Kinetics and mechanism of reduction of ferricytochrome c by glutathione and 
L-cysteine: A comparative study

U Subudhi, G B N Chainy and P Mohanty*

Department of Biotechnology, *Department of Chemistry, Utkal University, Bhubaneswar 751 004, India

Received 5 July 2005; revised 19 December 2005

The kinetics and mechanism of the reduction of ferricytochrome c [Cyt c(III)] by substrates namely glutathione (GSH) and L-cysteine (L-cys) have been investigated spectrophotometrically employing [substrate]₀ >> [Cyt c(III)]₀. The reaction exhibits first order dependence in [substrate]₀ and [Cyt c(III)]₀. The pseudo-first order rate constant increases with an increase in pH, indicating that the conjugate base form of the HCytc(III) is a better oxidant than the parent HCyt c(III). The electron transfer rate constants between the oxidants and GSH for both the k₁ and k₂ paths are found to be greater than that with L-cysteine. Hence, GSH is a better reductant of Cyt c(III) as compared to L-cysteine. A suitable mechanism has been proposed on the basis of experimental findings. The deprotonation constant for HCyt c(III) and the second order rate constants of k₁ and k₂ paths for the present reaction at 25°C have been determined.

Keywords: Ferricytochrome c, glutathione, L-cysteine, reduction

IPC Code: C07K14/80

Ferricytochrome c [Cyt c(III)] is a small (Mₚ ~12,400), relatively stable heme protein, found in the mitochondria of all aerobic organisms. It is a member of the respiratory chain complex that affects the oxidation of foodstuffs and the synthesis of ATP. During this process, the iron atom in Cyt c(III) is alternatively oxidized and reduced; consequently, the electron transfer properties of this protein are intimately related to its environment. The X-ray studies¹,² have shown that in addition to four porphyrin nitrogen, the heme iron is coordinated with histidine-18 imidazole and methionine-80 sulphur. The ligands attached to iron do not vary with the oxidation state of the Cyt c(III), but some changes in the formation of the polypeptide chain do occur².

A number of oxidizing and reducing agents have been used to probe the reactivity of Cyt c(III), in the hope that its behaviour in such reactions might advance our understanding of its function in vivo. Its reduction by chromium(II)³, dithionite⁴, hexamine-ruthenium(II) ion⁵, Fe(EDTA)²⁻⁶, glutathione⁷,⁸, ruthenium(II)⁹, peroxynitrite¹⁰ and vanadate¹¹ has been reported. Although reaction mechanism of the reduction of Cyt c(III) has been studied using the above substrates, the electron transfer reaction of Cyt c(III) with sulphur containing amino acids has not been investigated in detail.

In the present study, we investigated the reduction of Cyt c(III) with naturally-occurring reducing agents glutathione (GSH) and L-cysteine (L-cys). The tripeptide L-γ-glutamyl-L-cysteinyl-glycine or GSH is the major low molecular mass thiol compound in plants and animals¹² and its peptidic γ-linkage is thought to protect it from degradation by aminopeptidases. It has a role in signal transduction, gene expression, apoptosis, protein glutathionylation and nitric oxide (NO) metabolism¹³⁻²⁰. L-Cysteine is an important component of GSH and both are found in the mitochondria. Therefore, our main aim was to compare the reductant role of –SH group in GSH and L-cys.

Kinetics and mechanism of the reduction of Cyt c(III) by GSH have been previously investigated⁸. This study postulated the formation of a GSH-Cyt c(III) intermediate, in which the GSH most likely interacts with the edge of the heme moiety and the electron transfer takes place in a subsequent slower step. However, we differ from the above observations, as had there been a complex formation, it would have shown a hyperbolic type plot. But, in the present study, kₘₐₓ (sec⁻¹) vs [substrate] plot is linear, which does not support the intermediate complex formation between Cyt c(III) and the substrates.

*Author for correspondence
E-mail: prakashmohanty@rediffmail.com
Tel: (0674) –2381680; Fax: (0674)-2587389

Abbreviations: GSH, reduced glutathione; L-cys, L-cysteine; Cyt c(III), ferricytochrome c; DTNB, 5-5’-dithiobis-2-nitrobenzoic acid.
Materials and Methods

Horse heart ferriicytochrome c (used without any further purification), L-cysteine, glutathione and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from SRL, India.

An extinction coefficient of 1.06 × 10^5 M⁻¹ cm⁻¹ at 410 nm was used for spectrophotometric determination of Cyt c(III) concentration. Reduction of Cyt c(III) by GSH or L-cysteine was studied at 25°C in phosphate buffer (pH 6.0-7.6) and the reaction mixture contained 522 to 1572-fold excess of [glutathione] or [L-cysteine] over [Cyt c(III)].

Kinetic measurements were recorded on a Varian Cary 100 UV-visible spectrophotometer, by following the change in absorbance at 415 nm (Fig. 1). Conventional mixing technique was followed and pseudo-first order conditions were maintained in all the kinetic measurements. The values for the observed rate constants (k_{obs}) were determined from a weighed least-squares analysis of the time dependence of log (A_t - A_0), where A_0 and A_t represent the absorbance of the reaction mixture at 415 nm at infinite time and after time t, respectively. The reported rate data were reproducible within ±3%.

DTNB was used to determine the concentration of GSH and L-cysteine before and after the completion of the reaction. For the estimation of –SH group, a 2 ml mixture was prepared by taking 1.7 ml phosphate buffer (pH 7.4), 0.2 ml DTNB (6 mM) and 0.1 ml sample [Cyt c(III)] with GSH or L-cysteine before the reaction and after completion the rate reaction]. The absorbance was then measured spectrophotometrically at 412 nm taking the phosphate buffer (pH 7.4) with Cyt c(III) and DTNB as blank. From the above reaction stoichiometry of the reaction was determined.

Results and Discussion

The kinetic results obtained for the above reaction may be summarized as follows. With varying concentrations of [Cyt c(III)]_T in the range 1.59 to 3.18×10⁻⁶ mol dm⁻³ with [GSH]_T/[L-cysteine]_T = 1.66 × 10^3 mol dm⁻³, at pH = 7.2, the 10^5 k_{obs} (at 25°C) were found to be 50.8 and 15.8 sec⁻¹ for GSH and L-cysteine, respectively (Table 1). Furthermore, the second order rate constant k_2 (mol⁻¹ dm³ sec⁻¹) = k_{obs} /[reductant]_T for the electron transfer reaction by GSH and L-cysteine are 0.19 ± 0.06 and 0.07 ± 0.01, respectively. Constancy of k_2 indicates the fact that overall order of the reaction is two. As the order of the reaction is one with respect to [substrate]_T, the order would be one with respect to [Cyt c(III)]_T. The rate law is, therefore, given by Eq. (1):

\[
\text{Rate} = k_{obs} [\text{Cyt c (III)}]_T
\]

A plot of k_{obs} vs [GSH]_T/[L-cysteine]_T (Fig. 2) was linear when the concentrations of [GSH]_T and [L-cysteine]_T varied from 1.66 to 5.0 × 10⁻³ mol dm⁻³ at constant pH and fixed [Cyt c(III)]_T, indicating a first order dependence on [GSH]_T/[L-cysteine]_T. With [GSH]_T/[L-cysteine]_T = 1.66 × 10⁻³, [Cyt c(III)]_T = 3.18 × 10⁻⁶ mol dm⁻³, 10^5 k_{obs} (at 25°C) increased from 22.5 to 58.6 sec⁻¹ and 8.83 to 18.8 sec⁻¹ for GSH and...
L-cysteine respectively, when the pH of the reaction mixture was raised from 6.0 to 7.6 (Table 1). Under the studied pH range, two conformational forms of the cytochrome c namely HCyt c(III) and Cyt c(III) exist. Since pK₁, pK₂, pK₃ and pK₄ of GSH are 2.05, 3.47, 8.63 and 9.52 and pK₁, pK₂, and pK₃ of L-cysteine are 1.88, 8.15 and 10.29 respectively, the reductants that will participate in the electron transfer reaction are as follows:

\[ \text{HCyt c(III)}^+ \rightleftharpoons \text{Cyt c(III)} + \text{H}^+ \]  \hspace{1cm} (2)

\[ \text{HCyt c(III)}^+ + \text{GSH} \rightarrow \text{Products} \]  \hspace{1cm} (3)

\[ \text{Cyt c(III)} + \text{GSH} \rightarrow \text{Products} \]  \hspace{1cm} (4)

The rate law for the electron transfer reaction can be derived in the following manner:

\[ \text{Rate} = k_1 [\text{HCyt c(III)}^+] [\text{GSH}] + k_2 [\text{Cyt c(III)}][\text{GSH}] \]  \hspace{1cm} (5)

\[ K_d = [\text{Cyt c(III)}][\text{H}^+]/[\text{HCyt c(III)}^+] \]

\[ [\text{Cyt c(III)}] = K_d [\text{HCyt c(III)}^+]/[\text{H}^+] \]  \hspace{1cm} (6)

Substituting the value of [Cyt c(III)] from Eq. (6) in Eq. (5)

\[ \text{Rate} = k_1 [\text{HCyt c(III)}^+] [\text{GSH}] + k_2 \{K_d [\text{HCyt c(III)}^+]/[\text{H}^+]\} [\text{GSH}] \]

\[ = [\text{HCyt c(III)}^+] [\text{GSH}] \{k_1 + k_2 K_d/[\text{H}^+]\} \]  \hspace{1cm} (7)

\[ \text{[HCyt c(III)}^+]_T = [\text{HCyt c(III)}^+] + [\text{Cyt c(III)}] \]

\[ = [\text{HCyt c(III)}^+] + K_d [\text{HCyt c(III)}^+]/[\text{H}^+] \]

\[ = [\text{HCyt c(III)}^+] e \{(\text{H}^+) + K_d/\text{[H}^+]\} \]  \hspace{1cm} (8)

Substituting the value of [HCyt c(III)] in the Eq. (7)

\[ \text{Rate} = ([\text{HCyt c(III)}^+] T \times [\text{GSH}]

\[ \times (k_1 [\text{H}^+] + k_2 K_d)]/([\text{H}^+] + K_d) \]  \hspace{1cm} (9)

Since Rate = \(k_{obs}[\text{HCyt c(III)}^+] T\)  \hspace{1cm} (10)

\[ k_{obs} = [\text{GSH}] \{k_1[H^+] + k_2 K_d]/([H^+] + K_d) \]  \hspace{1cm} (11)

\[ k_{obs}/[\text{GSH}] = k_2' = (k_1[H^+] + k_2 K_d)/([H^+]+K_d) \]  \hspace{1cm} (12)

\[ k_2' = \text{Second order rate constant} \]

\[ k_2'[\text{H}^+] + K_d = [k_1[H^+] + k_2 K_d] \]  \hspace{1cm} (13)

\[ k_2'[\text{H}^+] + k_2 K_d = k_1[H^+] + k_2 K_d \]  \hspace{1cm} (14)

Electron transfer to the iron atom of Cyt c(III) reasonably could take place either by remote attack, probably at the heme edge or by adjacent attack at some point in the limited space available in the heme pocket. Earlier, it was suggested that both remote and adjacent attacks may occur with the reductant chromium(II). In the present study, \(k_{obs}\) vs [substrate] plot is linear for both the substrates, indicating that there is a little scope for formation of an intermediate complex during the electron transfer reaction and this is in agreement with first order dependance on [Cyt c(III)]ₚ.
The \( k_1 \) values were gradually changed in the equation by plotting \([H^+](k_2' - k_1)\) vs \( k_2' \) in such a way that the correlation coefficient becomes 0.99, with the best-fitting the slope and intercepts were calculated. Intercept/slope gives the value of \( k_3 \). The \( k_1 \) (best-fitted value), \( k_2 \) and \( K_d \) for both the substrates are shown in Table 1. The \( K_d \) values are comparable with the values obtained in the earlier work\(^{11}\).

Stoichiometry of the reaction shows that one mole of the substrate reacted with one mole of Cyt c(III) and the products of the reaction were GSSG and L-cystine. Nevertheless, reduction of Cyt c(III) by GSH is 2.7-fold faster than L-cysteine. The physiological significance of higher rate of reduction of Cyt c(III) with GSH in comparison to L-cysteine could not be explained at present. However, it may be mentioned here that as GSH is a tripeptide, the species in the activated state formed between GSH and Cyt c(III) is of lower potential energy than that of L-cysteine. Again, GSH lacks the toxicity associated with L-cysteine, making it suitable as a cellular “redox buffer” to maintain a given thiol/disulfide redox potential\(^{24}\). Possibly, that is why through evolution cell selects the GSH as better reductant than the L-cysteine.

Acknowledgement

The authors are indebted to University Grants Commission, Department of Biotechnology and Department of Science and Technology, New Delhi for the financial assistance.

References

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