Studies on the kinetics and mechanism of the oxidation of γ-glutamylcysteine by a Co(III)-bound superoxide

Ritu Mukherjee

Department of Chemistry, Dum Dum Motijheel College, Kolkata 700 074.

E-mail: ritu069@gmail.com

Abstract

In an aqueous acetate buffer solution, γ-glutamylcysteine (GGC), which is a precursor to glutathione, acts as a reducing agent and reduces the bridging superoxide in $[(NH_3)_5Co^{III}(O_2)Co^{III}(NH_3)_5]^{5+}$ (**1**). This reduction results in the formation of the corresponding peroxide in the complex, $[(NH_3)_5Co^{III}(O_2H)Co^{III}(NH_3)_5]^{5+}$ (**2**). During this process, the reductant (GGC) is oxidized and forms its disulfide form. The complex **2** that is formed subsequently undergoes rapid decomposition, leading to the formation of the final products such as Co^{II}, NH_3, and O_2. Complex **2** undergoes this decomposition process rather than reacting with a second molecule of GGC. When there is an excess of reductants compared to complex **1**, the reaction follows first-order kinetics and shows an inverse dependence on proton concentration. Additionally, the observed rate constant (k_o) values decrease as the ionic strength (I) of the medium increases. These observations suggest that thiolate anions are the reactive forms of the reductant in the reaction. The pK_a value of GGC (1.91) also supports this thiolate ion formation in the experimental pH range.

KEYWORDS: Cobalt(III), Superoxide, γ-glutamylcysteine, Redox, Kinetics, Mechanism.

Introduction

Glutathione (GSH) an abundant thiol antioxidant within cells, is widely recognized [1,2]. Numerous chronic and age-related ailments have been connected to a decrease in GSH levels within the cells [3,4]. Under conditions of oxidative stress, γ-glutamylcysteine (GGC), which serves as a precursor to glutathione (GSH), can restore diminished GSH levels by bypassing the regulation of GSH biosynthesis and supplying the essential substrate. [5,6]. In addition to its function as a substrate in the synthesis of GSH, GGC also contributes to shielding against oxidative stress by acting as an antioxidant and influencing the expression of protein(s) involved in antioxidant defense mechanisms [7,8]. In mammals, GGC exhibits a neuroprotective function in vivo, particularly in age-related disorders and diseases. [9, 10]. The oxidation of membrane lipids caused by free radicals and the resulting peroxidation, as well as oxidative harm to DNA, have been linked to various long-term health issues such as cancer, inflammation, degenerative brain diseases, and the aging process [11, 12]. Metalloenzymes usually initiate their oxygen activation mechanisms by binding to dioxygen, which leads to the transfer of electrons from the metal to O_2, producing a metal-superoxo compound [13,14]. Therefore, the reactions involving superoxide are of particular significance and attract special attention.. Metal coordinated superoxide can react with other molecules to form other ROS, such as hydroxyl radicals, which are highly reactive and can damage biomolecules [15]. Hence the kineic study of redox reaction between a metal coordinated superoxide and GGC is of great significance. The current study focuses on investigating the mechanism behind reduction of bridging superoxide ligand in the dinuclear complex, $[(NH_3)_5Co^{III}(O_2)Co^{III}(NH_3)_5]^{5+}$ (**1**), by GGC in the pH interval 4.3–5.45 maintained by acetate buffer.

Experimental

Materials

Cobalt(II) nitrate (Aldrich), γ- glutamylcysteine (Aldrich), perchloric acid (Merck), 2,6-pyridinedicarboxylic acid (dipicolinic acid, Aldrich) and sodium perchlorate (Aldrich), and were used without further purification. All the experimental solutions were prepared with freshly prepared double distilled water. μ-superoxobis[pentaamminecobalt(III)] chloride, $[(NH_3)_5Co^{III}(O_2)Co^{III}(NH_3)_5]Cl_5$, (**1**) was synthesised by the literature process [16]. The corresponding perchlorate salt was prepared from the chloride salt [17] and then subjected to recrystallization using a 10% HClO4 solution. [ε (M^{-1} cm^{-1}) at 670 nm: found 830, reported 838[16]. Only fresh stocks of γ- glutamylcysteine (GGC) were used for kinetic and stoichiometric studies. All the other materials utilized were of high-quality reagent grade and employed as received without additional purification steps.

Instrumentation

Absorbance and UV-VIS spectra were measured using a Shimadzu (1601 PC) spectrophotometer, employing a 1.00 cm quartz cell. The kinetics of the reaction were monitored in real-time within an electrically controlled, thermostated (25.0 ± 0.1 °C) cell housing (CPS - 240 A) at 670 nm, which corresponds to the visible absorption peak of compound **1**. The acid solutions' concentrations were determined through pH metric titration using a Metrohm 736-GP Titrino instrument. The pH of the reaction solution was measured utilizing a Toshniwal pH-meter (model CL-54, India). The reaction media were purged with argon to remove any dissolved gases before conducting the kinetic measurements. The first-order rate constants (k_o) were determined by fitting the time-dependent decay of the absorbance (A_t) of compound **1** to a

standard first-order exponential decay equation using non-linear least-squares fitting.

Kinetic measurement

Unless otherwise stated, the interactions between complex **1** and GGC were investigated within the pH range of 4.3-5.45, while maintaining a constant ionic strength (*I*) of 0.5 M using $NaClO_4$, and at a temperature of 25.0 °C. Complex 1 demonstrates remarkable stability against spontaneous decomposition and exhibits favorable reaction rates with GGC within the experimental acidity range. Existing literature indicates that metal ions, such as Cu^{2+}, can accelerate the oxidation of thiol molecules by forming complexes between Cu^{2+} and thiol compounds. [18]. In this study, dipicolinic acid (dpa), a widely recognized sequestering agent, was employed at a concentration of 2.0 mM to inactivate any metal ions present by forming chelates with them. [19]. The measured volume of dipicolinic acid (dpa) was combined with the reactant solution prior to the addition of the GGC solution. Results obtained from separate experiments confirm that neither compound **1** nor GGC molecules react with dpa. The advancement of the aforementioned reactions was tracked by observing the reduction in absorbance specifically at 670 nm, a wavelength where only compound **1** absorbs and no other reactants contribute. Figure 1 illustrates the time-resolved changes in the UV-VIS spectrum for the reaction mixture containing compound **1** and GGC. Kinetic experiments were conducted under conditions where the concentration of GGC was in excess, ensuring the maintenance of a pseudo first-order reaction. The observed rate constant (k_o) values were obtained by fitting the absorbance versus time data to a first-order non-linear least-squares model. Moreover, the extracted k_o values were graphed against various reaction parameters to investigate the relationship and dependency between ko and these parameters.

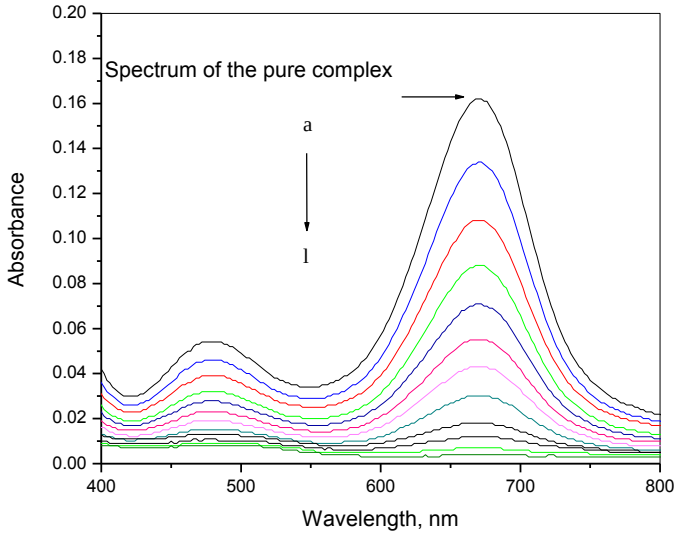


Fig. 1. Time-resolved spectra of 0.20 mM of **1** reacting with 0.02 M GGC. pH = 4.70 in acetate buffer (T_{OAc} = 0.20 M), *I* = 0.50 M ($NaClO_4$), T = 25.0 ^{o}C.. (a): spectrum of pure complex shown in black; (b) – (l): spectra of reaction mixtures.

Stoichiometry

The stoichiometry of the reaction was established by reacting a known excess of compound **1** with a predetermined limited amount of GGC. The equilibrium absorbance values were then calculated to determine the stoichiometric ratio between compound **1** and GGC.

Results and discussion

Stoichiometry and the reaction products

By employing an excess of [**1**], the remaining concentration of compound **1** was measured using spectrophotometry. The results indicated a stoichiometry of 1:1 between the change in concentration of GGC (Δ[GGC]) and the change in concentration of compound **1** (Δ[**1**]). The observed stoichiometry implies that the reaction product of GGC is its disulfide derivative. **1** is

commonly reduced to its predominant reduction product. $[(NH_3)_5Co^{III}(HO_2)Co^{III}(NH_3)_5]^{5+}$ (**2**) [20]. Spectral observations confirm that the reduction of **1** by divalent metal ions such as, V^{2+}, Eu^{2+} and Cr^{2+} in acidic conditions leads to the formation of a protonated intermediate peroxo complex structure. (**2**) [21]. **2** undergoes rapid decomposition to yield Co(II), NH_3, and O_2 [17, 20], but this decomposition does not significantly impact the overall kinetics. Furthermore, independent experiments have demonstrated that the disulfide products do not react with **1**. Therefore, the overall reaction can be described by the following equation, followed by the rapid decomposition of **2**.

$$2[(NH_3)_5Co^{III}(O_2)Co^{III}(NH_3)_5]^{5+} + 2C_8H_{13}N_2O_5SH \rightarrow 2[(NH_3)_5Co^{III}(HO_2)Co^{III}(NH_3)_5]^{5+} + (C_8H_{13}N_2O_5S\text{-}SO_5N_2C_8H_{13})$$

(**1**) (**2**)

Kinetics and Mechanism

In the acidic media utilized throughout the study, both the superoxo complex **1** and GGC remain stable. However, upon the addition of freshly prepared GGC to **1**, there is a gradual reduction in the absorbance of **1**. Under the given reaction conditions, GGC exhibited a gradual consumption of **1**, resulting in a decrease in absorbance over time. The reaction proceeded with excellent first-order kinetics. Notably, the first-order rate constants (k_o) displayed a linear increase with the concentration of GGC ([GGC]). (Table 1, Fig 2) Throughout the entire range of acidity investigated, the first-order rate constants (k_o) showed a significant inverse dependence on proton concentration (Table 1, Fig 3).

Table 1. Some representative first-order rate constants (k_o) for the reduction of **1** (0.20 mM) by GGC, T_{OAc} = 0.2 M, [dpa] = 2.0 mM , T = 25.0 ^oC.

[GGC], M	**pH**	***I* , M ($NaClO_4$)**	**$10^3 k_o$, s^{-1}**
0.02	4.20	0.50	0.91
0.02	4.35	0.50	1.15
0.02	4.50	0.50	1.3
0.02	4.75	0.50	2.8
0.02	4.90	0.50	4.1
0.02	5.10	0.50	6.2
0.04	4.50	0.50	2.2
0.06	4.50	0.50	2.9
0.08	4.50	0.50	4.5
0.1	4.50	0.50	3.8
0.02	4.50	0.25	1.8
0.02	4.50	0.70	1.1
0.02	4.50	0.85	0.9
0.02	4.50	1.0	0.6

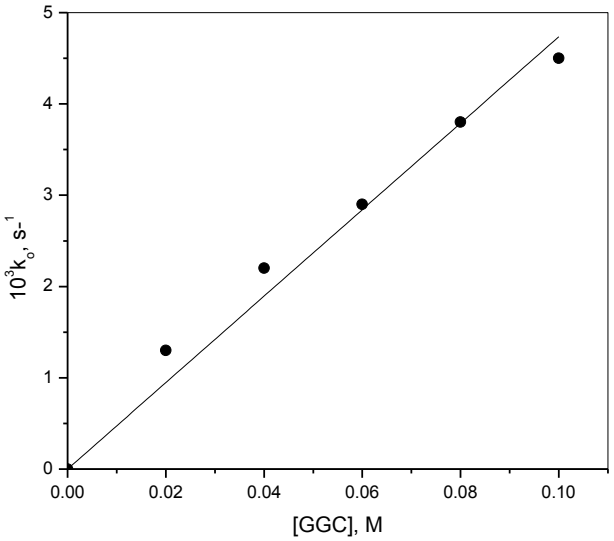


Fig. 2. Variation of k_o with [GGC]. [**1**] = 0.20 mM, pH = 4.50 (T_{OAc} = 0.20 M), I = 0.5 M ($NaClO_4$), [dpa] = 2.0 mM, T = 25.0 ^oC.

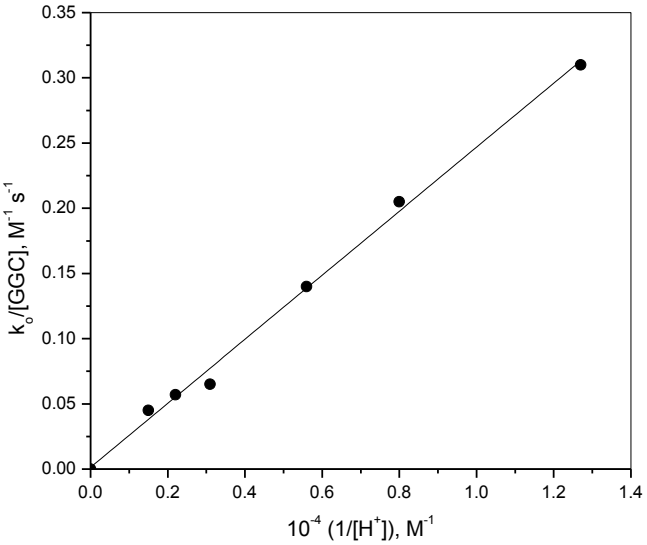
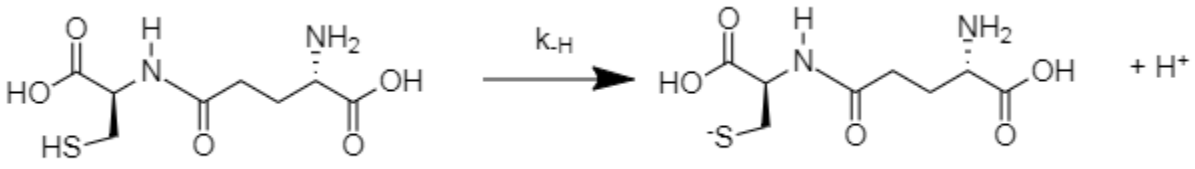


Fig. 3. Variation of k_o/[GGC] with 1/[H^+], [**1**] = 0.20 mM, [GGC] = 0.02 M**,** (T_{OAc} = 0.20 M), *I* = 0.50 M ($NaClO_4$), [dpa] = 2.0 mM, T = 25.0 ^oC.

Variation of k_o with [GGC] and [H^+]

The plots depicting the observed rate constants are as follows: k_o vs. [GGC] are shown in Figure 2. The observed rate constants exhibited a linear relationship with the concentrations of GGC ([GGC]). Importantly, the plots passed through the origin, indicating that the reactions followed first-order kinetics.

The considerable enhancement in the rate observed with $[H^+]^{-1}$ (as shown in Figure 3) cannot be attributed to the deprotonation of the superoxo complex. This is because previous studies on metal-bound superoxo complexes have indicated that the bridging superoxo group does not engage in any protic equilibria. [22]. Only the thiol component of GGC undergoes protic equilibria, and its corresponding pK_a value is determined to be 1.91. [6]. The pK_a value of GGC (1.91) indicates that it undergoes significant deprotonation, resulting in the formation of the thiolate species. GGC_{-H}. This observation suggests that the reactive reductants in the system are likely to be the corresponding deprotonated thiolate anions.



Variation of *k*o with *I*

The influence of changes in the ionic strength (*I*) of the reaction media on the rate constant (k_o) is particularly significant. It has been observed that an increase in the ionic strength of the reaction

media leads to a decrease in the rate values. (Figures 4, Table 1). Redox reactions between two species with opposite charges are indicated by the negative slopes observed in the plots. Given that oxidant **1** is cationic, the reactive reductant is expected to be anionic. This observation aligns with the conclusion drawn from the data of k_o versus $[H^+]$, providing further support for the involvement of thiolate anions, specifically GGC_{-H}, as the reactive reductant.

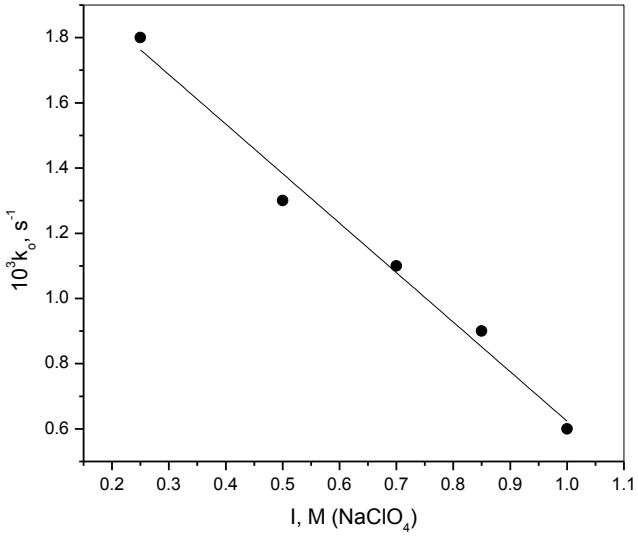
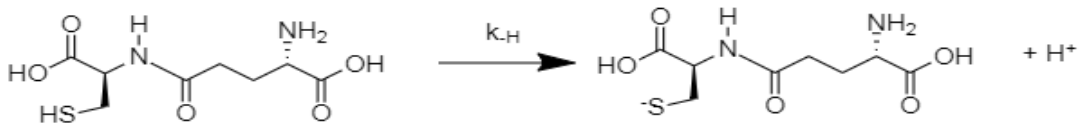


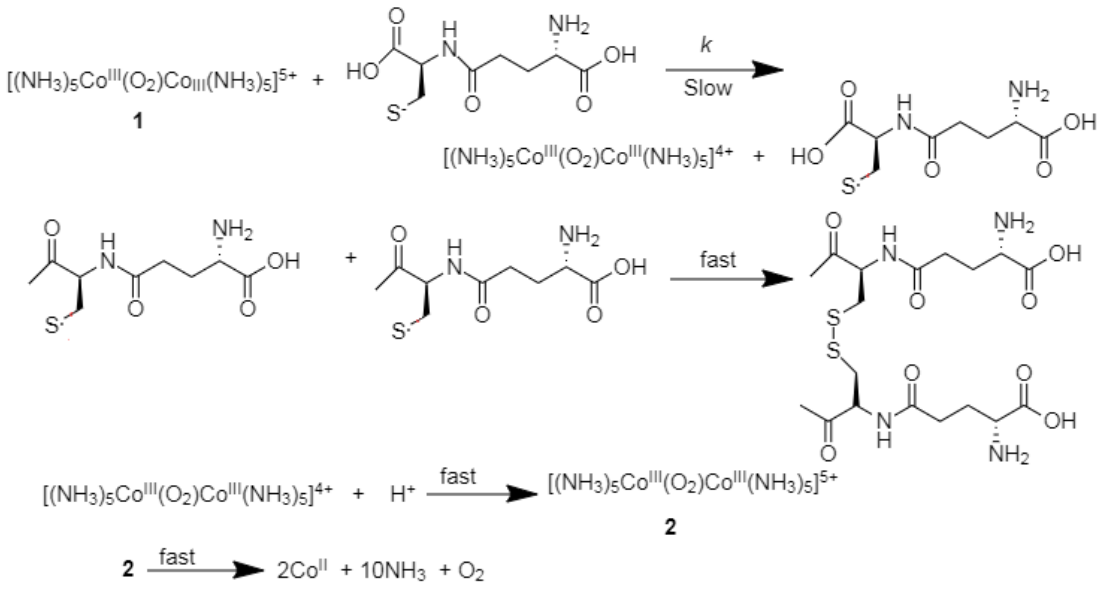
Fig. 4. Variation of k_o with I ($NaClO_4$), [**1**] = 0.20 mM, [GGC] = 0.20 M, pH = 4.50 (T_{OAc} = 0.20 M, [dpa] = 2.0 mM, T = 25.0 ^oC.

Proposed mechanism for the reactions

The uncatalyzed reactions show a linear relationship between the observed rate constant (k_o) and the concentration of thiol molecules ([GGC]). Additionally, the observed rate constant decreases when the concentration of protons ($[H^+]$) increases. Furthermore, based on the data obtained from studying the rate of the reaction in relation to ionic strength, it can be deduced that two reactive species involved in the rate-determining step have opposite charges. Additionally, when

the aqueous media was enriched with deuterium oxide (D2O), there was no significant change in the reaction rate. This lack of a solvent isotope effect suggests that there is no involvement of an electroprotic pathway in the reaction. [23]. Based on these observations, a proposed mechanism for the uncatalyzed reaction was presented in Scheme 1. In the given reaction conditions, GGC undergoes deprotonation to form GGC_{-H}. In the rate-determining step, GGC_{-H} reduces a molecule of 1 to produce its corresponding peroxo species. Simultaneously, GGC_{-H} itself undergoes oxidation, leading to the formation of the corresponding thiyl radical. In the subsequent rapid reactions, the peroxo complex acquires a proton in the presence of acidic conditions. Ultimately, the peroxo complex undergoes self-dissociation, resulting in the formation of Co(II), NH_3, and O_2. The thiyl radical of GGC_{-H} dimerizes to the corresponding disulphide.





Scheme 1

Scheme 1 leads to the eqn.

$k_o/[GGCl] = k_{-H}k/[H^+]$

A plot of the of $k_o/[GGC]$ against the reciprocal of $[H^+]$ showed a strong linear relationship, with a statistically insignificant intercept. (Fig. 3).

Conclusion

The biochemically important uncatalyzed oxidation of γ-glutamylcysteine (GGC), by superoxo complex **1** in acid media is studied in detail here. Based on the observations, it can be inferred that the redox reaction proceeds through the initial formation of the thiolate anion. This indicates that the reactions are catalyzed by a base. The effect of change in ionic strength of the media supports this mechanism and absence of media solvent effect rules out electroprotic path.

Acknowledgements

Department of Chemistry, Dum Dum Motijheel College is thankfully acknowledged for instrumental facilities.

Conflict of interest

The author declares that there is no conflict of interests regarding the publication of this article.

References:

1. A. Meister and M.E. Anderson, *Annu. Rev. Biochem.*, **52**, 711 (1983); https://doi.org/10.1146/annurev.bi.52.070183.003431
2. R. Brigelius-Flohe, *Free Radic. Biol. Med*., **27**, 951 (1999); https://doi.org/10.1016/s0891-5849(99)00173-2
3. H. Jay Forman, H. Zhang, and A. Rinna, *Mol Aspects Med.*, **30**, 1 (2009); https://doi.org/10.1016/j.mam.2008.08.006
4. A. Pastore, G. Federici, E. Bertini, F. Piemonte, *Clin. Chim. Acta*, 333, 19 (2003); https://doi.org/10.1016/S0009-8981(03)00200-6
5. N. Braidy, M. Zarka, B. E. Jugder, J. Welch, , T. Jayasena, D. K. Y. Chan, P. Sachdev, and W. Bridge, *Frontiers in Aging Neuroscience*, **11**, 177 (2019); https://doi.org/10.3389%2Ffnagi.2019.00177
6. M.H. Zarka, W.J. Bridge, *Redox Biology*. **11**, 631 (2017); https://doi.org/10.1016/j.redox.2017.01.014R
7. R. Quintana-Cabrera and J. P. Bolaños, *Biochem. Soc. Trans.*, **41**, 106 (2013); https://doi.org/10.1042/bst20120252
8. G. Ferguson, W. Bridge, *Arch. Biochem. Biophys.*, **593**, 12 (2016); https://doi.org/10.1016/j.abb.2016.01.017
9. C. Guo, L. Sun, X. Chen, and D. Zhang,. *Neural Regen. Res.*, **8**, 2003 (2013); https://doi.org/10.3969/j.issn.1673-5374.2013.21.009

10. J.B. Melo, P. Agostinho, C.R. Oliveira, *Neurol. Res.*, **45**, 117 (2003); https://doi.org/10.1016/S0168-0102(02)00201-8
11. C.M. Jones, M.J. Burkitt, *J. Am. Chem. Soc.*, **125**, 6946 (2003); https://doi.org/10.1021/ja034416z
12. V. Lobo, A. Patil, A. Phatak, and N. Chandra, *Pharmacogn Rev.*, **4** 118 (2010); https://doi.org/10.4103%2F0973-7847.70902
13. F. Nastri, M. Chino, O. Maglio, A. Bhagi-Damodaran, Y. Lu, and A. Lombardi, *Chem. Soc. Rev.*, **45**, 5020 (2016); https://doi.org/10.1039/C5CS00923E
14. W. Nam, *Acc. Chem. Res.*, **40**, 465 (2007); https://doi.org/10.1021/ar700131d
15. S. Fukuzumi, H. Ohtsu, K. Ohkubo, S. Itoh and H. Imahori, *Coord. Chem. Rev.*, **226**, 71 (2002) https://doi.org/10.1016/S0010-8545(01)00435-0.
16. R. Davies, M. Mori, A. G. Sykes and J.A. Weil, *Inorg. Synth,*, **12**, 197 (1982)
17. S.K. Saha, M.C. Ghosh, E.S. Gould, *Inorg. Chem.*, **31**, 5439 (1992); https://doi.org/10.1021/ic00052a020
18. R. Mishra, S. Mukhopadhyay, R. Banerjee, *Dalton Trans.*, **39**, 2692 (2010); https://doi.org/10.1039/B918582H
19. K.A. McCall, C.A. Fierke, *Anal. Biochem.*, **284**, 307 (2000); https://doi.org/10.1006/abio.2000.4706
20. S.P. Ghosh, S.K. Saha, R.N. Bose, J.W. Reed, M.C. Ghosh, E.S. Gould, *Inorg. Chem.*, , **32**, 2261 (1993);

https://doi.org/10.1021/ic00063a011

21. A.B. Hoffman and H. Taube, *Inorg. Chem*. **7**, 1971 (**1968)**;

    https://doi.org/10.1021/ic50068a003

22. T.-Y. Chen, P.-H. Ho, C.-J. Spyra, F. Meyer, E.Bill, S. Ye, and W.-Z. Lee, *Chem. Comm.*, **56**, 14821 (2020)

    https://doi.org/10.1039/D0CC05337F

23. J.A. Gilbert, S.W. Gersten, T.J. Meyer, *J. Am. Chem. Soc.*, **104** 6872 (1982);

    https://doi.org/10.1021/ja00388a107

24. R.A. Binstead, M.E. McGuire, A. Dovletoglou, W.K. Seok, L.E. Roecker, T.J. Meyer, *J. Am. Chem. Soc.*, **114**, 173 (1992);

    https://doi.org/10.1021/ja00027a025