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Thermodynamics of Iron(II) and Substrate Binding to the Ethylene-Forming Enzyme

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Abstract

The ethylene-forming enzyme (EFE), like many other 2-oxoglutarate (2OG)-dependent nonheme iron(II) oxygenases, catalyzes the oxidative decarboxylation of 2OG to succinate and CO₂ to generate a highly reactive iron species that hydroxylates a specific alkane C–H bond, in this case targeting L-arginine (Arg) for hydroxylation. However, the prominently observed reactivity of EFE is the transformation of 2OG into ethylene and three molecules of CO₂. Crystallographic and biochemical studies have led to several proposed mechanisms for this 2-fold reactivity, but the detailed reaction steps are still obscure. Here, the thermodynamics associated with iron(II), 2OG, and Arg binding to EFE are studied using calorimetry (isothermal titration calorimetry and differential scanning calorimetry) to gain insight into how these binding equilibria organize the active site of EFE, which may have an impact on the O₂ activation pathways observed in this system. Calorimetric data show that the addition of iron(II), Arg, and 2OG increases the stability over that of the apoenzyme, and there is distinctive cooperativity between substrate and cofactor binding. The energetics of binding of 2OG to Fe·EFE are consistent with a unique monodentate binding mode, which is different than the prototypical 2OG coordination mode in other 2OG-dependent oxygenases. This difference in the pre-O₂ activation equilibria may be important for supporting the alternative ethylene-forming chemistry of EFE.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

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Tables of thermodynamic data for iron(II)-buffer interactions and metal chelation, buffer ionization energies, iron(II)-EFE interaction in ACES and TES buffer, thermodynamic properties of 2OG binding to Fe·EFE, and thermodynamics of EDTA chelation of Fe·EFE, supporting figures associated with plots of buffer ionization versus observed and metal-buffer enthalpy, and several representative ITC data sets for EDTA titrated into Fe·EFE and iron(II) titrated into EDTA (PDF)

The authors declare no competing financial interest.

Ethylene is an important molecule for the formation of a variety of plastics and other materials.¹ Traditional methods for producing ethylene from petroleum and petroleum byproducts have serious impacts on the environment.² This fact has led to increasing efforts in the search for alternative, environmentally friendly methods for CH_2CH_2 production. Fortunately, ethylene is also a molecule found in biological systems, where it acts as a vital hormone for plant growth and development.³ Additionally, some plant pathogens, including several *Pseudomonas* strains, contain enzymatic pathways for generating ethylene that could be adapted to produce this compound in a more sustainable fashion.^{4–8} The ethylene-forming enzyme (EFE) from *Pseudomonas syringae* has been proposed as a “green” option for synthesizing ethylene,^{9–11} and a series of crystal structures of EFE have recently been reported,^{12,13} which give new mechanistic insight into this nonheme iron(II) protein.

EFE belongs to a family of iron(II) and 2OG-dependent oxygenases, which typically utilize 2OG and O_2 to generate a high-valent iron species that oxidatively activates a specific C–H bond to yield a C–OH;¹⁴ in this case, EFE hydroxylates the $\text{C}\delta$ atom of L-arginine (Arg) to form an intermediate that rapidly decomposes to guanidine and L-1-pyrroline-5-carboxylate (P5C) (Scheme 1, top).^{12,15} In EFE, however, a second reaction pathway is catalyzed at the same enzyme active site. In this case, O_2 is activated to convert 2OG into ethylene and three CO_2 molecules (Scheme 1, bottom). This 2-fold catalytic reactivity of EFE suggests there is remarkable flexibility in the active site pocket of this enzyme, which provides the means to perform both of these distinctive O_2 activation reactions.

The active site of EFE consists of an iron(II) binding motif housed in a cupin fold or antiparallel β barrel (Figure 1A).^{12,13} The nonheme iron(II) ion in EFE is bound by two histidine residues (H189 and H268) and the carboxylate side chain of an aspartate residue (D191). These ligands occupy one face of the octahedral coordination geometry, which has been dubbed the 2-histidine-1-carboxylate facial triad (Figure 1B).^{16,17} This geometry leaves the adjacent three coordination positions occupied by solvent that can readily be displaced by the typically bidentate cofactor, 2OG (Figure 1B), leaving one site for O_2 coordination. A series of secondary amino acid side chains are poised to stabilize Arg and 2OG binding. The side chains of E84, D191, Y192, and R316 along with the backbone carbonyl groups of V85 and T86 form a H-bonding network that positions Arg in the active site of EFE. Furthermore, the side chains of R171 and H268 stabilize the C1 carboxyl group and α -keto acid functionality, respectively. Additionally, R277 forms a salt bridge with the distal carboxylate of 2OG. Interestingly, there is crystallographic evidence that when the substrate Arg is not present 2OG exhibits an aberrant monodentate binding mode, and it is

this novel coordination mode that may contribute to the alternative chemistry that this enzyme can perform.¹²

Herein, we report a thermodynamic investigation of iron(II), substrate, and cofactor binding to EFE, where we focus on the complex series of equilibria involved in the pre-O₂ activation steps associated with the mechanism of this enzyme. The thermodynamic data collected here are compared to similar data reported for the pre-O₂ activation steps of the well-characterized 2OG-dependent dioxygenase, TauD, which hydroxylates taurine leading to its decomposition into aminoacetaldehyde and sulfite. Via comparison of the thermodynamic steps associated with the mechanism of EFE to the canonical mechanistic steps associated with TauD, significant differences can be readily identified, which give insight into the origins of the 2-fold reactivity found in EFE.

MATERIALS AND METHODS

Reagents and General Procedures.

L-Arg and 2OG were used as received from Sigma-Aldrich. All buffers, ethylenediaminetetraacetic acid disodium salt (EDTA), and metal salts were used as received from Fisher Scientific. The iron salts were in their +2 ionization states. Water was filtered through a Millipore Ultrapurification system and possessed a resistivity of ~18 MΩ. As a general convention, all EFE side chain amino acids are designated by their single-letter abbreviations whereas the substrate Arg is indicated by its three-letter abbreviation.

Overexpression and Purification of EFE.

The His-tagged form of EFE (His₆-EFE) was generated in *Escherichia coli* BL21 Gold (DE3) cells containing the pUC19-derived plasmid that harbored the gene encoding EFE (pUC19-efe-His₆).¹⁸ Cultures were supplemented with kanamycin (50 μg/mL) and grown overnight at 37 °C while being constantly shaken. The culture was induced by the addition of 0.2 mM isopropyl β-D-1-thiogalactopyranoside and incubated overnight at 20 °C. Cells were harvested by centrifugation at 6130g for 10 min and resuspended in 50 mM NaH₂PO₄ (pH 8.0) containing 500 mM NaCl and 10 mM imidazole. Cell-free extracts were obtained by lysing cells using sonication followed by centrifugation at 34220g for 20 min. The lysates were applied to a nickel-nitrilotriacetic acid (Ni-NTA) agarose column (10 mL, Qiagen), and the resin was eluted using 100 mL of buffer A to remove unbound proteins and 50 mL of 50 mM NaH₂PO₄ (pH 8.0) and 500 mM NaCl to obtain pure His₆-EFE. The sample was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the desired fractions containing EFE were pooled, concentrated, and exchanged into buffer composed of 50 mM NaH₂PO₄ (pH 8.0) and 500 mM NaCl by using a 10 kDa molecular weight cutoff Amicon Ultra-15 centrifugal filter unit (EMD Millipore). The poly-His tag was removed by incubating the concentrated protein with His₇-TEV238 protease for 16–18 h at 4 °C. The His₆ tag was separated from EFE using a Ni-NTA column, for which the flow-through fractions were concentrated and dialyzed into 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 8.0) containing 1 mM EDTA and 1 mM dithiothreitol (DTT). A 5% glycerol solution was added, and the purified EFE was stored in aliquots at –80 °C.

Samples for circular dichroism (CD), differential scanning calorimetry (DSC), and isothermal titration calorimetry (ITC) analyses were prepared as follows. EFE apoprotein was dialyzed for 18 h against a buffer system containing 50 mM HEPES, 100 mM NaCl, and 200 μ M EDTA (pH 8.0) to remove residual metal. The EFE apoprotein stock was then dialyzed twice against 1 L of buffer containing 25 mM HEPES (pH 7.4) for 3 h and one additional dialysis against the buffer (1 L) overnight. Stock solutions of Arg, 2OG, and $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ were dissolved using the dialysate buffer. All solutions were purged with Ar to eliminate oxidation reactions by providing an anaerobic environment. One equivalent of $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ was added anaerobically to EFE apoprotein to produce Fe-EFE. To shift the equilibrium in favor of the substrate–enzyme complexes, Arg and/or 2OG was added anaerobically to enzyme samples to produce final concentrations of 0.5 mM.

Circular Dichroism.

CD was used to monitor the secondary structure of EFE and its complexes. CD samples were diluted to a final concentration of 5 μ M. All the solutions including apoprotein, iron(II) salt, 2OG, and Arg were degassed for 30 min to remove oxygen. EFE and its complexes were prepared in an anaerobic environment and transferred into a cuvette with a rubber top. CD spectra of samples were measured from 190 to 250 nm by using an OLIS (Bogart, GA) DSM-20 CD spectrometer.

Differential Scanning Calorimetry.

DSC was used to assess the thermal stability of EFE complexes. DSC samples were diluted to a final concentration of 20 μ M and analyzed using a Calorimetry Sciences Nano-DSC instrument constructed with gold capillary cells for reference and sample solutions. A portable glovebox was used to cover the top of DSC, thus affording an anaerobic environment. Ar was purged through the glovebox for at least 30 min before samples were loaded into cells, and gas flow was maintained during the denaturation process. A scan rate of 2 $^{\circ}\text{C}/\text{min}$ was set to monitor the thermal denaturation of EFE and its related complexes.

Isothermal Titration Calorimetry.

The MicroCal VP-ITC instrumentation was sealed in an anaerobic chamber with a constant dinitrogen flow during the course of the experiment. A purified EFE apoprotein stock was dialyzed using three different buffers at pH 7.4 for 18 h and then diluted to a final concentration of 100 μ M. The dialysate and EFE protein were made anaerobic by purging Ar over the solutions. A 1 mM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ solution was prepared by using the degassed dialysis buffer. Thermodynamic data were collected using the following conditions: 25 $^{\circ}\text{C}$, 5 μ L injections of the iron(II) solution into a 1.5 mL cell containing the EFE solution to generate Fe-EFE, a stirring rate of 307 rpm, and injections over 6 s periods with 300 s spacings between injections. Chelation titration experiments were conducted with 50 μ M anaerobic Fe-EFE and 1 mM EDTA in 25 mM HEPES buffer (pH 7.4).

RESULTS

Structure of EFE and Its Complexes.

Currently, there are 13 published structures of EFE and related EFE complexes,^{12,13} which range from the apoprotein to quaternary complexes of Fe·EFE·2OG-substrate analogues. When overlaid, these structures show significant conservation of the EFE globular structure, with most of the secondary structures of the protein retained regardless of metal(II), 2OG, and substrate binding.^{12,13} These data give anecdotal evidence that the thermodynamic interactions of EFE with its metal ion and ligands investigated herein are derived from local structural perturbation rather than global dynamic changes. Because of constraints imposed by crystal contacts, the structures may not perfectly reflect the solution structure of EFE or its complexes, so these complexes were also examined by CD spectroscopy.

The CD spectra of various Fe·EFE complexes show a distinctive negative ellipticity feature near 225 nm (Figure 2), which is characteristic of the cupin fold.¹⁹ When iron(II), Arg, or 2OG was added to the protein, this feature red-shifted slightly and the intensity slightly decreased, indicating the cupin fold is slightly destabilized. This destabilization is likely due to subtle flexing of the cupin to accommodate metal ion or ligand binding, but this destabilization is likely only a minor contributor to the overall stability of these complexes. Changes in the CD signal between 210 and 220 nm for Fe·EFE complexes with 2OG and/or Arg could also indicate a slight increase in the random coil content in these samples. No precipitate was visible in any of the EFE samples studied. Overall, there appears to be little change in the structure of EFE and related EFE complexes, suggesting the thermodynamic parameters studied herein are primarily derived from local interactions within the protein as iron(II), Arg, and 2OG are bound.

Thermodynamic Study of Iron(II) Binding to EFE Analyzed by ITC.

The thermodynamic parameters associated with iron(II) binding to EFE were obtained by using ITC.^{20,21} From a single titration experiment, the observed enthalpy (H_{obs}) and binding constant (K_{obs}) can be directly measured, where the Gibbs free energy (G°) and entropy (S) terms associated with this process can be calculated through eqs 1 and 2:

$$\Delta G^\circ = -RT \ln K \quad (1)$$

$$\Delta G^\circ = \Delta H - T\Delta S \quad (2)$$

The data associated with the direct titration of iron(II) into the EFE apoprotein in 25 mM TES buffer at pH 7.4 were baseline-corrected and fit to a single binding event (Figure 3). The observed thermodynamic data, including the change in enthalpy (H_{obs}) and K_{obs} , were measured directly, and the change in entropy (S_{obs}) was calculated. The observed thermodynamic parameters for the direct titration of iron(II) into EFE using three different buffers are listed in Table 1.

The enthalpy change (H_{obs}) measured in these titrations describes the amount of heat released or absorbed in a reaction, where the H_{obs} term is the sum of all changes in

enthalpy associated with iron(II) binding to EFE. Dissecting this term into more fundamental thermodynamic quantities can yield a better understanding of the specific interactions that impact the global stability of the metalated protein. In this case, H_{obs} is effectively described as a complex series of competitive binding events involving the iron(II) ion, where the release of the metal ion from buffer ($H_{\text{Fe}\cdot\text{buffer}}$) and coordination to protein ($H_{\text{Fe}\cdot\text{EFE}+\text{H}}$) are generally linked to the release of protons to the bulk solution. The release of proton density leads to a buffer ionization enthalpy change ($H_{\text{ionization}}$), which is also a key part of H_{obs} . Equation 3 depicts the relationship between the competitive binding events in an ITC experiment.

$$\Delta H_{\text{obs}} + \Delta H_{\text{Fe}\cdot\text{buffer}} = \Delta H_{\text{Fe}\cdot\text{EFE}+\text{H}} + n_{\text{p}}(\Delta H_{\text{ionization}}) \quad (3)$$

By plotting the $H_{\text{ionization}}$ of the buffer system versus the $H_{\text{obs}} + H_{\text{Fe}\cdot\text{buffer}}$ terms for this system, one observes a linear relationship where the slope indicates the number of protons (n_{p}) released or consumed during the binding event. A plot of $H_{\text{obs}} + H_{\text{Fe}\cdot\text{buffer}}$ versus $H_{\text{ionization}}$ demonstrates the release of 1.0 proton during the binding event (Figure S1).

$H_{\text{Fe}\cdot\text{buffer}}$ was obtained via control ITC experiments of iron(II)–buffer complexes titrated into EDTA solutions (cf. Figure S2 and the thermodynamic cycles associated with Tables S1 and S2).

Using these data, a thermodynamic cycle was generated to model the equilibria associated with this complex ion binding event, thereby allowing us to estimate the enthalpy value associated with iron(II) binding to EFE. Similar models have been used to study metal ion binding to other nonheme iron(II) proteins in the literature.^{20,23} For example, deconvolution of the observed enthalpy (H_{obs}) in MOPS buffer resulted in a $H_{\text{Fe}\cdot\text{EFE}+\text{H}}$ of 0.9 ± 0.4 kcal/mol, shown in Table 2. Thermodynamic cycles of the observed enthalpy in two other buffers (ACES and TES) were obtained (Tables S3 and S4, respectively).

K_{obs} determined by ITC titration of iron(II) into EFE also is affected by additional binding events occurring in solution. This value is at least the product of the association constant for association of iron(II) with EFE (K_{a}) and the dissociation constant (K_{d}) for dissociation of iron(II) from the iron(II) buffer complex, which presents a challenge to measure directly. A more convenient and accurate method for measuring K_{a} is to determine the reciprocal value, i.e., the dissociation constant. $K_{\text{d}}(\text{Fe}\cdot\text{EFE})$ can be measured directly by titrating the well-characterized divalent metal chelate EDTA into Fe·EFE, where the observed equilibrium constant for this process (K_{obs}^*) is the product of $K_{\text{d}}(\text{Fe}\cdot\text{EFE})$ and $K_{\text{a}}(\text{Fe}\cdot\text{EDTA})$ (Table S7).

Because the Fe·EDTA binding equilibrium [$K_{\text{a}}(\text{Fe}\cdot\text{EDTA}) = 2.1 \times 10^{14}$] is known,²⁴ it is relatively easy to derive the $K_{\text{d}}(\text{Fe}\cdot\text{EFE})$ and then determine the association constant for iron(II) binding to EFE (K_{a}).

The integrated isotherm obtained by ITC chelation titration of EDTA into Fe·EFE in 25 mM TES buffer at pH 7.4 is shown in Figure S3. The integrated isotherm of the observed chelation was corrected by using control experiments, which reveal an exothermic process with one-site binding. Alternatively, these equilibria could be fit to a competitive binding model, which better corrects for activity (or effective concentration) of EFE apoprotein in

solution. When the competitive binding model is used, only a minor change in the fitted binding constant is produced, which is within the experimental error of the one-site binding model fit. To correct for heats of dilution, we performed control experiments to study titrations of EDTA into buffer solutions.^{20,23} The (K_{obs}^*) value for the chelation titration was $1.8 (\pm 0.3) \times 10^7$. Deconvolution of the series of equilibria taking place in the chelation experiment yielded a K_a for Fe-EFE of $1.2 (\pm 0.3) \times 10^7$.

DSC Analysis of EFE and Its Substrate Complexes.

For comparison to the ITC results, DSC was used to study the thermal denaturation processes of EFE and its related enzyme–substrate complexes. These thermal denaturation experiments are for the most part irreversible,^{25–27} but these data can be used to approximate the reversible unfolding process of the enzyme.^{28–30} The DSC-derived enthalpy terms are denoted with an asterisk indicating they are estimations of these enthalpy terms. The heat capacity (C_p) curves for the various EFE species are shown in Figure 4, and the corresponding thermodynamic data are summarized in Table 3. All DSC experimental data were best fit by simple two-state unfolding models.

The EFE apoprotein unfolding event fit well to a single two-state model, with a $T_{\text{m(EFE)}}$ of 39.2 ± 0.7 °C and a ΔH_{EFE}^* of 86.8 ± 1.0 kcal/mol (Figure 4, black dashed curve). For Fe-EFE (Figure 4, green dotted curve), a single two-state model was used to fit the unfolding event, which gave a $T_{\text{m(Fe-EFE)}}$ 42.9 ± 0.7 °C with an enthalpy value of 96.8 ± 1.1 kcal/mol. The difference in the unfolding enthalpies of the Fe-EFE versus EFE samples yields the enthalpy stabilization resulting from iron(II) binding to EFE (eq 4). The $\delta \Delta H_{\text{Fe binding}}^*$ for EFE is -10.6 ± 1.5 kcal/mol.

$$\delta \Delta H_{\text{binding}}^* = \Delta H_{\text{EFE}}^* - \Delta H_{\text{Fe-EFE}}^* \quad (4)$$

L-Arg was mixed with EFE and Fe-EFE, and the substrate complexes were subjected to thermal denaturation. The DSC melting curve of Fe-EFE·Arg was fit to a single two-state function, which gave a $T_{\text{m(Fe-EFE·Arg)}}$ of 44.7 ± 1.3 °C and an enthalpy of 86.9 ± 3.6 kcal/mol (Figure 4, red dashes and dots). Comparing these data to those of Fe-EFE, one can estimate an enthalpic penalty of 10.0 ± 3.8 kcal/mol associated with Arg binding to the iron(II)-containing complex; however, $T_{\text{m(Fe-EFE·Arg)}}$ was measured to be 1.8 ± 1.5 °C higher than $T_{\text{m(Fe-EFE)}}$. The K_d of 71 μM for Arg binding to Fe-EFE allows us to calculate the thermodynamic profile of this binding process (Table 4).¹⁸ The data indicate that Arg binding to Fe-EFE is favorable and entropically driven with an entropy change of $52.3 \text{ cal mol}^{-1} \text{ K}^{-1}$. The unfolding event of the EFE·Arg sample was fit to a single two-state model, unfolding at a $T_{\text{m(EFE·Arg)}}$ of 39.6 ± 0.1 °C, with an enthalpy of 92.5 ± 1.4 kcal/mol (Figure 4, solid orange line). Via comparison of the unfolding of EFE·Arg and EFE, the heat capacity curve of EFE·Arg indicates an enthalpy larger than that of EFE, with Arg binding to EFE providing -5.7 ± 1.8 kcal/mol in stability. This substrate–enzyme complex appears to unfold at almost the same temperature as the EFE apoprotein. The Arg binding site in EFE is in the proximity of the nonheme iron(II) center (Figure 1), but there is no direct coordination between Arg and the metal ion.

The substrate 2OG formed a complex with Fe-EFE, and the sample was thermally denatured to produce a C_p curve that fit well to a single two-state model (Figure 4, blue dashes). The unfolding event had a $T_{m(\text{Fe-EFE} \cdot 2\text{OG})}$ of 45.4 ± 0.6 °C with a $H^*_{\text{Fe-EFE} \cdot 2\text{OG}}$ of 109.8 ± 2.6 kcal/mol. Compared with that of Fe-EFE, the addition of 2OG provided 2.6 ± 0.9 °C and -13.0 ± 2.8 kcal/mol of stability. The T_m for the Fe-EFE·2OG complex increased when compared to that of the iron(II)-enzyme complex. The increase in enthalpy is likely the result of strengthening the intramolecular interactions surrounding the iron(II) center.

The quaternary complex, generated by mixing iron(II), EFE, Arg, and 2OG, was investigated by DSC (Figure 4, solid purple line). The symmetric unfolding event for this complex fit well to a single two-state model with a $T_{m(\text{Fe-EFE} \cdot 2\text{OG} \cdot \text{Arg})}$ of 49.3 ± 0.2 °C and a

$H^*_{\text{Fe-EFE} \cdot \text{Arg} \cdot 2\text{OG}}$ of 124.8 ± 0.1 kcal/mol (Table 3). Denaturation of this species shows an increase in its T_m and H^* over the corresponding values for the Fe-EFE·2OG species, where the T_m increased by approximately 4.0 ± 0.6 °C and the difference in enthalpy term suggests the Fe-EFE·2OG·Arg quaternary complex is -15.0 ± 2.6 kcal/mol more enthalpically stable than the Fe-EFE·2OG complex. Combining these data with the known binding constants,¹⁸ we obtained a full thermodynamic profile for Arg binding to Fe-EFE·2OG (Table 4), where this enthalpically driven reaction has a Gibbs free energy of -6.1 kcal/mol. When compared with that of Fe-EFE·Arg, the addition of 2OG provided 4.5 ± 1.3 °C and -38.0 ± 2.6 kcal/mol of stability. This enthalpy stabilization is approximately 3 times larger than 2OG binding to Fe-EFE. Using the K_d for 2OG binding in the presence of Arg, a free energy of -6.3 kcal/mol was calculated with a $-T \Delta S$ term of 31.7 kcal/mol (Table 4).

DISCUSSION

The orchestrated binding of iron(II), substrate, and cofactor to EFE organizes the active site of this protein for O₂ activation, and the details of these thermodynamic processes may provide insight into the key mechanistic step(s) that leads to Arg hydroxylation or ethylene formation.

Deconvoluting the Enthalpy Value of Iron(II) Binding to EFE.

By considering the local environment surrounding the iron(II) binding site, a model can be developed to further deconvolute the enthalpy term ($H_{\text{Fe-EFE}+\text{H}}$) to a more simplified iron(II) binding enthalpy ($H_{\text{Fe-EFE}}$). This process is performed by modeling the local interactions that likely contribute to this term (shown in eq 5), where $H(\text{His-H})\text{EFE}$ represents the ionization of histidine and $H(\text{Fe-OH}_2)\text{EFE}$ represents the ionization of an iron(II)-coordinated water molecule.

$$\Delta H_{\text{Fe} \cdot \text{EFE} + \text{H}} = \Delta H_{\text{Fe-EFE}} - \Delta H_{(\text{His-H}) \cdot \text{EFE}} - \Delta H_{(\text{Fe-OH}_2) \cdot \text{EFE}} \quad (5)$$

Iron(II) binding to H189, H268, and D191 releases approximately ~1.0 proton to solution. We hypothesize that the proton release event is associated with both partial deprotonation of H189 and H268 and the ionization of water coordinated to the iron(II) ion. At pH 7.4, we estimate that 96% of histidine side chains are deprotonated at pH 7.4, which suggests that only ~0.1 proton will be released from H189 and H268, resulting in 0.72 kcal/mol in

enthalpic instability for iron(II) binding. Additionally, solvated metal ions like iron(II) are charge-stabilized through ion pairing in solution, where anions and buffer molecules are likely to help stabilize divalent metal ions in solution. However, once divalent metal ion is bound to a protein, this Lewis acid moiety impacts the ionization constants of local residues and is generally stabilized through ion pairing to negatively charged amino acids. In the case of Fe-EFE, the iron(II) is stabilized by D191, but this metal ion will also have an impact on the pK_a of the coordinating water molecules. We estimate that the remaining 0.9 proton released to solution is likely from partial deprotonation of the coordinated water(s), resulting in an endothermic enthalpy change (+12.0 kcal/mol) that also stabilizes the divalent iron(II) in EFE. Once these local equilibria are considered, this leaves an iron(II) binding enthalpy ($H_{\text{Fe-EFE}}$) of -11.8 ± 0.4 kcal/mol in MOPS buffer (Table 5). Deconvolution of the iron(II) binding data in ACES and TES can be found in Tables S5 and S6, respectively. A list of the enthalpy values determined in three different buffers is shown in Table 6, along with the average, buffer-independent $H_{\text{Fe-EFE}}$ measured at pH 7.4.

By using the corrected K_a for iron(II) binding to EFE and the deconvoluted $H_{\text{Fe-EFE}}$ term (-11.8 ± 0.7), the corrected G° and $-T \Delta S$ values were determined to be -9.6 ± 0.2 and 2.2 ± 0.7 kcal/mol, respectively. These data are consistent with the iron(II) binding process that is mainly enthalpically driven with only a minor entropic penalty associated with this metal ion coordination event. A well-studied protein with a similar iron(II) binding site involving a 2-His-1-carboxylate facial triad, TauD, also followed a similar trend, where the thermodynamic parameters found for iron(II) binding to TauD were -10.1 ± 0.03 , -12.0 ± 0.3 , and 1.9 ± 0.3 kcal/mol for G , H , and $-T \Delta S$, respectively.²⁰

DSC Analysis of EFE and Its Substrate Complexes.

The $\delta H^*_{\text{Fe binding}}$ for EFE, -10.6 ± 1.5 kcal/mol, is within experimental error of the ITC-derived iron(II) binding enthalpy (-11.9 ± 0.7 kcal/mol) discussed above. The systematic error in these DSC measurements suggests some instability in the apoprotein under the experimental conditions used. Regardless, there is reasonable consistency between the DSC and ITC data, which gives credibility to the thermodynamic terms generated from DSC-derived data even though these unfolding events are irreversible.²⁸

Interestingly, Arg binding to the apoenzyme is enthalpy-driven ($H_{\text{EFE-Arg}} \sim -5.7$ kcal/mol), but when iron(II) is present, Arg binding is entropy-driven ($H_{\text{Fe-EFE-Arg}} \sim 10.0$ kcal/mol). In both cases, Arg binds in a similar fashion, suggesting that H-bonding networks and charge-charge interactions between the substrate and EFE drive Arg binding. However, when iron(II) is present, these exothermic interactions are overwhelmed by endothermic processes, where binding is made favorable through an increase in disorder. There are no major structural changes observed between the crystallographically characterized Fe-EFE and Fe-EFE-Arg structures,¹² so a reasonable assumption can be made that the binding of Arg to Fe-EFE results in significant reorganization of water networks within the active site of this enzyme. We theorize that the enthalpy penalty and favorable entropy terms associated with this event result from breaking the H-bonding network of the solvating waters surrounding the iron(II) center and the release of these water molecules to bulk solution upon Arg binding, although further studies are needed to confirm this notion.

Crystallographically characterized Fe-EFE·2OG complexes show 2OG binding to the iron(II) center in a monodentate fashion through either carboxylate of 2OG, although there is spectroscopic evidence supporting an equilibrium between monodentate and bidentate binding mode for 2OG in Fe-EFE 2OG.¹² The enthalpy value (−13.0 kcal/mol) established for 2OG binding to Fe-EFE is consistent with a monodentate binding mode in solution, where classical bidentate 2OG binding to related 2OG-dependent oxygenases show a much stronger enthalpy-driven process (−30.1 kcal/mol).²⁸ Additional interactions leading to the exothermic 2OG binding event result from the formation of hydrogen bonding networks between 2OG and local residues, including R277 and R171. When combined with known values for the K_d of 2OG to Fe·EFE (our ITC-obtained value), a favorable Gibbs free energy (−7.2 kcal/mol) and an unfavorable change in entropy (−22.8 cal mol^{−1} K^{−1}) can be calculated (Table 4).

The favorability of Arg binding to Fe-EFE·2OG ($\Delta H \sim -15$ kcal/mol) may result from structural changes, with 2OG undergoing a prominent rearrangement that allows bidentate coordination to the iron(II) in the presence of Arg.¹² In addition, binding of Arg is associated with shifting of the metal-coordinating carboxylate oxygen of D191 and the creation of a twisted peptide bond between D191 and Y192. We expect that this rearrangement and the H-bonding network generated in the Fe-EFE·2OG·Arg complex provide the stability to drive this reaction forward.

The thermodynamic data collected in this study provide the ability to generate a thermodynamic cycle describing the interactions among EFE, iron(II), 2OG, and Arg (Figure 5). This complex equilibrium shows significant cooperativity between parallel binding events, where iron(II) impacts Arg binding and Arg impacts 2OG binding. This interplay among the metal ion, substrate, and cofactor is somewhat expected when direct interactions occur. For example, one anticipates interdependence between iron(II) and 2OG, because 2OG coordinates directly to the iron(II) ion. The enthalpy changes associated with Arg binding to different EFE complexes in solution demonstrate that the local environment and solvating waters control how these complexes form in solution.

Diverging from the Typical 2OG-Dependent Oxygenase Mechanism.

EFE, like other 2OG-dependent oxygenases, has a cupin fold that houses the iron(II) active site and supports the targeted hydroxylation of a biological molecule. Interestingly, EFE also catalyzes a unique fragmentation of 2OG to produce ethylene and three molecules of CO₂. Several possible mechanisms have been proposed for this unique reaction,^{12,13,15} but no consensus has been reached. The data presented here parallel the thermodynamic data reported for TauD,²⁸ the best characterized 2OG-dependent oxygenase. Identifying major differences in the energetics of iron(II), substrate, or 2OG binding to EFE compared to TauD may give insight into how EFE can support the aberrant oxidative fragmentation of 2OG.

The energy landscapes for EFE and TauD are plotted in Figure 6, with the resting enzyme energetic states plotted at 0 kcal/mol for G , H , and $-T \Delta S$ terms as a point of reference, and all energies are normalized with respect to these assignments. For both EFE and TauD, iron(II) binding is spontaneous with similar free energy terms. The enzymes use the same ligands to bind iron(II), but subtle perturbations in the local structure may tune the free

energy of the metal ion binding event. The tertiary and quaternary complexes of both these enzymes also form spontaneously. Again, there is little difference in the free energy landscape between EFE and TauD (Figure 6, top panel, blue and red traces, respectively). When considering the enthalpy landscapes for the ternary states (Figure 6, middle panel), there appears to be a significant difference in the magnitude of the enthalpy term for substrate and cofactor binding to EFE and TauD when generating the ternary complexes. In both enzymes, substrate binding is an endothermic event, where the change in enthalpy of taurine (the substrate for TauD) binding to Fe·TauD ($\Delta H \sim 26$ kcal/mol) is almost 2-fold more endothermic than for the corresponding Arg binding to Fe·EFE event ($\Delta H \sim 10$ kcal/mol). Without a major structural perturbation, these endothermic processes are likely due to solvation changes within the active site of these proteins. One possible explanation is that taurine is significantly smaller than Arg, and the ensemble of conformations available to Arg may limit solvent organization in the active site. Perhaps a more compelling situation is observed when comparing the Fe·EFE·2OG and Fe·TauD·2OG ternary complexes. The strongly exothermic stabilizing force associated with 2OG binding to Fe·TauD ($\Delta H \sim -30.1$ kcal/mol) is driven primarily by the bidentate coordination mode and the related charge-stabilizing interactions between the anionic charges of 2OG and cations from the protein. In Fe·EFE, 2OG binds with a much weaker enthalpy ($\Delta H \sim -13.0$ kcal/mol), consistent with the monodentate binding mode observed crystallographically. Formation of the quaternary complex in both systems is again highly exothermic, generating the prototypical O₂-activating species. Generally, the changes in entropy terms mirror those observed by studying the changes in enthalpy.

The monodentate 2OG binding to Fe·EFE appears to be one unique aspect of this enzymatic system, where 2OG has been shown to bind the iron(II) in a monodentate fashion with either the C1 or C5 carboxylates. Scheme 2 shows the Fe·EFE·2OG species with 2OG binding in a general monodentate mode, where the C1 or C5 carboxylate can coordinate the metal (the blue carbonyl appears in this position only when the C1 carboxylate is bound). Although neither the Fe·EFE·2OG complex with a bidentate 2OG nor the Fe·EFE·2OG·Arg complexes with a monodentate 2OG binding mode were identified in this thermodynamic study (structures shown in the light blue box), they seem to be reasonable intermediate steps toward the formation of Fe·EFE·2OG·Arg where 2OG coordinates in a bidentate mode. Arg binding eventually leads to a single five-coordinate Fe·EFE·2OG·Arg species, with 2OG bound in a bidentate fashion. In part, this is due to a hydrogen bond that is formed between the substrate Arg and D191, which leads to a change in metal coordination and the formation of a D191-Y192 twisted peptide bond. The resulting species is similar to the prototypical O₂-activating intermediate in 2OG-dependent oxygenases, allowing this species of EFE to perform 2OG decarboxylation and Arg hydroxylation; however, the positioning of D191 is distinct from that of other enzymes in this family, and the open coordination site for dioxygen binding is directed far from Arg. These subtle active site rearrangements are important to the Arg hydroxylation mechanism, but they are also likely to be key steps in ethylene formation, where Arg binding to EFE is necessary for ethylene formation.¹⁸

From a mechanistic perspective, either of the monodentate 2OG binding modes in Fe·EFE·2OG or the unique structural features of the quaternary complex are potential features that are responsible for the dual activity of the enzyme. It is from these complicated,

interdependent equilibria that O₂ activation leads to the penultimate oxidized complex [either a bicyclic iron(IV)-peroxo species (shown in Scheme 2) or an iron(II)-persuccinic acid species (not illustrated)], which generates the iron(IV)-oxo used for Arg hydroxylation; it remains unclear which of these oxidized species undergoes the alternative fragmentation pathway to generate CO₂ and ethylene.^{15,18,32} Given the limited kinetic and spectroscopic data available for EFE, further studies are required to define the precise mechanism for ethylene formation to develop a sustainable method of production of ethylene based upon EFE.

CONCLUSION

Thermal unfolding data for EFE and its enzyme–substrate complexes indicate that intramolecular interactions increase the structural stability in this protein system. Deconvolution of the DSC data allowed for the generation of a thermodynamic cycle for iron(II), 2OG, and Arg binding to EFE (Figure 5). The cycle clearly shows the complexity of the interactions between the metal ion and substrates in the monomeric unit of protein. Binding of both substrates at the active site has a significant impact on the global stability of the complex. These data compare well with those from thermodynamic studies of TauD but highlight the unique 2OG binding process in Fe- EFE. It is feasible that this highly stabilized monodentate binding mode of 2OG in EFE contributes as a critical component in supporting ethylene formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

2OG	2-oxoglutarate
EFE	ethylene-forming enzyme
ITC	isothermal titration calorimetry
DSC	differential scanning calorimetry
CD	circular dichroism
EDTA	ethylenediaminetetraacetic
Arg	L-arginine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
P5C	L- 1-pyrroline-5-carboxylate

MOPS	3-morpholinopropane-1-sulfonic acid
ACES	N(2-acetamido)-2-aminoethanesulfonic acid
TES	2-{[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino}ethanesulfonic acid
TauD	taurine-dependent dioxygenase

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Figure 1.

Key features of the EFE monomer structure. (A) The protein binds iron(II) [substituted with manganese(II) in this structure, Protein Data Bank entry 5V2Y] in the center of the characteristic cupin barrel. (B) Active site cavity with bound substrates. The H189, H268, and D191 metal ligands are colored green. The carbon atoms of 2OG are colored yellow, and the carbon atoms of L-arginine (Arg) are colored magenta. The residues stabilizing 2OG are indicated with orange carbon atoms, whereas the residues stabilizing Arg are shown with their purple carbon atoms. Throughout this study, the protein-derived amino acids will be

abbreviated with single-letter amino acid codes, whereas the substrate Arg will be designated by its three-letter code for the sake of clarity.

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Figure 2.
CD spectra of 5 μ M EFE complexes: EFE apoprotein (black), Fe·EFE (purple), EFE·Arg (orange), Fe·EFE·Arg (red), Fe·EFE·2OG (green), and the quaternary complex (blue) consisting of EFE with bound iron(II), Arg, and 2OG.

Figure 3. Representative raw heat and integrated isotherm for iron(II) binding to EFE in 25 mM TES buffer (pH 7.4) at 25 °C.

Figure 4.

Heat capacity curves for the thermal denaturation of EFE species: EFE apoprotein (black dashes), Fe·EFE (green dots), EFE·Arg (solid orange line), Fe·EFE·Arg (red dashes and dots), Fe·EFE 2OG (blue dashes), and the quaternary complex (solid purple line) consisting of EFE with bound iron(II), Arg, and 2OG.

Figure 5.

Summary of enthalpy changes measured by DSC. ΔH terms associated with each equilibrium are shown in parentheses and have units of kilocalories per mole. Exothermic processes are shown with blue equilibrium arrows, whereas the two endothermic processes are shown with red arrows.

Figure 6.

Comparison of the energetic reaction coordinates from enzyme apoprotein to the quaternary complex (4°) of EFE (blue) and the related enzyme TauD (red). Iron(II)-protein refers to the iron(II)-bound forms of EFE and TauD, and “3°” refers to either of two types of tertiary complexes associated with these enzymes, where dashed lines show the profile for the substrate.

Scheme 1.
Reactions Catalyzed by the Ethylene-Forming Enzyme (EFE)

Scheme 2.
Proposed Equilibria in *EFE* Prior to the Steps of O_2 Activation

Table 1.Observed Thermodynamic Parameters of Iron(II) Binding to EFE at pH 7.4^a

25 mM buffer	$H_{\text{ionization}}$	b (kcal/mol)	H_{obs} (kcal/mol)	K_{obs}	$-T \Delta S_{\text{obs}}$ (kcal/mol)
MOPS		-5.04	-2.3 ± 0.2	$(4.8 \pm 0.7) \times 10^5$	-5.5 ± 0.3
ACES		-7.27	-4.1 ± 0.1	$(1.6 \pm 0.3) \times 10^6$	-4.4 ± 0.2
TES		-7.7	-7.1 ± 0.5	$(1.2 \pm 0.4) \times 10^6$	-1.6 ± 0.1

^aThe error associated with the data presented represents one standard deviation from the mean of three or four independent experiments.^bBuffer ionization enthalpy values are from ref 22.

Table 2.

Thermodynamic Cycle for Iron(II) Binding to EFE in MOPS Buffer

reaction	coefficient	<i>H</i> (kcal/mol)
$\text{Fe}^{2+}\cdot\text{MOPS} \rightleftharpoons \text{Fe}^{2+} + \text{MOPS}$	1.0	1.8 ^a
$\text{MOPS} + \text{H}^+ \rightleftharpoons \text{MOPS-H}^+$	1.0	-5.0 ^b
$\text{Fe}^{2+} + \text{EFE-H}^+ \rightleftharpoons \text{Fe}^{2+}\cdot\text{EFE} + \text{H}^+$	1.0	0.9
$\text{MOPS} + \text{Fe}\cdot\text{MOPS} + \text{EFE-H}^+ \rightleftharpoons \text{Fe}\cdot\text{EFE} + \text{MOPS-H}^+$		-2.3 ^c

^aCalculated from control experiments of EDTA chelating Fe^{2+} in a MOPS buffer. Value taken from Table S2.^bValue taken from ref 22.^cData from Table 1.

Table 3.

Thermodynamic Data for the Thermal Denaturation of Fe-EFE Species As Measured by DSC

sample	T_m (°C)	H^* (kcal/mol)
EFE	39.2 ± 0.7	86.8 ± 1.0
EFE-Arg	39.6 ± 0.1	92.5 ± 1.4
Fe-EFE	42.9 ± 0.7	96.8 ± 1.1
Fe-EFE-Arg	44.7 ± 1.3	86.9 ± 3.6
Fe-EFE-2OG	45.4 ± 0.6	109.8 ± 2.6
Fe-EFE-2OG-Arg	49.3 ± 0.2	124.8 ± 0.1

Table 4.

Thermodynamic Properties of Substrate Binding to EFE

substrate	G (kcal/mol)	δH (kcal/mol)	$-T \Delta S^a$ (kcal/mol)
iron (binding to EFE)	-9.6^b	-11.8 ± 0.7^b	+2.2
2OG (binding to Fe-EFE)	-7.2^b	-13.0 ± 2.8^c	+6.8
Arg (binding to Fe-EFE)	-5.6^d	$+10.0 \pm 3.8^c$	-15.6
2OG (binding to Fe-EFE-Arg)	-6.26^d	-38.0 ± 2.8^c	+31.74
Arg (binding to Fe-EFE-2OG)	-6.1^d	-15.0 ± 2.6^c	+8.93

^a Calculated from G and δH values reported here using the equation $G = \delta H - T \Delta S$, where the δH term is estimated to be equal to the enthalpy of binding.

^b Value determined from ITC experiments shown in Table S8 and Figure S4

^c Thermodynamic parameters derived from DSC data collected in this study.

^d Calculated values based on the K_d reported by Martinez et al.¹⁸

Table 5.

Deconvolution of the Enthalpy of Iron(II) Binding to EFE in MOPS Buffer

reaction	coefficient	<i>H</i> (kcal/mol)
$\text{EFE} \cdot \text{FeOH}_2 \rightleftharpoons \text{EFE} \cdot \text{FeOH} + \text{H}^+$	0.9	13.3 ^a
$\text{EFE} \cdot (\text{His} \cdot \text{H}^+) \rightleftharpoons \text{EFE} + \text{H}^+$	0.1	7.2 ^b
$\text{Fe}^{2+} + \text{EFE} \rightleftharpoons \text{Fe} \cdot \text{EFE}$	1.0	-11.8
$\text{Fe}^{2+} + \text{EFE} \cdot \text{H} \rightleftharpoons \text{Fe} \cdot \text{EFE} + \text{H}^+$	1.0	0.9 ^c

^aRepresents the ionization of water within the $\text{H}_2\text{O} \cdot \text{Fe}^{2+} \cdot \text{EFE}$ complex involving deprotonation to the $\text{HO} \cdot \text{Fe}^{2+} \cdot \text{EFE}$ species.

^bValue taken from ref 31.

^cData from Table 2.

Table 6.

Average Enthalpy Values in Each Buffer for Iron(II) Binding to EFE at pH 7.4

buffer	$H_{\text{Fe-EFE}}$ (kcal/mol)
MOPS	-11.8 ± 0.4
ACES	-12.1 ± 0.2
TES	-11.6 ± 0.5
average	-11.8 ± 0.7