

Article

Understanding (*R*) Specific Carbonyl Reductase from *Candida parapsilosis* ATCC 7330 [CpCR]: Substrate Scope, Kinetic Studies and the Role of Zinc

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Abstract: CpCR, an (*R*) specific carbonyl reductase, so named because it gave (*R*)-alcohols on asymmetric reduction of ketones and ketoesters, is a recombinantly expressed enzyme from *Candida parapsilosis* ATCC 7330. It turns out to be a better aldehyde reductase and catalyses cofactor (NADPH) specific reduction of aliphatic and aromatic aldehydes. Kinetics studies against benzaldehyde and 2,4-dichlorobenzaldehyde show that the enzyme affinity and rate of reaction change significantly upon substitution on the benzene ring of benzaldehyde. CpCR, an MDR (medium chain reductase/dehydrogenase) containing both structural and catalytic Zn atoms, exists as a dimer, unlike the (*S*) specific reductase (SRED) from the same yeast which can exist in both dimeric and tetrameric forms. Divalent metal salts inhibit the enzyme even at nanomolar concentrations. EDTA chelation decreases CpCR activity. However, chelation done after the enzyme is pre-incubated with the NADPH retains most of the activity implying that Zn removal is largely prevented by the formation of the enzyme-cofactor complex.

Keywords: MDR—medium-chain reductase/dehydrogenase; ADH—alcohol dehydrogenase; enzyme kinetics; EDTA (Ethylenediaminetetraacetic acid) chelation; ultrafiltration

1. Introduction

The MDR superfamily is a part of the oxidoreductase class and contains a family of zinc-dependent alcohol dehydrogenases [1]. MDRs are hypothesized to have evolved from SDR (short-chain reductases/dehydrogenases) superfamily and later incorporated zinc atoms within themselves to facilitate divergence in catalytic abilities [2]. CpCR belongs to MDR superfamily and is reported to catalyse reductions of ketoesters, ketones and aldehydes leading to the production of some important pharmaceutical precursors [3]. It is one of the important enzymes present in *Candida parapsilosis* ATCC 7330, which is a well-known whole-cell biocatalyst [4]. CpCR, a heterodimer (PDB: 4OAO), has two different Zn atoms viz. catalytic Zn and the structural Zn. The former is coordinated to two Cys, His and a water while the latter is coordinated to four Cys residues and lies away from the active site. Aldehyde reduction by various ADHs from horse liver, human liver and *Saccharomyces* sp. is well established [5–7]. CpCR reduces aliphatic and aromatic aldehydes with higher activity compared to other carbonyl substrates.

Even though a lot of literature on understanding the role of Zn in MDR superfamily exists [8–17], still there is some ambiguity in the function of structural Zn [8,9,16,17]. Chelation studies with multi-dentate ligands, like EDTA and 1,10-phenanthroline on ADHs, indicate that they significantly affect the activity by chelating one of the Zn atoms [9,17,18]. Dithiothreitol (DTT) at higher

concentrations is known to cause heat lability of yeast ADH (YADH) by changing the Zn stoichiometry in the enzyme [9]. Cofactor binding to the liver ADH (LADH) induces a large conformational change where the two domains (catalytic and cofactor binding domains) rotate around 10 degrees to close the active site cleft [19]. A similar observation was made in alcohol dehydrogenase from *Arabidopsis thaliana* but the mechanism is different from that of LADH [20]. It is also established that the conformational change induced by cofactor binding requires the presence of the nicotinamide part of NAD(P)H, while the binding of ADP-ribose does not induce such a change [21]. Recently, cofactor binding to various ADHs was studied using circular dichroism wherein the orientation of nicotinamide ring of the cofactor at the active site could be observed [22]. Another study on cofactor binding shows that NAD⁺ and NADH adopt different structures in water, but both fit in the enzyme's active site in a semi-extended conformation [23]. These studies are essential in understanding the initial step (binding of the cofactor to the active site) of the reactions catalysed by NAD(P)H-dependent ADHs. Cofactor switching is also an important aspect in obtaining enzymes with better catalytic ability and applications in metabolic engineering [24–27]. However, the effect of this changed configuration upon cofactor binding on enzyme activity has not been probed systematically to date. This is of importance because in nature most enzymes exist bound to their natural cofactor as evidenced by typically low K_d values of the cofactor [22].

In this study we used various concentrations of EDTA for chelation studies against CpCR and employed ultrafiltration for rapid removal of EDTA. To the best of our knowledge this is the first report to elucidate the kinetic characteristics of cofactor-enzyme complex.

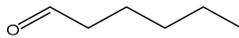
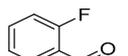
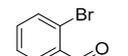
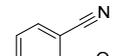
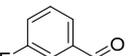
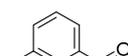
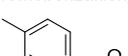
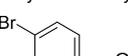
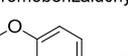
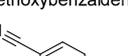
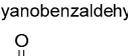
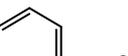
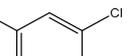
2. Results and Discussion

2.1. Purification of CpCR, Expanding Its Substrate Scope and Kinetic Studies

The purification protocol was modified, keeping in mind the yield and the stability of the enzyme. Compared to the previous protocol [28], the modified protocol increased the yield ten times and fold purification by 3.7 times. CpCR, an MDR, is a Zn-containing enzyme and it is very important that the Zn coordination stays unaffected by the buffer conditions in which it is purified/stored. Earlier purifications of CpCR had DTT in the buffers to maintain a reducing environment for the four free cysteine residues present in the enzyme. DTT, a reducing agent is known to reduce the Cys residues coordinated to the zinc and release it [9]. CpCR and YADH belong to the same MDR superfamily. Thus, the enzyme was purified without the addition of DTT in the buffering system and the effects were clear with the increase in the activity by more than three times. The presence of MgCl₂ in the storage buffer was also omitted as the Mg ion does not have any significant interactions with the protein surface (PDB: 4OAQ). HEPES replacing Tris-HCl buffer was based on the fact that the pH of the Tris buffer is sensitive to changes in temperature.

Our previous study reported an asymmetric reduction of ketones and ketoesters by CpCR, but the activity with aldehydes was better [3]. Thus, in this work, a detailed study of aldehydes, i.e., aliphatic and various substituents of benzaldehyde as the substrates for CpCR, was carried out. Aldehyde reduction is NADPH-specific. Aliphatic aldehydes show 60–70% activity as that of benzaldehyde (Table 1). Any substitution on any position of benzaldehyde decreases the activity, due to electronic and steric effects. 2, 4-dichlorobenzaldehyde (substrate 15) shows the least activity. Among the *ortho* and *para* substituted benzaldehydes, electron withdrawing groups like NO₂ and CN (substrates 13 & 12) show less activity as they can destabilise the benzene ring. Bromo and fluoro substitutions along with electron donating groups such as CH₃ and O-CH₃ at *ortho* and *para* positions (substrates 4, 9, 10 and 11) show comparatively better activity than substrates which contain electron-withdrawing groups (substrates 6, 12 and 13). It is expected that substitutions on *ortho* and *para* positions behave similarly but in the case of *ortho* bromo (substrate 5), steric effects dominate. Substrate 9 shows comparatively better activity than substrates 10 and 11 due to the presence of the smaller methyl group.

Table 1. The specific activity of CpCR against various aldehydes that were not reported earlier.

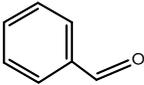
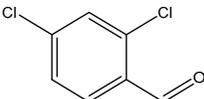
Entry	Substrate	Specific Activity (U mg ⁻¹) ¹
1	 Propanal	24.98 ± 1.06
2	 Pentanal	19.29 ± 0.59
3	 Hexanal	21.9 ± 0.64
4	 o-Fluorobenzaldehyde	23.06 ± 0.94
5	 o-Bromobenzaldehyde	6.76 ± 0.63
6	 o-Cyanobenzaldehyde	21.17 ± 0.73
7	 m-Fluorobenzaldehyde	6.53 ± 0.82
8	 m-Bromobenzaldehyde	20.79 ± 0.76
9	 p-Methylbenzaldehyde	26.46 ± 0.92
10	 p-Bromobenzaldehyde	21.0 ± 0.53
11	 p-Methoxybenzaldehyde	19.69 ± 0.97
12	 p-Cyanobenzaldehyde	18.63 ± 0.72
13	 p-Nitrobenzaldehyde	13.35 ± 0.69
14	 Benzaldehyde	33.96 ± 1.02 ²
15	 2,4-dichlorobenzaldehyde	3.8 ± 0.64

¹ One unit of the enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of NADPH per minute at 30 °C. ² Previously reported [3]. Put here for comparative analysis with other substrates.

The kinetic parameters, i.e., enzyme affinity and the catalytic rates of CpCR with two different substrates (benzaldehyde and 2,4-dichlorobenzaldehyde) show drastic differences in activity (Table 2).

The dramatic decrease in activity with 2,4-dichlorobenzaldehyde could be because of its poor fit in the substrate cleft as reflected in >20 fold higher K_m value as compared to that of benzaldehyde. A similar decreased activity can be seen in case of the *ortho* and *para* substituted benzaldehydes (Table 1). The presence of two Cl substitutions (inductive electron withdrawing groups) in 2,4-dichlorobenzaldehyde cause significant instability of the benzene ring which does not favour the reduction of the carbonyl group. Thus, both electronic and steric factors can explain the low activity of compound seen in entry 15, Table 1. Overall, the affinity of CpCR towards benzaldehyde decreases with increase in substitution on the benzene ring.

Table 2. Kinetics of CpCR.

Entry	Substrate	V_{max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$)	K_m (mM)
1	 Benzaldehyde	34 ± 0.9	0.29 ± 0.04
2	 2,4-dichlorobenzaldehyde	3.88 ± 0.56	7.14 ± 0.04

2.2. Oligomeric State of CpCR

The crystal structure of CpCR (PDB: 4OAQ) shows that the enzyme is a hetero dimer. SDS-PAGE indicates the presence of a 40 kDa sub unit in the buffer [19]. Gel filtration chromatography data indicates that CpCR is indeed a dimer of 80 kDa (Figure 1A) and remains so even at higher concentrations (Figure 1B). SRED from *Candida parapsilosis* ATCC 7330 exists in other oligomeric states unlike CpCR [29].

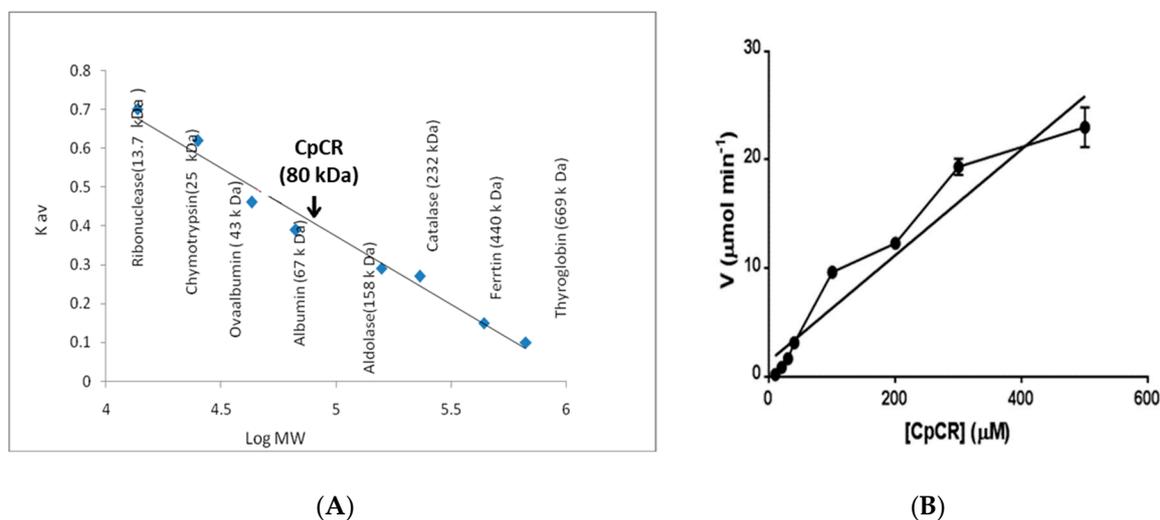


Figure 1. Oligomeric state of CpCR. (A) A plot of Log Mol. Wt. vs. K_{av} shows the calibration curve of the known standards including and the molecular weight of CpCR; (B) A plot of velocity of reaction vs. micro molar concentration of CpCR.

2.3. Effect of Chelating Agent EDTA and Divalent Metal Salts on Activity of CpCR

2.3.1. Effect of Time on Chelation

Addition of EDTA to the enzyme solution in a 1:3 enzyme: EDTA mole ratio, resulted in a drop of the specific activity of the enzyme immediately by 30% (from around 36 U/mg to 25 U/mg). Prolonged

incubation of the mixture for up to 120 min showed that the specific activity remained around 25 U/mg for the entire duration. Furthermore, this experiment when repeated with a four-fold increased EDTA concentration (1:12—enzyme: EDTA mole ratio) over a lesser duration of 30 min showed the exact same trend (Figure S1). This indicates that EDTA binding to zinc in the enzyme is a fast process, and since the equilibrium is established, prolonged incubation is unnecessary.

2.3.2. Removal of EDTA

Different amounts of EDTA were incubated with a fixed concentration of enzyme in order to determine the effective concentration of EDTA necessary to remove zinc from the enzyme. However, it was found that despite the wide range of mole ratio tested, the specific activity of the protein samples containing EDTA remained the same (Figure S2). This was indicative of the fact that although EDTA binds to the enzyme quickly, it has to be removed in order to remove the zinc. This is consistent with what has been reported in literature [11,17,18].

The specific activities obtained after EDTA removal from samples by dialysis shows a decreasing trend in specific activity with increasing EDTA content (results not shown), indicating that the EDTA removal is necessary for zinc removal. A similar trend was observed in CpCR2 where the loss of activity of the enzyme is a function of loss of catalytic Zn [17]. However, the dialysis method was time consuming (12 h) and not feasible for this enzyme as it is not very stable. The specific activities obtained after EDTA removal from samples by ultrafiltration shows a similar decreasing trend using dialysis (Figure 2A). This method takes 3 h, and is likely to be associated with a gradual loss of specific activity which has to be taken into account. The protein recovery from this method is very high and, therefore, this method was optimized for use in future experiments.

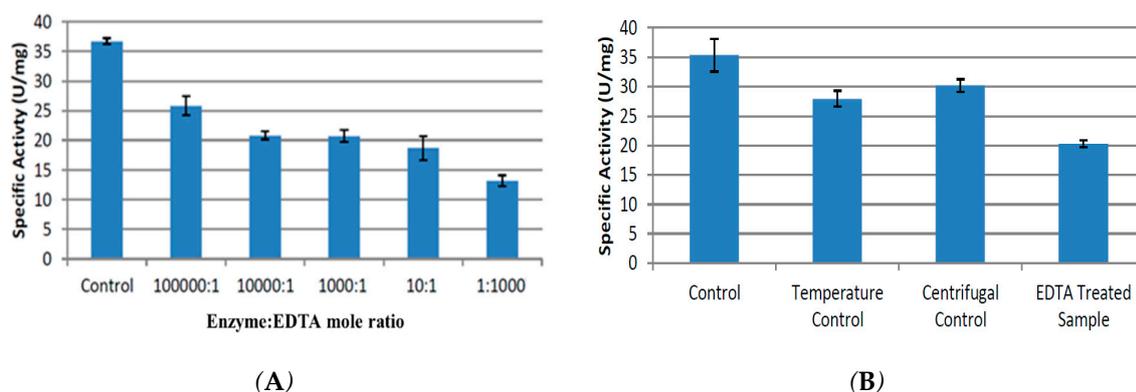


Figure 2. Activity of CpCR after chelation with EDTA (A) Effect of EDTA on CpCR activity at various concentrations; (B) Change in specific activity of CpCR before and after incubation at 4 °C.

Prolonged incubation during the EDTA removal on the enzyme sample was also studied. Two controls were designed to check spontaneous loss of activity—a temperature control that was placed at 4 °C for three hours and a centrifugal control that was placed in the filtration unit without EDTA treatment. The specific activity values were measured for all the controls and a sample treated with 10:1 enzyme:EDTA (Figure 2B). It was seen that there is a decrease of specific activity from 35 U/mg to 30 U/mg due to the three-hour incubation. However, the loss due to EDTA treatment was much more significant.

2.3.3. Inhibition of CpCR by Divalent Metal Salts

Attempts were made to restore specific activity of the enzyme samples treated with EDTA by addition of zinc chloride. Surprisingly, it was found that the addition of external zinc decreased the specific activity of not only the EDTA treated sample but also of the control sample without any EDTA (Figure 3A). This has been previously reported for carboxypeptidase-A and could be caused

by bridging of the water molecule bound to the catalytic zinc to the external zinc thereby preventing substrate entry [30]. It was confirmed that this phenomenon is not just specific to zinc but to divalent ions of size similar to zinc as shown in Figure 3B. Reports on inhibition of ADHs by divalent metal ions suggest that the inhibition can be pH dependent and the mechanism mainly involves the replacement of native metal ion present in the catalytic site or by the coordination of added divalent metal ions with the sulphhydryl groups of the enzyme. The inhibition can be reversed by addition of EDTA to remove the excess Zn ions [31–34]. Currently we are investigating the mechanism of inhibition of CpCR by such divalent metal salts.

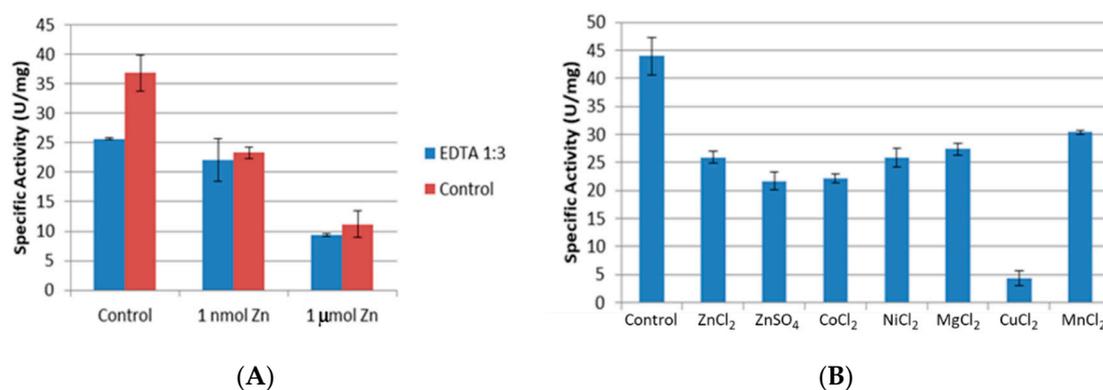


Figure 3. Effect of Zn²⁺ and other divalent metals on activity of CpCR. (A) Effect of Zn²⁺ (ZnSO₄) on CpCR activity at various concentrations; (B) Specific activity of CpCR influenced by various divalent metal ions.

2.4. Cofactor Pre-Treatment Prevents CpCR Activity Loss

Pre-treatment of enzyme samples with cofactors NADPH/NADP⁺ prevented the loss of specific activity upon EDTA treatment in a concentration-dependent manner (Figure 4A). This is also reported with 1,10-phenanthroline when YADH is pre-incubated with the cofactor [18]. It was seen that pre-treatment with 1 mM NADP⁺ gave 50% more specific activity as compared to the EDTA treated sample without cofactor treatment, while 2 mM NADPH retained the entire specific activity of the initial control. This may be due to retaining zinc and not allowing EDTA to access it possibly a result of structural changes due to cofactor binding.

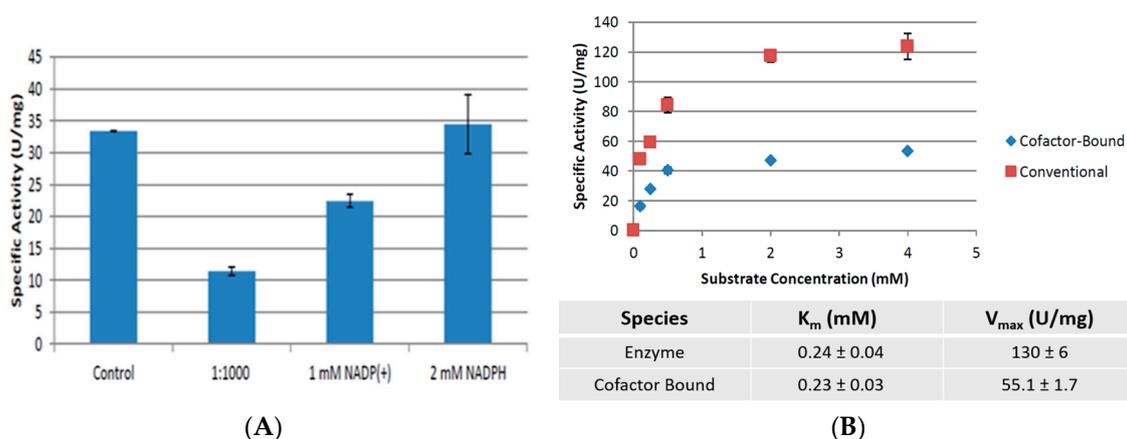


Figure 4. Effect of NADPH incubated CpCR on its activity. (A) Effect of incubation of different concentrations of NADPH on CpCR activity; (B) Kinetics of CpCR, NADPH bound vs. unbound.

The kinetics of the cofactor-bound enzyme were determined by the enzyme obtained from the modified purification protocol presented in this study with changes in the kinetic parameters duly noted. It was observed that the K_m value for both cofactor bound and unbound CpCR remained

more or less constant at 0.23 mM, implying that substrate binding remained largely unchanged in the cofactor-bound enzyme. However, there was a two-fold decrease in V_{\max} of cofactor-bound enzyme (Figure 4B). The structure of CpCR is significantly similar to LADH (PDB: 1HLD) with a p -value of 1.41×10^{-13} [35]. The decreased V_{\max} value may also be attributed to the structural changes in the cofactor bound enzyme resulting in narrowing of the catalytic cleft and hindering the entry of the substrate [36]. Overall, the conversion of the *apo* enzyme to the *holo* form seems to affect the rate of the reaction significantly even though the enzyme affinity towards the substrate is retained.

3. Materials and Methods

3.1. Chemicals and Media

All the chemicals and media were purchased from SRL, Chennai, India and HiMedia, Mumbai, India. AKTA protein purification system and GST affinity column were purchased from GE Healthcare Life Sciences, Bangalore, India. The ultra-centrifugal filters were obtained from Merck, Mumbai, India.

3.2. Enzyme Expression and Purification

The overexpression and purification of CpCR were performed as per the reported methodology using GST affinity chromatography [28]. For all the experiments, except cofactor binding studies, the enzyme obtained from this protocol was used. Slight modifications to the protocol were done to obtain better yield and stability of the enzyme. They include: 1. Removal of DTT and $MgCl_2$ from all the buffers and replacing the Tris HCl with HEPES buffer. 2. Cell disruption was done using a 150 W ultra-sonicator. 3. The cleared lysate was loaded onto the GST column at a flow rate of 1 ml min^{-1} . 4. The use of a superdex column was skipped. The yield and fold purifications were obtained by checking the specific activity of CpCR against benzaldehyde.

The composition of the buffers used are as follows: Equilibration and wash buffer: 50 mM HEPES pH 7.5, 500 mM NaCl, 2.5% glycerol; Elution buffer: 50 mM HEPES pH 7.5, 20 mM Glutathione; Desalting buffer: 20 mM HEPES pH 7.5, 50 mM NaCl.

3.3. Specific Activity, Substrate Scope and Kinetic Studies of CpCR

The protocol used for determining the specific activity of the enzyme against different substrates was the same as reported previously [3]. The activity of CpCR against benzaldehyde substituents and aliphatic aldehydes was checked. Substrate concentrations varying from 0–4 mM were used to determine the specific activity of CpCR and the Lineweaver–Burk plot gave the K_m and V_{\max} of CpCR against the specific substrates.

3.4. Oligomeric State of CpCR

Gel filtration chromatography was done to find out the oligomeric state of CpCR [37]. Mixture of standard proteins containing ribonuclease (13.7 kDa), chymotrypsin (25 kDa), ovalbumin (43 kDa), albumin (67 kDa), aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa) were used to calibrate the Sephadex 200 HR column. A plot of Log Mol. Wt. and K_{av} was made to calculate the molecular weight of CpCR.

A plot of velocity ($\mu\text{moles min}^{-1}$) vs. concentration of CpCR (μM) was made to find the presence of higher oligomeric state of CpCR at its higher concentrations of up to 500 μM .

3.5. Treatment of CpCR with EDTA, Divalent Metal Salts

Benzaldehyde was used as the substrate to check the activity of CpCR against the effects of EDTA and other metal salts. The mixture containing the ratio of 1:3 and 1:12 (number of moles of CpCR to the number of moles of EDTA) was checked for the activity instantaneously and compared to the untreated CpCR. Another experiment with the same mixtures incubated for up to two hours at 4 °C with the activity checked every 15 min from the start of incubation was also done. Additionally, different ratios

of the number of moles of CpCR to EDTA were tried to see the instantaneous effect on the activity of CpCR.

Ultrafiltration was done to remove the low Mol. Wt. EDTA from the above mixtures to later determine the change in the activity of CpCR. The chelator treated protein (100–500 µg) in a volume < 500 µL was placed in 0.5 mL filter (Amicon, 10 kDa mol. wt. cutoff) and the volume was made up to 500 µL using a desalting buffer. The samples were then concentrated at 14,000 g at 4 °C for 30 min, following which the volume was again made up to 500 µL. This process was repeated for a total of six times over three hours. At the end of the process, the enzyme was recovered and its concentration was determined by Bradford's method [38] followed by its activity assay.

The addition of micro- and nanomolar concentrations of ZnSO₄ to the EDTA treated CpCR was also studied. The metal salt in question was directly added to the assay solution from a 1 M stock to obtain the desired concentration after addition of substrate and enzyme to determine the activity spectrophotometrically. Effects of divalent metal salts like CoCl₂, NiCl₂, MgCl₂, CuCl₂, MnCl₂ and ZnCl₂ at nanomolar concentrations on the CpCR activity were noted.

3.6. CpCR—Cofactor Binding Studies

For this study, the modified protocol for purification was used. NADPH was added to 100 µg of the protein from 10 mM and 20 mM stock of cofactor to a final concentration of 1 mM and 2 mM respectively, and the solution was incubated at 4 °C for one hour. Following this, the sample was treated with EDTA maintaining a 1:1000 enzyme: EDTA mole ratio. The EDTA was removed by ultra-centrifugation, and the specific activity of the sample was determined. Kinetic studies of CpCR against benzaldehyde were compared with the CpCR pre-incubated with 2 mM NADPH.

4. Conclusions

CpCR substrates include substituted benzaldehydes and aliphatic aldehydes. Substituted benzaldehydes showed lower activity as compared to benzaldehyde. The oligomeric state of the enzyme was confirmed to be dimeric at all concentrations, in agreement with the crystal structure. In chelation studies with EDTA, a decrease in enzyme activity with an increase in EDTA concentration is seen after removal of the EDTA from the solution by ultrafiltration. All the divalent metal ions inhibit the activity of CpCR even at nanomolar concentrations. The protocol for CpCR purification was modified to obtain 10 times more yield and > 3-fold purification and used in studying the cofactor binding studies. The pre-incubation of CpCR with cofactor makes the enzyme resist the Zn removal by EDTA chelation and retains activity. The *apo* and *holo* forms of CpCR do not differ in their affinity towards benzaldehyde but differ in their reaction rates.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4344/9/9/702/s1>; Figure S1. Time study of chelation; Figure S2. Concentration based chelation without removing the chelator.

Author Contributions: Conceptualization, A.C., V.K.K. and D.C.; Experiments carried out by, V.K.K. and D.C.; Formal analysis, A.C., V.K.K. and D.C.; Writing—original draft preparation, V.K.K.; Writing—review and editing, A.C.; Supervision, A.C.

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Conflicts of Interest: The authors declare no conflict of interest.

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