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# $^{18}O$ Kinetic Isotope Effects in Non-Heme Iron Enzymes: Probing the Nature of Fe/$O_2$ Intermediates

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## Abstract

Reported here are the competitive $^{18}O/^{16}O$ kinetic isotope effects ($^{18}O$ KIEs) on $k_{cat}/K_m(O_2)$ for three non-heme iron enzymes that activate $O_2$ at an iron center coordinated by a 2-His-1-carboxylate facial triad: taurine dioxygenase (TauD), S-(2)-hydroxypropylphosphonic acid epoxidase (HppE), and 1-aminocyclopropyl-1-carboxylic acid oxidase (ACCO). The comparison of the measured $^{18}O$ KIEs with calculated $^{18}O$ equilibrium isotope effects ($^{18}O$ EIEs) reveals an excellent correlation with the proposed mechanisms for these enzymes. $^{18}O$ KIEs of 1.0104 ± 0.0002 (TauD), 1.0120 ± 0.0002 (HppE), and 1.0215 ± 0.0005 (ACCO) suggest the formation in the rate limiting step of $O_2$ activation of an $Fe^{III}$-alkylperoxo, $Fe^{III}$-OOH, and $Fe^{IV}$=O species, respectively. By probing only the steps from initial $O_2$ binding up to and including the first irreversible step of $O_2$ activation, the measured $^{18}O$ KIEs can be a valuable companion to pre-steady state kinetic analyses in studying the complex catalytic mechanisms of non-heme iron enzymes.

The $O_2$-activating, non-heme iron enzymes catalyze a wide range of oxygenation and oxidation reactions with important biological implications, such as DNA repair, hypoxic response, collagen biosynthesis, and histone demethylation.[1] Most of these enzymes contain a single iron center coordinated by two His and one Asp/Glu residues in a tridentate binding motif referred to as "2-His-1-carboxylate facial triad". Understanding the $O_2$-activation process for these enzymes may provide key insights into the source of their divergent substrate specificity despite similarly coordinated active site metal centers.[1]

Several recent studies have employed the measurement of competitive $^{18}O/^{16}O$ kinetic isotope effects ($^{18}O$ KIEs) on $k_{cat}/K_m(O_2)$ for $O_2$-activating metalloenzymes, in order to probe the early steps involved in $O_2$ activation and to reveal the nature of the metal/$O_2$ intermediate formed in the rate determining step (RDS) of $k_{cat}/K_m(O_2)$.[2,3] Reported here are the $^{18}O$ KIEs for three non-heme iron enzymes that activate $O_2$ at an iron center coordinated by a 2-His-1-carboxylate facial triad: taurine dioxygenase (TauD), S-(2)-hydroxypropylphosphonic acid (S-HPP) epoxidase (HppE), and 1-aminocyclopropyl-1-carboxylic acid oxidase (ACCO). These $^{18}O$ KIE measurements allow, for the first time, a direct comparison of the $O_2$ activation process by non-heme iron enzymes employing different substrates and coreductants.[1]

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Supporting Information Available: Protein expression and purification procedures, $^{18}O$ KIE experimental details, and $^{18}O$ EIE calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

TauD is an α-ketoglutarate (αKG)-dependent non-heme iron enzyme that catalyzes the hydroxylation of taurine in bacteria.[4] Its mechanism has been extensively investigated and several Fe/$O_2$ intermediates have been characterized, including a high-valent $Fe^{IV}$=O species.[5–7] The RDS of $k_{cat}/K_m(O_2)$ in TauD is proposed to be the attack of an $Fe^{III}$-$OO^{\bullet-}$ species on αKG to form a cyclic alkylperoxo intermediate, which then undergoes oxidative decarboxylation to form the $Fe^{IV}$=O species. The measured $^{18}$O KIE for TauD is 1.0102 ± 0.0002 at 30 °C (Figure 1).[8]

HppE is a reductase-dependent non-heme iron enzyme that catalyzes the epoxidation of S-HPP, the last step in the biosynthesis of the antibiotic fosfomycin.[9] The mechanism of HppE is not as well known as for TauD, formation of an $Fe^{III}$-OOH species being proposed to involve either a hydrogen atom transfer (HAT) from S-HPP or proton-coupled electron transfer (PCET) from the reductant.[10] The measured $^{18}$O KIE for HppE is 1.0120 ± 0.0002 at 25 °C, using FMN in the presence of NADH as the reductant (Figure 1).[8] This value is similar to the one obtained for TauD, suggesting a similar oxidation state for the Fe/$O_2$ intermediate formed in the RDS.

ACCO is an ascorbate-dependent non-heme iron enzyme that catalyzes the last step in ethylene biosynthesis, an important plant hormone.[11] Recent steady-state kinetic studies of ACCO suggest that substrate oxidation occurs after the RDS of $O_2$ activation, which most probably involves the formation of an $Fe^{IV}$=O species.[12] The $^{18}$O KIE for ACCO is 1.0215 ± 0.0005 at 25 °C (Figure 1), one of the largest values measured for $O_2$-activating metalloenzymes.[13]

Competitive $^{18}$O KIEs on $k_{cat}/K_m(O_2)$ reflect changes in the oxygen bond order that occur in all steps from initial $O_2$ binding up to and including the first irreversible step.[19] To help interpret the measured $^{18}$O KIEs, calculated $^{18}$O equilibrium isotope effects ($^{18}$O EIEs) can be obtained from vibrational frequencies of reactants and products,[14] following the formalism developed by Bigeleisen and Mayer.[20] These calculated $^{18}$O EIEs can be used as upper limits for the measured $^{18}$O KIEs (assuming a negligible isotope effect contribution from the reaction coordinate frequency),[21] allowing a direct comparison between model reactions and experimental values. Using known frequencies for $Fe^{III}$-$OO^{\bullet-}$,[15] $Fe^{III}$-OOH,[1] and $Fe^{IV}$=O species,[7] the relevant Fe/$O_2$ intermediates for the studied enzymes,[1,22] we calculated $^{18}$O EIEs of 1.0080, 1.0172, and 1.0287, respectively (Table 1).[8] In addition, $^{18}$O EIEs of 1.0187 and 1.0143 were calculated for an Fe-alkylperoxo species, $Fe^{III}$-$OO^{t}Bu$,[16,17] and an Fe-peroxocarbonate species,[18] respectively, the latter being similar to a proposed intermediate in TauD.[6]

The comparison of the measured $^{18}$O KIEs with calculated $^{18}$O EIEs reveals an excellent correlation with the proposed mechanisms for these enzymes (Scheme 1). In TauD, the formation of the cyclic alkylperoxo intermediate in the rate determining step is supported by the measured $^{18}$O KIE of 1.0102 ± 0.0002, higher than the calculated $^{18}$O EIE for formation of an $Fe^{III}$-$OO^{\bullet-}$ species (1.0080) and the measured $^{18}$O EIE for Mb (1.0054 ± 0.0006, Table 1),[14] but lower than the calculated $^{18}$O EIE for an Fe-peroxocarbonate species (1.0143). The $^{18}$O KIE of 1.0120 ± 0.0002 for HppE is similar to the measured $^{18}$O EIE of 1.0113 ± 0.0005 for Hr[14] and lower than the calculated $^{18}$O EIE of 1.0172 for an $Fe^{III}$-OOH species (Table 1), suggesting a rate determining formation of an $Fe^{III}$-OOH species.[23] The relatively small measured $^{18}$O KIEs in TauD and HppE could have been diminished vs. calculated $^{18}$O EIEs due to the presence of a partially rate limiting $O_2$ binding step. This appears not to be the case for HppE, where preliminary data support a $^{18}O/^{16}O$ discrimination free from kinetic complexity.[23] For TauD, a kinetically significant $O_2$ binding step cannot be ruled out,[6] yet the excellent agreement between the measured $^{18}$O KIE and the calculated $^{18}$O EIE (Table 1) strongly suggests a rate limiting formation of the cyclic alkylperoxo intermediate. Thus, in both TauD and HppE the observed $^{18}$O KIEs are concluded to be mechanistic in origin.

For ACCO, the large $^{18}O$ KIE of 1.0215 ± 0.0005 (lower than only one calculated $^{18}O$ EIE of 1.0287, Table 1) implies a significant change in the oxygen bond order and points toward $Fe^{IV}$=O species formation as the RDS of $O_2$ activation (Scheme 1).[13] In ACCO, the oxidation of the $Fe^{III}$-$O_2^{\bullet-}$ species to $Fe^{III}$-OOH by ascorbate is proposed to be reversible, similar to the reversible $O_2$ binding observed in hemerythrin.[14] Interestingly, the initial inner sphere reduction of $O_2$ and formation of an Fe-$OO^{\bullet-}$ species does not appear to be rate limiting for any of these enzymes, in accordance with a recently proposed reversible $O_2$ binding as a requisite for the specific reactivity of non-heme iron enzymes.[24] This is in contrast to a rate limiting and irreversible outer sphere activation of $O_2$ by glucose oxidase.[2,3]

The studies reported herein reveal the ability of $^{18}O$ KIE measurements to dissect the complex catalytic mechanisms of non-heme iron enzymes. By probing only the steps from $O_2$ binding to the first irreversible step of $O_2$ activation, these measurements offer a valuable companion to pre-steady state kinetic analyses, which investigate the nature of the intermediate immediately preceding the RDS. In the present case, measured $^{18}O$ KIEs for three non-heme iron enzymes that use different reductants in the course of $O_2$ activation have been directly related to each enzyme's distinct chemical mechanism.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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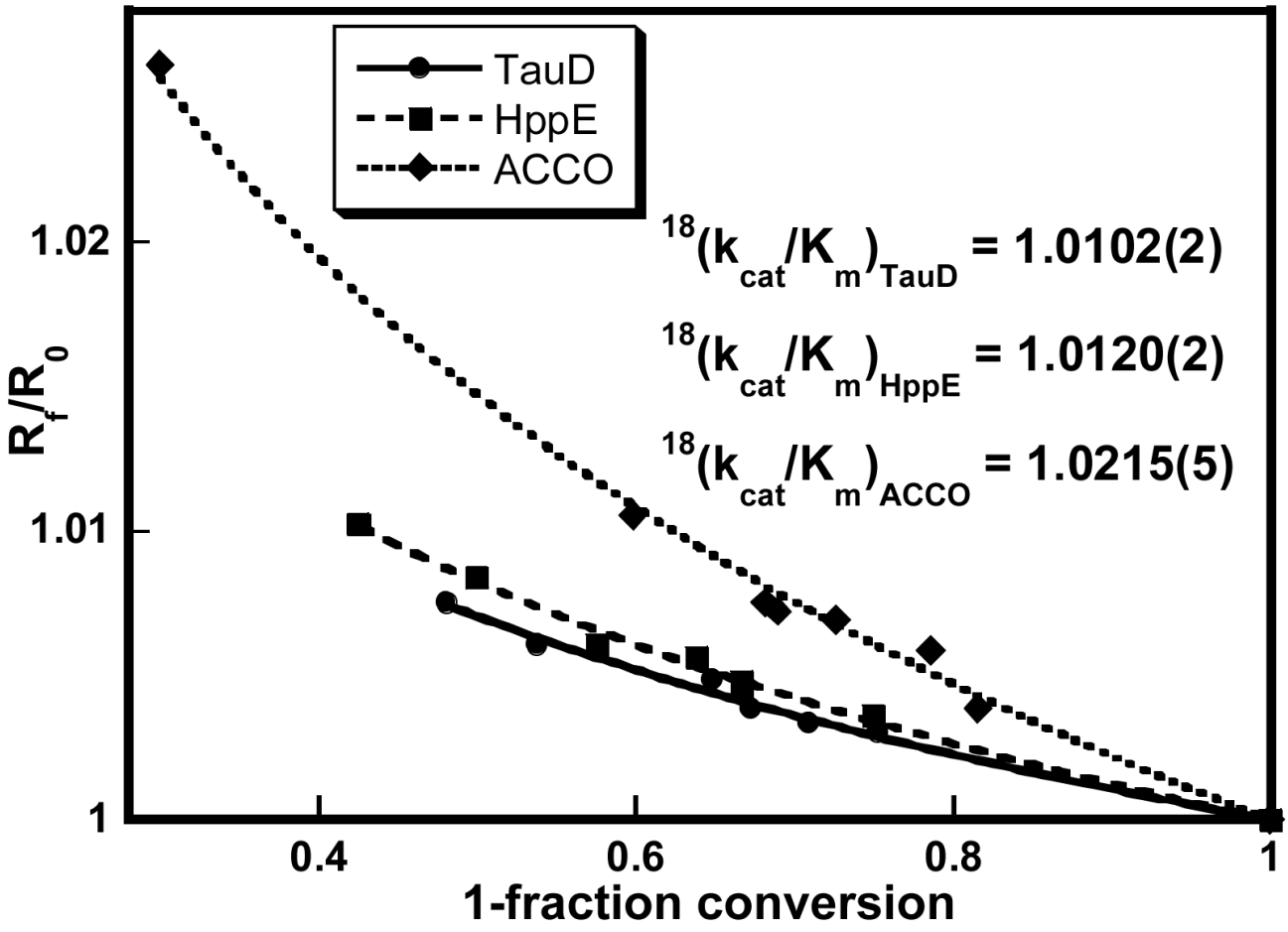
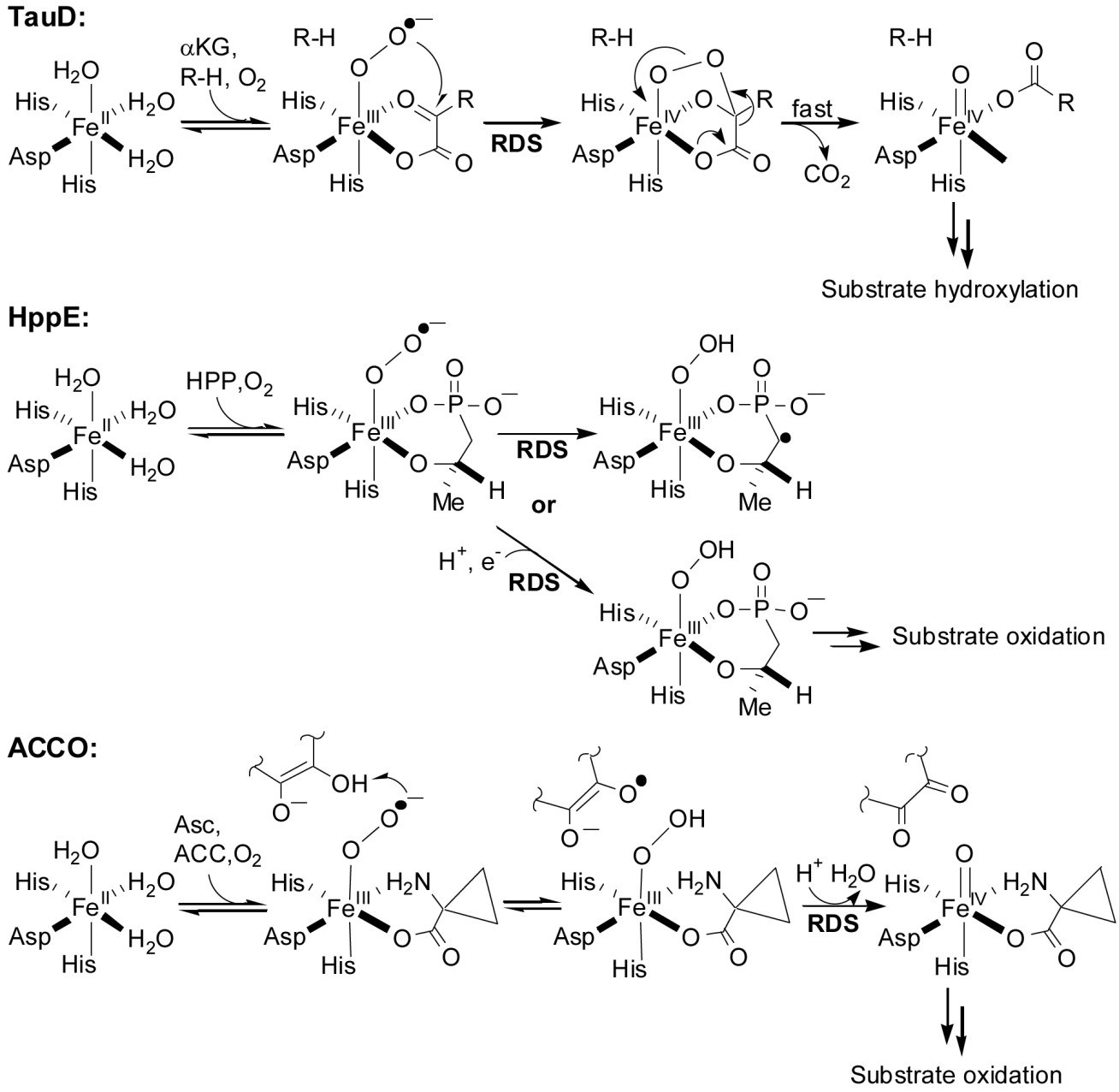



**Figure 1.**

Isotope fractionation plots for TauD (●), HppE (■), and ACCO (◆). The fits for obtaining $^{18}O$ KIEs are shown in solid, dashed, and dotted lines, respectively.[8] Conditions: TauD: 0.2 μM TauD, 0.4–0.6 mM $O_2$, 2 mM taurine, 2 mM αKG, 0.2 mM ascorbate, 50 mM BisTris pH 6.2, 30 °C; HppE: 10μM HppE, 0.4–0.6 mM $O_2$, 1 mM S-HPP, 11 μM FMN, 1.5 mM NADH, 20 mM Tris-HCl pH 7.5, 25 °C; ACCO: 5 μM ACCO, 0.3–0.5 mM $O_2$, 3 mM ACC, 20 mM ascorbate, 20 mM $NaHCO_3$, 100 mM NaCl, 100 mM MOPS pH 7.2, 25 °C.



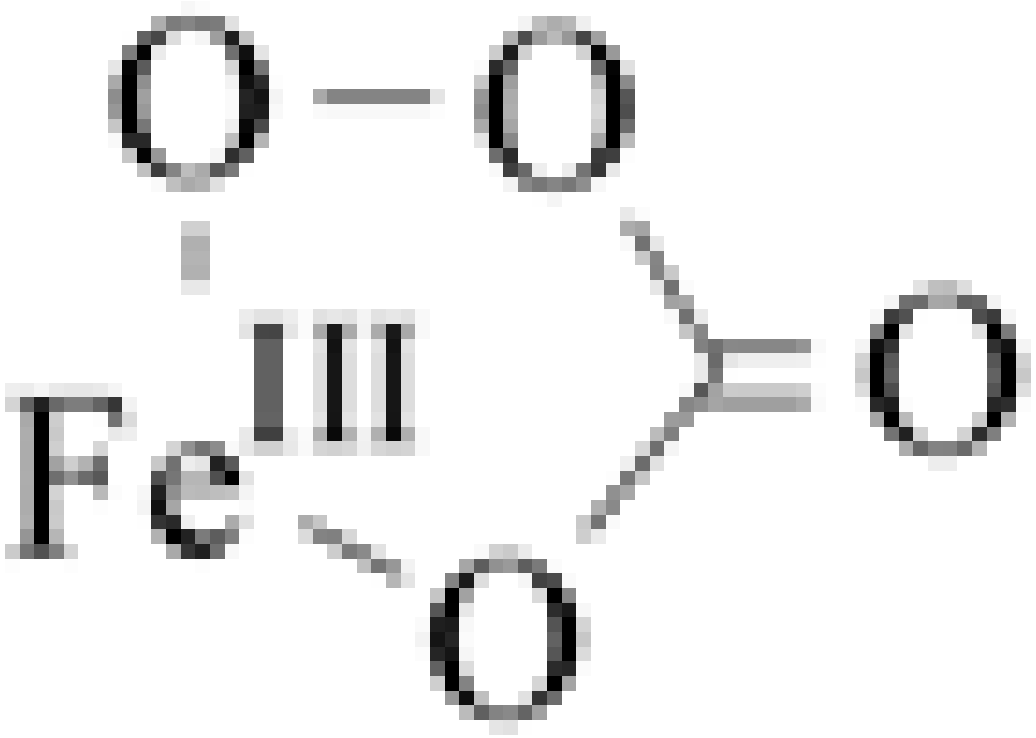

**Scheme 1.**

Proposed mechanisms of $O_2$ activation for TauD, HppE, and ACCO (RDS = proposed rate determining step of $k_{cat}/K_m(O_2)$, R-H = taurine, Asc = ascorbate).

**Table 1**

Vibrational frequencies ($cm^{-1}$) of $Fe/O_2$ species, the corresponding calculated $^{18}O$ EIEs, and experimental $^{18}O$ E/KIEs.

| molecule | Frequency ($cm^{-1}$) mode | $\nu_{16\text{-}16}$ | $\nu_{18\text{-}18}$ | $^{18}O$ EIE (calc)[a] | $^{18}O$ EIE (expt)[b] | $^{18}O$ KIE (expt)[c] |
|---|---|---|---|---|---|---|
| $Fe^{III}$-$OO^{\bullet-}$ | Fe-O[d] | 555 | 526 | 1.0080 | 1.0054 (Mb) | ND[e] |
| | O-O[d] | 1136 | 1100[f] | | | |
| $Fe^{III}$-OOH | Fe-O[g] | 621 | 599 | 1.0172 | 1.0113 (Hr) | 1.0120 (HppE) |
| | O-O[g] | 844 | 820[f] | | | |
| | O-H[h] | 3539 | 3527 | | | |
| | O-O-H[h] | 1205 | 1199 | | | |
| $Fe^{III}$-$OO^{t}Bu$ | Fe-O[i] | 637 | 612 | 1.0187 | ND | ND |
| | O-O[j] | 860 | 829[f] | | | |
| | O-$^{t}$Bu[j] | 746 | 738 | | | |
| | Fe-O[k] | 547 | 524 | 1.0143 | ND | 1.0102 (TauD) |



| molecule | Frequency ($cm^{-1}$) mode | $\nu_{16-16}$ | $\nu_{18-16}$ | $^{18}$O EIE (calc)[a] | $^{18}$O EIE (expt)[b] | $^{18}$O KIE (expt)[c] |
|---|---|---|---|---|---|---|
| | O-O[k] | 884 | 863[f] | | | |
| | O-C[k] | 965 | 946 | | | |
| | O-C-O[k] | 728 | 710 | | | |
| $Fe^{IV}$=O | Fe-O[l] | 821 | 787 | 1.0287 | ND | 1.0215 (ACCO) |

[a] The $^{18}$O EIEs were calculated using the Bigeleisen-Mayer equation and known frequencies (Ref 8).

[b] Measured $^{18}$O EIEs for $O_2$-binding proteins myoglobin (Mb) and hemerythrin (Hr) (Ref 14).

[c] This work.

[d] Ref 15.

[e] Not determined.

[f] $\nu_{18-16}$ was calculated as follows: $\nu_{18-16} = (\nu_{16-16} \nu_{18-18})^{1/2}$ (Ref 8).

[g] Ref 1.

[h] Ref 14.

[i] Ref 16.

[j] Ref 17.

[k] Ref 18.

[l] Ref 7.