2-Alkylmalonic Acid: Amphiphilic Chelator and a Potent Inhibitor of Metalloenzyme

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The present investigation demonstrates the potential of 2-alkylmalonic acid amphiphile as inhibitor of metalloenzymes like *Taq* DNA polymerase and α-amylase. A dose-dependent inhibition of *Taq* DNA polymerase was observed when a polymerase chain reaction (PCR) was performed in the presence of amphiphiles while in the case of α-amylase the inhibition was found to be independent of the inhibitor concentration. Control experiments revealed that both the chelating as well as the amphiphilic nature of the inhibitor was essential for enzyme inhibition. The fluorescence intensity and lifetime of α-amylase were also found to decrease in the presence of the amphiphiles. Steady-state fluorescence quenching studies suggested that removal of the metal ion from the enzyme leads to a decrease in the solvent accessibility of tryptophans, indicating change in the tertiary structure of the protein. It is proposed that removal of metal ion from the active sites of the enzyme by the amphiphilic compound possibly leads to disruption of the native conformation of the enzyme which is responsible for loss of its activity.

Introduction

Malonic acid and its derivatives are an important class of bioactive molecules, and they have been used to study the topography of the active center of the dicarboxylate transporter in intact rat liver mitochondria.[1] These compounds were shown to act as inhibitors of succinate transport[2] and fatty acid biosynthesis. The compounds were also tested for their ability to induce interferon-gamma (IFNγ) in cultures of human peripheral blood leukocytes, for the treatment of obesity,[3] as a lubrication performance in polar base oils, and for methyl oxidation in the animal body.[4] Malonic acid itself forms uncharged monomalonato complexes with divalent cations.[5] Substituted malonic acid has been investigated and successfully used as chelating units in magnesium-selective ionophores.[6] From an extensive bibliographic search in the Cambridge Structural Database, it was found that an overwhelming majority of the magnesium complexes showed an octahedral coordination of magnesium by the ligand atoms. Alkyl-substituted malonic acids have also been used as metal extraction reagents for quantitative extraction of metals.[7] The strong metal-binding ability of these compounds suggests that they hold considerable promise as potential inhibitors of metalloenzymes like polymerase and amylase which require Mg^{2+} and Ca^{2+} ion, respectively, for their structural stability and enzymatic activity.[8]

DNA polymerase enzyme has long been recognized as a significant therapeutic target for infectious microorganisms, and a number of compounds showing promising inhibitory activity toward the enzyme have been synthesized and characterized.[9,10] The choice of the polymerase enzyme as a therapeutic target assumes special significance owing to the emergence of antibiotic resistant strains, which is a constant challenge in modern health care and therapy of infectious diseases. Additionally, polymerase antagonists have been the center of attraction owing to their potential application as antiproliferative agents against tumor cells.[11] Chemotherapy plays a key role in the treatment of many tumors. In this context, DNA polymerases represent important cellular targets in the development of anticancer and antiviral agents.[12] α-Amylase is a glycoprotein[13] which breaks down long-chain carbohydrates such as starch, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose, and limit dextrin from amylopectin. It is produced mainly in the pancreas and the glands that make saliva. When the pancreas is diseased or inflamed, amylase is released into the blood. The excessive amylase in the blood may be implicated in digestive tract problems.[14] Currently, there is considerable interest in developing inhibitors for digestive enzymes such as α-amylase and other intestinal glucosidases as they can significantly influence breakdown of ingested starch and other carbohydrates and regulate absorption of glucose into the bloodstream. Such inhibitors could impact control of blood glucose levels and have significant implications in the treatment of type 2 diabetes.

It is quite clear from the aforementioned facts that there is an increasing demand in developing potent inhibitors of metalloenzymes and in this context synthesis of novel antagonistic amphiphilic compounds and evaluation of their inhibitory effect on candidate metalloenzymes assumes great significance. Such an endeavor would eventually lead to the generation of potent agents for effective therapy of infectious diseases and cancer. In the present study, we have conducted experiments to explore the potential of such amphiphilic compounds as an inhibitor of metalloenzyme for which we have selected *Taq* DNA polymerase and α-amylase as model enzymes. Our investigation reports the effect of amphiphilic malonic acid compounds in chelating magnesium and calcium and its subsequent inhibitory effect on *Taq* polymerase enzyme in polymerase chain reaction (PCR), and α-amylase in the starch digestion process. Steady-state and nanosecond time-resolved fluorescence studies on α-amylase and its composites with the

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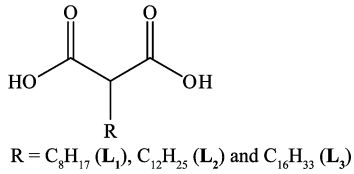
SCHEME 1: Structure of the Amphiphiles (L_1, L_2, and L_3)



R = C_8H_{17} (L_1), $C_{12}H_{25}$ (L_2) and $C_{16}H_{33}$ (L_3)

amphiphilic compounds have been also described to understand the conformational changes associated with removal of metal ion from the active site of the enzyme along with starch digestion study by UV−visible spectroscopy. A possible mechanism of enzyme inhibition mediated through the strong metal chelating property of the amphiphiles has been suggested.

Experimental Section

Materials. Commercially available diethyl malonate and alkyl halides were obtained from Merck, India, and Sigma, USA. The compounds were 99.9% pure and used as received without any further purification. Commercially available pure enzymes were purchased from Sibenzyme, Novosibirsk, Russia, and Fluka, Germany, while the water-soluble starch powder was purchased from Merck, India. Ultrapure water (Milli-Q system, Millipore, USA) was used in all the experiments.

Synthesis of the Amphiphiles. 2-Octylmalonic acid (L_1), 2-dodecylmalonic acid (L_2), and 2-hexadecylmalonic acid (L_3) were prepared following the literature method.[12,15] The structure of the amphiphiles is shown in Scheme 1.

Methods. Scanning electron micrograph (SEM) images of samples glued on an aluminum stub and gold sputtered were obtained by means of a LEO-1430 VP electron microscope. FT-IR spectra were recorded at 4 cm^{-1} resolution with 10 scan using a Perkin-Elmer Spectrum One FT-IR spectrometer from 4000 to 450 cm^{-1}. A background spectrum was measured for pure KBr. The absorption spectra were recorded on a Perkin-Elmer Lambda-25 UV−visible spectrophotometer using 10 mm path length quartz cuvettes at 298 K, in the range of 400−900 nm wavelengths. Fluorescence measurements were taken on a FSP-920 spectrofluorimeter (Edinburgh Instruments) using 10 mm path length quartz cuvettes with the slit width of 2 nm at 298 K. The enzyme solution was excited at 290 nm wavelength. Background intensities of the buffer blanks in which α-amylase was reconstituted were subtracted from each sample spectrum to account for any contribution due to the solvent. Steady-state fluorescence anisotropy measurements were performed on FSP-920 spectrofluorimeter (Edinburgh Instruments). The tryptophan fluorescence of α-amylase was recorded over 300−450 nm wavelengths by exciting at 295 nm using an excitation slit width of 2 nm. The anisotropy values were averaged over an integration time of 5 s, and a maximum number of three measurements were made for each sample. All anisotropy values of amylase in the presence of amphiphiles are the mean values of three individual determinations. The degree r of anisotropy in the tryptophan fluorescence of α-amylase was calculated using eq 1 at the peak of the protein fluorescence spectrum, where I_{VV} and I_{VH} are the fluorescence intensities of the emitted light polarized parallel and perpendicular to the excited light, respectively, and $G = I_{HV}/I_{HH}$ is the instrumental grating factor.

$$r = (I_{VV} - GI_{VH})/(I_{VV} + 2GI_{VH}) \quad (1)$$

Time-resolved intensity decays of the protein were measured using a Life Spec II spectrofluorimeter (Edinburgh Instruments) at 298 K. The sample was excited by Pico-quant 290 nm LED source and the decay was measured through 50 ns time scale at a time resolution of 0.0122 ns/channel. The decay curves were analyzed by FAST software using discrete exponential method (eq 2), provided by Edinburgh Instruments along with the instrument, where α_i is the initial intensity of the decay component i, having a lifetime τ_i.[16]

$$I(t) = \sum \alpha_i \exp(-t/\tau_i) \quad (2)$$

Polymerase Chain Reaction (PCR) Studies. The potency of the compounds, viz., 2-octylmalonic acid (L_1), 2-dodecylmalonic acid (L_2), and 2-hexadecylmalonic acid (L_3) as magnesium chelators and potential inhibitors of *Taq* DNA polymerase enzyme was ascertained by performing polymerase chain reaction (PCR). A 23.0 mM stock solution of the compounds (L_1, L_2, and L_3) was prepared in ultrafiltered water (pH ∼ 9.0). Experiments were performed to study the effect of saturation of the compounds with magnesium chloride as follows: in one set of experiment (set A), varying concentrations of the compounds (4.5, 3.6, 2.7, 1.75, and 0.83 mM) were preincubated overnight at room temperature with 1.5 μL of a 25.0 mM stock solution of magnesium chloride. In a parallel set of experiment (set B), varying concentrations of the compounds were preincubated overnight at room temperature with 1.5 μL of ultrafiltered water instead of magnesium chloride. Reaction mixtures from sets A and B were subsequently included in a PCR reaction mixture. Genomic DNA from *Escherichia coli* MTCC 433 was isolated using the bacterial genomic DNA isolation kit (Sigma, USA) and used as template DNA. The oligonucleotide primers used for PCR amplification included the universal primers specific for the domain *Bacteria* and the sequences of the forward and reverse primers were 8F, 5′-AGAGTTTGATCCTGGCTCAG-3′, and 1492R, 5′-GGTTACCTTGTTACGACTT-3′. PCR amplification was carried out in a total reaction volume of 25.0 μL, which contained contents of the reaction mixtures obtained from set A or set B, 2.0 μL of template DNA sample (50 ng), 2.5 μL of PCR buffer (60 mM Tris-HCl pH 8.5, 1.5 mM $MgCl_2$, 25.0 mM KCl, 10.0 mM 2-mercaptoethanol, 0.1% Triton X-100), 200.0 μM of each deoxynucleoside triphosphate (dNTP), 1.0 unit of *Taq* DNA polymerase (Sibenzyme, Novosibirsk, Russia), and 50.0 pmol each of forward and reverse primer. Template DNA was initially denatured at 94 °C for 2.0 min. Subsequently, a total of 35 amplification cycles were carried out in a programmable thermal cycler (Gene Amp Gold PCR System, Applied Biosystems, USA). Each cycle consisted of denaturation for 1.0 min at 94 °C, primer annealing for 1.0 min at 55 °C, and extension for 1.0 min at 72 °C. The last cycle was followed by a final extension at 72 °C for 10 min. PCR products were analyzed by agarose (1%) gel electrophoresis.[17]

Enzymatic Starch Digestion Studies. The potency of the compounds as calcium chelators and potential inhibitors of α-amylase (from hog pancreas, Fluka) was ascertained by performing starch digestion studies. As porcine α-amylase is likely to lose its activity at pH ∼ 9.0, a 23.0 mM stock solution of the compounds (L_1, L_2, and L_3) was prepared in 1:1 (v/v) mixture of ultrafiltered water and DMSO. In one set of experiment (set A), the enzyme solution (1.0 mg/mL in 10.0 mM phosphate buffer of pH 7.0) was preincubated overnight at room temperature with equal amounts of DMSO (v/v) instead of the amphiphilic compounds solution to check the effect of DMSO on enzymatic activity. In a parallel set of experiment

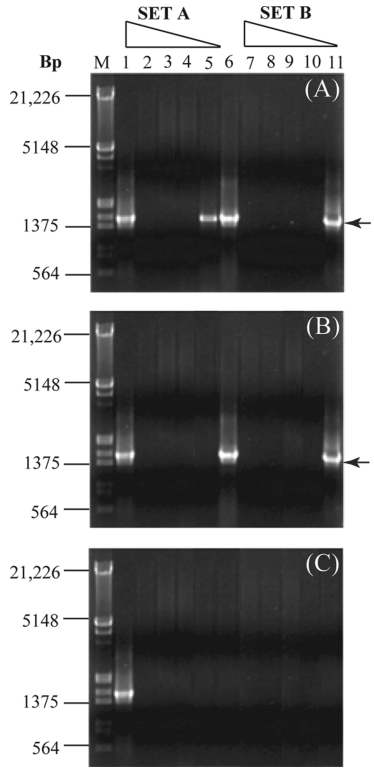


Figure 1. Agarose gel electrophoresis of amplicons obtained from PCR reaction mixtures containing (A) amphiphile $\mathbf{L}_1$, (B) amphiphile $\mathbf{L}_2$, and (C) amphiphile $\mathbf{L}_3$. Lane 1: amplicon obtained from control PCR reaction using ultrafiltered water (pH ~ 9.0) and without amphiphile. Lanes 2−6: amplicons obtained from experiment set A. Lanes 7−11: amplicons obtained from experiment set B. Arrow indicates an amplicon of 1485 bp.

(set B), varying concentrations of the compounds (4.5, 3.6, 2.7, 1.75, and 0.83 mM) were preincubated overnight at room temperature with 100.0 μL of a 1.0 mg/mL of enzyme solution prepared in 10.0 mM phosphate buffer of pH 7.0. For the starch digestion kinetics studies, 100.0 μL of the preincubated enzyme solution with DMSO (set A) was added to 36.0 mL of the starch solution having a starch concentration of 0.05 mg/mL. An equivalent amount of preincubated enzyme solution with compound (set B) was added to 36.0 mL of starch solution having concentration of 0.05 mg/mL to compare the kinetics of starch digestion by the enzyme in presence of the compounds. Aliquots (2.0 mL) from each of the reaction mixtures were withdrawn at regular time intervals and to each of these 10.0 μL of iodine solution (Gram's iodine, HiMedia) was added and the UV−visible spectra of the solutions were subsequently recorded. For kinetics data, the absorbance at 580 nm was plotted as a function of time.

Results and Discussion

Effect of Amphiphiles on the Enzymatic Activity of *Taq* DNA Polymerase. Figure 1 depicts the results obtained from PCR in presence of the amphiphiles $\mathbf{L}_1$, $\mathbf{L}_2$, and $\mathbf{L}_3$. It is evident from the figure that when varying concentrations of the amphiphile $\mathbf{L}_1$ (4.5, 3.6, 2.7, 1.75, and 0.83 mM) were preincubated with 1.5 μL of 25.0 mM of magnesium chloride (experiment set A), the PCR reaction was completely inhibited at high concentrations of $\mathbf{L}_1$ (lanes 2−4). However, when the concentration of $\mathbf{L}_1$ was as low as 1.75 and 0.83 mM (lanes 5, 6), the PCR reaction was positive as evidenced from the presence of amplicons in the lanes. A plausible explanation for these results is as follows: in the experiment set A, decreasing concentrations of the amphiphile $\mathbf{L}_1$ were incubated with 1.5 μL of 25.0 mM magnesium chloride for saturation of the amphiphile. The concentration of magnesium chloride (1.5 mM) used was suboptimal for complete saturation when higher concentrations of $\mathbf{L}_1$ (4.5, 3.6, and 2.7 mM) was used during preincubation. Hence, the amphiphiles still retained additional magnesium ion binding ability at these concentrations. Consequently, in the PCR reaction, the amphiphile was able to sequester magnesium ions from the active site of the enzyme *Taq* polymerase as well as additional magnesium ions present in the PCR buffer (1.5 mM magnesium is present in the buffer). In the context of DNA polymerase activity, the role of the metal ion (magnesium in the case of *Taq* polymerase) in mediating the nucleotidyl transfer has been well established. Two magnesium ions present in the active site of *Taq* polymerase are in contact with the conserved aspartate residues of the enzyme (Supporting Information, Figure S4). Further, one of the metal ions is involved in the activation of the 3′-OH of the DNA primer for attack of the nucleotide α-phosphate, while the other metal ion presumably stabilizes the displaced pyrophosphate moiety.[13,18] Thus, the role of the magnesium ions is pivotal in the nucleotidyl transfer mechanism and synthesis of the new DNA strand. Further, the structure of the amphiphile $\mathbf{L}_1$ suggests that it is a potent magnesium chelator as similar compounds based on malonic acid have been shown to have a high propensity to bind magnesium ion.[16] Hence, the detrimental effect of amphiphile $\mathbf{L}_1$ in PCR can be accounted in terms of its inherent magnesium chelating property and subsequent inhibitory effect on *Taq* polymerase activity. It may also be mentioned that collectively the results obtained from set A clearly indicate that the inhibition of the polymerase enzyme, which in turn stalled the PCR reaction, is a function of the effect of saturation of the amphiphile $\mathbf{L}_1$ with magnesium chloride and is determined by the concentration of the amphiphile used. When lower concentration of $\mathbf{L}_1$ (1.75 and 0.83 mM) was used in the experiments, the amphiphile was fully saturated by magnesium during the preincubation step. Subsequently, this rendered the amphiphile $\mathbf{L}_1$ ineffective to further chelate magnesium ions that were complexed with *Taq* polymerase enzyme or present in the PCR buffer. Hence, as expected, a positive PCR reaction ensued and amplicons of the expected size of 1485 bp were obtained (lanes 5, 6).

The effect of saturation of the amphiphiles with magnesium chloride and corresponding inhibition of polymerase activity in PCR is further corroborated by the results obtained in experiment set B. It is clear from Figure 1 that there was considerable inhibition of the PCR reaction when $\mathbf{L}_1$ was used at varying concentrations without any saturation step. A complete inhibition of the PCR reaction could be observed when $\mathbf{L}_1$ was used at concentrations ranging from 4.5 to 1.75 mM (lanes 7−10). It can be construed that at these concentrations $\mathbf{L}_1$ is able to effectively bind magnesium and reduce its accessibility for the enzyme. As a result, the enzyme activity is hampered and the progress of the PCR reaction is significantly affected. At the lowest concentration of $\mathbf{L}_1$ (0.83 mM) the amphiphile is unable to chelate magnesium sufficiently enough so as to inhibit the enzyme and halt the reaction. Hence, under these conditions, PCR reaction yields a positive amplicon (lane 11). The magnesium chelating ability of the amphiphilic compounds and the consequential inhibition of *Taq* polymerase in PCR reaction are clearly established.

It may be noted that a high pH of ~9.0 was chosen for the PCR study so as to ensure that all the malonic acid ($\mathbf{H_2L}$) head

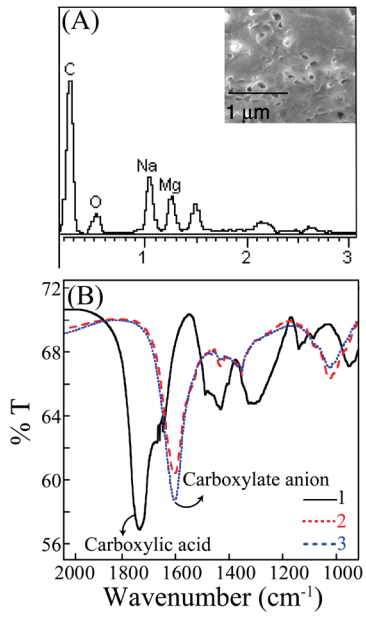


Figure 2. (A) EDX and inset: SEM image of the precipitate from PCR reaction in the presence of $\mathbf{L}_3$. (B) FT-IR spectra of the amphiphile $\mathbf{L}_3$ before and after PCR reaction. 1, amphiphile $\mathbf{L}_3$; 2, preformed Mg complex; 3, precipitate obtained from PCR reaction.

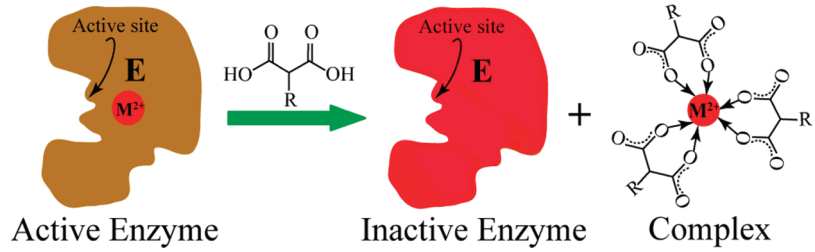
groups are in the malonate ($\mathbf{L}^{2-}$) form, so as to exert stronger metal chelating properties.[19] At this pH, the precipitation of magnesium hydroxide was not observed. This precipitation was probably prevented since all the malonic acid ($H_2\mathbf{L}$) is essentially in the malonate ($\mathbf{L}^{2-}$) form. Furthermore, the stability constant of the Mg complex is high, which facilitates the formation of the magnesium(II) malonate complex.[20] The possible role of the high pH being a cause of inhibition of the enzyme activity was negated by the fact that a positive amplicon was obtained in a control reaction when ultrafiltered water of pH ~9.0 was used to set up a conventional PCR reaction in the absence of the amphiphiles (Figure 1, lane 1).

Characterization of the Amphiphile−Metal Complex. When amphiphile $\mathbf{L}_2$ was used in a similar experimental setup, amplicons could only be obtained in the presence of the lowest concentration of amphiphile $\mathbf{L}_2$ used in experiment sets A and B (Figure 1B, lanes 6 and 11). The PCR reaction was completely inhibited at high concentrations of $\mathbf{L}_2$ (Figure 1B, lanes 2−5). Thus, the presence of the amphiphile $\mathbf{L}_2$ resulted in the inhibition of enzyme in the same manner as $\mathbf{L}_1$, discussed above. When the amphiphile $\mathbf{L}_3$ was used in a similar experimental setup, amplicons could not be obtained from any of the PCR experimental samples except for the control reaction (Figure 1C). Thus, the presence of the amphiphile $\mathbf{L}_3$ resulted in complete inhibition of the enzyme independent of its concentration. Further, when the amphiphile $\mathbf{L}_3$ was used, a precipitate was observed in all the tubes (at all concentrations) following PCR. Both the amphiphiles ($\mathbf{L}_2$ and $\mathbf{L}_3$) are capable of forming complex with magnesium. However, we observed that in the case of $\mathbf{L}_3$ an insoluble complex was formed which precipitated out of the reaction mixture, possibly because of the hydrophobicity. In order to study the amphiphile−metal complex formation, we performed SEM followed by EDX (energy-dispersive X-ray) analysis, which revealed the presence of magnesium in the precipitate (Figure 2A). Further, FT-IR analysis of the precipitate obtained from the reaction mixture indicated the formation of magnesium−amphiphile [Mg**L**] complex when compared with the result obtained with pure preformed complex (Figure 2B). The peak at around 1720 cm^{-1} in spectra 1 is due to the carboxylic acid, while in spectra 2 and 3 the peak at around 1600 cm^{-1} is due to the carboxylate ion that can form hydrogen bond and account for the formation of complex between the amphiphiles and magnesium. Collectively, these results clearly indicated that the amphiphile $\mathbf{L}_3$ possessed strong magnesium-binding ability and the complex formed thereof fell out of solution, leading to dramatic depletion of magnesium ions in the reaction mixture which eventually caused complete inhibition of the PCR. On the basis of the magnesium ion binding ability of the amphiphilic compounds and their implications on inhibiting *Taq* polymerase activity, we propose a mechanism of action of these potent polymerase inhibitors as depicted in Scheme 2.

Effect of Amphiphiles on the Enzymatic Activity of α-Amylase. The inhibitory effect of the amphiphiles on the activity of α-amylase was studied by following the UV−visible absorption of starch−iodine solution[21] in the presence of pure enzyme only and in the presence of the enzyme−amphiphile composites separately. Figure 3, A and B, represents the time-dependent decrease in absorbance of the starch−iodine complex in the presence of pure enzyme and amphiphile−enzyme composites. It is evident from Figure 3A that in the case of pure enzyme a systematic decrease in the absorbance of starch−iodine solution was observed with time, indicating rapid digestion of starch by α-amylase. However, in the case of the amphiphile $\mathbf{L}_1$−enzyme composites, hydrolysis of starch was completely abolished as no significant change in the absorbance of the starch−iodine solution could be observed (Figure 3B). This indicated a profound inhibition of enzyme activity by the amphiphile, and the inhibition was unequivocally observed irrespective of the alkyl chain length of the amphiphilic compounds. From Figure 3C it is clear that the kinetics of starch digestion by pure α-amylase in buffer as well as in buffer−DMSO mixture (1:1) was comparable. Hence, the activity of the enzyme was retained in presence of DMSO. Further, from Figure 3C, the inhibitory effect of the amphiphiles on the activity of α-amylase was apparent as the typical decrease in the absorbance of starch−iodine solution was not observed in the case of the enzyme−amphiphile composites. We have also studied the enzymatic activity of amylase by varying the concentration of the amphiphiles (Supporting Information, Figure S3). Interestingly, unlike *Taq* DNA polymerase, the activity of amylase was inhibited even at a very low concentration of the amphiphiles (0.83 mM) which is comparable to the known inhibitors of amylase reported in the literature.[22] This indicates that a very low concentration of the amphiphile was sufficient to chelate the metal from the enzyme which resulted in the loss of activity. We have also pursued the starch agar plate test for ascertaining the enzymatic activity of the enzyme−amphiphile composite vis-à-vis the pure enzyme. The clear zone obtained around the wells in starch agar plate indicates amylase activity. The results of such tests are shown in Figure 3D, where zones 1 and 2 are due to amylase only in buffer and buffer−DMSO mixture, respectively. It is also evident from the figure that the enzyme−amphiphile composite failed to produce any zone (zone 3), indicating the ability of the amphiphiles to act as a potent inhibitor of amylase. As clear from the figure, the zone of activity associated with the amylase in buffer−DMSO mixture was same as that of pure enzyme in buffer, indicating the enzyme retained its activity in presence of DMSO. Collectively, the starch agar tests corroborate earlier results.

Role of Chelating and Amphiphilic Nature in Enzyme Inhibition. In order to gain further insight on the mechanism of enzyme inhibition caused by the amphiphilic compounds,

SCHEME 2: Schematic Model Showing the Extraction of Metal from the Active Sites of the Enzyme by the Amphiphile (Where R = C_8H_{17}, $C_{12}H_{25}$, or $C_{16}H_{33}$)



we pursued additional experiments. The acid group of the amphiphile which is responsible for chelating metal ion was modified to the corresponding ester by reaction with ethanol. The modified amphiphilic compound failed to inhibit amylase activity. The control samples with only diethyl malonate and only malonic acid were also unable to inhibit the enzyme under identical experimental conditions (Figure 4A−C). These experiments clearly established the fact that the malonic acid headgroup as well as the amphiphilic nature of the compound is indispensable for the inhibition of enzyme; i.e., the removal of either chelating headgroup or the hydrocarbon chain from the compound obliterates its ability to inhibit the enzyme. Essentially, interactions of the enzyme with the amphiphilic compounds lead to the removal of metal ion from the active sites of the enzyme, which possibly results in disruption of the native conformation of the enzyme and concomitant loss of activity.[23] Conventionally, binding of the inhibitor with enzyme molecule is generally regarded as a critical step for manifestation of enzyme inhibition. However, literature reports suggest that metal chelators can also play the role of potent enzyme inhibitors by selectively removing metal ions from the active site of such enzymes.[24] Further, addition of Ca^{2+} to the enzyme solution incubated with amphiphile was unable to recover the enzymatic activity (Figure 4D), supporting the fact that the inhibition was irreversible in nature. Irreversible inhibition of enzyme by metal chelation has also been reported earlier for M17 leucine aminipeptidase.[25] In order to verify the possible structural changes of the protein in the presence of the amphiphiles, we have also measured the steady-state fluorescence anisotropy (r) of the enzyme and enzyme−amphiphile composites at room temperature. Table 1 shows the variation of the fluorescence anisotropy (r) of amylase upon interactions with amphiphiles. The anisotropy of pure amylase increases from 0.068 in native solution to 0.085, 0.102, and 0.113 upon interactions with amphiphiles $\mathbf{L}_1$, $\mathbf{L}_2$, and $\mathbf{L}_3$, respectively, which suggests the increased rigidity of the surrounding environment of the fluorophore. From the results indicated in Table 1, it can also be observed that the value of anisotropy for enzyme−amphiphile composites are more than for the pure enzyme, indicating the loosening of the globules, which reduces the rotational diffusion coefficient for the enzyme molecules and leads to an increase in anisotropy. This was further supported by steady-state and lifetime fluorescence measurements, as described in the next section.

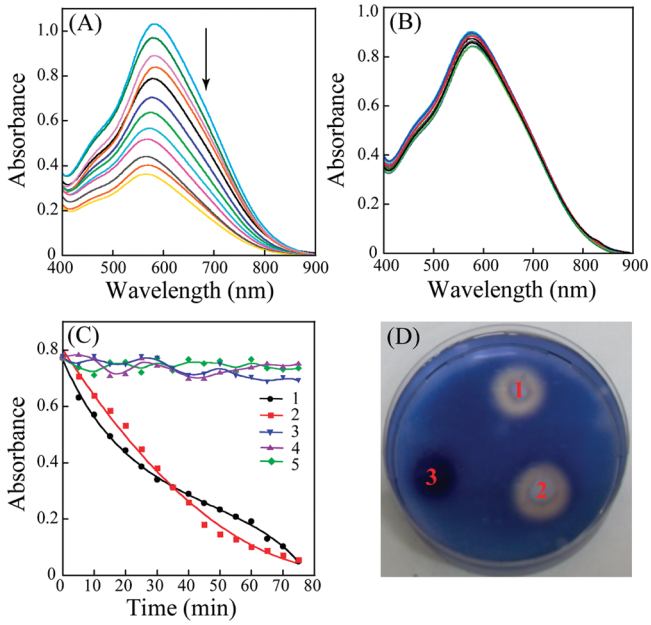


Figure 3. UV−visible spectra of the starch digestion by (A) α-amylase and (B) α-amylase-amphiphile $\mathbf{L}_1$ composite. (C) Comparative kinetics study of starch digestion by pure α-amylase in buffer (1), pure α-amylase in buffer−DMSO mixture (2), α-amylase−amphiphile $\mathbf{L}_1$(3), α-amylase−amphiphile $\mathbf{L}_2$(4), and α-amylase−amphiphile $\mathbf{L}_3$ (5) as followed by the absorbance at 580 nm. (D) Starch agar plate assay for enzymatic activity of pure α-amylase in buffer (1), pure α-amylase in 1:1 mixture of buffer and DMSO (2), and α-amylase−amphiphile $\mathbf{L}_1$composites (3).

Probing Molecular-Level Interactions of Amphiphile with α-Amylase. To get a molecular insight of the nature of amphiphile−α-amylase interactions, we performed fluorescence spectroscopy. In proteins, tryptophan (Trp) fluorescence is widely used as a tool to monitor changes in its structures and to draw inferences regarding local structure and dynamics.[26] α-Amylase contains both tyrosine and tryptophan residues, and the change in its conformation or microenvironment caused by metal ions or inhibitors can be detected by fluorescence spectroscopy. The wavelength of emission maximum for Trp depends on its microenvironment. Specifically, a low polarity, hydrophobic microenvironment is characterized by $\lambda_{max} \sim 331$ nm, while for Trp in an aqueous phase λ_{max} is 350−353 nm. The effect of the amphiphiles on the photophysical properties of the enzyme was studied by steady-state and lifetime fluorescence measurements. The fluorescence spectrum of amylase exhibits strong emission with maximum at 340 nm when excited at 290 nm. Excitation wavelength of 290 nm was taken to avoid the contribution from the tyrosine residues. A 10 nm blue shift in λ_{max} of amylase relative to that of Trp in an aqueous phase suggests that the Trp residues in amylase are located in hydrophobic environment. As shown in Figure 5A, the presence of amphiphiles caused a reduction of fluorescence intensity accompanied by a blue shift in λ_{max} by 5 nm upon complexation with the enzyme. This blue shift of emission maximum along with decrease in intensity indicates that interaction of amphiphiles with the enzyme changes the polarity of microenvironment of Trp residues with probable increase in hydrophobicity in its vicinity and this phenomenon is seen when the protein is in either native or structurally disrupted states.[27]

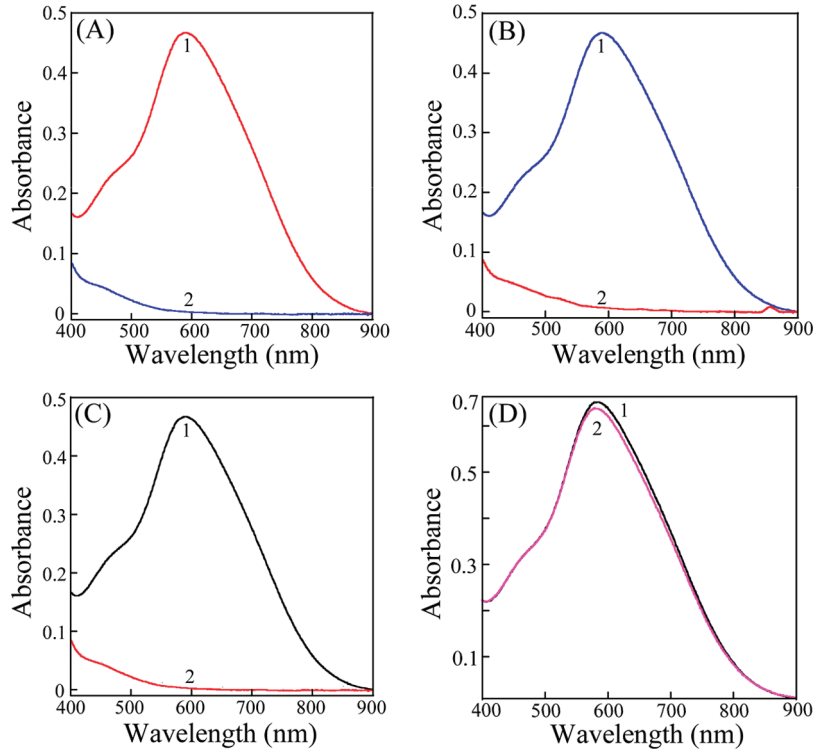


Figure 4. UV−visible spectra of starch digestion by (A) α-amylase−2-octyldiethyl malonate; (B) α-amylase−diethyl malonate; (C) α-amylase−malonic acid composites at 0 min (1) and after 90 min (2) in an aqueous buffer−DMSO mixture (1:1). (D) Addition of $CaCl_2$ to the solution of amylase−amphiphile $\mathbf{L}_1$ composites. The concentrations of the enzyme and diethyl malonate used were 1 mg/mL and 4.5 mM, respectively.

TABLE 1: Anisotropy (r) and Fluorescence Lifetime (τ) Parameters of Pure α-Amylase[a] and α-Amylase−Amphiphile[b] Composites

sample	λ_{em} (nm)[c]	anisotropy (r)	τ_1 (ns)	τ_2 (ns)	α_1	α_2	χ^2
α-amylase in buffer + DMSO[d]	340	0.068	4.52	1.57	0.011	0.012	1.001
α-amylase + amphiphile $\mathbf{L}_1$	335	0.085	2.98	0.62	0.012	0.019	1.000
α-amylase + amphiphile $\mathbf{L}_2$	335	0.102	3.13	0.51	0.014	0.018	1.003
α-amylase + amphiphile $\mathbf{L}_3$	335	0.113	3.27	0.69	0.014	0.028	1.002

[a] [amylase] = 1 mg/mL. [b] [amphiphile] = 4.5 mM. [c] λ_{ex} = 290 nm. [d] 1:1 mixture of buffer and DMSO.

To explain the variation of blue shift and decrease in the intensity of amylase−amphiphile composites compared to the pure amylase, we conducted the time-resolved fluorescence experiment with α-amylase in the absence and presence of the amphiphiles. The fluorescence lifetime decay profiles of the pure amylase and amylase−amphiphile composites are shown in Figure 5B. The fluorescence lifetimes and the various statistical parameters used to check the goodness of fit are given in Table 1. The two time constants of amylase alone in buffer DMSO mixture (1:1) are 4.52 and 1.57 ns, while in the presence of the amphiphiles the fluorescence lifetimes of the Trp residues have decreased significantly. A decrease of lifetime from 4.52 (τ_1) to 2.98 and from 1.57 (τ_2) to 0.62 ns results due to the addition of amphiphile $\mathbf{L}_1$. Thus, the decrease of fluorescence lifetime was a clear consequence of the significant interactions between the amphiphiles and enzyme. It is clear from the lifetime measurements that fluorescence decay of amylase has significant impact due to the presence of amphiphiles. Therefore, metal-ions-induced fluorescence quenching of amylase is attributed to the complex formation between amphiphiles and metal ion, and this binding perturbs the microenvironment around the Trp residues and causes the fluorescence quenching of enzyme. Removal of metal ion has significant impact on the polarity of microenvironment of Trp residue as indicated by change for the maximum emission wavelength of Trp after addition of amphiphiles. The aforementioned results indicate that the Trp residues in the enzyme are positioned in the vicinity of Ca^{2+} binding sites and removal of the divalent cation by the amphiphilic compounds probably induces a change in the conformation of the active site in the protein and abolishes its activity. In corroboration of these results, the data shown in the present study also indicates that the metal ion not only plays an essential role in the catalytic mechanism but also has a great impact on the structural features of the enzyme.

On the basis of the cumulative results obtained for amphiphile−α-amylase interactions, we propose the following mechanism for amphiphile-mediated enzyme inhibition. α-Amylase consists of a single polypeptide chain of about 475 amino acid residues, has two −SH groups and four disulfide bridges, and contains a tightly bound Ca^{2+}.[28] α-Amylases have long been known to require at least one Ca^{2+} ion per enzyme molecule for maintaining their tertiary structure and catalytic activity.[26,8] Ligands that bind the Ca^{2+} ion in α-amylase belong to domains A and B of the enzyme (Supporting Information, Figure S4) and the active site cleft is located between these two domains. Essentially, the Ca^{2+} ion appears to stabilize the active site cleft by inducing an ionic bridge between domains A and B. The divalent metal ion is octahedrally coordinated by four amino

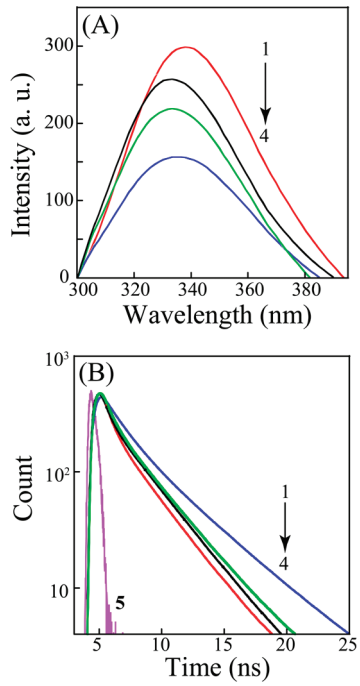


Figure 5. (A) Steady-state fluorescence emission spectra and (B) time-resolved fluorescence spectra of pure α-amylase and α-amylase−amphiphile composites 1:1 in buffer−DMSO mixture. Trace 1−4: α-amylase only, α-amylase + $\mathbf{L}_3$, α-amylase + $\mathbf{L}_2$, and α-amylase + $\mathbf{L}_1$. Trace 5: IRF. The concentrations of the amylase and amphiphile used were 1 mg/mL and 4.5 mM, respectively.

acid residues of the enzyme, viz., Asn-100, His-201, Asp-159, and Asp-167 from the domains A and B, while its coordination sphere is completed by water molecule (Supporting Information, Figure S5). In the present investigation, we have shown that the addition of the amphiphiles inhibits the enzymatic activity of α-amylase, presumably due to chelation of the metal present in the active site of the enzyme and subsequent formation of a metal complex. In addition, binding of the amphiphile to the enzyme molecule followed by sequestration of Ca^{2+} results in a concomitant loss of activity as depicted in Scheme 2.

Conclusions

The present studies have systematically shown the effect of the amphiphilic compounds in chelating the metal from the active sites of *Taq* polymerase and α-amylase enzyme and their subsequent inhibitory effect in polymerase chain reaction (PCR) and enzymatic assay of starch−iodine complex. We have also provided evidence of magnesium−amphiphile complex formation during the process of enzyme inhibition by IR spectroscopy and EDX analysis, while the complexation of the amphiphiles with calcium ion in α-amylase was demonstrated by UV−visible and fluorescence spectroscopy. In the present case of enzymatic process, it is essentially the disruption of the native three-dimensional structures of the enzymes due to the removal of metal ion from its active sites by the amphiphiles which was responsible for the loss of its activity. The amphiphilic compounds hold considerable promise as small molecule inhibitors of metal-dependent enzymes such as DNA polymerase and α-amylase. The strong structural homology among DNA polymerases could further motivate investigations to ascertain the inhibitory effect of these amphiphiles against other polymerases. In future, we also intend to investigate the spectrum of inhibitory activity of the amphiphiles against other metalloenzymes as well as explore the antimicrobial activity of the compounds.

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Supporting Information Available: Information about control experiments and sample characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

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