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Ca^{2+}-Induced Changes of Surfactin Conformation: A FTIR and Circular Dichroism Study

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Previous NMR studies on surfactin proposed two γ or β-turn-containing conformers while recent CD studies described β-sheets and α-helices in surfactin. Since these data were not obtained in the same conditions, the conformation of surfactin was reinvestigated by FTIR spectroscopy, a diagnostic method for β-sheets. In trifluoroethanol, the FTIR spectra of surfactin and its diester are compatible with γ and/or β-turn(s) and the differences in their CD spectra show the importance of the Glu[1] and Asp[5] COOH groups in stabilizing the lipopeptide conformation. The calcium-induced spectral changes of both lipopeptides suggest a first binding of the divalent ions to the surfactin COOH groups (until calcium-lipopeptide mole ratio reached 1) followed by bulk conformational changes (at higher mole ratios). In Tris buffer at pH 8.5, the FTIR amide I band shape, without the typical 1610–1628 and 1675–1695 cm^{-1} bands, ascertains the absence of β-sheets. © 2001 Academic Press

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Although surfactin (SF) had been discovered about 30 years ago (1), its biosynthesis (2–4), its biological effects (5–7) and its interaction with artificial membranes (8–10) are still on actuality because of its potent applications (11–14). SF is a cyclic lipopeptide (Fig. 1) produced by various strains of *Bacillus subtilis.* It was proposed that the two polar groups of Glu and Asp residues of SF could be involved in divalent ion chelation (15–16) since the methylation of these carboxyl residues affected its biological and surfactant properties (5, 8, 17). The conformation of SF in dimethylsulfoxide (DMSO) was studied by NMR analysis combined with molecular modeling (18). Two conformers were proposed: S1 with a single intramolecular hydrogen bond, fixing a β-turn and S2 with three hydrogen bonds, two of them corresponding to γ-turns. A FTIR spectroscopy of SF in different solvents ascertained the presence of a hydrogen-bonded C═O group involved in a β-turn structure (19). Other studies on SF in aqueous solutions showed an increase of the β-sheet formation by heating and a Ca^{2+}-induced formation of α-helices (13). Since these conformational studies performed with different methods and with various solvents, our purpose was to reinvestigate the conformation of SF by using FTIR spectroscopy, a diagnostic method for β-sheets (20, 21) and by CD spectroscopy, a method sensitive for the presence of helices and turns (22–24).

MATERIALS AND METHODS

Materials. Calcium perchlorate (tetrahydrate, 99%), used in titrations, was purchased from Aldrich, ^2H_2O (99.9%) from Merck (Darmstadt, Germany) and trifluoroethanol (TFE) (NMR grade) from Aldrich. Acidic SF was purified from *B. subtilis* according to (19). SF was methylated on the Glu and Asp residues as in (16). The purity of SF and its diester (SFDM) was tested by TLC on silica gel 60 in chloroform-methanol-water (65:25:4, v/v/v) and chloroform-methanol-30% NH_4OH (13:5:1, v/v/v), respectively.

Infrared spectroscopy. FTIR spectra were recorded with a Bruker IFS-55 FTIR spectrometer at a 2 cm^{-1} resolution using 0.20–0.25 mm pathlength CaF_2 cells. During data acquisition, the spectrometer was continuously purged with dry air. The solvent spectrum was deducted from the sample spectrum taken under the same conditions. The contribution in the amide I region of traces of water in TFE (H—O—H deformation band at 1633 cm^{-1}) was removed on the basis of the antisymmetric O—H stretching vibration appearing at 3688 cm^{-1} in this solvent. Each FTIR spectrum is representative of at least three independent measurements. In some cases, the FTIR spectrum was decomposed into individual bands by the Levenberg-Marqardt nonlinear curve-fitting algorithm as weighted sums of Lorentzian and Gaussian functions on the basis of the FSD analysis of the complex band contour. The concentrations of SF and SFDM were 2 mM in TFE or in Tris buffer (10 mM Tris–HCl, pH 8.5) in ^2H_2O.

CD spectroscopy. CD spectra were recorded in TFE, TFE-water (1/1, v/v), and Tris buffer on a Jobin-Yvon Mark VI dichrograph (calibrated with epiandrosterone) at room temperature in 0.02 cm

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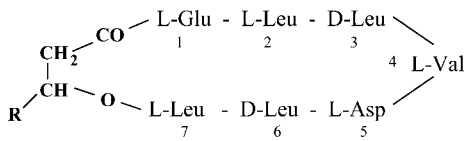

FIG. 1. Primary structure of surfactin. The amino acid numbers are related to their position in the heptapeptide moiety and the β-hydroxy fatty acid is indicated by bold letters.

cells. The sample concentrations ranged between 0.5–0.766 mM. The spectra obtained as an average of 3 scans were smoothed by the Savitzky-Golay algorithm (25).

RESULTS AND DISCUSSION

Influence of Calcium on the CD Spectra Measured in TFE

SF in TFE shows a CD spectrum dominated by a broad positive band centered at ~218 nm and a negative one at 198 nm (Fig. 2a). The positive band may reflect a strong spectral contribution of the $n\pi^*$ transition of D-Leu[3] and D-Leu[6] residues. Another possible explanation is the adoption of γ_D^{inv} turns which are in mirror image relationship with γ_L^{inv} turns characterized by a red-shifted broad negative CD band at the same wavelength. Thus, γ-turns centered at D-amino acids are expected to show a positive $n\pi^*$ band. SFDM in TFE has a CD spectrum very different to that of SF (Fig. 2b). The SFDM spectrum shows a broad negative CD band at ~220 nm, with a negative shoulder near 200 nm. Also, a positive band turns up below 190 nm. The spectral features indicate the presence of β-sheet conformation and/or β-turn(s), probably of type II, and/or γ_L^{inv} turn(s) (22). The spectral differences between SF and SFDM clearly show the importance of the Glu[1] and Asp[5] COOH in stabilizing the conformation of the SF backbone.

The addition of calcium to SFDM gives rise to profound conformational changes. Increasing calcium concentration induced (i) the progressive appearance of a positive band centered at 215 nm associated with the gradual disappearance of the broad negative band centered at 220 nm and (ii) a shift of the negative band at ~200 nm to 190–195 nm. The presence of an isodichroic point indicates an equilibrium between two conformers.

The addition of calcium to SF gives rise to more complex conformational changes: (i) until the ratio of calcium to peptide (r_{Ca}) reached 1, the CD spectrum of SF correlates with that of SFDM without calcium except for the presence of the positive band at ~195 nm, while (ii) at r_{Ca} higher than 1, the calcium-induced spectral changes of SF are more similar to those observed with SFDM. These results indicate that at $r_{Ca} \leq 1$ calcium binds the COOH of Glu[1] and Asp[5] of SF and at $r_{Ca} > 1$ the ion binding induces SF conformational changes as it does with SFDM.

Influence of Calcium on the FTIR Spectra Measured in TFE

The IR spectrum of SF in TFE (Fig. 3a) shows a wide band centered at 1730 cm^{-1}, while the IR spectrum of SFDM indicates a narrower band centered at 1725 cm^{-1} (Fig. 3b). The deconvolution of SF and SFDM spectra indicates the presence of a component band at 1742–1743 cm^{-1}, corresponding to the absorbance of the lactone ring C═O of both compounds and a component band at 1724–1723 cm^{-1}, corresponding to the absorbances of the COOH of Glu[1] and Asp[5] for SF and the $COOCH_3$ of Glu[1] and Asp[5] for SFDM. This last result is unexpected because ester C═O groups generally absorb at higher wavenumber than COOH groups. The only explanation would be that TFE, a hydrogen bond donor solvent, forms strong hydrogen bonds with the C═O of the Asp and Glu methylesters in SFDM. In the amide I region, the asymmetric band deconvolution gives a main band at 1659 or 1660 cm^{-1} with a high-frequency contribution at 1674 or 1682 cm^{-1} and low-frequency contributions at 1642 or 1653 and 1621 or 1629 cm^{-1} for SF or SFDM, respectively. These latter bands are compatible with both γ- and/or β-turn(s) but not with the β-sheet structure previously identified by CD spectroscopy.

The addition of calcium to SF in TFE (Fig. 4a) gives rise to significant changes in the 1750–1700 cm^{-1} region. The ν_{CO} band, corresponding to the absorptions of both the lactone ring and the COOH of Glu[1] and Asp[5], is shifted to higher frequency at a r_{Ca} from 0.75 to 1 and the ν_{CO} band symmetry is lost. Furthermore, the shifts observed in the 1600–1500 cm^{-1} region are mostly due to the COO^- group(s) but the amide II frequency is also affected. Both the higher frequency shift in the 1750–1700 cm^{-1} region and the appearance of a shoulder at ~1570 cm^{-1} are likely due to binding of calcium to the COOH group(s). In the amide I region, the Ca^{2+}-induced changes reflect the peptide backbone modifications. The dominant band at ~1640 cm^{-1} can be associated with β-turn(s) and the low frequency one at ~1630 cm^{-1} is compatible with the presence of C_7 H-bonding or amide(s) complexing Ca^{2+}. Ca^{2+}-titration suggests the formation of a 1:1 SF/Ca^{2+} complex in equilibrium with uncomplexed SF (presence of an isosbestic point).

The addition of calcium to SFDM (Fig. 4b) gives rise to spectral changes until $r_{Ca} = 1$ and no significant shifts were obtained after $r_{Ca} = 2$. Concerning the lactone and ester ν_{CO} absorptions, there is only a small shift to higher frequency. In the amide I region, a main component band appears at 1648 cm^{-1}, accompanied with a broad high frequency shoulder. The 1648 cm^{-1} band is compatible with weakly H-bonded turn(s). In the amide II region, the shift to higher frequency of the main component band at 1543 cm^{-1} and the shoulder at ~1560 cm^{-1} reflect also the presence of two types of

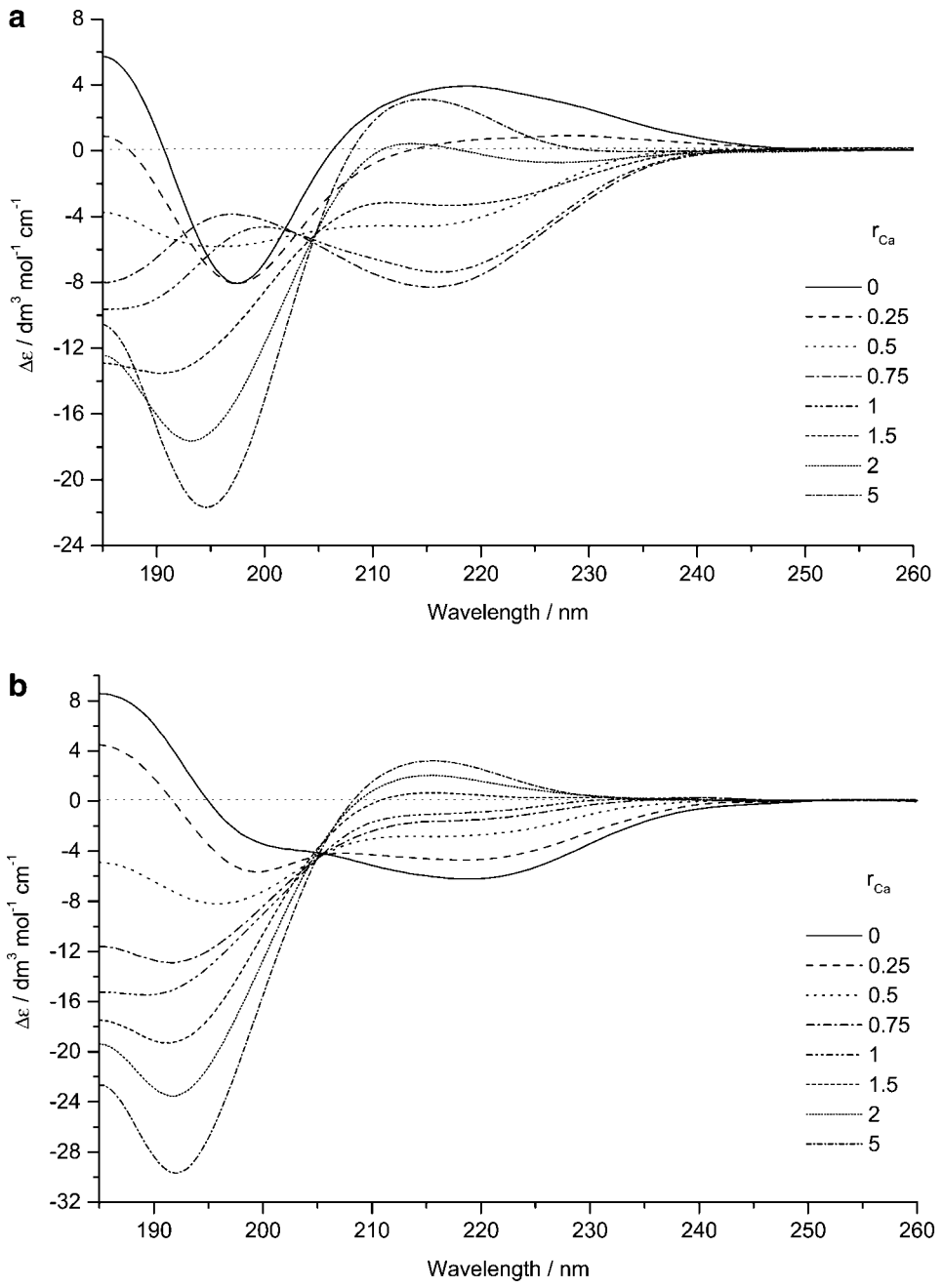

FIG. 2. CD spectra of surfactin (a) and surfactin dimethylester (b) in TFE at different Ca^{2+}/peptide ratios (r_{Ca}).

amide groups. These spectral changes suggest bulk conformational modifications due to the Ca^{2+}-binding to amide groups. Furthermore the FTIR evidence of an equilibrium between two conformers during the Ca^{2+}-titration of SFDM and SF (presence of an isobestic point) corroborates the CD results.

CD and FTIR Spectra Measured in Aqueous Solution

The CD spectrum of SF in a TFE/water (1:1, v/v) mixture indicates that SF undergoes a dramatic conformational change (Fig. 5). The broad positive band centered at ~218 nm and the negative one at 198 nm in TFE is replaced by a negative band at 222 nm with a shoulder at 198 nm and a positive band at ~190 nm in the TFE/water mixture. This spectral behaviour can be interpreted as the formation of β-sheet micelles. The shape of the spectrum is unchanged in the presence of a great excess of Ca^{2+} ions (r_{Ca} = 15) except the band intensity decreases, which could be interpreted as the precipitation of the micelles. This is in agreement with the well-known low solubility of SF at the pH of water.

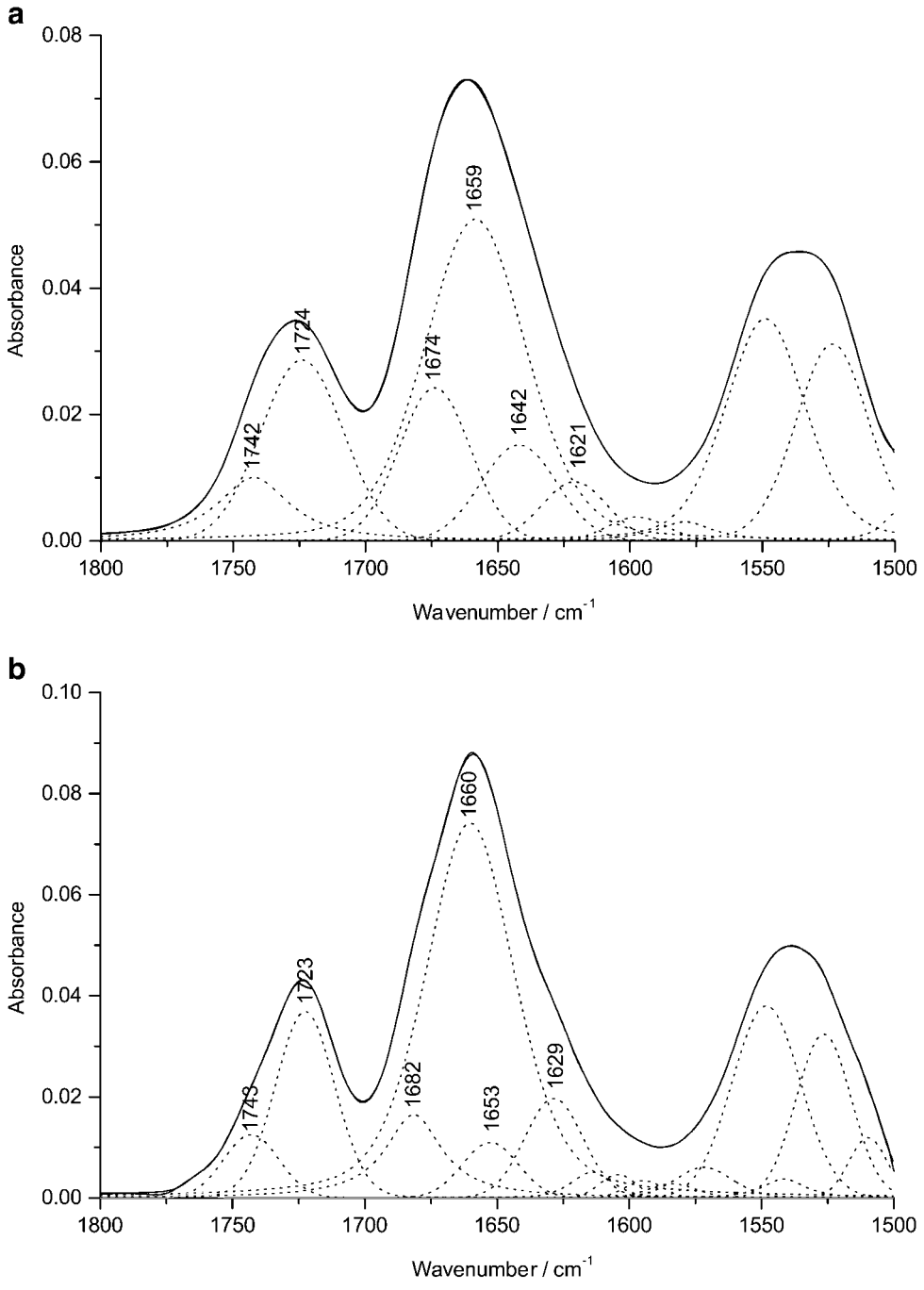

FIG. 3. Curve-fitted FTIR spectra of surfactin (a) and surfactin dimethylester (b) in TFE.

Indeed similar calcium-induced CD spectral changes have been observed when surfactin was dissolved in buffer at pH 7.5 (13).

The CD spectrum of SF in 10 mM Tris buffer (pH 8.5) does not exhibit the bands observed when SF was dissolved in the TFE/water mixture but gives a positive band centered at ~230 nm, a negative band at ~200 nm (Fig. 5), looking more like the CD spectrum of SF in TFE (Fig. 2a). The small positive band at ~190 nm could be interpreted if few β-sheet micelles (observed when SF is dissolved in TFE/water mixture) are in equilibrium with the turn containing conformer observed in TFE. Similar CD spectrum had been previously observed at a concentration of 0.11 mM in 10 mM Tris buffer at pH 8.3 (11). Addition of Ca^{2+} ions (r_{Ca} = 1) gives rise to a CD spectrum looking like that of SF without divalent ion, except for the lower amplitude of the positive band, showing no precipitation of the li-

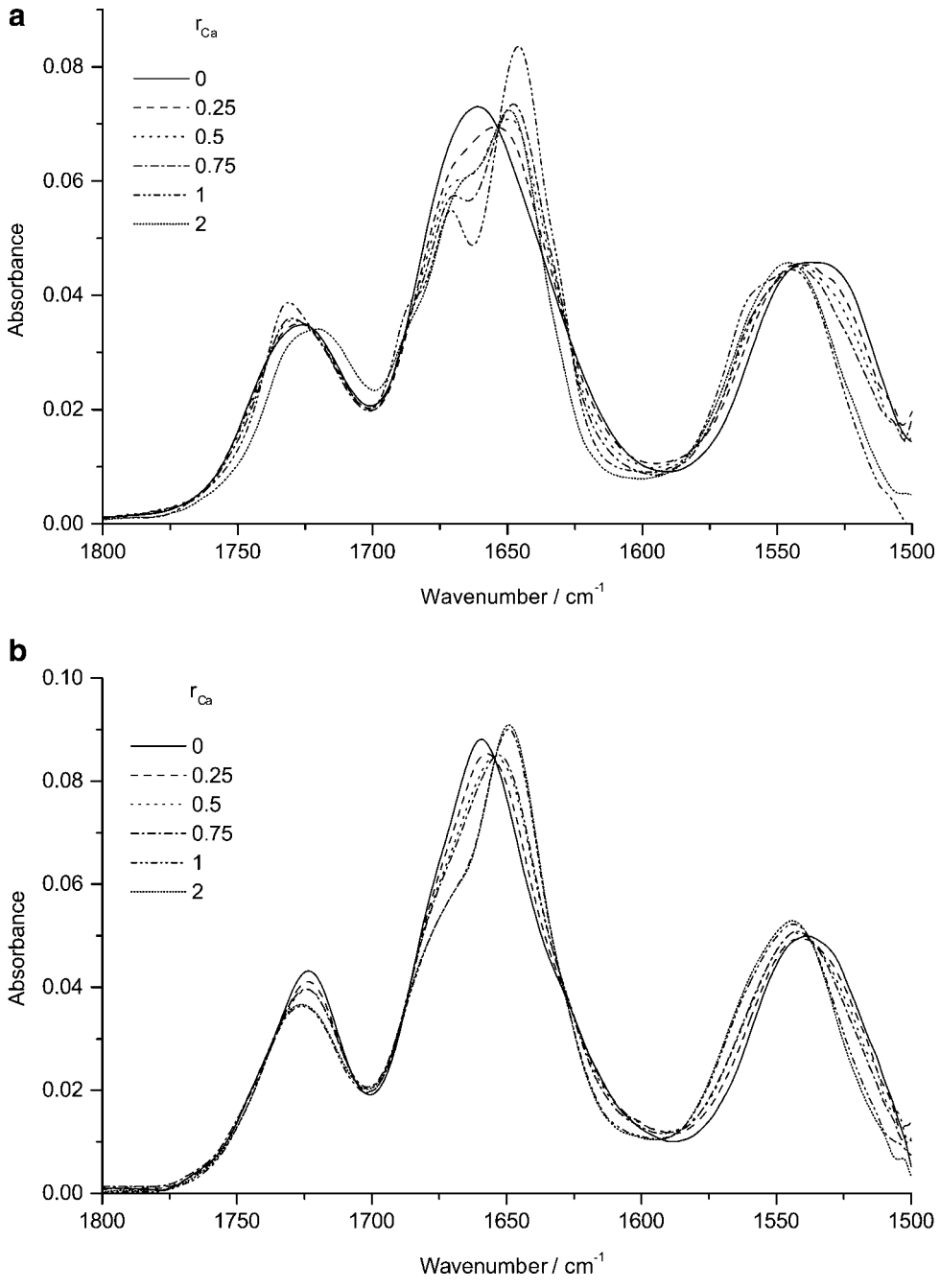

FIG. 4. FTIR spectra of surfactin (a) and surfactin dimethylester (b) in TFE at different Ca^{2+}/peptide ratios (r_{Ca}).

popeptide (Fig. 5). These calcium-induced conformational changes in buffer are less important than those observed in TFE, indicating stronger interactions between the Asp and Glu COO^- with water molecules than between the Asp and Glu COOH with TFE molecules.

The FTIR spectrum of SF in the Tris buffer is given in Fig. 6. The most important changes, as compared with SF in TFE (Fig. 3a), are the shift of the amide I band from 1659–1660 cm^{-1} to 1639 cm^{-1}, the decrease of the 1730 and 1537 cm^{-1} bands and the appearance of a 1570 cm^{-1} band. Similar spectra were obtained when the SF concentration in Tris buffer was reduced until 0.5 mM. The decrease of the 1730 cm^{-1} band and the concomitant increase of the 1570 cm^{-1} band can be interpreted as deprotonation of the Asp and Glu COOH (26), while the decrease of the 1537 cm^{-1} band corresponds to the H/^{2}H exchanges of NH groups. Furthermore, the amide I band shape, without the 1610–1628 and 1675–1695 cm^{-1} bands typical of the presence of aggregates (27), ascertains the absence of β-sheets in SF dissolved in the Tris buffer.

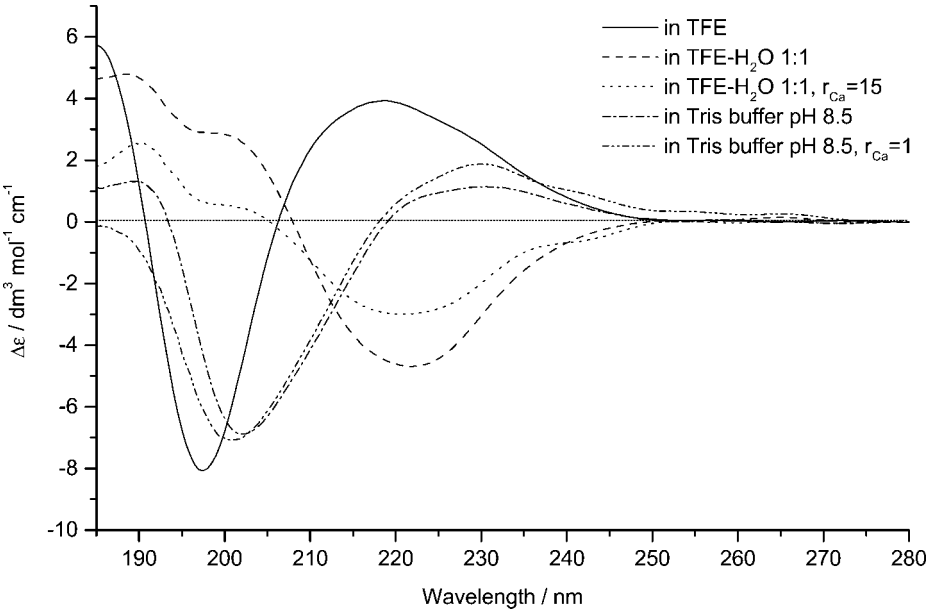

FIG. 5. Comparison of CD spectra of surfactin obtained in TFE, TFE-H_2O (1:1 mixture, in the absence or the presence of Ca^{2+} ions at r_{Ca} = 15) and Tris buffer (at pH 8.5, in the absence or the presence of Ca^{2+} ions at r_{Ca} = 1).

CONCLUSION

Based on our comparative CD and FTIR spectroscopic studies in different solvents with or without Ca^{2+} ions, SF looks like a chimeric molecule, having the unique ability of adopting strongly different conformations depending on the conditions. The differences between the CD and FTIR spectra of SF and SFDM put emphasis on the COOH groups of Glu^1 and Asp^5 in stabilizing the backbone conformation of the ring. On the other hand, these carboxyl groups are also responsible for Ca^{2+} binding at low concentration ($r_{Ca} < 1$). In TFE, a structure-promoting solvent, both SF and SFDM exist as a mixture of conformers featuring different β and/or γ-turn structures. This finding is in agreement with the results of a NMR analysis of the backbone conformation of SF in DMSO (18). When TFE is replaced by aqueous solution at pH 8.5, meanwhile our CD studies of SF could be interpreted by the presence of few β-sheet micelles, the use of FTIR spectros-

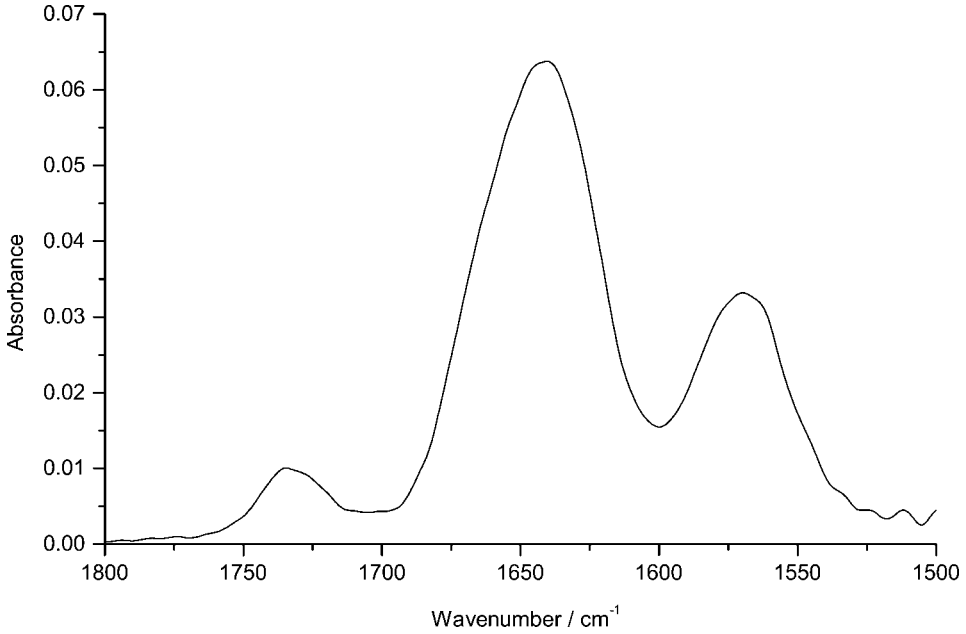

FIG. 6. FTIR spectrum of surfactin in ^2H_2O in the presence of 10 mM Tris buffer (pH 8.5).

copy, a diagnostic method for β-sheets, gives a FTIR spectrum, which excludes the presence of significant amount of conformers with a β-sheet backbone or the formation of β-sheet micelles.

The chimeric structure of SF having chelator COOH groups, fatty acid side chains with micelle-forming ability and a highly flexible conformation with a cyclic depsipeptide ring may give an explanation for the biological activity of SF. Indeed, the COOH groups can strongly bind Ca^{2+} or related cations which are essential for many biological processes. Such a strong binding of divalent ions by SF explain the inhibition of some enzymes (cyclic AMP phosphodiesterase, alkaline phosphatase) which need divalent ions for their activity (15, 16). Meanwhile, another process, involving a direct interaction of the highly flexible peptide ring or the fatty acid side chains with the protein, would explain the activator effect of surfactin on the platelet cytosolic phospholipase A2, which does not need Ca^{2+} ions (7).

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REFERENCES

1. Kakinuma, A., Hori, M., Isono, M., Tamura, G., and Arima, K. (1969) Determination of fatty acid in surfactin and elucidation of the total structure of surfactin. *Agric. Biol. Chem.* **33,** 973–976.
2. Reuter, K., Mofid, M. R., Marahiel, M. A., and Ficner, R. (1999) Crystal structure of the surfactin synthetase-activating enzyme sfp: A prototype of the 4′-phosphopantetheinyl transferase superfamily. *EMBO J.* **18,** 6823–6831.
3. Symmank, H., Saenger, W., and Bernhard, F. (1999) Analysis of engineered multifunctional peptide synthetases. Enzymatic characterization of surfactin synthetase domains in hybrid bimodular systems. *J. Biol. Chem.* **274,** 21581–21588.
4. Weinreb, P. H., Quadri, L. E., Walsh, C. T., and Zuber, P. (1998) Stoichiometry and specificity of in vitro phosphopantetheinylation and aminoacylation of the valine-activating module of surfactin synthetase. *Biochemistry* **37,** 1575–1584.
5. Kracht, M., Rokos, H., Ozel, M., Kowall, M., Pauli, G., and Vater, J. (1999) Antiviral and hemolytic activities of surfactin isoforms and their methyl ester derivatives. *J. Antibiot.* **52,** 613–619.
6. Zhu, X., Munoz, N. M., Kim, K. P., Sano, H., Cho, W., and Leff, A. R. (1999) Cytosolic phospholipase A2 activation is essential for beta 1 and beta 2 integrin-dependent adhesion of human eosinophils. *J. Immunol.* **163,** 3423–3429.
7. Kim, K., Jung, S. Y., Lee, D. K., Jung, J. K., Park, J. K., Kim, D. K., and Lee, C. H. (1998) Suppression of inflammatory responses by surfactin, a selective inhibitor of platelet cytosolic phospholipase A2. *Biochem. Pharmacol.* **55,** 975–985.
8. Morikawa, M., Hirata, Y., and Imanaka, T. (2000) A study on the structure-function relationship of lipopeptide biosurfactants. *Biochim. Biophys. Acta* **1488,** 211–218.
9. Grau, A., Gomez Fernandez, J. C., Peypoux, F., and Ortiz, A. (1999) A study on the interactions of surfactin with phospholipid vesicles. *Biochim. Biophys. Acta* **1418,** 307–319.
10. Deleu, M., Paquot, M., Jacques, P., Thonart, P., Adriaensen, Y., and Dufrene, Y. F. (1999) Nanometer scale organization of mixed Surfactin/Phosphatidylcholine monolayers. *Biophys. J.* **77,** 2304–2310.
11. Yakimov, M. M., Giuliano, L., Timmis, K. N., and Golyshin, P. N. (2000) Recombinant acylheptapeptide lichenysin: high level of production by *Bacillus subtilis* cells. *J. Mol. Microbiol. Biotechnol.* **2,** 217–224.
12. Golyshin, P. M., Fredrickson, H. L., Giuliano, L., Rothmel, R., Timmis, K. N., and Yakimov, M. M. (1999) Effect of novel biosurfactants on biodegradation of polychlorinated biphenyls by pure and mixed bacterial cultures. *New Microbiol.* **22,** 257–267.
13. Osman, M., Hoiland, H., Holmsen, H., and Ishigami, Y. (1998) Tuning micelles of a bioactive heptapeptide biosurfactant via extrinsically induced conformational transition of surfactin assembly. *J. Pept. Sci.* **4,** 449–58.
14. Vollenbroich, D., Pauli, G., Ozel, M., and Vater, J. (1997) Antimycoplasma properties and application in cell culture of surfactin, a lipopeptide antibiotic from *Bacillus subtilis*. *Appl. Environ. Microbiol.* **63,** 44–49.
15. Bortolato, M., Besson, F., and Roux, B. (1997) Inhibition of alkaline phosphatase by surfactin, a natural chelating lipopeptide from *Bacillus subtilis*. *Biotechnol. Lett.* **19,** 433–435.
16. Hosono, K., and Suzuki, H. (1983) Acylpeptides, the inhibitors of cyclic adenosine 3′, 5′-monophosphate phosphodiesterase. III. Inhibition of cyclic AMP phosphodiesterase. *J. Antibiot.* **36,** 679–683.
17. Thimon, L., Peypoux, F., Das, B. C., Wallach, J., and Michel, G. (1994) Selective esterification of surfactin: preparation and properties of surfactin methylesters. *Biotechnol. Appl. Biochem.* **20,** 415–423.
18. Bonmatin, J. M., Genest, M., Labbé, H., and Ptak, M. (1994) Solution three-dimensional structure of surfactin: a cyclic lipopeptide studied by ^{1}H-NMR, distance geometry, and molecular dynamics. *Biopolymers* **34,** 975–986.
19. Ferré, G., Besson, F., and Buchet, R. (1997) Conformational studies of the cyclic L,D-lipopeptide surfactin by Fourier Transform Infrared Spectroscopy. *Spectrochim. Acta Part A* **53,** 623–635.
20. Krimm, S., and Bandekar, J. (1986) Vibrational spectroscopy and conformation of peptides, polypeptides and proteins. *Adv. Protein Chem.* **38,** 181–364.
21. Susi, H., and Byler, M. (1987) Fourier transform infrared study of proteins with parallel β-chains. *Arch. Biochem. Biophys.* **258,** 465–469.
22. Perczel, A., and Hollósi, M. (1996) Circular Dichroism and the Conformational Analysis of Biomolecules (Fasman, G. D., Ed.), pp. 285, Plenum Press, New York.
23. Likó, Z., Botyánszki, J., Bódi, J., Vass, E., Majer, Z., Hollósi, M., and Süli-Vargha, H. (1996) Effect of Ca^{2+} on the secondary structure of linear and cyclic collagen sequence analogs. *Biochem. Biophys. Res. Commun.* **227,** 351–359
24. Laczkó, I., Hollósi, M., Vass, E., Hegedüs, Z., Monostori, E., and Tóth, G. K. (1998) Conformational effect of phosphorylation on T cell receptor/CD3 zeta-chain sequences. *Biochem. Biophys. Res. Commun.* **242,** 474–479.
25. Savitzky, A., and Golay, M. J. E. (1964) Smoothing and differentiation of data by simplified least squares procedures. *Anal. Chem.* **36,** 1627–1639.
26. Goormaghtigh, E., Cabiaux, V., and Ruysschaert, J. M. (1994) Subcellular Biochemistry (Harris, J. R., Ed.), Vol. 23, pp. 329, Plenum Press, New York.
27. Jackson, M, and Mantsch, H. H. (1995) The use and misuse of FTIR spectroscopy in the determination of protein structure. *Crit. Rev. Biochem. Mol. Biol.* **30,** 95–120.