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Thermodynamics and Kinetics of Reduction of Fe(III) Acetohydroxamic Acid Complex by Ascorbic Acid

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Abstract. Kinetic and thermodynamic studies of reduction of Fe(III) acetohydroxamic acid ([Fe(III)-AHA]) complex by ascorbic acid (AA) was performed at pH values ranging from 3.00-4.50 ± 0.1 (ionic strength 0.2) and temperature 05.0 - 25.0 ± 0.5 °C. The redox reactions were studied, spectrophotometrically, under pseudo first order conditions of [AA] over the [Fe(III)-AHA] under the experimental conditions. The redox reaction was found to be highly pH dependent. The values of 2nd order rate constants (k_2), at 25 °C and pH 3.0, 3.5, 4.0 and 4.5 were found out to be 2.3920, 2.1550, 2.0122, 1.7596 $M^{-1}s^{-1}$, respectively. At 20 °C the values were 1.7290, 1.4000, 1.3400, 1.2650 $M^{-1}s^{-1}$ at pH 3.0, 3.5, 4.0 and 4.5, respectively. The results at 15 °C and pH 3.0, 3.5, 4.0 and 4.5 were 1.1410, 0.9459, 1.0390, 0.9126 $M^{-1}s^{-1}$, respectively. While, the values of rate constants at 10 °C were 0.7847, 0.7697, 0.6810, 0.7096 $M^{-1}s^{-1}$ and at 5 °C were 0.6167, 0.5106, 0.4775, 0.4833 $M^{-1}s^{-1}$, respectively. In addition to that, the following rate law was evaluated. Rate = $dp/dt = k_2$ [Fe(III)-AHA] [AA]. Moreover, the thermodynamic activation parameters of the reaction were also determined. The values of $\Delta Ea^{\#}$ at pH 3.0, 3.5, 4.0 and 4.5 were found out to be 22.4058, 16.3243, 19.9636, 14.6050 J/mol, respectively. While, the values of $\Delta H^{\#}$ were 58.5056, 42.6258, 52.1288, 38.1363 J/mol, respectively at pH 3.0 to 0 4.5. Values of $\Delta S^{\#}$ were also evaluated as -23.0297, -68.7567, -37.8287, -84.1377 J/mol/K at pH 3.0, 3.5, 4.0 and 4.5, respectively.

Keywords: acetohydroxamic acid, ascorbic acid, hydroxamate, siderophore, thermodynamics

Introduction

Siderophores are low molecular weight organic molecules that are produced by microorganisms under iron-stress conditions. Siderophores have been the core interest for many years, amongst the biological scientists due to the fact that they help boost the uptake of iron to the microbial cells. These siderophores, with respect to their three main classes i.e., hydroxamate, catecholate and carboxylate share extensive areas of research. Under aerobic environment, the Fe(III) form of iron is insoluble, due to its low ksp and hence is inaccessible at physiological pH (7.40). Under such conditions, microorganisms synthesise siderophores that have high affinity for Fe(III). They chelate Fe(III) and these complexes are then transported to cytosol, where ferric iron is reduced to ferrous iron and becomes available to microorganism. In recent years, siderophores have attracted much attention due to its potential roles in different fields (Saha *et al*., 2016). Three different siderophores mycobactins (Francis *et al*., 1949), ferrichrome (Neilands, 1952) and coprogen (Hesseltine *et al*., 1952) were isolated as growth factors during 1949 to 1952. By the end of the 1960s, several hydroxamate siderophores were recognized. So, there are three main classes of siderophores like catecholate, hydroxamate and hydroxycarboxylate that have high affinity for iron(III) (Xiao *et al*., 1992).

Iron is the most important and abundantly available element but its extremely low solubility in the form of $Fe(OH)_3$ ($Ksp=10^{-39}$) (Neilands, 1952; Francis *et al*., 1949) is very challenging for its bioavailability. The total soluble form of Fe(III) containing hydroxyl species is 10^{-10} M (Hesseltine *et al*., 1952). Iron is very valuable for all living organisms (Crichton, 2001; Xiao *et al*., 1992) as it performs variety of functions in biology. To cope up this low solubility problem, microorganisms are very efficient to synthesize the iron carrier compounds known as siderophores. These microorganisms capture, store and transfer the iron (Sandy and Butler, 2009; Dhungana and Crumbliss, 2005; Raymond *et al*., 2003; Pierre *et al*., 2002; Stintzi *et al*., 2000; Albrecht-Gary and Crumbliss, 1998). Very high Fe(III) complex formation constants (logβ =30-50) (Andrews *et al*., 2003; Pierre *et al*., 2002; Howard, 1999; Guerinot, 1994) for these complexes reveal the reduction

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mechanism pathways from Fe(III) to Fe(II) (Harrington and Crumbliss, 2009; Boukhalfa *et al*., 2000; Kwak, and Rhee, 1992; Cooper *et al*., 1978) proceeded by some biological reductants (Matzanke *et al*., 2004; Hallé and Meyer, 1992; Monzyk and Crumbliss, 1979; Neilands, 1952).

On biological grounds, ascorbic acid has immense values in performing many key functions in the body (Packer and Fuchs, 1997). Its tremendous role as a biological redutant exhibits to protect different components of the cell from oxidative damage. In this regard, it scavenges different radicals and damaging oxygen involving requisitions (Arrigoni and Tulio, 2002; Tolbert *et al*., 1975).

Desferrioxamine B, a hydroxamate siderophore, has long been used for the treatment of iron overload conditions in β-thalassemia (Raymond *et al*., 1982). Acetohydroxamic acid (Fig. 1a), a model, synthetic monohydroxamic acid, can be used as a ligand to investigate the chemistry of Fe(III) with hydroxamate siderophores. This study was conducted by Alvin Crumbliss and his students (Monzyk and Crumbliss, 1979). The complexes of iron(III) with AHA have high stability constants and hence are very stable as compared to iron(II) complexes. So, the reduction (Bezkorovainy, 1980) of iron(III) in [Fe(III)-AHA] complex can provide a suitable pathway for release of iron from this complex (Crosa, 1989). A comparison of stability constants of Fe^{+2} and Fe^{+3} siderophore complexes is given in Table 1.

Acetohydroxamic has long been used as a drug to treat UTI (urinary tract infections). Keeping in view the strong complexation between Fe(III) and AHA, biological significance of AA and AHA, a study of reduction of [Fe(III)-AHA] complex by ascorbic acid (AA) is reported here. This study is aimed at the removal and

Table 1. Stability constants for some iron siderophore complexes (Boukhalfa and Crumbliss, 2002)

Siderophores	Log[(β Fe(III))]	Log[(β Fe(II))]
Enterobactin	49.0000	23.9100
Pyoverdin	30.8000	9.7800
Ferrichrome A	32.0000	9.9100
Ferrichrome E	32.5000	11.1600
Ferrichrome B	30.6000	10.2900
Aerobactin	22.5000	4.8600
Acetohydroxamic acid	28.2900	11.2000

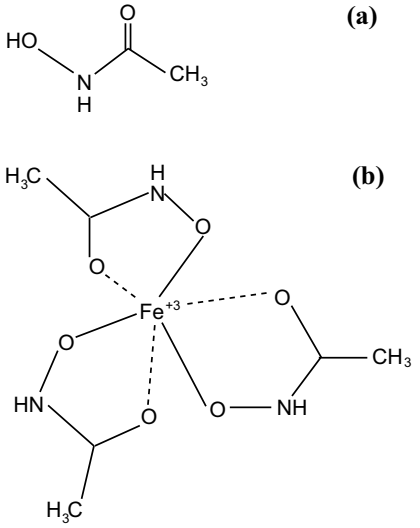

Fig. 1. (a) Acetohydroxamic acid (b) Iron(III) acetohydroxamic complex.

subsequent recovery of Fe(III) from this complex and was carried out through visible spectrophotometry.

For the present study, [Fe(III)-AHA] complex (Fig. 1b) was synthesized in solution under varying conditions of pH and temperature, and its subsequent reduction by ascorbic acid (AA) was studied.

Materials and Methods

In the present study the complexes of Fe(III) with AHA were prepared and reduced further by AA at different pH of the solution. Figure 2 shows the absorption spectra of these complexes at different pH. The colour change from intense to colourless exhibits the extent of the reduction.

Analytical grade chemicals were used throughout the experiments. For the preparation of solutions, each time distilled de-ionized water was used. This de-ionized water was also boiled, degassed and cooled in air tight containers.

Preparation of solutions. ***Preparation of stock solution of $Fe(NO_3)_3.9H_2O$ and standardisation.*** An approximately 1.01×10^{-2} M stock solution of Fe(III) was prepared by dissolving 1.01 g of $Fe(NO_3)_3.9H_2O$ (Riedel de Haen) in 250 mL volumetric flask.

i- The solution was acidified with 1.5 mL of 0.05 M HNO_3 before making it up to the mark.

ii- This solution was standardised by Fe-opt method (Ford-Smith and Sutin, 1961; Bandemer

and Schaible, 1944; Fortune and Mellon, 1938; Saywell and Cunningham, 1937).

The actual concentration was found to be 1.00×10^{-2} M with 5% impurities. This solution was used as a stock solution for preparation of complex solutions at desired pH.

Acetohydroxamic acid solution. This solution was prepared as per requirement by dissolving the calculated and accurately weighed amount of AHA (Wako) in the buffer solutions of respective pH i.e., 3.0, 3.5, 4.0, and 4.5.

Ascorbic acid solution (Wako). Ascorbic acid was prepared freshly in the buffer solutions of desired pH, before each use. This solution was degassed and purged with N_2 for 10-15 min, especially, to remove oxygen gas.

Buffer solutions. Formate buffer solutions of pH 3.0, 3.5, 4.0 and acetate buffer of pH 4.5 were prepared in deionized water. The ionic strength of buffer solutions was 0.2 M which was maintained by NaCl and KCl.

Preparation of formate buffers. Format buffers of pH 3.0, 3.5, and 4.0 were prepared by taking 100.0 mL of standardized 1.6 M NaOH (Merck). Formic acid (TEDIA) was added drop wise to this solution till the pH values 3.0, 3.5 and 4.0 were obtained. To make the ionic strength equal to 0.2 M, 4.68 g of NaCl (Merck) were added to the same volumetric flask before making up the volume up to 2000.0 mL, using de-ionized water.

Preparation of acetate buffer. Acetate buffer of pH 4.5 was prepared in the same way except that acetic acid (Merck) was used instead of formic acid. In this case the ionic strength was maintained using 5.96 g of KCl (Avonchem).

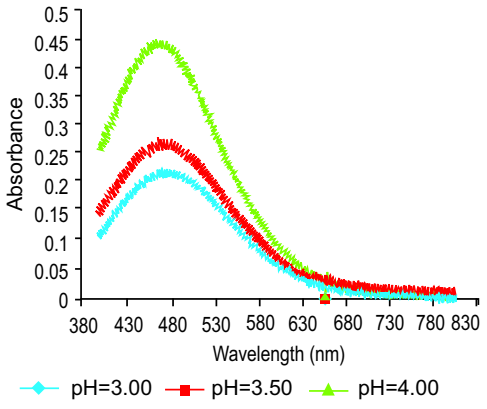

Fig. 2. Sample absroption spectrum of [Fe(III)-AHA] at different pH. [Fe(III)-AHA] = 2.0×10^{-4} M, μ = 0.2 M.

Formation of [Fe(III)-AHA] complex. [Fe(III)-AHA] complex was prepared by mixing Fe(III) solution and AHA solution of known concentrations. The concentration of AHA solution was kept 5 times over the concentration of Fe(III) to ensure the complete complexation in form of 1:3 (M:L). Molar extinction coefficients (ε) of [Fe(III)-AHA] complex were calculated at different pH and temperature as shown in Table 2.

Instrumentations. Diode array spectrometer model HP 8452A, logger pro 3.2 and stopped flow model RX-2000 were used to follow the reaction and record the absorbance and changes in absorbance during the kinetic experiments. Analytical balance model TE214S Sartorius was used for weighing in the entire research work. The re-circulating water chiller model 470 was used for the maintenance of temperature. It allows the effective re-circulating water chilling system with a temperature display. The temperature control is the most important part of the chiller and can be set manually as per requirement of the experiment.

Kinetic experiments. The kinetics of reduction of Fe(III)-hydroxamic acid complexes by ascorbic acid, under the pseudo first order conditions was studied spectrophotometrically. These reactions were monitored at λ_{max} of the complexes through diode array spectrophotometer in a compatibility of stopped flow. The other conventional spectrophotometer is not effective for such kind of measurements because the reaction between Fe(III)-AHA and AA was found to be too fast to be observed by manual mixing.

The drive syringes with equal volumes of both the reagents were filled or loaded and then stayed for 15-20 min to maintain the temperature through water circulating bath. The cuvette of the stopped flow was set into the cuvette holder of photodiode array spectrophotometer. Thus both syringes are operated simultaneously as it reaches to the trigger block. Different sets of reactions were observed under different conditions till 4-5 half lives.

The rate of reduction of Fe(III)-acetohydroxamic acid complexes by ascorbic acid was observed in a range of concentration from 1.5×10^{-3} to 2×10^{-2} M at a particular temperature 05.0 to 25.0 ± 0.5 °C, pH 3.00 - 4.50 ± 0.1 with ionic strength 0.2 M and at the λ_{max} of complex at respective pH.

Results and Discussion

Stoichiometry of the [Fe(III)-AHA] complex. The complex formation reaction between Fe^{+3} and AHA has already been studied extensively by Kazmi and McArdle (1981). Those studies have shown a linear dependence of k_{obs} on AHA. Moreover, the formation of $[Fe(AHA)_2]$ was discussed that supports the formation of $[Fe(AHA)]^{+2}$. The studies show that the stoichiometry of complex depends upon the pH of the solution. At lower pH (Nisar and Kazmi, 2006) such as 3.00 and 3.50, $[Fe(III)\text{-}(AHA)_2]$ species may exist whereas $[Fe(III)\text{-}(AHA)_3]$ may exist at higher pH, such as, 4.00 and 4.50. So, the pH will be a key parameter in deciding the stoichiometry of the [Fe(III)-AHA] complex.

The reduction of [Fe(III)-AHA] complex by ascorbic acid was investigated spectrophotometrically under pseudo 1st order conditions, over a range of pH 3.00 to 4.50. The preparation of the complex solution was carried out in the buffers of respective pH. The values of λ_{max} for [Fe(III)-AHA] complex at pH 3.0, 3.5, 4.0 and 4.5 are given in Table 2.

$$Fe(III) + n(CH_3CONHOH) \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}}$$

$$CH_3CONHO^- \longrightarrow [(CH_3CONHO)_nFe] \text{ ..... } 1$$

It was previously observed that as the pH values are increased, λ_{max} increases (Kazmi and McArdle, 1981) significantly.

$$Fe(III)\,(CH_3CONHO)^{+(3-n)_n} + H^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}}$$

$$[(CH_3CONHO)_{n-1}(F(III)] + CH_3CONHO$$

$$[(CH_3CONHO)_{n-1}(F(III)] + HAsc^- \xrightarrow{k_2} Fe(II) + Asc\cdot + (n\text{-}l)CH_3CONHOH$$

In the above equation 1, n is representing the number of moles of AHA; 1, 2 or 3 depending on the pH. The maximum possibility for the complexation i.e. 1:3 increases as we keep on increasing the value of pH. The k_{obs} values were determined through the linear regression analysis of the raw data. The slopes of the plots of $\ln(A_t\text{-}A_\alpha)/(A_o\text{-}A_\alpha)$ vs t correspond to the values of the pseudo first order rate constant (k_{obs}) according to equation 2.

$$\ln(A_t\text{-}A_\alpha)/(A_o\text{-}A_\alpha) = k_{obs} \cdot t \text{ ..... } 2$$

We obtained a straight line by plotting $\ln(A_t\text{-}A_\alpha)/(A_o\text{-}A_\alpha)$ versus time for many half lives and out of these sample plots one plot of kinetic runs is given in Fig. 3. For the reactions at pH 3, k_{obs} values are tabulated in Table 3. The values of k_{obs} were plotted against the [AA] and gave a straight line suggesting that the reaction is first order with respect to the concentration of ascorbic acid. These plots of k_{obs} verses concentration of ascorbic acid for each corresponding pH are shown in Fig. 4.

The plots of k_{obs} *vs* [AA] were found to be linear, indicating a simple 1st order pathway for reduction of Fe^{+3} to Fe^{+2} in the complex. The values of rate constants are given in Table 4.

Table 2. Molar absorptivity of Fe(III) acetohydroxamic complex at different pH

pH	λ_{max}	ε (M/cm)
3.00	480	1046.5000
3.50	470	1196.0000
4.00	472	1410.5000
4.50	468	1635.0000

Table 3. Values of k_{obs} (s^{-1}) for the reduction of Fe(III)-AHA by AA at pH = 3.00

[AA] M	k_{obs} (s^{-1})				
	25 °C	20 °C	15 °C	10 °C	05 °C
0.02	4.2768	3.2500	2.2928	1.4630	1.2000
0.015	3.5300	2.7500	1.9190	1.2060	1.0300
0.01	2.9092	2.2500	1.4928	1.0525	0.8800
0.005	2.5647	1.6850	1.2018	0.8237	0.7661
0.0025	2.0122	1.2650	0.9126	0.7096	0.6167
0.0015	1.7392	1.0250	0.6495	-	0.5695

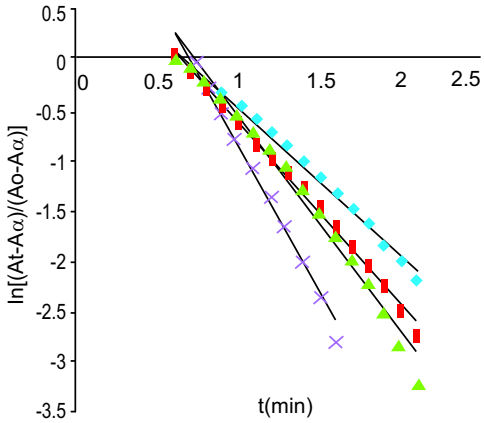

Fig. 3. Sample plot for $\ln(At\text{-}A\alpha)/A_0\text{-}A\alpha$ versus t.

It has already been reported that at lower pH 3.00 and 3.50, the formation of bis (acetohydroxamato)-Fe(III) complex is more feasible (Nisar and Kazmi, 2006; Kazmi and McArdle, 1981). In the presence of suitable reducing agent, [Fe(III)-$(AHA)_2$] complex undergoes reduction to [Fe(II)-$(AHA)_2$] complex easily.

Table 4. Values of rate constants at different pH and temperatures

T (°C)	pH	k_2 (M/s)	k_o
5	3.00	32.72	0.5492
	3.50	64.65	0.352
	4.00	58.89	0.357
	4.50	59.20	0.294
10	3.00	41.91	0.610
	3.50	92.77	0.480
	4.00	108.3	0.392
	4.50	96.24	0.507
15	3.00	82.99	0.664
	3.50	88.7	0.926
	4.00	167.6	0.492
	4.50	130.9	0.722
20	3.00	116.8	0.985
	3.50	178.5	0.976
	4.00	192.9	0.942
	4.50	150.1	1.408
25	3.00	127.0	1.690
	3.50	185.3	1.285
	4.00	235.1	1.890
	4.50	164.5	1.665

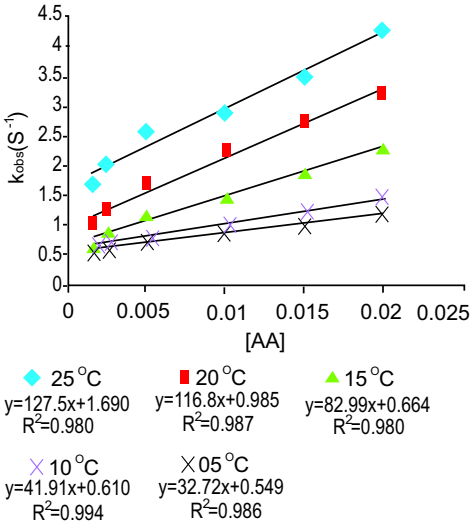

Fig. 4. Sample plot between k_{obs} (s^{-1}) and [AA] at pH 3.00 and different temperature [Fe(III)-AHA] = 2.0×10^{-4} M; λ_{max} = 480 nm; pH = 3.00; μ = 0.2 M; T = 5.0 - 25.0 ± 0.5 °C.

At higher pH, 4.00 and 4.50, the reduction process becomes more challenging because of the change in stoichiometry from1:1 to 1:3, through 1:2 hence, the value of k_2 must decrease. But in the present study, the trends in the values are in reverse order that is, instead of decreasing, the values of rate constants are increasing as shown in Table 4.

The two competing factors are very important and responsible for these trends in k_2 values at different pH. One factor is the stoichiometry of the complex and the second, is the redox potential of the reducing agent at different pH. At low pH, 1:1 & 1:2 complexation dominates over 1:3 species, which are easier to be reduced. On the basis of equation 1, as the pH is raised n goes from 1 to 3 making its reduction a challenging task for the reducing agents. While, the E° value of AA is also highly pH dependent (Daniel *et al*., 1985). Values of formal potential are available (Daniel *et al*., 1985) and show an increased reducing power with the rise of pH. Trends in the values of k_2 from Table 4 indicate that the increased redox potential of AA with pH is the dominant factor in this case.

k_o is insignificant as $k_o <<< k_2$
Hence, $k_{obs} = k_o + k_2$ [AA]

Here k_2 depends upon the concentration of ascorbic acid but k_o is independent. Under the conditions $k_o <<< k_2$

$k_{obs} = k_2$[AA]
Rate = k_{obs} [Fe(III)-AHA]
Rate = k_2 [Fe(III)-AHA] [AA]

On the basis of all above observations, our suggested mechanism for the given reaction is as:

$$AAH \rightleftharpoons AA^- + H^+$$

$$AA^- \longrightarrow AA^{\cdot} + e^-$$

$$2AA^{\cdot} \longrightarrow 2AA$$

$$AAH + [Fe(III)\text{-}(AHA]_n \xrightarrow{RDS} AA^{\cdot} + [Fe(II)\text{-}AHA]_n$$

$$2AA^{\cdot} \xrightarrow{Fast} AAAA$$

Here, AA = Ascorbic acid
Rate = -d[Fe(III)-$(AHA)_n$]/ dt = k [AA] [Fe(III)-$(AHA)_n$]
Rate = k_{obs} [Fe(III)-$(AHA)_n$]
So, $k_{obs} = k_2$ [AA]

Thermodynamic studies. Thermodynamic parameters (ΔEa, $\Delta H^{\#}$ and $\Delta S^{\#}$) of the reaction were determined through Arhenius (equation 3) and Eyring (equation 4) plots and the values are tabulated in Table 5.

$$-\ln k = \ln A + (-Ea/RT) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \; 3$$

Equation 3 is the equation of a straight line whose slope is $-Ea/R$. This helps in determining the activation energy from values of rate constant at different temperatures, by plotting ln*k* as a function of 1/*T*.

$$\ln k/T = -\Delta H^{\#}R/T + \ln kB/h + \Delta S^{\#}/R \quad \ldots\ldots\ldots\ldots \; 4$$

where:

k is the rate constant
kB is the Boltzmann's constant (1.381 x 10^{-23} J/K),
T is the absolute temperature in Kelvin (K) and
h is Planck's constant (6.626 x 10^{-34} Js).

The values for $\Delta H^{\#}$ and $\Delta S^{\#}$ can be determined from kinetic data obtained from a plot of lnk/T vs. 1/T. The equation is a straight line with negative slope, $-\Delta H^{\#}/R$, and a y-intercept, $\Delta S^{\#}/R + \ln k_B/h$. $\Delta H^{\#}$ is changing from +58.5056 kJ/mol to +38.1363 by changing the pH from 3.00 to 4.50 but $\Delta S^{\#}$ goes from -23.0297 to -84.1376 as shown in Table 5. Here the negative sign of $\Delta S^{\#}$ supports an associative mechanism for the redox reaction. Negative values for $\Delta S^{\#}$ indicate that entropy decreases on forming the transition state, which often indicates an associative mechanism in which two reaction partners form a single activated complex (Johnstone and Nolan, 2015).

Table 5. Activation parameters $\Delta Ea^{\#}$, $\Delta S^{\#}$ and $\Delta H^{\#}$ at different pH

pH	$\Delta Ea^{\#}$	$\Delta H^{\#}$ J/mol	$\Delta S^{\#}$ J/mol/K
3.00	22.4058	58.5056	-23.0297
3.50	16.3243	42.6258	-68.7568
4.00	19.9636	52.1288	-37.8287
4.50	14.6050	38.1363	-84.1377

Conclusion

The results of this study have shown that increasing the pH of the medium also increases the reduction of the said complex. Moreover, the trends in the values of rate constants with increasing pH show that the physiological pH could enhance the reduction of [Fe(III)-AHA] complex. If acetohydroxamic acid administered during UTI, chelates iron stores present in the body, a proper dose of vitamin C might be helpful in reducing this complex and maintaining iron stores of the body. So, it is recommended that vitamin C should be given to the patients taking acetohydroxamic acid (lithostate) drug to treat urinary tract infections.

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