

Supplementary data

Inactivation of Human Angiotensin Converting Enzyme by Copper Peptide Complexes Containing ATCUN Motifs.

Nikhil H. Gokhale and J. A. Cowan*

Evans Laboratory of Chemistry, Ohio State University, 100 West 18th Avenue, Columbus, Ohio 43210

SUPPORTING MATERIAL

1. Materials and Methods

1.1 Determination of Kinetic Constants. Determination of K_m and V_{max} values was carried out using the same fluorescence assay as described earlier. Reactions were carried out for 30 min at 37 °C. Initial rates for the hydrolysis of substrate (V_o) were determined by following the change in fluorescence (relative fluorescence units/min, RFU/min), plotted as a function of substrate concentration ($[S]$) and fit to the Michaelis-Menten equation $V_o = V_{max} [S] / ([S] + K_m)$, using Origin software (Microcal) to determine K_m . Substrate concentration was varied from 0 to 20 μ M (Figure SM1 to SM4).

1.2 Determination of IC_{50} values for rhACE inhibitors using, Mca-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH substrate. Reaction mixtures (100 μ L) contained 50 mM HEPES buffer, 300 mM NaCl and 10 μ M $ZnCl_2$ (pH 7.4), 10 μ M substrate and 1nM (13.1 ng) rhACE. Concentrations of GGH, KGHK, YIHFP, Cu^{2+} , $Cu(GGH)^-$, $Cu(KGHK)^+$, $Cu(YIHFP)^-$, were varied from 0 to 100 μ M. Prior to the start of the enzymatic reaction (by addition of substrate) the inhibitors were pre-incubated with the enzyme for 45 min. Reactions were run in the wells of polystyrene 96-well microplates (Corning) as described previously. Fluorescence was measured for 70 min. and the background rate determined for samples containing no rhACE was subtracted from all reactions to calculate the initial rates in RFU/min. Initial rate data were plotted as percentage activity, relative to uninhibited control reactions, as a function of inhibitor concentration (Figure SM5 to SM9).

1.3 Determination of the Type of Inhibition. Reaction mixtures (100 μ L) contained 50 mM HEPES buffer, 300 mM NaCl, 10 μ M $ZnCl_2$ (pH 7.4) and 1nM (13.1 ng) rhACE. Two sets of reactions were performed at distinct substrate concentration (5 and 10 μ M) with varying concentrations of inhibitor (0 to 20 μ M). Prior to the start of the enzymatic reaction (by addition of substrate) the inhibitors were pre-incubated with the enzyme for 45 min. Reactions were run in the wells of polystyrene 96-well microplates (Corning). Fluorescence change was measured for 30 min as described previously. Initial rate data were plotted versus inhibitor concentration and a Dixon plot (inverse of initial velocity versus inhibitor concentration) was generated using Origin (Microcal).

1.4 Determination of Inhibition Constants. For the competitive inhibitors the enzyme-inhibitor dissociation constant (K_i) was determined by graphical methods by use

of a Dixon plot, as well as by the equation below, where IC_{50} , $[S]$ and K_m have units of molar concentration.

$$K_I = IC_{50} / (1 + [S]/K_m)$$

1.5 Determination of the Inhibition Mechanism under Oxidative Reaction

Conditions. Enzymatic reactions performed in the presence of dioxygen and ascorbate are defined as oxidative conditions, while all other reactions described herein that contained no ascorbate are defined as hydrolytic. Reaction mixtures (100 μ L) contained 50 mM HEPES buffer, 300 mM NaCl, 10 μ M ZnCl₂ (pH 7.4), 10 μ M ascorbate (L-ascorbic acid, prepared in deionized water) and 1 nM (13.1 ng) rhACE. The concentration of inhibitor was set at K_I . Prior to the start of the enzymatic reaction (either by addition of substrate or the enzyme) the inhibitors were pre-incubated with the enzyme for 45 min. Two sets of reactions were run in the wells of polystyrene 96-well microplates (Porvair) and fluorescence change (relative fluorescence units/min, RFU/min) was measured for 30 min as described previously.

Set I: Monitoring the progress curve for the enzymatic reaction under oxidative conditions:

Following prior pre-incubation of the enzyme with the inhibitor, the enzymatic reaction was initiated by simultaneous addition of the substrate and ascorbate, and the progress of the reaction monitored by formation of product as reflected by the observed change of fluorescence. The initial velocity (V_o), obtained from the progress curve in the presence and absence of the inhibitor, was plotted versus time as described. Respective control reactions had either ascorbate present or absent (Figure SM10 and Figure SM11).

Set II: Enzyme deactivation under oxidative conditions in the presence and absence of inhibitor:

To obtain a true rate of enzyme deactivation by the inhibitor under oxidative conditions the enzyme was pre-incubated with the inhibitor for 45 min. Ascorbate was added, followed by a preincubation time, and aliquots of the reaction mixture were taken at various time intervals and the residual enzyme activity estimated using the 96-well plate assay described previously. Initial velocity (V_o) obtained in the presence and absence of inhibitor was plotted as a function of time and fitted to the first-order rate equation to obtain k_{obs} .

Figures for Supplementary Material.

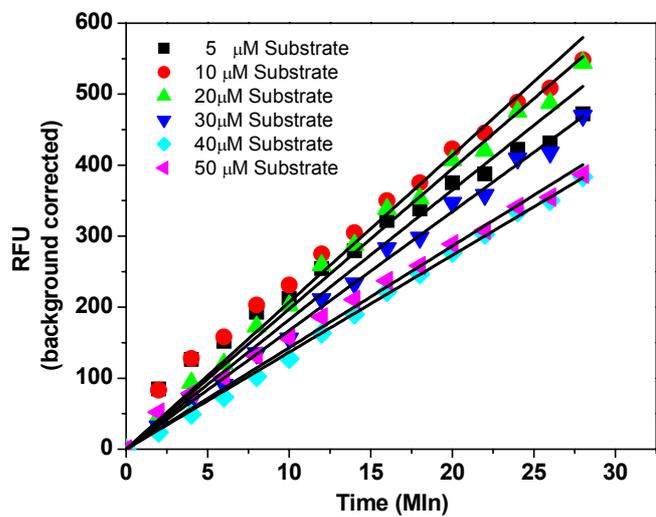


Figure SM1

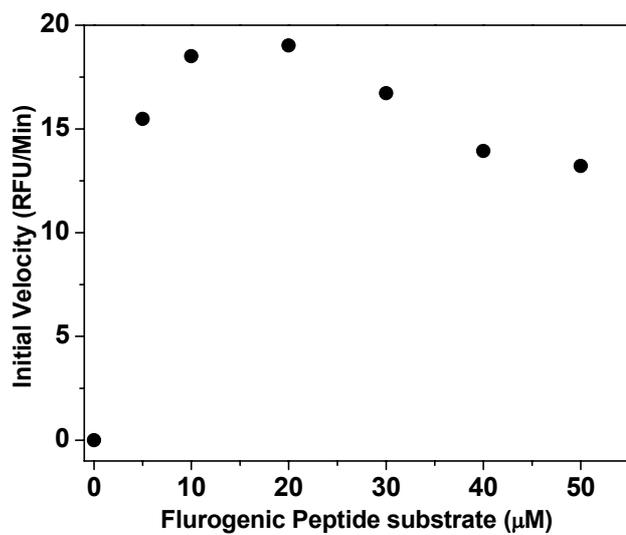


Figure SM2

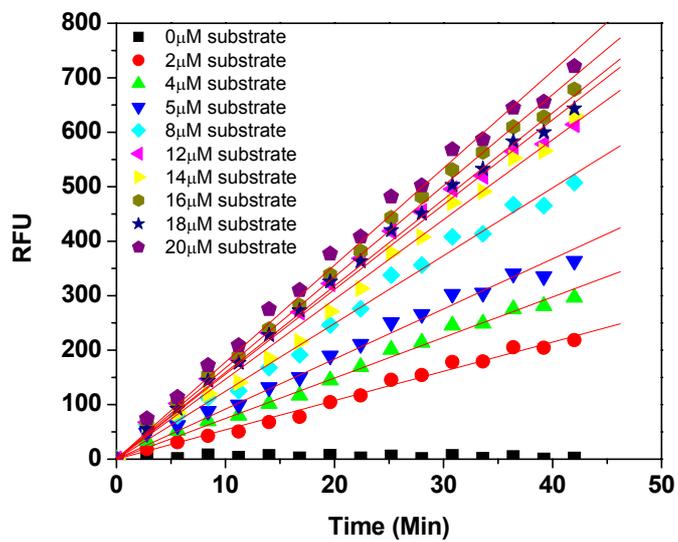


Figure SM3

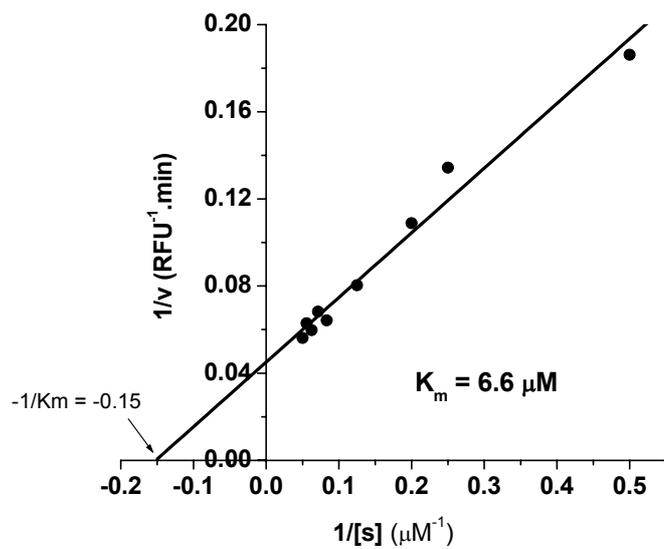
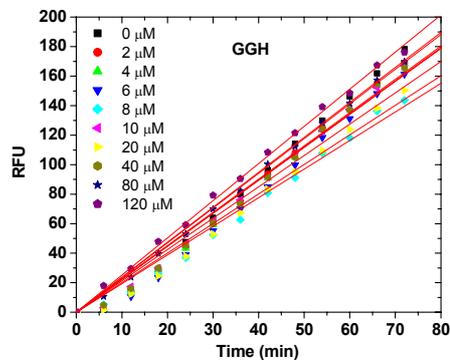
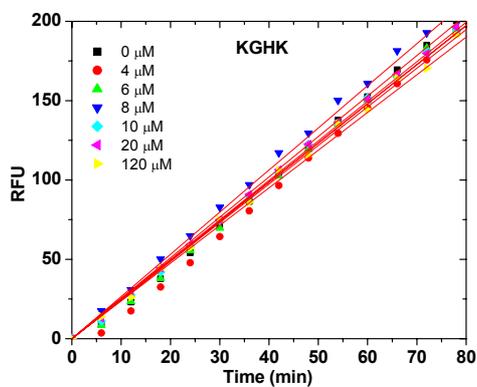


Figure SM4

Dose Dependence GGH



Dose Dependence KGHK



Dose Dependence YIHPF

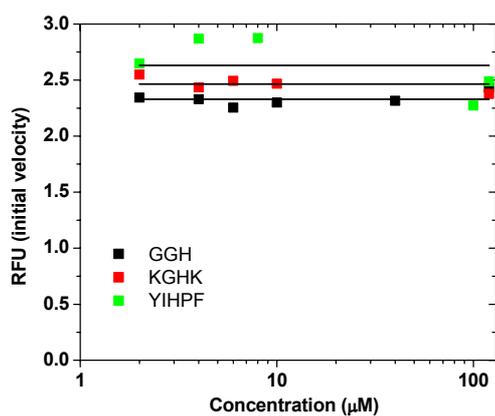
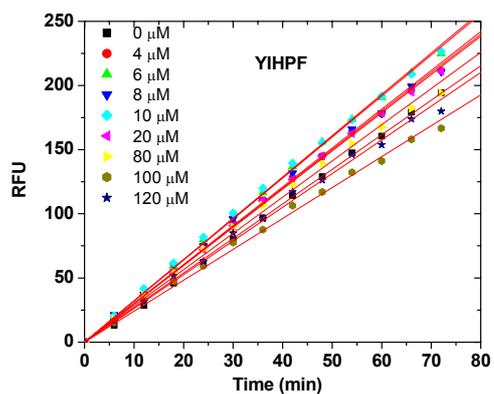


Figure SM5

For Cu²⁺

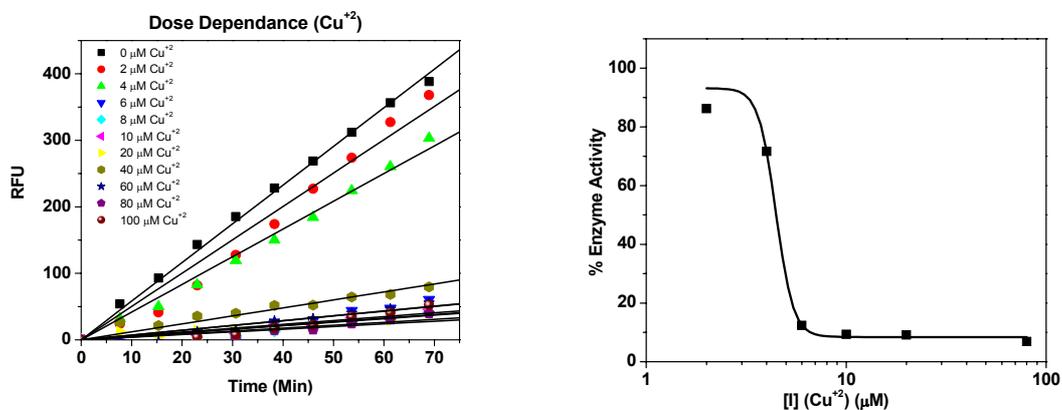


Figure SM6
For Cu(GGH)⁻

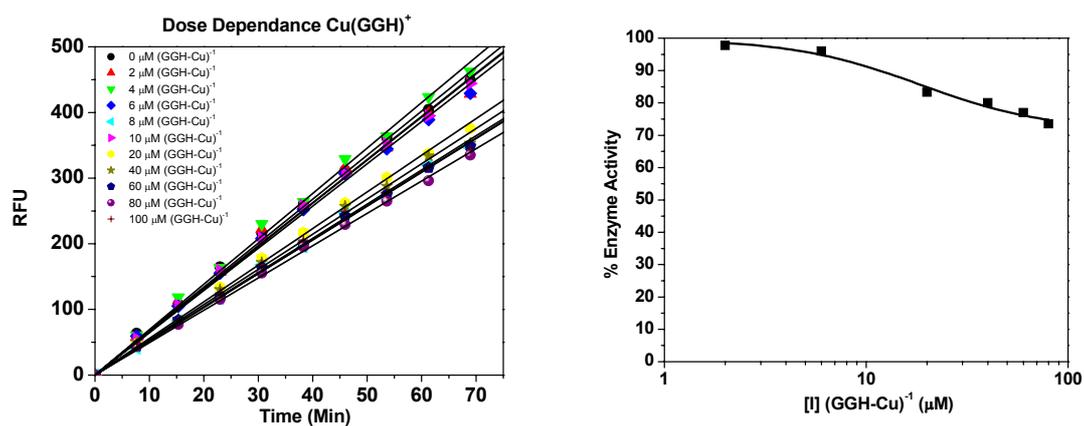


Figure SM7
For Cu(KGHK)⁺

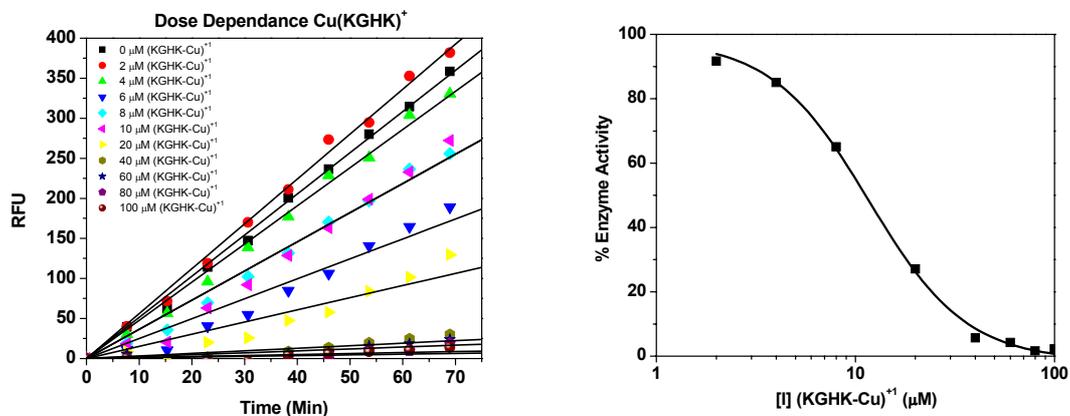


Figure SM8
For Cu(YIHPF)⁺

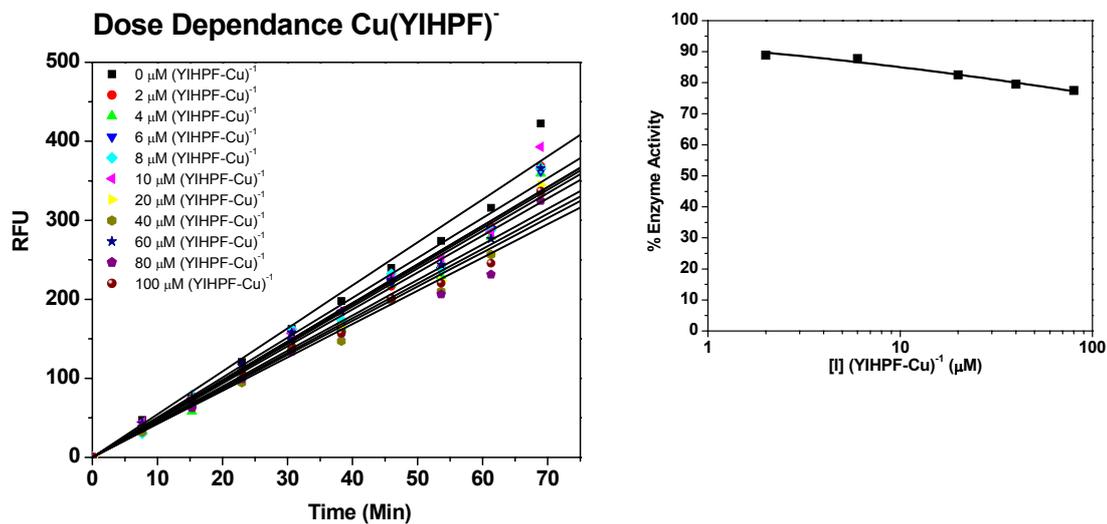


Figure SM9

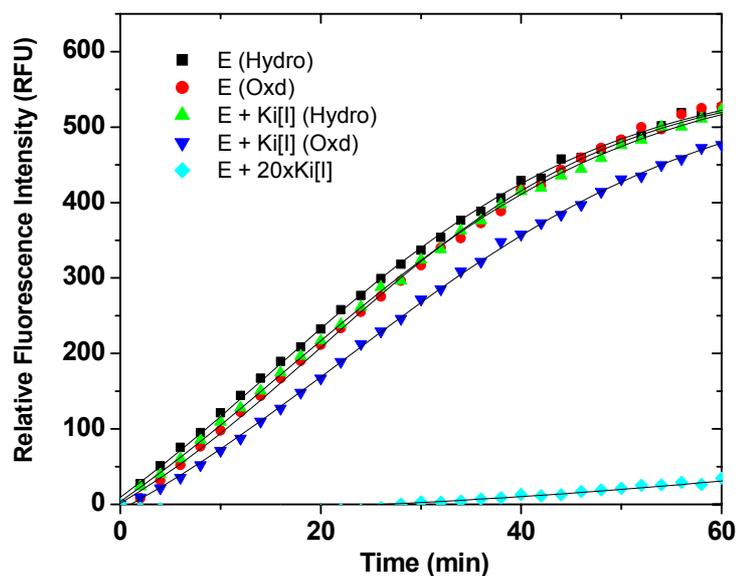


Figure SM10. Plot of RFU with time (min) under various experimental conditions (progress curve), where E(hydro) is the hydrolytic control, E(Oxd) is the oxidative control, E + K(I)(Hydro) contains the inhibitor at a concentration of $4\mu\text{M}$ under hydrolytic conditions, E + K(I)(Oxd) contains the inhibitor at a concentration of K_i under oxidative conditions, and E + $20\times K(I)$ contains the inhibitor at a concentration 20-fold higher than K_i under oxidative conditions

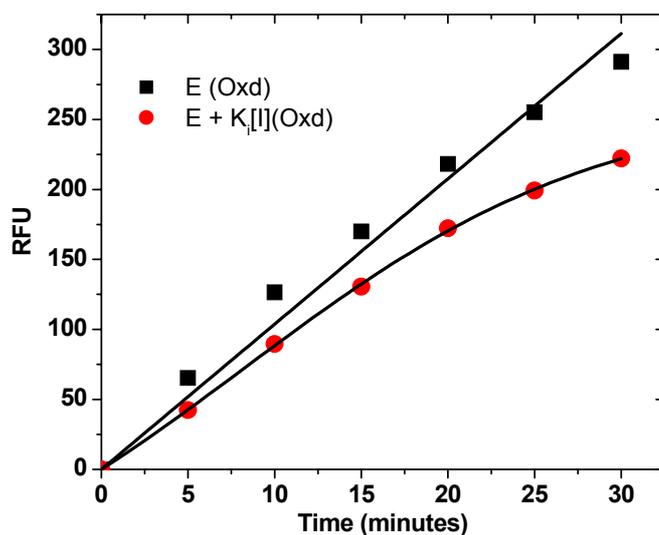


Figure SM11. Plot of RFU with time (min) under oxidative experimental conditions (progress curve), where E (Oxd) is the oxidative control, E + K(I)(Oxd) contains the

Supplementary Material for Chemical Communications
This journal is © The Royal Society of Chemistry 2005

inhibitor at a concentration of K_1 under oxidative conditions.