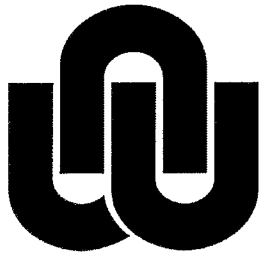

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POTCHEFSTROOMKAMPUS

Molecular characterisation of a recombinant bovine glycine N-acyltransferase

Christoffel Petrus Stephanus Badenhorst, Hons. B.Sc

Division for Biochemistry, School of Physical and Chemical Sciences, North-West University, Potchefstroom Campus, Potchefstroom, 2520, South Africa.

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Supervisor: Prof. A.A van Dijk

Nothing in medicine makes sense except in the light of biology

- Charles R. Scriver

Nothing in biology makes sense except in the light of evolution

- Theodosius Dobzhansky

Abstract

Conjugation of glycine to organic acids is an important detoxification mechanism. Metabolites of aspirin and industrial solvents, benzoic acid found in plant material and many endogenous metabolites are detoxified by conjugation to glycine. The enzyme responsible for glycine conjugation, glycine N-acyltransferase (GLYAT), is investigated in this study. The enzyme is also important for the management of organic acidemias which are inherited metabolic diseases.

However, not all organic acids can be efficiently detoxified by GLYAT. Consequently, some organic acidemias, such as propionic acidemia, are difficult to treat. We hypothesise that a novel variant of GLYAT might be designed that can effectively detoxify propionic acid and several other organic acids. This novel GLYAT might eventually be used as a recombinant therapeutic enzyme for the treatment of organic acidemias. A thorough understanding of the mechanisms of substrate binding and catalysis by the enzyme is needed to design such a novel enzyme. This understanding is lacking at present. The first step to investigating the mechanics of substrate binding and catalysis is the development of a recombinant enzyme expression system. Amino acids in the protein can then be altered using site-directed mutagenesis, to study the importance of individual amino acid residues to enzyme function. No system for the expression of a biologically active recombinant human GLYAT has yet been developed. In a recent study in our laboratory, it was shown that bovine GLYAT could be expressed in a partially soluble and enzymatically active form, using expression at 15 °C and chaperone co-expression. The enzyme could not be investigated in detail because no tags for purification were fused to the protein.

In this study the bovine GLYAT was expressed with a C-terminal histidine tag for affinity purification using the same system. It was confirmed that the recombinant bovine GLYAT was enzymatically active and it could be partially purified. Two major proteins were present after purification. The identity of the co-purifying protein is unknown. The enzyme reaction kinetics of the partially purified recombinant bovine GLYAT and of GLYAT isolated from bovine liver was determined and compared. The kinetic parameters of the two enzymes were similar and correlated with the values reported in the literature.

The recombinant bovine GLYAT was used to elucidate the catalytic mechanism of the enzyme. A putative catalytic residue, E226, was identified on the basis of biochemical arguments and

bioinformatic analyses. This proposed catalytic residue was mutated by means of site-directed mutagenesis. The E226Q mutant recombinant bovine GLYAT enzyme was compared to the wild type bovine GLYAT with regard to reaction kinetics and pH dependence of the reaction. The results suggested that bovine GLYAT uses the E226 residue as a general base catalyst to remove a proton from glycine in the reaction mechanism. This is the first time a mechanism for GLYAT activity has been worked out.

Benzoyl-coenzyme A is a substrate of the GLYAT reaction. It is used as a reagent in GLYAT activity assays and kinetic investigations. Since this compound is very expensive, a method was adapted from the literature for the cost effective in-house synthesis of this compound. Three biosynthetic enzymes from *Escherichia coli* were cloned, sequenced, expressed and purified and then used for the synthesis of benzoyl-coenzyme A from benzoyl-pantetheine. The conversion from benzoyl-pantetheine to benzoyl-coenzyme A was stochiometric. After purification a yield higher than 75% was obtained. The benzoyl-pantetheine used was first synthesised from benzoic acid and pantethine by acylation under reducing conditions. All steps could be performed in a single tube. The method does not require purification of the benzoyl-pantetheine before use in the enzymatic synthesis of benzoyl-coenzyme A, minimising loss of material.

To summarise, a recombinant bovine GLYAT with a C-terminal histidine tag was expressed and partially purified. The kinetic properties of the recombinant bovine GLYAT corresponded to the properties of GLYAT extracted from bovine liver. The recombinant bovine GLYAT was used to elucidate the catalytic mechanism of GLYAT by means of site-directed mutagenesis. This demonstrates the power of the recombinant expression system to studying the importance of specific amino acid residues. Benzoyl-coenzyme A was synthesised using a cost effective method adapted from the literature. In the future, photoaffinity labelling will be used to identify residues that constitute the substrate binding site of GLYAT and site-directed mutagenesis will then be used to investigate their function. It may then become possible to attempt to rationally design a GLYAT with altered substrate specificity.

Opsomming

Die konjugering van organiese sure met glisien is 'n belangrike detoksifiserings meganisme. Metaboliete van aspirien en industriële oplosmiddels, bensoaat wat in plant materiaal voorkom en verskeie endogene metaboliete word deur glisien-konjugering gedetoksifiseer. Die ensiem wat die reaksies kataliseer, glisien N-asieltransferase (GLIAT), word in hierdie studie ondersoek. Die ensiem is ook belangrik vir die behandeling van verskeie oorerflike siektes van die metabolisme van organiese sure.

Glisien N-asieltransferase kan egter nie alle organiese sure detoksifiseer nie. Daarom is sommige defekte van organiese suur metabolisme, soos propioonsuur-urie, moeilik om te behandel. Ons hipotese is dat 'n variant van GLIAT ontwerp kan word wat propioonsuur en ander organiese sure effektief sal kan detoksifiseer. So 'n GLIAT mag dalk ontwikkel word as 'n terapeutiese ensiem vir die behandeling van defekte van die metabolisme van organiese sure. Om so 'n gemodifiseerde ensiem te ontwerp, moet die meganismes van substraatbinding en katalise deeglik verstaan word. Op die oomblik verstaan ons nie genoeg nie. Die ontwikkeling van 'n rekombinante ensiem uitdrukkingsisteem is die eerste stap na 'n beter begrip van hierdie meganismes. So 'n sisteem kan gebruik word om spesifieke aminosuur veranderinge te maak en dus die funksie van individuele aminosure te bestudeer. Tot dusver is nog geen sisteem vir die uitdrukking van oplosbare rekombinante mens GLIAT met ensiem aktiwiteit ontwikkel nie. In 'n onlangse studie in ons laboratorium is bevind dat bees GLIAT in 'n oplosbare ensiematies aktiewe vorm geproduseer kan word as dit teen 15 °C saam met chaperone uitgedruk word. Omdat hierdie proteïen geen herkenningspunt vir suiwering bevat het nie, kon dit nie gesuiwer word en in detail bestudeer word nie.

In hierdie studie is 'n rekombinante bees GLIAT met 'n C-terminale histidien herkenningspunt in die selfde sisteem uitgedruk. Dit het bevestig dat die rekombinante bees GLIAT ensiematies aktief is. Die rekombinante bees GLIAT is gedeeltelik gesuiwer. Na die suiwering was twee proteïene teenwoordig. Die identiteit van die tweede proteïen is nog onbekend. Die gedeeltelik gesuiwerde rekombinante bees GLIAT is kineties gekarakteriseer en vergelyk met GLIAT wat uit beeslewer berei is. Die twee ensieme se kinetiese eienskappe was soortgelyk en het goed gekorreleer met gepubliseerde waardes.

Die rekombinante bees GLIAT is gebruik om die katalitiese meganisme van die ensiem uit te werk. 'n Potensiële katalitiese residu, E226, is met behulp van biochemiese argumente en bioïnformatika geïdentifiseer. Die voorgestelde katalitiese residu is gemuteer deur gebruik te maak van punt-spesifieke mutagenese. Die pH-afhanklikheid en ensiem-kinetika van die E226Q mutant van rekombinante bees GLIAT en die wilde tipe rekombinante GLIAT is met mekaar vergelyk. Uit die resultate blyk dit dat die E226 residu van bees GLIAT betrokke is by proton-verwydering in die katalitiese meganisme. Dit is die eerste keer dat 'n meganisme vir die GLIAT reaksie uitgewerk is.

Benzoiel-koënsiem A is 'n substraat van die GLIAT reaksie. Dit word gebruik as 'n reagens in GLIAT ensiemtoetse en in kinetiese eksperimente. Omdat die verbinding baie duur is, is 'n metode saamgestel uit die literatuur om dit self goedkoop te kan sintetiseer. Drie ensieme van *Escherichia coli* is gekloneer en hulle nukleïensuurvolgorde bepaal. Die ensieme was uitgedruk, gesuiwer en gebruik om benzoiel-koënsiem A van benzoiel-pantetien te sintetiseer. Benzoiel-pantetien is heeltemal omgeskakel na benzoiel-koënsiem A. Na suiwering was die opbrengs gewoonlik meer as 75%. Die benzoiel-pantetien is vooraf eers vanaf benzoësuur en pantetien gesintetiseer deur pantetien onder reduserende toestande te asileer. Al die stappe is in 'n enkele buis gedoen. Dit was nie nodig om die benzoiel-pantetien te suiwer voor gebruik in die sintese van benzoiel-koënsiem A nie. Sodoende gaan geen materiaal verlore in die sintese nie.

Om op te som, 'n rekombinante bees GLIAT met 'n C-terminale histidien herkenningspunt is uitgedruk en gedeeltelik gesuiwer. Die kinetiese eienskappe van die rekombinante ensiem het ooreengestem met die van die beeslewer ensiem. Die rekombinante uitdrukkingsisteem is gebruik om die katalitiese meganisme van GLIAT uit te werk deur spesifieke mutante te maak en te karakteriseer. Dit demonstreer die krag van die rekombinante uitdrukkingsisteem om die belangrikheid van spesifieke aminosure van GLIAT te ondersoek. Benzoiel-koënsiem A is gesintetiseer deur 'n metode wat saamgestel is uit literatuurgegewens en gebruik maak van redelik goedkoop reagense. In die toekoms sal foto-affiniteit merking gebruik word om aminosure in die aktiewe setel van GLIAT te identifiseer. Hierdie aminosure van die rekombinante ensiem sal dan gemuteer word en die invloed daarvan bestudeer word. So sal die inligting wat nodig is om uiteindelik die ensiem se substraatspesifisiteit te kan manipuleer, bekom word.

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