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PROLYL-4-HYDROXYLASE OXYGEN ACTIVATION AND ASCORBATE PROTECTION

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Abstract

It has long been known that ascorbate is necessary to the human diet for the proper formation of collagen. Without ascorbate, the enzyme prolyl-4-hydroxylase becomes inactivated and is no longer able to form the 4-hydroxyproline residues needed for stable collagen. However, the exact mechanism of inactivation has remained unknown. Using a P4H from PBCV-1, it was possible to determine enzymatic interactions with ascorbate. The results of this study demonstrate that ascorbate is able to reduce both singly and doubly oxidized iron species. By a one electron reaction, ascorbate reduces the inactive P4H·Fe(III) complex that forms by the adventitious oxidation of P4H·Fe(II). Alternatively, ascorbate can reduce the P4H·Fe(IV) intermediate. Presumably, the P4H·Fe(IV) is reduced by two single electron reduction reactions to P4H·Fe(III) then P4H·Fe(II). The reduction of P4H·Fe(IV) is of particular significance because P4H self-hydroxylation can occur when no substrate is present, a process called the "untriggered" reaction. By reducing the extremely reactive P4H·Fe(IV) intermediate that may form during the untriggered reaction, ascorbate is able to prevent enzymatic self hydroxylation and therefore inactivation.

This study also sought to elucidate binding interactions between ascorbate, αKG, and P4H. It was shown that ascorbate and αKG directly compete with each other for binding to P4H.

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Introduction

Post-translational modifications of proteins are mechanisms by which cells can expand their repertoire of protein building blocks beyond the 20 amino acids incorporated by the ribosome. The effects of the post-translational modifications can be categorized into two major groups: signaling and functional changes. The hydroxylation of proline C4, giving 4(*R*)-hydroxyproline (Hyp) (Figure 1), can serve as an important example because it can be classified into both of these categories depending on the context.

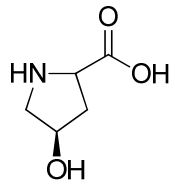

Figure 1
4(*R*)-hydroxyproline

The signaling functionality of proline C4 hydroxylation has been linked primarily to cellular oxygen sensing in metazoans, including humans (1). The enzymes involved, prolyl hydroxylase domains (PHDs), require oxygen for their hydroxylation chemistry. An important member of this class is PHD2. When oxygen is present, PHD2 catalyzes the hydroxylation of proline residues 402 and 564 in the α subunit of the human transcription factor, hypoxia-inducible factor 1 (HIF-1). The hydroxylation of these residues causes HIF-1α to be degraded by the proteasome after marking by the ubiquination pathway. However, if oxygen levels are low, PHD2 can no longer function optimally. Unmodified HIF-1α is not degraded by the cell. It can bind to its partner subunit, HIF-β, and the co-activator, p300. Binding of this complex to the hypoxia responsive element (HRE) site on DNA results in altered gene expression that allows the cell to respond to the low oxygen environment (4). One of the effects of HIF-1α activation, angiogenesis, is of particular interest for cancer research. It has been shown that tumors require the increased formation of blood vessels to supply the needed amount of oxygen and nutrients (4). Therefore, there has been focus on determining pathways that may inhibit HIF-1α activation, such as PHD2.

In contrast to the recent developments on the role of hydroxylation of specific proline residues at C4 in cellular signaling, the importance of hydroxyproline to the functional stability in collagen has long been known. Collagen is a structural protein that takes the shape of a long triple helix. The individual peptide chains have repeating glycine-x-y units, where y is often Hyp. In the case of collagen, the proline is converted to Hyp by prolyl-4-hydroxylase (P4H). Hyp appears to have two different roles in the function of collagen (17). First, Hyp helps to stabilize the collagen super structure, particularly with regards to temperature. Collagen lacking Hyp has a melting temperature (T_m) of 26 ^oC, while the collagen containing Hyp has a T_m of 39 ^oC (2). Without these hydroxyl groups on the proline, collagen quickly denatures at temperatures found in the human body. Hydroxyproline also facilitates cellular secretion of procollagen, the building block of collagen. Therefore, if the hydroxylation of the proline residues does not occur, procollagen will not be excreted from cells efficiently and the extracellular matrix of collagen will not be properly assembled.

Both PHD2 and P4H are members of a larger group of enzymes known as Fe(II)- and α-ketoglutarate-dependent dioxygenases (3,9). As their name suggests, these enzymes are characterized by their use of a single iron ion that functions as a catalytic cofactor in the active site. Additionally, members of this family use oxygen and α-ketoglutarate (αKG) as cosubstrates in the hydroxylation of their prime substrates. Much is known about the specific mechanistic cycle of this class of enzymes (Figure 2). The “resting” form of the enzyme is the reduced Fe(II) state with three coordinated H_2O molecules, termed the binary complex. αKG and substrate then bind in an ordered-sequential mechanism (19). First, αKG binds in bidentate fashion, displacing two water molecules (16). This produces the anaerobically stable ternary complex. The ternary complex is characterized by an absorption feature centered near 520 nm due to the

metal-to-ligand charge transfer between the Fe(II) and αKG. After αKG binding, the substrate binds, leading to the release of the final water (22). The resultant enzyme state, called the quaternary complex, generally retains an absorption spectrum similar to that of the ternary complex. In the case of P4H, there is no change in the absorption spectrum upon binding of the proline containing peptide substrate to the ternary complex (9). Upon addition of oxygen, the enzyme cleaves the dioxygen molecule and decarboxylates the αKG to succinate. This process produces the most important mechanistic feature of this class of enzymes, the Fe(IV)-oxo (ferryl) species (intermediate J in Figure 2) (18). This ferryl complex has the ability to abstract a hydrogen atom from the substrate to form a ferric hydroxide complex and a substrate radical. The substrate radical then adds to the coordinated hydroxyl radical equivalent to form the hydroxylated product. The products, succinate and the hydroxylated substrate, are then released by the enzyme to reform the binary complex.

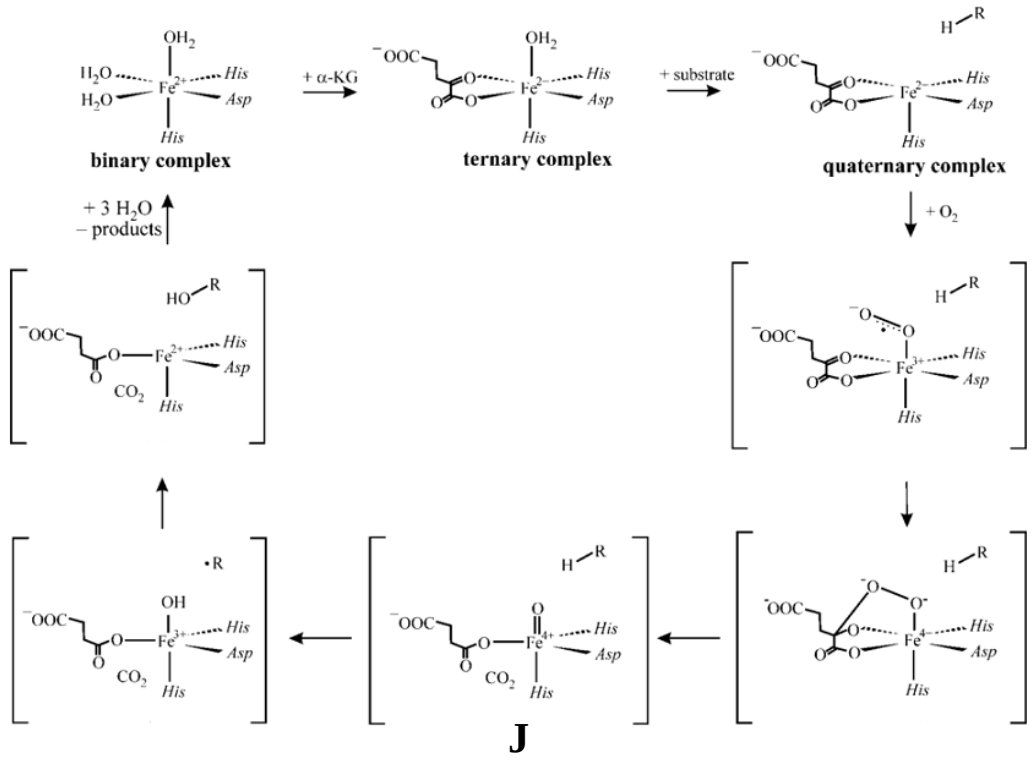

Figure 2
Consensus mechanism for members of the Fe(II)- and αKG-dependent dioxygenase family. Taken and modified from (9).

Previously, a thorough mechanistic study was conducted with a P4H from *Paramecium bursaria Chlorella* virus-1, PBCV-1 P4H. While the biological targets of this enzyme are unknown, it was found to hydroxylate the proline residues of certain small peptides, such as $(PAPK)_3$, with unusual efficiency. This feature makes this particular P4H useful for study because it obviates the use of a large substrate complex similar to procollagen (7). Hoffart *et al*. demonstrated, using a combination of stopped-flow absorption and freeze-quench Mössbauer experiments, that PBCV-1 P4H does indeed form the canonical Fe(IV) species (9). Additionally, titrations revealed the formation of both ternary and quaternary complexes as intermediates. In contrast to other Fe(II)- and αKG-dependent dioxygenases, the binding of peptide substrate to form the quaternary intermediate did not cause a shift in the absorption maximum, nor did it

cause an increase in αKG binding affinity. Instead, binding of the peptide caused a decrease in the molar absorptivity of the complex, called a hypochromic shift.

Enzymes of the α-ketoglutarate dioxygenase family are optimally activated for turnover only when the substrate is present, an effect termed "substrate triggering". In substrate triggering for this family, activation of the Fe(II) to react efficiently with O_2 occurs after the substrate binds. Conversely, if the substrate is not present, oxygen activation is much slower, but can still occur in the "untriggered" reaction. The untriggered reaction is believed to be a major source of enzyme inactivation. While the specific mechanism is not completely known, it is hypothesized that the Fe(IV) forms in the untriggered reaction (11,13). As there is no substrate present, the Fe(IV) instead targets a nearby amino acid residue (Figure 3). Often times, the residue is either a tyrosine or tryptophan. The hydroxylation allows the iron to return to its reduced Fe(II) state, but the enzyme is now inactivated due to the hydroxylated amino acid residue (13). If the Fe(II) is subsequently oxidized by dissolved O_2 to Fe(III), the residue then coordinates to the Fe(III) in the active site to produce a blue or green chromophore (13,20). Studies on the untriggered reaction of chick embryo P4H have previously determined that enzymatic inactivation occurs rapidly. Within 60 seconds of the reaction's start, the activity of P4H had been reduced to 8% (5). Additionally, EPR analysis has shown that the relative amount of Fe(III) reaches a maximum within 300 seconds of reaction initiation (Figure 4). Without doubt, the untriggered reaction is detrimental to sustained enzymatic activity.

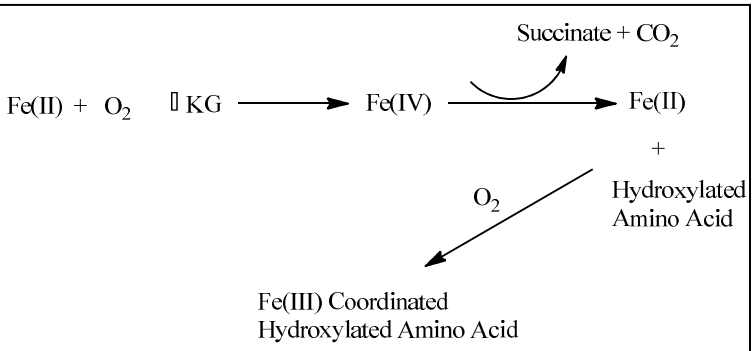

Figure 3
Hypothesized mechanism for the formation of the hydroxylated amino acid residue and subsequent formation of the Fe(III) coordinated hydroxylated amino acid chromophore.

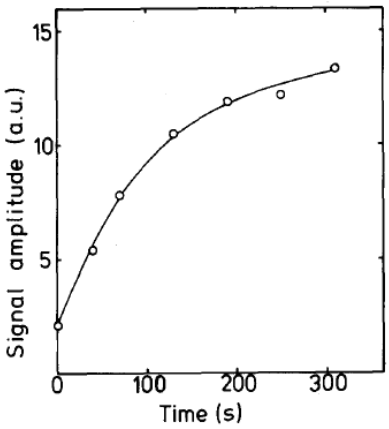

Figure 4
Intensity of Fe(III) signal as monitored by EPR vs. time. Taken and modified from (5).

Curiously, experiments in guinea pigs, one of the few animals unable to synthesize ascorbate, identified P4H as having a functional ascorbate dependence (17). Ascorbate, also known as vitamin C, is a biologically important single-electron reductant. Humans also lack the ability to synthesize ascorbate, requiring them to consume ascorbate in the diet. Ranging from bone deformities to abnormal bleeding, the symptoms of ascorbate deficiency, or scurvy, are widely recognized (12).

As seen in Figure 2, the proposed mechanism for P4H does not include ascorbate in the reaction. However, many sources often show ascorbate as part of an electronically unbalanced reaction (Figure 5). In the accepted mechanism, the four-electron reduction of dioxygen is balanced with a two-electron oxidation of proline and the two-electron oxidation in the decarboxylation of αKG to succinate and CO_2. The commonly depicted two-electron oxidation of ascorbate is not balanced by the reduction of any substrate and is therefore inaccurate. Additionally, ascorbate is often referred to as a cofactor or coenzyme; both designations are

inaccurate (21). As demonstrated by Tuderman *et al*., ascorbate is not used stoichiometrically in the catalytic reaction.

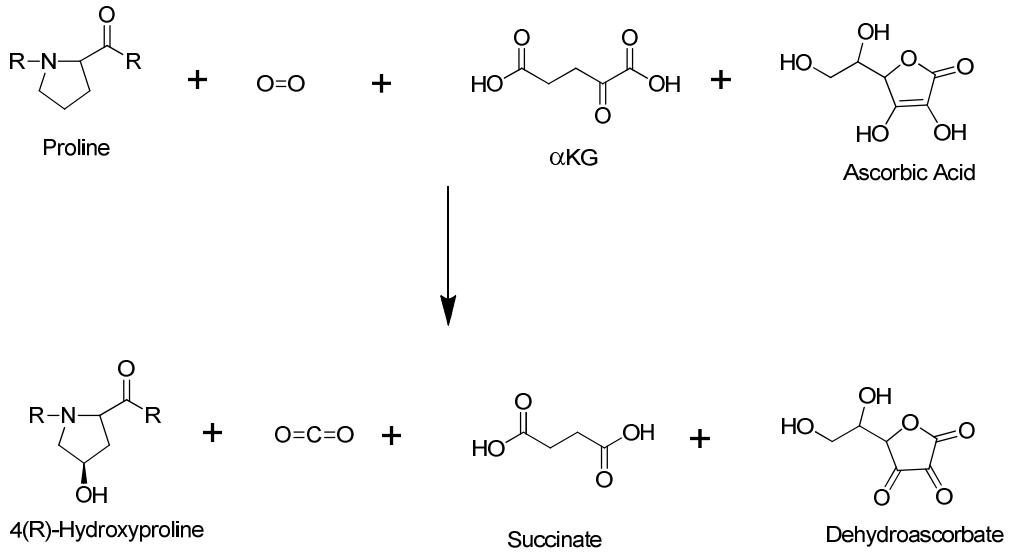

Figure 5
Electronically Unbalanced Reaction. The two electron oxidation of ascorbate to dehydroascorbate is unaccounted for in the products.

It is hypothesized that ascorbate is effective both in keeping the iron reduced in the active site of P4H and also in preventing the deleterious effects of the untriggered reaction. Together, these properties of ascorbate could confer the ability to keep P4H and other enzymes in this family active for long periods of time. While the mechanism of action is largely unknown, many theories have been devised to explain the role(s) of ascorbate. It could reverse iron oxidization by reducing any Fe(III) sites that are generated by non-enzymatic oxidation of the active Fe(II) form. The iron reduction would return the enzyme to its active state. This hypothesis is supported by an EPR-spectroscopic study of chick embryo P4H, in which the gradual increase in intensity of the hallmark g = 4.3 signal associated with high-spin Fe(III) was observed (5).

While previous studies have established that ascorbate can reactivate the enzyme through iron reduction, the specific mechanism of this process is unknown. For example, it has been suggested that ascorbate could reduce the Fe(III) after it disassociates from the enzyme, and the rebinding of the Fe(II) could then reactivate P4H (5). Conversely, it is also possible that ascorbate could reduce the Fe(III) in situ, while the metal is still bound. In addition to the unknown mechanism, the kinetics of the ascorbate reduction of P4H·Fe(III) have not been explored.

Another hypothesis of ascorbate action is centered around reduction of the Fe(IV) that may form during the untriggered reaction. Reduction of this species would prevent any detrimental self-hydroxylation reactions. This hypothesis is consistent with data that suggests that ascorbate is being stoichiometrically consumed in the uncoupled turnovers (6). If the ascorbate was utilizing a single-electron reduction mechanism, semidehydroascorbate would form and quickly disproportionate into ascorbate and dehydroascorbate (Figure 6). This mechanism would lead to one ascorbate utilized for every two untriggered reactions. Conversely, Fe(IV) reduction would require two electrons, and therefore two ascorbate equivalents, and yield the observed 1:1 ratio of ascorbate consumption to untriggered reactions (8). The final method of action could involve limiting the rate of oxygen activation, preventing the formation of the reactive iron species when substrate is not present. This study sought to determine which of the proposed mechanisms of activation of P4H by ascorbate are operant and which are the most important.

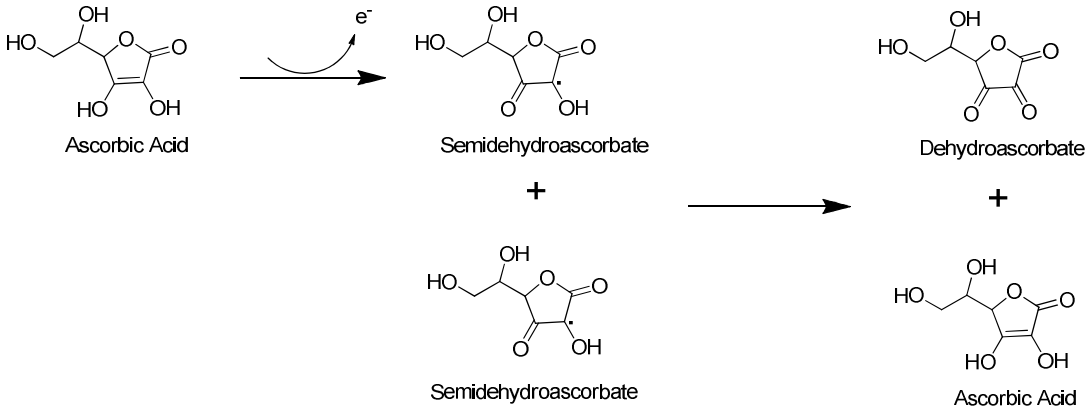

Figure 6
One electron oxidation of ascorbate.

Materials and Methods

Chemicals

EDTA and sodium hydroxide were purchased from EMD Chemicals (Darmstadt, Germany). Sodium ascorbate was purchased from Spectrum Chemical Mfg. Corp. (New Brunswick, NJ). Sodium chloride and ethanol were purchased from VWR International (Bridgeport, NJ). Ferrous ammonium sulfate, Tris-base, and hydrochloric acid were purchased from J.T. Baker (Phillipsburg, NJ). All other materials were purchased from Sigma Corp. (St. Louis, MO).

Materials

Frozen stocks of *E. coli* BL21 (DE3) containing a His_6 PBCV-1 P4H pET15b plasmid (Figure 7) were obtained from previous work on P4H (9). A peptide, $(PAPK)_3$, was used as a substrate (9). $(PAPK)_3$ was synthesized by Anne Stanley in the Macromolecular Core Facility of the Penn State Milton S. Hershey Medical Center (Hershey, PA).

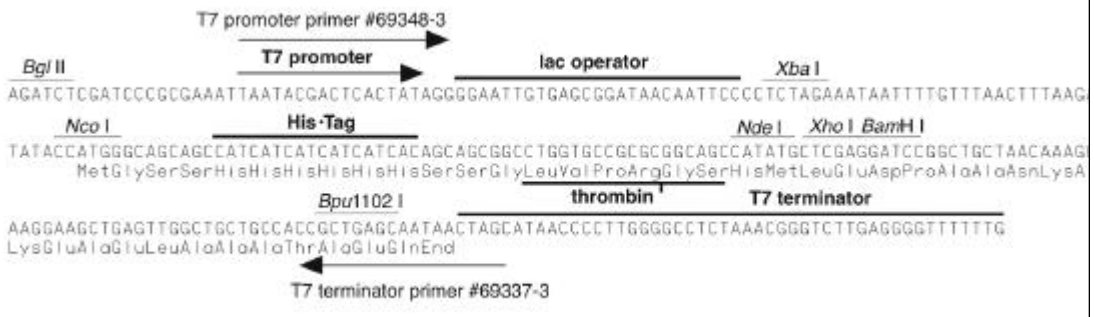

Figure 7
pET15b Expression Vector from Novagen (Darmstadt, Germany).

Over-Expression of P4H in *E. coli*

A frozen stock *E. coli* BL21 (DE3) was plated onto 100 μg/mL Luria-Bertani (LB) agar media (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 14 g agar dissolved in 1 L double distilled H_2O at pH 7.4). The plate was incubated at 37 ^oC overnight. A single colony from the plate was used to inoculate 1 L of 100 μg/mL ampicillin contained rich LB media (35 g tryptone, 20 g yeast extract, and 5 g NaCl dissolved in 1 L ddH_2O at pH 7.4). The flask was allowed to grow with rigorous shaking overnight at 37 ^oC. The 1 L of cells was used to seed 60 L of rich LB. The cells were incubated in the fermenter at a constant pH (7.4) at 37^oC and were continuously oxygenated and stirred. The culture was grown to an OD_{600} of 2. The culture was cooled to 18 ^oC and inoculated with 400 μM IPTG. The cells were incubated overnight and then pelleted in a continuous flow centrifuge. The cell paste was collected and stored at -80 ^oC.

P4H Purification

All steps in the purification procedure were carried out at 4 ^oC unless otherwise noted. 50 g of wet cell mass was resuspended in 200 mL of Buffer A (20 mM Tris-Cl, 500 mM NaCl, pH 8.0). The cells were then lysed by two passes through a french pressure cell. The lystate was centrifuged at 50,000 *g* for 1 hour. The cleared lysate was then loaded onto a 50 mL Ni(II)-nitrilotriacetate agarose resin. The column was washed with 200 mL 60 mM imidazole in Buffer A. The protein was eluted with 500 mM imidazole, and fractions were collected. The P4H-containing fractions were then dialyzed against 50 mM Tris-Cl, 10% glycerol, and 1 mM EDTA at pH 8.0 overnight. Dialysis was repeated without EDTA in the buffer, called Buffer B. The enzyme was concentrated to 5 mL and loaded onto a GE Healthcare Life Sciences Sephacryl S-200 gel filtration column (Piscataway, NJ). The protein was eluted at 0.5 mL/min with Buffer B,

and fractions were collected. Those fractions with the highest purity were pooled and concentrated. P4H was frozen and stored at -80 ^{o}C.

Mass Spectrometry Sample Preparation and Analysis

The samples were prepared at room temperature with Fe(II) added last to initiate the reaction (Figure 8). Reactions were quenched in a 1:1 ratio with ethanol after 10 minutes to denature P4H and stop the reaction. P4H was removed from the reaction mixture by using 10K Millipore Microcon Centrifugal Filter Units (Billerica, MA). The flow-through from the filtration unit was directly injected into a Waters Micromass ZQ mass spectrometer (Milford, MA) for analysis. For peptide determination, a mobile phase of 50% HPLC grade methanol and 0.05% triethylamine in water was used in the positive ion mode. For succinate quantification, deuterium labeled succinate was added to the mixture directly after the ethanol quench. The same mobile phase was used, with signal monitored in the negative mode.

Protein Deoxygenation

The protein was deoxygenated as described by Parkin, *et al.* (15).

Buffer Deoxygenation

Buffer B was deoxygenated on vacuum line by cycling four times between 10 minutes vacuum and 10 minutes of 1 atmosphere argon. After the last cycle, the buffer was left under argon atmosphere for 30 minutes.

αKG Titration to Determine Binding Affinity

Anoxic stocks of ascorbate and αKG were prepared in a glove box by dissolving in O_2-free Buffer B. A stock of ferrous ammonium sulfate was made and quickly mixed with deoxygenated P4H and ascorbate to the desired concentration (Figure 9B). The mixture was added to a covered quartz cuvette and used as a blank for an Agilent 8453 UV-Visible Spectrophotometer (Santa Clara, CA). αKG was titrated into the cuvette and absorbance readings were taken. The titration continued until no absorbance change could be seen upon addition of αKG.

Monitoring Binding of αKG by Stopped-Flow Absorption Spectrophotometry

Anoxic stocks of αKG and ascorbate were perpared in a glove box with deoxygenated Buffer B. They were mixed to the appropriate concentrations (Figure 10) with deoxygenated P4H. The stock of ferrous ammonium sulfate was quickly made and immediately aliquoted into the enzyme mixture to ensure that no oxygen could contaminate the Fe(II). The syringes were loaded and the solutions were allowed to cool to 4 ^{o}C for 15 min. The ram drive mixed the solutions together, and the change in absorbance at 520 nm was monitored in photomultiplier tube (PMT) mode of an Applied Photophysics SX20 Stopped-Flow Spectrometer (Surrey, UK) using a slit width of 1 mm.

Stopped-Flow Absorption Spectroscopy to Monitor Reduction of P4H·Fe(III)

Anoxic solutions of ferrous ammonium sulfate were prepared in the glove box and immediately added to O_2 free P4H to yield an appropriate final concentration (Figure 11A,B). A stock of potassium ferricyanide was prepared and aliquoted into the P4H·Fe(II)mixture. This

sample was incubated for five minutes to allow Fe(II) to be oxidized to Fe(III). Stocks of αKG and ascorbate were created as before with deoxygenated Buffer B, and added to the syringes as described (Figure 11A,B). As before, the stopped-flow syringes were loaded, and the solutions cooled to 4 ^{o}C for 15 min. Actuation of the drive mixed the two solutions, and the absorbance was observed in PMT mode with a slit width of 1 mm.

UV Visible Absorption Spectroscopy to Monitor Reduction of P4H·Fe(III)

Stocks and P4H were prepared as in the stopped-flow reduction experiment and mixed to give to the appropriate concentrations (Figure 11D). The P4H, Fe(II), potassium ferricyanide, and αKG mixture was added to a quartz cuvette and used as a blank for an Agilent 8453 UV-Visible Spectrophotometer. The ascorbate was added and the change in absorbance was monitored every 10 seconds.

Mass Spectrometry to Monitor Ascorbate Reduction of P4H·Fe(IV)

The samples were prepared at 4 ^{o}C with Fe(II) added last to initiate the reaction (Figure 12). Because very high concentrations of ascorbate were known to completely remove oxygen from solution, the samples were incubated under an atmosphere of pure O_2. Specifically, the samples were cycled between three seconds of vacuum followed by reoxygenation three times and then left under oxygen for 10 minutes. The samples were quenched by the addition of an equivalent volume of ethanol.

Results

Ascorbate Activation of P4H for Turnover

Without clear data showing the need for ascorbate for optimal catalysis by PBCV-1 P4H, it was important to first establish dependence of activity on the presence of the reductant. Figure 8 illustrates the a requirement for ascorbate for productive turnover by the enzyme, where each hydroxylation of the $(PAPK)_3$ substrate was viewed as a successful turnover. Specifically, minimal turnover was observed at ascorbate concentrations ranging from 0-10 µM. However, at 100 µM ascorbate, the enzyme was observed to affect many turnovers, producing both singly and doubly hydroxylated products. At ascorbate concentrations of 1 mM and 2 mM, there appeared to be a maximal level of turnover with very little difference between these two concentrations. The effect of ascorbate concentrations even greater than 2 mM is considered below.

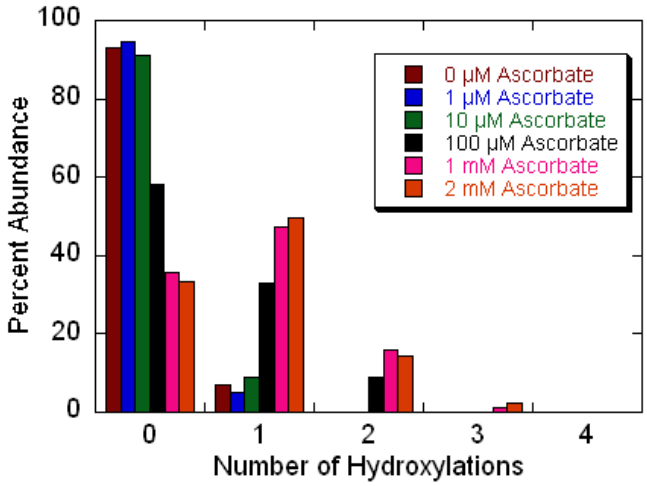

Figure 8
Ascorbate dependent increase in number of substrate hydroxylations after 10 minutes. Samples were prepared with 1 µM P4H, 0.7 µM Fe(II), 1 mM αKG, 1 mM $(PAPK)_3$, and ascorbate. A single hydroxylation was observed as a shift in the peptide peak of the mass spectrometer by 16 m/z. Each additional hydroxylation resulted in a further increase of 16 m/z.

αKG and Ascorbate Titrations

Generally, addition of the prime substrate to the ternary Enzyme·Fe(II)·αKG complex causes a shift in the absorbance of the chromophore (18). Additionally, it has been observed that the substrate can allow for a decrease in the K_D for αKG complex by causing a conformational change that traps the αKG in the active site (18). For P4H, the development of the 520 nm absorption of the ternary complex upon addition of increasing concentration of αKG was monitored both in the absence and in the presence of 10 mM ascorbate. As seen in Figure 9A, the presence of ascorbate had no major affect on the absorption spectrum of the complex. This observation was expected, as no shift in absorption occurs with the addition of the peptide substrate (9). However, the K_D for αKG seemed to increase slightly in the presence of ascorbate: 323 ± 49 µM without ascorbate compared to 448 ± 12 µM with 10 mM ascorbate (Figure 9B). This increase in K_D suggests that ascorbate could inhibit αKG binding rather than promote.

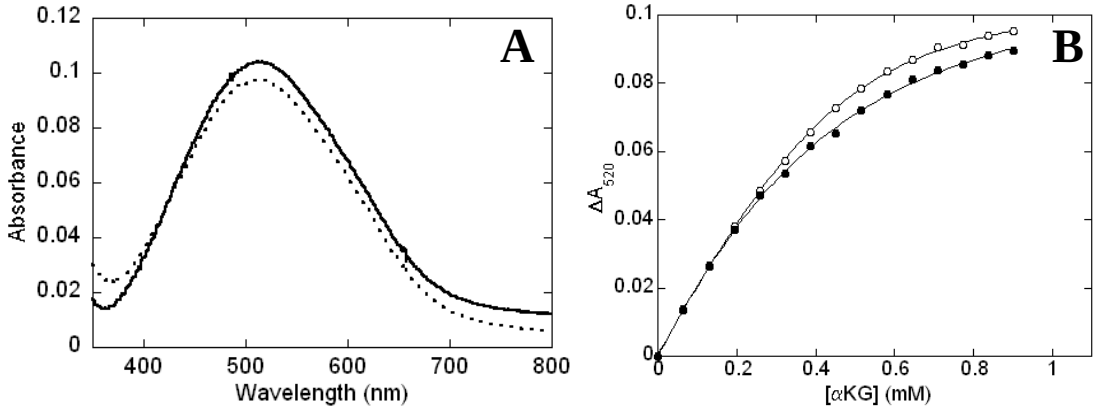

Figure 9

(A) Absorption spectra of an anoxic solution of 0.5 mM P4H, 0.35 mM Fe(II), and 0.9 mM αKG with 10 mM ascorbate (solid trace) or 0 mM ascorbate (dotted line).

(B) Titration of αKG into 0.5 mM P4H and 0.35 mM Fe(II) with 10 mM ascorbate (open circles) or 0 mM ascorbate (filled circles). The points were fit by the quadratic equation for binding (solid line).

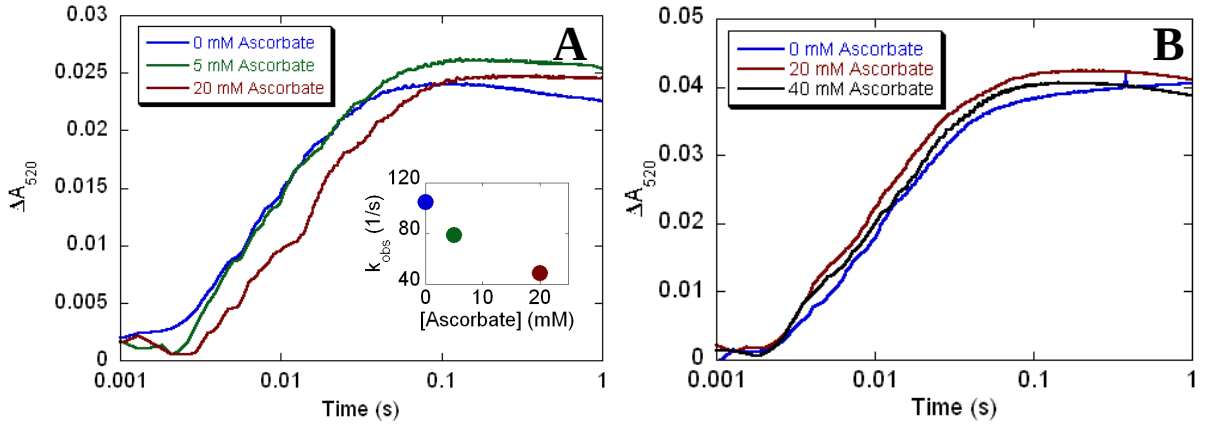

Figure 10
(A) Inhibition of αKG binding rate when P4H·Fe(II) was preincubated with ascorbate. A solutions containing 0.8 mM P4H, 0.6 mM Fe(II), and varying ascorbate concentrations was mixed with 1.6 mM αKG anaerobically at 4 ^{o}C.

(A inset) k_{obs} for each concentration of ascorbate was determined using an exponential fit.

(B) No inhibition of αKG binding rate when αKG was preincubated with ascorbate. A solution containing 0.8 mM P4H and 0.6 mM Fe(II) was mixed with s solution 1.6 mM αKG and varying ascorbate concentrations anaerobically at 4 ^{o}C.

αKG and Ascorbate Binding Kinetics

The kinetics of αKG binding to the TauD·Fe(II) complex had previously been used as a probe to determine the order of substrate binding (14). Therefore, stopped-flow absorption spectroscopy was used to help determine whether ascorbate inhibits αKG binding, as is suggested by the increase in K_D observed by titration. To monitor αKG binding, a solution of Fe(II)-bound P4H containing varying concentrations of ascorbate was mixed with a solution of αKG. Presumably, the rate of formation of the ternary complex is determined by monitoring the development of the 520 nm absorption band. As seen in Figure 10A, ascorbate has a significant retarding effect on αKG binding to the Fe(II)-bound enzyme. With increasing concentrations of ascorbate, the rate constant for formation of the ternary complex decreases (Figure 10A inset).

This observation suggests that ascorbate interferes with binding of αKG to the enzyme's active site. However, the presence of ascorbate had no effect on the rate constant for αKG binding when enzyme was mixed with a solution containing αKG (Figure 10B). Therefore, the αKG is able to bind faster to the P4H·Fe(II) complex, suggesting that the K_D of αKG is much less than the K_D of ascorbate.

Ascorbate Reduction of the P4H·Fe(III) Complex and αKG Inhibition Thereof

Reduction of the P4H·Fe(III) complex, which was formed by oxidation of the P4H·Fe(II) complex with the one-electron oxidant ferricyanide, was shown to have a clear dependence on both αKG and ascorbate concentration (Figure 11). Increasing concentrations of ascorbate increase the rate of reduction, as monitored by the change at 318 nm (Figure 11A). Inclusion of varying concentrations of αKG in the enzyme solution slows the reduction reaction (Figure 11B). At reaction times longer than 10 seconds (Figure 11D), the samples with the higher concentrations of αKG were seen to return to the fully reduced state. This observation is consistent with the hypothesis that αKG may be bound to the active site thereby preventing P4H·Fe(III) reduction. With high concentrations of αKG, the premixed P4H·Fe(III)·αKG state is favored, which can not be reduced. After the initial population of αKG-free P4H·Fe(III) is reduced (Figure 11A), the αKG slowly dissociates, allowing the remaining P4H·Fe(III) to be reduced (Figure 11D). The P4H·Fe(II) can then rebind αKG to form the 520 nm P4H·Fe(II) ·αKG ternary complex (Figure 11D inset). By determining the change in absorbance at 318 nm after the first phase of reduction using an exponential fit, it was possible to indirectly monitor the concentration dependence of αKG binding to P4H·Fe(III). These observations were plotted and fit to calculate a K_D for αKG to P4H·Fe(III), 746 ± 79 μM (Figure 11C).

It should also be noted that the P4H·Fe(III) complex was determined to be stable. If the Fe(III) was able to dissociate from the active site it would complex with the reduced potassium ferricyanide, potassium ferrocyanide, to form a deep blue pigment, Prussian blue. The lack of Prussian blue formation indicated that Fe(III) remains sequestered in the enzyme active site. In contrast to the prevention of Prussian blue formation in the presence of P4H, a control without enzyme in solution was shown to yield the Prussian blue pigment.

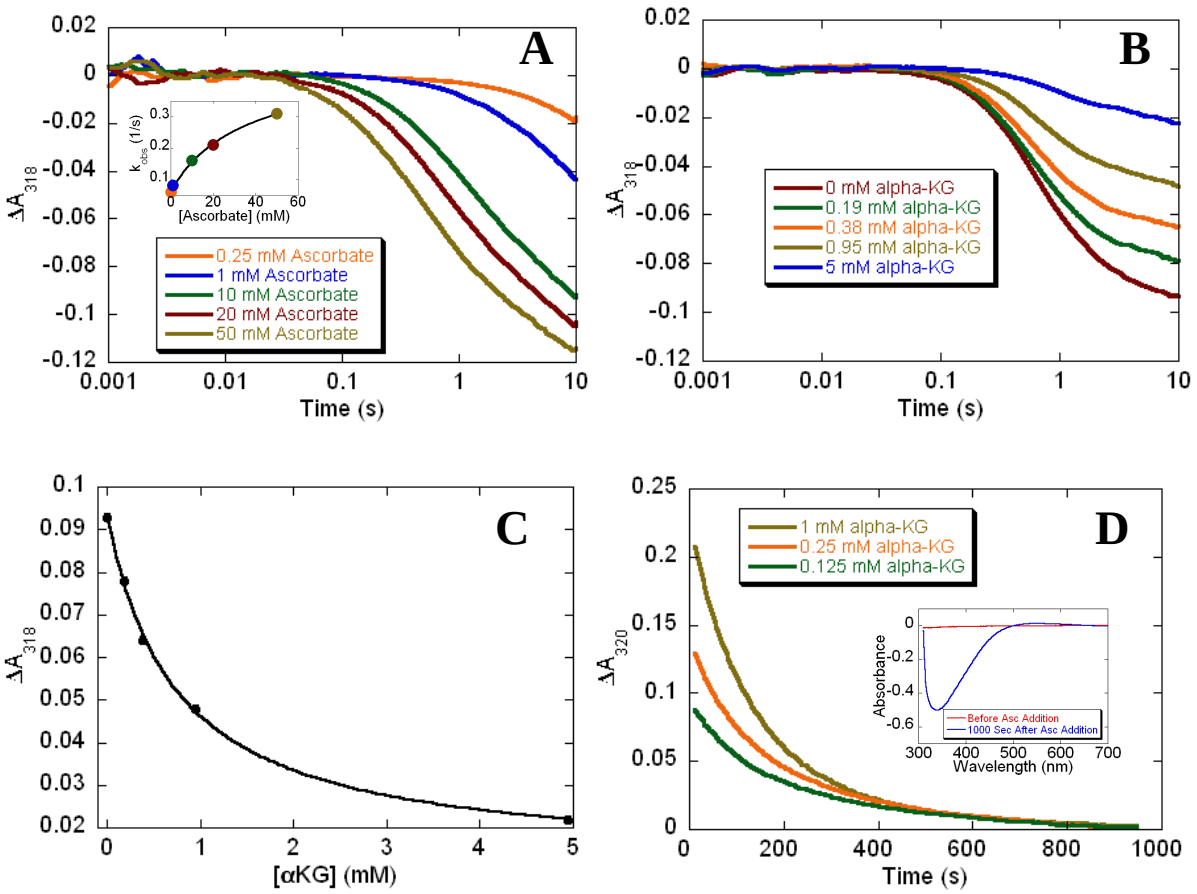

Figure 11

(A) Dependence of the reduction of P4H·Fe(III) on ascorbate. A solution containing 0.25 mM P4H, 0.19 mM Fe(II), and 0.19 mM potassium ferricyanide was mixed with an equal volume of an ascorbate solution.

(A inset) k_{obs} for each concentration of ascorbate was determined using an exponential fit.

(B) Inhibition of P4H·Fe(III) reduction by αKG. A solution containing 0.25 mM P4H, 0.19 mM Fe(II), 0.19 mM potassium ferricyanide and αKG was mixed with an equal volume of a 20 mM ascorbate solution.

(C) Change in absorbance at 320 nm due to reduction of P4H·Fe(III) not bound with αKG. The values represent the change in absorbance of the first phase of reduction (B) as determined by exponential fit. The points were fit to a hyperbolic curve to determine K_D of αKG from P4H·Fe(III).

(D) αKG inhibition of P4H·Fe(III) reduction after 10 seconds. 0.25 mM P4H, 0.19 mM Fe(II), 0.19 mM potassium ferricyanide, 10 mM ascorbate, with αKG.

(D inset) Absorption spectrum of the 1 mM αKG sample from (D) before addition of ascorbate and 1000 seconds after addition of ascorbate.

Ascorbate Inhibition of P4H Turnover

Non physiologically high concentrations of ascorbate were used to probe if ascorbate can reduce the hydrogen abstracting Fe(IV) complex. It should be noted that these experiments are in direct contrast with those that sought to show that ascorbate keeps P4H active when present at low concentrations (Figure 8). As seen in Figure 12A, high concentrations of ascorbate do indeed inhibit proline hydroxylation, consistent with reduction of the ferryl intermediate. To determine if the decrease in hydroxylated product is caused by enzyme inactivation or Fe(IV) reduction, succinate production was monitored. Because succinate is produced as a byproduct in the reaction concomitantly with the Fe(IV) species, it could be used an indirect measure of formation of the Fe(IV) species. Quantification of succinate (Figure 12B) suggested that, regardless of ascorbate concentration, the amount of succinate produced remained almost constant, providing evidence for formation of the ferryl intermediate and subsequent reduction by ascorbate. An alternative explanation for ascorbate inhibition is that it might compete with $(PAPK)_3$ as a substrate. This possibility was tested by increasing the concentration of peptide to determine if ascorbate inhibition was reversed. The subsequent mass-spectrometric analysis revealed that increasing concentration of $(PAPK)_3$ failed to reverse ascorbate inhibition of hydroxylation (Figure 12C). Therefore, ascorbate does not compete with $(PAPK)_3$ in binding. These results strengthen the hypothesis that ascorbate prevents productive turnover via a mechanism that is independent of $(PAPK)_3$ concentration, such as Fe(IV) reduction.

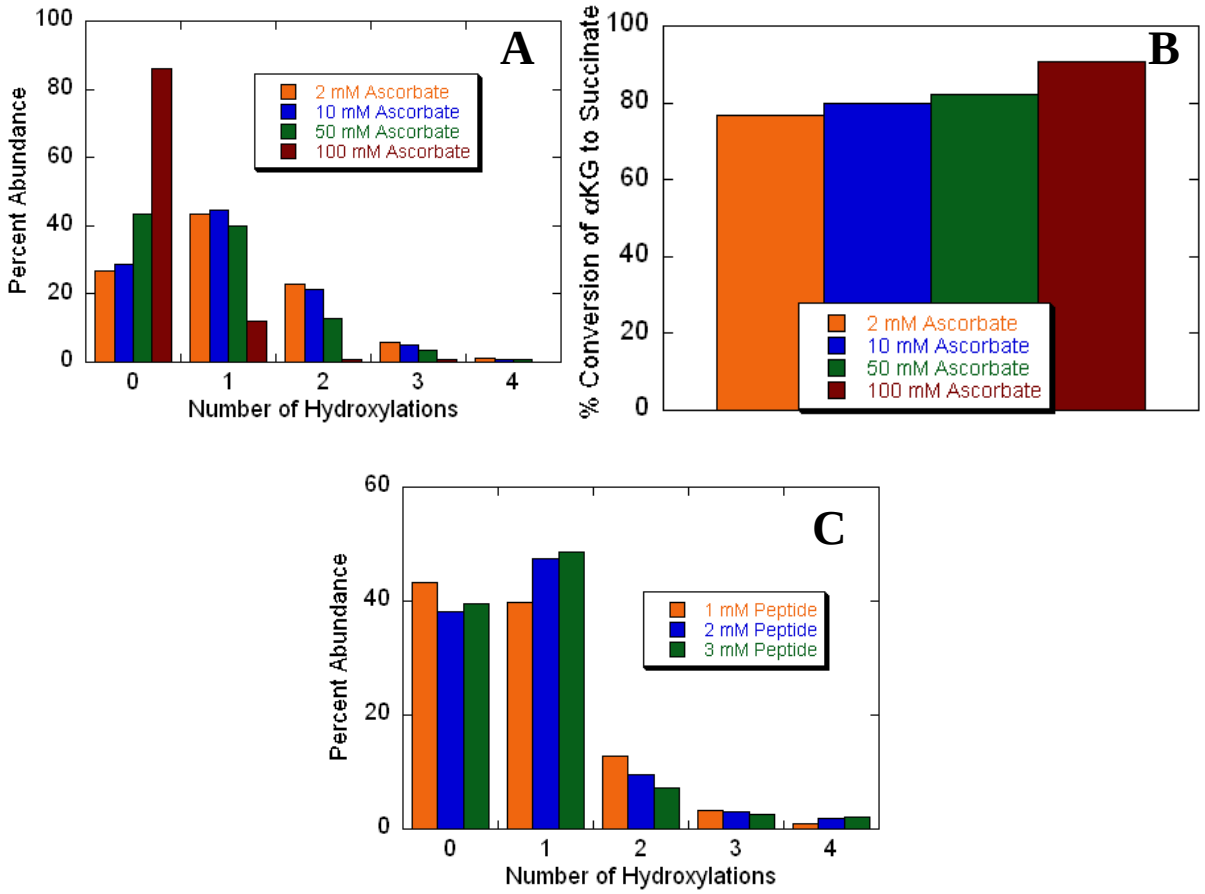

Figure 12

(A) Ascorbate dependent inhibition of productive turnover. The relative intensities were normalized to 100 for the amount of unhydroxylated substrate. Samples were prepared with 5 μM P4H, 3.5 μM Fe(II), 900 μM αKG, 1 mM $(PAPK)_3$, and ascorbate.

(B) Percent conversion of αKG to succinate at varying ascorbate concentrations. The intensities were standardized against 900 μM deuterium labeled succinate to determine the amount of conversion of αKG to succinate. The samples were prepared with 5 μM P4H, 3.5 μM Fe(II), 900 μM αKG, 1000 μM $(PAPK)_3$, and ascorbate.

(C) Effect of peptide concentration on ascorbate inhibition. The relative intensities were normalized to 100 for the amount of unhydroxylated substrate. Samples were prepared with 5 μM P4H, 3.5 μM Fe(II), 900 μM αKG, 50mM ascorbate, and $(PAPK)_3$ peptide substrate.

Discussion

While the importance of ascorbate as a vitamin has been recognized for centuries, its mode of action has remained largely unknown (12). Therefore, this study sought to define the mechanisms of interaction between ascorbate and P4H. While the P4H homolog used for this study, PBCV-1 P4H, is not the form found in humans, it was selected as a model after an ascorbate dependence was established (Figure 8). With the need for ascorbate shown, the binding relationships with αKG and P4H were elucidated. The results of anaerobic titrations suggest that ascorbate does not bind as a substrate, which would have produced a change in the absorption spectrum or a diminution of the K_D for αKG. Instead, ascorbate may actually impede αKG binding, as the K_D of αKG was observed to increase marginally from 323 ± 49 μM without ascorbate to 448 ± 12 μM in the presence 10 mM of the reductant (Figure 9B). The binding relationships were also characterized by monitoring formation of the ternary complex at 520 nm with the use of stopped-flow absorption spectroscopy. When the ascorbate was allowed to form a binding equilibrium with P4H before mixing with αKG, the rate of αKG binding decreased (Figure 10A). Conversely, if the ascorbate was present with the αKG before mixing, no effect on the kinetics of the αKG binding was observed (Figure 10B). The binding kinetics of these two complementary experiments suggest that ascorbate and αKG bind in mutually exclusive manner. Also, αKG must have a much lower K_D than ascorbate, because it is able to outcompete ascorbate for binding to P4H. While the competition between these two molecules is clear, the molecular reason for αKG binding inhibition is currently unknown. Among the possibilities, αKG and ascorbate could share an identical binding site; αKG and ascorbate could have overlapping binding sites; or αKG and ascorbate could each cause a conformational change that impedes binding of the other.

Majamaa *et al*. had previously suggested that ascorbate binds to P4H. By the use of ascorbate mimicking inhibitors, ascorbate had been shown to directly interact with P4H to ensure sustained activity. However, the results of this present study are the first to show directly that ascorbate can bind to the P4H·Fe(II) complex. Additionally, the observation of competition between αKG and ascorbate helps to define the characteristics of the ascorbate binding site.

While the binding of ascorbate to P4H is important, determining the chemistry that ascorbate performs is key to truly understanding its function. Previously, it was suggested that two distinct reduction mechanisms could occur, single electron reduction of Fe(III) to Fe(II) or reduction of Fe(IV) to Fe(III), subsequently followed by reduction to Fe(II) (8). Because previous studies had been conflicting in their conclusions, experiments were designed to conclusively distinguish between the two mechanisms of ascorbate action. By generating P4H·Fe(III) anoxically with potassium ferricyanide, it was possible to observe single electron reduction in real time. Stopped-flow spectrophotometric analysis demonstrated that ascorbate is efficient at reducing the Fe(III) in the enzyme's active site (Figure 11A). However, the binding characteristics of ascorbate create a caveat for true biological conditions. As shown previously, ascorbate and αKG are mutually exclusive in binding P4H. This fact was again observed in ascorbate reduction of P4H·Fe(III). With αKG present and bound to the enzyme, ascorbate could not reduce P4H·Fe(III) (Figure 11B). Only after αKG dissociation can the reduction of P4H·Fe(III) by ascorbate be effected.

Previously, de Jong *et al*. (1982) determined that ascorbate can diminish the EPR signal associated with Fe(III). Whether the iron was bound to P4H or free in solution was unknown. Additionally, their data suggested that some fraction of the Fe(III) could not be reduced, even after 30 seconds. The results of this present study help to explain these previous observations of

ascorbate reduction. It was shown that the P4H·Fe(III) complex was stable and able to be reduced by ascorbate. Therefore, iron is primarily bound to P4H and not free in solution when it is reduced. Additionally, αKG and ascorbate binding interactions explained the presence of Fe(III) signal even after 30 seconds. These EPR experiments had excess αKG in the samples. The αKG probably bound to the P4H·Fe(III) complex, preventing ascorbate reduction, giving the observed results. Incubation of only 30 seconds was not enough time for the αKG to dissociate from the oxidized species.

The alternative mechanism of ascorbate action, Fe(IV) reduction, was also explored in this present study. By using high concentrations of ascorbate in the reaction mixture, productive hydroxylation of $(PAPK)_3$ was inhibited (Figure 12A). However, the amount of αKG decarboxylated to succinate remained almost constant, irrespective of ascorbate concentration (Figure 12B). Therefore, we conclude that ascorbate acts on P4H between αKG decarboxylation and substrate hydroxylation by reducing the Fe(IV) species. There are two mechanisms by which the reduction can occur with the substrate present. The first relates to the αKG and ascorbate binding relationship. Once αKG is decarboxylated to succinate, it could possibly dissociate. If ascorbate is present, it could then bind to the enzyme and reduce the resulting Fe(IV) by an inner sphere mechanism. The second mechanism of reduction would rely on the highly reactive nature of the Fe(IV). It is possible that the Fe(IV) has high enough oxidation potential that the ascorbate might not even need to bind to its favored site. Instead, the ascorbate might reduce by an outer sphere mechanism.

The efficiency of ascorbate reduction of the Fe(IV) may depend largely on the kinetics of ferryl iron formation and the rate of its reaction. Because the enzyme is undergoing triggered turnover in these experiments, the Fe(IV) species forms rapidly and then reacts quickly, because

it can efficiently cleave the carbon-hydrogen bound of the substrate. In the untriggered reaction, the rate of oxygen activation and accumulation of the Fe(IV) intermediate would be lesser. Additionally, the expected half life of the Fe(IV) species should increase, because the prime substrate for the enzyme is absent, forcing the ferryl iron to instead react with an internal amino acid residue. Presumably, ascorbate could intercept and reduce the Fe(IV) under these conditions, preventing the detrimental self hydroxylation reaction.

Ultimately, this study has offered insight into the mechanism of ascorbate action. In fact, the results have shown that ascorbate is able to reduce both P4H·Fe(III) and P4H·Fe(IV) species. This versatility explains its biological importance in the continuous turnover of P4H. Research has shown that ascorbate may be important not just for P4H, but also for the entire class of Fe(II)- and αKG-dependent dioxygenases (20). Therefore, sustained ascorbate deprivation may be deleterious not only to the stable synthesis of collagen, but other cellular functionalities as well. For example, ascorbate may have an analogous role in keeping JmjC, an enzyme important for histone demethylation, active. Furthermore, ascorbate may modulate PHD2 mediated HIF-1α hydroxylation, and by extension prevent cancer propagation by controlling angiogenesis.

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PJAS Scholarship	2007- Present
Science Alumni Award	2010
Greek Catholic Union Scholarship	2007- Present
Tershak Scholarship	2009
Eagle Scout	

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