SULPHATION OF A CELL SURFACE GLYCOPROTEIN FROM *VOLVOX CARTERI*

Evidence for a membrane-bound sulfokinase working with PAPS

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1. Introduction

Sulphated glycoproteins have been implicated in the control of cell growth and differentiation: fibronectins [1,2]; glycoproteins in developing sea urchin embryos [3]; and a surface glycoprotein from *Volvox carteri* [4]. This *Volvox* glycoprotein, of 185 000 app. M_r [4] appears to be involved in the control of the differentiation of the organism during embryogenesis. Although PAPS or APS must be involved as sulphate donor in the synthesis of such molecules, the incorporation of sulphate from PAPS into a sulphated glycoprotein has been demonstrated only in [5]. It was therefore of interest to partially characterize the sulphate-transfer system operative in *Volvox*.

2. Materials and methods

The growth of *Volvox carteri f. nagariensis* female strain HK10, pulse labelling of *Volvox* in vivo with [^{35}S]sulphate, SDS–polyacrylamide gel electrophoresis on 6% gels, fluorography and the preparation of *Volvox* sonicates were as in [4,6]. During sonication, gonidia were rapidly destroyed and, although a few somatic cells retained their shape, they were permeable to trypan blue. Membrane and particulate material was isolated by centrifugation of 1 ml samples of sonicate on 4 ml cushions of 20% sucrose at 110 000 × *g* for 70 min.

Abbreviations: APS, adenosine 5′-phosphosulphate; PAPS, 3′-phosphoadenosine 5′-phosphosulphate

2.1. *Preparation of PAPS and APS*

PAPS was synthesized using the yeast system [7] and isolated by chromatography on DEAE-cellulose, eluting with a linear gradient of 0.15–0.6 M triethylammonium bicarbonate. PAPS was well separated from the adenine nucleotides. Thin-layer chromatography on PEI-cellulose in 0.2 M collidine, 0.1 M acetic acid, 1.2 M NaCl showed only 2 radioactive components, sulphate and PAPS. Two preparations of [^{35}S]PAPS were used. The first had spec. act. 7.2×10^9 cpm/μmol and contained 4.6% free sulphate, while the second had spec. act. 2.0×10^{10} cpm/μmol and 8.3% free sulphate.

APS was prepared by treatment of PAPS with alkaline phosphatase and identified by chromatography on PEI-cellulose in 0.7 M LiCl and 0.3 M ammonium bicarbonate, using commercial APS as a standard.

2.2. *In vitro incorporation of inorganic sulphate*

Volvox sonicate was prepared in 400 μl 0.05 M Tris-acetate buffer containing 10 mM $MgCl_2$, 10 mM cysteine, 1 μM sodium sulphate and 10 μM carbonylcyanide *p*-trifluoromethoxyphenylhydrazone, at pH 8.0. Then 200 μCi carrier-free [^{35}S]sulphate and either 40 μl 0.1 M ATP or water were added. After 2 h at 30°C, the membrane fraction was centrifuged, suspended in 500 μl 0.05 M Tris buffer (pH 8.0) containing 200 μg subtilisin and, following digestion for 1 h, the samples were dried, denatured in SDS–mercaptoethanol and electrophoresed.

2.3. *In vitro incorporation of sulphate from PAPS*

Volvox was sonicated in a 0.05 M Tris–acetate

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buffer containing 0.1 M sucrose, 0.1 M NaF, 2 mM EDTA, 1 mM sodium sulphate, 10 mM cysteine at pH 8.0. Incubations were for 2 h at 30°C with PAPS at 3 μM. Subtilisin treatment and electrophoresis were performed as above.

2.4. *In vitro synthesis of PAPS by* Volvox

Incubations were in 1 ml Tris-acetate buffer containing 10 mM $MgCl_2$ and 0.1 M sucrose at pH 8.0. Synthesis was initiated by addition of 100 μl 0.1 M ATP and 100 μl 5 mM sodium sulphate, containing ^{35}S as a tracer. After 1 h at 30°C reaction was stopped by the addition of 3 ml ethanol and PAPS partially purified on DEAE-cellulose. Subsequently, samples were chromatographed on PEI-cellulose as before and the synthesized PAPS counted directly.

3. Results

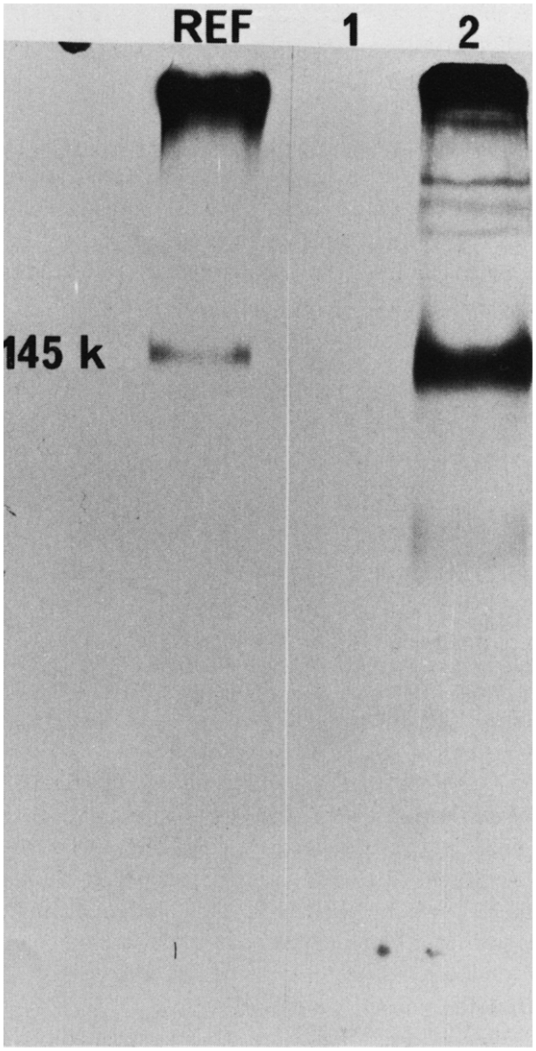
All experiments were performed with synchronized *Volvox* populations in the 4 cell stage of embryogenesis, at which stage rapid sulphate incorporation into a sulphated surface glycoprotein is observed in vivo [4]. In all experiments, product formation or sulphate incorporation is expressed as 'per 100 ml *Volvox*', that is, relative to the activity measured in the material isolated from 100 ml *Volvox* culture suspension containing ~15 colonies/ml.

3.1. *In vitro incorporation of inorganic sulphate*

Incubation of *Volvox* sonicates with inorganic sulphate and ATP led to the incorporation of sulphate into a number of high M_r products, most notably a band of diffuse material with an electrophoretic mobility close to that of the 185 000 M_r glycoprotein synthesized in vivo. If the material was treated with subtilisin following the labelling experiment, then this glycoprotein was not observed, and instead another band of labelled material was found with a mobility corresponding to the 145 000 M_r glycopeptide produced by the same treatment of in vivo labelled 185 000 M_r glycoprotein. Up to 0.05 pmol sulphate was found in this component which will be referred to as the 145 kA component. In the absence of ATP there was no detectable incorporation of sulphate (fig.1). Since subtilisin treatment gave a more identifiable product, this step was used routinely.

3.2. *In vitro transfer of sulphate from PAPS to 145 kA*

Incubation of *Volvox* sonicates with PAPS led to labelling of the 145 kA component. Incorporation of sulphate by hydrolysis of the added PAPS and subsequent activation of the sulphate can be completely excluded: (i) activation of sulphate is exceedingly unlikely in the presence of fluoride and EDTA and in the absence of ATP and magnesium; (ii) 1 mM sulphate was always present in the buffers used. This would dilute any liberated [^{35}S]sulphate to an indetectable specific activity.



ig.1. SDS–polyacrylamide gel electrophoresis of *Volvox* nicates mixed with: (1) [^{35}S]sulphate; (2) [^{35}S]sulphate + ATP. The lane labelled 'Ref' was obtained by in vivo labelling with sulphate and subsequent subtilisin treatment.

The 145 kA component was frequently more diffuse than the product of in vivo labelling, the 145 000 M_r glycoconjugate, and it occasionally had a slightly higher electrophoretic mobility. However, the two appear identical (145 kA may be a poorly synthesized version of the 145 000 M_r glycoprotein):

(1) Material with mobility close to that of 185 000 M_r protein disappears after subtilisin treatment, and 145 kA is produced – this parallels the in vivo results.

(2) 145 kA is the major discrete glycoprotein band which is sulphate labelled in the membrane fraction of *Volvox* in the 4 cell stage. The same is true for the 145 000 M_r glycoprotein in vivo.

(3) Treatment of 145 000 M_r glycoprotein with 1 M NaOH at 96°C for 1 h produces a single, discrete, sulphate-containing product of ~100 000 app. M_r. When 145 kA was eluted from gels following electrophoresis and hydrolysed in the same way, a single labelled band of 100 000 M_r was produced.

Under these conditions, the mean incorporation of sulphate into 145 kA was 0.20 pmol/100 ml *Volvox* (mean of 8 detn., 0.10–0.50 pmol). In in vivo pulse experiments, the mean was 1.4 pmol/100 ml *Volvox* (mean of 7 detn). An accurate comparison of the 2 systems is difficult, since neither is likely to represent optimal incorporation. The in vitro sulphation is, however, not strikingly inefficient, particularly since preliminary results indicate that the in vitro activity is unstable.

3.3. *APS is not an intermediate in the sulphation*

Incubation of *Volvox* sonicates with 3 μM PAPS and 3 μM APS in parallel experiments showed the normal incorporation of sulphate from PAPS. None was observed from APS. Thus incorporation from PAPS by preliminary hydrolysis to APS does not occur.

3.4. Volvox *synthesizes PAPS in vivo*

Volvox suspensions were sonicated following normal in vivo pulse labelling with sulphate. PAPS could be identified in the soluble material as in section 2. The amounts were small: ⩽0.01 pmol PAPS/100 ml *Volvox*.

3.5. *Transfer of sulphate from PAPS to 145 kA occurs in a particulate system*

Volvox sonicates were separated into membrane and supernatant fractions by centrifugation through 20% sucrose. These 2 fractions were then retained, and material within the sucrose discarded. Incubations were then made with PAPS and: (i) whole sonicate; (ii) resuspended membrane fraction; (iii) membrane fraction to which was added supernatant, equivalent to that in whole sonicate. Sulphate incorporation in the 3 incubations was, respectively, 0.105, 0.107 and 0.105 pmol/100 ml (mean of 2 expt). There is no evidence for the requirement of a soluble sulphokinase (fig.2).

To eliminate the possibility that sulphate incorporation occurred solely on cell ghosts, sonicates were centrifuged sequentially at 500 × *g* for 10 min, 10 000 × *g* for 20 min and 110 000 × *g* for 60 min, and the fractions incubated separately with PAPS. Sulphate incorporation into 145 kA occurred almost exclusively in the 500 × *g* to 10 000 × *g* material.

3.6. *Enzymes synthesizing PAPS in* Volvox *are partially particle bound*

In view of the above result, it was of interest to

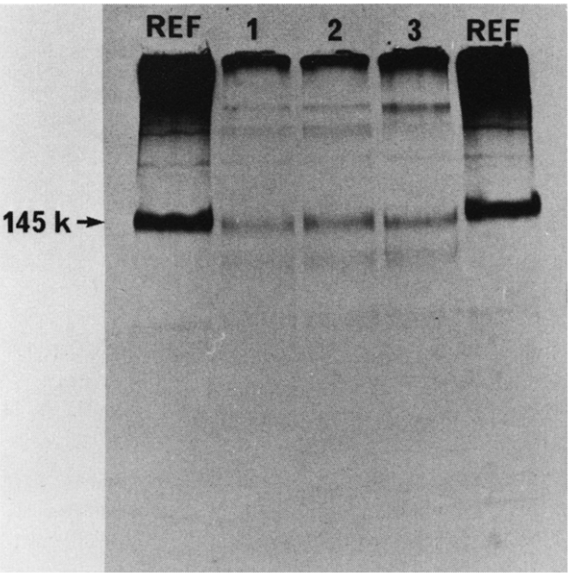


Fig.2. Sulphated proteins following incubation of [^{35}S]PAPS with: (1) *Volvox* sonicate; (2) particulate material isolated from the sonicate; (3) recombined particulate and soluble material. The lanes labelled 'Ref' were obtained by in vivo labelling with sulphate and subsequent subtilisin treatment.

see if the enzymes synthesizing PAPS were also membrane-bound. Therefore whole homogenate, resuspended membrane fraction and supernatant or soluble fraction were incubated with sulphate and ATP as in section 2. Typical net synthesis rates for PAPS were: whole sonicate, 8.9 nmol/100 ml *Volvox;* membrane fraction, 2.2 nmol/100 ml; supernatant, 3.0 nmol/100 ml. In the absence of added ATP, whole sonicate synthesized $\leqslant 1\%$ of the above amount of PAPS.

4. Discussion

The results demonstrate that sonicates of *Volvox* incorporate sulphate into high M_r material, including the cell surface glycoprotein which was proposed to be involved in the control of cell differentiation during embryogenesis. The reaction is dependent on ATP. As evidence that PAPS is the sulphate donor, it has been shown that PAPS is synthesized in vivo and in vitro, and that sulphate is transferred in vitro from PAPS to acceptors. Although APS is known to be a sulphate donor in phototrophic bacteria, plants and green algae [7–11], it is not involved here.

The enzyme or enzymes catalyzing the transfer of sulphate from PAPS to protein are particle-bound; synthesis of PAPS seems to be partially particle bound. This suggests that a closely coordinated system incorporates inorganic sulphate into membrane glycoproteins.

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