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Aggregation properties of mycolic acid molecules in monolayer films: a comparative study of compounds from various acid-fast bacterial species

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Abstract

Three kinds of mycolic acids (MAs) (α-, keto and methoxy-MAs) extracted from several species of mycobacteria were used to prepare monolayer films on water, and the surface pressure–area (π–A) isotherms of the monolayers have been compared, so that the monolayer characteristics of the MAs as in cell walls would be revealed, since the monolayer molecular aggregation is related to drug permeability via the molecular packing. It was expected that the limiting molecular areas of the isotherms would be changed only a little, which reflects the minor difference in chemical structure and conformation of the mycobacteria. Nevertheless, the results are largely different from the expectation, and two greatly different patterns of the limiting molecular area have been observed. In a new model for elucidation of the results, two parts in an MA molecule are separately considered, and both contributions to the molecular unfolding by the monolayer compression have been suggested. This model is found to be useful to totally understand the isotherm behaviors of MAs. The relationship between monolayer properties and chemical structures for MAs has been summarized for the first time.

Keywords: Mycolic acid; Molecular aggregation property; Chain folding; Molecular conformation

1. Introduction

For many years, tuberculosis has been a leading dread among infectious diseases. Several effective medicines were developed so that the biosynthesis of cell wall would be inhibited, and the number of patients gradually decreased by about 5.3% a year from 1953 in USA [1]. This success made people believe that tuberculosis was a disease of the past. Nevertheless, in turn, the number of patience suddenly began to increase from 1985, and the increase rate of the reported case was about 20% higher than the expected roadmap [1]. This is because multidrug resistance bacteria turned out, and they quickly spread over HIV patients who have very weak physical strength [2].

To chemically target the infectious disease, structures and physicochemical properties of the cell envelope of *Mycobacterium tuberculosis* (*M. tb*) have been considered to be important keys for the development of a new anti-disease drugs. There have been proposed, in fact, various strategies to inhibit the duplications of the bacteria. The most historically famous drug, streptomycin, which is one of the aminoglycoside compounds, works to disrupt the synthesis of bacterial protein [3]. Sulfonamides are known to block the production of DNA via inhibition of biosynthesis of tetrahydrofolic acid. Cell-wall targeting is also an important strategy, and cycloserine is effective in this approach to inhibit incorporation of D-alanine into peptidoglycan precursor. On the other hand, isoniazid has another approach that it inhibits synthesis of 'mycolic acids' (MAs). Since MA is an acid-fast bacteria-specific compound that is one of the important constituents of cell envelope, this inhibition directly disturbs the duplication of the cells. In any case, the access of drug compounds to the cell is a key factor to consider the therapy of tuberculosis.

The cell envelope of *M. tb* has roughly ascribed that it has a model structure like a stratified layers that consists of plasma membrane, peptidoglycan, arabinogalactan and the most outer layer. The outer layer contains MAs, some of which are covalently bonded to trehalose that is further bonded to the arabigalactan layer. The peptidoglycan layer is bridged to the arabigalactan layer by some proteins [4–6]. In this fashion, the cell envelope of *M. tb* has a thick

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and dense structure to protect the cell; it is considered very few paths by lipoarabinomannan and some large protein with pore inside work as canals to take nutrition from outside the cell. Therefore, the access of drug molecules into the cell would generally be very difficult.

Among the high-density layers, the role of the mycobacteria-specific compound, MA, is particularly not known and its physical property is also unclear in many aspects. The general molecular structure of MA is schematically presented in Chart 1. The repeat numbers of *l*, *m*, *n*, and *k* are variable, which depend on bacterial species, but the total number of $l+m+n$ is known to range from 42 to 51 [8,10], while *k* takes only two values as 21 and 23 [7]. Therefore, an MA molecule has a largely asymmetric structure about the hydrophilic head group (C–C with COOH and OH). The long-chain part is sometimes called 'mero' group [8] (Chart 1). The mero group has two alternative groups represented by X and Y in Chart 1. Both X and Y have a cis-cyclopropyl group for the most hydrophobic MA, α-MA. Depending on species and strains, however, X in MA can be replaced by a C=C bond that has both trans and cis conformations. For other species and strains of mycobacteria, the X part can be changed to be keto, methoxy and others. In this manner, the mero group varies greatly depending on bacterial species and strains, which is highly complicated.

In this fashion, the molecular property of α-MA in terms of surface chemistry has been studied in relation to molecular fine structure and conformations. Nonetheless, comparative study of different strains of MAs has never been reported in a similar interest. In the present study, precious MAs extracted from several strains of mycobacteria have been readily obtained, and a comparative study of measurements of π–*A* isotherms has been performed. As a result, it has been found that limiting molecular areas of the isotherms were categorized into major two patterns, which correspond to the double- and triple-chain molecules. The difference in the unfolding of the mero group has been discussed by introducing a new model of unfolding mechanism.

In the present paper, the experimental results are discussed, as if the analytes were pure compounds, while each of the analyte is a mixture of homologues that arose from living cells. Of note, however, is that the aim of this paper is to present new findings of monolayer properties of different mycolic-acid species. Therefore, the discussion will be made within elucidations of the major characteristics of the monolayers.

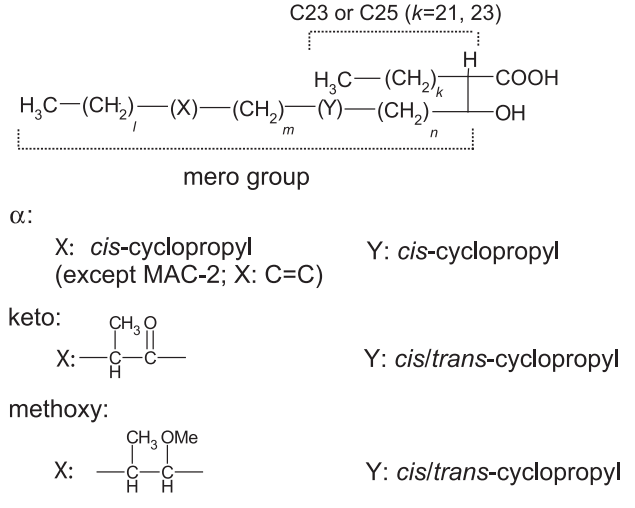

Chart 1. General molecular structure of MA.

2. Materials and methods

2.1. Materials

The MAs used in the present study were kindly provided by Dr. Motoko Watanabe, who was a faculty member of Tokyo College of Pharmacy, with analytical data (MS and NMR). Each methyl ester of MA was prepared from defatted freeze-dried cell mass by alkaline hydrolysis followed by methylation, which was an established method [9]. The details of the source of the cell mass were mentioned elsewhere [10]. Silica gel column chromatography and preparative thin layer chromatography on Merck 5744 plates with diethyl ether/hexane (6:100 v/v) were used for purification. The methyl esters of the MAs were hydrolysed with KOH in 2-propanol as described [10], and the resulting acids were subjected to silica gel column chromatography with hexane–ethyl acetate (5:1 v/v) to remove the epimer produced during the hydrolysis and to prepare MA specimen.

Since each sample was extracted from natural cells, the sample was obtained as a mixture of a few similar homologues. Even when chemical composition was common for the components, the chain length of the mero group varied reflecting homologues. Therefore, the molecular weight was calculated as an average value of the components by considering the ratio of the components. The molecular weight of each component and the component ratio were verified by a Perspective Biosystems Voyager Rs, MALDI/TOF-MS (matrix: 2,5-dihydroxybenzoic acid) spectrometer, and it is presented in each figure. The source species of mycobacteria used for the sample preparation were *M. tb*, *M. tb* Canetti, *M. bovis BCG* Tokyo, *M. avium intracellulare* complex (MAC), and *M. kansasii*. The strain of each species is *M. tb* K, *M. tb* Canetti MNC1485, *M. bovis* BCG Tokyo172, MAC MNC10 and *M. kansasii* 10-01, respectively, and details of all the strains and culture conditions are described in the papers by Watanabe et al. [8,10]. In this series, the last two compounds belong to the atypical mycobacteria. For the details of the strains, the reader is referred to literature [7]. In the present study, two kinds of α-MAs were available for MAC. One of them has the same mero group as other α-MAs, but the other one has a different mero group, in which the cyclopropyl group at

the position X (Chart 1) is substituted by the C=C bond with a ratio of 4:1 of trans and cis conformers. The former and latter MAC-related MAs are named MAC-1 and MAC-2, respectively, in this paper.

Solvent for the isotherm measurements was chloroform that was a spectra-grade reagent purchased from Dojindo (Kumamoto, Japan). Concentration of the solutions was in the range of 0.95 to 1.30 mg ml^{-1}, and the experimental error of each concentration was evaluated to be within ± 2%.

2.2. Surface pressure–area (π–A) measurements

All π–A isotherm measurements were performed on a Kyowa Interface Science (Saitama, Japan) HBM LB apparatus. The initial surface area of spread monolayer was 900 cm^2, and the compression rate by a Teflon-coated moving barrier was 14.0 cm^2 min^{-1} that corresponds to ca. 4.8 $Å^2$ min^{-1} $molecule^{-1}$. The pure water used for the experiments was generated by a Millipore (Molsheim, France) Milli-Q Labo water purifier equipped with a microfilter (Millipak-40) that has micropores of 0.22 μm to remove unexpected organic contaminants. The resistivity of the pure water was 18.3 MΩ·cm, and the surface tension was 72.8 mN m^{-1} at 25 °C. The temperature of the water was fixed at 25 °C, and pH was about 6.2 (no buffer).

For the first trace of the isotherm, the hysteresis (cyclic isotherm) was measured for each analyte. Every analyte exhibited no hysteresis below the collapse surface-pressure, which suggested a stable monolayer formation on water. After the hysteresis check, the isotherms were measured to obtain limiting molecular areas as presented in this paper.

3. Results and discussion

3.1. α-MAs

Monolayer properties of five α-MAs arising from four strains were investigated by measuring π–A isotherms. Fig. 1

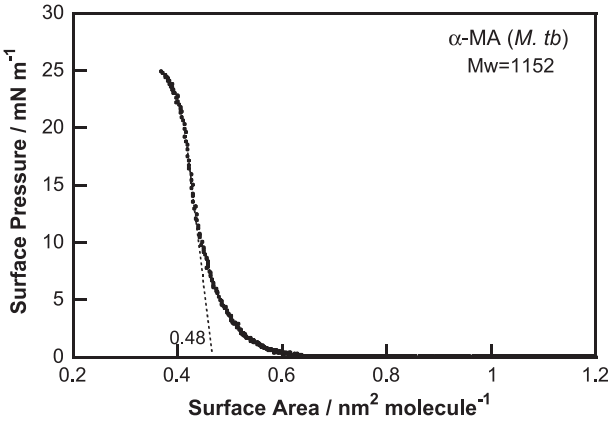

Fig. 1. Surface pressure–area isotherm of α-MA (*M. tb*) monolayer on pure water measured at 25 °C.

Table 1
Analytical results of π–A isotherms for five α-MA monolayers

Origins	Lift-off molecular area ($nm^2 molecule^{-1}$)	Collapse surface pressure (mN m^{-1})	Limiting molecular area ($nm^2 molecule^{-1}$)
M. tb	0.63	20	0.48
M. tb Canetti	0.60	40	0.47
M. bovis BCG Tokyo	0.65	30	0.47
MAC-1	0.68	25	0.51
MAC-2	0.90	15	0.57

presents a π–A isotherm of a monolayer of α-MA extracted from *M. tb*. The pressure begins to go up at around the surface area of 0.63 nm^2 $molecule^{-1}$ (lift-off area) during the monolayer compression, and the collapse surface pressure is found at around 20 mN m^{-1}, which is not so high for a monolayer of double-chain molecule [11,12]. The limiting molecular area is obtained to be 0.48 nm^2 $molecule^{-1}$ (Table 1), which suggests that the molecules are fully extended by the lateral compression, so that the cross section corresponding to a double-chain molecule is obtained. Since the decompression and recompression of the monolayer below the collapsed pressure gave almost an identical isotherm to the first run, it was suggested that the monolayer was not collapsed, and a single monolayer was readily prepared on water. Therefore, the limiting molecular area can be used as the cross-section area of a single molecule in the monolayer.

The same measurements were performed for α-MA derived from *M. tb* Canetti, and *M. bovis BCG* Tokyo species (isotherms not shown). The lift-off and limiting molecular areas of the two compounds are summarized in Table 1. The only meaningful difference is that the collapse surface pressure of the monolayer for *M. tb* is lower than other compounds. This suggests that the stiffness of the monolayers of α-MA varies considerably [13], although the chemical structure of the α-MA itself is believed to be essentially common irrespective of strains. All the results above are for MAs that have a short-chain part of C25.

The same analysis of α-MA monolayer of MAC-1, however, gave an apparently different result as shown in Fig. 2 and Table 1. This α-MA is characterized by the short-chain part that has C23 in length. Since other MAs used above have a C25 chain in the short-chain part, the difference of the isotherms should be considered carefully. The lift-off point is a little larger than the previous compounds, and the isotherm exhibits a small plateau region at about 4 mN m^{-1} after a short linear part. After the plateau, a clear linear part appears, which corresponds to the solid state of the monolayer, and it collapses at about 27 mN m^{-1}. A difference of this monolayer as referred to the previous compounds is that the limiting molecular area is obtained at 0.51 nm^2 $molecule^{-1}$, which is notably larger than the previous results that are about 0.47 nm^2 $molecule^{-1}$. A good reproducibility (within ± 2%) of the

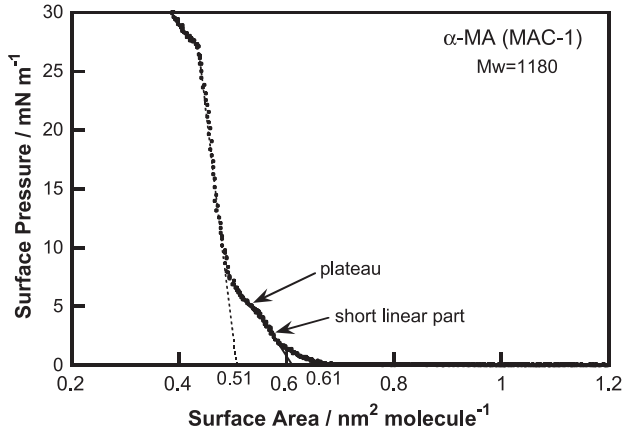

Fig. 2. Surface pressure–area isotherm of α-MA (MAC-1) monolayer on pure water measured at 25 °C.

experiments confirmed this value. Since the α-MA from MAC-1 has the same mero group, this difference should be attributed to the influence of the short-chain part (C23). The reader would be skeptical to the discussion of the chain length, since the present experiments were not performed with pure compounds, but with homologues. As described in the previous paper, however, C25 has an extraordinary strong aggregation property, which corresponds to the stiff-domain formation in the monolayer. This means that the monolayer is not homogeneous, and the limiting molecular area can be evaluated by linear summation of each domain area. Therefore, the present discussion would be no problem.

One of the important characteristics of α-MA is that the molecule is most hydrophobic [7,9] in comparison to other types of MAs (keto, methoxy, etc.) that have other functional groups than the cyclopropyl group in the mero group. If the mero group had a hydrophilic group that has a large dipole, the group would cause 'molecular friction' when the lateral pressure unfolds the mero group, since the strong inter-molecular force between the large dipole would make the extension difficult. It is considered, along this interpretation, that the hydrophobic mero group of the α-MA monolayers has small molecular friction, which would make the extension of the mero group easy. This molecular friction model is useful as found for keto-MAs as mentioned later. Nevertheless, it is impossible to understand the larger limiting area of MAC-1 than those of *M. tb* with the molecular friction model only.

In our previous study, a fatty acid of C25 was found to form extraordinary stiff monolayer domains on water as if the molecules were metal salts, and it showed an extraordinarily strong molecular aggregation property [14]. The shorter fatty acids (C22–C24) were, on the other hand, found to form ordinary monolayers that had characteristics of free acids, and they exhibited a significantly large liquid-expanded region in the isotherms. It was concluded that the C25 fatty acid monolayer has much higher molecular interaction property than that of shorter molecules.

Therefore, the slight difference in chain length of C23 and C25 for the MAs should be critical to the property of monolayer formation on water. This chain-length effect would also play an important role in MAs when they are spread on water, since C25 in the short-chain part would particularly be interacted with the mero group in the molecule. In other words, the chain folding or extension of the mero group would be governed by the intramolecular interaction through the chain-length effect.

When the mero group is extended to be a single chain, the linear part of the mero group would be stabilized by the effective interaction with the short-chain part, which is schematically presented in Fig. 3. The C25 hydrocarbon chain would positively be interacted with the mero group to make the mero group be extended. In other words, the MAs of the *M. tb* and *M. bovis BCG* series would be extended easier than the MAs of atypical acid-fast bacteria (MAC, *M. kansaii*, etc.) that have the C23 chain.

Now, we are ready to understand why α-MA of MAC-1 exhibits a different isotherm pattern as presented in Fig. 2. The limiting molecular area roughly corresponds to three times the cross-section area of a simple single hydrocarbon chain (ca. 0.2 nm^2 $molecule^{-1}$) [14], which suggests that the α-MA is of the doubly folded mero group and the C23 hydrocarbon chain (three hydrocarbon chains in total) at a low surface pressure. When the surface pressure goes up above 5 mN m^{-1}, the mero group is extended by the high

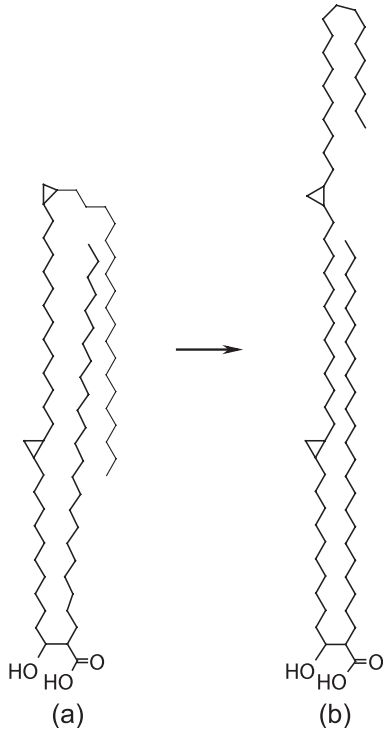

Fig. 3. Scheme of the chain unfolding of α-MA by lateral compression. (a) Triple-chain molecule, in which the short-chain part is partly interacted with the mero group. (b) The mero group is extending by the pressure, and the short-chain part is not readily interacted with the linear part of the mero group.

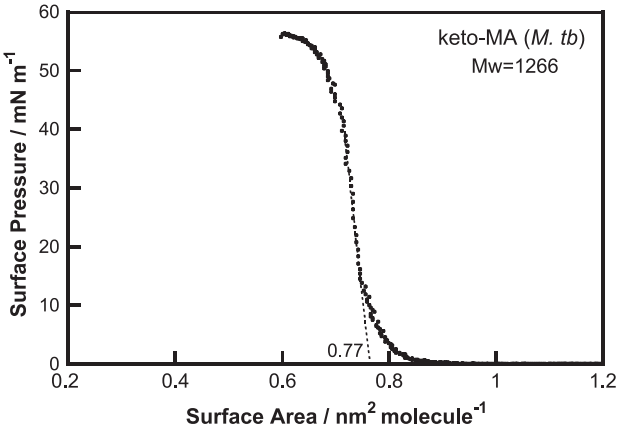

Fig. 4. Surface pressure–area isotherm of keto-MA (*M. tb*) monolayer on pure water measured at 25 °C.

pressure. In this fashion, the α-MA of 'MAC-1' exhibits two steps about the plateau region for the film compression, and the chain extension occurred later than the MAs of *M. tb* and *M. bovis* series. This is due to the molecular aggregation property of the C25 and C23 hydrocarbon chains. As for MAC-1, the short-chain part (C23) in the α-MA is not enough to help the mero group be extended by a low surface pressure, and the triple-chain molecule is compressed as it is, which corresponds to the short linear part of below the plateau (Fig. 2). When the surface pressure above 5 mN m^{-1} is applied to the monolayer, however, the mero group begins to be unfolded because of the 'low molecular friction'. It is of note that the limiting molecular area of the isotherm is apparently larger than the MAs of *M. tb* and *M. bovis BCG* (Table 1). This suggests that all the folded mero groups are not fully extended even at a high surface pressure, and some portion of the mero group may remain folded in the monolayer, which makes the average molecular cross-section area larger.

Another α-MA from MAC (MAC-2), which has a C=C bond at the X position in Chart 1 instead of the cis cyclopropyl group, was also investigated by π–A isotherm measurements (Table 1). This α-MA contains a lot of trans C=C bonds of 80%, and it is expected that this compound would give a larger cross section than other compounds due to the disorder of the mero group caused by this trans conformation. As expected, the lift-off area is found at about 0.90 nm^2 $molecule^{-1}$, which is much larger than others, and the limiting molecular area is 0.57 nm^2 $molecule^{-1}$, which is also significantly larger than others. This cross section, however, still suggests that the α-MA has a double-chain form like other α-MAs, since the area is apparently smaller than the triple-chain form that would give at least 0.6 nm^2 $molecule^{-1}$ [9]. Therefore, the expansion may be due to the disorder by the trans C=C bonds. The evaluation of the enlargement of limiting molecular area by use of the trans/cis ratio is, however, difficult because the influence by the substituted group on the molecular conformational change is not easy to evaluate. A problem that remained here is that the increase of the limiting molecular area for the MA of MAC-1 is difficult to explain. MAC-1 has a common mero group with other α-MAs, but the short-chain part is slightly shorter.

3.2. Keto-MAs

A π–A isotherm of a keto-MA arisen from *M. tb* was measured, which is presented in Fig. 4. Although the α-MA for Fig. 1 and this keto-MA were extracted from the identical strain, Fig. 4 exhibits a largely different isotherm from Fig. 1. The limiting molecular area (0.77 nm^2 $molecule^{-1}$; Table 2) is significantly larger than those of the α-MA (near 0.5 nm^2 $molecule^{-1}$). The collapse surface pressure reaches 40 mN m^{-1}, which suggests that this monolayer is more stable than that of the α-MA. The shape of the isotherms, however, exhibits a common shape for both α- and keto-MAs and no plateau region appears.

The same analysis was performed for another keto-MA derived from *M. tb* Canetti as done for α-MA (Table 2). The limiting molecular area of the monolayer of this compound is also fairly large (0.80 nm^2 $molecule^{-1}$), which is comparable to the previous result in Fig. 4. As found in Tables 1 and 2, the α-MA taken from the same strain (*M. tb* Canetti) exhibited a largely compressed property for the monolayer (0.47 nm^2 $molecule^{-1}$). Therefore, the large limiting molecular area of the keto-MA strongly suggests that the substituted chemical group (keto) in the mero group in MAs largely influences the final molecular conformation after the compression. For the monolayers of α-MAs, the variance of limiting molecular areas ranged from 0.47 to 0.57 nm^2 $molecule^{-1}$, which corresponds to the scale of cross-section area of double-chain molecules like dipalmitoylphosphatidyl choline (DPPC; 0.52 nm^2 $molecule^{-1}$) [15]. On the other hand, the limiting molecular area of the keto-MAs is apparently beyond this range, which could not be explained by the cross section of a double-chain molecules model. Therefore, it is suggested that MAs having keto group have triple-chain forms, which is possibly caused by molecular folding of the mero group as reported in our previous paper [9]. In the folded molecule, the mero group is doubly folded, which would structurally be supported by the short-chain part through the hydrophobic interaction.

Table 2
Analytical results of π–A isotherms for five keto-MA monolayers

Origins	Lift-off molecular area ($nm^2molecule^{-1}$)	Collapse surface pressure (mN m^{-1})	Limiting molecular area ($nm^2molecule^{-1}$)
M. tb	0.90	40	0.77
M. tb Canetti	1.2	25	0.80
M. bovis BCG Tokyo	1.0	25	0.75
MAC-1	0.95	40	0.74
M. kansasii	0.90	40	0.77

The isotherm measurements were also performed for monolayers of keto-MAs (*M. bovis BCG* Tokyo, MAC-1, and *M. Kansasii* species) as presented in Table 2. Since MAC-2 has no keto-MA component, only MAC-1 was studied. Similar isotherms were yielded from the three monolayers, and the limiting molecular areas are also similar to each other, which are found at ca. 0.75 nm^2 $molecule^{-1}$. These limiting molecular areas are comparable to the previous results for *M. tb* and *M. tb* Canetti strains, which supports the triple-chain model for keto-MA molecules in a monolayer. In this manner, it has been found that MA monolayers exhibit two types of limiting molecular areas that correspond to double- and triple-chain molecules.

Keto group has a large dipole due to the large electron negativity of the oxygen atom in the C=O bond. If the model of molecular unfolding mechanism discussed earlier were correct, keto-MAs would be difficult to extend by lateral monolayer compression because of the large molecular friction raised by the large dipole effect. The large limiting areas obtained for all the keto-MAs in this study are consistent with expectations by the molecular model.

In this manner, for keto-MAs, the molecular friction model due to the keto group is considered to govern the molecular unfolding property, and contribution of the short-chain part is negligibly small. The large molecular limiting areas correspond to triple hydrocarbon chains. Although both MAC and *M. kansasii* species have a C23 hydrocarbon chain for the short-chain part, the limiting molecular areas are relatively near to other MAs. The minor differences in the limiting molecular area are difficult to explain at present. A minor impurity may probably play an unexpected role to give the slightly varying results, since the compounds are not synthesized, but chemically extracted from living cells.

3.3. Methoxy-MAs

It has been found that α- and keto-MA monolayers give different range of the limiting molecular area, and this tendency is commonly found for various species and strains. The next interest was, therefore, what limiting molecular area would be yielded from another type of MA monolayer. For this purpose, methoxy-MA was used for the monolayer analyses, since the methoxy MA is also one of the major chemical compounds found in the cell envelope of acid-fast bacteria.

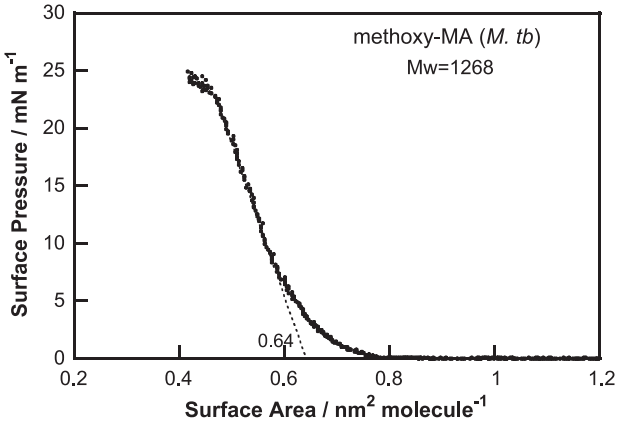

Fig. 5. Surface pressure–area isotherm of methoxy-MA (*M. tb* Canetti) monolayer on pure water measured at 25 °C.

Table 3
Analytical results of π–A isotherms for two methoxy-MA monolayers

Origins	Lift-off molecular area ($nm^2 molecule^{-1}$)	Collapse surface pressure ($mN\ m^{-1}$)	Limiting molecular area ($nm^2 molecule^{-1}$)
M. tb	0.80	20	0.64
M. tb Canetti	0.75	17	0.71

First, a monolayer of methoxy-MA taken from *M. tb* is presented in Fig. 5. This isotherm exhibits a lift-off area at about 0.8 nm^2 $molecule^{-1}$ followed by a linear part that seems to correspond to the liquid-expanded state of the monolayer. The limiting molecular area obtained from this isotherm is 0.64 nm^2 $molecule^{-1}$, which is an intermediate value between the areas of α- and keto-MA monolayers (Tables 1 and 2). A similar result was obtained for a methoxy-MA from *M. tb* (Table 3). The limiting molecular area for *M. tb* was 0.71 nm^2 $molecule^{-1}$. Since the compounds of *M. tb* Canetti and *M. tb* have been considered to have almost identical chemical structure, it is difficult to understand the difference in limiting molecular areas. Regardless, it should be noticed that both limiting molecular areas fall between the areas of α and keto compounds.

Before closing this discussion, it should be noted that the isotherms of the monolayers of α-MA exhibited a relatively larger liquid-expanded region before attaining the solid-state region than keto- and methoxy-MAs. Although the liquid-expanded region of a α-MA does not show a plateau, this region is considered to be a region in which molecular conformational change happens during the compression, since this region is much longer than that found for keto- and methoxy-MAs. On the other hand, the linear part of the isotherms for α-MAs is considered to correspond to the solid-state region, which makes the hydrocarbon chains fully extended to give the double-chain cross section. This conformational change is under investigation by infrared spectroscopy, which will be presented elsewhere.

4. Conclusion

A model of molecular unfolding mechanism has been proposed, so that the discrimination of the two cases would be possible. At a low surface pressure, the mero group in all MAs is folded. When an external surface pressure is applied to the film, the unfolding process is influenced by two major factors. One of them depends on a substituted group at the

position X in the mero group. The molecular unfolding of keto-MA would be effectively inhibited by the strong molecular interactive force. This force was represented as a molecular friction in this paper.

The other factor is the length effect of the short-chain part in the MA. The slight difference in length (C23 and C25) is considered to largely influence the molecular aggregation property, and this effect would also influence the stabilization of the unfolded mero group.

In this manner, the balance of the two strongly correlated physical parameters would govern the molecular conformation of MA under high surface pressure, which would be useful to understand the regulation mechanism of the drug permeability of the cell envelope.

Acknowledgements

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