ARTICLE

Interaction Between Cytochrome c and the Hapten 2,4-Dinitrofluorobenzene by Electrospray Ionization Mass Spectrometry

Bo Wu[a], Yan-qiu Chu[a], Zhao-yun Dai[b], Chuan-fan Ding[a*]

a. Physical Chemistry Institute, Department of Chemistry, Fudan University, Shanghai 200433, China
b. Infectious Disease Department, Huadong Hospital, Shanghai 200040, China

(Dated: Received on December 12, 2007; Accepted on February 20, 2008)

Allergic contact dermatitis is a delayed hypersensitivity reaction, which results from skin exposure to low molecular weight chemicals such as haptens. To clarify the pathogenic mechanism, electrospray ionization mass spectrometry (ESI-MS) and hydrogen/deuterium (H/D) exchange, as well as UV spectroscopy, were applied to determine the interaction between the model protein cytochrome c (cyt c) and the hapten 2,4-dinitro-fluorobenzene (DNFB). The ESI-MS results demonstrate that the conformation of cyt c can change from native folded state into partially unfolded state with the increase of DNFB. The equilibrium state H/D exchange followed by ESI-MS further confirms the above results. UV spectroscopy indicates that the strong-field coordination between iron of heme (prosthetic group) and His18 or Met80 of cyt c is not obviously affected by the hapten.

Key words: Interaction, Cytochrome c, 2,4-dinitro-fluorobenzene, ESI-MS

I. INTRODUCTION

Allergic contact dermatitis (ACD) [1] is one of the leading causes for occupational diseases affecting about 5% of men and 11% of women in industrialized countries. In affected individuals, it has a serious impact on their quality of life. ACD is a delayed (type IV) hypersensitivity reaction, the first phase of which is the induction of skin sensitization, a chemical-specific priming of the immune system, manifested by the occurrence of erythema and oedema at the site of secondary skin contact with non-protein chemicals, i.e. hapten oxazolone. The prevailing viewpoint in immunology is that the chemical haptens with small molecular weight are not large enough to stimulate an immune response directly and must combine with skin proteins to produce covalent adducts.

Over past decades, great efforts have been made to investigate the protein haptenation of skin sensitizers [2]. For instance, Gorbachev *et al.* reviewed the researches on the allergic contact dermatitis and discussed the mechanism of T cell priming by hapten-presenting Langerhans cells and how the priming environment influenced the development of these hapten-specific T cells to different functional phenotypes during sensitization for the ACD response [3]. Recently, Saint-Mezard *et al.* found the occurrence of ACD, as well as its magnitude and duration, was controlled by the functions of CD8 effector T cells and CD4 regulatory T cells [4]. The ACD was considered as a breakdown of cutaneous immune tolerance to haptens. Furthermore, Li *et al.* proposed that ACD induced by 2,4-dinitro-fluorobenzene (DNFB) was the T cell mediated delayed hypersensitivity [5]. As a hapten (semi-antigen), DNFB could interact with skin keratin to form complete-antigen. It stimulated the T cells to differentiate, proliferate and became sensitized lymphatic cells, which redistributed in the whole skin by lymphatic and blood circulation. When a sensitized lymphatic cell met the same type of hapten again, it differentiated into the effector T cell, which led to ACD. However, when the hapten DNFB first penetrates the epidermis into the dermis, in which thousands of skin proteins exist, the situation is unclear, since the interaction mechanism between DNFB and many proteins has not been understood yet and more work needs to be done.

The model protein candidate chosen for the research between skin protein and hapten should meet two requirements: well characterized in the physiological condition and closely relevant to human skin. Cytochrome c (cyt c) is an essential redox protein in the presence of the mitochondria, which is ubiquitously present in cells of all human organisms. It functions as an electron transporter in the energy-yielding respiratory chain and participates in the process of apoptosis. Thus, it exists in dermal cells, which have close relations to the disease. To the best of our knowledge, cyt c is one of the proteins which can be easily extracted. It is a single-domain, mainly helical globular protein containing 104 amino acids and one covalently attached heme group [6]. Its structure in native state is characterized by three major amphipathic helices (the N-terminal, the C-terminal and the 60 s helices) and two minor 3_{10} helices [7]. As a result of its favourable physical and chemical properties, cyt c has been used extensively as a model protein for kinetic and equilibrium studies of conformational changes [8]. Horse cytochrome c is homologous

*Author to whom correspondence should be addressed. E-mail: cfding@fudan.edu.cn, Tel.: +86-21-65105204

to human cytochrome c, so they have similar biological properties and functions. Thereby it can be used as a representative protein to perform the experiments.

A variety of techniques and models have been applied to the research of protein structures and conformations [9-14], including X-ray crystallography, circular dichroism (CD), nuclear magnetic resonance (NMR), Fourier-transform infrared (FT-IR), Raman, ultraviolet (UV)-visible absorption, and fluorescence. Recently, mass spectrometry (MS) has been added to this family because of its unrivaled speed, sensitivity, stoichiometry and low sample consumption [15]. Among the various methods of ionization, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) are two soft ionization modes, which can produce intact gas phase ions from protein samples [16]. Compared to MALDI, ESI has two particular advantages for protein conformational studies [17]. First, the sample preparation is easy and the proteins can be directly injected from solutions; second, multiply charged ions are typically produced. By virtue of these characteristics, molecular weights of large molecules can be measured with high accuracy. Based on the fact that the charge state distribution (CSD) produced in ESI spectra is greatly related to the protein solution phase conformational states, the research on CSD can play a critical role in protein unfolding studies.

In this work, we explore the interaction of cytochrome c and the hapten DNFB *in vitro* by using ESI-MS and ultraviolet spectroscopy. The equilibrium state H/D exchange is also measured by ESI-MS to investigate the protein conformational transitions as a result of the concentration changes of DNFB.

II. EXPERIMENTS

All the ESI-MS analyses were performed using a Perkin-Elmer SCIEX API III triple quadrupole mass spectrometer with a ion-spray source, in the positive-ion mode. The spray tip potential was operated at 4.8 kV. The ion-spray interface is equipped with a nitrogen stream directed against the sample spray. Ultraviolet spectroscopy was detected using a Shimadzu UV-2450 ultraviolet spectrophotometer.

Horse heart cytochrome c (12384Da) was purchased from Acros Organics (Phillipsburg, New Jersey, USA). DNFB was obtained from Sino-pharm Chemical Reagent Co. Ltd (P. R. China) as analytical pure reagent. They were used without further purification. For ESI-MS measurements, the cyt c concentration was kept constant at 10 μmol/L and a stoichiometry of DNFB was added. The experimental solution compositions were on a volume/volume basis. Solutions were all injected at room temperature. The pH of solution was kept at neutral to simulate physiological conditions in a skin environment. For the H/D exchange experiments in ESI-MS, cyt c (10 μmol/L) is dissolved in D_2O and incubated with different amounts of DNFB (0%, 0.05%, 0.1%). The 9+ and 14+ charge states (shown in Fig.1) are recorded by ESI-MS at the initial time and measured again after they are left overnight to reach equilibrium. The exchanged numbers can be calculated by comparing the experimental results at different times.

III. RESULTS AND DISCUSSION

ESI mass spectra for cyt c in different DNFB concentrations are depicted in Fig.1. The mass spectrum of cyt c at neutral pH is shown in Fig.1(a). After mixing DNFB (186.1 Da) with cyt c, the peaks of m/z for the mixed solution in Fig.1(b) and 1(c) remain in the same positions as those appearing in Fig.1(a). With its most important characteristic of soft ionization, ESI can hold noncovalent complexes from solutions [18]. This shows that the addition of DNFB does not lead to the shift of the peaks. Therefore, it can be inferred that DNFB does not complex with cyt c in the above conditions. However, the addition of such small amounts of DNFB into cyt c solutions dramatically influences the CSD displayed in the ESI-MS spectra. The protein without DNFB shows a relatively narrow CSD between 7+ and 12+, with the greatest intensity at 9+ (Fig.1(a)). As shown in Fig.1(b), the addition of 0.05% DNFB leads to a bimodal CSD with the emergence of

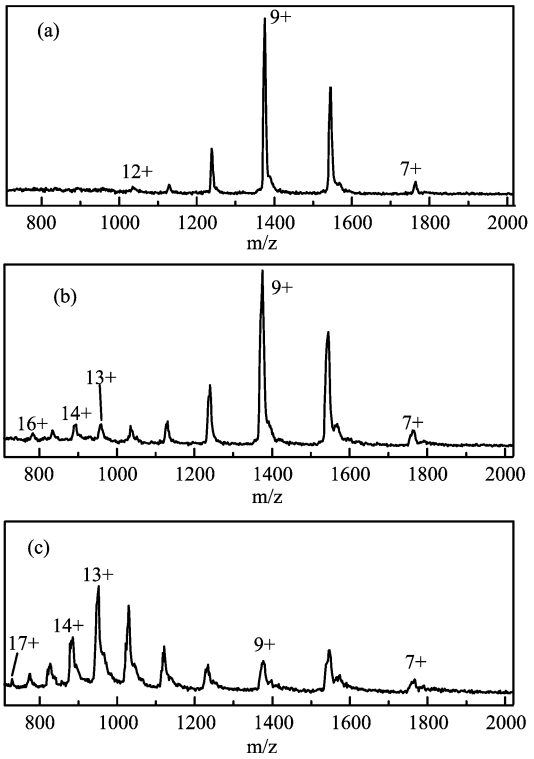

FIG. 1 Electrospray ionization mass spectra of cytochrome c (10 μmol/L) in water containing (a) 0, (b) 0.05%, and (c) 0.1% DNFB.

DOI:10.1088/1674-0068/21/03/217-220

another higher CSD centered at 13+ with low intensity. Such a bimodal character is often attributed to a mixture of partially unfolded and folded conformers coexisting in solution [19]. With the increase of DNFB concentration up to 0.1%, the intensity of higher CSD becomes more intense and the intensity of lower CSD centered at 9+ becomes weaker. Further increasing the DNFB concentration, precipitates begin to produce in mixed solution and no protein peak is identified in any mass spectrum.

Numerous results prove that the dramatic shifts in the CSD are associated with folding and unfolding changes in protein samples [17]. In the positive mode, the multiply charged ions observed in the spectra are formed as a result of proton attachment to available basic sites in the protein molecule. The number of charge attached to protein molecules during ESI is strongly influenced by the 3-dimensional conformation of the polypeptidic chain in the sprayed solution [20]. Thus, the conformation dependence of protein CSD is a powerful and routinely used tool for monitoring protein conformational changes in solution. Since Chowdhury *et al.* first used this method in the field [20], the effects of pH [21], heat [22], intramolecular disulfide bonds [23] as well as solution composition [19] have been largely studied by ESI-MS. In this work, we keep the pH and temperature constant so that the only variant relative to protein conformation is solvent composition. It is known that hydrophobic interaction plays a key role in maintaining protein 3D structure. Organic reagents usually weaken hydrophobic interactions [24]. In the native state, globular proteins such as cyt c are tightly folded with a hydrophobic core. DNFB is in essence a small organic molecule, the polarity of which is far away from water. With the increase of DNFB, cyt c tends to unfold to suit the new solvent polarity. Generally speaking, a protein in an unfolded conformation has more basic sites available for protonation compared with the same protein in a folded conformation. According to the CSD variation results, it is believed that the existence of 0.05% DNFB accounts for the high charge state. When the amount of DNFB reached 0.1%, the conformation of cyt c changed from its native folded state into a partially unfolded state.

It is emphasized that ESI-MS selectively monitors changes in the tertiary structure of proteins [25], so the appearance of another higher CSD indicates that the tertiary structure of cyt c is significantly destabilized with the addition of DNFB. Cyt c undergoes a highly cooperative, apparently a two-state, unfolding transition between pH 3 and pH 2 [17]. It is pointed that the completely unfolded protein induced by acid is centered on the 17+ or 18+ ion peaks [25,26]. Additionally, two partially folded forms, centered respectively on the 11+ and 14+ ion peaks, are also described by Grandori [26]. It is known that characterization of partially folded intermediates contributes to understanding protein folding mechanisms [27,28]. Here, we provide an effective and simple way to produce cyt c intermediates. In order to acquire quantitative information about the protein conformational changes, some average charge states of cyt c are calculated and listed in Table I. According to the published literature [29], the average charge state $\overline{Z}$ can be obtained from the mass spectra for cyt c at different conditions using the following equation:

$$\overline{Z} = \frac{\sum_i z_i I_i}{\sum_i I_i} \tag{1}$$

where the z_i and I_i represent the charge number and the intensity of the peak at the charge state i, respectively.

TABLE I The average charge state ($\overline{Z}$) and exchanged hydrogen number of cyt c

Solution	$\overline{Z}$	Hydrogen number	
		9+	14+
cyt c	8	139	
cyt c+0.05%DNFB	10	163	177
cyt c+0.1%DNFB	12	152	170

When the protein extends from native folded state into unfolded state, the solvent accessibility will increase. Consequently, the $\overline{Z}$ will rise. With the addition of DNFB into cyt c solution, the average charge state $\overline{Z}$ monotonically increases from 8.0 (Fig.1(a)), 9.0 (Fig.1(b)) to 12.0 (Fig.1(c)), which also indicates that the conformation of cyt c changed from its native state to unfolded state [25].

The H/D exchange experiments were also carried out to further understand the conformational changes of cyt c in the presence of DNFB. The results are listed in Table I. It is reported [19] that horse heart cyt c contains a total of 198 exchangeable hydrogens. In the native folded state (without DNFB), 139 hydrogens of cyt c exchange. With the addition of 0.05%DNFB, the exchanged hydrogens of 9+ charge state increase to 163 and that of 14+ are 177. The 9+ and 14+ peaks represent two charge state envelopes. This suggests that cyt c exhibits more flexible conformation and is changed into a partially unfolded state. When the concentration of DNFB increases to 0.1%, exchanged hydrogens of each conformer decrease to 152 and 170, respectively. This suggests that cyt c adopts a protected structure at this concentration of DNFB. The bimodal distribution shown in the ESI-MS spectrum represents two different conformations of cyt c [30] that do not interconvert in solution. In our work, the lower CSD, centered at 9+, exchanges more slowly than a more unfolded state, represented by the high CSD centered at 14+.

The UV absorption spectroscopy is used to monitor changes in the accessibility of the heme group to the solvent. It has been found [8,17] that the prosthetic group (heme) is covalently bound to the polypeptide through

DOI:10.1088/1674-0068/21/03/217-220

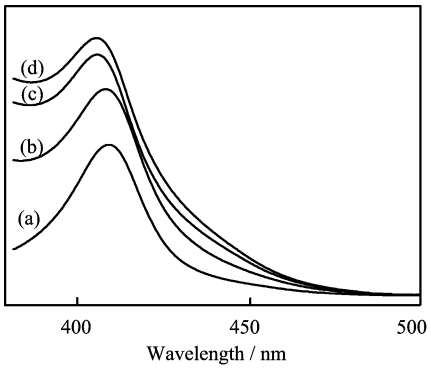

FIG. 2 UV absorption spectra of cyt c in water containing (a) 0, (b) 0.05%, (c) 0.1%, (d) 0.2% DNFB.

thioether linkage with two cysteine residues. In the native state, the iron in the heme coordinates two axial ligands, histidine (His18) and methionine (Met80), and adopts a low-spin configuration having a Soret absorption maximum at 408 nm (Fig.2(a)). With the increase of DNFB, the peaks undergo a blue shift and finally result in an absorption maximum at 404 nm (Fig.2(d)). The unfolding of the protein produces a high-spin complex with a Soret absorption maximum between 390 and 385 nm. Based on the above results, the strong-field coordination between prosthetic iron with either His18 or Met80 of cyt c is not influenced significantly.

IV. CONCLUSION

In summary, investigation on the interaction mechanism of the hapten and skin protein *in vitro* was carried out by using ESI-MS, H/D exchange as well as UV spectroscopy techniques. The preliminary results of ESI-MS reveal that the conformation of cyt c changes from native folded state into partially unfolded state with the increase of the amount of DNFB. The increase of the number of exchangeable hydrogens offered by cyt c in the equilibrium state H/D exchange is characterized by ESI-MS, which further confirmed the above results. According to the results of UV spectroscopy, it is recognized that the strong-field coordination between iron of heme (prosthetic group) and His18 or Met80 of cyt c is not affected obviously by the addition of DNFB. We also provide an effective way to produce cyt c intermediates.

V. ACKNOWLEDGMENT

This work was supported by the National Natural Science Foundation of China (No.20473020).

[1] M. Karsak, E. Gaffal, R. Date, L. Wang-Eckhardt, J. Rehnelt, S. Petrosino, K. Starowicz, R. Steuder, E. Schlicker, B. Cravatt, R. Mechoulam, R. Buettner, S. Werner, V. D. Marzo, T. Tuting, and A. Zimmer, Science **316**, 1494 (2007).
[2] M. Aleksic, C. K. Pease, D. A. Basketter, M. Panico, H. R. Morris, and A. Dell, Toxicol. in Vitro **21**, 723 (2007).
[3] A. V. Gorbachev and R. L. Fairchild, Critical Reviews in Immunol. **21**, 451 (2001).
[4] P. Saint-Mezard, A. Rosieres, M. Krasteva, F. Berard, B. Dubois, D. Kaiserlian, and J. F. Nicolas, Euro. J. Dermatol. **14**, 284 (2004).
[5] Y. Q. Li, J. Wang, Q. Xie, J. Y. Wang, and X. M. Zhang, Chin. J. Pharmacol. Toxicol. **19**, 383 (2005).
[6] G. W. Bushnell, G. V. Louie, and G. D. Brayer, J. Mol. Biol. **214**, 585 (1990).
[7] R. Grandori, I. Matecko, and N. Muller, J. Mass Spectrom. **37**, 191 (2002)
[8] R. Varhac, M. Antalik, and M. Bano, J. Biol. Inorg. Chem. **9**, 12 (2004).
[9] B. Z. Lu, C. X. Wang, and B. H. Wang, Chin. J. Chem. Phys. **16**, 117 (2003).
[10] T. E. Creighton, Biochem. J. **270**, 1 (1990).
[11] N. Berova, K. Nakanishi, and R. W. Woody, *Circular Dichroism: Principles and Applications,* New York: Wiley, (2000).
[12] Y. Bai, T. R. Sosmick, L. Mayne, and S. W. Englander, Science **269**, 192 (1995).
[13] R. Vogel and S. Friedrich, Biochemistry **41**, 3536 (2002).
[14] A. Ozdemir, L. K. Lednev, and S. A. Asher, Biochemistry **41**, 1893 (2002).
[15] Z. Y. Dai, Y. Q. Chu, B. Wu, L. Wu, and C. F. Ding, Acta Pharmacol. Sin. **29**, 759 (2008).
[16] X. M. Cai and C. Dass, Curr. Org. Chem. **7**, 1841 (2003).
[17] R. Grandori, Curr. Org. Chem. **7**, 1589 (2003).
[18] H. J. Lu, Y. L. Guo, and P. Y. Yang, J. Am. Soc. Mass Spectrom. **15**, 1612 (2004).
[19] K. R. Babu, A. Moradian, and D. J. Douglas, J. Am. Soc. Mass Spectrom. **12**, 317 (2001).
[20] S. K. Chowdhury, V. Katta, and B. T. Chait, J. Am. Chem. Soc. **112**, 9012 (1990).
[21] L. Konermann and D. J. Douglas, Rapid Commun. Mass Spectrom. **12**, 435 (1998).
[22] U. A. Mirza, S. L. Cohen, and B. T. Chait, Anal. Chem. **65**, 1 (1993).
[23] J. A. Loo, C. G. Edmonds, H. R. Udseh, and R. D. Smith, Anal. Chem. **62**, 693 (1990).
[24] H. Lu, M. Buck, S. E. Radford, and C. M. Dobson, J. Mol. Biol. **265**, 112 (1997).
[25] L. Konermann and D. J. Douglas, Biochemistry **36**, 12296 (1997).
[26] R. Grandori, Protein Sci. **11**, 453 (2002).
[27] P. J. Privalov, J. Mol. Biol. **258**, 707 (1996).
[28] T. E. Creighton, N. J. Darby, and J. Kemmink, FASEB J. **10**, 110 (1996).
[29] A. T. Iavarone, J. C. Jurchen, and E. R. Williams, Anal. Chem. **73**, 1455 (2001).
[30] D. S. Wagner and R. J. Anderegg, Anal. Chem. **66**, 706 (1994).

DOI:10.1088/1674-0068/21/03/217-220