Natural Poly(L-malic acid): NMR Shows a Poly(3-hydroxy acid)-Type Structure

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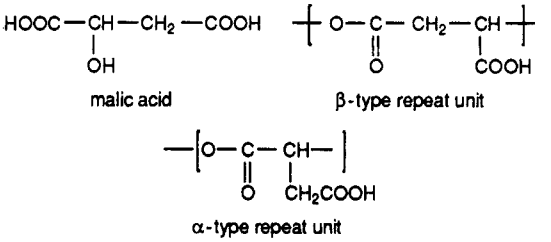
ABSTRACT: Poly(L-malic acid) was recently isolated for the first time from *Physarum polycephalum*, a myxomycete for which it plays the role of a coordinator during DNA replication. To determine the chain structure of this new natural polymer, its high-resolution ^1H and ^{13}C NMR spectra were compared with those of other poly(malic acids), namely, synthetic poly(β-L-malic acid) and poly(α-L-malic acid-*co*-β-L-malic acid). Both synthetic and natural poly(malic acids) as well as their mixture displayed similar ^1H and ^{13}C NMR spectra. In contrast, poly(α-L-malic acid-*co*-β-L-malic acid) oligomers exhibited different NMR characteristics. These findings confirmed that natural poly(L-malic acid) belongs to the family of poly(3-hydroxy acid)-type aliphatic polyesters. It was further deduced from resonance stereosensitivity that the stereoregularity of natural poly(β-L-malic acid) is very high.

Introduction

Interest in polymers made by microorganisms has been growing during the past decade, especially in the case of microbial polymers. Temporary therapeutic applications, biodegradable packaging materials, and investigation of the interactions between artificial polymeric materials and living species are three areas where such polymers appear as worthwhile compounds, because they can basically degrade up to complete assimilation by living organisms.[1] Moreover, understanding the strategies of production and digestion of microbial polymers by microorganisms in their natural environment is a critical step toward the discovery and the development of new living species respecting polymers and copolymers.

In the field of microbial polymers, attention has been primarily paid to poly(β-hydroxybutyrate) and related materials generally referred to as poly(β-hydroxyalkanoates).[2,3] The formation of natural aliphatic polyesters in procaryotic organisms is stimulated by unbalanced growth conditions, these polyesters subsequently serving as either energy or carbon storage products. The synthesis of an osmotically inert macromolecule also could have a survival function for the cell in the case of environmental stress conditions.[4] Recently, a new natural polyanion has been isolated from plasmodia extracts and from the culture medium of *Physarum polycephalum*.[5] This polyanion was identified as the neutralized form of a poly(L-malic acid) and referred to as nat-PMLA. A polymeric compound made of L-malic acid had already been detected in *Penicillium*.[6] The myxomycete *Physarum polycephalum*, a slime mold, grows normally on rotten material on the soil. It has been showed that nat-PMLA inhibits homologous DNA polymerase α of *Physarum polycephalum*.[5] The chemical structure of this polyanion was previously achieved by chemical group analysis, IR spectroscopy, and NAD reduction of the ultimate degradation product (acidic hydrolysis) in the stereospecific reaction catalyzed by malate dehydrogenase.[5] However, the type of ester linkage in nat-PMLA had not been determined conclusively. Indeed, alternative ester linkages of α- or β-types are possible in polymer chains derived from malic acid.



Synthetic poly(β-malic acid) has been known for more than a decade, and stereopolymers of L- and D-malic acid (syn-PMLA^{x}H$_{100}$ with $50 < x \leq 98$ according to enantiomeric composition and H$_{100}$ for the fully protonated form) were prepared from aspartic acid enantiomers[7] and malic acid enantiomers.[8] Both L-(*S*)-aspartic acid and L-(*S*)-malic acid yielded poly(β-L-malic acid), syn-PMLA^{95}H$_{100}$, which produced L-malic acid by *in vitro* degradation.[9-11] A poly(L-malic acid) polymer was also synthesized by Fujino et al.[12] by direct condensation of malic acid. ^1H NMR showed that α and β ester linkages were present in this polymer, which will be referred to as α,β-PMLA^{100}H$_{100}$ assuming ee = 100%.

In this paper, we report the results of a detailed investigation of high-resolution ^1H and ^{13}C NMR spectra aimed at ascertaining the poly(β-hydroxy acid) structure of the chain of natural poly(L-malic acid). To make the demonstration consistent, three different approaches were considered: (a) comparison between ^1H and ^{13}C NMR spectra of syn-PMLA, nat-PMLA, and their 50/50 w/w mixture, (b) complete assignment of the α,β-PMLA^{100}H$_{100}$ spectrum by using 2D ^1H NMR, and (c) study of a mixture

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of nat-PMLAH$_{100}$ and α,β-PMLA^{100}H$_{100}$ and comparison with previous spectra.

Experimental Section

Chemicals. Racemic PMLA^{50}H$_{100}$ and optically active syn-PMLA^{95}H$_{100}$ were prepared respectively from racemic aspartic acid and L-malic acid as described previously.[8-10]

Poly(α-L-malic acid-*co*-β-L-malic acid), α,β-PMLA^{100}H$_{100}$, was prepared by dehydrative polycondensation of 5 g of L-malic acid at 130 °C (7 h) under vacuum (2×10^{-2} mmHg). The resulting crude material was purified by solubilization in acetone and precipitation in diethyl ether to yield 0.7 g of a white powder. ^{1}H NMR (CD_3COCD_3): δ 3.00–3.11 (m, CH_2), 5.54–5.65 (m, CH). The molecular weight was 3000 as determined by size exclusion chromatography in dioxane (polystyrene standards).

Natural poly(L-malic acid), nat-PMLA, was prepared at the University of Regensburg by extraction from the cellular material and the culture supernatant of plasmodial *Physarum polycephalum* as previously described by Fischer et al.[5] The myxomycete is easily grown in its plasmodial life cycle stage ("microplasmodia") on laboratory and larger scales in axenic cultures, typically in amounts of 500 g per 500 mL of nutrient broth (2-L Erlenmeyer) on a rotary shaker in the dark. The cells are harvested after 3–5 days simply by passage through a cotton on nylon cloth as a sieve which retains the cells. The supernatant is used directly for the preparation of poly(L-malic acid). The cells are ruptured in a kitchen mixer in 20 mM citrate, pH 4.5, proteins are denaturated for 10 min at 50 °C and removed by centrifugation, and the supernatant is subjected to purification of nat-PMLA exactly as in the case of the culture medium. The product from the culture medium is of high purity, and that from cells may contain small amounts of proteins. Because of the instability of nat-PMLA, purification should be carried out in the cold at 4 °C and should not last for longer than a few days. Under optimal conditions of growth and harvest the culture medium contains 1 g of nat-PMLA in 2.5 L. Cells contain 1 mg of nat-PMLA/g. Thus, in a 2.5-L culture a total of $5 \times 500 \times 1$ mg + 1000 mg = 3500 mg of nat-PMLA is potentially available. Our purification may, at best, yield 20% of this amount, that is 700 mg of nat-PMLA from a 2.5-L culture.

NMR Spectra. All poly(malic acid) compounds were dissolved in CD_3COCD_3 (acid form) or D_2O (Na salt form). ^{1}H and ^{13}C NMR spectra were recorded at 305 K on Bruker WM-250 (250 MHz) or AM-400 (400 MHz) spectrometers using 5-mm NMR tubes. ^{1}H and ^{13}C chemical shifts are reported in ppm with tetramethylsilane as the internal reference. The 2D ^{1}H,^{1}H COSY NMR spectra were recorded at 400 MHz with sweep widths of 1400 Hz into 1024 data points in f_2.[13] The 90° pulse was 14 μs, the relaxation delay was 4 s, and 128 fids were recorded with eight scans and two dummy scans. Data were zero-filled to 512 points in f_1 prior to double Fourier transformation with unshifted sinebell window functions in both dimensions.

Results and Discussion

In a first attempt to compare nat-PMLA and syn-PMLA, the NMR spectra of nat-PMLA, syn-PMLA^{95}H$_{100}$, and syn-PMLA^{50}H$_{100}$ were recorded under similar conditions. The high enantiomeric excess of syn-PMLA^{95}H$_{100}$ was confirmed by comparison of the ^{13}C NMR spectra of this optically active polymer and of the corresponding racemic PMLA^{50}H$_{100}$, both in the H and Na^+ forms as shown in Table I. Line narrowing by the enhanced resolution technique and magnification of the field scale revealed the presence of fine structures for resonances corresponding to the main-chain carbon atoms or the pendant carboxylic carbon atoms.

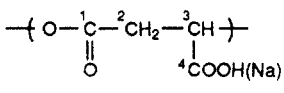

Table I. ^{13}C NMR: Carbonyl Carbon Atom Resonances of Poly(malic acid) Compounds

sample	chem shift (ppm) C_1	C_2	C_3	C_4
			69.54	
PMLA^{50}H$_{100}$[a]	169.30[b]	36.43[b]	69.44	170.00
	169.01[c]	36.32[c]	69.42	
			69.32[d]	
syn-PMLA^{95}H$_{100}$[a]	169.03[c]	36.31[c]	69.35[d]	170.00
nat-PMLAH$_{100}$[a]	169.07[c]	36.33[c]	69.28[d]	170.03
syn-PMLA^{95}H$_{100}$ + nat-PMLAH$_{100}$[a,e]	168.96[c]	36.31[c]	72.27[d]	170.01
PMLA^{50}Na$_{100}$[f]	171.93	36.30	72.13	175.77[c]
			71.87[c]	175.69[b]
syn-PMLA^{95}Na$_{100}$[f]	171.91	36.24	71.88[c]	175.79[c]
nat-PMLANa$_{100}$[f]	171.98	36.34	71.95[c]	175.78[c]

[a] CD_3COCD_3 solvent. [b] Racemic diads. [c] Meso diads. [d] Isotactic triads. [e] In a 1/1 molar ratio. [f] D_2O solvent.

Peaks corresponding to meso and racemic diads and isotactic triads have been assigned previously by comparison with data obtained from a series of stereocopolymers.[14] The methine carbon atom (C_3) of racemic poly(β-malic acid), PMLA^{50}H$_{100}$, displayed four peaks in acetone as expected from a typical triad effect. The same methine carbon atom in racemic poly(β-malic acid) in the salt form, PMLA^{50}Na$_{100}$ (water as solvent), displayed two peaks as expected from a typical diad effect.

Optically active synthetic PMLA in acetone (syn-PMLA^{95}H$_{100}$) and in water (syn-PMLA^{95}Na$_{100}$) showed only one C_3 resonance corresponding to isotactic diads. This finding agreed well with the absence of splitting for C_1 and C_2 (syn-PMLA^{95}H$_{100}$) and C_4 (syn-PMLA^{95}H$_{100}$), in contrast to what was previously observed for PMLA^{50}H$_{100}$ and PMLA^{50}Na$_{100}$. The ^{13}C NMR spectra of nat-PMLA also exhibited only one very fine peak for each of the four carbon atoms present in the monomer unit, in agreement with the very high enantiomeric excess found in the natural polymer macromolecular chain.[5] The four peaks were located at chemical shifts very close to those of syn-PMLA, suggesting similar structures. Similarity was confirmed by considering an equimolar mixture of syn-PMLAH$_{100}$ and nat-PMLAH$_{100}$, which showed perfect matching of all peaks.

High-resolution ^{1}H NMR spectra of the different poly(malic acids) were also recorded (Figure 1). syn-PMLA^{95}H$_{100}$ displayed a resolved spectrum due to the high enantiomeric excess (≈95%) of L-malic acid enantiomer in the macromolecular chain. C*H* and C*H*$_2$ resonances gave a well-defined ABX system (Figure 1a), contrary to those of racemic PMLA^{50}H$_{100}$.[7] In the case of nat-PMLAH$_{100}$, similar features were observed (Figure 1b). The higher optical purity of nat-PMLA agreed well with the better resolution of the ^{1}H NMR spectra as previously shown.[5] The 50/50 w/w syn-PMLA^{95}H$_{100}$/nat-PMLAH$_{100}$ mixture reflected no difference (Figure 1c), as the case in the ^{13}C NMR spectrum. One could presume that the α-enchainments should have resulted in at least different coupling parameters for the ABX system due to different spatial arrangements. This point was demonstrated by considering the NMR spectra of a poly(α-L-malic acid-*co*-β-L-malic acid), α,β-PMLA^{100}H$_{100}$, which was prepared by direct condensation of L-malic acid as described by Fujino.[12] The ^{1}H NMR spectrum at 250 MHz in acetone displayed two large peaks with lines at 5.65 and 5.54 ppm and lines at 3.01 and 3.11 ppm (Figure 2b). Partial assignment was made by mixing poly(α-L-malic acid-*co*-β-L-malic acid) and syn-PMLA^{95}H$_{100}$ in a 50/50 w/w ratio (Figure 2a). The corresponding ^{1}H NMR spectrum showed

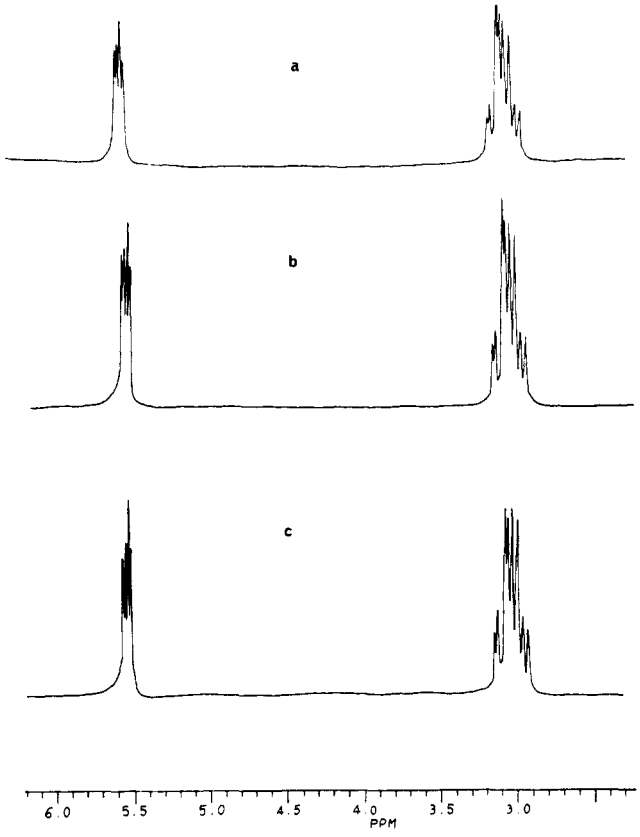

Figure 1. ^{1}H NMR spectra (250 MHz in deuterated acetone): (a) syn-PMLA^{95}H$_{100}$; (b) nat-PMLAH$_{100}$; (c) 50/50 w/w mixture of syn-PMLA^{95}H$_{100}$ and nat-PMLAH$_{100}$.

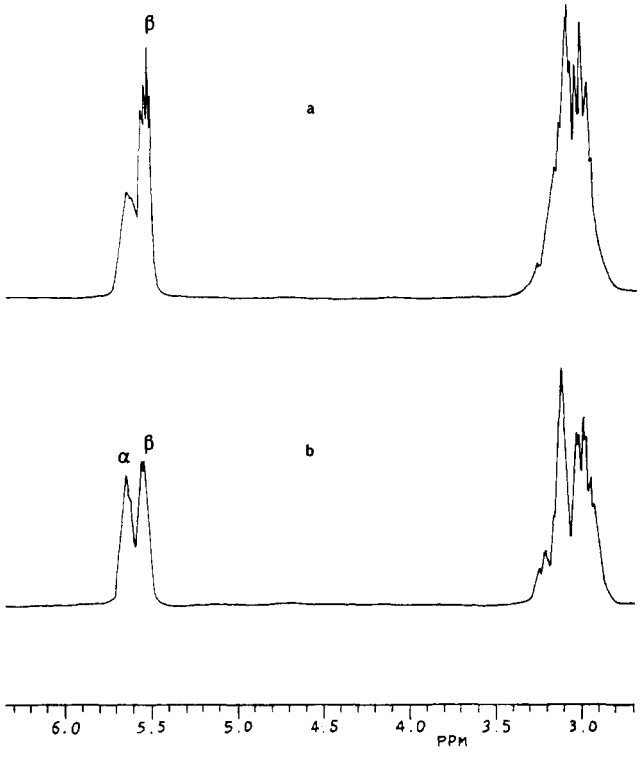

Figure 2. ^{1}H NMR spectra (250 MHz in deuterated acetone): (a) 50/50 w/w mixture of α,β-PMLA^{100}H$_{100}$ and syn-PMLA^{95}H$_{100}$; (b) α,β-PMLA^{100}H$_{100}$.

the superposition of C*H* resonances at 5.54 ppm for syn-PMLA^{95}H$_{100}$ and α,β-PMLA^{100}H$_{100}$ oligomers. These resonances were assigned to the β-structure. The peak at 5.65 ppm, which was not present in the spectrum of syn-PMLA^{95}H$_{100}$, was assigned to the α-structure. syn-PMLA^{95}H$_{100}$ C*H*$_2$ resonances and C*H*$_2$ peaks of α,β-

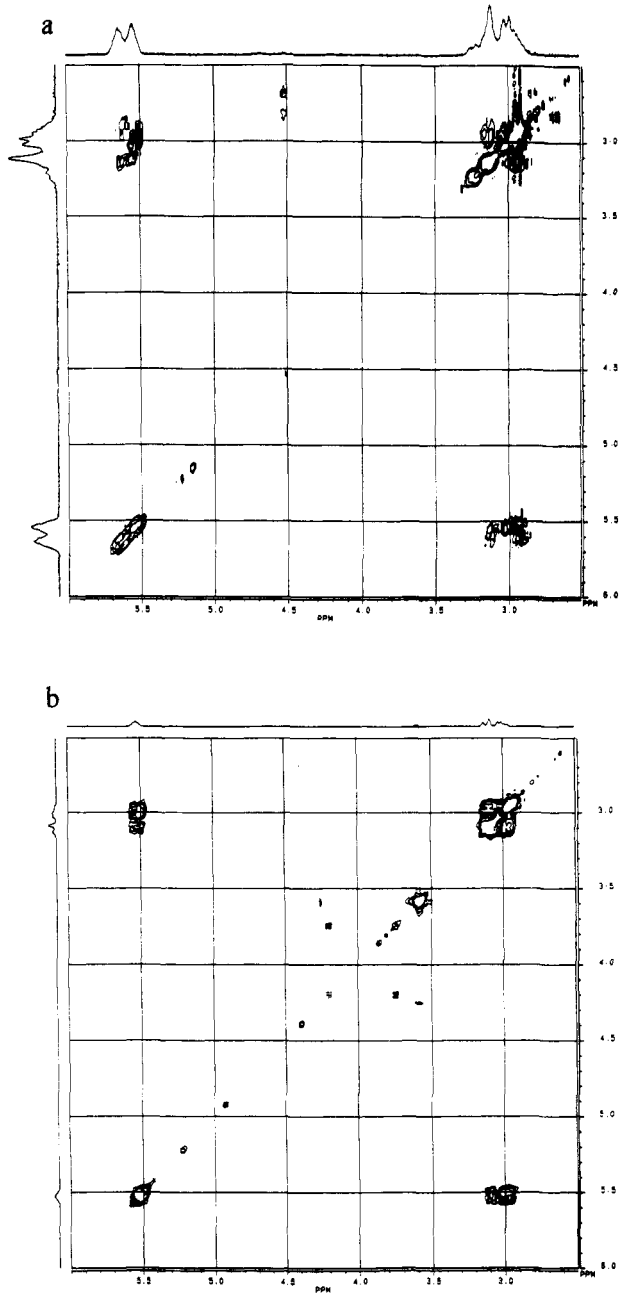

Figure 3. 2D ^{1}H NMR spectra (400 MHz in deuterated acetone): (a) α,β-PMLA^{100}H$_{100}$; (b) PMLA^{50}H$_{100}$.

PMLA^{100}H$_{100}$ overlapped but did not match. To complete the assignment of the α,β-PMLA^{100}H$_{100}$ spectrum and to assign resonances due to the CH$_2$ α-type structure, 2D COSY homonuclear shift-correlated NMR spectra of β-PMLA^{50}H$_{100}$ and α,β-PMLA^{100}H$_{100}$ were obtained and compared (Figure 3).

The 2D COSY spectrum of α,β-PMLA^{100}H$_{100}$ showed two superimposed ABX systems. As the CH signals (X part of the ABX system) present different chemical shifts, the chemical shifts of the A and B parts of the α- and β-type repeat units in α,β-PMLA^{100}H$_{100}$ could be obtained from the examination of the cross peaks corresponding to the CH signals, which appeared at *ca.* 2.90 and 3.15 ppm for the CH$_2$ of the α-structure and at *ca.* 3.00 and 3.10 ppm for the CH$_2$ of the β-type repeat units in α,β-PMLA^{100}H$_{100}$ (analogous to those obtained from β-PMLA^{50}H$_{100}$, Figure 3b). These results showed conclusively that the assignments deduced from the mixture of α,β-PMLA^{100}H$_{100}$ and syn-PMLA^{95}H$_{100}$ were correct.

Last, but not least, the mixture of nat-PMLAH$_{100}$ and α,β-PMLA^{100}H$_{100}$ (50/50 w/w) gave a ^{1}H NMR spectrum

which was in total coincidence with that of (syn-PMLA^{95}H$_{100}$ + α,β-PMLA^{100}H$_{100}$). Resonances of nat-PMLA coincided with the part of the α,β-PMLA^{100}H$_{100}$ spectrum assigned to β-type repeat units. From these three series of experiments, one can definitely conclude that natural poly(malic acid) is an aliphatic polyester of the poly(3-hydroxy acid) type.

This finding agreed well with the fact that syn-PMLA^{95}-Na$_{100}$ and nat-PMLANa$_{100}$ exhibited similar biological activity, namely, endogeneous inhibitions of DNA polymerase. *Physarum polycephalum* in a very potent and highly efficient manner. However, the stereoregularity does not seem to be critical as racemic PMLA^{50}H$_{100}$ had the same activity in *Physarum polycephalum*. It is worth noting that poly(β-L-malic acid) can be obtained from the biomass by three different routes, namely, chemical synthesis from aspartic acid and malic acid and biological synthesis by microorganisms such as *Penicillium* or *Physarum polycephalum*. However, the chemical route appears more versatile as it opens the way to a larger number of derivatives by changing enantiomeric composition and chiral center distribution within the polymer chain or by modifying the nature of the ester pendant groups via copolymerization and/or chemical modification of PMLA^{x}H$_{100}$.

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