Kinetics of Oxidation of Amino Acids by Alkaline Hexacyanoferrate(III) †

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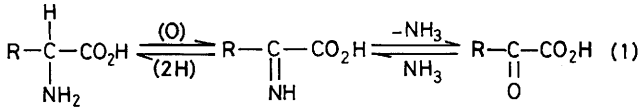
The kinetics of oxidation of amino acids (lysine, arginine, and histidine) by alkaline hexacyanoferrate(III) has been studied at constant ionic strength over the temperature range 318—338 K. The rate was dependent on the first powers of the concentrations of substrate and oxidant, but independent of the concentration of the alkali in the range studied. The reaction proceeds by way of the α-imino acid, formed in a rapid step, which then undergoes hydrolysis to give the corresponding α-keto acid.

The fact that important naturally occurring amino acids have α-hydrogen atoms suggests that the biosynthesis and degradation of amino acids occur by way of α-imino acids and α-keto acids [equation (1)].



(1)

The oxidation of amino acids is of utmost importance, both from a chemical point of view and its bearing on the mechanism of amino acid metabolism. The present investigation forms part of our broad programme of studying mechanistic aspects of the oxidation of amino acids by potassium hexacyanoferrate(III) in alkaline media. The oxidation of amino acids lysine, arginine, and histidine were studied at constant ionic strength, under a nitrogen atmosphere.

Experimental

The substrates (BDH) were found to be chromatographically pure, but were further assayed by the acetous perchloric acid method,[1] and aqueous solutions were used for kinetic studies. All other compounds employed were E. Merck samples. The ionic strength of the system was kept constant at a high value (0.5 mol dm^{-3}) using a concentrated solution of sodium perchlorate. Triply distilled water was used throughout. All reactions were performed under a nitrogen atmosphere.

Kinetic Measurements.—The rate studies were made under pseudo-first-order conditions with an excess of amino acid over the oxidant. Aqueous solutions of potassium hexacyanoferrate(III) were prepared in aqueous NaOH, and $NaClO_4$ solution was added to adjust the ionic strength. The two solutions were separately thermostatted at 328 K for 1 h, under nitrogen, and then mixed in equal volumes by syringing into the spectrophotometric cell. The kinetics was followed by monitoring the disappearance of $[Fe(CN)_6]^{3-}$, spectrophotometrically, at 420 nm (UV 26, Beckman). At this wavelength the absorption due to hexacyanoferrate(II) was negligible.[2] The course of the reaction was studied for two half-lives. The rate constants were evaluated from linear (r = 0.990) plots of log[oxidant] against time, and were reproducible to within ±3%.

Stoicheiometry and Product Analysis.—Varying ratios of hexacyanoferrate(III) to amino acid, in the presence of 0.5 mol dm^{-3} NaOH (ionic strength adjusted by the addition of $NaClO_4$) were equilibrated at 328 K for 24 h, under nitrogen.

Table 1. Characterisation of product

Amino acid	Keto acid (oxidation product)	2,4-Dinitrophenylhydrazone derivative (m.p./°C)
Lysine	ε-Amino-α-oxohexanoic acid	212
Arginine	δ-Guanidino-α-oxovaleric acid	216
Histidine	β-Imidazolylpyruvic acid	190

Estimation of the residual oxidant showed that 1 mol of amino acid consumed 2 mol of hexacyanoferrate(III), corresponding to the stoicheiometry (2).

$$RCH(NH_2)CO_2H + 2[Fe(CN)_6]^{3-} + 2OH^- \longrightarrow RCOCO_2H + NH_3 + 2[Fe(CN)_6]^{4-} + H_2O \quad (2)$$

Using the same experimental conditions that were used for the kinetic determinations, solutions of substrate and oxidant, in NaOH (ionic strength adjusted by the addition of the requisite amount of $NaClO_4$), were mixed and kept at 328 K for 24 h under nitrogen.

(*i*) The evolution of ammonia was shown by partial distillation of the reaction mixture. The ammonia formed was absorbed in an excess of standard acid (0.5 mol dm^{-3} HCl). The excess of acid was then back-titrated (against base) in the presence of methyl red indicator.[3]

(*ii*) The reaction mixture was extracted with diethyl ether, washed with water, dried over anhydrous $MgSO_4$, and then concentrated. The product obtained was the corresponding keto acid, which was detected by spot tests,[4] in agreement with earlier work.[5]

(*iii*) The product obtained was treated with an acidic solution of sodium hydrogensulphite, and cooled in ice. 2,4-Dinitrophenylhydrazine solution (0.05 mol dm^{-3}, 25 cm^3) was added and the mixture allowed to stand overnight at 0 °C. The solid compound formed was filtered off, dried, recrystallised from a mixture of ethyl acetate and light petroleum (b.p. 60—80 °C), and weighed as the corresponding 2,4-dinitrophenylhydrazone derivative of the keto acid (Table 1). The yields in all cases were between 70 and 80%.

E.S.R. Measurements.—Using the requisite reaction conditions, the radicals were generated, in a flow system, by mixing

† *Non-S.I. unit employed:* G = 10^{-4} T.

Table 2. Rate data for the oxidation of amino acids at 328K (I = 0.5 mol dm^{-3})

10^2[Substrate]	10^3[$K_3Fe(CN)_6$]	[NaOH]	$10^4 k_{obs.}$/s^{-1}		
	mol dm^{-3}		Lysine	Arginine	Histidine
1.0	1.0	0.5	1.5	1.0	1.6
2.5	1.0	0.5	3.8	2.8	4.2
5.0	1.0	0.5	7.5	5.5	8.3
10.0	1.0	0.5	15.0	10.0	16.0
25.0	1.0	0.5	38.0	28.0	41.0
1.0	0.75	0.5	1.6	1.0	1.7
1.0	0.50	0.5	1.5	1.2	1.5
1.0	0.25	0.5	1.6	1.0	1.5
1.0	0.10	0.5	1.5	1.0	1.7
1.0	0.05	0.5	1.5	1.2	1.6
1.0	1.0	0.75	1.5	1.0	1.7
1.0	1.0	0.25	1.6	1.1	1.8
1.0	1.0	0.10	1.5	0.9	1.6
1.0	1.0	0.05	1.6	1.0	1.7
1.0	1.0	0.01	1.5	1.0	1.7

Table 3. Effect of temperature, and activation parameters

	$10^4 k_{obs.}$/s^{-1}		
T/K (±0.1°)	Lysine	Arginine	Histidine
318	0.7	0.4	0.9
323	1.0	0.8	1.3
328	1.5	1.0	1.6
333	2.4	2.0	2.7
338	3.6	3.3	4.3
E/kJ mol^{-1}	44 ± 2	49 ± 2	43 ± 2
$\Delta H^{\ddagger}$/kJ mol^{-1}	41 ± 2	46 ± 2	40 ± 2
log A	4.6	4.5	4.0
$\Delta S^{\ddagger}$/J K^{-1} mol^{-1}	−170 ± 4	−160 ± 4	−175 ± 4

[Substrate] = 1 × 10^{-2}, [$K_3Fe(CN)_6$] = 1 × 10^{-3}, [NaOH] = 0.5, I = 0.5 mol dm^{-3}.

Table 4. Solvent isotope effect at 328 K

	$10^4 k_{obs.}$/s^{-1}		
Amino acid	k_{H_2O}	k_{D_2O}	k_{H_2O}/k_{D_2O}
Lysine	1.5	1.6	0.94
Arginine	1.0	1.1	0.91
Histidine	1.6	1.8	0.89

Concentrations as in Table 3.

the substrate and oxidant, by volume, in an e.s.r. sample tube just outside the cavity of the spectrometer (E 4, Varian). The mixture was placed under high vacuum in order to expel dissolved oxygen, and the sample tube was placed in the cavity of the spectrometer. The conditions for obtaining the spectra at 77 K were as follows: scan range 4 000 G, field set 3 300 G, modulation amplitude 6.3 G, microwave frequency 9.45 GHz, time constant 0.3 s, scan time 4 min.

Results and Discussion

Kinetic Results.– The rate of the reaction was observed to be dependent on the first powers of the concentrations of substrate and oxidant, but was independent of the concentration of alkali in the range studied (Table 2). Plots of $k_{obs.}$ against a 25-fold range of concentration of substrate were linear passing through the origin, suggesting a first-order dependence of the rate of oxidation on the concentration of the substrate. This was further seen by the constant values of k_2, the second-order rate constant.

When a constant concentration of substrate (large excess) was used, the pseudo-first-order rate constant, $k_{obs.}$, did not show any appreciable variation with changing concentration of oxidant, indicating a first-order dependence of the reaction on the oxidant concentration (Table 2).

Under the present experimental conditions, the rate law can be expressed as in equation (3). A linear correlation (r = 0.996)

$$\text{Rate} = -\text{d}[Fe(CN)_6^{3-}]/\text{d}t = k_{obs.}\,[\text{Amino acid anion}][Fe(CN)_6^{3-}] \quad (3)$$

between log $k_{obs.}$ and the reciprocal of temperature in the range 318—338 K was observed for all the amino acids. The rate data and the activation parameters are shown in Table 3.

The addition of hexacyanoferrate(II) ions in the concentration range 1 × 10^{-4}—5 × 10^{-3} mol dm^{-3} did not have any effect on the rates of these oxidation reactions, indicating that the reaction between the substrate and oxidant (the electron-abstraction step) was irreversible.

Variations in the ionic strength of the medium using $NaClO_4$ (I = 0.01—0.50 mol dm^{-3}) did not have any effect on the rates of these reactions, neither did the addition of salts such as NaCl, $NaNO_3$, KNO_3, Na_2SO_4, and $MgSO_4$ (concentration range 1 × 10^{-4}—5 × 10^{-3} mol dm^{-3}).

The radicals generated from the oxidation of each of the substrates, using a flow method (E 4, Varian), gave five-line e.s.r. spectra, with intensity ratios of 1:3:4:3:1.

Solvent isotope studies in D_2O gave k_{H_2O}/k_{D_2O} values close to unity (Table 4). Even though OD^- is a stronger base than OH^-, the k_H/k_D value indicated that OD^- did not influence the kinetics of the reaction. Since the rate of the reaction was independent of the concentration of alkali, in the range studied, the ratio k_{H_2O}/k_{D_2O} should be close to unity. This has been observed.

The linear correlation between log (rate) at 318 and 338 K for the reactions (r = 0.997) shows that an isokinetic relationship exists in the oxidation of these amino acids by hexacyanoferrate(III). The value of the isokinetic temperature is 459 K. An isokinetic relationship is a necessary condition for the validity of linear free-energy relationships. It also implies that all these

Table 5. Ionisation constants[6] and pH values at the isoelectric points, pH_i [$=(pK_2 + pK_3)/2$]

Amino acid	pK_1	pK_2	pK_3	pH_i
Lysine	2.18	9.12	10.53	9.82
Arginine	2.17	9.04	12.48	10.76
Histidine	1.82	6.00	9.17	7.59

Table 6. Kinetic isotope effect at the α-carbon atom

	$10^5 k_{obs.}/s^{-1}$		
Amino acid	$RCH(NH_2)CO_2^-$	$RCD(NH_2)CO_2^-$	k_H/k_D
Lysine	15.0	1.8	8.3
Arginine	10.0	1.2	8.3
Histidine	16.0	2.0	8.0

Concentrations as in Table 3.

amino acids are oxidised by the same mechanism, and the changes in the rates are governed by both the entropy and enthalpy of activation.[6]

Mechanism.—The rate of the reaction between the substrate and hexacyanoferrate(III), in alkaline medium, was dependent on the first powers of the concentrations of both the amino acid and the oxidant (Table 2).

The dissociation of amino acids depends upon the pH of the medium. It is well known that amino acids exist as zwitterions in aqueous solution. In strongly acidic or alkaline media, the following equilibria (4) exist. In alkaline solution, the zwitterion

$$\underset{\text{cation}}{RCH(\overset{+}{N}H_3)CO_2H} \overset{OH^-}{\underset{H^+}{\rightleftharpoons}} \underset{\text{zwitterion}}{RCH(\overset{+}{N}H_3)CO_2^-} \overset{OH^-}{\underset{H^+}{\rightleftharpoons}} \underset{\text{anion}}{RCH(NH_2)CO_2^-} \quad (4)$$

is converted into the anion, $RCH(NH_2)CO_2^-$, which is the reactive species under the present experiment conditions. The pK_a values for the system (5) have been reported.[7] The

$$RCH(\overset{+}{N}H_3)CO_2^- \rightleftharpoons RCH(NH_2)CO_2^- + H^+ \quad (5)$$

ionisation constants[8] and the pH values at the isoelectric points of lysine, arginine, and histidine at 25 °C are given in Table 5. Since all the kinetic studies were carried out at high concentrations of NaOH, it may be assumed that the amino acids would be completely dissociated into their anions, $RCH(NH_2)CO_2^-$.

The radicals, generated from the oxidation of each of the substrates, gave five-line e.s.r. spectra having intensity ratios of 1:3:4:3:1. These radical intermediates were formed by loss of a hydrogen atom from the carbon atom of the methylene group. The number of lines can be accounted for by the radical species $R\dot{C}(NH_2)CO_2^-$, which has two equivalent protons and a nitrogen atom, all having nearly equal coupling constants (2.4 G).

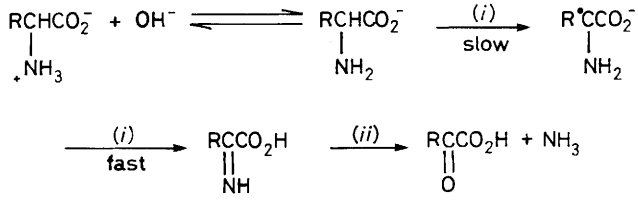
Since potassium hexacyanoferrate(III) is a one-electron oxidant, it would be justified to postulate that the reaction between the substrate and oxidant would give rise to a radical intermediate, analogous to enzymatic oxidation reactions which are also known to proceed *via* radical intermediates.[9] This would suggest that hexacyanoferrate(III), as a chemical oxidant, is capable of simulating enzymatic behaviour.

The kinetic isotope effect caused by deuteriating the α-carbon atom was studied. The k_H/k_D values were between 8.0 and 8.3 (Table 6), indicating that, in the rate-determining step, the C–H bond undergoes fission to give the radical species $R\dot{C}(NH_2)CO_2^-$, which has been characterised by e.s.r. spectroscopy.

In the present investigation, the salient experimental observations are as follows: (*a*) the rate of the reaction is dependent on the first powers of the concentrations of substrate and oxidant, and independent of the concentration of alkali in the range studied; (*b*) the lack of any effect on the rate of the reaction of the addition of hexacyanoferrate(II) ions; (*c*) favourable enthalpy and entropy factors, characteristic of radical processes; (*d*) detection of radical intermediates, characterised by e.s.r. spectroscopy; and (*e*) kinetic isotope effect at the α-carbon atom, indicating fission of a C–H bond in the slow step of the reaction.

The subsequent steps involved the rapid reaction of the radical intermediate with the oxidant, yielding the imine compound, which underwent hydrolysis to give the corresponding keto acid and ammonia. No other intermediate(s) could be isolated from the reaction mixture.

The reaction sequence for the oxidation of these amino acids (lysine, arginine, and histidine) by potassium hexacyanoferrate(III), in alkaline medium, is shown in the Scheme. The



Scheme. (*i*) $[Fe(N)_6]^{3-}$; (*ii*) water

ε-amino group of lysine was unaffected during the course of the oxidation reaction, in agreement with an earlier experimental observation.[10] The products obtained in each case (the corresponding α-keto acids) were isolated and characterised.

This mechanistic pathway for the oxidation of amino acids to the keto acids, *via* intermediate formation of the imino acid, is well established in the synthesis of α-keto acid esters.[11]

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