The intersubunit lock-and-key motif in human glutathione transferase A1-1: role of the key residues Met⁵¹ and Phe⁵² in function and dimer stability

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The dimeric structure of certain cytosolic GSTs (glutathione S-transferases) is stabilized by a hydrophobic lock-and-key motif at their subunit interface. In hGSTA1-1 (human class Alpha GST with two type-1 subunits), the key consists of two residues, Met⁵¹ and Phe⁵², that fit into a hydrophobic cavity (lock) in the adjacent subunit. SEC (size-exclusion chromatography)–HPLC, far-UV CD and tryptophan fluorescence of the M51A and M51A/F52S mutants indicated the non-disruptive nature of these mutations on the global structure. While the M51A mutant retained 80% of wild-type activity, the activity of the M51A/F52S was markedly diminished, indicating the importance of Phe⁵² in maintaining the correct conformation at the active site. The M51A and M51A/F52S mutations altered the binding of ANS (8-anilino-naphthalene-1-sulphonic acid) at the H-site by destabilizing

helix 9 in the C-terminal region. Data from urea unfolding studies show that the dimer is destabilized by both mutations and that the dimer dissociates to aggregation-prone monomers at low urea concentrations before global unfolding. Although not essential for the assembly of the dimeric structure of hGSTA1-1, both Met⁵¹ and Phe⁵² in the intersubunit lock-and-key motif play important structural roles in maintaining the catalytic and ligandin functions and stability of the GST dimer.

Key words: 8-anilino-naphthalene-1-sulphonic acid (ANS) binding, conformational stability, glutathione S-transferases (GST), human class Alpha GST with two type-1 subunits (hGSTA1-1), lock-and-key motif.

INTRODUCTION

During their folding pathway, cytosolic GSTs (glutathione S-transferases, EC 2.5.1.18), a supergene family of multifunctional proteins (for a review, see [1]), form obligate homo- or heterodimers with subunits from only within the same gene class (e.g. Alpha, Mu or Pi) [2]. Interactions at the subunit interface therefore play important roles in specifying molecular recognition between subunits and in stabilizing the dimeric structures of these proteins [3]. Crystal structures of GSTs reveal the involvement of both polar and non-polar moieties in forming intersubunit contacts between domain 1 of one subunit and domain 2 of the adjacent subunit and also reveal that approx. 14% of the subunit solvent-accessible surface area becomes buried at the dimer interface [4].

Based on the features of their crystal structures, GST subunit interfaces exist as two major groups. In the first group, the interface is mainly hydrophobic with a curved topography, whereas, in the second group, the interface is mainly hydrophilic with a flat topography [5]. The first group, the subject of the present study, is characterized by a hydrophobic lock-and-key (or ball-and-socket) motif that forms an interdigitated interface anchoring the two subunits together at both ends of the dimer interface. In this motif, a hydrophobic key in a loop in domain 1 of one subunit inserts into a non-polar pocket (lock) between helices 4 and 5 of the adjacent subunit [see Figure 1 for hGSTA1-1 (human class Alpha GST with two type-1 subunits)]. The type and number of hydrophobic residues making up the key of this motif varies among the different GST classes, which for hGSTA1-1 consists of the side chains of Met⁵¹ and Phe⁵² (Figure 1). The absence of a hydrophobic lock-and-key motif in certain GST classes (Beta, Delta, Kappa, squid Sigma, Tau and Theta [5–7]), as well as the structurally similar yeast prion Ure2p [8], may possibly be compensated for by an increase in electrostatic interactions at their dimer interfaces [9]. Interestingly, the crystal structures of

several class Sigma GSTs have indicated that these enzymes do in fact possess a lock-and-key motif, which, in some cases, resembles that of the other GSTs [10,11]. Therefore the absence of a lock-and-key motif at the subunit interface is no longer a useful means of differentiating the class Sigma GSTs from the other GSTs.

Although the lock-and-key motif appears not to be essential for the folding and assembly of GST dimers, it has been demonstrated to play a significant role in maintaining dimer stability and the proper conformation of functional sites in Alpha, Mu and Pi class GSTs [12–17]. Disruption of the motif (or part thereof) results in destabilized dimers with reduced or abolished catalytic activity and altered ligandin functions, the effects being dependent on the replacement residue. Recently, it has been proposed that the F52A mutation in rGST (rat GST) A1-1 produces a stable monomeric species in equilibrium with the dimer [15]. This, however, is in contrast with our findings that the GSTA1 monomer is thermodynamically unstable [12,18]. Since the intersubunit lock-and-key motif in GSTA1-1 consists of two residues (Met⁵¹ and Phe⁵²) and the contribution of Phe⁵² towards stability and function is known [12,15], the contributions of Met⁵¹ alone and of Met⁵¹ and Phe⁵² together were investigated in the present study. In addition to characterizing the structural and functional status of these mutants, urea denaturation methods, together with the use of various probes, were employed to determine the conformational stabilities of the proteins.

EXPERIMENTAL

Materials

ANS (8-anilino-naphthalene-1-sulphonic acid) and CDNB (1-chloro-2,4-dinitrobenzene) were purchased from Sigma (St. Louis, MO, U.S.A.). GSH was from ICN Biomedicals (Aurora, OH, U.S.A.). Ultrapure urea was from Merck (Darmstadt,

Abbreviations used: ANS, 8-anilino-naphthalene-1-sulphonic acid; CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione S-transferase; hGSTA1-1, human class Alpha GST with two type-1 subunits; rGST(M), (class Mu) rat GST; SEC, size-exclusion chromatography.

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Figure 1 Ribbon representation of the crystal structure of homodimeric hGSTA1-1

Met⁵¹ (ball-and-stick) and Phe⁵² (stick), comprising the lock-and-key motif of hGSTA1-1, are shown at the dimer interface. This Figure was generated using Pymol [49].

Germany). The restriction enzymes EcoRV and PvuII were purchased from Amersham Biosciences (Little Chalfont, Bucks., U.K.). All other reagents were of analytical grade.

Plasmids and mutagenesis

The hGSTA1-1 expression plasmid, pKHA1 [19], was a gift from Professor B. Mannervik (Uppsala University, Uppsala, Sweden). The M51A and M51A/F52S mutants were introduced into the hGSTA1-1 coding region of the pKHA1 expression plasmid using the QuikChange™ Site-directed Mutagenesis kit purchased from Stratagene (La Jolla, CA, U.S.A.). The oligonucleotide primers used with the kit to incorporate the M51A and M51A/F52S mutations were M51A forward primer, 5'-GA AAT GAT GGA TAT CTG **GCC** TTT CAG CAA GTG CC-3', and M51A reverse primer, 5'-GG CAC TTG CTG AAA **GGC** CAG ATA TCC ATC ATT TC-3'. The underlined nucleotide sequence represents the translationally silent mutation encoding the EcoRV diagnostic restriction site that was engineered to aid the identification of the mutant plasmid DNA. The nucleotides in bold represent the mutation that generates the Met → Ala substitution. The same set of primers was used to generate the double M51A/F52S mutant but using the cDNA encoding the F52S hGSTA1-1 mutant enzyme as template [12]. cDNA encoding either the M51A or the M51A/F52S mutant enzymes were sequenced to confirm the incorporation of the mutations into the pKHA1 plasmid. DNA sequencing was performed by Inqaba Biotechnical Industries (Pty) (Pretoria, South Africa).

Expression and protein purification

Both GST mutants were expressed in *Escherichia coli* BL21(DE3) cells containing the pLysS plasmid and transformed with either the M51A or the M51A/F52S mutant plasmids. Cells were grown in LB (Luria–Bertani) broth supplemented with 100 µg/ml ampicillin and 35 µg/ml chloramphenicol at 37°C. When a D_{600} of 0.7 was reached, protein expression was induced with 1.0 mM IPTG (isopropyl β-D-thiogalactoside) and allowed to continue overnight at 37°C. Cells were harvested by centrifugation at

5000 g for 20 min, then resuspended in 10 mM sodium phosphate, 1 mM EDTA and 0.02 % (w/v) sodium azide, pH 7.5, frozen for 2 h at –70°C, thawed and sonicated. Following centrifugation, the supernatant was loaded on to a CM-Sepharose column pre-equilibrated with 10 mM sodium phosphate buffer containing 1 mM EDTA and 0.02 % (w/v) sodium azide, pH 7.5. The column was washed with ten column-volumes of buffer to remove unbound proteins and the bound GST proteins were eluted with a linear salt gradient of 0–0.3 M NaCl prepared in the phosphate buffer. The presence of the GST proteins was monitored by 15% SDS/PAGE [20] and fractions identified to have pure hGSTA1-1 were pooled and concentrated by ultrafiltration with a PM-10 membrane (10 kDa molecular mass cut-off; Millipore, Bedford, MA, U.S.A.). The protein solution was then dialysed against 20 mM sodium phosphate buffer containing 1 mM EDTA and 0.02 % (w/v) sodium azide, pH 6.5. Unless stated otherwise, all experiments described hereafter were performed in this buffer. The oligomeric status of the purified M51A and M51A/F52S hGSTA1-1 proteins was assessed by SEC (size-exclusion chromatography)–HPLC. Protein concentrations were determined spectrophotometrically at 280 nm using a molar absorption coefficient of 38 200 M^{–1} · cm^{–1} [21].

Enzyme activity

The catalytic activity of the M51A and M51A/F52S hGSTA1-1 proteins was determined using the standard GSH/CDNB S-conjugation assay [22]. Activity was measured at 340 nm in 0.1 M potassium phosphate, 1 mM EDTA and 0.02 % (w/v) sodium azide, pH 6.5, with final concentrations of 1 mM GSH, 1 mM CDNB and 3 % (v/v) ethanol. All reactions were conducted at 20°C with a Jasco V-550 UV/visible spectrophotometer. Linear progress curves were observed and the concentration of 1-(S-glutathionyl)-2,4-dinitrobenzene produced was determined using the molar absorption coefficient of 9600 M^{–1} · cm^{–1} [22]. All reactions were corrected for non-enzymatic reaction rates.

Spectroscopic measurements

Far-UV CD spectroscopy was performed at 20°C on a Jasco J-810 spectropolarimeter using a path length of 2 mm. The spectra were an average of ten runs from 250 to 200 nm. Protein samples were in 20 mM sodium phosphate buffer, pH 6.5, containing 1 mM EDTA and 0.02 % (w/v) sodium azide. Mean residue ellipticities, $[\theta]_{m.r.w.}$, were calculated from the following equation:

$$[\theta]_{m.r.w.} = (\theta \times M_{m.r.w.}) / (10 \times c \times l)$$

where θ is the ellipticity signal in millidegrees after subtraction of the solvent baseline, $M_{m.r.w.}$ is the mean residue molecular mass, c is the protein concentration in mg/ml and l is the path length in cm [23].

The tryptophan fluorescence properties (excitation at 295 nm) of the M51A and M51A/F52S mutants were measured at 20°C on a PerkinElmer LS50B luminescence spectrometer. Protein samples were in 20 mM sodium phosphate buffer, pH 6.5, containing 1 mM EDTA and 0.02 % (w/v) sodium azide. All fluorescence data were corrected for the buffer. Rayleigh scattering by protein samples was measured by setting the excitation and emission wavelengths to 295 nm.

The binding of the amphipathic dye, ANS, to the M51A and M51A/F52S mutants was monitored by fluorescence enhancement. A final concentration of 200 µM ANS in 20 mM sodium phosphate buffer, pH 6.5, was added to 2 µM protein. ANS was excited at 390 nm, and the emission spectrum was recorded from

Figure 2 Typical emission spectra of ANS in complex with the wild-type and mutant hGSTA1-1 proteins

The spectra of ANS binding to the wild-type (wt), M51A, F52S and M51A/F52S protein are indicated. Experiments were performed by adding 200 μ M ANS in 20 mM sodium phosphate buffer, pH 6.5, to 2 μ M protein. ANS was excited at 390 nm, and the emission spectra were measured (arbitrary units).

400 to 600 nm. All emission spectra for protein/ANS samples were corrected for the contribution of free ANS.

All spectroscopic measurements were performed in triplicate in order to ensure reproducibility of the data.

Urea-induced unfolding studies

Unfolding studies were performed in triplicate at 20 °C in 20 mM sodium phosphate buffer, pH 6.5, containing 1 mM EDTA and 0.02 % (w/v) sodium azide. Protein samples (0.2–10 μ M) were incubated with urea (0–8 M) for 1 h to allow unfolding/refolding to go to completion [12]. Samples were subsequently analysed by far-UV CD, tryptophan fluorescence and ANS-binding. The concentrations of urea stock solutions were checked using refractometry [24].

RESULTS

Structural and functional properties of M51A and M51A/F52S hGSTA1-1

Both mutants were soluble during their expression in *E. coli* at 37 °C, yielding 20–25 mg of pure GST per litre of culture. SEC–HPLC indicated that, at 1–10 μ M, both mutants are dimeric (54 kDa) without the presence of a monomeric species (results not shown). Furthermore, a comparison of their far-UV CD and tryptophan fluorescence spectra with those of the wild-type enzyme indicates very little difference in secondary and tertiary structures between the mutant and wild-type proteins (results not shown).

The specific activities of M51A and M51A/F52S were 80 % and 4 % respectively of that for the wild-type enzyme. Typical fluorescence emission spectra of ANS bound to the wild-type, M51A, F52S and M51A/F52S forms of hGSTA1-1 are shown in Figure 2. In the absence of protein, ANS displayed an emission maximum at 530 nm and a low quantum yield owing to quenching by water [25]. When bound to protein, the emission wavelength maximum shifted to 469 nm (wild-type), 470 nm (M51A), 468 nm (F52S) and 465 nm (M51A/F52S), accompanied by an enhanced quantum yield of ANS fluorescence.

Conformational stability of M51A and M51A/F52S hGSTA1-1

Far-UV CD and tryptophan fluorescence were used to monitor the unfolding of the M51A and M51A/F52S proteins, as shown in

Figure 3 Urea-induced equilibrium unfolding of the wild-type and mutant hGSTA1-1 proteins

Unfolding transitions of (A) 1 μ M M51A hGST A1-1 and (B) 1 μ M M51A/F52S hGST A1-1 monitored by far-UV CD (○) and fluorescence (●). Trp²¹ was selectively excited at 295 nm and the emission intensities at 332 nm (folded protein) and 358 nm (unfolded protein) were measured and plotted (means $\pm$ S.D.) as a ratio for each urea concentration. The urea-induced equilibrium unfolding of the wild-type hGSTA1-1 (dotted line) [26] monitored by Trp²¹ fluorescence is also included.

Figure 3. For comparative purposes, the urea-induced unfolding curve for wild-type hGSTA1-1 [26] is included. At a protein concentration of 1 μ M, each unfolding curve is characterized by a single sigmoidal transition with overlapping far-UV CD and tryptophan fluorescence signals. The unfolding curves of the M51A and M51A/F52S mutants, however, do not coincide with that of the wild-type protein and are shifted to lower urea concentrations [C_m = 4.3 M urea (M51A), C_m = 4.2 M urea (F52S/M51A) and C_m = 4.5 M urea (wild-type), where C_m is the value of the midpoint of the urea unfolding transition curves]. The slopes of the transition regions are also less steep than that for the wild-type protein. Fitting of the unfolding data to a two-state model ($N_2 \leftrightarrow 2U$, where U is the unfolded state), as described previously for the wild-type protein [27], yielded $\Delta G(H_2O)$ (the change in free energy of unfolding in the absence of denaturant) values of 16.1 ± 0.9 kcal/mol (1 cal $\approx$ 4.184 J) and 14 ± 0.7 kcal/mol for M51A and M51A/F52S respectively. The corresponding m -values (the dependence of ΔG on denaturant concentration) derived from the slopes of the transition regions are 2.0 ± 0.2 kcal/mol per M urea (M51A) and 1.5 ± 0.2 kcal/mol per M urea (M51A/F52S). The unfolding transition of M51A displays a weak dependence on protein concentration (Figure 4A), while that of M51A/F52S does not (Figure 4B).

At protein concentrations higher than 1 μ M, M51A and F52S/M51A hGSTA1-1 are prone to aggregate within the unfolding transition. This is demonstrated by their monophasic unfolding transitions becoming biphasic, accompanied by enhanced

Figure 4 Protein-concentration-dependence of the unfolding transition

Protein-concentration-dependence of the unfolding transition of (A) the M51A and (B) M51A/F52S mutant hGSTA1-1 proteins at 0.2 (Δ), 1 ($\bullet$), 2 ($\circ$) and 10 ($\blacktriangledown$) μ M. Excitation was at 295 nm and emission intensity was measured at 332 nm (folded protein) and 358 nm (unfolded protein). The inset shows the Rayleigh scatter in arbitrary units (excitation and emission at 295 nm).

light scattering as the protein concentration increases (Figure 4). The protein-concentration effect is more pronounced for the M51A/F52S mutant. The presence of protein aggregates is also demonstrated by the binding of the anionic dye, ANS, a probe used to monitor the appearance and disappearance of hydrophobic patches on proteins [28] (Figure 5). The peak of ANS-binding at approx. 4.6 M urea for the double mutant is consistent with its larger amount of aggregates, as shown by light scattering (Figure 4). Furthermore, both mutants display a loss of ANS-binding within the spectroscopic pre-transition region, with the double mutant showing a loss at lower urea concentrations (Figure 5).

DISCUSSION

Unlike most GSTs that have a hydrophobic lock-and-key motif at the dimer interface, crystallographic data indicate that the key of the class Alpha GST motif is made up of two residues, Met⁵¹ and Phe⁵² (Figure 1). Although the lock-and-key motif is not essential for dimer assembly and the global tertiary and secondary structures of the subunits, its disruption has an adverse impact on the functionality and stability of the GST dimer (the present study and [12–17]). The hydrophobic lock is a large pocket formed by Met⁹⁴, Tyr⁹⁵, Gly⁹⁸, Arg¹³¹, Tyr¹³², Ala¹³⁵, Phe¹³⁶ and Val¹³⁹ between helices 4 and 5. Although truncation of either Met⁵¹ or Phe⁵²

Figure 5 Effect of urea on ANS binding

The effect of urea on the binding of ANS to M51A ($\bullet$) and M51A/F52S ($\blacktriangledown$). ANS (200 μ M) was added to 2 μ M protein in 20 mM sodium phosphate buffer, pH 6.5, pre-equilibrated with 0–8 M urea. ANS was excited at 390 nm, and the emission intensity was monitored at 480 nm.

decreases the packing density within this pocket, removal of the phenyl ring of Phe⁵² has the greatest impact on the van der Waals contacts between the key and the lock. Met⁵¹ and Phe⁵² reside in the loop between helix 2 and strand 3 of GSTA1-1 [29]. Helix 2 and this loop form part of the G-site (GSH-binding site) and provide four residues (Arg⁴⁵ in helix 2, and Gln⁵⁴, Val⁵⁵ and *cis*-Pro⁵⁶ in the loop) that interact with glutathione via hydrogen bonds. The major contribution that Phe⁵² makes towards maintaining a proper conformation at the G-site is demonstrated by the lower activity of the F52S [12], F52A [15] and M51A/F52S mutants (the present study), when compared with the activity of M51A and the wild-type. The M51A mutation reduces the activity by only 20% compared with the wild-type. Mutating the key residues should alter not only the conformation and dynamics of the loop but also the dynamics of helix 2, as demonstrated for the corresponding region of rGSTM1-1 (class Mu rGST with two type-1 subunits) by hydrogen-exchange MS [30]. These mutation-induced changes at the G-site would explain, at least in part, the reduced activity of the mutants, but what about the hydrophobic H-site (hydrophobic-substrate-binding site) to which electrophilic substrates (e.g., CDNB) bind? Class Alpha GSTs have an extended C-terminal tail that forms a dynamic helix (helix 9) at the H-site [29,31]. Although not directly involved in the chemical mechanism of catalysis, the C-terminal region is an important determinant of substrate selectivity [32–35], the binding of substrates [36], rate-determining steps [37], desolvation of the active site [38] and the pK_a of the catalytic tyrosine residue [36,39]. Furthermore, this region is an important determinant of ligandin function, i.e. the binding of non-substrate ligands [26,40,41]. Given the flexibility of the C-terminal region, its conformational dynamics impact significantly on the enzyme's functions [26,42]. Since the dynamics of helix 9 are regulated by tertiary packing interactions between it and helix 2 [26,42], the enhanced mobility of helix 2, induced by the M51A and/or F52S mutations, should also enhance the mobility of helix 9. Consequently, this will have an impact on the binding of electrophilic substrates to the H-site. This view is supported by our ANS-binding data. ANS, an anionic dye, binds the H-site of hGSTA1-1 with a stoichiometry of one dye molecule per site, and has been used to probe structural changes at the H-site [26,40,41,43]. The spectroscopic properties of ANS bound to the various forms of hGSTA1-1 indicate that its binding site is non-polar as expected (emission maximum wavelength is blue-shifted to that for ANS in water), but that the solvent accessibility

of the site is affected by mutating the key residues, as demonstrated by the altered fluorescence intensity of ANS. Disruption of the intersubunit lock-and-key motif of rGSTM1-1 has also been shown to affect the extent of solvent exposure of its ANS-binding site [13]. Furthermore, unfolding studies show that both mutants begin to lose their ability to bind ANS at low concentrations of urea (C_m at 3.7 M urea for M51A and at 2.5 M urea for M51A/F52S) (Figure 5). These findings are indicative of the unfolding of helix 9, which occurs before global unfolding [26,27,40]. For the wild-type protein, the C_m of the unfolding of helix 9 is 4.1 M urea. Therefore disruption of the lock-and-key motif in class Alpha GSTs not only affects the G-site, as observed for other GST classes [13,14], but also has a substantial impact on the structure of the H-site via the destabilization of helix 9.

At low concentrations of M51A and M51A/F52S hGSTA1-1 ($\leq 1 \mu\text{M}$), their unfolding is reversible and, like that of the wild-type [27], displays a simultaneous and monophasic loss of secondary and tertiary structures. However, when compared with the wild-type unfolding transitions, the mutants unfold at lower concentrations of urea and with reduced co-operativity, indicative of reduced dimer stabilities. While a $\Delta G(\text{H}_2\text{O})$ of 27.5 kcal/mol for wild-type [27] is in agreement with that calculated for a dimer (26.6 kcal/mol) [44], the $\Delta G(\text{H}_2\text{O})$ values for the mutants are similar to those obtained for monomeric unfolding intermediates observed for class Mu [13,45] and class Sigma [46] GSTs. Similarly, an m -value of 4.2 kcal/mol per M urea for wild-type [27] is close to the calculated value of 4.8 kcal/mol per M urea for a concerted dissociation and unfolding of the dimeric protein [47], whereas the M51A and M51A/F52S mutants exhibit significantly lower m -values. These m -values, as well as that for the F52S mutant (2.1 kcal/mol per M urea; [12]), correspond more closely to that calculated for the unfolding of a monomeric form of hGSTA1-1 (2.5 kcal/mol per M urea) than to those obtained for class Mu [13,45] and Sigma [46] monomeric intermediates. Although our native SEC-HPLC data do not indicate the presence of monomers at protein concentrations of 1–10 μM and the spectroscopic probes do not indicate the presence of intermediates during urea-induced unfolding, the presence of a monomeric unfolding intermediate is suggested by the low $\Delta G(\text{H}_2\text{O})$ - and m -values obtained for the lock-and-key mutants (see [48]). Monomeric unfolding intermediates of other GSTs have been shown to share very similar spectroscopic properties to those of their native dimers [13,45,46], making the detection of these intermediates difficult. Recently, Vargo et al. [15] found that the F52A mutation in rGSTA1-1 resulted in the formation of a mixture of dimeric and monomeric species in the absence of denaturant. Given the destabilizing effect of lock-and-key mutations on the quaternary structure of GSTs, it is likely that the M51A and M51A/F52S mutants dissociate to aggregation-prone monomers at low concentrations of urea before their global unfolding. Evidence for a dissociation step within the spectroscopic pre-transition region (i.e. at urea concentrations between 0 and 3 M) is provided by the independence of the unfolding transition of M51A/F52S on protein concentration, indicating that the mutant dimer is either absent or not highly populated within the transition region. The weak protein-concentration-dependence of the unfolding transition of M51A also suggests dissociation of the dimer before global unfolding and that some dimer may be present within its transition. This is consistent with the M51A dimer being more stable than the double-mutant dimer given that fewer van der Waals contacts are disrupted at the lock-and-key motif of the single mutant.

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Received 1 July 2005/20 September 2005; accepted 29 September 2005

Published as BJ Immediate Publication 29 September 2005, doi:10.1042/BJ20051066