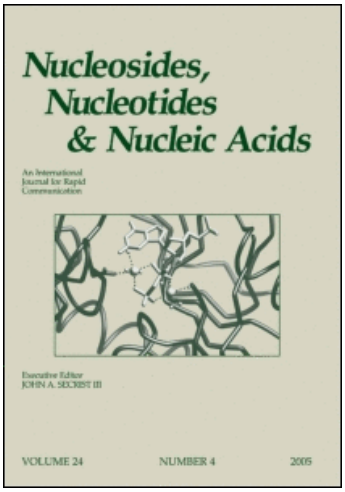

Nucleosides, Nucleotides and Nucleic Acids

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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Online publication date: 09 August 2003

To cite this Article Vincenzetti, S. , Costanzi, S. , Cristalli, G. , Mariani, P. , Quadrini, B. , Cammertoni, N. and Vita, A.(2003) 'Intersubunit Interactions in Human Cytidine Deaminase', Nucleosides, Nucleotides and Nucleic Acids, 22: 5, 1535 — 1538
To link to this Article: DOI: 10.1081/NCN-120023028
URL: http://dx.doi.org/10.1081/NCN-120023028

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS
Vol. 22, Nos. 5–8, pp. 1535–1538, 2003

Intersubunit Interactions in Human Cytidine Deaminase

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ABSTRACT

In order to design new efficient cytidine based drugs, an intersubunit interactions study related to the active site has been performed on the wild-type cytidine deaminase (CDA) and on the mutant enzyme F137W/W113F. F137 is the homologous to the *Bacillus subtilis* CDA F125 involved in the subunit interactions. In presence of the dissociating agent SDS, wild-type human CDA dissociate into enzymatically inactive monomers without intermediate forms via a non-cooperative transition. Extensive dialysis or dilution of the inactivated monomers restores completely the activity. The presence of the strong human CDA competitive inhibitor 5-fluorozebularine disfavour dissociation of the tetramer into subunits in the wild-type CDA but not in mutant enzyme F137W/W113F.

Key Words: Subunit; Human; Cytidine deaminase.

Human cytidine deaminase (CDA, EC 3.5.4.5) is a tetrameric enzyme involved in the pyrimidine salvage pathways, composed of identical 15 kDa subunits[1] each containing an essential zinc atom in the active site coordinated by three cysteine

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DOI: 10.1081/NCN-120023028
1525-7770 (Print); 1532-2335 (Online)

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residues[2] (C65, C99, C102). Recent studies[3] showed that the residues F137 and F36 may be important in stabilizing hydrophobic interaction between the ligand and the enzyme and thereby facilitate the catalytic process. These studies have been useful to clarify the role of specific amino acid residues in the active site of human CDA, but more information are needed to understand the catalytic mechanism and the molecular organization of the human enzyme. The knowledge of CDA mechanism of catalysis could be very useful to increase the efficacy of the cytidine based drugs, for example designing new molecules not deaminated and therefore not pharmacologically inactivated by cytidine deaminase.

The aim of the present study was the identification of the residues involved in the intersubunit contacts and their eventual role in the assembly of the active site(s) in order to understand the structure-function relationship of the tetrameric enzyme. At this purpose the four subunits of human cytidine deaminase were dissociated by adding small amount of SDS, therefore changes in the catalytic activity and in the hydrodynamic properties of the enzyme were followed. In *B. subtilis* CDA,[4] it was demonstrated that residue F125 (homologous to F137 of the human CDA) is quite close to the subunit interface of each dimer forming the tetrameric structure. Since amino acid replacement at the subunit interface may affect the enzyme function we investigated the effect of the mutation F137W on the dissociation process.

RESULTS AND DISCUSSION

A time course of the inactivation of CDA enzyme by low concentration of SDS (ranging from 0.35 to 1.73 mM) was performed, in order to reach different molar SDS/enzyme ratios. The inactivation of both wild-type and the F137W/W113F mutant enzyme was non-cooperative. This agrees with earlier kinetic data, which indicated that each subunit acts independently of the other.[1] Dissociation of the enzyme into monomers results in complete loss of activity, but full retention of the catalytical zinc ion. Wild-type CDA reached complete inactivation at a molar SDS/enzyme ratio of about 800, whereas the F137W/W113F mutant enzyme seemed more sensitive and was completely inactivated at a molar SDS/enzyme ratio of 250 (data not shown). Size-exclusion HPLC of human wild-type CDA at increasing concentrations of SDS also indicated a dissociation of the enzyme to monomers. Up to 0.35 mM SDS (SDS/CDA ratio = 22) the enzyme is present as an active tetramer. By increasing the SDS concentration, the 280 nm absorbance peak shifted to the position of the monomer, with a simultaneous decrease in the enzymatic activity indicating that the monomeric form of wild-type CDA is completely inactive (Fig. 1).

The dissociation was reversible as extensive dialysis or dilution of the SDS-treated sample with buffer without SDS restores completely the enzyme activity. The dissociation of human CDA into subunits was also studied by 15% PAGE performed at different SDS concentrations. The wild-type CDA was a tetramer up to a molar SDS/enzyme ratio of 61 and complete its dissociation into monomer at an SDS/enzyme ratio of 616. With the F137W/W113F mutant enzyme the dissociation process was significantly faster: at an SDS/enzyme ratio of 31, the mutant enzyme was present only in the tetrameric form, whereas it existed completely as monomer at an SDS/enzyme ratio of 154 (data not shown). Semi-quantitative analysis of the

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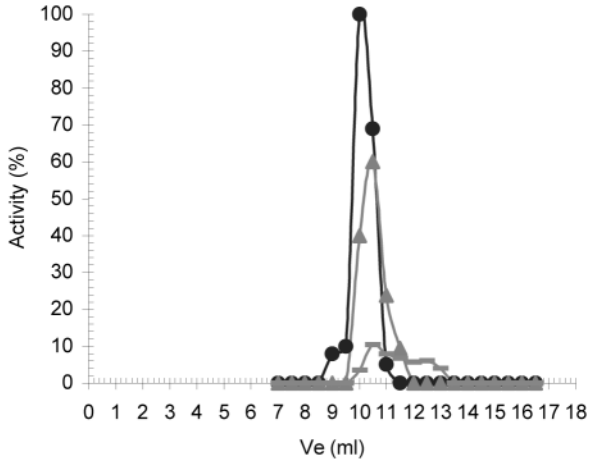

Figure 1. Gel filtration of wild- type CDA in presence of various SDS concentrations: (●) no SDS and 0.35 mM SDS; (▲) 0.52 mM SDS; (–) 0.7 mM SDS.

PAGE images by the QUANTISCAN software allowed the calculation of the SDS/enzyme ratio that caused 50% dissociation of the tetrameric form of the enzyme into monomer. As shown in Fig. 2a this value was 316 for the wild-type cytidine deaminase and 69 for the mutant enzyme F137W/W113F.

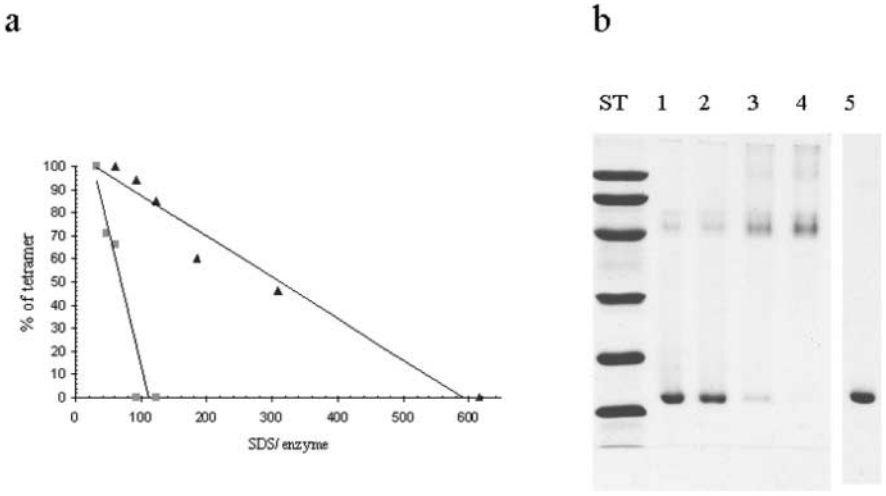

Figure 2. a) Percent of the enzyme present as the tetrameric form at various molar SDS/enzyme ratios Wild-type CDA (●), F137W/W113F mutant enzyme (▲). The results were obtained by scanning a series of 15% PAGE performed at different SDS concentrations. b) Effect of F-ZEB on SDS-promoted dissociation of tetrameric human CDA. St: standards (Bio-Rad); 1,2,3,4, wild-type CDA in the presence of 0.06; 0.6; 6; 60 μM of F-ZEB; lane 5: mutant enzyme in the presence of 60 μM of F-ZEB.

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The presence of the strong CDA competitive inhibitor 5-fluoro-zebularine (F-ZEB, 5-fluoropyrimidine-2-one ribonucleoside) affects wild-type CDA subunit dissociation. In the wild-type CDA in fact at a molar SDS/enzyme ratio of 200 is predominantly present the monomeric form, but addition of increasing amount of F-ZEB ranging from 0.06 to 60 μM, resulted in the disappearance of the monomer and increased amounts of the tetrameric form (Fig. 2b). This may indicate that a residue(s) involved in creating the active site may also participate in subunit interaction. On the other hand the presence of F-ZEB did not affected the mutant enzyme F137W/W113F: this suggest that F137 from one subunit may contribute to the active site of another subunit.

CONCLUSION

Our results indicate a structural involvement of F137 in the contact between subunits in human CDA. This assumption was confirmed also by our structural model of human cytidine deaminase, based on the know crystal structure of *E. coli* CDA[5] (see Costanzi et al., this issue), in which is shown that F137 residue from subunits C contributes to the generation of the uridine-binding pocket and its location between subunits A and C explains further the contribution by this residue to the stabilization of the CDA quaternary structure. It is also evident from the quaternary structure that other amino acidic residues, highly conserved among homotetrameric CDAs,[4] are located at the subunits interface: F36, R103, Q104 and L133 between subunits A and C; Y33, located between subunits A and D.

Site-directed mutagenesis on the highly conserved residues Y60 and Y33 (corresponding to the *E. coli* Y633, Y206 and *B. subtilis* Y48, Y21 respectively) will clarify how this residue may participate to the intersubunit interactions together with the F137 residue.

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