# Reduced Thioredoxin Inhibits Melanin Biosynthesis: Evidence for the Formation of a Stable Bis-cysteinate Complex with Tyrosinase

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(Received July 7, 1987)

Recent kinetic experiments provided a molecular basis for the regulation of melanin biosynthesis through the inhibition of tyrosinase by reduced thioredoxin [1]. Also the natural electron donor for thioredoxin, thioredoxin reductase, has been implicated in the regulation of skin pigmentation in the human population [2–6]. Since low molecular weight thiols can inhibit tyrosinase non-specifically by the reduction of its binuclear $Cu^{II}$-active site, it was important to examine the specific mechanism for the protein–protein interaction between reduced thioredoxin and tyrosinase using low temperature ESR.

Pure tyrosinase and hemocyanin are diamagnetic copper proteins as a consequence of spin-pairing for the two $Cu^{II}$ atoms in their respective active sites [7]. At low concentrations thiols such as β-mercaptoethanol uncouple the two copper atoms in tyrosinase presumably by the reduction of one $Cu^{II}$ to $Cu^{I}$, followed by the coordination of the resulting thiyl radical to the second $Cu^{II}$ to yield a paramagnetic $Cu^{II}$–thiolate complex. Uncoupling of these diamagnetic spin-paired $Cu^{II}$ atoms by small thiolate molecules in tyrosinase is demonstrable spectroscopically by the formation of a green colored complex with a charge-transfer band at 355 mμ, as well as by ESR ($g'' = 2.19$) [8]. This reaction between thiols and tyrosinase does not occur if the enzyme is first reduced to the $Cu^{I}$ complex with hydroxylamine. This result shows that the reduction of $Cu^{II}$ by thiolate is a necessary prerequisite for the formation of the parmagnetic $Cu^{II}$ complex. Stopped-flow ESR has been used to study the coordination of the thiolate form of cysteine at pH 9.5 to simple $Cu^{II}$ triglycinate peptides [9]. The transient formation of monocysteinyl–$Cu^{II}$ ($g'' = 2.17$) and biscysteinyl–$Cu^{II}$ ($g'' = 2.14$) have been characterized by ESR. Therefore this model system provided us with important information on $Cu^{II}$–thiol complexation. The active site of reduced thioredoxin has the possibility of forming either the unstable mono, or more stable, bisthiolate complex with tyrosinase.

Mushroom tyrosinase (12.5 mg/ml) ($M_r$ 125 000) was dissolved in 0.1 M $KH_2PO_4$ buffer at pH 6.5 containing 1.0 mM L-tyrosine. The reaction with tyrosine was inhibited after one minute by the addition of a 10 fold excess of reduced thioredoxin (12.5 mg/ml) ($M_r$ 12 500). Thioredoxin was reduced enzymatically with 50 mM NADPH and 0.1 mg of *E. coli* thioredoxin reductase. The ESR spectrum of the reduced thioredoxin/tyrosinase inhibitor complex is presented in Fig. 1. This ESR spectrum resembles that reported for the model biscysteinyl–$Cu^{II}$ complex [9]. It can now be concluded that reduced thioredoxin reacts with the binuclear $Cu^{II}$ center of tyrosinase to reduce one $Cu^{II}$ atom and form a stable bisthiolate with the second $Cu^{II}$ site. This result presents additional *in vitro* evidence in support of the NADPH/thioredoxin reductase/thioredoxin/tyrosinase feedback mechanism for the inhibition of melanin biosynthesis [2–6].

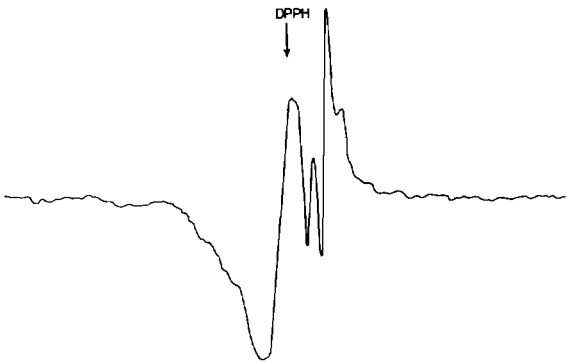


Fig. 1. ESR spectrum at 3.2 K of the reduced thioredoxin/tyrosinase inhibitor complex ($g'' = 2.15$) (mod. frequency 100 kHz, microwave frequency 9.228 GHz, microwave power 0.2 mW).

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0020-1693/88/$3.50 