30 March 2001

ELSEVIER

Chemical Physics Letters 337 (2001) 79–84

CHEMICAL PHYSICS LETTERS

www.elsevier.nl/locate/cplett

Conformational change of electrosprayed cytochrome *c* studied by laser-induced fluorescence

Shozo Ideue [a], Kunio Sakamoto [a], Kenji Honma [a,*], David E. Clemmer [b]

[a] *Department of Material Science, Himeji Institute of Technology, Kamigori, Hyogo 678-1297, Japan*
[b] *Department of Chemistry, Indiana University, Bloomington, IN 47405, USA*

Received 6 December 2000; in final form 2 January 2001

Abstract

The laser-induced fluorescence technique is applied to electrosprayed cytochrome *c* (cyt-*c*). While no fluorescence was observed for the electrospray of the solution of neutral pH, the fluorescence ascribed to the tryptophane residue was observed for the spray of the acidified solution. The relative intensity of the fluorescence is observed to increase with the distance from the end of the spray needle. This result suggested that the highly charged cyt-*c* ions which preserve the nonfluorescent compact forms in solution make a conformational change by the loss of solvent in the electrospray and the tryptophane residue becomes more fluorescent.

1. Introduction

Since the development of methods to put large biomolecules in the gas phase, [1,2] their conformation can be studied under specific hydration and charged state conditions [3]. Physical and chemical methods have been combined with mass spectrometric studies to obtain conformational information on gas-phase proteins, [4–8] and these studies provide basic information about the folding–unfolding processes of proteins in solution such as roles of the number of charges and solvents on the conformational changes. However, while almost exclusively mass spectrometric techniques are used in the gas-phase studies, most of the conformational properties of proteins in solution have been studied by optical methods, such as circular dichroism, Raman scattering, absorption, and fluorescence techniques [1]. Therefore, the application of these optical methods to gas-phase proteins is required to obtain conformational information directly relevant to those in solution.

Cytochrome *c* (cyt-*c*) is one of well studied proteins with respect to its folding–unfolding process in solution [9]. This protein contains one tryptophane residue and its fluorescence has been used as a sensitive measure for the conformational change of the protein [10]. The tryptophane fluorescence is almost completely quenched in the native form by energy transfer to the heme [11,12], however, unfolding of the protein increases the heme-tryptophane distance and consequently enhances the fluorescence yield [13]. The gas-phase ions of cyt-*c* formed by electrospray ionization

* Corresponding author. Fax: +81-791-58-0132.
E-mail address: honma@sci.himeji-tech.ac.jp (K. Honma).

[1] See for example articles in special issue on Protein Folding [9].

0009-2614/01/$ - see front matter
PII: S0009-2614(01)00153-1

have been also studied quite extensively. Conformational information has been accumulated for its specific charged states by chemical and physical methods. Drift tube studies have observed that the cross-sections of cyt-*c* ions increase with the number of positive charges (corresponding to the number of protons attaching to the cyt-*c* molecule) and have suggested that the conformation of the ions changes from the native like compact form to the elongated form such as a random coil at around 7+ or 8+ charge state [4–8].

Here, we applied the laser-induced fluorescence technique to study the fluorescence of the tryptophane residue in electrosprayed cyt-*c*. Combination of fluorescence detection techniques with the electrospray ion source has been started recently and was first applied for some dye molecules [14–17]. These studies have provided important information on the processes in the electrospray plume. The study presented here is the extension of this technique to the detection of proteins in the electrospray plume. Although we could not specify the charged state and extent of hydration of cyt-*c* because the LIF measurement is limited to the spray in the air, we believe that it is an important step to apply the fluorescence detection technique to the protein in vacuum under controlled hydration and charged conditions.

2. Experimental

The experimental setup is shown in Fig. 1. The solution of a sample in a 50:50 mixture of water and methanol was electrosprayed from a spray needle (stainless steel, 0.2 mm o.d.) to a counter electrode. Positive high voltage (typically 3.5 kV) is applied to the spray needle and the typical distance between the needle and the counter electrode is 25 mm. A collimated fourth harmonic (266 nm) of a Nd:YAG laser (Spectra Physics DCR-11) irradiated the spray and fluorescence from the excitation region was collected by a lens and dispersed by a monochromator (Spex 270M). The dispersed fluorescence was detected by a photomultiplier tube (Hamamatsu R-928) and its output was amplified and fed into a boxcar integrator (SRS-250). The laser intensity was monitored by a fast photodiode and its signal was integrated by another boxcar integrator and used for normalization of the LIF intensity.

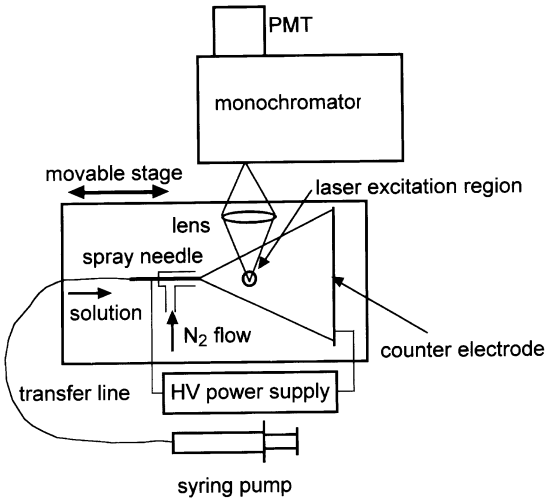

Fig. 1. Schematic diagram of the experimental setup. Excitation laser beam axis is perpendicular to this figure.

Both the spray needle and the counter electrode are mounted on a stage driven by a micrometer and the distance of the excitation and observation point from the spray needle can be changed continuously with 0.1 mm accuracy. The typical spot size and power of the laser were 2 mm dia. and 0.2 mJ/pulse, respectively. For the measurements of fluorescence lifetimes, the third harmonic output (264 nm) of an amplified mode-locked titanium sapphire laser (Mira, Coherent) was used for excitation and the time evolution of the fluorescence was measured by a streak camera (Hamamatsu C4334). The temporal resolution was limited by the streak camera to $\sim$10 ps.

Since the electrospray plume studied here is in the air and O_2 may quench the photoexcited species, a flow of N_2 can be applied through a $1/4''$ tee as shown in Fig. 1. The effect of the N_2 flow was measured by the lifetime of the electrosprayed $Ru(bpy)_3^{2+}$ ion. The long lifetime identical to that of the deoxidized solution was observed with the N_2 flow, while the lifetime measured in the air was much shorter presumably because of the quenching by O_2 [18].

Samples of horse heart cytochrome *c* and L-tryptophane were both obtained from Sigma Chemical and used without further purification. Methanol (>99.7%) and deionized water were obtained from Kanto Chemical and used to prepare 50/50 (v/v) solutions. The solutions were not degassed and showed a pH = 6.7–6.8 as measured with a pH meter (TOA electronics, HM-5S) equipped with a glass electrode. Acetic acid (>99.7%) was obtained from Wako Chemical and used to acidify solutions.

3. Results and discussion

3.1. Dispersed fluorescence

The dispersed fluorescence (DF) spectrum was measured for the electrosprayed tryptophane solution (5×10^{-3} M). The result is shown in Fig. 2a and the spectrum peaking around 350 nm is identical to that of the solution measured by a conventional fluorescence spectrometer. The fluorescence spectra of electrosprayed cytochrome *c* (1.2×10^{-4} M) were measured for two different solutions, i.e., unacidified and acidified solutions. The former is the solution without adding any acid (pH = 6.8) and the spectrum is shown in Fig. 2b. No fluorescence is observed in Fig. 2b except for the weak contribution of the tail of the scattered laser light at the shorter wavelength. The spectrum shown in Fig. 2c is the DF spectrum measured for the electrospray of the solution with 0.3% acetic acid (pH = 3.6). This spectrum shows a fluorescence band peaking around 350 nm. Although this band is slightly broader than that of the electrosprayed tryptophane (Fig. 2a), it is ascribed to the fluorescence of the tryptophane residue in the electrosprayed cyt-*c*. DF spectra for the acidified cyt-*c* solution were measured at several distances from the end of the needle, and no significant difference was observed. The results observed here, i.e., no fluorescence in the electrospray of the unacidified solution and the tryptophane fluorescence in that of the acidified solution, are consistent with the fluorescence property of cyt-*c* in solution. In room temperature solution, the tryptophane fluorescence is almost completely quenched by the heme in the native form

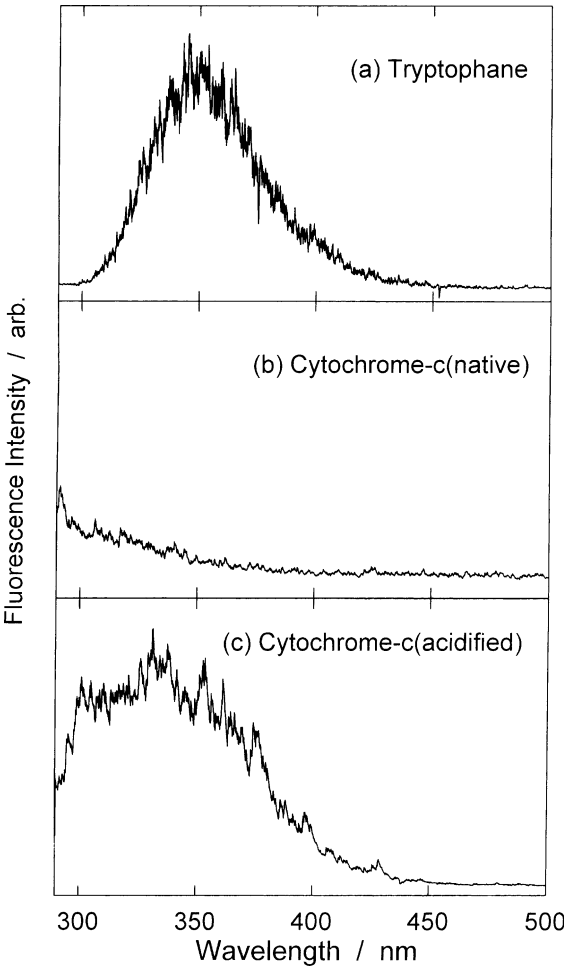

Fig. 2. Dispersed fluorescence spectra of electrosprayed (a) tryptophane (b) cytochrome c (unacidified) and (c) cytochrome c (acidified by 2.5% acetic acid) solutions measured by 266 nm excitation. The spectra were taken at 10 mm from the top of the spray needle.

[11,12], however, unfolding by acid increases the heme-tryptophane distance and consequently enhances the fluorescence yield [13].

One unique property of the electrospray is that the solvation state is changed through evolution of the spray. Since charged droplets formed at the electrically biased needle lose solvents by evaporation, ions with fewer solvent molecules can be sampled by increasing the distance from the needle. The effect of desolvation was studied by the fluorescence intensity at 350 nm as a function of the distance from the needle and the results are

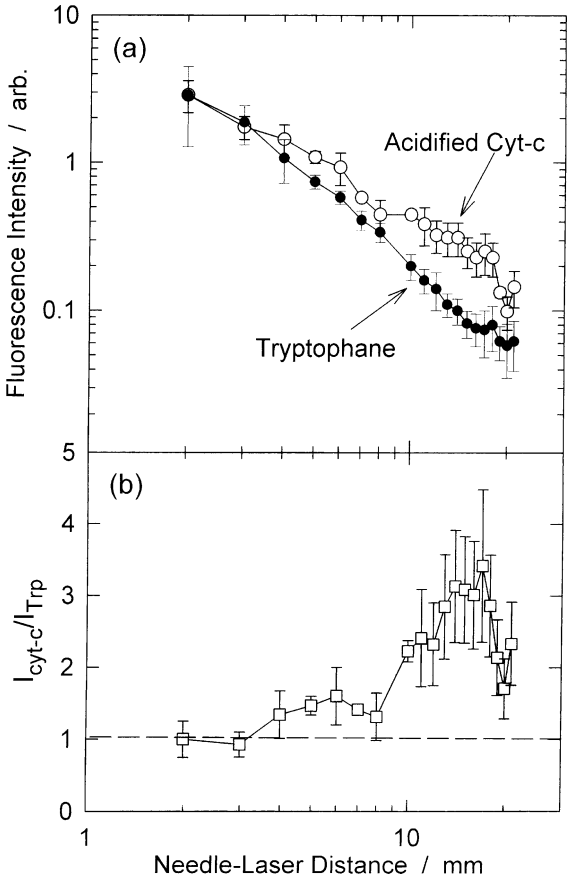

Fig. 3. Plots of tryptophane fluorescence intensities against the distance from the top of the spray needle. (a) Relative intensities at 350 nm are measured for electrosprayed tryptophane and cytochrome c (acidified). (b) Fluorescence intensity of cytochrome c is normalized with respect to that of tryptophane. Error bars show 1σ of the measurements.

summarized in Fig. 3. Relative intensities are shown in Fig. 3a for the tryptophane and acidified cyt-*c* solutions. Although both intensities decrease with the distance from the needle as expected from the spatial expansion of the spray plume, [16] the acidified cyt-*c* shows a slightly milder decrease. Assuming that the fluorescence of tryptophane is not affected by the loss of solvents, [19] [2] the intensities of acidified cyt-*c* were normalized with respect to those of tryptophane, I_{cyt-c}/I_{trp}, and shown in Fig. 3b. This plot clearly shows the enhancement of fluorescence in acidified cyt-*c* with increasing distance from the needle, suggests that the fluorescence yield increases with the loss of solvents. The relative intensity was measured with the N_2 flow to clarify the effect of O_2 in air. Because the N_2 flow slightly changes the shape of the electrospray plume, the absolute intensity of the fluorescence is changed by the N_2 flow. However, the relative fluorescence intensity shows almost identical dependence on the distance from the spray needle.

3.2. Fluorescence lifetime

The relative fluorescence yield shown in Fig. 3b is given by $I_{cyt-c}/I_{trp} = n_{cyt-c}\Phi_{cyt-c}/n_{trp}\Phi_{trp}$, where $n_{cyt-c}, n_{trp}, \Phi_{cyt-c}$, and Φ_{trp} are the densities of fluorescent cyt-*c* and tryptophane and the fluorescence quantum yields of cyt-*c* and tryptophane, respectively. Therefore, the observed increase of the relative fluorescence yield is ascribed to either the increase of Φ_{cyt-c}/Φ_{trp} or n_{cyt-c}/n_{trp}, i.e., the increase of the fluorescence yield in each fluorescent cyt-*c* or the increase of the number of fluorescent cyt-*c*.

The fluorescence quantum yield of the sprayed cyt-*c* can be estimated from the fluorescence lifetimes. The fluorescence decays were measured at 3 mm from the spray needle for the electrospray of the tryptophane and acidified cyt-*c* solutions. Although the lifetime measurement at longer distance from the needle is expected to provide more direct information about the fluorescence quantum yield, the fluorescence of cyt-*c* at longer distance was so weak that we could not accomplish the lifetime measurement. The results are summarized in Fig. 4. Dispersed fluorescence in the range of 350–360 nm is collected for these measurements. The fluorescence decays of both electrosprayed tryptophane and cyt-*c* solutions were characterized by single exponential decay functions, and their lifetimes, τ_{cyt-c} and τ_{trp}, are determined to be 3.4 ± 0.3 ns and 1.9 ± 0.3 ns, respectively. Although tryptophane fluorescence shows a nonexponential decay in solution, [20,21] the time

[2] Actually, the lifetime of tryptophane slightly decreases as increasing the distance. This may reflect pH change through electrospray suggested by Gatlin and Turecek [19] and Van Berkel et al. [15].

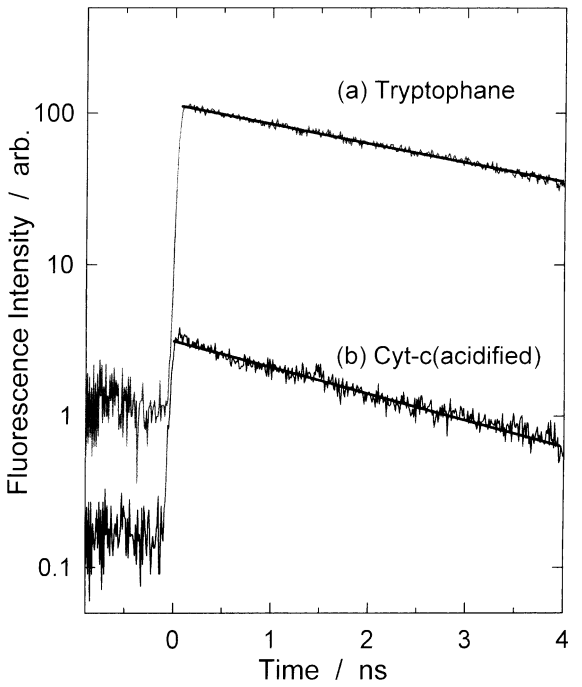

Fig. 4. Time profiles of fluorescence detected in the electrosprayed tryptophane (a) and acidified cyt-*c* (b). Solid lines are best fits of single exponential functions for the decays.

evolutions are characterized by single exponential functions for both electrosprayed tryptophane and cyt-*c* (acidified) solutions in this experiment [3]. The lifetime of tryptophane determined in the electrospray is identical to the major component of its lifetime in aqueous solution of pH = 7 [20,21].

From these values, the relative quantum yield of electrosprayed cyt-*c*, $\Phi_{\text{cyt}-c}/\Phi_{\text{trp}}(=\tau_{\text{cyt}-c}/\tau_{\text{trp}})$, is established to be 0.6 at 3 mm from the needle. Because this is significantly smaller than that estimated for a random coil, 0.8 [22] a 30–40% increase of the fluorescence yield is possible if the conformation of fluorescent cyt-*c* changes to the random coil by increasing the distance from the needle and by losing solvents. However, the increase shown in Fig. 3b is almost greater than 100%, which cannot be explained only by the increase of the fluorescence yield of each fluorescent cyt-*c*. Therefore, the number of fluorescent cyt-*c* must also be increased by the loss of solvents through the electrospray.

The experimental results obtained here and the acid-induced conformational changes studied in solution and in vacuum are summarized as the following. (1) In acidified solution, cyt-*c* takes unfolded structure in which the distance between the tryptophane residue and heme is increased and the tryptophane fluorescence is not quenched completely [10]. (2) In vacuum, it has been confirmed that highly protonated cyt-*c* ions, peak population around 16+, are formed by the electrospray of acidified solution [8,23]. Drift time measurements and molecular dynamics studies have suggested that these highly protonated cyt-*c* ions have an unfolded conformation [6,7]. (3) The results obtained in this study show that the tryptophane fluorescence is enhanced by the loss of solvation through the electrospray, and the enhancement is ascribed to the increase of the number of fluorescent cyt-*c*.

The fluorescence observed in the early stage of the electrospray, near the spray needle, is probably identical to that observed in the acidified solution, i.e., fluorescence from cyt-*c* unfolded by acid. The highly protonated and unfolded cyt-*c* observed in vacuum are still solvated in the spray studied here. The solvents reduce intramolecular repulsive forces, then allow some of highly protonated cyt-*c* to have compact conformations which are not fluorescent [24]. These highly protonated but compact cyt-*c* could not be observed by the fluorescence detection in the early stage of the spray as well as in solution. The loss of the solvents may induce their conformational changes to fluorescent cyt-*c*, which could be responsible to the enhanced fluorescence at the larger distance from the spray needle.

In the fluorescence studies of cyt-*c*, the quenching of the tryptophane fluorescence is assumed to be due to proximity to heme. It may be better to note the possibility of quenching by oxygen in air. Oxygen may bound in a high affinity site adjacent to the tryptophane and quench the tryptophane fluorescence in the compact form of cyt-*c*. The conformational change of cyt-*c* may increase the distance between oxygen and tryptophane residue or release oxygen to enhance the

[3] χ^2 values of both decays indicate no significant improvement for double exponential fittings.

fluorescence. However, as described in the previous section, replacement of air with N_2 shows no significant change in the relative intensity of fluorescence and its dependence on the distance from the needle. Therefore, it is concluded that the quenching arising from oxygen is unlikely.

Here, we successfully achieve the fluorescence detection of electrosprayed cyt-*c*. These results provide unique information about the conformational change of cyt-*c* which can connect gas-phase mass spectrometric studies and optical studies in solutions. Although the study here is limited to the electrospray plume in air, it is an important step to apply the fluorescence detection technique to the protein in vacuum under controlled hydration and charged conditions.

Acknowledgements

We thank Prof. Takagi (Himeji Institute of Technology) for his help in the lifetime measurements. Financial support from the Ministry of Education, Science, Sports, and Culture (Contract No. 1020629) is acknowledged.

References

[1] J.B. Fenn, M. Mann, C.K. Meng, S.F. Wong, C.M. Whitehouse, Science 246 (1989) 64.
[2] P. Kebarle, L. Tang, Anal. Chem. 65 (1993) 972.
[3] C.S. Hoaglund-Hyzer, A.E. Counterman, D.E. Clemmer, Chem. Rev. 99 (1999) 3037.
[4] M.F. Jarrold, Acc. Chem. Res. 32 (1999) 360.
[5] F.W. McLafferty, Z. Guan, U. Haupts, T.D. Wood, N.L. Kelleher, J. Am. Chem. Soc. 120 (1998) 4732.
[6] D.E. Clemmer, R.R. Hudgins, M.F. Jarrold, J. Am. Chem. Soc. 117 (1995) 10141.
[7] K.B. Shelimov, D.E. Clemmer, R.R. Hudgins, M.F. Jarrold, J. Am. Chem. Soc. 119 (1997) 2240.
[8] K.B. Shelimov, M.F. Jarrold, J. Am. Chem. Soc. 118 (1996) 10313.
[9] Protein Folding (special issue), Acc. Chem. Res. 31 (1998) 11.
[10] M.-F. Jeng, S.W. Englander, J. Mol. Biol. 221 (1991) 1045.
[11] W.R. Fisher, H. Taniuchi, C.B. Anfinsen, J. Biol. Chem. 248 (1973) 3188.
[12] J. Vanderlooi, M. Erecinska, Eur. J. Biochem. 60 (1975) 199.
[13] Y. Goto, L.J. Calciano, A.L. Fink, Proc. Natl. Acad. Sci. 87 (1990) 573.
[14] X.Fr.D. Chiller, A. Monnier, H. Bill, F.O. Gulacar, A. Buchs, S.A. McLuckey, G.J. Van Berkel, Rapid Commun. Mass Spectrom. 10 (1996) 299.
[15] G.J. Van Berkel, F. Zhou, J.T. Aronson, Int. J. Mass Spectrom. Ion Processes 162 (1997) 55.
[16] S. Zhou, A.G. Edwards, K.D. Cook, G.J. Van Berkel, Anal. Chem. 71 (1999) 769.
[17] S. Zhou, K.D. Cook, Anal. Chem. 72 (2000) 963.
[18] K. Sakamoto, S. Ideue, K. Honma, to be published.
[19] C.L. Gatlin, F. Turecek, Anal. Chem. 66 (1994) 712.
[20] J.W. Petrich, M.C. Chang, D.B. McDonald, G.R. Fleming, J. Am. Chem. Soc. 105 (1983) 3824.
[21] E.F. Gudgin-Templeton, W.R. Ware, J. Phys. Chem. 88 (1984) 4626.
[22] C.-K. Chan, Y. Hu, S. Takahashi, D.L. Rousseau, W.A. Eaton, J. Hofrichter, Proc. Natl. Acad. Sci. 94 (1997) 1779.
[23] S.K. Chowdhury, V. Katta, B.T. Chait, J. Am. Chem. Soc. 112 (1990) 9012.
[24] H. Theorell, A. Akesson, J. Am. Chem. Soc. 63 (1941) 1818.