Comparison of the Thermal Stability of Acid Phosphatase Conjugated with Ficoll and with Modified Ficolls

HEIDI KILL BLOMHOFF and
TERJE B. CHRISTENSEN

Institute of Biochemistry, Postbox 1041, Blindern,
University of Oslo, Oslo, Norway

The thermal stability of proteins may be enhanced by covalent attachment to carbohydrate. This applies to naturally occurring glycoproteins^{1,2} as well as to synthetic conjugates.^{3,4} Little evidence has been presented to explain the reason(s) for the stabilising effect although Marshall³ has suggested that synthetic conjugates are stabilised due to internal, covalent, crosslinks formed by carbohydrate.

The present experiments were carried out in order to obtain further insight into the causes of stabilisation and the results showed that partial methylation of Ficoll prior to conjugation with acid phosphatase reduced the stabilising effect. This was not the case for partial acetylation and the results suggest that the hydrophilic nature of the polysaccharide may be of importance for the stabilisation.

Experimental. Wheat germ phosphatase (EC 3.1.3.2) was obtained from Koch Light Laboratories, cyanogen bromide and dimethyl sulfate from Fluka AG, acetic anhydride from Merck AG, Ficoll-70 from Pharmacia Fine Chemicals and sodium metaperiodate from Riedel-de-Haen AG.

Methylation of Ficoll was carried out according to the classical Haworth procedure.⁵ 2 g Ficoll was dissolved in 10 ml water, dimethyl sulfate added at a rate of 4 ml/h and 30% sodium hydroxide at the rate of 8 ml/h. The first sample was withdrawn after 5 min and the second after 15 min. The temperature was 20 °C. The samples were worked up in the usual way, dialysed against distilled water and freeze dried.

Acetylation was effected by dissolving 5 g Ficoll in 35 ml acetic anhydride at 20 °C. After 5 min the first sample was taken and the temperature raised to 35 °C. The second sample was taken after 3 min at this temperature. The reagent was removed by dialysis at 10 °C, first against tap water and then against distilled water before the samples were freeze dried.

The approximate degree of substitution in Ficoll was determined by comparing the uptake of periodate by the substituted and the unsubstituted polysaccharide. Increased degree of substitution

lowers the content of vicinal hydroxyl groups and in turn the susceptibility to periodate oxidation. The consumption of periodate in the oxidation reaction was determined from the absorbance at 223 nm.⁶ The degree of substitution in the methylated and acetylated Ficolls were determined to be: MeFicoll₁ 19%, MeFicoll₂ 45%, AcFicoll₁ 34% and AcFicoll₂ 55%.

Conjugation of acid phosphatase with polysaccharide was carried out by the cyanogen bromide method.⁷ 50 mg solid cyanogen bromide was added to a solution of 100 mg polysaccharide in 20 ml dilute sodium hydroxide, pH 11.0. After the activation of the polysaccharide, the coupling reaction took place at 0 °C in 0.1 M borate buffer, pH 8.0, for 18 h. 10 mg enzyme was used.

Acid phosphatase was assayed by a method based on the method of Verjee⁸ using *p*-nitrophenyl phosphate as substrate. The reaction was stopped by addition of 0.25 M sodium carbonate solution and the absorbance read at 400 nm.

Three methods were used to test if the conjugation reaction had taken place to a reasonable extent. The first one was based on the solubility of the enzyme in 6% (w/v) perchloric acid. Conjugation with polysaccharide caused a marked increase in the solubility. The second test was based on the failure of the polysaccharide to migrate on electrophoresis unless attached to acid phosphatase. Slabs of 5% polyacrylamide were used with 0.2 M phosphate, pH 7.2. Duplicate tracks were run of each sample, one being stained for protein by Coomassie brilliant blue, the other for carbohydrate by the periodate-Schiff method.⁹

The third method involved determination of molecular weight by sedimentation equilibrium in the analytical ultracentrifuge. Registration was by photoelectric scanner at 280 nm. The partial specific volume of acid phosphatase was assumed to be 0.72 ml/g and that of the conjugates 0.68 ml/g.

Results and discussion. Judging by the increases in molecular weight and in perchloric acid solubility it is evident that reasonable conjugation has been achieved although it is not complete (Table 1). Conjugation was also confirmed by the results of electrophoresis which showed that polysaccharide co-migrated with protein in the conjugate preparations but not in the controls where polysaccharide was mixed with the enzyme.

The results of the thermal inactivation experiments are shown in Table 2. Acetylated and unsubstituted Ficolls induced stability on conjugation with acid phosphatase whereas the methylated Ficolls had little effect.

A control experiment showed that the presence of 50 mg/ml of unconjugated and unsubstituted Ficoll did not enhance the thermal stability at 60 °C. We shall consider some possible explanations of the

Table 1. Characterisation of acid phosphatase and the conjugates.

Preparation	Average molecular weight ^a (Daltons)	Solubility in 6% perchloric acid (%)	Relative specific activity (%)
Acid phosphatase	30 000	34	100
Acid phosphatase–Ficoll	55 000	84	87
Acid phosphatase–MeFicoll ₁	66 000	74	84
Acid phosphatase–MeFicoll ₂	82 000	74	88
Acid phosphatase–AcFicoll ₁	117 000	86	87
Acid phosphatase–AcFicoll ₂	105 000	86	89

^a Unconjugated polysaccharide in the preparations did not influence the results because optical registration was at 280 nm.

Table 2. Thermal inactivation of acid phosphatase and the conjugates. Heating was carried out at 60 °C in 0.2 M acetate, pH 5.0, at a protein concentration of 0.1 mg/ml. Duplicate samples were taken at intervals, frozen down and assayed later for enzyme activity. The results quoted are the mean of duplicate assays. The ratio (by weight) of polysaccharide to protein was always 10, whether the two were conjugated or mixed.

Time at 60 °C (min):	Remaining enzyme activity (%)			
	0	30	60	120
Control preparations				
Phosphatase	100	20	11	3
Phosphatase + Ficoll	100	19	11	4
Phosphatase + AcFicoll ₁	100	23	12	5
Phosphatase + MeFicoll ₁	100	23	13	5
Conjugate preparations				
Phosphatase–Ficoll	100	41	26	18
Phosphatase–AcFicoll ₁	100	37	25	17
Phosphatase–AcFicoll ₂	100	37	25	16
Phosphatase–MeFicoll ₁	100	28	16	8
Phosphatase–MeFicoll ₂	100	27	15	6

stabilising effect in relation to our results. In the discussion below, *intramolecular* crosslink signifies that the polysaccharide forms a bridge within one protein molecule. *Intermolecular* crosslink signifies a bridge between two protein molecules.

Intramolecular, covalent, crosslinks. Marshall³ has suggested that synthetic protein-polysaccharide conjugates owe their enhanced stability to the presence of intramolecular, covalent, crosslinks formed by the polysaccharide. This explanation, however, is not valid for natural glycoproteins nor does it seem to fit our results. Thus the partially acetylated and methylated Ficolls should be able to form crosslinks to about the same extent. Yet, only the methylated Ficolls failed to induce stability.

Marshall's hypothesis seems plausible and may well be true in many cases, but there must also be other causes for the stabilising effect.

Intramolecular, non-covalent, crosslinks. Another possibility is that the bound carbohydrate can establish close association with the protein surface by forming a number of hydrogen bonds with hydrophilic amino acid sidechains. This might be considered as intramolecular, non-covalent crosslinking and could conceivably lead to enhanced thermal stability. An effect of this type would be similar to the well-known substrate-induced stabilisation of enzymes caused by non-covalent binding of the substrate at the active site of the enzyme.

Intermolecular, covalent crosslinks. When proteins are linked to polysaccharides there will normally be two or more potential sites of attachment in all polymer molecules. Aggregates consisting of several molecules of each kind may therefore arise (superpolymers) and the enhanced thermal stability be due to the presence of intermolecular, covalent bonds.³ However, we consider this an unlikely explanation of our results since the average molecular weights of the conjugates were rather low (Table 1). This indicates the presence of only small amounts of superpolymers.

Prevention of aggregation. Glycosylation of proteins may reduce the tendency to aggregate at elevated temperature^{1,10,11} and the observed stability could possibly be due to this effect. To test this, the preparations were analysed for molecular weight by sedimentation equilibrium before and after heating at 60 °C for 2 1/2 h. Only small increases were observed and the different conjugate preparations showed similar behaviour. We therefore consider prevention of aggregation to be an improbable explanation of our results.

Effects on protein hydration. Both hydration¹ and dehydration⁴ have been suggested as causes of thermal stabilisation of proteins, and we feel rather uncertain as to the importance of this parameter for proteins in solution. However, it seems plausible that a bound polysaccharide in close association with the protein surface might affect the hydration in local areas and possibly the thermal stability. An effect of this nature should be sensitive to the introduction of hydrophobic groups in the polysaccharide and is thus compatible with our results.

Chemical alteration of amino acid sidechains. The cyanogen bromide method of coupling involves free amino groups in the protein and the alteration of these groups might affect the thermal stability. However, this change in the protein is the same whether the polysaccharide is methylated, acetylated or unsubstituted and could not adequately explain the results obtained.

Having considered various possible explanations for the stabilising effect of bound carbohydrate, we believe "crosslinking" by hydrogen bonds between polysaccharide and protein to be the most probable one. It is also possible that effect on protein hydration are important. Both explanations are compatible with the experimental results.

Further experiments should be carried out using other enzymes and other modified polysaccharides in order to test if our observations have general validity.

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