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Succinylation of cytochrome *c* investigated by electrospray ionization mass spectrometry: Reactive lysine residues

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ABSTRACT

The horse heart cytochrome *c* contains nineteen lysine residues that constitute 18% of the amino acid residues in the protein. These lysine residues are widely spread throughout the structure of the protein. Acylation of cytochrome *c* with succinic anhydride produces different partially succinylated cytochrome *c* species due to acylation of various lysine residues present in the protein. Partially succinylated species present in the reaction solutions were detected by electrospray ionization mass spectrometry (ESI-MS). ESI-MS shows that a maximum of seven lysine residue are readily modified on addition of excess succinic anhydride to the protein. Lysine residues that are readily succinylated are identified by ESI-MS detection of the peptide fragments produced by the trypsin digestion of partially succinylated cytochrome *c* solutions, and by peptide mass mapping. The lysine residues, viz. K86, K79, K72, K60, K39, K27 and K25 were identified as the most reactive residues. These lysine residues are in the locations in the protein that form turn or loop structures. The present results show that the lysines in the helical regions are much less reactive than those in the flexible loops or in the turn regions of the protein structure. It is proposed that the lower reactivity of the lysine residues present in the helical regions may be due to the higher rigidity of the helical regions.

1. Introduction

The chemical modification of the amino acid residues of proteins is important in understanding the post-translational modifications of proteins, and in the study of the structure and function of proteins [1–13]. Lysine is an important amino acid residue that is suitable for chemical modification by acylation reaction. It has been shown that acylation of simple peptides like insulin generates charge ladders in capillary electrophoresis, which is useful for determination of the charge and the relative basicity of the side chain amine groups [4–6].

Acylation of lysine residue is a nucleophilic substitution reaction, in which the side chain ε-amine group of lysine is the nucleophilic attacking group, resulting in formation of an amide bond as shown in Scheme 1 [4–7]. Post-translational modification of the lysine residues such as acetylation or methylation by the enzymes acyl- or methyl-transferase, involves the participation of the side chain ε-amino group of the lysine residues as the nucleophilic attacking group [7,8,14,15].

In the horse heart cytochrome *c*, there are nineteen lysine residues, which are widely distributed on the surface of the protein, and constitute 18% of the amino acid residues in the total sequence of the horse heart cytochrome *c* [16–22]. These lysine residues contribute to the net positive charge of the protein and effect electrostatic interaction of cytochrome *c* with cytochrome *c* oxidase, during the respiratory electron transfer [16–22]. In general, the chemical modification of lysine residues in this protein by acylating agents such as succinic anhydride is important in the study of structure and function of proteins [4–6].

It has been shown that all the nineteen lysine residues of cytochrome *c* could be succinylated by maintaining the pH of the reaction around 7, and gradually increasing the concentration of the succinic anhydride [1,2,4]. The pH of the reaction solution is maintained by addition of base as hydrolysis of the un-reacted succinic anhydride tends to decrease the pH, which is not favorable for acylation of the lysine residues (Scheme 1). Moreover, not all the 19 lysine residues of cytochrome *c* are simultaneously succinylated by direct addition of succinnic anhydride to the folded cytochrome *c* [1,2,4]. However, the sequence specific identity of the lysine residues that are modified by addition of succinic anhydride to the folded form of the cytochrome *c* is not known.

Electrospray ionization mass spectrometry (ESI-MS) is a recognized as versatile tool for the study of chemical modification of proteins [23]. Recently we have shown a direct correlation between the ESI-charge states and the folded structure of cytochrome *c* and other proteins in aqueous solution [24–27]. ESI-MS of cytochrome *c* has been reported earlier [24–27]. Acylation of multiple lysine

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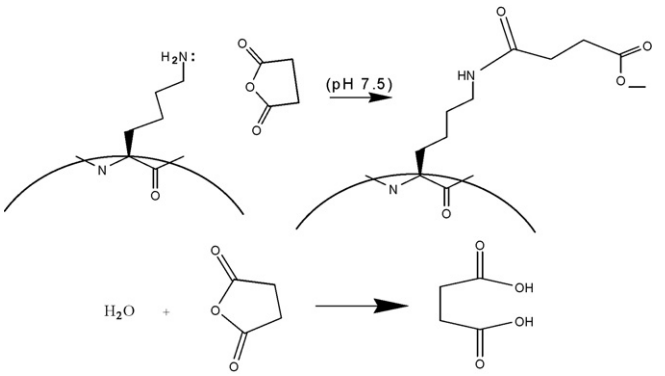


Scheme 1. Acylation of lysine residue by succinic anhydride (acylating agent). The curved line represents the surface of the protein. The ε-amine of the lysine residue attacks the carbonyl group of the acylating agent. This chemical modification involves the nucleophilic substitution of the lysine residues.

residues of cytochrome *c* changes the molecular mass of the protein that can be detected in the ESI-mass spectra of cytochrome *c*. Here, we report sequence specific identification of the lysine residues that are succinylated by the addition of succinic anhydride (as the acylating agent) to the folded form of the horse heart cytochrome *c*. Various partially succinylated species produced in the reaction were detected by ESI-MS. Succinylated lysine residues were identified by peptide mass mapping of the peptide fragments produced in the trypsin digestion of various partially succinylated cytochrome *c* solutions. The results show that among the nineteen lysine residues of cytochrome *c*, there are only seven lysine residues of the protein that are readily modified on the addition of succinic anhydride to the folded form of the protein. These reactive lysine residues are located in the regions of cytochrome *c* which form the turn or loop structure. The results have been described in the light of understanding the position specific reactivity of the surface exposed lysine residues in the protein.

2. Materials and methods

Horse heart cytochrome *c* (type VI A) and ammonium acetate were obtained from Sigma, USA. Cytochrome *c* was purified by Sephadex G-25 size exclusion chromatography using 3 mM ammonium acetate (pH ~7.5) as the eluting solution. The concentration of cytochrome *c* was determined from the absorption spectrum of the reduced protein, using an extinction coefficient of 29 mM^{-1} cm^{-1} at 550 nm [28,29]. The purity of the protein was checked by SDS PAGE. Mass spectrometric grade trypsin (trypsin gold) obtained from Promega was used for the digestion of cytochrome *c* and the succinylated cytochrome. Trypsin was dissolved in ice cooled 10 mM acetic acid and always kept on ice before use. The strength of the trypsin solution was 1 μg/μl. Succinic anhydride was obtained from Qualigens, India.

2.1. Succinylation of cytochrome c

To 500 μl of 1 mM cytochrome *c* in 3 mM ammonium acetate (pH 7), appropriate concentration of succinic anhydride was added and the mixture was vortexed. The solution was diluted 100-fold with 3 mM ammonium acetate for mass spectral measurements. The reaction of succinic anhydride with the lysine residue is a diffusion controlled process and incubation of the reaction mixture for ~30 min to overnight did not affect the results. The final pH of the reaction mixture was pH ~6 in presence of 100 mM succinic anhydride.

2.2. Trypsin digestion of native and succinylated derivative of cytochrome c

To 50 μl of the cytochrome *c* (1 mM) or succinyated cytochrome *c* solutions, 15 μl of acetonitrile is added and the pH of the solution is adjusted to pH 8.5 by addition of dilute ammonia solution [30–33]. Then trypsin solution was added to the respective solution, such that the weight ratio of protein:trypsin was 50:1. After the addition of trypsin, the samples were kept inside an incubator at 37 °C, for 12 h.

Electrospray ionization mass spectrometry (ESI-MS) and collision induced dissociation (ESI-MS/CID) studies were carried out using a Thermo Finnigan LCQ Deca Electrospray quadrupole ion trap mass spectrometer [24]. The flow rate of the protein solution was maintained at 5 μl/min. Capillary temperature was maintained at approximately 200 °C and the capillary voltage was kept at 31 V. The ion-spray voltage was maintained at 4.5 kV and the ion optics was tuned to get maximum ion count. The instrument was separately calibrated both in the normal-mass range (0–2000 *m/z*) and in the high-mass range (0–4000 *m/z*)[24]. Collision induced dissociation (CID) spectra were obtained by mass selecting the corresponding

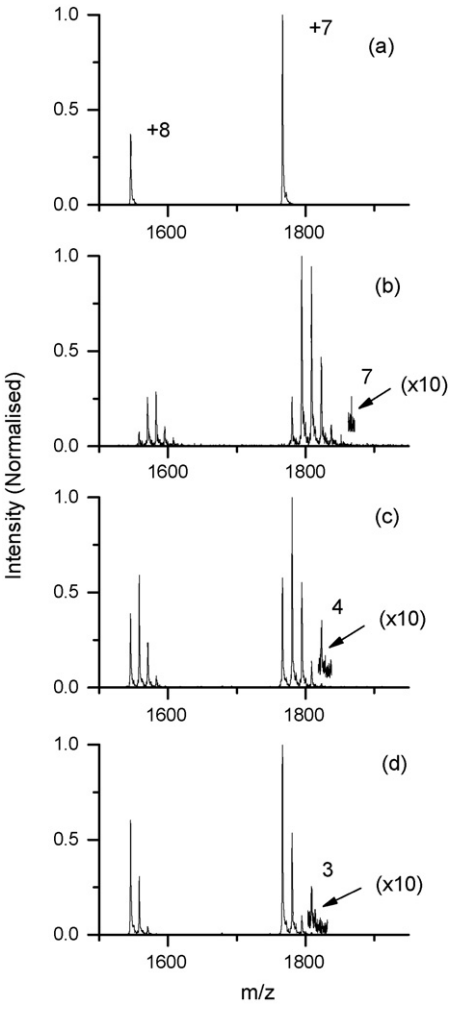


Fig. 1. ESI-mass spectra of unmodified and various partially succinylated cytochrome *c*. The +7 (1766.5 *m/z*) and +8 (1545.7 *m/z*) charge states for the unmodified protein (a) unmodified cytochrome *c*. (b) Cytochrome *c* (1 mM) treated with 100 mM succinic anhydride. The peak corresponding to cytochrome *c* with seven lysine modification is enlarged ten times (c) with 25 mM succinic anhydride. The peak corresponding to cytochrome *c* with four lysine modification is enlarged ten times (d) with 10 mM succinic anhydride respectively. The peak corresponding to cytochrome *c* with three lysine modification is enlarged ten times.

peptide ion and subjecting the selected mass ions to 30–40% of normalized collision energy. The concentration of the protein used for recording ESI-MS was ~10 μM. Tryptic digest solutions were directly infused into the ESI-mass spectrometer [24].

3. Results and discussion

The ESI-mass spectrum of the unmodified cytochrome *c* solution shows sharp peaks corresponding to +7 and +8 charge states as shown in Fig. 1a. It has been shown that that the folded form of cytochrome *c* solution produce +7 or +8 as the major charge states. Earlier studies [2,4,5] suggested that acylation of a large number of lysine residues in the protein may affect the structure of the protein as the modification of the lysine would change in the charge of the residue. The CD (circular dichroism) spectrum of the protein remained unchanged before and after the reaction with the succinic anhydride indicating that there was no significant change in the structure of the protein on modification of the lysine residues in the present case. The mass spectral results also show that the charge state distribution in ESI-MS spectrum of the protein was not affected even at the highest concentration (100 mM) of succinic anhydride, indicating that the secondary as well as the tertiary structure of the protein was unaffected by succinylation in the present conditions [24,26]. The addition of succinic anhydride to the folded cytochrome *c* solution would result in the succinylation of any of these surface exposed lysine residues, with an increase in the mass of the protein by 100.1 amu for a single lysine modification. The number of modifications of lysine residues in cytochrome *c* was found to vary with the amount of succinic anhydride added to the solution which are detected in the ESI-mass spectra shown in Fig. 1b–d. These mass spectral results indicate that seven lysine residues of cytochrome *c* are predominantly modified on the addition of excess succinic anhydride (~100 mM) to the folded cytochrome *c* solution (Fig. 1b). Incubation of the reaction mixture for overnight also did not show any change in the mass spectrum of the protein indicating that even ~100-fold excess of succinic anhydride cannot modify all the lysine residues in the protein under the present conditions. It is however, important to note that formation of small concentrations of higher level of acylation (succinylation) of the protein that was beyond the detection limit of the mass spectrometer cannot be ruled out in the present case.

Table 1
Position of the cleavage sites and the peptides fragments by trypsin digestion of unmodified cytochrome *c*.

Position of cleavage site	Resulting peptide sequence	Peptide length	Peptide mass [Da]
5	GDVEK	5	546.578
7	GK	2	203.241
8	K	1	146.189
13	IFVQK	5	633.789
22	CAQCHTVEK	9	1619.571
25	GGK	3	260.293
27	HK	2	283.330
38	TGPNLHGLFGR	11	1168.321
39	K	1	146.189
53	TGQAPGFTYTDANK	14	1470.558
55	NK	2	260.293
60	GITWK	5	603.719
72	EETLMEYLENPK	12	1495.664
73	K	1	146.189
79	YIPGTK	6	677.798
86	MIFAGIK	7	779.008
87	K	1	146.189
88	K	1	146.189
91	TER	3	404.423
99	EDLIAYLK	8	964.126
100	K	1	146.189
C-terminal	ATNE	4	433.418
Partially digested (99)	TEREDLIAYLK	11	1349.72

The lysine residues that are succinylated on addition of succinic anhydride to the folded cytochrome *c* protein solution were identified by tryptic digestion of the modified cytochrome *c*, and by peptide mass mapping [30,33]. It is well known that the arginine and lysine residues of a protein are cleaved by trypsin [30,33], and the possible peptide fragments that could be formed in the sequence of cytochrome *c* on digestion with trypsin are shown in Table 1. The folded unmodified cytochrome *c* also showed a partially proteolysed fragment as shown in Table 1, which also could

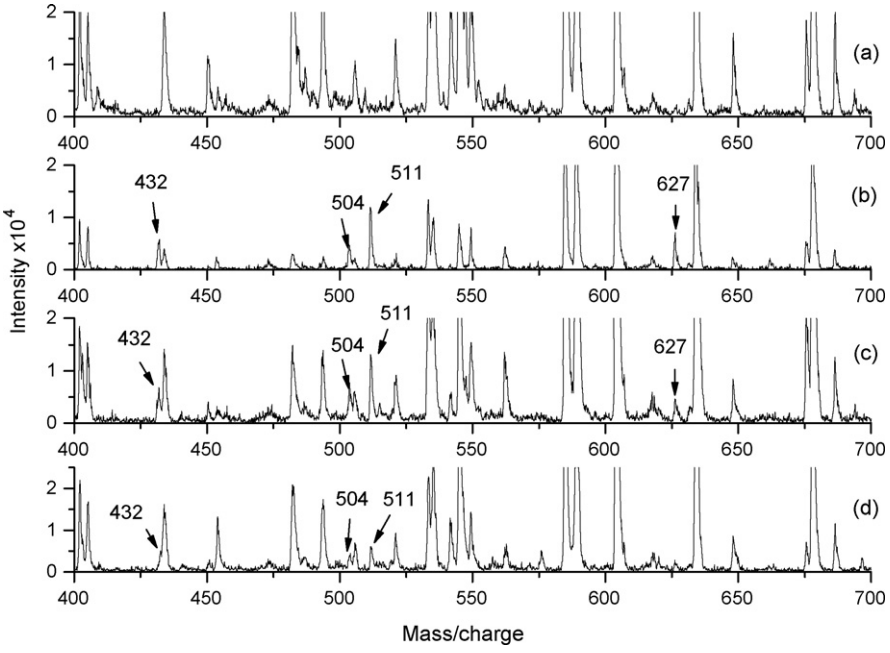


Fig. 2. ESI-mass spectra of the tryptic digest of unmodified and various partially succinylated cytochrome in the range of 400–700 *m*/*z* (a) unmodified cytochrome *c*. (b) Cytochrome *c* (~1 mM) treated with 100 mM succinic anhydride respectively (c) with 25 mM succinic anhydride. (d) With 10 mM succinic anhydride. *m*/*z* values of the peaks corresponding to the peptide fragments containing the succinylated lysine residues are shown by arrows.

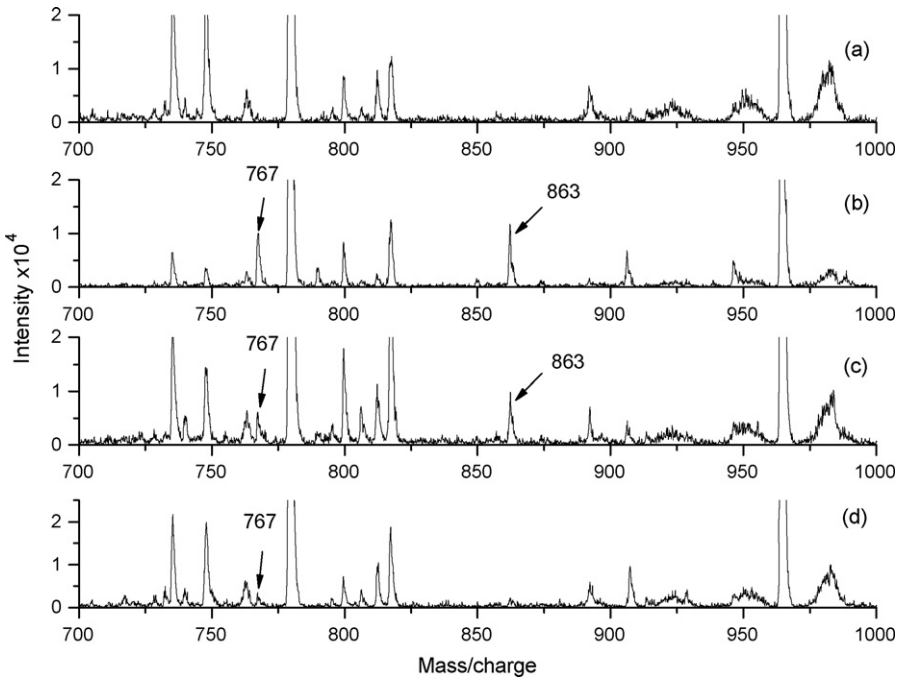


Fig. 3. ESI-mass spectra of the tryptic digest of unmodified and various partially succinylated cytochrome in the range of 700–1000 *m*/*z* (a) unmodified cytochrome *c*. (b) Cytochrome *c* (~1 mM) treated with 100 mM succinic anhydride (c) with 25 mM succinic anhydride, (d) with 10 mM succinic anhydride respectively. The *m*/*z* values of the peaks corresponding to the peptide fragments containing the succinylated lysine residues are shown by arrows.

be identified and characterized. The lysine residues that are chemically modified are not susceptible to digestion by trypsin [30,33]. Hence, only the unmodified lysine residues, and arginine residues of the partially succinylated cytochrome *c* species are digested by trypsin to produce peptide fragments that contain succinylated lysine residues. These peptide fragments are detected by ESI-MS, and the peptide mass mapping provided the sequence specific identity of the succinylated lysine residues [30,33].

The ESI-MS spectra of the tryptic digest of unmodified cytochrome *c* are shown in Figs. 2a, 3a and 4a, in which the peaks corresponding to the peptide fragments of cytochrome *c* generated by trypsin, and the degraded (autolysis) products of trypsin are observed [34,35]. The partially proteolysed peptide TEREDLIAYLK was also observed in the proteolysis of unmodified cytochrome *c* (Fig. 4a) as well as of the succinylation reaction mixture of the protein (Fig. 4b–d). Some of the peptide fragments were also found

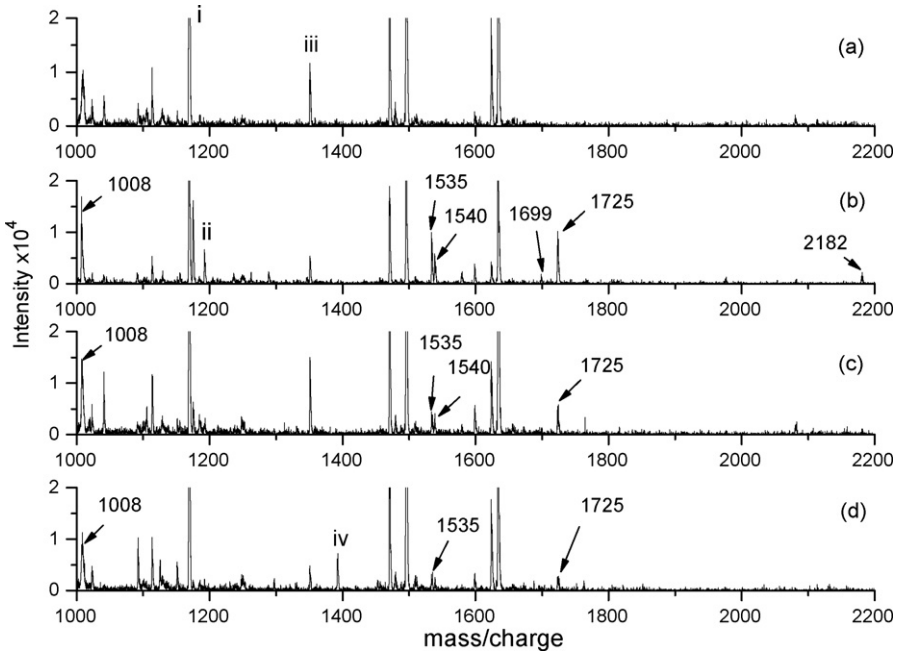


Fig. 4. ESI-mass spectra of the tryptic digest of unmodified and various partially succinylated cytochrome in the range of 1000–2200 *m*/*z* (a) unmodified cytochrome *c*. (b) Cytochrome *c* (~1 mM) treated with 100 mM succinic anhydride (c) with 25 mM succinic anhydride. (d) With 10 mM succinic anhydride respectively. *m*/*z* values of the peaks corresponding to the peptide fragments containing the succinylated lysine residues are shown by arrows. The labels (i) and (ii) represent the isotopic and sodium adduct peaks for the peptide fragment TGPNLHGLFGR (1169 *m*/*z*) respectively. The labels (iii) and (iv) are the peaks corresponding to the peptide fragment TEREDLIAYLK (1350 *m*/*z*) and its potassium adduct respectively.

Table 2
m/*z* values of the new peaks observed from the tryptic digest of different partially succinylated cytochrome *c*. Different partially succinylated cytochrome *c* species are produced by the reaction between 1 mM cytochrome *c* and with 10, 25 and 30 mM succinic anhydride.

m/*z* values of succinylated peptides observed for the reaction of cytochrome *c* with succinic anhydride[a]			Succinylated lysine residue with sequence number, charge state of the peptide, sequence of the peptide fragment that contain the succinylated lysine residue. Succinylated lysine is represented in italic. The average molecular weight of the peptide in the unmodified form is given in parentheses
10 times excess of succinic anhydride	25 times excess of succinic anhydride	100 times excess of succinic anhydride	
511	511	511	K27, +3, H*K*TGPNLHGLFGR
767	767	767	K27, +2, H*K*TGPNLHGLFGR
1535	1535	1535	K27, +1, H*K*TGPNLHGLFGR (1433.60)
432	432	432	K72, +4, EETLMEYLENP*K*K
863	863	863	K72, +2, EETLMEYLENP*K*K
1725	1725	1725	K72, +1, EETLMEYLENP*K*K (1623.80)
1540(w)	1540(i)	1540(s)	K79, +1, YIPGT*K*MIFAGIK (1438.76)
504	504	504	K86, +2, MIFAGI*K*K
1008	1008	1008	K86, +1, MIFAGI*K*K (907.15)
626(w)	627(i)	627(s)	K25, +1, GG*K*HK (525.58)
–	–	1699	K39, +1, *K*TGQAPGFTYTDANK (1598.69)
–		2182	K60, +1, GITW*K*EETLMEYLENPK (2081.32)

[a] Values of *m*/*z* are rounded to the nearest number for clarity. (w), (i) and (s) represents the weak, intermediate and strong intensity of the peaks in the spectra.

to form sodium or potassium adducts such as the TGPNLHGLFGR and TEREDLIAYLK peptide fragments. The alkali metal ion (sodium or potassium) adducts become more significant in the digestion mixture of the modified protein (Fig. 4b–d), possibly due to the presence of the alkali metal ions as impurity in the base added for neutralisation of the reaction mixture. Cytochrome *c* solution that was modified by addition of excess succinic anhydride (Fig. 1b, 100 mM succinic anhydride to ~1 mM cytochrome *c*) was digested by trypsin and the ESI-MS spectra of the tryptic digest is shown in Figs. 2b, 3b and 4b. In Figs. 2b, 3b and 4b extra new peaks are observed in comparison with the mass spectral results of the unmodified cytochrome *c* (i.e., Figs. 2a, 3a and 4a respectively). The *m*/*z* values of these extra new peaks (excluding the potassium adducts of some of the unmodified peaks) are tabulated in Table 2, and these peaks are also marked in Figs. 2b, 3b and 4b. These new peaks are due to the peptides fragments that contain a lysine residue in the succinylated form. Charge assignments and mass analysis, i.e. peptide mass mapping were performed and the results are complied in Table 2, which shows the charge states, and sequences of the peptide fragments containing the succinylated lysine residue. These results show that the seven lysine residues, viz. K86, K79, K72, K60, K39, K27 and K25, in the sequence of cytochrome *c* are predominantly succinylated with excess (100 mM) succinic anhydride. Thus, the above results show that the lysine residues at positions 86, 79, 72, 60, 39, 27 and 25 of cytochrome *c* are highly susceptible for the succinylation, out of the total of nineteen lysine residues present in the protein, on the direct addition of excess succinic anhydride to the cytochrome *c* solution.

Partially succinylated cytochrome *c* solution containing the mono-succinylated cytochrome *c* as the predominant species was observed on decreasing the amount of succinic anhydride (25 mM succinic anhydride to ~1 mM cytochrome *c*), as shown in Fig. 1c. The ESI-mass spectrum of the tryptic digest of this solution is shown in Figs. 2c, 3c and 4c. These results show that only the 86, 79, 72, 27 and 25 lysine residues are succinylated in presence of 25 mM succinic anhydride, and the intensities of the peaks corresponding to the succinylation of 60 and 39 lysine residues that are seen in the samples containing 100 mM succinic anhydride, are not significant. On further decreasing the concentration of the succinic anhydride (10 mM succinic anhydride to ~1 mM cytochrome *c*), only the mono-succinylated species was significant with trace amounts of di and tri succinylated species, as shown in Fig. 1d. Again, interestingly, the ESI-mass spectra of the tryptic digest of this solution clearly show the peaks corresponding to the modification of the lysine residues 86, 79, 72, 27 and 25 as shown in Figs. 2d, 3d and 4d, however, the intensities of these peaks are low compared to those obtained in the earlier cases, discussed above. Though only the mono-succinylated species is found to be significantly present in this solution (Fig. 1d), the ESI-mass spectra of the tryptic digest clearly show that 86, 79, 72, 27 and 25 lysine residues are succinylated. This fact suggests that these five lysine residues have almost similar reactivity towards succinic anhydride, and they are readily acylated on direct addition of succinic anhydride to the cytochrome *c* in the folded form. The result also reveals the random acylation of these lysine residues leading to a predominantly mono-succinylated form (Fig. 1d) of the protein. Attempts to identify the reactivity order among the reactive lysine residues K86, K72, K79, K27 and K25 by further reducing the concentration of succinic anhydride, were not successful and <10 mM succinic anhydride added to ~1 mM cytochrome *c*, also gave very similar results as discussed above.

The collision induced dissociation (CID) fragmentations of the peaks (observed from the tryptic digest) corresponding to the succinylated peptide fragments were studied. Fig. 5 shows a typical CID spectrum of a lysine modified peptide for the succinylated peptide MIFAGIK$_{(86)suc}$ K$_{(87)}$ with peak at ~1008 *m*/*z*, in which the lysine 86 is succinylated. The molecular mass of the y and b ions in the CID spectrum could unambiguously assign the peptide sequences and reveal the assignment of the lysine residues that are succinylated (see Scheme 2A). Fragmentation corresponding to loss of the succinyl group is shown in Scheme 2B. Such fragmentation occurs at

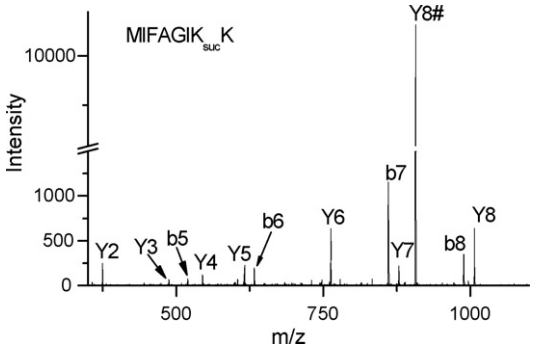
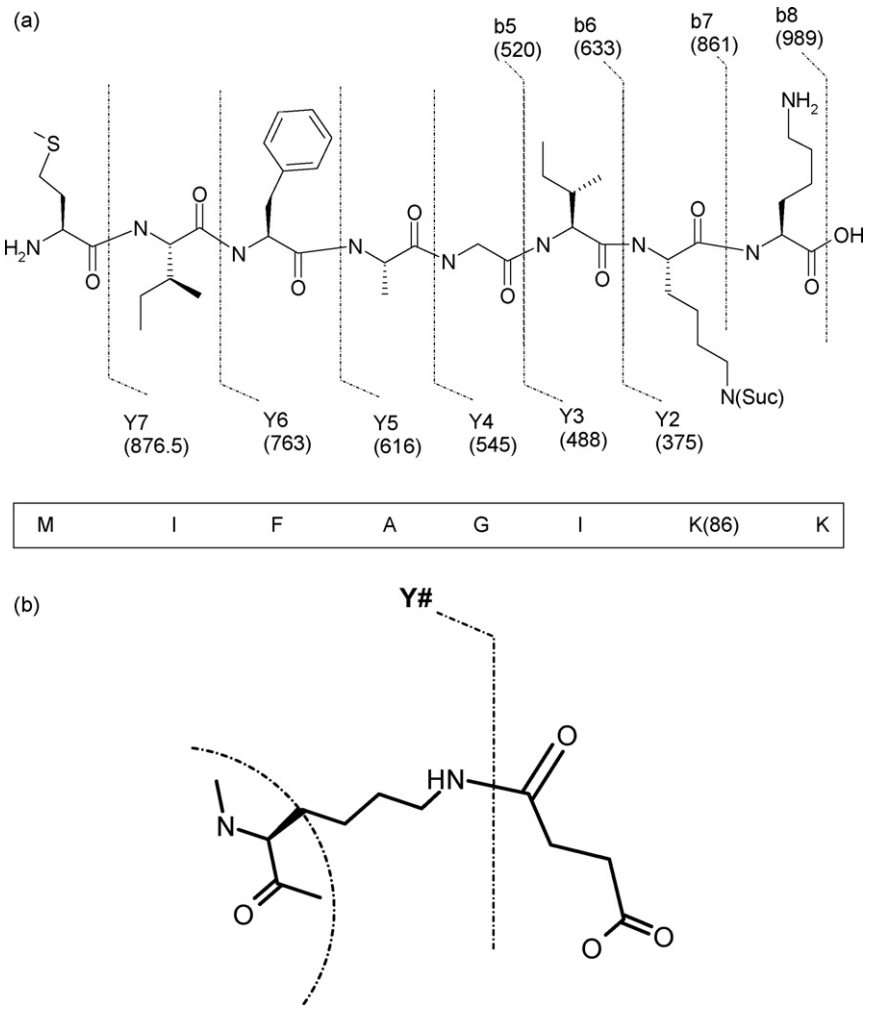


Fig. 5. ESI-MS/CID spectrum of the peptide MIFAGIK$_{86}$K in which the lysine 86 residue is succinylated. The respective y and b ions as shown Scheme 2, are marked in the spectrum. The CID spectrum was obtained by selecting the +1 charge state peak (at 1007 *m*/*z*) of the peptide MIFAGIK$_{86}$K and fragmented with 35% normalized collision energy.



Scheme 2. (A) Collision induced dissociation (CID) of the peptide MIFAGIK86K which contains the lysine 86 residue in the succinylated form. The respective y and b ions formed in this peptide are marked with their *m*/*z* in parentheses. The y ions consist of neutral carboxy end and protonated amine end. The b ions consist of neutral amine end and positive charge at the carbonyl end. The b7 ion confirms that lysine 86 is succinylated. This peptide is obtained due to the cleavage at the lysine 79 and lysine 87. (B) Formation of the Y# ion in the CID spectrum of succinylated peptides. The Y# ion is formed due to the cleavage at the ε-amide bond of the lysine residue which is succinylated.

the ε-amide bond of the modified lysine residue producing succinyl anion and the peptide cation (y# ion). The peak observed at 907 *m*/*z* is due to the loss of the succinyl group from the modified lysine (Fig. 5). The molecular mass assignment of the y and b ions confirm that the lysine 86 is succinylated and lysine 87 is unmodified (Scheme 2A).

It has earlier been observed that the proteins with multiple modification sites, usually produce several partially modified species [1,4]. Thus, the present results clearly show that the seven lysine residues 86, 79, 72, 60, 39, 27 and 25 are more susceptible for succinylation than the other lyisne residues, although the crystal structure of cytochrome *c* shows that almost all the lysine residues are well exposed on the surface of the protein. The crystal structures of cytochrome *c* obtained from the Research Collaboratory for Structural Bioinformatics (RCSB, http://pdbbeta.rcsb.org/pdb/) reported under ambient conditions (PDB: 1HRC) and under low-salt conditions (PDB: 1CRC) were analysed to determine the surface accessibility of various residues in the protein. The crystal structures were analysed using the WHATIF Web server [36] (http://swift.cmbi.ru.nl/servers/html/index.html) and the results of the relative surface accessibility of the amine nitrogen (Nz) in the lysine residues are shown in Fig. 6. Fig. 6 shows that the surface accessibility of some of the lysine residues depends on the salt concentration in the medium. Nevertheless, the amine nitrogen (Nz) atoms of the lysine residues reside predominantly at the surface of the protein. The sequence and the secondary structure of cytochrome *c* are shown in Fig. 7a. Interestingly, it is observed that the lysine residues 86, 79, 60, 39, 27 and 25 are not in the helical regions of the protein, and K72 is in the short helical turn formed by

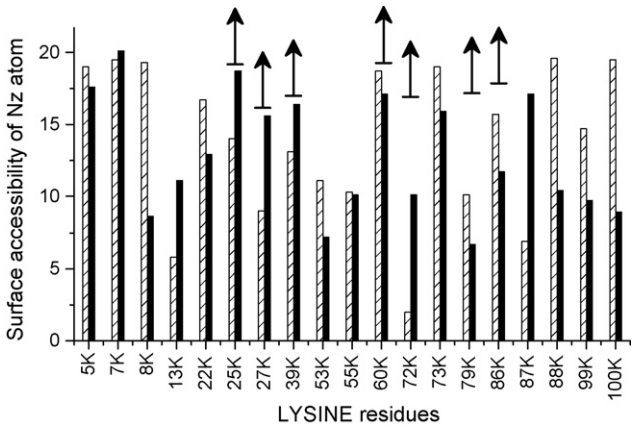


Fig. 6. Surface accessibility of the amine nitrogen (Nz) of the lysine residues in horse heart cytochrome *c* obtained from analyses of the crystal structures of the protein in ambient condition (PDB: 1HRC,) and in low-salt conditions (PDB: 1CRC, ■). Arrows indicate the reactive lysine residues.

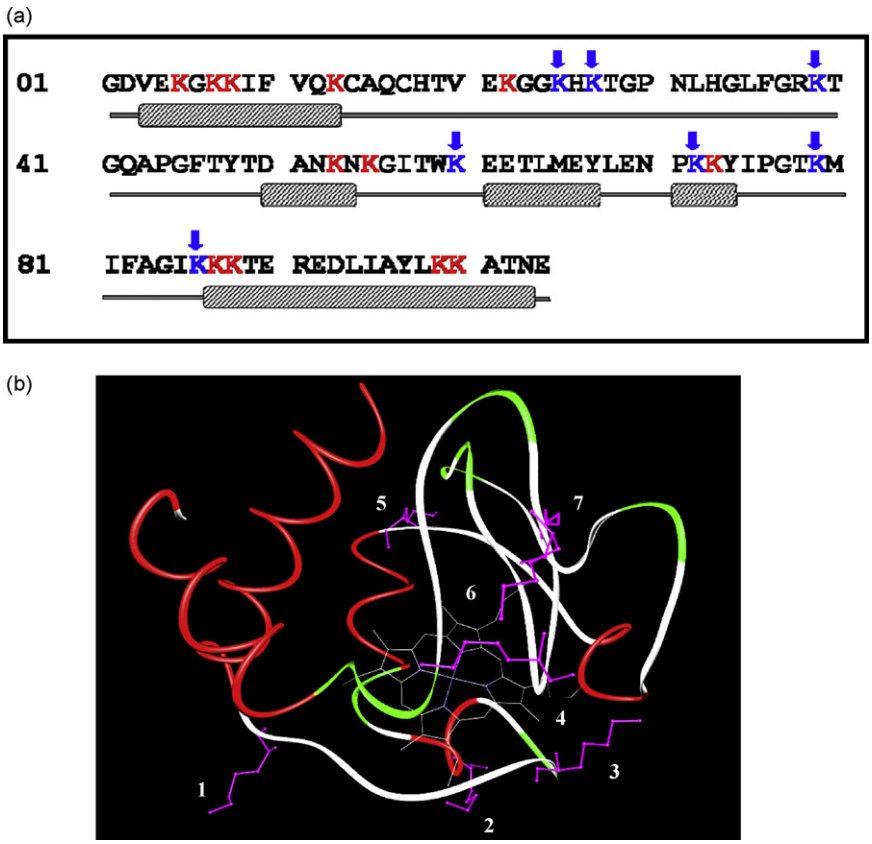


Fig. 7. (a) Sequence of the cytochrome *c* (PDB: 1HRC) showing the secondary structure (helical region is shown by cylinders and the remaining parts, mostly beta turns, are shown by thin lines). All the lysine residues are exposed to the surface. The reactive lysine residues are colored in blue and marked by arrows. Lysine residues that are not readily modified are colored in red. (b) Structure of cytochrome *c* showing the locations of the reactive lysine residues (1-K86, 2-K72, 3-K79, 4-27, 5-K60, 6-K25, and 7-K39) in the protein.

the four residues, viz. P71, K72, K73 and K74. On the other hand, the remaining lysine residues that are relatively less susceptible for succinylation, viz. K5, K7, K8, K13, K53, K73, K88, K99, K100 are located in the middle and K55, K87 in the end of helical regions, while only K22 is not in the helical region. Thus, the present results indicate that the lysine residues in the helical parts of the cytochrome *c* are not readily modified by succinylation, whereas the lysine residues that are in the flexible turns are highly susceptible for modification (Fig. 7b). The helical segments of the proteins are generally more rigid and the lysine residues residing in helical segments may have higher contribution towards the overall stability of the protein but they would be much less flexible compared to those residing in turns or loop regions of the molecule. Thus, lysine residues of cytochrome *c* in the loops are highly susceptible for succinylation while those in the helical segments are relatively less reactive to the modification. Earlier studies have shown that lysine 72 is often involved in the post-translational modification (tri-methylation), and the structure of the protein shows that this lysine residue is located in a short helical turn, which is relatively flexible [7,8,14,15].

4. Conclusions

Cytochrome *c* consists of nineteen lysine residues distributed on the surface of the protein, which are exposed. The mass spectral results show that only seven lysine residues are modified on addition of succinic anhydride. The ESI-mass spectra of the tryptic digest of the partially succinylated solutions, and peptide mass mapping were performed to identify the lysine residues that are modified by succinic anhydride. These seven lysine residues namely K86, K79, K72, K60, K39, 27 and 25 are readily succinylated. The results show that 7 out of 19 lysines residues in cytochrome *c* (40% of the lysine residues) are reactive, when the folded form of the protein is reacted with succinic anhydride. These reactive lysine residues are in the flexible part of the cytochrome *c* molecule. The present results show that the lysine residue that are in the helical structure are not involved in the modification but only those which are in the sequence that form the turn or loop are reactive for acylation.

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