Polysialic Acid
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STUDIES OF THE O-ACETYLATION AND (IN)STABILITY OF POLYSIALIC ACID

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SUMMARY:
Bacterial polysaccharides that consist of polysialic acid (PSA) can be O-acetylated. Expression of this O-acetylation in K1+ E. coli correlates with the expression of a specific O-acetyltransferase activity. This enzyme uses acetyl-Coenzyme A as a donor and shows a marked preference for polymers of length >12-14. Although the enzyme is very stable in the crude state, it becomes extremely labile upon further purification. This destabilization can be reproduced by exposing the crude enzyme to a bacteriophage endosialidase specific for PSA. It appears that the enzyme requires the continuous presence of PSA for its stability.
Another interesting property of PSA is its propensity to undergo depolymerization under mildly acidic conditions. However, a search of the literature indicates that the primary linkage unit of PSA (Siaα2-8Sia, which is found also in gangliosides) must be quite stable under such conditions. This paradox is being investigated to determine if it is due to selective destabilization of the internal linkages of the polymer. Regardless of the mechanism(s) involved, this instability must be taken into account in exploring the chemistry and biology of PSA in various systems.

BACTERIAL POLYSIALIC ACIDS CAN BE O-ACETYLATED

Polysialic acid (PSA) is an unusual homopolymer of sialic acid (Sia) found in certain animal glycoproteins e.g. the Neural Cell Adhesion Molecule (N-CAM), and in the capsular polysaccharides of certain pathogenic bacteria (Troy, 1992; Rutishauser et al., 1988). In the latter case, the polymer is known to be subject to O-acetylation at the 7- or 9-positions. This occurs in some, but not all, strains of E. coli and meningococcus (Orskov et al., 1979; Arakere et al., 1991; Vann et al., 1978). O-acetylation is also found in the PSA of fish egg glycoproteins (Kitajima et al., 1988; Iwasaki et al., 1990). In the case of N-CAM, the possibility of O-acetylation has not been investigated.

Pathogenic K1+ E. coli bearing the Siaα2-8Sia PSA homopolymer can be fixed in an O-acetyl negative or an O-acetyl-positive state. Alternatively, some strains undergo a form variation between the O-acetyl-positive and O-acetyl-negative states. This form variation has been noted to be rather

rapid, occurring as frequently as every 1/30 doublings (Orskov et al., 1979). We have investigated the enzymatic basis of the O-acetyl-positive state and of the O-acetyl form variation.

THE O-ACETYL POSITIVE STATE CORRELATES WITH THE EXPRESSION OF A SPECIFIC O-ACETYLTRANSFERASE ACTIVITY

A simple assay for the O-acetyltransferase was developed, that uses PSA as an acceptor, and [^{3}H]acetyl CoA as the radioactive donor molecule. Following incubation, the macromolecular product is separated from the radioactive donor molecule by gel filtration (Higa et al., 1988; Vann et al., 1978). The radioactive product was confirmed to be O-acetylated PSA by sequential digestion with endo- and exosialidases. The monomeric product that resulted proved to be a mixture of [^{3}H-acetyl]7- and 9-O-acetyl-N-acetyl-neuraminic acid (the latter being the predominant product). Significant activity of this enzyme was found in E. coli strains known to be O-acetyl positive (C375:K1,D698:K1), and not in strains known to be O-acetyl negative (016:K1,C940:K1) or K1 negative (HB101:K12,JM103:K1) (Higa et al., 1988).

THE O-ACETYL FORM VARIATION IS CORRELATED WITH FLUCTUATIONS IN THE LEVEL OF EXPRESSION OF THE O-ACETYLTRANSFERASE

By picking random clones from D698:K1(known to show the O-acetyl form variation), we followed the expression of the O-acetyl-transferase through several generations. Progeny of a colony positive for enzyme expression were mostly positive, but the occasional negative one (~1/30-40) gave mostly negative progeny, with an occasional positive one. This "flip-flop" was followed through four generations. Thus, the pattern of expression of the O-acetyltransferase indicates that it is the determinant of O-acetyl form variation. The genetic mechanism by which the form switch occurs is currently unknown.

PARTIAL PURIFICATION AND CHARACTERIZATION OF THE O-ACETYLTRANSFERASE

The enzyme activity was solubilized by Triton X-100 (0.05%), and was found to be quite stable at 4°C. The crude preparation showed significant endogenous acceptor activity, presumably arising from PSA present in the bacteria that were extracted. The activity was partially purified by chromatography on DEAE-cellulose, giving ~100-fold purification over total cellular protein. This step also freed the enzyme from almost all detectable endogenous acceptor. Although the enzyme was very stable at this step, all attempts to purify it further failed (the probable reasons for this phenomenon are discussed below). Further characterization of the enzyme was therefore done with

material from the DEAE step. We found that the enzyme had a pH optimum of 7.0 - 7.5, was not dependent on divalent cations, and showed an apparent Km of 300 μM for AcCoA. Coenzyme A was inhibitory, giving half-maximal inhibition at 100 μM (Higa et al., 1988).

THE O-ACETYLTRANSFERASE PREFERENTIALLY O-ACETYLATES HIGH-MOLECULAR WEIGHT POLYSIALIC ACIDS

The apparent Km of the transferase for PSA was 3.7 mM (expressed as sialic acid concentration). However, this substrate (commercial "colominic acid") is actually a mixture of PSA fragments of varying sizes, with an average of ~15 residues. Gel filtration analysis of the radiolabeled reaction product indicated that it was rather large. To explore this further, we studied the radioactive reaction product by MONO-Q HPLC, which separates fragments of different sizes. We found that the enzyme preferentially acetylates the polymers with a DP>14. Thus, the enzyme appears to recognize some feature of PSA that is confined to very large polymers (Higa et al., 1988). This may be related to the secondary structure of PSA discussed by others in this book.

THE O-ACETYLTRANSFERASE IS STABLE IN THE CRUDE STATE, BUT EXTREMELY LABILE TO FURTHER PURIFICATION

After the DEAE purification step, the enzyme is very stable at 4°C (>5 years) and even at 60°C (>1 hour). However, all further attempts to purify the enzyme failed because it became unstable. It was puzzling that this instability became manifest upon using many different purification methods that rely on entirely different principles (e.g. cation exchange, dye-matrix affinity, Coenzyme-A affinity and gel filtration). All attempts to add-back fractions from various steps failed to regain the activity. This change in stability is best explained by the findings presented below, that indicate that the stability of the transferase is dependent upon continous association with PSA (Higa et al., 1988).

THE O-ACETYLTRANSFERASE APPEARS TO BE STABILIZED BY CONSTANT ASSOCIATION WITH POLYSIALIC ACID

We reasoned that if the O-acetyltransferase was stabilized by constant association with PSA, the activity should be destabilized by treatment with the phage endosialidase specific for PSA. This proved to be the case. If the transferase was first treated with endosialidase at 57°C, and then cooled 37°C, most of the activity was lost. This was clearly due to the activity of the endosialidase itself (and not a contaminating protease), because the effect could be completely protected against by the presence of competing PSA in the initial incubation (Higa et al., 1988). These data indicate that

the transferase may be stabilized by constant association with PSA of endogenous origin. This might explain why the enzyme is stable to the DEAE purification step, in which PSA would be expected to co-elute from the column. Thus, this step would presumably result in co-purification of endogenous PSA, which can bind to this anion exchange column. On the other hand, the fact that the endosialidase could not inactivate the transferase at 37°C of this, it is somewhat surprising that activity is so easily lost during attempts at further purification at 4°C. This paradox remains unresolved, and suggests that other unknown factors may also contribute to the destabilization of the enzyme.

THE TRANSFERASE CAN O-ACETYLATE POLYSIALIC ACIDS OF EMBRYONIC N-CAM

The E. coli O-acetyltransferase was found to be able to O-acetylate the PSA chains of chicken embryonic N-CAM. All of the radioactively labeled products were found to have a molecular weight of >210 Kda, indicating that the enzyme specifically recognized the molecules with the longest length of PSA (Higa et al., 1988). This suggests that the enzyme could be used as a probe to detect such structures in a variety of systems. Since the other available probes (e.g. antibodies, endosialidase) recognize PSA of shorter lengths (8-10 residues) (Finne et al., 1985; Hallenbeck et al., 1987; Häyrinen et al., 1989; Hekmat et al., 1990; Lackie et al., 1990), the O-acetyltransferase could theoretically be used to further differentiate adult from embryonic N-CAM.

PARADOXICAL FINDINGS CONCERNING THE STABILITY OF THE Siaα2-8Sia LINKAGE OF POLYSIALIC ACIDS

During our studies of the O-acetyltransferase, we noted that PSA has a tendency to break down gradually into smaller fragments even when stored in the freezer for prolonged periods of time. This problem was particularly evident when the samples were slightly acidic. Other observations mentioned in the literature indicate that PSA is a very labile molecule. The PSA in N-CAM is easily degraded when preparing samples for SDS-PAGE gels (i.e. boiling in pH 6.5 Laemli buffer). Thus samples are usually heated at 60°C to minimize this problem. On the other hand, PSA is sometimes deliberately degraded into smaller fragments for analysis by incubation at moderately low pH (Kitazume et al., 1992). In some instances, even boiling at close to neutral pH is used to obtain partial breakdown of PSA (Troy, 1979). Paradoxically, the primary α2-8 linked Sia linkage unit of PSA is reported to be more stable than other Sia linkages, being difficult to degrade in hot sulfuric acid (Troy, 1979; Nadano et al., 1986). Similarly, the α2-8 linked Sia residues of gangliosides are notoriously difficult to release with acid hydrolysis.

Is the long PSA intrinsically more unstable than short PSA? Taken together, the evidence from the literature would suggest that this is indeed the case. We have carried out some preliminary comparisons that confirm and extend this phenomenon. Further studies are required to define the exact extent of difference, and the point at which increasing length results in decreased stability.

POSSIBLE EXPLANATIONS FOR THE INSTABILITY OF POLYSIALIC ACID

The following possibilities must be considered:

1. Polysialic acids are well known to develop secondary structure as the polymer size increases (Kabat et al., 1988; Jennings et al., 1989; Häyrinen et al., 1989; Yamasaki et al., 1991; Brisson et al., 1992) (see also other chapters in this book). It is possible that some unknown feature of this secondary structure results in destabilization of the glycosidic bond.
2. Polysialic acids are well known to develop internal lactones (Lifely et al., 1981). It is possible that formation of these five-membered rings results in destabilization of the glycosidic bond.
3. Acidic polymers can undergo changes in the pKa of internal acidic groups as they increase in length (Katchalsky, 1947). It is possible that this may favor an intra-molecular general acid catalysis involving a protonated carboxyl group and the immediately adjacent glycosidic bond (Karkas et al., 1964). If this is so, the mechanism would be similar to that of glycosidases, in which glutamic or aspartic acid residues with a high pKa are involved in catalysis (Tull et al., 1991; Rouvinen et al., 1990 ; Reddy and Maley, 1990). It is also possible that some combination of these mechanisms is involved. We are currently investigating each of these possibilities.

IS THE INSTABILITY OF PSA SUFFICIENT TO BE BIOLOGICALLY RELEVANT?

From the practical point of view, the instability of PSA at mildly acidic pH should be taken into account by all investigators designing *in* vitro studies involving these molecules. The question arises whether the instability is sufficient to be physiologically relevant in vivo. The precise pH values at which long-lasting stability is achieved need to be defined. It is quite possible that, in certain specialized situations, the extracellular pH might be low enough to be significant (e.g. in renal tissues, in infections, in hypoxic states, and in tumors). Likewise, at the pH values encountered in certain intracellular compartments (endosomes and lysosomes), PSA might be destabilized. In this regard, it is worthy of note that the following questions about N-CAM remain unanswered:

1. Is N-CAM normally internalized, or is it confined to the cell surface?
2. Is the length of the PSA on N-CAM molecules stable after synthesis?
3. Does the PSA on N-CAM turn over faster than the protein itself?
4. What is the mechanism for the switch from the embryonic to adult form of N-CAM seen in many systems? Does this always involve new synthesis of N-CAM molecules?
5. What is the mechanism for terminal degradation of the PSA of N-CAM?

The answers to these questions may require a better understanding of the instability of PSA. Our current efforts are directed at elucidating this fundamental issue.

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