Solvent Induced Disulfide Bond Formation in 2,5-dimercapto-1,3,4-thiadiazole

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Disulfide bond formation is the decisive event in the protein folding to determine the conformation and stability of protein.[1] Many evidences have been shown for the role of disulfide bond formation in protein folding as a transient intermediate.[2] It is worthwhile to mention here that there are recent reports on the co-solvent assisted disulfide bond formation in the production of biologically active α-Conotoxin ImI by methanol.[3] The disulfide bonds must be formed between Cys2-Cys8 and Cys3-Cys12 of pro α-Conotoxin ImI to produce naturally occurring α-Conotoxin ImI.[3] This α-Conotoxin is a selective antagonist of α7 nicotinic acetylcholine receptors.[3] Thus it is very interesting to observe the disulfide formation *in vitro*. Can solvent alone do this excellent biological job without any added reagent? To answer this question we have done a bench work for the oxidation of sulfhydryl groups to disulfide by taking a hetero aromatic dithiol, 2,5-dimercapto-1,3,4-thiadiazole (DMcT) in different solvents. Alhough DMSO is known for the oxidation of sulfhydryl oxidation to disulfide in the presence of added reagents,[4] we found disulfide products with good yield in methanol in the absence of reagents when compared to DMSO and other solvents. Methanol offers good solubility of DMcT and easy isolation of disulfide products.

We have observed a colour change for the methanolic solution of DMcT from pale yellow to intense yellow within three hours. On standing, the pale yellow colour of the methanolic solution of DMcT was further intensified to bright yellow fluorescent colour and then started to fade after 48h due to the formation of a pale yellow color solid in the solution. The pH of the fresh methanolic solution of DMcT is 2.1 and the pH of the solution increases as the time increases and attained a constant value of 6.8 after 48h. Figure 1A shows the UV-Vis spectra of DMcT in methanol recorded at various time intervals. The fresh methanolic solution of DMcT shows predominant peaks at 260 nm and 340 nm due to electronic transitions involving charge transfer hydrogen bonded (CTHB) type complex[5] and $n \rightarrow \pi^*$ transition,[6] respectively. The intensities of the peaks at 340 nm and 260 nm were decreased after 3 h and a new absorbance band was appeared at around 390 nm responsible for the color intensification due to $n \rightarrow \pi^*$ transition (Fig 1A curve b). After 48h, the solution did not show any peak at 260 nm due to the transition involving CTHB type complex in addition to enormous decrease in intensity of peak at 340 nm indicating that DMcT molecules were converted into some products which were settled as a pale yellow solid in the solution. The disappearance of peak at 260 nm indicates that the –S-H groups may be converted into -S-S- bond via deprotanation of sulfhydryl groups.

To characterize the products formed in solution, we checked the 48h old methanolic solution with Thin Layer Chromatography (TLC) and found two spots corresponding to parent DMcT and a compound with higher molecular weight than DMcT. The pale yellow solid collected from the methanolic solution was found to be soluble in DMSO and DMF. EI Mass spectrum of pale yellow solid shows peaks at m/z 368 and 298.

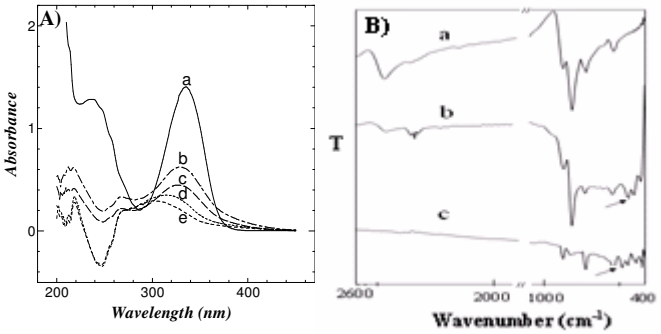


Figure 1. A) UV-Vis spectra of methanolic solution of 1mM DMcT a) fresh solution b) 3h c) 48h d) 72h and e) 96h old solutions. **B)** FT-IR spectra of a) DMcT b) 3h old methanolic solution of DMcT and c) pale yellow color solid from methanolic solution of DMcT.

The m/z 298 confirms the presence of dimer DMcT and the molecular ion peak at m/z 368 shows the presence of trimer minus CS_2. The formation of –S-S– bond was further confirmed by FTIR spectrum of the pale yellow solid. When compared to FT-IR spectrum of DMcT, spectra of 3h old solution of DMcT in methanol (Figure 1B) and the pale yellow solid (Fig 1B curve c) clearly showing a new weak absorption band at 471 cm^{-1} due to –S-S– bond formation[7] and the absorption band at 2475 cm^{-1} of –S-H_{str}[8] is undetectable for the pale yellow solid since most of the -S-H groups were consumed for the formation of disulfide. All other stretching frequencies of DMcT were retained for the pale yellow solid. From the above results we concluded that the formed pale yellow solid is the mixture of disulfide dimer and disulfide trimer of DMcT.

The next task is to reveal the nature of existence of DMcT in methanol and then the mechanism of formation of disulfide dimer and disulfide trimer in methanol. We have carried out the electrochemical studies of methanolic solution of DMcT at different time intervals. Figure 2 shows the cyclic voltammograms (CVs) recorded for the methanolic solution of DMcT at different time intervals. CV of fresh methanolic solution of DMcT shows three oxidation peaks at 0.29V, 0.69V and 0.86V corresponding to the oxidations of $DMcT^{2-}$, $DMcT^{-}$ and DMcT, respectively (Figure 2a).[9] Close examination of the CV indicates that the predominant existence of $DMcT^{2-}$ and $DMcT^{-}$, make us to conclude that the DMcT molecules were deprotanated to $DMcT^{2-}$ and $DMcT^{-}$ immediately after dissolved it in methanol.

The oxidation peaks corresponding to $DMcT^{2-}$ and $DMcT^{-}$ decreases when the CVs were recorded after 3h and 72h

(curves 2b and 2c). The presence of disulfide species in methanolic solution of DMcT was confirmed by running the CVs of 48h old methanolic solution of 1mM DMcT from 0V to -0.8V where electrochemical oxidation of $DMcT^{2-}/DMcT^{-}$ is not possible (Figure 2 inset (b)) and fresh methanolic solution of DMcT in the presence of excess triethylamine (TEA) (Figure 2 inset (a)). It has been reported that DMcT or $DMcT^{-}$ or $DMcT^{2-}$ oxidized to form dimer disulfide or polymer disulfide, but the reduction of both occurs at the same potential.[10] As we can see from Figure 2 (inset) that the reduction potential of 48h old methanolic solution (Figure 2 inset(b)) matches with the reduction potential of dimer/polymer disulfide peak of DMcT/TEA (Figure 2 inset (a)), suggesting the presence of disulfide containing species in the 48h old methanolic solution of DMcT.

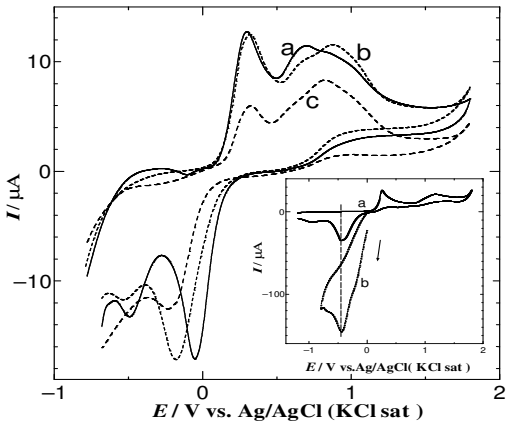
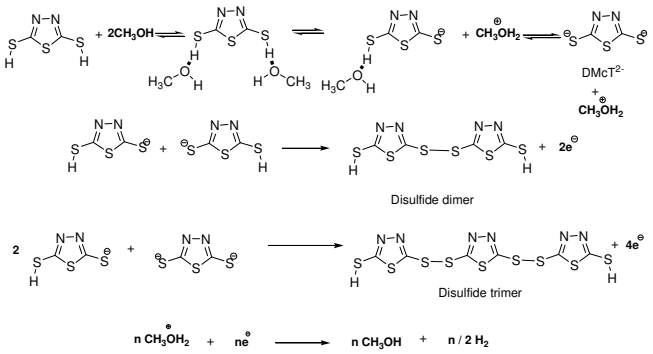


Figure 2. Cyclic voltammograms of 1mM DMcT in methanol at various time intervals. a) Fresh solution b) 3h old solution c) 72h old solution. Inset: Cyclic voltammograms of a) 1mM DMcT in methanol with excess TEA b) 48h old solution of 1mM DMcT in methanol.

From the above results we conclude that the oxidation of sulfhydryl groups to disulfide bond was induced by methanol. It is suggested that initially hydrogen bonding between the oxygen atom of methanol and hydrogen atom of sulfhydryl group of DMcT was formed. In the second step, proton transfer from the sulfhydryl group of DMcT to methanol leads to the formation of $DMcT^{-}$ and $DMcT^{2-}$. The protonated methanol molecules back donating the proton to harvest two electrons from $DMcT^{2-}$ and $DMcT^{-}$ and hence disulfide bond formation takes place (Scheme 1). The released two electrons from deprotanated molecules of DMcT during the course of disulfide dimer or trimer formation utilized for the molecular hydrogen formation. Since the reaction is extremely slow we could not observe hydrogen evolution. The formed trimer separates out along with some dimer as a pale yellow solid due to very low solubility in methanol.

The formation of disulfide bond in methanol can be explained by the proton donor and acceptor properties of the solvents.[11] The donor number[12] of solvent plays a main role in the present work. The high donor number of methanol (19.0)[13] implying good basicity and proton acceptor characters.[11] DMcT behaves as strong acid in solvents having good basicity and proton acceptor characters.[10] In those solvents the dissociation of thiol groups of DMcT is facilitated. Methanol facilitate the auto oxidation of sulfhydryl groups to disulfide by deprotanation. The formation of disulfide bond was not observed in chloroform and acetonitrile because of the poor proton donor and acceptor properties of these solvents.[9]

Scheme 1



Note: The hydrogen bonds between methanol and DMcT are shown in bold.

Since DMcT contains two acidic sulfhydryl groups and intuitive to form disulfide bond, it is possible to incorporate the DMcT in between two cyesteine units of a protein via disulfide bond formation, there by we can alter the conformation of the protein. Further the slow rate of formation of disulfide bond has to be accelerated by changing from pure methanol solvent to mixture of methanol. These works are currently underway in our laboratory.

Acknowledgment. Palanisamy Kalimuthu thanks UGC, NewDelhi for the award of Junoir Research Fellowship. SAJ thanks the Department of Science and Technology, New Delhi for financial support under the Fast Track Scheme for Young Scientists (SR/FTP/CS-09/2002).

Supporting Information Available: The detailed experimental procedure, pH values of solutions, colour changes of DMcT solution and mass spectrum of the products available as pdf file.

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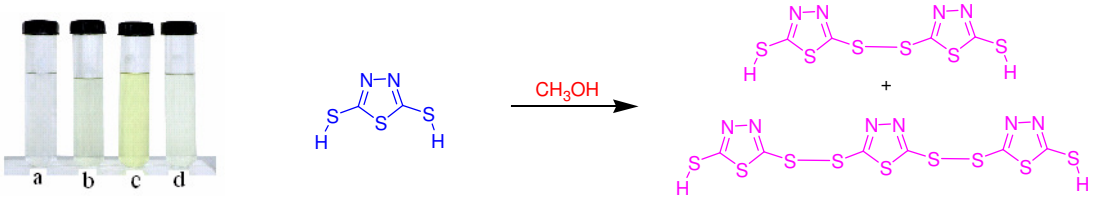

Disulfide bond formation is the decisive event in the protein folding to determine the conformation and stability of protein. To achieve this disulfide bond formation *in vitro*, we took 2,5-dimercapto-1,3,4-thiadiazole (DMcT) as a model compound. We found that disulfide bond formation takes place between two sulfhydryl groups of DMcT molecules in methanol. UV-Vis, FT-IR and mass spectroscopic as well as cyclic voltammetry were used to monitor the course of reaction. We proposed a mechanism for the solvent induced disulfide bond formation on the basis of the results we obtained.