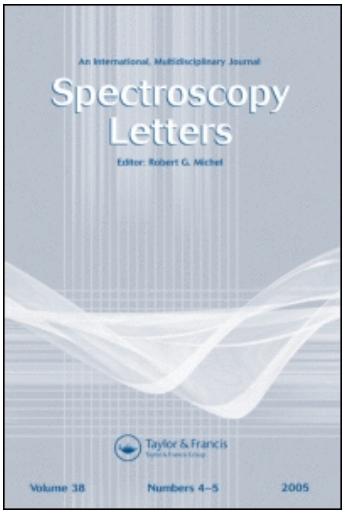

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On the Horse Heart Cytochrome C Api Spectrum

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ON THE HORSE HEART CYTOCHROME C API SPECTRUM

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Abstract

The silver salt cleavage of the Cytochrome C Horse Heart (CCHH) protein studied with API lead to identification of the deheminised parent protein, the heme fraction and two small peptides corresponding to cleavage at aminoacid 65. The API spectrum run in D_2O/CH_3OD showed 196 hydrogen exchange for CCHH and 188 hydrogen exchange for H_2O/CH_3OH.

Introduction

One of the most popular proteins used for API-ESI experiments for both specific peptide studies as well as for spectrum calibration purposes is Cytochrome C Horse Heart (CCHH) offered by several commercial suppliers at high 86-95% LC purity level (1). This compound is displaying multicharged molecular ions, the most intense corresponding to $9H^+$ to $16H^+$ reported in almost fifty papers (2).

The average mass, calculated from the multicharged ions reported in these papers, is 12360 Da. In our search for potential application of MS-MS API experiments for direct sequence determination, we cleaved the heme group of this protein. The amino acid sequence established for this free protein (3) is ca 650 amu lower than the calculated value for 104 amino acid with N-terminal acetyl and heme group (mass by sequence of 11702-Da).

The difference of ca 600 amu observed in protein mass spectrometry is usually related to or bound via the NAD co-factor radical or to the heme porphyrine residue unremoved during isolation. In the case of CCHH the evidence sometimes overlooked by mass spectrometrists is the fact that this compound is the hemoprotein and its good protonation of the CCHH ($9\text{-}16H^+$) should be related tot the basic heme group presence.

In this study we wanted to present a deheminised protein spectrum as well as report the observation on its protonation level.

The spectra of both Sigma CCHH in CH_3OH-H_2O and CH_3OD-D_2O solutions (at 5.5 pH) and the CCHH after the cleavage of heme group were recorded on Nermag R1010 quadrupole mass spectrometer.

The silver salt treatment, according to K.G. Paul's method, is used to realize this cleavage (4).

The spectrum of CCHH without heme group (Table 1) showed nine protonated molecular ions ($7H^+$ to $15H^+$) displaying an average mass of 11702 Da which together with 42 Da for an acetyl group is matching the Margoliash sequence (3). The important amount of $(Ag_2SO_4)_n$ Ag^+ (n = 1 - 5) ion resulting from the cleavage reagent was observed in the water solution requiring further purification and removal of this salt.

The comparison of CH_3OH/H_2O and CH_3OD/D_2O spectra for both CCHH indicates the presence of 196 protons exchanged (under pH 5.5) comparing to some 188 protons for CCHH without heme group.

The investigation of silver slat cleavage solution indicated presence of three series of ions. First the ion at 617.6 was accounted for the protonated heme group ion. This ion displays a characteristic natural abundance pattern. The absence of this ion in some previously reported spectra is related to the removal from the original CCHH of heme and the N-acetyl terminal group, probably during sliver salt treatment in the independent reaction (not related to the cysteine-heme bond cleavage). The 617 Da signal is washed completely out when the excess of silver sulfate and $(Ag_2SO_4)_n$ Ag^+ ion is removed.

However this signal is present in most of aged HHCC methanol-water solutions especially those slightly acidified and was overlooked in most of papers because of the recorded spectra range or its low intensity in freshly prepared solution of HHCC. The recording of API spectrum on preconcentrated fraction containing heme 617 ion also shows low intensity ions heme-Fe (562) followed by weak 517 ($-CO_2H$), 514 and terminal C-end 405 ions of unconfirmed structure and 365 ion (tripeptide 102-104 with its intense sodium adduct).

Two other weak ion series show polyprotonated species corresponding to smaller mass peptides 1 and 2 at 2796 ± 1.2 and 3294.7 ± 1.5 Da respectively (Table 2). The

TABLE 1

PI API SPECTRA OF CCHH DERIVATIVES

Solvent # of protons	CCHH CH_3OH – H_2O (1:1)	CCHH CH_3OD – D_2O (1:1)	CCHH Peptide without heme CH_3OH – H_2O (1:1)
7			1681.0
8			1474.5
9	1374.3	1395.1	1313.0
10	1237.3	1257.2	1180.5
11	1124.65	1142.6	1063.6
12	1031.0	1048.45	980.5
13	951.78	969.6	900.2
14	883.67	905.3	835.2
15			782.4
Average mass	12360.5	12556.7	11742.0(9)* 11740.4(8)* 11733.6(7)*
* Calculated for 9 ions, 8 ions, 7 ions respectively.			
Average mass difference in two solvent systems	196H^+		

- The most intense ions have been framed.

TABLE 2

SILVER SALT CLEAVAGE FRACTIONS

# of protons	Heme	Peptide 2	Peptide 1
1	617,6	-	-
2	-	1648,0	1398,8
3	-	1099,2	933,0
4	-	824,8	700,1
Average Mass	617,6	3294,7	2796,0

examination of the CCHH sequence as established by Margoliash (3) shows that the cleavage at position 65 or 80 lead to two peptides of this mass range responding for the peptide 1 to 65 to 88 sequence and for peptide 2 from 54 to 81 sequence with demethylation of methionine. Such a preliminary identification of both peptides also indicated that the cleavage of the S-CH_3 bond of methionines has been achieved under silver salt reaction. It is worthy to notice that both fragment peptides, primarily identified in this study resulted form cleavages in the area where the trypsin or chymotrypsin cleavages are observed (5,6). The synthesis of both fragment peptides from HHCC via controlled enzymatic cleavages is actually attempted (7).

Experimental

Mass Spectroscopy

The ES-API electrospray ionisation spectra were recorded on Nermag R 1010 (Riber) quadrupole mass spectrometer equipped with Analytica of Branford Inc. commercial source. The quadrupole pressure was 10^{-5} torr, transfer capillary used has 0.57 ID (0.7 torr). The calculations of data were done on HP-Chem Station (HP G10432) with MS Chem Station Software (DOS series). The peptide solutions 1-5 nMole were prepared in CH_3OH/H_2O 50:50 or in deuterated solvents as indicated, the syringe introduction 2µl min^{-1}.

All spectra quoted in this paper are available on request from the author (CKJ).

Proteins

The HHCC (95% minimum) was purchased from Sigma Chemicals and was not purified (millimolar extinction coefficient of 29.5 (ca) at 550 nm conformed to type VI HHCC).

Silver Salt Cleavage of the Prosthetic Group of HHCC

The HHCC without heme was prepared from the Cytochrome (10^{-3} Mole) with silver nitrate (2.10^{-1} Mole) in glacial acetic acid (5 mL) at 60°C for 90 minutes. The resulting protein was precipitated with acetone (0-5°) dissolved in water, reprecipitated and dialysed again, freeze-dried, dissolved in small quantity of water and filtrated through desalting cone, then analysed. Because of this double desalting, the solution does not show the silver ion content. The cleavage method was adopted from Paul's procedures (4).

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