microsomes with millimolar concentrations of DEP at 21C resulted in a rapid loss of 5'-deiodinase activity. The time course of enzyme inactivation by different DEP concentrations at pH 6.5 is shown in Fig. 1. Inhibition by DEP followed pseudo first order kinetics during the first 6 min of reaction. The rate of inactivation was also determined over the pH range 6.5 - 8.5 (Fig. 2).

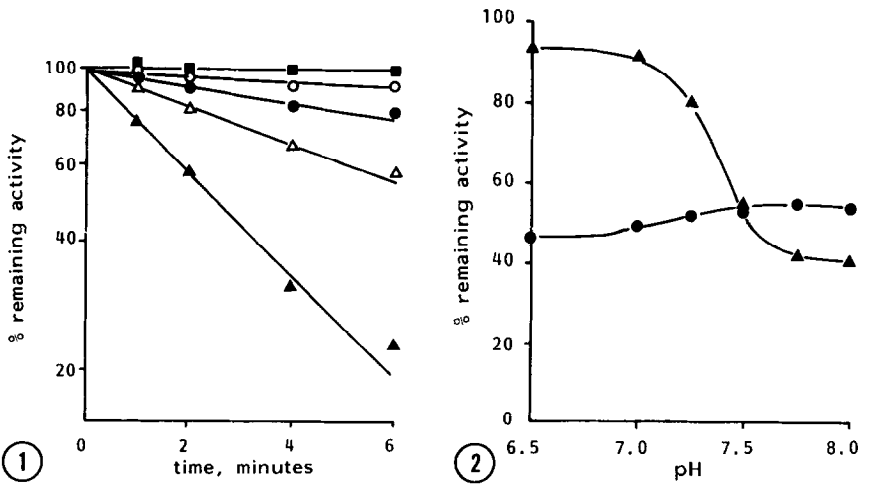


Fig. 1 Inactivation of $rT_3$ 5'-deiodinase activity by reaction of rat liver microsomes for various times at pH 6.5 and 21C without (■) or with 0.3 (o), 0.6 (●), 1.2 (△) or 3 (▲) mM DEP.

Fig. 2 Inactivation of $rT_3$ 5'-deiodinase activity by DEP or RB-induced photo-oxidation as a function of pH. The percentage remaining activity was determined after reaction of rat liver microsomes for 2 min with 0.6 mM DEP (▲) or after photo-oxidation for 4 min with 1 uM RB (o).

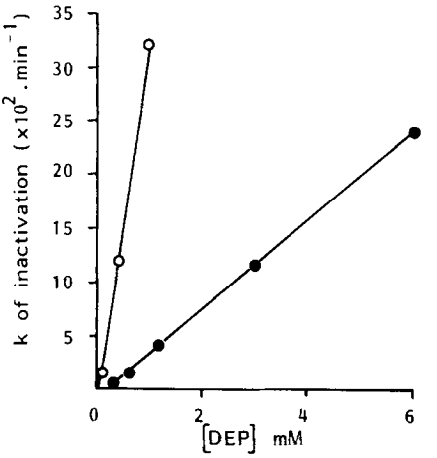


Fig. 3 First order rate constant (k) of inactivation of $rT_3$ 5'-deiodinase activity by DEP as a function of the DEP concentration at pH 6.5 (●) or pH 8.0 (o).

Between pH 7.0 and 7.8 there was a sharp increase in the rate of enzyme inactivation by DEP to remain constant thereafter. Plots of inactivation rate constants as a function of DEP concentration were linear (Fig. 3) and yielded second order rate constants for the simple bimolecular reaction of DEP with enzyme. Values for the second order k amounted to 40 $M^{-1}$ $min^{-1}$ at pH 6.5 and 530 $M^{-1}$ $min^{-1}$ at pH 8, corroborating the pH profile shown in Fig. 2. Carbethoxylated microsomes were treated with 0.5 M $NH_2OH$ to exclude the possibility that inactivation was due to reaction of DEP with lysine or cysteine residues. Table 1 demonstrates that, especially after limited reaction with DEP, $NH_2OH$ treatment resulted in

Table I

Reversal of DEP inactivation of 5'-deiodinase activity by hydroxylamine

| DEP Concentration (mM) | % remaining activity before and after treatment with $NH_2OH$ | |
|---|---|---|
| | BEFORE | AFTER |
| 0.0 | 100 | 100 |
| 0.6 | 74 | 93 |
| 1.2 | 38 | 90 |
| 3.0 | 13 | 55 |
| 6.0 | 5 | 23 |

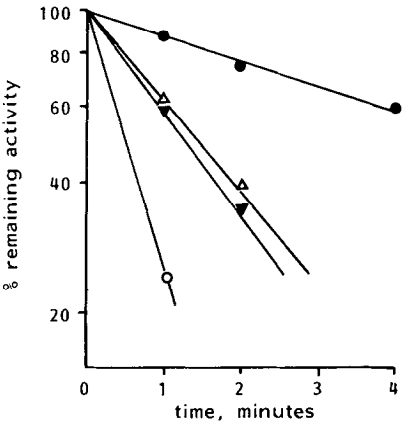


Fig. 4 Inactivation of $rT_3$ 5'-deiodinase activity by reaction of rat liver microsomes for various times at pH 6.5 and 21C with 6 mM DEP in the absence (o) or the presence of 1 μM RB (●) (in the dark) 2 μM rT3 (▲) or 2 μM IOP (▼).

substantial recovery of deiodinase activity. To localize the residue(s) subject to carbethoxylation, reaction with DEP was carried out in the presence of 2 μM $rT_3$ or IOP or 1 μM RB. The concentration of these compounds carried over from the preincubation mixtures were insufficient to affect enzyme activity in the subsequent assay. This was substantiated in appropriate control experiments. Fig. 4 shows that the substrate and both competitive inhibitors provided partial protection against inactivation by DEP.

Reaction with RB. Deiodinase activity was also strongly inhibited by photo-oxidation sensitized by low concentrations of RB. Direct addition of RB to the deiodinase assay mixtures, incubated in the dark, also resulted in inhibition of enzyme activity. This inhibition was reversible by simple dilution, and Lineweaver-Burk analysis of the data demonstrated that RB was a competitive inhibitor with a $K_i$ value of 60 nM (not shown). Care was taken, therefore, that following photo-oxidation mixtures were diluted sufficiently as to prevent "dark" inhibition by RB in the deiodinase assay. At the concentrations of RB used in the preincubations no effect on enzyme activity was observed without irradiation. The semi-logarithmic plots of the percentage enzyme activity remaining as a function of time of irradiation at different RB concentrations were linear

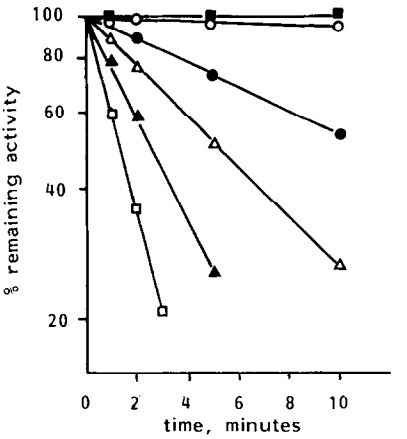


Fig. 5 Inactivation of $rT_3$ 5'-deiodinase activity by irradiation of rat liver microsomes for various times at pH 7.2 and 21C in the absence (■) or the presence of 0.1 (○), 0.3 (●), 0.5 (△), 1 (▲) or 2 (□) μM RB.

(Fig. 5), showing that photo-oxidation follows pseudo first order kinetics. From the linear replot of the inactivation constants versus RB concentration, the second order rate constant was calculated as 125 $mM^{-1}$ $min^{-1}$. To prevent the possible modification of cysteine and methionine residues, 3 mM DTT was usually included in the reaction mixtures. It was found that 5 mM DTT and 5 mM sodium azide provided only 40-50% protection against RB-sensitized photo-oxidation. Fig. 2 demonstrates that there is only a slight decrease in the rate of photo-inactivation by RB between pH 7 and 8. Inactivation in the presence of DTT was partially (45%) prevented by addition of 50 μM IOP to the irradiation mixtures.

## DISCUSSION

Reaction with DEP. The imidazole ring of His is, at neutral or slightly basic pH, by far the preferred target for DEP (21). Although other residues may also react with DEP, the results strongly indicate that the inactivation occurs through modification of a His residue near or at the active site. The second order k of 40 $M^{-1}$ $min^{-1}$ at pH 6.5 and 530 $M^{-1}$ $min^{-1}$ at pH 8.0 agrees with inactivation rates reported for several other proteins through DEP-mediated His modifications (14,15,22).

This rate is usually slower than the value of 1200 $M^{-1}$ $min^{-1}$ reported for the reaction of free His with DEP (23). Furthermore, $NH_2OH$ does not regenerate free amino groups or sulfhydryl groups from carbethoxylated lysine or cysteine residues (10). Thus, the observed reactivation with $NH_2OH$ agrees with His modification. The failure of hydroxylamine to fully reactivate the modified enzyme at higher DEP concentrations may be due to the reaction of a second DEP molecule with the carbethoxylated His residue, which is followed by a Bamberger reaction to open the imidazole ring (10). The protection by substrate or competitive inhibitors against inactivation indicates that the His residue is located at or near the active site. From the pH profile it is concluded that the reacting residue has a pK of 7.3, which is in the range of reported pK values of 6.2 to 7.5 for His residues (11,13).

Photo-oxidation with RB. In the photodynamic inactivation of deiodinase activity induced by RB, four processes need to be distinguished.

a. Incubation of $rT_3$ with RB in the presence of light resulted in a rapid release of labelled iodide from the outer ring. Destruction of substrate did not occur in the dark. This phenomenon interfered with the test of the possible protective effect of substrate and other halogenated compounds against enzyme inactivation by RB. Only a high concentration of IOP gave partial protection, and degradation of IOP in the reaction mixtures seems likely.

b. In the dark controls we observed competitive inhibition of deiodinase activity by RB that reflects the structural relationship with iodothyronines as is the case with phenolphthalein dyes (24). Interestingly, the $K_i$ for RB (0.06 μM) is less than the $K_m$ for $rT_3$ (0.1 μM), making RB one of the most potent inhibitors of deiodination.

c. Photo-oxidation reactions can proceed by two distinct mechanisms. Type II mechanism is that in which the energy of the sensitizer is transferred to molecular oxygen producing singlet oxygen ($^1O_2$) (25,26). $^1O_2$ is the reactive species in enzyme inactivation by the type II mechanism.

As compared to other dyes, RB and the frequently used dye methylene blue, give rise to high $^1O_2$ production rates (25), which may be quenched by scavengers such as azide and DTT (25,26). Since the present experiments were carried out in the presence of DTT, inactivation must have taken place by a mechanism other than via production of $^1O_2$.

d. In the type I mechanism there is a direct reaction of triplet sensitizer with susceptible residues in the enzyme, followed by reaction of these residues with molecular oxygen. Inactivation by RB was slightly faster below pH 7 possibly due to complex formation of the anionic dye with a protonated His residue. It is expected that such complex formation is a saturable reaction at concentrations above the $K_i$ value for RB (60 nM). Saturation of RB-induced photo-inactivation, however, was not observed. This may be caused by the use of high microsomal protein concentrations resulting in a lowering of the free RB concentration for interaction with the enzyme.

In conclusion, the present study suggests that there is an essential His residue in the active site of rat liver iodothyronine deiodinase. It is speculated that this residue forms a hydrogen bond with the catalytically important SH group, resulting in an increased nucleophilicity of the latter.

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