Dissociation and Reconstitution Studies of a Broad Substrate Specific Multimeric Alcohol Oxidase Protein Produced by *Aspergillus terreus*

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A multimeric alcohol oxidase from *Aspergillus terreus* was dissociated and simultaneously deflavinated into catalytically inactive FAD-free subunits when incubated with 0.74 M β-mercaptoethanol (β-ME) for 8 h at 4°C. This dissociation process had traversed through two FAD-associated intermediate proteins, between these one of them showed the enzyme activity. On removal of β-ME, the multimeric apoprotein was regenerated, which was, however, catalytically inactive. Reactivation of the FAD supplemented apoprotein was accomplished only after incubating with the substrate. This catalytic reactivation was a slow process as evident from the prolonged FAD emission quenching. The dissociation and re-association phenomena were demonstrated by using dynamic light scattering, size exclusion chromatographic, confocal laser scan microscopic and native PAGE analyses. The solvent effect caused by the high concentration of β-ME is attributed to the observed dissociation and linked deflavination of these multimeric alcohol oxidase protein particles.

INTRODUCTION

The alcohol oxidases are known as complex multimeric proteins (*1*). Considering the importance of these oxidases for biocatalytic and biosensor applications research on these redox enzymes has been accelerated to unveil the mechanism on biogenesis and assembly of the subunits in these multimeric proteins complex (*2–6*). The short chain alcohol oxidase, commonly known as alcohol oxidase, reported from the methylotrophic yeasts is studied to a greater extent and described mostly as octameric protein complex (*7–11*). The information on protein chemical characteristics of the alcohol oxidase produced by filamentous fungi is limited and, among these limited reports hetero-oligomeric characteristics of these oxidases have been reported (*12–14*). The molecular mass of the alcohol oxidases are largely vary from 145 to 600 kDa (*10*, *15–18*). Recently, we have isolated a hetero-oligomeric alcohol oxidase consisting of five different non-covalently bound subunits from *Aspergillus terreus* (*14*). Intriguingly this multimeric alcohol oxidase bears the catalytic activity for different short-chain, long-chain, secondary and aromatic alcohol substrates. Very high aggregating tendency of the protein stands as hindrance on elucidating the structure-function relationship of this broad substrate specific alcohol oxidase. To investigate the functional role of the individual subunit proteins and their nature of assembly, developing a benign technique to dissociate this aggregated multimeric protein into the subunit level and subsequent investigation on the dissociated proteins on their function and reasemblance may be a useful approach as reported for few other cases (*19–23*). Usually dissociation of the aggregated or multimeric protein into stable soluble forms has been a challenging task. Although few dissociating agents have been reported (*24*, *25*) the degree of dissociation is strongly dependent on the conditions applied and property of the proteins. Hence, searching a suitable dissociating agent for this fungal enzyme is warranted to augment understanding on the structural and functional insight of these alcohol oxidase proteins. In this paper we are reporting a simple and highly efficient method for dissociation and simultaneous separation of FAD of this flavin-dependent multimeric alcohol oxidase protein produced by *A. terreus*. Furthermore, reconstitution of the apoprotein and subsequent assembling of the reconstituted apoprotein with the flavin to a functionally active enzyme have also been demonstrated and reported here.

MATERIALS AND METHODS

Isolation and Purification of Alcohol Oxidase—The culture conditions of *A. terreus* MTCC 6324 used in this

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study and the procedure for isolation of the alcohol oxidase from the microsomal membrane by differential centrifugation are described earlier (*26*). The enzyme was separated and solubilized from the microsomal membrane using 0.5% (v/v) 3-[(3-cholamidopropyl) dimethyl ammonio] propane-1-sulfonic acid (CHAPS). The protein was then purified initially by 35% (w/v) ammonium sulfate precipitation followed by DEAE sepharose ion exchange chromatography. The steps for purification and functional characterization were described in detail in our earlier paper (*14*). The purified native protein was used throughout this investigation.

Enzymatic Assay of Alcohol Oxidase—Alcohol oxidase activity was assayed using HRP-coupled assay method monitoring H_2O_2 production at 405 nm for 2,2′-azino-*bis*[3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt (ABTS) radical at 30°C ($\varepsilon_{405} = 18,400 \, \mu M^{-1} cm^{-1}$) (*27*) and also by measuring substrate dependent oxygen consumption using biological oxygen monitor with Clark-type polarographic oxygen electrodes (*26*).

Dissociation and Deflavination Studies—Gel filtration chromatographic analysis

Enzyme protein (150–170 μg in 100 μl) was treated with a suitable concentration of the chemical (dissociating or deflavinating) reagents for different time periods (1 min to 8 h) at 4°C under either static or agitation condition, except KBr treatment, where the reaction mixture was dialysed against KBr buffer (2 M KBr in 0.25 M potassium phosphate, pH 7.5, 0.3 mM EDTA, 5 mM β-mercaptoethanol (β-ME) and 20% glycerol) for 4 days at 4°C (*28*). The mixture was applied to HPLC gel filtration column TSK G3000 SW (0.75×30 cm, 10 μM, Tosoh corp. Japan) and then eluted isocratically with 50 mM Tris–HCl buffer, pH 8.0 at flow rate 1 ml/min. The eluted fractions were scanned simultaneously at $UV_{280\,nm}$ for protein and at 525 nm emission filter for flavin (at 450 nm excitation). A control devoid of the chemical reagent was also run and analysed to compare the results.

Dynamic light scattering analysis

Experiments were carried out in a 201 Spectroscatter dynamic light scattering (DLS) instrument (RiNA GmbH) to measure the mean radius particle size distribution of the protein samples for investigating the aggregation and dissociation profile of the sample protein. Before analysis the sample was subjected to $20,000 \times g$ for 20 min, to remove any turbidity. Data were acquired at 20°C, repeated 20 times and averaged.

Confocal laser scanning microscopic analysis

The protein subjected to dissociation or re-association was stained with Nile red, a specific fluorescent dye for lipids and hydrophobic patches of proteins and then observed under CLSM (Zeiss LSM 510, UK). The fluorescent images were photographed using similar spectral settings as described earlier (*14*).

Native PAGE and SDS–PAGE analysis

Native PAGE and SDS–PAGE analysis of the dissociated and re-associated proteins were done following the

method of Laemmli (*29*). The holoenzyme molecular mass was determined through native PAGE following the standard Ferguson plot method (*30*). Different percentages of native PAGE gels (4%, 5%, 6% and 7.5%) were run to determine the molecular weight of the native protein. The final slope value obtained in the graph between slope ($-\log K_r$) against molecular weight of the standards ($r^2 = 0.9872$) was used to calculate the native protein molecular weight (*14*). Gels were stained with standard silver nitrate staining method. The SDS–PAGE protein markers used were rabbit muscle phosphorylase b (97.4 kDa), rabbit muscle fructose-6-phosphate kinase (85 kDa), human transferrin (76.1 kDa), BSA (66 kDa), rabbit muscle lactate dehydrogenase (36.5 kDa), respectively.

Re-association Studies of Alcohol oxidase—The dissociating agent was removed from the treated protein sample by dialysis and then examined the re-association of the proteins following the similar Gel filtration, CLSM, native PAGE and SDS–PAGE methods as stated above. For Gel filtration chromatographic analysis the dissociated protein fractions were pooled, dialysed against 50 mM Tris buffer, pH 8.5 for 4 h at 4°C (100 ml × 2) to remove the chemical reagent and again run gel filtration column separately under similar conditions as stated above to investigate the re-association process. The tubes containing protein peaks from this filtration were pooled, concentrated and assayed for alcohol oxidase activity. In gel filtration chromatography the dissociation and re-association of the protein were demonstrated on the basis of the retention times of the eluted protein peaks.

Absorbance and Fluorescence Studies of Proteins—Reaction mixtures containing 50 mM Tris buffer, pH 8.0 with varying re-associated protein concentration (5–25 μg/ml) were prepared. The protein sample was incubated with 10 mM *n*-heptanol and then absorbance at $UV_{280\,nm}$ (Cary 100Bio, Varian) was recorded for different time periods. The fluorescence study was carried out using spectrofluorometer (Fluoromax Jobin Vyon, Horiba) equipped with 450 nm excitation filter and 465–600 nm emission filter (excitation slit width 3 nm and emission slit width 6 nm, scan rate 0.5 nm/s). For fluorescence quenching study the re-associated protein samples was incubated with *n*-heptanol and FAD at 25°C. A control without the substrate was also studied simultaneously.

Analytical Methods—Protein estimation was done following Bradford method using BSA as standard (*31*). All the data presented in this study were the mean of three or more experiments with a variation within 10%.

RESULTS AND DISCUSSION

The microsomal alcohol oxidase protein purified through DEAE sepharose anion exchanger was reported to be lipoprotein in nature with high aggregating property to an average mean radius particle size of 180 nm (*14*). The aggregated multimeric protein when treated with β-ME at an optimum concentration of 0.74 M for 8 h at 4°C,

the protein was dissociated into smaller molecular masses as evident from the longer retention times of the eluted proteins P_2, P_3 and P_4 from the HPLC gel filtration column while untreated protein (marked as P_1) was eluted at low retention time (Fig. 1A). Hereafter, the untreated protein in all the analysis is termed as native protein. The dissociation was also evident from the loss of 180 nm (radius) protein particles in the treated fractions observed through DLS analysis. No significant distribution of particles with mean radius higher than
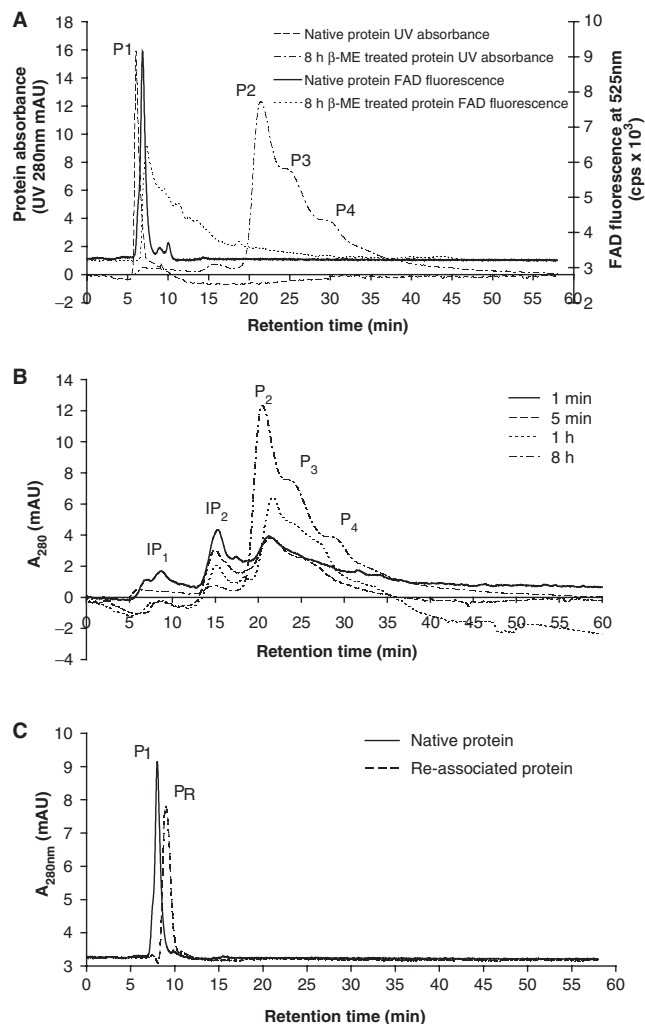

Fig. 1. **(A) Gel filtration chromatograms of native protein with and without treatment with β-ME. Primary (Y) axis: protein absorbance and secondary (Y) axis: FAD fluorescence intensity.** P_1: untreated native protein, P_2–P_4: β-ME dissociated protein peaks. (B) β-ME-mediated dissociation of alcohol oxidase at different incubation periods. IP_1 and IP_2: intermediate dissociated protein peaks. P_2–P_4: dissociated protein peaks. The intensity of 8 h treated sample was consciously multiplied by a factor of 0.2 to visualize other peaks of different incubation periods (1 min to 1 h). (C) Re-association studies of dissociated protein peaks using gel filtration chromatography. The dissociated peak fractions (P_2–P_4) obtained by β-ME-mediated dissociation were pooled, dialysed and concentrated, and then loaded on HPLC gel filtration column, a single peak (P_R) with lower retention time was generated.

4 nm was detected in the β-ME treated protein samples. The purified native protein, which is basically a lipoprotein, when stained with Nile red and observed under CLSM showed intense red fluorescent particles (Fig. 2A). However, no fluorescent particles were detected when the protein was treated with β-ME, indicating the dissociation of the native protein particles (Fig. 2B). No interference of β-ME on the fluorescence caused by the Nile red was observed. A control protein, HRP ($Mr = 45$ kDa), which do not have any hydrophobic domains and contains uniformly distributed medium size protein particles, did not show any fluorescence staining similar to the Fig. 2B.

FAD fluorescence recorded simultaneously during elution of the proteins through gel filtration with and without treatment with β-ME clearly showed deflavination of the dissociated proteins (Fig. 1A). A sharp fluorescent peak was observed when FAD was associated with the native protein, whereas the free FAD formed by deflavination of the protein showed a broad trailing fluorescence peak because of lack of its globular nature. After collecting the protein peaks the FAD fluorescence intensity of the dissociated protein fractions (pooled fraction from P_2 to P_4) and the un-dissociated protein (P_1) was recorded and found that nearly 87% of the total FAD of the native protein was detached while incubated with the β-ME. These results showed that the aggregated multimeric protein entity was simultaneously dissociated and deflavinated when incubated with β-ME at the concentration and time stated above.

At a low incubation time (<8 h) of the protein with β-ME, two additional intermediate dissociated protein peaks, IP_1 and IP_2 were detected (Fig. 1B). Concomitant increase in peak heights of P_2–P_4 with the reduction of IP_1 and IP_2 height upon increasing the β-ME treatment time was observed. This indicates that the aggregated multimeric protein was first dissociated into two intermediate states corresponding to the protein IP_1 and IP_2. The intensity of P_2 was increased at a rate of 0.06 mAU/min while incubated with the β-ME at the concentration stated above; whereas, the intensity of the IP_1 and IP_2 were decreased with increasing treatment time and vanished completely. We also observed that FAD was associated with these two intermediate protein peaks. Distribution of FAD in IP_1 and IP_2 were 72% and 28%, respectively, out of the total FAD (cps units) detected in these two proteins obtained after incubating the native protein with β-ME for a period of 5 min.

The dissociated protein fractions (P_2–P_4) were pooled and dialysed to remove β-ME and then concentrated. The concentrated fraction was loaded on the gel column and run under similar parameters as stated earlier. A single re-associated protein peak (P_R) was obtained and no additional peaks were detected in the subsequent elution (Fig. 1C). This indicates that upon removal of β-ME, the dissociated proteins were re-associated to a single multimeric protein entity. The inhibitory role of β-ME on re-association was confirmed by the fact that when the dissociated proteins were pooled, concentrated and then filtered through the gel column without removing the β-ME no protein peak corresponding to P_R was obtained, instead the peaks with retention time similar to P_2–P_4 were regenerated. The re-association process was
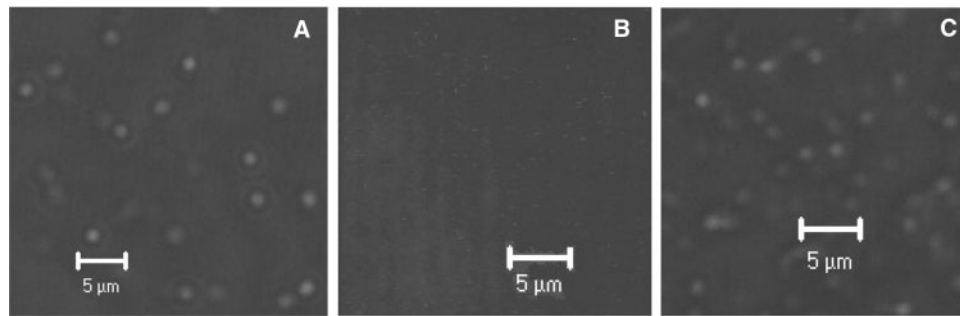
Fig. 2. **Dissociation and re-association studies of the protein particles with CLSM using fluorescence staining.** (A) The native protein. (B) Native protein incubated with β-ME. (C) Re-associated protein formed after removal of β-ME. Bar = 5 μM.

confirmed by CLSM study using Nile red staining, where intense red fluorescent protein particles were regenerated after removing β-ME from the dissociated protein fractions (Fig. 2C). Sequential removal of β-ME concomitantly increased the re-associated protein particles in the sample observed under CLSM following Nile red staining.

The dissociation and re-association process of the alcohol oxidase protein were also demonstrated by analysing the protein samples in native PAGE at 7% gel concentration. The retention factor (R_F) values of the protein bands corresponding to IP_1, P_R and purified native protein were nearly similar (0.10). The R_F value of the IP_2 protein band (0.46) was comparatively higher than the above proteins thus indicated lower molecular mass of the IP_2 protein than IP_1 and native proteins. The R_F values of the pooled protein fractions, P_2–P_4, (0.98) were far higher than the native protein and appeared immediately before the dye front due to the movement of these small protein masses together at low gel concentration. Results showed that the protein P_2–P_4 are the dissociated proteins formed from the purified native protein by the action of β-ME.

While computing the subunit masses of 85, 63, 43, 27 and 13 kDa for the total native protein molecular mass of 269 ± 5 kDa (14), probability of the presence of two 43 kDa subunits in the native protein was indicated. The molecular mass of the IP_1 was 267 kDa. The P_R protein is equivalent to IP_1 protein in native PAGE and gel filtration. These P_R and IP_1 showed enzyme activity as shown later, whereas, IP_2 did not show any activity. To investigate the molecular masses, these dissociated proteins were analysed by SDS–PAGE (Fig. 3). The β-ME treatment time for collecting the intermediate proteins entities, IP_1 and IP_2, was 5 min, and for the dissociated protein peaks (P_2–P_4) was 8 h. The protein IP_1 contained all except the 27 kDa along with an additional intense band at 25 kDa. Among these bands 85 kDa protein band was very weak and clearly visible only when the concentration of the loaded protein is high. The most likely reason for the formation of 25 kDa is the partial degradation of 27 kDa protein at the high β-ME concentration used for the incubation. The exact cause of this partial degradation is not yet known. The re-associated protein (P_R) also contained similar protein subunits like IP_1. The IP_2 fraction, which contained 85, 63, 25 (weak) 13 kDa (weak) bands, did not contain 43 kDa band. Thus, unlike IP_1, IP_2 did not contain all the subunit proteins
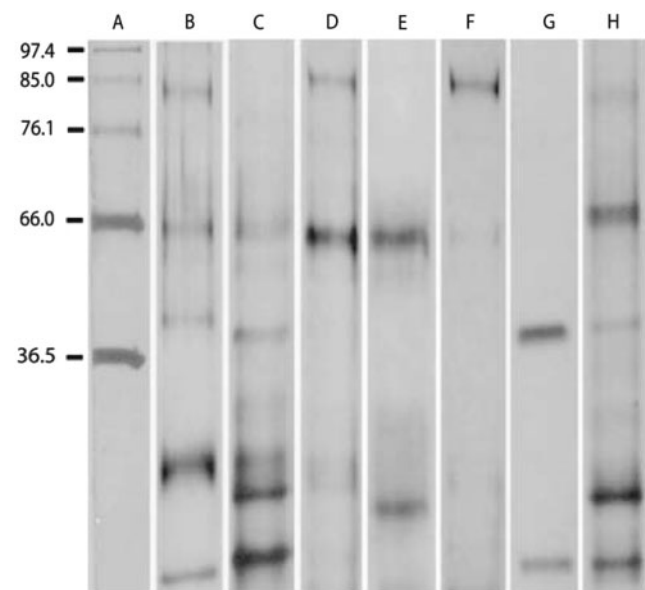

Fig. 3. **SDS–PAGE analysis of alcohol oxidase proteins.** (A) Molecular weight markers. (B) Native protein. (C) IP_1. D: IP_2. E: P_2. F: P_3. G: P_4. H: P_R.

present in the native protein; this is attributed as the possible cause for the lack of enzyme activity in this intermediate IP_2 protein. The band profile of the peaks is P_2, 66 and 25 kDa; P_3, 85 kDa; P_4, 43 and 13 kDa. During the process of elution through the gel column the concentration of β-ME goes down, which to a certain extent resuscitate native environment, thus likely to promote partial re-association of the dissociated proteins because of their inherent high aggregating tendency. As a result, the dissociated proteins were not entirely resolved in the fractions into single protein level. This dissociation study could not be accomplished through either cation or anion exchange chromatography because of the interference of the high concentration of β-ME on protein binding to the resins. While with hydrophobic interaction chromatography, all the β-ME dissociated proteins were eluted as an unresolved broad peak.

Both IP_1 and P_R had the enzyme activity, however, the activity of the P_R was obtained only when the protein was mixed with 50 μM FAD (since P_R was reconstituted

from the pooled fractions P_2 to P_4 that nearly void FAD) and incubated at 4°C with the respective substrates (10 mM) for at least 12 h. No enzyme activity was detected in IP_2 and other individual fractions P_2–P_4 (after collecting the central fractions of each and removing the β-ME by dialysis) even after prolong incubation with the FAD and substrates. It may be inferred from the above findings that the catalytic activity of this multimeric protein is lost upon dissociation. A comparative enzyme activity of the native protein, IP_1 and P_R with different substrates are shown in Table 1. The partial degradation of 27 kDa protein, as detected in IP_1 and P_R, did not completely arrest the enzyme activity of these proteins, though, the activity was reduced significantly. Above results also implied that the β-ME mediated dissociation is nearly a reversible process since the alcohol oxidase activity of the re-associated protein was regained, though, albeit lower than the native protein.

The flavin in flavoenzymes is known to mediate the transfer of electron from the redox centre to the terminal electron acceptor, which is oxygen in the present case, through its isoalloxazine ring during the catalytic process as reported in case of cholesterol oxidase (*32*). The event involves change of FAD emission fluorescence, which may be linked to the functional activity of this enzyme. When the native or IP_1 protein was treated with the substrate (10 mM n-heptanol), the emission intensity of FAD was quenched to nearly 90% with quenching rate, $K_{obs} \approx 2.7$–$3 \, \text{min}^{-1}$ immediately after addition of the substrate to the reaction media. However, when the FAD emission of P_R was studied by mixing it with FAD (50 μM) (since P_R was prepared from the fractions that nearly void FAD) and substrate a prolong incubation time (minimum 12 h) was required to exert the similar changes ($K_{obs} \approx 0.03 \, \text{min}^{-1}$) (Fig. 4). No such change of FAD fluorescence emission of P_R was observed in absence of the substrate. This showed that reconstitution of the FAD to the apoenzyme (P_R) is induced by the substrate and this induction is a slow process. To investigate the conformational change of the alcohol oxidase during substrate induced reconstitution of FAD, $UV_{280 \, \text{nm}}$ absorptions were recorded by varying protein concentration at a saturated substrate concentration and FAD (50 μM). The absorbance was decreased at a level of 0.0038 AU/μg protein when incubated the mixture for 30 min and at 25°C. No significant change of native protein absorption upon incubating with the substrate was observed. This change in $UV_{280 \, \text{nm}}$ absorbance is probably due to the change in conformations evolved in the proper reorganization of the subunits during the substrate-induced reconstitution of the FAD that resulted in catalytically active alcohol oxidase protein. The drastic change of conformation of alcohol oxidase protein during FAD release was also demonstrated by other (*33*). Contrary to the decrease in absorbance during the reactivation, many folds increase in the absorbance during the process of dissociation was observed (Fig. 1B). For instance the $UV_{280 \, \text{nm}}$ absorbance of the dissociated protein P_2 was nearly 5-fold higher than IP_1 at their respective highest absorbance. This dissociation process likely to expose the UV-absorbing polar aromatic amino acid residues of the subunits in this membrane-bound

Table 1. **Comparison of alcohol oxidase specific activity of the native, intermediate dissociated protein (IP_1) and re-associated protein (P_R).**

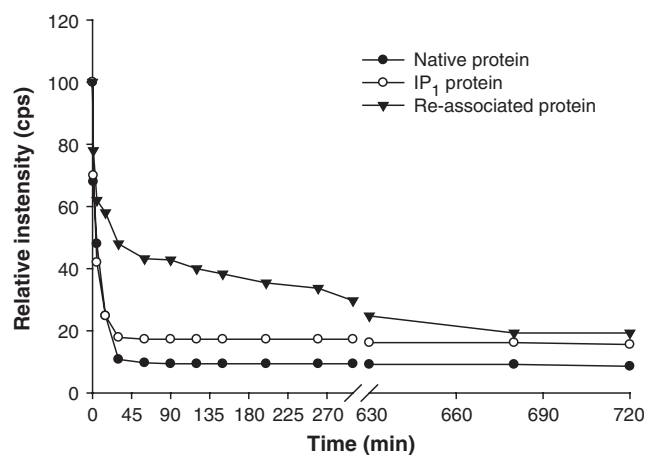| Alcohol substrate	Specific activity (U/mg protein)		
	Native protein	IP_1 protein	P_R protein
n-Heptanol	305 ± 0.3	190 ± 0.8	96 ± 1.3
n-Dodecanol	62 ± 0.1	49 ± 0.3	42 ± 0.5
Phenyl-3-propanol	250 ± 0.2	183 ± 1.0	45 ± 0.2
$R(-)$2-octanol	63 ± 0.3	37 ± 0.1	Trace

Fig. 4. **FAD fluorescence quenching study of native protein, IP_1 and P_R proteins after incubating with the substrate**. P_R was additionally pre-treated with 50 μM FAD.

multimeric protein thus increased the absorbance; whereas, substrate-induced reconstitution process buried these polar aromatic amino acids to the core of this hydrophobic protein that resulted in decrease the absorbance. Highly hydrophobic nature of this membrane protein is demonstrated earlier (*14*).

Deflavination and dissociation of the protein entity were studied separately using deflavinating agents, KBr (*28*), and TCA (*33*), and dissociating agents, glycerol (*24*), and DMSO (*25*), following the similar parameters reported in these protocols. The extent of deflavination of the purified native protein obtained with KBr and TCA was 24% and 60%, respectively. DMSO was ineffective in dissociating this alcohol oxidase, rather, 50% DMSO was inhibitory to this enzyme as no residual activity of the native enzyme was detected after treatment with this reagent. Ethylene glycol, a structurally similar compound to β-ME, could not dissociate the protein even at its molar concentrations. Unlike β-ME, no inhibitory effect of ethylene glycol on the enzyme activity was observed. Thus, we are reporting the role of β-ME as both dissociating and deflavinating agent for this multimeric protein. β-ME treatment could dissociate nearly 87% flavin from the purified protein entity as demonstrated by us.

That the subunit proteins of this multimeric alcohol oxidase are not linked by disulphide bond was demonstrated by us earlier since the SDS–PAGE separated the subunits of the protein alike with or without treating the sample with β-ME (*14*). Thus, breakage of inter-peptide

disulphide bonds is not the cause for the dissociation of this multimeric protein. The β-ME likely to destabilize the multimeric protein under the anaerobic and partially non-polar microenvironment created by its high concentration used in this investigation that promote the sub-unit detachment and eventual dissociation of this lipoprotein. The solvent effect of β-ME at high concentration is an established fact demonstrated in several cases (34, 35). This assumption is justified by the findings that removal of the β-ME from this dissociated fraction triggers the re-association of the subunit protein that led to the formation of the P_R protein. Expulsion of the solvent led to the preferential hydration and interaction among the subunits that simultaneously promote the re-association of the subunit proteins. Sequential removal of the β-ME by dialysis concomitantly increase the re-association as evident from the CLSM studies of the protein stained with Nile red.

SUPPLEMENTARY DATA

Supplementary data are available at *JB* online.

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CONFLICT OF INTEREST

None declared.

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