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Biochemical analysis of mucin glycoproteins in paediatric colonic mucus

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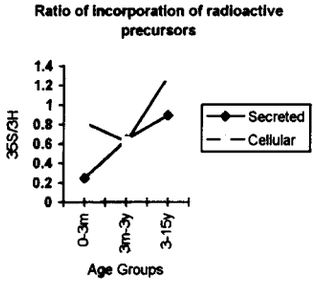
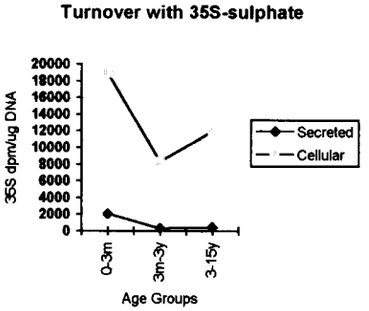
The luminal surface of the gastrointestinal tract is covered throughout by a water insoluble mucus gel layer. In the colon this layer serves as a lubricant, protects against mechanical trauma, and acts as the first line of defence against potential colonic pathogens, foreign compounds and toxins by providing a physical and diffusion barrier (1-3). It is a dynamic system maintained by continual mucosal cell synthesis and degraded, among other factors, by colonic bacterial enzymes(4). The major component of this gel are high molecular weight glycoproteins (mucins) which give it characteristic viscoelastic properties (1-3). This gel layer exhibits changes with nutrition (5). The changes associated with age related development have not been well characterized. We studied developmental changes in mucins in children 0-15 years. We used an established organ culture method (6) in which colonic mucosa was cultured with radioactive mucin precursors [35S]-sulphate and [3H]-glucosamine. After 24 hours secreted and cellular fractions were collected. These fractions were then passed over columns of Sepharose CL2B to obtain the high molecular weight mucin fraction. The ratio of incorporation [35S]/[3H] was measured in the mucin peak. The turnover of individual radioisotopes was quantified by relating radioactivity to mucosal DNA content. Purified mucins were then vacuum blotted on PVDF membranes and and tested against Wheat germ agglutinin, 2 lectins, and 5 antimucin antibodies to identify peripheral epitopes. Wheat germ aggultinin reacts with N-acetylglucosamine and sialic acids and is a good general purpose reagent for mucin detection. 91.9H is a monoclonal antibody against human sulphated mucin (7). Reactivity of individual mucins was quantified by scanning the membranes for optical density. Total mucin turnover was assessed by relating the optical density to the DNA content of the colonic epithelial tissue. The patients were divided into three age groups 0-3 months, 3 months-3 years, and 3-15 years.

RESULTS



Secreted: Mann Whitney test: <3 months v 3m-3y: p=0.026
A trend of an increasing ratio (i.e sulphate incorporation relative to glucosamine) with age is present in both fractions. This is statistically significant in the <3 months v 3months-3 years age groups in the secreted fraction revealing a qualitative mucin change.



Secreted: Mann Whitney test: <3 months v 3m-3y:p=0.019
Cellular: Mann Whitney test: <3 months v 3m-3y:p=0.04

The turnover of mucin with [35S]-sulphate was highest in the youngest age group and significant in both fractions. Turnover with [3H]-glucosamine was also significantly increased in the youngest age group in both fractions. This reflects changes in quantity of radiolabelled mucin. Experiments with wheat germ agglutinin also showed significantly higher turnover in both secreted and cellular mucins in the youngest age group suggesting rapid turnover in early life. An increased content of sulphated mucin with age is implied from the ratio measured in the radiolabelling experiments and confirmed with 91.9H reactivity which is significantly higher in the oldest age group. The absence of any mucin change in the age groups using reagents to detect sialic acids, suggests that changes in the radiolabel ratio are due to the sulphate, as [3H]-glucosamine will be incorporated into sialic acids. This may reflect an altered mucin protective function in older children. Radiolabelling experiments show higher values for the cellular fractions while the reverse is true with the WGA assay, showing that only part of the total mucin is labelled in the 24 hours reflecting the long period required for colonic mucin maturation. This is the first characterization of the changes in mucin glycoproteins during early development.

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Evidence that the active site in type II dehydroquinase from *Streptomyces coelicolor* is near the single tryptophan

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The enzyme dehydroquinase (EC 4.2.1.10) catalyses the dehydration of 3-dehydroquinate to 3-dehydroshikimate. The reaction is common to two metabolic pathways:- the shikimate pathway for the synthesis of aromatic compounds and the catabolic quinate pathway of fungi [1]. DHQases fall into two distinct classes distinguished by non-homologous sequences and by various biophysical criteria [2]. The type I enzymes (dimers of subunit M_r about 27 000) involve a covalent imine intermediate to catalyse a *cis* elimination. Several important amino acids which are involved in the catalytic mechanism have been identified [3]. By contrast the type II DHQases do not involve an imine intermediate, catalyse a *trans* elimination and are usually dodecameric of subunit M_r about 16 000. At present there is relatively little information available concerning the mechanism of the type II enzymes, though a conserved arginine residue (Arg-23) has been implicated on the basis of chemical modification experiments [4]. In order to identify the possible location of the active site of the enzyme, we have examined the fluorescence properties of the single tryptophan (Trp-66) in each subunit of the type II DHQase from *Streptomyces coelicolor*.

The emission maximum of the enzyme, when excited at 290 nm, is 341 nm. This value is significantly higher that that of the single tryptophan in each subunit of the type I enzyme from *Salmonella typhi* [1], and indicates that Trp-66 in type II DHQase is significantly exposed to solvent [5]. This conclusion is confirmed by measurements of the quenching of the fluorescence by succinimide, which can be used to provide a measure of the accessibility and local dynamics of Trp-66 [6]. The value for the Stern-Volmer constant (K_{sv}) is 3.2 M^{-1} (Table 1), a value much higher than that (0.25 M^{-1}) for the single tryptophan in each subunit of the type I enzyme from *S. typhi* [1]. According to data fron circular dichroism and fluorescence, addition of 4 M GdnHCl leads to the complete loss of the native secondary and tertiary structure of the type II DHQase. Under these conditions there is no significant increase in the K_{sv} value; the effect of viscosity is taken into account by measuring the effect of 4 M GdnHCl on the K_{sv} value for the model compound, NATA (Table 1). The lack of effect of denaturation of the enzyme provides additional evidence for the high degree of accessibility of Trp-66 in native type II DHQase.

On addition of substrate (dehydroquinate) to the enzyme, a substrate/product mixture (15/1 in favour of the product dehydroshikimate [1]) is formed. As shown in Table 1, when this substrate/product mixture (5 mM) is added to type II DHQase, there is a reduction in the value of K_{sv} by more than 50%. Addition of the mixture to the model compound NATA has only a small effect on the value of K_{sv}. Addition of the mixture to the enzyme has only a very small effect on the far UV circular dichroism spectrum, indicating that there are no substantial effects on the secondary structure of the enzyme. From these results it is clear that the single tryptophan (Trp-66) in each subunit of the type II DHQase from *S. coelicolor* is highly accessible both to solvent and to the quencher succinimide. The large reduction in accessibility to succinimide on addition of the substrate/product mixture indicates that Trp-66 is at or near to the active site of the enzyme. In view of the fact that this tryptophan is not conserved in type II DHQases from a number of species, it is unlikely to play a crucial role in the catalytic mechanism. Nevertheless, the results presented here should assist the structural studies currently in progress [7] in helping to define the location of the active site.

Abbreviations used:- DHQase, dehydroquinase; GdnHCl, guanidinium chloride; NATA, N-acetyltryptophan amide

Table 1. Quenching of fluorescence by succinimide

The values shown are those of the Stern-Volmer constants obtained in 50 mM Tris/HCl buffer, pH 7.5, at 25°C. In the table, the term substrate refers to the substrate/product mixture.

Sample	K_{sv} (M^{-1})
DHQase	3.18
DHQase + 4 M GdnHCl	2.29
DHQase + 5 mM substrate	1.25
NATA	11.5
NATA + 4 M GdnHCl	9.06
NATA + 5 mM substrate	9.72

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