EVIDENCE FOR FREE RADICAL INVOLVEMENT IN THE HYDROXYLATION OF PROLINE: INHIBITION BY NITRO BLUE TETRAZOLIUM

Rajendra S. BHATNAGAR and Tsan Z. LIU

Laboratory of Connective Tissue Biochemistry, School of Dentistry, University of California, San Francisco, California 94122, USA

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1. Introduction

The enzyme, protocollagen proline hydroxylase (PPH), hydroxylates specific proline residues in the collagen sequence to hydroxyproline in the presence of molecular O_2, ascorbate and Fe^{2+} [1, 2]. There is an absolute requirement for α-ketoglutarate which undergoes oxidative decarboxylation in stoichiometric proportions to the hydroxyproline formed [3]. The role of Fe^{2+} and ascorbate in this reaction has not been adequately described. We have recently reported that the Fe^{2+} activates the enzyme by binding to the apoprotein [4, 5]. We describe here, an inhibition of proline hydroxylation by the dye, nitro blue tetrazolium (NBT) which undergoes rapid reduction by scavenging free radicals. The inhibition by NBT is competitive with respect to the enzyme, suggesting that free radicals are involved in the hydroxylation of proline, probably in the reduction of the ferri-enzyme to ferro-enzyme.

2. Materials and methods

L-[3, 4-$^3H_2$]proline (4.8 Ci/mmole) was purchased from New England Nuclear. Nitro blue tetrazolium (grade III) was a product of Sigma Chemicals.

Tritium-labeled protocollagen was prepared as described elsewhere [4]. Protocollagen proline hydroxylase was prepared from decapitated 12-day old chick embryos by a procedure adapted from Halme et al. [6] up to the DEAE-cellulose chromatography step.

Enzyme activity was assayed as described previously [4]. The reactions involving nitro blue tetrazolium were carried out in the dark to avoid extensive photoreduction of the dye. [^{3}H]HO formed during the reaction was collected by vacuum distillation and the radioactivity of [^{3}H]HO was used as an index of hydroxylation.

3. Results and discussion

Nitro blue tetrazolium is reduced by the superoxide radical O_2^- with the formation of blue formazan [7, 8] and it has been used as a detector of O_2^- formation [9]. The requirement of ascorbate and Fe^{2+} for the hydroxylation of proline suggested that the reaction might involve the formation of free-radicals. To examine this possibility, the reaction was carried out in the presence of several concentrations of NBT. The results from this experiment are presented in fig. 1. Increasing concentrations of NBT caused increasing inhibition of hydroxylation of proline.

In all experiments with NBT, the dye was present at the beginning and the reaction was started by the addition of ascorbic acid. An examination of the time-course (fig. 2) of hydroxylation in the presence of 0.5 mM NBT showed that near maximal inhibition (75%) occurred at 5 min and the inhibition did not exceed 85% at time points where maximal hydroxylation was seen in the controls.

The inhibition of proline hydroxylation was partially reversed when additional Fe^{2+} and ascorbic acid were introduced into the system (table 1). Since Fe^{2+} and ascorbic acid generate free-radicals, the reversal was apparently caused by the sudden flux of free-

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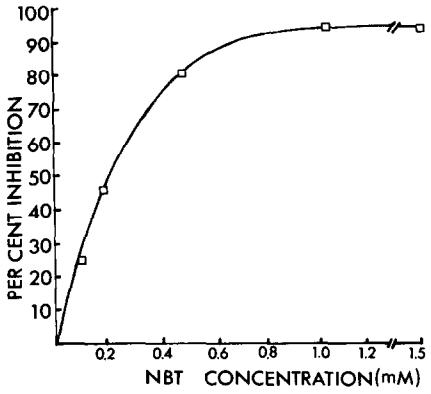

Fig. 1. Concentration curve of the nitro blue tetrazolium inhibition of protocollagen proline hydroxylase. The components of incubation were those described in table 1. Percent inhibition by NBT at various concentrations used was obtained by comparing with the activity of a non inhibited control.

radicals, in the presence of diminished concentrations of unreduced dye. These data strongly suggest the involvement of free-radicals in proline hydroxylation.

In order to examine the nature of the inhibitory effect, competition for free-radicals between the dye and the enzyme was determined (fig. 3). In order to limit the number of possible variables, this experiment was carried out with enzyme which was activated by pre-incubation with Fe^{2+} [5]. As seen in fig. 3, the inhibition of proline hydroxylation was competitive, with the dye and the enzyme competing, presumably for the free-radicals. These results support the involvement of free radicals in the hydroxylation of proline by PPH.

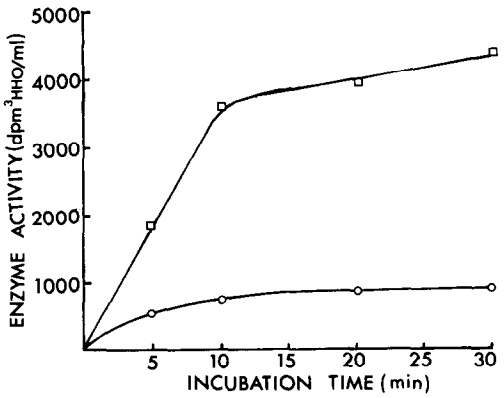

Fig. 2. Time course of the nitro blue tetrazolium inhibition of procollagen proline hydroxylase (o—o—o): In the presence of 0.5 mM NBT and (□—□—□): in the absence of NBT.

Table 1

Reversibility of NBT inhibition of protocollagen proline hydroxylase by Fe^{2+} and ascorbate.

	Time of incubation (min)	Total [^{3}H]HO formed (dpm)	% of Control
Complete System	5	4,239	-
+NBT, 0.5 mM	5	1,269	30
+NBT, 0.5 mM +Fe^{2+}, 0.5 mM +Ascorbate, 2.5 mM	5 + 5*	2,039	48
Complete System	10	8,685	-
+NBT, 0.5 mM	10	1,876	20
+NBT, 0.5 mM +Fe^{2+}, 0.5 mM +Ascorbate, 2.5 mM	10 + 10*	3,675	42
Complete System	20	9,334	-
+NBT, 0.5 mM	20	1,974	21
+NBT, 0.5 mM +Fe^{2+}, 0.5 mM +Ascorbate, 2.5 mM	10 + 20*	4,013	43
Complete System	30	12,085	-
+NBT, 0.5 mM	30	2,340	19
+NBT, 0.5 mM +Fe^{2+}, 0.5 mM +Ascorbate, 2.5 mM	10 + 30*	6,042	50

Complete system consisted of tritiated protocollagen (4.4 X 10^5 dpm), 1.4 mg enzyme, 0.1 mM α-ketoglutarate, 0.1 mM ferrous ammonium sulfate and 0.2 mM Tris-HCl, pH 7.4 and the reaction was started by the addition of 0.5 mM ascorbate in a final volume of 2.0 ml. The reaction was stopped by the addition of TCA to a concentration of 5%. *Indicates that the complete system was incubated with NBT for 5 or 10 min and then further incubated for 5, 10, 20 or 30 min after the addition of excess Fe^{2+} and ascorbate.

Hydroxylation reactions involving a concomitant decarboxylation of α-ketoglutarate proceed by a dioxygenase type mechanism since one atom from the molecule of O_2 is incorporated into each of two cosubstrates [10]. It has been proposed that in the initial step of such reactions, O_2 may bind to Fe^{2+}

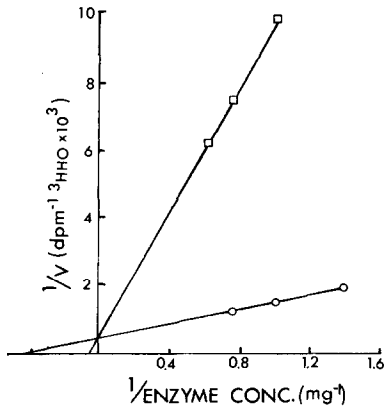

Fig. 3. Double reciprocal plot of the initial velocity against enzyme concentration. (○—○—○): In the absence of NBT and (□—□—□): in the presence of 0.5 mM NBT.

to form complexes of the type $Fe^{2+} <^{O}_{O}$ and that during the transfer of oxygen atoms to the substrate or substrates, Fe^{2+} may be oxidized to Fe^{3+} [11]. The Fe^{3+} must be reduced back to Fe^{2+} before another hydroxylation can occur. The superoxide radical O_2^-, formed as a result of a reaction between Fe^{2+}, O_2 and ascorbic acid in a reducing site on the enzyme may reduce the Fe^{3+} involved in the O_2-binding site. The hydroxylase reaction may be written in an abbreviated form as follows:

$$E \cdot Fe^{2+} + O_2 \rightarrow E\ Fe <^{O}_{O} \quad (1)$$

oxygen-binding site

$$E \cdot Fe <^{O}_{O} + \alpha\text{-ketoglutarate} + \text{peptidyl proline} \rightarrow$$

$E \cdot Fe^{3+}$ + succinate + CO_2 + peptidyl hydroxyproline (2)

$$E \cdot Fe^{3+} + O_2^- \rightarrow E \cdot Fe^{2+} + O_2 \quad (3)$$

The superoxide radical, O_2^- may be generated at a reducing site from the interaction of Fe^{2+} and ascorbate.

Recent observations in our laboratory have confirmed the formation of Fe^{3+} during the reaction (manuscript in preparation) and the reduction of $E \cdot Fe^{3+}$ by enzymatically generated O_2^- in the absence of ascorbic acid is currently under investigation. Observations on the reductive activation of tryptophan 2, 3-dioxygenase by O_2^- have been reported by Brady et al. [12] and Hirata and Hayaishi [13]. We suggest that O_2^- may provide a general mechanism for reductive activation of enzymes which catalyze the reductive fixation of O_2 through the mediation of a metal prosthetic group.

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