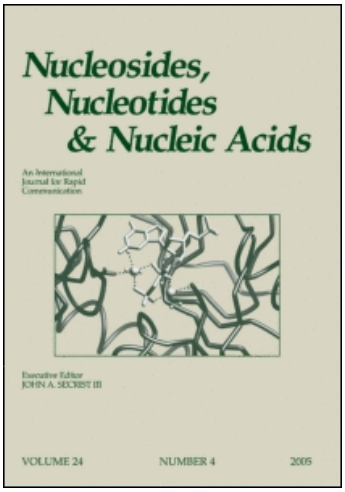

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STUDIES ON THERMAL STABILITY OF HUMAN CYTIDINE DEAMINASE

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□ *The thermal stability of human cytidine deaminase (CDA), an enzyme involved in pyrimidine metabolism was investigated. With this in view, the residues R68 and Y60, supposed to be involved in the intersubunit interactions and in the catalytic site of CDA, were mutated to glutamine and glycine, respectively. Thermal stability experiments were performed on the purified mutants by means of circular dichroism and enzymatic assays. The results obtained should be useful for designing more efficient cytidine based drugs for chemotherapy.*

Keywords Cytidine deaminase; thermal stability experiments; circular dichroism

INTRODUCTION

Cytidine deaminase (CDA) is an enzyme involved in the salvage pathways of pyrimidine metabolism, where it catalyses the hydrolytic deamination of cytidine and/or deoxycytidine into uridine and/or deoxyuridine. Due to its ability to inactivate by deamination several antitumoral and antiviral cytosine nucleoside analogues, such as cytosine arabinoside, 5-azacytidine,[1] the enzyme has been the subject of several studies. In fact, knowledge of the human CDA active site should be useful for designing more efficient cytidine based drugs for antiviral or anticancer chemotherapy. Previous studies on the catalytic site of the tetrameric CDA indicated that the active site of one monomer is formed by the contribution of residues coming from the other three.[2] We focused our attention on the residues Y60

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and R68, that are supposed to be involved in the intersubunit interactions and also in the active site;[3] thus, by site-directed mutagenesis on human CDA cDNA, we obtained the mutants Y60G and R68Q. After purification, circular dichroism (CD) and thermal stability experiments were performed on the mutant enzymes, in order to reveal conformational changes in the protein structure induced by these point mutations. The results indicated that the mutant Y60G is an enzyme with significantly increased stability with respect to the wild-type CDA; in fact, CD studies on the Y60G mutant CDA revealed a more stable tertiary and quaternary structure against thermal denaturation. R68Q mutant CDA proved to be less thermo-stable with respect to wild-type CDA.

Furthermore, CD studies revealed that the arginine in position 68 plays a crucial role in subunit interactions and also participates in the catalytic event.

RESULTS AND DISCUSSION

In order to establish the role of tyrosine 60 and arginine 68 on the human CDA active site, the Y60G and R68Q mutants were obtained by site directed mutagenesis on wild-type CDA cDNA. After expression, each mutant enzyme was purified to homogeneity by using the CV6 affinity column, and kinetic studies were performed[4] by means of the natural substrates, cytidine, and deoxycytidine, and some cytosine nucleoside analogs (cytosine arabinoside, 5-I-deoxycytidine, 5-aza-cytidine, 5-methyl deoxycytidine). The mutant proteins Y60G and R68Q showed almost the same kinetic behaviour as the wild-type enzyme, indicating that the mutations did not have a dramatic effect on the catalytic event (data not shown). The effect of temperature on activity and stability of CDA wild-type and on the mutant enzymes R68Q and Y60G was also investigated by incubating each enzyme at temperatures ranging from 5°C to 75°C for 6 minutes and then assaying it at 37°C. Wild-type CDA is a thermo-stable enzyme showing an optimum of activity at about 58°C and a decrement in enzyme activity at temperatures higher than 70°C. Y60G showed a mean of 75% residual activity for temperatures ranging from 15°C to 65°C with three peaks of maximum activity at about 30°C, 46°C and 62°C. R68Q mutant CDA proved to be less thermo-stable than the wild-type CDA, with a marked decrement of its stability at temperatures higher than 65°C (data not shown). The temperature dependence of V_{max} and K_m values of Y60G and R68Q mutants was analysed by using the empirical Arrhenius equation ($\ln V_{max} = \ln A - E_a/RT$) and the van't Hoff equation ($\ln K_m = -\Delta H/RT + \Delta S/R$). While a linear Arrhenius plot was observed for the R68Q mutant, in agreement with wild-type CDA[4], a discontinuous one was obtained when plotting the $\ln V_{max}$ of the Y60G mutant *vs* 1/T (Figure 1). The discontinuity displayed by Y60G can be explained by assuming either that E_a for this mutant CDA is dependent on T, or that

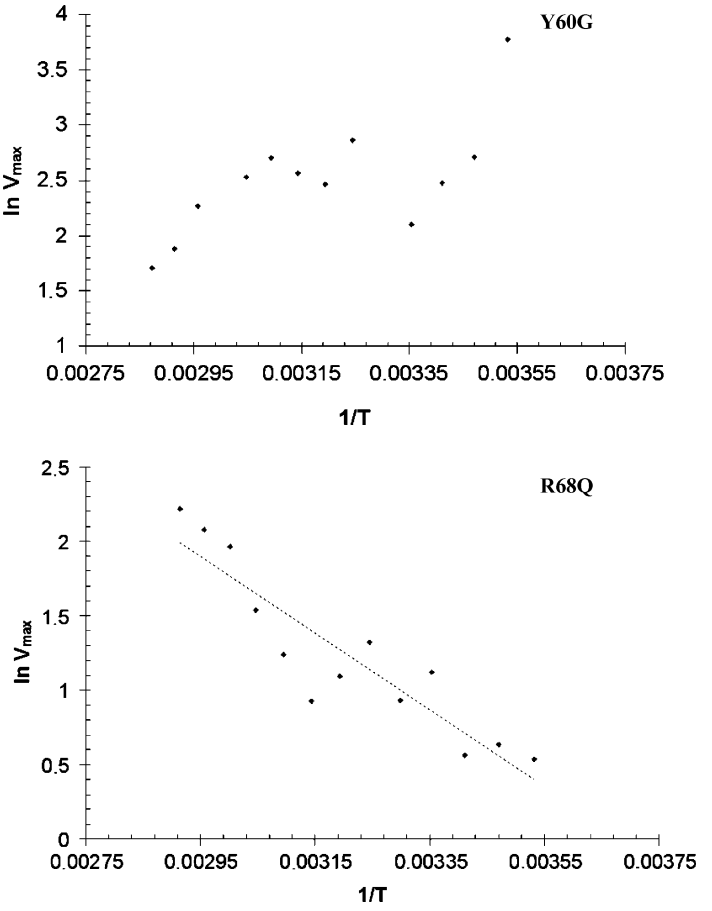

FIGURE 1 Arrhenius plots for Y60G and R68Q mutant enzymes showing the temperature dependence of V_{max}. Temperature is expressed in Kelvin; the kinetic data are the mean of three replicates.

the rate constant used to build the plot may be a composite of other rate constants. In the first case, a structural change induced by the temperature could be at the origin of this behavior.

By means of the van't Hoff plots, in Figure 2, a view of the binding energetics that accompanies the formation of the enzyme-substrate complex was obtained for the two mutated CDAs. The van't Hoff plot of R68Q mutant displayed two temperature regions in which a different energetics of substrate binding was observed: in the temperature range 10–45°C the binding enthalpy was endothermic (3.2 kcal/mol), while in the 50–70°C range it was exothermic (−13.38 kcal/mol). The relatively low positive enthalpic value in the low temperature range indicates that the binding process is entropically driven (hydrophobic surface burial); on the other hand, at high temperatures it seems that the substrate binding is accompanied by strong non-covalent interactions (H-bonds, salt bridges, etc.). A perturbation of the

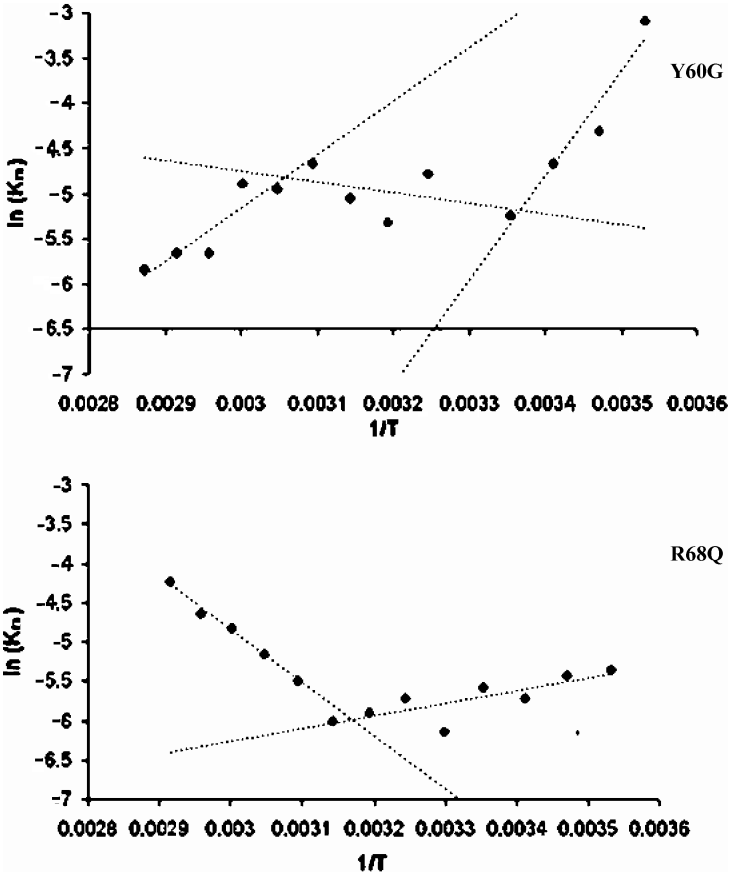

FIGURE 2 Temperature dependence of K_m for Y60G and R68Q mutant CDAs described by van't Hoff plots. Temperature is expressed in Kelvin; the kinetic data are the mean of three replicates.

binding pocket structure around 45°C could be responsible for the observed results. Similar results were obtained from the van't Hoff plot of the Y60G mutant shown in Figure 2; in this plot, there were at least two breaks of linearity, approximately at 25°C and 55°C. The enthalpy of substrate binding evaluated by this plot was endothermic (23 kcal/mol) in the temperature interval 10–25°C, became exothermic (−2.34 kcal/mol) between 25°C and 55°C, and switched again to a positive value (11.73 kcal/mol) in the high temperatures range 55–70°C. These results suggest that rearrangements of the protein structure around the catalytic pocket induced by temperature are promoted by the replacement of tyrosine 60 by a less rigid residue such as glycine.

Figure 3 shows the CD spectra (Jasco J710 spectropolarimeter) in the far UV region (200–250 nm) of wild-type and mutant CDAs obtained at increasing temperatures (from 5°C to 90°C). Wild-type CDA proved to be stable up to 60–70°C with a slight loss of secondary structure; at 80°C and 90°C, there

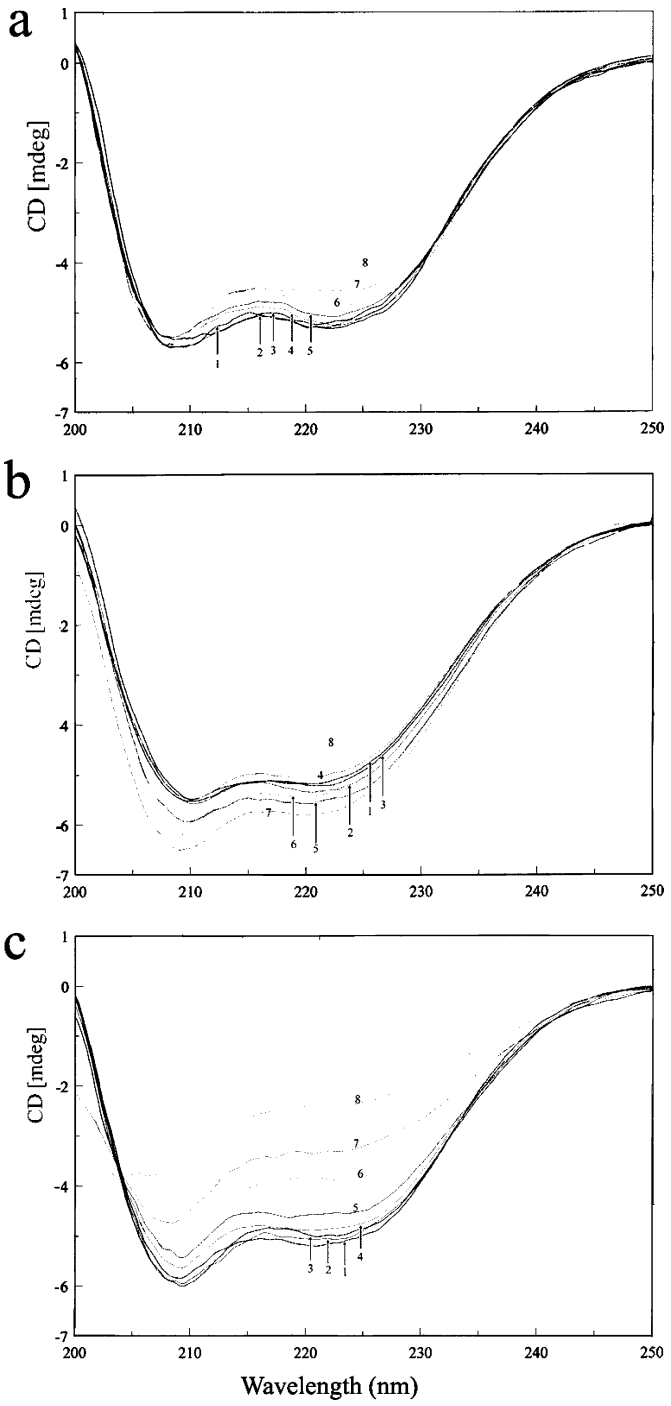

FIGURE 3 Temperature dependent CD spectra in the far UV-region. Wild-type CDA (a), Y60G (b) and R68Q (c). Temperatures: 5°C (1); 20°C (2); 40°C (3); 50°C (4); 60°C (5); 70°C (6); 80°C (7); 90°C (8). The buffer was 20 mM Tris/HCl pH 7.6, 1 mM DTT. Protein concentration was 2 μM; cell length 2 mm.

was a decrease of ellipticity at 222 nm and a greater negative signal at 208 nm, that reflected a monomerization process of the enzyme. Also in the case of R68Q mutant, there was a marked decrease of ellipticity that may be due to a monomerization process correlated to the overall secondary structure denaturation. In the Y60G mutant enzyme, a first increase of secondary structure was evident at 60–70°C, and a second one at 80°C, whereas the protein was denaturated at 90°C. A possible explanation of this different behaviour may be that, in wild-type CDA, tyrosine 60 induces a rigidity in the tertiary and quaternary structure due to the possibility of hydrogen bonds and/or aromatic interactions. In the mutant Y60G, the lack of these constraints did not affect the overall structure and function, but it could be responsible for a minor rigidity with a consequent step by step rearrangement of secondary structures and local hydrophobic collapses induced by the increased temperature.

CONCLUSIONS

The mutation of R68 to glutamine showed that arginine, with its two electrostatic bonds, plays an important role in intersubunit interactions and in the correct tertiary folding of each monomer in wild type CDA. Y60G mutant enzyme displayed an increased overall structure stability against thermal denaturation with respect to the wild-type enzyme, confirming that mutation does not affect the quaternary structure of the enzyme, but, as evidenced by the three peaks of optimum temperature, allows the enzyme to readjust its binding pocket conformation in presence of increasing temperatures.

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