The serine acetyltransferase from *Escherichia coli*

Over-expression, purification and preliminary crystallographic analysis

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Received 11 October 1990; revised version received 25 October 1990

An expression vector has been constructed which increases the expression of serine acetyltransferase (SAT) from *E. coli* to 17% of the soluble cell protein. A novel purification procedure, using dye-affinity chromatography, allows purification of SAT to homogeneity. The enzyme has been crystallised from polyethylene glycol, in the presence of L-cysteine (an inhibitor of SAT). The crystals which diffract to beyond 3.0 Å resolution are of the tetragonal spacegroup $P4_12_12$ (or $P4_32_12$) with cell dimensions $a=b=123$ Å, $c=79$ Å. Since ultracentrifugation and gel-filtration experiments indicate that purified SAT is a tetramer, there appears to be one-half tetramer in the asymmetric unit ($V_m=2.55$ Å^3/Da).

Crystallisation; Acetyltransferase; *cysE*

1. INTRODUCTION

Serine can be converted to cysteine by a wide variety of organisms including bacteria [1,2], fungi [3], and higher plants [4]. The pathway comprises two steps:

L-serine + acetyl-coenzyme A → *O*-acetyl-L-serine + coenzyme A (1)

O-acetyl-L-serine + sulphide → L-cysteine + acetate (2)

Step 1 is catalysed by serine acetyltransferase (SAT; EC 2.3.1.30) while step 2 is catalysed by *O*-acetyl-L-serine (thiol) lyase (EC 4.2.99.8). The SAT gene has been cloned from *E. coli* [5] and expressed at 0.2% soluble cell protein. Purification protocols have also been described previously [1,2,5].

Serine acetyltransferase shows a number of interesting enzymological properties. Firstly, the enzyme probably functions in vivo as a tightly bound complex with *O*-acetyl-L-serine (thiol) lyase, since the two enzymes copurify even after a number of diverse purification procedures (e.g. salt precipitation, ion-exchange, gel filtration) [1]. The bifunctional complex has been referred to as 'cysteine synthase' [2]. The stability of this complex, in both *Salmonella typhimurium* and *E. coli*, is reduced by the presence of *O*-acetyl-L-serine - the product of the SAT catalysed reaction.

Serine acetyltransferase is inhibited by cysteine [1]. Steady-state kinetic measurements suggest the inhibition to be competitive with respect to coenzyme A, a somewhat surprising finding given the lack of structural similarity between the two compounds. The inhibition constant K_i, is very low (1 μM), whereas the K_m for serine is three orders of magnitude weaker (1 mM). Nonetheless, in the absence of cysteine, no significant inhibition of the enzyme is apparent until a serine concentration of 5 mM. Thus the enzyme is able to distinguish between two remarkably similar compounds with a very high degree of specificity. In order to investigate the structural basis for this specificity, SAT has been over-expressed, purified, and crystallised as a prelude to studies by X-ray diffraction.

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2. EXPERIMENTAL

2.1. *Enzyme assays*

These were performed according to [1] by monitoring the increase in absorbance at 412 nm due to the reaction of the CoA thiolate anion with 5,5-dithiobis(2-nitrobenzoate) (DTNB). For assays in the presence of cysteine (which reacts with the DTNB), the cleavage of the thioester bond of acetyl-coenzyme A was monitored directly by the absorbance change at 232 nm [1].

2.2. *Recombinant DNA techniques*

Plasmid pWT2 was kindly provided by Prof. A. Bock, Munich University. Unless otherwise stated, all in vitro DNA manipulations were carried out according to [6]. A 3.7 kb *Bam*HI fragment was excised from pWT2 and inserted into the *Bam*HI site of pUC19 [7] to give pSAT1 (Fig. 1). A 800 bp *Sau*96I fragment was removed from pSAT1 and purified by extraction from low melting point agarose. The 5′ overhangs were removed by digestion with mung bean nuclease (New England Biolabs) in accordance with the supplier's instructions. After the blunt-ended fragment had been further digested with *Cla*I, the resulting 550 bp and 250 bp fragments were separated by agarose gel electrophoresis. In a separate incubation, pSAT1 was digested with *Cla*I and *Pst*I, to excise a 1.5 kb *Cla*I/*Pst*I fragment. In

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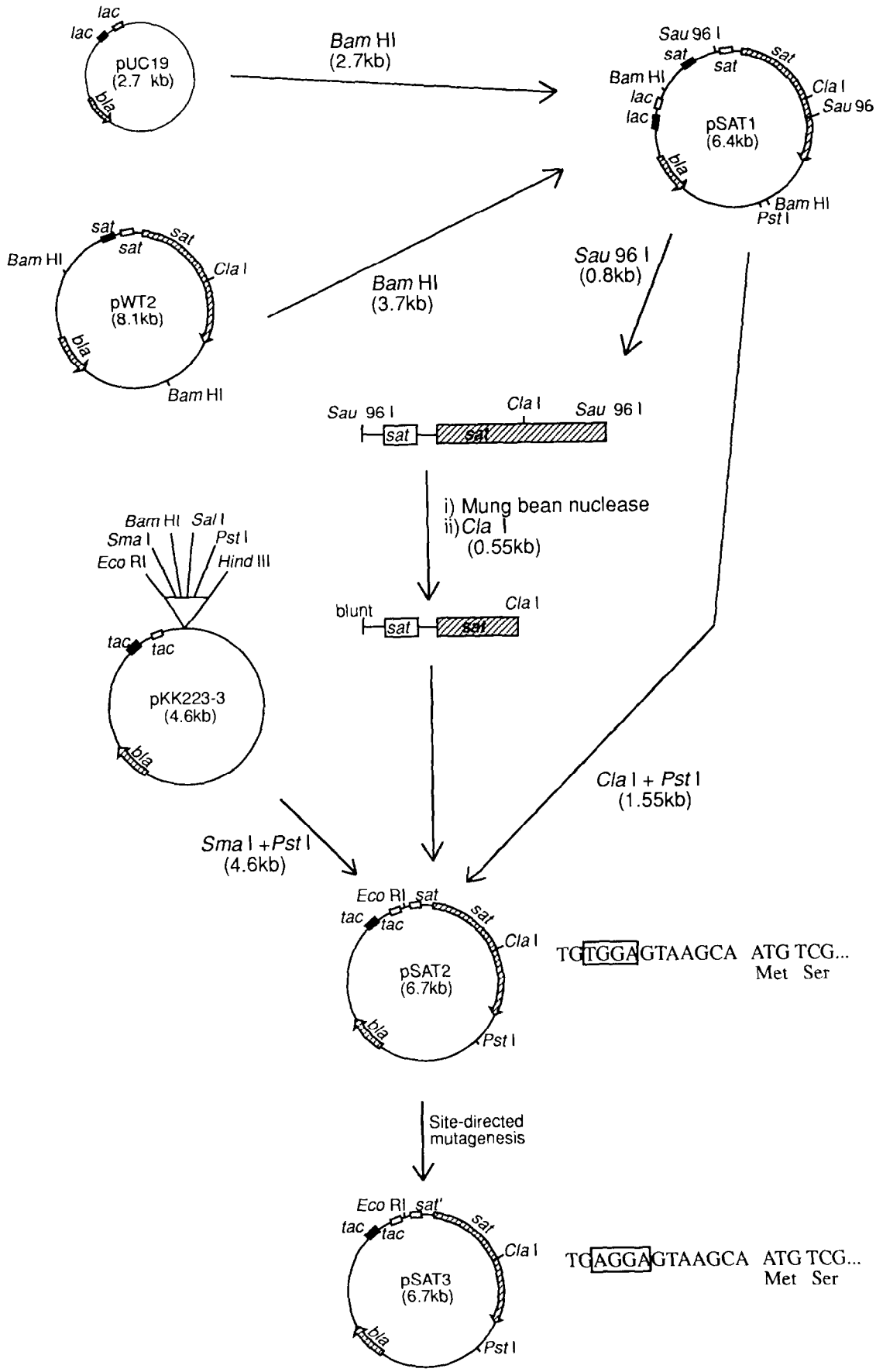
00145793/90/$3.50


lac
lac
pUC19
(2.7 kb)
bla
Bam HI
(2.7kb)
Sau 96 I
sat
sat
sat
Bam HI
lac
lac
Cla I
Sau 96I
pSAT1
(6.4kb)
bla
Bam HI
Pst I
sat
sat
sat
Bam HI
Cla I
pWT2
(8.1kb)
bla
Bam HI
Bam HI
(3.7kb)
Sau 96 I
(0.8kb)
Sau 96 I
Cla I
Sau 96 I
sat
sat
i) Mung bean nuclease
ii) Cla I
(0.55kb)
Bam HI Sal I
Sma I
Pst I
Eco RI
Hind III
tac
tac
pKK223-3
(4.6kb)
bla
blunt
Cla I
sat
sat
Cla I + Pst I
(1.55kb)
Sma I + Pst I
(4.6kb)
Eco RI sat
tac
tac
sat
Cla I
pSAT2
(6.7kb)
bla
Pst I
TGTGGAGTAAGCA ATG TCG...
Met Ser
Site-directed
mutagenesis
Eco RI sat'
tac
tac
sat
Cla I
pSAT3
(6.7kb)
bla
Pst I
TGAGGAGTAAGCA ATG TCG...
Met Ser

a tripartite ligation, the 550 bp (5′-blunt/*ClaI*) and 1.5 kb *ClaI*/*PstI* fragments were ligated into the plasmid pKK223-3 (Pharmacia), between the *SmaI* and *PstI* sites, to give pSAT2.

Plasmid pSAT3 was prepared by site-specific mutagenesis according to [8] using the 550 bp *EcoRI*/*ClaI* fragment from pSAT2. The mutated DNA fragment was sequenced and then used to replace the equivalent fragment in pSAT2 to give pSAT3.

2.3. *Preparation of Cibacron-blue Sepharose*

This was prepared by mixing 5 g of Cibacron-Blue F3GA (Sigma) with 100 ml of Sepharose 6B (Pharmacia) in 100 ml of sodium carbonate buffer at pH 9.5. The gel was incubated overnight at 60°C, and then washed in 6 M urea, 2 M sodium chloride until dye could no longer be washed from the gel. The Cibacron-blue gel was then equilibrated in 20 mM Tris-HCl (pH 7.5), and stored in the same buffer (containing 0.01% sodium azide) at 4°C.

2.4. *Protein purification*

Because of the moderate cold lability of this enzyme [1], all procedures were performed at room temperature unless otherwise stated. Protein was prepared from cultures of *E. coli* (JM 101), harbouring pSAT3. Cultures (typically 2 × 500 ml) were grown in 2YT broth [6] at 37°C with agitation. Ampicillin was added to a concentration of 100 μg/ml. In addition, 1 mM L-cysteine and 10 mM L-serine were added to assist cell growth (see below). Cultures were grown until $A_{600} = 0.5$, and then induced by the addition of isopropyl-thiogalactoside to 1 mM. A further addition of L-cysteine and L-serine (1 and 10 mM, respectively) was made at this stage. Cells were harvested by centrifugation, and resuspended in 20 mM Tris · HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol. The cells were disrupted by sonication and a cell-free extract was prepared by centrifugation (10 000 × g) at 4°C. This cell extract was heated to 60°C in a water-bath set at 70°C, and then maintained at 60°C for 5 min. The solution was cooled on ice for 5 min and then centrifuged to remove the precipitated protein. Ammonium sulphate (0.35 g/ml extract) was added to the supernatant to precipitate a protein fraction which included SAT. The precipitate was collected by centrifugation, and resuspended in at least 100 ml of 20 mM Tris-HCl (pH 7.5). This sample was then applied to a 40 ml Cibacron-blue Sepharose column at a flow rate of 50 ml/h, and washed exhaustively with 0.1 M NaCl, 20 mM Tris-HCl (pH 7.5). The SAT was eluted with 20 mM Tris-HCl (pH 7.5) containing 100 μM L-cysteine. Only fractions with an A_{280} greater than 0.15 were pooled. The eluted protein was concentrated to 10 mg/ml by ultrafiltration, and then stored at room temperature after the addition of Tris-HCl (pH 7.5) to a final concentration of 100 mM.

2.5. *Molecular mass determinations*

Ultracentrifugation experiments were performed at 25°C using protein at a concentration of 0.5 mg/ml in 100 mM Tris-HCl (pH 7.5) containing 100 μM L-cysteine.

Gel filtration experiments were carried out using an FPLC system (Pharmacia) at 25°C. Protein (0.5 ml at 0.2 mg/ml) was passed through a Superose 6 and Superose 12 HR 10/30 column in series, at a flow rate of 0.4 ml/min. Elution volumes were calibrated using a number of molecular weight standards (see Fig. 2). Experiments were performed in 0.15 M KCl, 50 mM potassium phosphate (pH 7.5) in the presence and absence of 100 μM L-cysteine.

2.5. *Crystallisation*

Crystals were prepared by vapour diffusion [9], in hanging drops, from polyethylene glycol (PEG) 1000. Equal volumes of protein solution (at 10 mg/ml) and reservoir solution were mixed and left to equilibrate for 2–4 weeks at room temperature. After this time crystals were observed in drops over wells containing 8–16% PEG 1000, 10 mM Tris · HCl (pH 7.5).

3. RESULTS AND DISCUSSION

3.1. *Over-expression*

Previous studies [5] demonstrated that clones containing the plasmid pWT2 express SAT at around 0.2% of the soluble cell protein when grown in minimal medium to an A_{420} of 1.0. Although some 40-fold higher than expression from the chromosomal gene, the low cell density requires that large volumes of culture be grown in order to prepare sufficient quantities of SAT for structural studies. In order to improve the level of expression of SAT, a 3.7 kb *Bam*HI fragment was subcloned from pUC19 to give plasmid pSAT1. Strains of *E. coli* containing these plasmids have approximately 10-fold increased copy number over pWT2 (data not shown) and show an increased level of expression of SAT, by a factor equivalent to the increase in copy number. Nonetheless, such host/vector systems still only yield 2–3 mg SAT/litre of cell culture. The subcloning of the SAT gene into pKK223-3, which carries the powerful IPTG-inducible TAC promoter [10], both increases expression levels and has the advantage of providing some control over expression. Hence, clones carrying pSAT2 not only expressed SAT at 5 mg/litre of culture wtihout induction, but were capable of 10 mg/litre when induced by the addition of IPTG. The poor agreement between the ribosome binding site (just 5′ of the SAT gene in pSAT2) and that of the consensus sequence from other highly expressed genes in *E. coli* [11] was identified as the limitation to the level of expression of the gene. Consequently, site-specific mutagenesis was used to alter the ribosome binding site to one which more closely matched the consensus sequence. The resultant plasmid (pSAT3) showed increased expression levels of SAT to 30 mg/litre after induction, but this increased level of expression appeared to be harmful since cultures failed to reach high densities. A possible explanation is that the over-expression of SAT results in a reduced intracellular concentration of serine (because it becomes acetylated) which then impairs the rate of protein synthesis. In order to overcome this problem, 1 mM cysteine and 10 mM serine were added to the culture medium to inhibit SAT and to ensure the availability of serine. The addition of either serine or cysteine alone was not sufficient to sustain growth to high cell density. Cultures grown in the presence of additional cysteine and serine did grow to high cell density and produced SAT at a level of around 100 mg/litre.

Fig. 1. Preparation of pSAT3. Hatched regions indicate DNA coding for proteins (*bla* = β-lactamase; *sat* = serine acetyltransferase; *lac* = DNA derived from the lactose operon). Open boxes indicate ribosome binding sites, while filled boxes indicate promoter sequences. The modification of the Shine Dalgarno sequence at the 5′ end of the *sat* gene in pSAT2 which was made to prepare pSAT3 is indicated.

Table I

Purification of SAT

Sample	Volume (ml)	Protein (mg)	Activity (μmol/min)	Specific activity (μmol/min/mg)	Yield (%)
Cell extract	60	735	8820	12.0	100
Heat extract	58	319	6950	21.8	79
$(NH_4)_2SO_4$	160	232	5660	24.4	64
SAT pool	47	58	4150	71.6	47

3.2. *Purification of SAT*

Procedures for the purification of SAT which have been described previously [1,2,5] involve a large number of steps and are consequently very time consuming, and the reported yields are rather low (0.2–9%). We present here an alternative method of purification which enables rapid purification of SAT in high yield, and with a high specific activity (Table I).

It is known that a number of proteins which bind compounds containing an AMP moiety will bind the dye Cibacron-blue. SAT was found to bind very tightly to Cibacron-blue Sepharose, but can be eluted in a number of different ways. When the bound protein was eluted from the column with a salt gradient, the SAT was eluted in a broad peak with a consequent dilution of the protein. In addition the eluted protein, although purified considerably over the starting material, was observed to be rather impure by SDS polyacrylamide gel elctrophoresis. Elution from the column with a salt step directly, eluted the SAT in a sharper peak but at an even lower purity (data not shown). Since L-cysteine shows competitive inhibition kinetics, with respect to acetyl-coenzyme A [1], it seemed an attractive strategy to use the inhibitor to elute the bound SAT from the dye-column. Although SAT purified in this way still eluted in a rather broad peak (typically greater than one column volume), protein was very pure. The specific activity of the eluted protein was 70–80 U/mg - some 2–3-fold higher than that prepared by other methods [1,2,5] possibly because of the speed of the purification procedure (typically 6 h from cells to pure protein).

The kinetic properties of SAT prepared by this method were the same as those described for SAT purified by other methods [1,2,5].

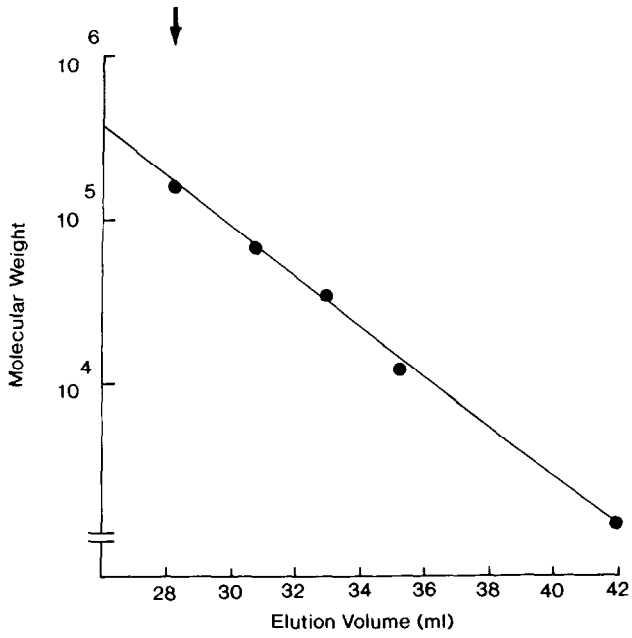


Fig. 2. FPLC gel-filtration of SAT. Standards used were immunoglobulin G (160 000 Da), bovine serum albumin (66 000 Da), β-lactoglobulin (35 000 Da), cytochrome *c* (12 000 Da) and vitamin B_{12} (1400 Da). The elution point of SAT (in both the presence and absence of cysteine) is indicated by the arrow.

3.3. *Oligomeric structure of purified SAT*

Ultracentrifugation and gel-filtration experiments were carried out to determine the quaternary structure of purified SAT from estimates of the native molecular mass of the protein. Ultracentrifugation experiments in the presence of 100 μM L-cysteine indicated a molecular mass of 125 000 Da. This result was in reasonable agreement with the gel-filtration experiments, which suggested a molecular mass of 160 000 Da. The molecular mass appeared to be independent of the presence or absence of cysteine (Fig. 2). From these results, taken in conjunction with the crystallographic data presented below, we deduce that purified SAT (subunit M_r = 29 261) is a tetramer.

3.4. *Crystallography*

Crystals were obtained after 2–4 weeks at room temperature, and are shown in Fig. 3. Typically these reached a maximum size of 0.25 × 0.15 mm. X-Ray analysis of these crystals showed diffraction to beyond 3.0 Å resolution using Synchrotron radiation (Daresbury, UK), but showed signs of disorder at high resolution. When viewed down the long axis of the crystal, the diffraction exhibited 4-fold symmetry with a primitive lattice. Systematic absences in this zone and others identified the spacegroup as $P4_12_12$ or $P4_32_12$ with $a = b = 123$ Å and $c = 79$ Å. The number of SAT subunits in the asymmetric unit can be estimated according to [12]. With one subunit, V_m would be implausibly high at 5.1 Å^3/Da, while with three subunits, V_m would be rather low at 1.7 Å^3/Da. Hence the most likely situation is one in which there is one-half SAT tetramer in the asymmetric unit resulting in a V_{max} of

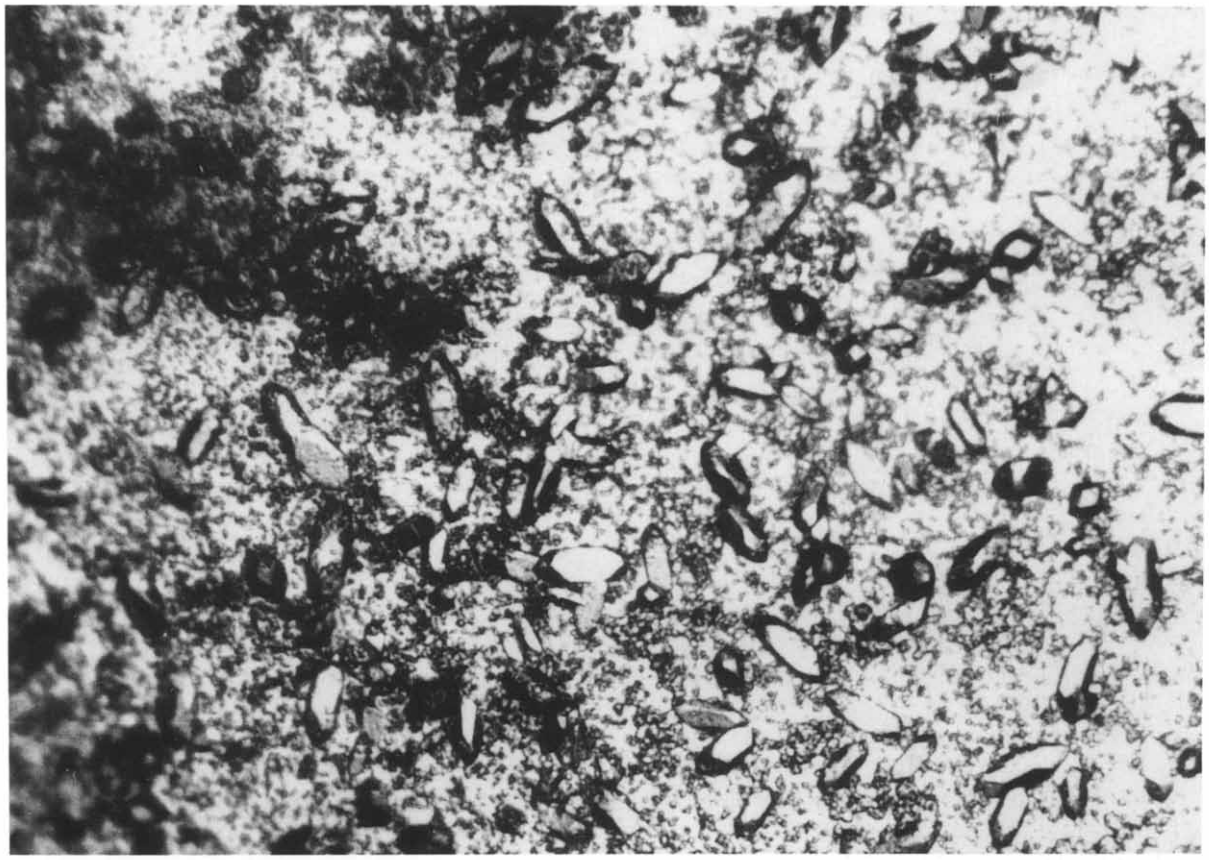


Fig. 3. Crystals of SAT grown from polyethylene glycol. The largest crystals grew to a size of 0.25 × 0.15 × 0.15 mm.

2.55 $Å^3$/Da. Because part of the molecular symmetry of the SAT tetramer is expressed crystallographically, the tetramer must sit on a crystallographic two-fold axis. The positions of the other two-fold axes of the tetramer should be readily determined using the rotation function [13], and will aid the interpretation of derivative Patterson maps.

Acknowledgements: We wish to thank Dr A.J. Rowe for carrying out the ultracentrifugation experiments, J. Keyte for synthesising oligonucleotides, and Prof. A. Bock for the gift of plasmid pWT2. We also gratefully acknowledge support from the Wellcome Trust, SERC, and the Nuffield foundation.

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