Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Cloning, purification, crystallization and preliminary X-ray crystallographic analysis of the biosynthetic *N*-acetylornithine aminotransferases from *Salmonella typhimurium* and *Escherichia coli*

V. Rajaram,[a] K. Prasad,[a] P. Ratna Prasuna,[a] N. Ramachandra,[a] S. R. Bharath,[a] H. S. Savithri[b] and M. R. N. Murthy[a]*

[a]Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India, and [b]Department of Biochemistry, Indian Institute of Science, Bangalore 560 012, India

Correspondence e-mail: mrn@mbu.iisc.ernet.in

Received 14 June 2006
Accepted 23 August 2006

Acetylornithine aminotransferase (AcOAT) is a type I pyridoxal 5′-phosphate-dependent enzyme catalyzing the conversion of *N*-acetylglutamic semialdehyde to *N*-acetylornithine in the presence of α-ketoglutarate, a step involved in arginine metabolism. In *Escherichia coli*, the biosynthetic AcOAT also catalyzes the conversion of *N*-succinyl-L-2-amino-6-oxopimelate to *N*-succinyl-L,L-diaminopimelate, one of the steps in lysine biosynthesis. It is closely related to ornithine aminotransferase. AcOAT was cloned from *Salmonella typhimurium* and *E. coli*, overexpressed in *E. coli* and purified using Ni–NTA affinity column chromatography. The enzymes crystallized in the presence of gabaculine. Crystals of *E. coli* AcOAT (eAcOAT) only diffracted X-rays to 3.5 Å and were twinned. The crystals of *S. typhimurium* AcOAT (sAcOAT) diffracted to 1.9 Å and had a dimer in the asymmetric unit. The structure of sAcOAT was solved by the molecular-replacement method.

1. Introduction

N-Acetylornithine aminotransferase (AcOAT; EC 2.6.1.11) is one of the enzymes involved in the biosynthesis of arginine. It catalyzes the conversion of *N*-acetylglutamate semialdehyde (NAGSA) to acetylornithine (AcOrn; Riley & Glansdorff, 1983; Rossi *et al.*, 1977; Albrecht & Vogel, 1964; Heimberg *et al.*, 1990; Fig. 1). AcOAT belongs to the fold-type I (α family) subgroup II family of pyridoxal 5′-phosphate (PLP) dependent enzymes in which the transamination takes place at a distal amino group (Mehta *et al.*, 1993). The mechanism of catalysis is similar to that of other aminotransferases, with two coupled half-reactions (John & Fowler, 1976; Williams *et al.*, 1982). In the first half-reaction, a semialdehyde and pyridoxamine 5′-phosphate (PMP) are formed. PMP is then converted back to PLP in the second half-reaction, which involves the conversion of α-ketoglutarate to glutamate. The substrate-specificity of these enzymes arises only with respect to the amino-group donor. AcOAT

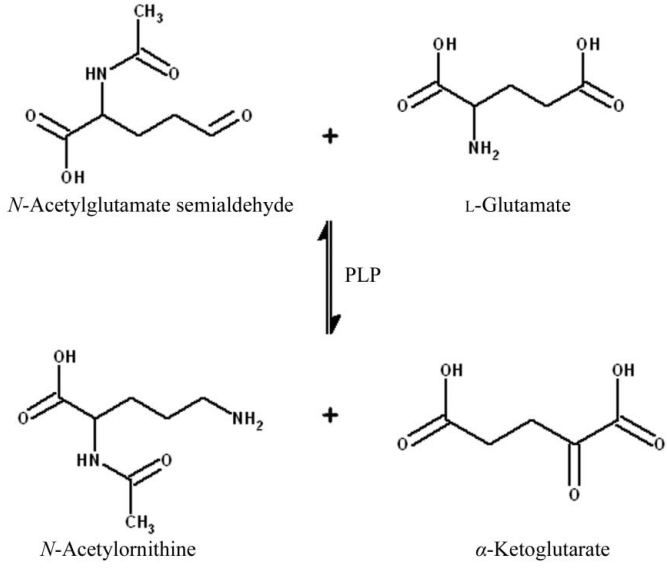
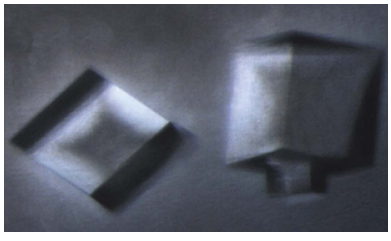


Figure 1
Reaction catalyzed by AcOAT.



is homologous to ornithine aminotransferase (OAT). The two enzymes differ in their substrates only by an acetyl group. OAT is highly specific for ornithine (Orn). In contrast, AcOAT can accept both AcOrn and Orn as substrates (Billheimer & Jones, 1974; Billheimer *et al.*, 1976, 1979). In *Escherichia coli*, biosynthetic AcOAT also catalyses the conversion of *N*-succinyl-L-2-amino-6-oxopimelate to *N*-succinyl-L,L-diaminopimelate, one of the steps in lysine biosynthesis. Therefore, AcOAT appears to be involved in both arginine and lysine biosynthesis (Ledwidge & Blanchard, 1999).

Gabaculine (5-amino-1,3-cyclohexadienylcarboxylic acid; Gcn), a naturally occurring organic compound first isolated from *Streptomyces toyocaenis*, is known to act as an irreversible inhibitor of γ-amino butyric acid aminotransferase (GABA-AT) and OAT (Rando, 1977; Jung & Seiler, 1978). In addition to OAT and GABA-AT, it also inhibits other PLP-dependent enzymes such as L-alanine transaminase, L-aspartate transaminase and D-amino-acid transaminase (Soper & Manning, 1982). It has been reported that the inhibition by Gcn is related to the enzymatic exchange of β-protons of the substrate and it could act as a potent inhibitor of AcOAT. A comparison of the structure of AcOAT with those of OAT (Shah *et al.*, 1997; Shen *et al.*, 1998; Storici, Capitani, Muller *et al.*, 1999) and other ω-aminotransferases (Storici, Capitani, De Biase *et al.*, 1999; Storici *et al.*, 2004; Hennig *et al.*, 1997) could provide information on their substrate-specificity and structural similarity. Here, we report cloning, purification and crystallization of AcOATs from *Salmonella typhimurium* and *E. coli*.

2. Materials and methods

2.1. Cloning

The AcOAT gene (*argD*) was PCR-amplified from *S. typhimurium* genomic DNA using KOD Hi-Fi DNA polymerase (Novagen) and specific sense (5′-**GCT*AGC***CATATGGCAACTGAACAAACGG-CTATTACG-3′; *Nhe*I site in bold, *Nde*I site in italics) and antisense (5′-**GGATCC**TTACTCGAGGGCCAGCACCTTCCCTACCG-3′; *Bam*HI site in bold) primers. The PCR-amplified fragment was digested with *Nhe*I and *Bam*HI. It was then ligated with pRSET 'C' vector previously digested with the same restriction enzymes. The genomic DNA and plasmid DNA isolation for cloning were carried out according to Sambrook & Russell (2001). The clone thus obtained (PR-sArgD) was confirmed by DNA sequencing. Such a cloning strategy resulted in the expression of *S. typhimurium* AcOAT (sAcOAT) with 14 additional amino acids from the vector including six histidines at the N-terminus, which facilitated purification by Ni–NTA affinity chromatography. AcOAT from *E. coli* (eAcOAT) was also cloned (PR-eArgD), expressed and purified in a similar manner, except that sense (5′-**GCTAGC**CATATGGCAATTGAACAAACA-GCAATTAC-3′) and antisense (5′-**GGATCC**TTACTCGAGCGCC-CCAACCACCTTCGC-3′) primers corresponding to the 5′ and 3′ ends of the eAcOAT gene and the genomic DNA of *E. coli* as the template were used to amplify the gene.

2.2. Overexpression and purification

The recombinant PR-sArgD and PR-eArgD clones were transformed into BL21 (DE3) pLys S cells and plated on LB-agar plates with ampicillin. The preinoculum prepared from a single colony was transferred to a large culture (500 ml) of Terrific broth containing 50 µg ml^{-1} ampicillin and incubated at 310 K until the OD at 600 nm reached 0.6. The expression of enzyme was induced with 0.3 m*M* IPTG and the cells were allowed to grow for a further 5–6 h at 303 K. The cells were pelleted and resuspended in 50 m*M* Tris pH 8.0 with 300 m*M* NaCl, 20% glycerol and 1% Triton-X100 (extraction buffer).

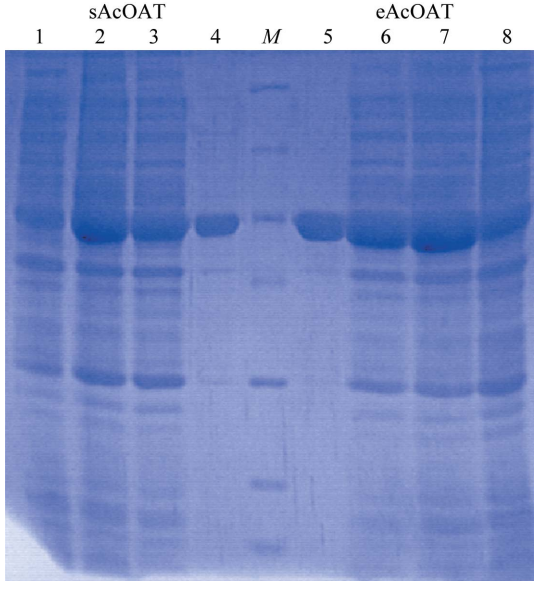


Figure 2
SDS–PAGE analysis of sAcOAT (lanes 1–4) and eAcOAT (lanes 5–8) during purification. Proteins were analysed on 12% SDS–PAGE and stained with Coomassie blue. Lanes 1 and 5, crude cell lysates before induction; lanes 2 and 6, crude cell lysates after 0.3 m*M* IPTG induction; lanes 3 and 7, soluble fraction; lanes 4 and 8, purified eAcOAT/sAcOAT after affinity column chromatography; lane *M*, molecular-weight markers. The bands in lane *M* correspond to the following molecular weights (kDa) from top to bottom: 116.0, 66.2, 45.0, 35.0, 25.0, 18.4 and 14.4.

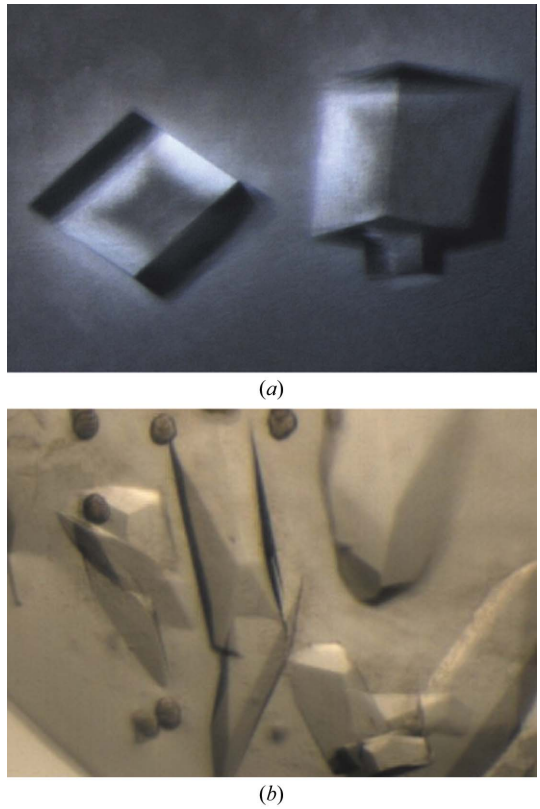


Figure 3
Crystals of (*a*) sAcOAT, (*b*) eAcOAT.

The cells were sonicated with intermittent cooling until the solution became clear and soluble; insoluble fractions were separated by centrifugation. Fractions along with marker proteins were run on 12% SDS–PAGE (Laemmli, 1970) to monitor the expression of sAcOAT and eAcOAT. sAcOAT and eAcOAT were purified from the soluble fraction using Ni–NTA affinity column chromatography following the manufacturer's protocol and dialyzed against buffer containing 20 m*M* Tris pH 8.0 with 200 m*M* NaCl. The molecular weight and purity of the enzymes were checked on 12% SDS–PAGE and a MALDI–TOF mass spectrometer. The enzyme was concentrated by several cycles of low-speed centrifugation using a 10 kDa molecular-weight cutoff Centricon (Amicon) to the required final concentration for crystallization. Protein concentration was estimated by the Bradford method (Bradford, 1976).

2.3. Crystallization, data collection and processing

sAcOAT and eAcOAT were crystallized at room temperature using the hanging-drop vapour-diffusion method. Crystallization attempts were carried out with Hampton Research Crystal Screens I and II, Index Screen Salt Rx Screen and by using conditions under which similar PLP-dependent enzymes have been crystallized (Shah *et al.*, 1997; Storici, Capitani, De Biase *et al.*, 1999; Hennig *et al.*, 1997). Also, screening for crystallization was performed in the presence of 1 m*M* Gcn. X-ray diffraction data were collected at 100 K on a Rigaku RU-200 rotating-anode X-ray generator equipped with a MAR Research imaging-plate detector. 20% ethylene glycol was used as the cryoprotectant. The data were indexed, integrated and scaled using *DENZO* and *SCALEPACK* from the *HKL*-2000 suite (Otwinowski & Minor, 1997). Structure-factor analysis for the presence of twinning was performed using the program *SFCHECK* from the *CCP*4 program suite (Vaguine *et al.*, 1999).

3. Results and discussion

3.1. Cloning, overexpression and purification

AcOATs from *S. typhimurium* and *E. coli* were overexpressed and purified by Ni–NTA affinity column chromatography as described in §2. Analysis of the purified enzymes by SDS–PAGE and MALDI–TOF for both sAcOAT and eAcOAT showed molecular weights close to the expected molecular weight of 46 kDa (Fig. 2).

3.2. Crystallization and data collection

Crystals suitable for collecting X-ray diffraction data for sAcOAT were obtained in the presence of 1 m*M* Gcn using 15 mg ml^{-1} protein in 0.1 *M* HEPES pH 7.0 in the presence of 20% PEG 3350 and 0.5 *M* ammonium acetate (Fig. 3*a*). These crystals were very sensitive to air exposure and developed multiple cracks. However, crystals were stable when flash-frozen in liquid propane immediately after soaking them in 20% ethylene glycol as the cryoprotectant. Data collected from crystals of sAcOAT extended to 1.9 Å resolution. The crystals belonged to space group $P2_12_12$, with a dimer in the asymmetric unit (Matthews coefficient of 2.0 Å^3 Da^{-1}; Matthews, 1968). Unit-cell parameters and data-collection statistics for sAcOAT are shown in Table 1. A molecular-replacement search for the orientation and position of sAcOAT with the program *AMoRe* (Navaza, 1994) using the human OAT monomer as the model revealed a unique solution. Placement of the model on the basis of the solution obtained revealed good packing with no short contacts. The resulting model could be successfully refined. Further refinement, model building and analysis of the resulting structure are in progress.

Crystals of eAcOAT suitable for collecting X-ray diffraction data were obtained in the presence of Gcn using 25 mg ml^{-1} enzyme in 0.1 *M* Tris pH 8.5 with 0.7 *M* trisodium citrate dihydrate as the precipitant (Fig. 3*b*). Most of the eAcOAT crystals only diffracted X-rays to a resolution of 3.5 Å. Although the unit-cell parameters were very similar for most of the crystals and appeared to correspond to a trigonal system, many of them could only be processed in space group *P*1. After screening many crystals, two data sets were acceptable. One of the data sets belonged to space group *P*1 (data 1) and the other to space group *P*321 (data 2). Analysis of both data sets using the *SFCHECK* program from the *CCP*4 suite (Vaguine *et al.*, 1999) indicated that data 1 was twinned with a twinning fraction of 0.35, whereas data 2 was untwinned. The twin operator in data 1 was h, $-h - k$, $-l$ (Fig. 4). The trigonal cell contained three dimers (Matthews

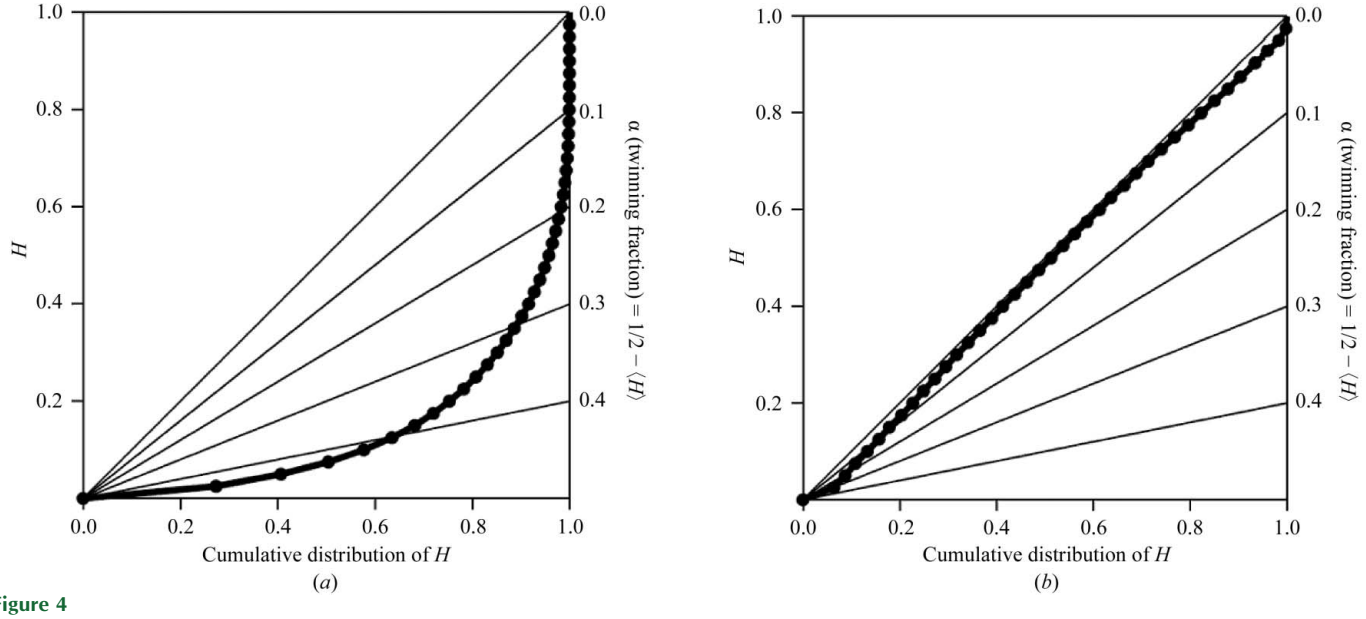


Figure 4
Structure-factor analysis illustrating the presence of partial twinning in triclinic eAcOAT crystals. $H = |I_{h1} - I_{h2}|/(I_{h1} + I_{h1})$. (*a*) *P*1 form of eAcOAT. Twin operator h, $-h - k$, $-l$. Twinning fraction 0.353. (*b*) *P*321 form of eAcOAT. Twin operator $-h$, $-k$, l. Twinning fraction 0.010.

Table 1
Data-collection and processing statistics.

Values in parentheses are for the last resolution shell.

		eAcOAT	
	sAcOAT	Data 1	Data 2
Ligand used	Gabaculine	Gabaculine	Gabaculine
Space group	$P2_12_12$	$P1$	$P321$
Unit-cell parameters			
a (Å)	97.07	151.05	150.42
b (Å)	65.49	151.09	150.42
c (Å)	111.97	226.127	227.00
α (°)	90	89.90	90
β (°)	90	90.00	90
γ (°)	90	120.00	120
Resolution (Å)	30–1.90 (1.97–1.90)	30–3.4 (3.52–3.40)	30–3.5 (3.62–3.50)
$R_{\rm merge}$† (%)	7.1 (39.3)	9.8 (36.4)	12.7 (37.7)
Compleness (%)	91.8 (80.7)	96.5 (96.8)	97.5 (99.3)
$\langle I/\langle\sigma(I)\rangle\rangle$	19.0 (3.4)	6.4 (2.1)	9.6 (3.0)
No. of reflections	693015	1403079	576135
Unique reflections	51290	227244	37294
Mathews coefficient (Å^3 Da^{-1})	2.0	2.8	2.8
No. of molecules in ASU	2	36	6

† $R_{\rm merge} = \sum I_{hj} - \langle I_h\rangle|/\sum\langle I_{hj}\rangle$, where I_{hj} is the jth observation of I_h and $\langle I_h\rangle$ is its mean intensity.

coefficient 2.8 Å^3 Da^{-1}), whereas the $P1$ cell was unusually large and could accommodate 36 molecules in the asymmetric unit with the same Matthews coefficient. Table 1 shows the data-collection statistics.

Although eAcOAT and sAcOAT are homologous with 91% sequence identity, they crystallize under entirely different conditions with different space groups. Comparison of AcOAT with OAT could provide information on the structural basis of substrate specificity and their evolutionary relationship.

The intensity data were collected at the X-ray facility for Structural Biology at the Molecular Biophysics Unit, Indian Institute of Science, supported by the Department of Science and Technology (DST) and the Department of Biotechnology (DBT) of the Government of India. Dr P. S. Satheshkumar is acknowledged for assistance during cloning. VR acknowledges the Council for Scienctific and Industrial Research (CSIR), Government of India for the award of a scholarship. MRN and HSS thank DST and DBT for financial support.

References

Albrecht, A. M. & Vogel, H. J. (1964). *J. Biol. Chem.* **239**, 1872–1876.
Billheimer, J. T., Carnevale, H. N., Leisinger, T., Eckhardt, T. & Jones, E. E. (1976). *J. Bacteriol.* **127**, 1315–1323.
Billheimer, J. T. & Jones, E. E. (1974). *Arch. Biochem. Biophys.* **161**, 647–651.
Billheimer, J. T., Shen, M. Y., Carnevale, H. N., Horton, H. R. & Jones, E. E. (1979). *Arch. Biochem. Biophys.* **195**, 401–413.
Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
Heimberg, H., Boyen, A., Crabeel, M. & Glansdorff, N. (1990). *Gene*, **90**, 69–78.
Hennig, M., Grimm, B., Contestabile, R., John, R. A. & Jansonius, J. N. (1997). *Proc. Natl Acad. Sci. USA*, **94**, 4866–4871.
John, R. A. & Fowler, L. J. (1976). *Biochem. J.* **155**, 645–651.
Jung, M. J. & Seiler, N. (1978). *Biol. Chem.* **253**, 7431–7439.
Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
Ledwidge, R. & Blanchard, J. S. (1999). *Biochemistry*, **38**, 3019–3024.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mehta, P. K., Hale, T. I. & Christen, P. (1993). *Eur. J. Biochem.* **214**, 549–561.
Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Rando, R. R. (1977). *Biochemistry*, **16**, 4604–4610.
Riley, M. & Glansdorff, N. (1983). *Gene*, **24**, 335–339.
Rossi, J. J., Vender, J. & Berg, C. M. (1977). *Biochem. Genet.* **15**, 287–296.
Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*, 3rd ed. Cold Spring Harbour, NY, USA: Cold Spring Harbour Laboratory Press.
Shah, S. A., Shen, B. W. & Brünger, A. T. (1997). *Structure*, **5**, 1067–1075.
Shen, B. W., Hennig, M., Hohenester, E., Jansonius, J. N. & Schirmer, T. (1998). *J. Mol. Biol.* **277**, 81–102.
Soper, T. S. & Manning, J. M. (1982). *J. Biol. Chem.* **257**, 13930–13936.
Storici, P., Capitani, G., De Biase, D., Moser, M., John, R. A., Jansonius, J. N. & Schirmer, T. (1999). *Biochemistry*, **38**, 8628–8634.
Storici, P., Capitani, G., Muller, R., Schirmer, T. & Jansonius, J. N. (1999). *J. Mol. Biol.* **285**, 297–309.
Storici, P., De Biase, D., Bossa, F., Bruno, S., Mozzarelli, A., Peneff, C., Silverman, R. B. & Schirmer, T. (2004). *J. Biol. Chem.* **279**, 363–373.
Vaguine, A. A., Richelle, J. & Wodak, S. J. (1999). *Acta Cryst.* D**55**, 191–205.
Williams, J. A., Bridge, G., Fowler, L. J. & John, R. A. (1982). *Biochem. J.* **201**, 221–225.