Morten K. Grøftehauge,^{a*}
Martin Ø. Therkelsen,^b Rolf
Taaning,^c Troels Skrydstrup,^c
J. Preben Morth^d and Poul
Nissen^{b*}

^aDepartment of Chemistry, Durham University,
South Road, Durham DH1 3LE, England,

^bDepartment of Molecular Biology,
Aarhus University, Gustav Wieds Vej 10C,
8000 Aarhus C, Denmark, ^cCenter for Insoluble
Protein Structures (inSPIN), Department of
Chemistry and the Interdisciplinary Nanoscience
Center, Aarhus University, Langelandsgade 140,
8000 Aarhus C, Denmark, and ^dCentre for
Molecular Medicine Norway, University of
Oslo, Forskningsparken, 0349 Oslo, Norway

Correspondence e-mail:
m.k.groftehaug@dur.ac.uk, pn@mb.au.dk

Received 25 March 2013

Accepted 4 July 2013

PDB References: EF-Tu, 4h9g; 4lbv; 4lbw; 4lby;
4lbz; 4lc0

Identifying ligand-binding hot spots in proteins using brominated fragments

High-quality crystals of *Thermus thermophilus* EF-Tu in the GTP-bound conformation at 1.7–2.7 Å resolution were used to test 18 small organic molecules, all brominated for confident identification in the anomalous difference maps. From this relatively small collection, it was possible to identify a small molecule bound in the functionally important tRNA CCA-end binding pocket. The antibiotic GE2270 A is known to interact with the same pocket in EF-Tu and to disrupt the association with tRNA. Bromide could be located from peaks in the anomalous map in data truncated to very low resolution without refining the structure. Considering the speed with which diffraction data can be collected today, it is proposed that it is worthwhile to collect the extra data from fragment screens while crystals are at hand to increase the knowledge of biological function and drug binding in an experimental structural context.

1. Introduction

As macromolecular X-ray crystallography becomes more widespread and high-throughput, the ratio of structural data to biochemical and cell biological data is rapidly increasing. Small organic molecules tend to bind in ‘hot spots’ (Barelier *et al.*, 2010), and these hot spots are commonly binding sites for substrates or signalling molecules but can also be allosteric regulation sites or owing to the protein having multiple native conformations. More rarely, they indicate a protein–protein binding site. Obtaining knowledge about these hot spots greatly increases the value of a given protein structure. As an alternative to experimental procedures for the identification of ligand–interaction sites, software such as *FTMap* uses *in silico* search routines to identify structural hotspots for ligand binding (Brenke *et al.*, 2009; Landon *et al.*, 2009).

Elongation factor Tu (EF-Tu) is the most plentiful protein in bacteria and was the first GTPase to have its structure determined (Morikawa *et al.*, 1978; Jurnak *et al.*, 1980; Kjeldgaard & Nyborg, 1992; Berchtold *et al.*, 1993; Kjeldgaard *et al.*, 1993). The primary function of EF-Tu is in the elongation cycle of protein synthesis. GTP-bound EF-Tu forms a ternary complex with aminoacylated transfer RNA (aa-tRNA; Nilsson & Nissen, 2005), which interacts with the A site of a translating ribosome. Cognate codon–anticodon interaction on the ribosome triggers the hydrolysis of GTP to GDP. EF-Tu–GDP undergoes a large conformational change and is released from the ribosome, while aa-tRNA binding is accommodated at the peptidyl-transferase centre. EF-Tu–GDP is reactivated to the GTP-bound form by the nucleotide-exchange factor EF-Ts.

Four chemical classes of antibiotic EF-Tu inhibitors have been isolated from natural sources. Kirromycin and enacyloxin both prevent the dissociation of EF-Tu from tRNA and ribosome after the hydrolysis of GTP by stabilizing a GTP-like conformation and are in effect ribosome inhibitors, while pulvomycin and GE2270 A act by disrupting the association of EF-Tu and aa-tRNA (Parmeggiani & Nissen, 2006). However, these antibiotics are large and complex natural products and have not found significant use.

Fragment-based lead discovery has become an increasingly important tool in drug discovery and in the development of tool compounds and chemical probes. The combination of high-throughput methods with highly sensitive biophysical methods such as surface plasmon resonance, nuclear magnetic resonance and X-ray crystallography has allowed researchers to screen libraries of increasingly smaller molecules and to identify even very low affinity binders, which can subsequently be developed into drug leads (Rees *et al.*, 2004; Hajduk & Greer, 2007; Chessari & Woodhead, 2009; Warr, 2009; Murray & Blundell, 2010; Murray *et al.*, 2012; Scott *et al.*, 2012; Chilingaryan *et al.*, 2012). While it is possible to perform fragment-based drug discovery without X-ray structures, it has mostly been performed with structures determined at high to moderate resolution (2.5 Å). There is often a discrepancy between the fragment hit sets found by different methods from the same library, possibly because the binding affinities are so close to the limit of detection. Therefore, while X-ray crystallographic screening has the lowest throughput, successful pre-screening does not guarantee a protein–fragment complex structure and would be likely to reduce the total number of fragment-bound structures.

Bromide has an anomalous signal at both copper-anode and synchrotron wavelengths, with an f'' of 1.3 e at the 1.5418 Å wavelength X-rays produced by copper anodes and an f'' of 3.8 e at energies above the Br *K* edge at 0.92 Å. While a synchrotron delivers the strongest signal and the quickest data collection, the convenience and value of in-house data collection should not be underestimated. When a protein model is available, heavy-atom site location is greatly aided.

A brominated compound, in particular a bromoarene, is a perfect starting point for growing or merging fragments to create a larger scaffold and for further structural elaborations in a structure–activity relationship (SAR) study (Antonyamy *et al.*, 2009) *via* palladium-catalyzed cross-coupling reactions such as the Mizoroki–Heck (Mizoroki *et al.*, 1971; Heck & Nolley, 1972), Suzuki–Miyaura (Miyaura & Suzuki, 1995) and Sonogashira (Sonogashira *et al.*, 1975) reactions or the corresponding carbonylative versions (Brennfürher *et al.*, 2009; Hermange *et al.*, 2011). The use of aryl bromides in these couplings means that bromo fragments are often available from chemical vendors in the ‘building blocks’ section. Additionally, dedicated brominated fragment libraries are readily available from a few vendors.

For buried bromides that interact directly with the protein, leaving no space to grow the scaffold *via* coupling, the following substitutions can be attempted to extend the structure–activity relationship: other halides, nitrile, trifluoromethyl, methyl or hydrogen. However, aryl chlorides, bromides and iodides are unique in that they have the ability to form halogen bonds with hydrogen-bond acceptors through the partial positive charge of the ‘sigma hole’ opposite the carbon bond (Wilcken *et al.*, 2013). The strength of the halogen bond increases with the size of the halide and can be tuned by substituents on the aromatic system.

We have identified the binding site of 5-bromo-2-furancarboxylic acid both from the anomalous signal of bromide and by determining the structure of EF-Tu in this complex.

2. Materials and methods

2.1. Purification and crystallization of EF-Tu–GDPNP

Escherichia coli BL21(DE3) cells were transfected with a pKK223-3 plasmid containing the *Thermus thermophilus* EF-Tu gene *tufA* (Blank *et al.*, 1995). The cells were grown in 2×TY medium at 310 K

to an OD₆₀₀ of 0.6, induced with 0.1 mM IPTG and then grown for a further 5 h before harvesting. The cells were lysed with an Avestin EmulsiFlex-C5 high-pressure homogenizer in 0.1 mM PMSF and cell debris was removed by S30 centrifugation. The supernatant was heated for 15 min at 333 K in a water bath and denatured *E. coli* proteins were removed by centrifugation. Soluble *T. thermophilus* EF-Tu was precipitated by ammonium sulfate at 70% saturation. Further purification was performed by ion-exchange (HiTrap Q FF), hydrophobic interaction (HiTrap Phenyl HP) and gel-filtration (Superdex 75) chromatography into 150 mM KCl, 25 mM Tris–HCl pH 7.8. Treatment with 2 units mg^{−1} alkaline phosphatase and a fivefold surplus of guanylyl-β,γ-iminodiphosphate (GDPNP; a non-hydrolyzable GTP analogue) yielded a GTP-like conformation.

Rectangular crystals of up to 1500 μm in length were obtained in 1.6–2.1 M ammonium sulfate, 15% sucrose, 0.1 M Tris–HCl pH 7.6 using 2 μl + 2 μl sitting drops.

2.2. Fragment collection and cocktail formulations

A small improvised collection of brominated compounds was assembled from a larger chemical library for organic synthesis (Department of Chemistry, Aarhus University). The primary considerations in the design were size, availability and the presence of bromide. Many brominated compounds are reactive, but potential reactivity was not used as a parameter for exclusion. The cocktails were manually assembled for distinct morphology of expected electron densities. A total of 18 different fragments were tested in six cocktails containing the individual compounds at approximately 5 mM concentration (see Supplementary Table S1¹).

2.3. Cryoprotection and fragment soaking

The fragments were weighed out to give a final concentration of 5 mM and were mixed in cocktails in dry form before being dissolved in cryobuffer consisting of 2.65 M ammonium sulfate, 25% sucrose, 0.1 M Tris–HCl pH 7.6. Owing to the difficulty in scooping out exact amounts, the actual concentrations were then calculated and are given in Supplementary Table S1.

Crystals were soaked for 60 s in cryobuffer–fragment cocktail.

2.4. Data collection

Data collection was performed using an in-house rotating-anode source (Enraf–Nonius, now Bruker) equipped with a MAR345 image-plate detector or on beamline I911-3 at MAX-lab, Lund, Sweden using a MAR CCD 225 detector. Maximum diffraction limits ranged from 2.7 to 2.2 Å for fragment cocktails *D*, *E* and *F* at the home source and from 2.0 to 1.7 Å for fragment cocktails *A*, *B* and *C* at MAX-lab. The space group was *C*2 with one copy in the asymmetric unit and 62% solvent content. The data sets were processed in *XDS* (Kabsch, 2010) and scaled in *AIMLESS* (Evans & Murshudov, 2013) using *xia2* (Winter, 2010). Data sets were truncated to give a mean $I/\sigma(I) > 2$ as suggested by *AIMLESS*.

2.5. Structure determination and refinement

All data sets were originally phased using the EF-Tu–GDPNP complex (PDB entry 1eft; Kjeldgaard & Nyborg, 1992) for molecular replacement with *Phaser* (McCoy *et al.*, 2007) in *phenix.automr* (Adams *et al.*, 2010) followed by calculation of the anomalous peaks using *phenix.find_peaks_holes*. The structures were remodelled in

¹ Supplementary material has been deposited in the IUCr electronic archive (Reference: WD5212).

Table 1 Data-collection and processing statistics for the structure obtained using cocktail *B*.

Values in parentheses are for the highest resolution bin. Each data set was collected from individual single crystals. The statistics anomalous completeness, anomalous multiplicity, R_{merge} (within $I+/I-$), R_{merge} (all $I+/I-$) and DelAnom correlation between half-sets are from *AIMLESS*. All other statistics are from *PHENIX*.

PDB code	4h9g
Space group	C2
Wavelength (Å)	0.9000
Data-collection temperature (K)	100
No. of images	360
Oscillation (°)	0.5
Resolution range	33.34–1.93 (1.999–1.93)
Unit-cell parameters (Å, °)	$a = 147.23$, $b = 98.64$, $c = 39.76$, $\beta = 96.07$
Total reflections	161427 (16024)
Unique reflections	161427 (16024)
Completeness (%)	99.84 (99.88)
Multiplicity	3.8 (3.8)
Anomalous completeness (%)	90.6 (88.6)
Anomalous multiplicity	1.8 (1.8)
R_{merge} (within $I+/I-$) (%)	8.0 (50.4)
R_{merge} (all $I+/I-$) (%)	10.4 (60.9)
DelAnom correlation between half-sets	0.236 (0.026)
$\langle I/\sigma(I) \rangle$	10.05 (2.30)
CC _{1/2}	0.994 (0.731)
CC*	0.999 (0.919)
$R_{\text{work}}/R_{\text{free}}$ (%)	15.43/18.42
R.m.s. deviation from ideal	
Bond lengths (Å)	0.008
Angles (°)	1.16
Average <i>B</i> factors (Å ²)	
Protein	28.80
Solvent	40.30
Ligands	40.70
Wilson <i>B</i> factor (Å ²)	22.46
Ramachandran (%)	
Favoured	98.0
Outliers	0.0

Figure 1

EF-Tu structure with the anomalous map at 3.5σ calculated at 6 Å resolution from the molecular-replacement solution. The position of the brominated fragment is obvious even before structure refinement. GTP-binding domain 1 is in red with GDPNP bound, domain 2 is in green with BFA bound and domain 3 is in cyan. Ligands are displayed as ball-and-stick models with C atoms in white, O atoms in red, Br atoms in purple and N atoms in blue. Magnesium ion is shown as a green sphere and ammonium ion is shown as a blue sphere.

Coot (Emsley *et al.*, 2010) and refined in *phenix.refine* with one TLS group for each domain and individual *B* factors. Waters were added in *Coot* and validated by *phenix.refine* procedures. Maps were inspected for bound ligands that did not show up in the anomalous map. The data collected from fragment cocktail *B* were diligently refined and remodelled with maximum-entropy maps (Gull & Daniell, 1978). The structure from fragment cocktail *B* was used as an input model for *phenix.autobuild* for the five other data sets. Simulated annealing and *phenix.autobuild* were equally successful in removing bias. After refinement and remodelling, the statistics and geometry were better for the five models obtained using the fragment cocktail *B* structure.

Coordinates for the fragment cocktail *B* structure were deposited with PDB entry 4h9g and data statistics are presented in Table 1. PDB codes and statistics for the five other structures are given in Supplementary Table S2.

2.6. FTMap analysis

PDB entry 1eft was submitted to the *FTMap* server (Brenke *et al.*, 2009) and the results were aligned and manually compared with the full structures of PDB entries 1ttt (Nissen *et al.*, 1995), 2bvn (Parmeggiani, Krab, Watanabe *et al.*, 2006), 1ha3 (Vogele *et al.*, 2001), 2c77, 2c78 (Parmeggiani, Krab, Okamura *et al.*, 2006), 1efu (Kawashima *et al.*, 1996) and 2wrn (Schmeing *et al.*, 2009), which are complexes of EF-Tu with tRNA-Phe, with enacyloxin, with aurodox/methylkirromycin, with GE2270 A, with pulvomycin, with EF-Ts and with tRNA^{Thr} with kirromycin and the ribosome, respectively.

3. Results and discussion

3.1. Structure quality

Data collection was optimized for time-efficient screening with complete coverage of Bijvoet pairs for anomalous difference Fourier analysis. Diffraction data sets were obtained for all cocktail soaks. Six structures were obtained and refined, yielding R_{free} factors ranging from 18.4 to 21.3%. In five structures no additional ligands were identified and their data and structure statistics are given in Supplementary Table S2. Cocktail *B* produced a hit compound, and statistics for the data and for the structure obtained are detailed in Table 1. In no structures was there any evidence of covalent modification of EF-Tu or non-brominated ligand hits.

3.2. A 5-bromo-2-furancarboxylic acid binding site

The anomalous difference map (calculated at 6 Å resolution with phases directly from molecular replacement) for the cocktail *B* crystal shows a peak of 7.4σ distinct from noise peaks, which do not exceed $\sim 3.8\sigma$ (largest noise peak). The anomalous difference Fourier peak superimposes with residual density in the $2F_o - F_c$ map indicating bound 5-bromo-2-furancarboxylic acid (BFA) with bromide in a hydrophobic cavity (see Figs. 1 and 2). BFA has the molecular formula $\text{H}_3\text{C}_5\text{O}_3\text{Br}$, a molecular mass of 191 Da and a total of nine non-H atoms; for a graphical representation, see Supplementary Table S1. The group occupancy of BFA refined to 0.70.

Calculating the anomalous peaks at various resolution cutoffs revealed that the site was identified down to 11 Å resolution (Fig. 3). However, at this peak height it would not be possible to separate it from background noise if the site were not already known. We do not know why the peak height decreases with data truncated to 8 Å resolution.

The binding pocket is highly conserved (Nissen *et al.*, 1995; Altschul *et al.*, 1997): Ile231, Val237, Glu271 and Leu289 are

Figure 2

Binding site of BFA. BFA is shown using the same colour scheme as in Fig. 1. The black density around the bromide is the same anomalous density as in Fig. 1 but is calculated at 5σ , and the blue density is the $F_o - F_c$ density from the final model displayed at 1.0σ . BFA is buried in a hydrophobic environment with the carboxylic acid exposed to solvent, a non-optimal halogen bond to Gly269 (distance, 3.8 Å; angle, 165°) and Glu271 stacking onto the aromatic ring. Ile231 has adopted a different rotamer from the EF-Tu-GDPNP structure (PDB entry left) to accommodate the ligand.

completely conserved and Gly287, Gly269 and Phe229 are almost completely conserved in all bacterial EF-Tu sequences, while neighbouring residues with side chains that are turned away from BFA are not conserved (Thr230, Val270, Thr232 and Leu288). Thus, only conserved residues interact with BFA (see Fig. 2).

Fig. 4 illustrates how the binding of BFA overlaps with that of aminoacyl-tRNA (PDB entry 1ttt; Nissen *et al.*, 1995), while Fig. 5 shows the overlay of BFA and the antibiotic GE2270 A (PDB entry 2c77; Parmeggiani, Krab, Okamura *et al.*, 2006). It does not overlap with any of the other known EF-Tu-targeting antibiotics (Parmeggiani, Krab, Watanabe *et al.*, 2006; Parmeggiani & Nissen, 2006). These observations point to the binding pocket for the aminoacylated end of tRNA as a druggable site compatible with multiple scaffolds and *vice versa*: i.e. the fragment-based approach has pinpointed a functionally important site.

Figure 3

Anomalous signal from Br calculated using truncated data. Data truncation at 12 Å resolution or worse failed to locate the bromide ion.

The soak times were very short, inspired by quick-soak heavy-atom derivatization. It is possible that the BFA occupancy might have been higher with a longer soak time. The stability of protein crystals during

Figure 4

Overlap between the BFA site and the functional site. BFA (transparent spheres) superposes with the adenosine of the CCA motif of Phe-tRNA^{Phe} (cartoon representation, magenta C atoms; phenylalanine in stick representation) from PDB entry 1ttt.

soaking varies greatly between different proteins, so we used the gentlest approach.

3.3. Differences from previously published EF-Tu–GDPNP structures

Comparing our BFA-complexed structure and PDB entry 1eft, the r.m.s.d. is 0.343 Å and very few differences were noticed, but we did observe a change of the rotamers of Ile231 and Leu289 to better accommodate the BFA ligand. Interestingly, the same rotamers are observed in the Phe-tRNA^{Phe}–EF-Tu–GDPNP ternary complex in PDB entry 1ttt, which contains adenine in the same pocket (see Fig. 4). Glu271 apparently interacts through its carboxylic acid with the aromatic ring of BFA. Both PDB entries 1eft and 1ttt reveal the first and second residues at the N-terminal end, but we only observed density for the second residue in our structure and in a different position. Additionally, we suggest that HOH412 of PDB entry 1eft is an ammonium ion and we identified eight sulfate ions, all of which we ascribe to the very high ammonium sulfate concentration of the crystallization conditions and are thus without physiological significance *per se*. Two backbone O atoms, a water molecule and Asp51 and Glu56 coordinate the ammonium ion. Both coordinating side chains are well ordered but lack a counterion. Ammonium is a hydrogen-bond donor and carries a charge, thus forming strong bonds to the two carboxylic acids. Magnesium ions would be coordinated with much shorter bond distances and potassium ions would provide more electrons than are evident from the electron density. There is no sodium in the crystallization conditions or protein buffer. The sulfates are all near the guanidinium functional groups of arginines; two of them appear to be involved in crystal contacts. The density is too large and continuous for water molecules and chloride would be too far from the protein to be able to form hydrogen bonds and salt bridges. In the structure from the crystal soaked in cocktail A there is what appears to be a chloride ion in the position occupied by bromine in the BFA structure. The electron-density peak is too high for water, but none of the other structures reported have evidence of anything other than a water molecule at this position and neither

does the structure 1eft. It is quite likely to be an artefact, but it should be kept in mind when applying brominated fragment screening.

3.4. FTMap clusters and comparison to functional sites

FTMap mimics multiple solvent crystal structures (Mattos *et al.*, 2006) to identify hot spots by docking organic solvent molecules onto the entire surface of a protein structure and returning ‘clusters’ based on sites in which multiple small organic molecules dock in the same area. It ranks the clusters by calculated average free energies of binding.

For PDB entry 1eft, *FTMap* returns ten clusters and clusters 1–9 correlate to functional sites. The BFA site itself is inaccessible owing to a rotamer change of Ile231 between 1eft and the BFA structure, but next to it are the first- and seventh-ranked clusters in the amino-acid binding pocket. Clusters 2, 3, 5 and 8 overlap with the binding sites of the antibiotics enacyloxin (PDB entry 2bvn) and kirromycin (PDB entries 1ha3, 2wrn and 1ob2). Cluster 4 locates into the site of the β - and γ -phosphates of GDPNP. Clusters 6 and 7 overlap with GE2270 A when superposing domains 2 and 3, but not when superimposing the GTPase domain. GE2270 A binds in the interface between domains 1 and 2, inducing a slightly different conformation from the native EF-Tu–GTP complex. Cluster 9 occupies a site that is important in the switch mechanism between the GDP-bound and GTP-bound conformations. Supplementary Figs. S1–S4 show these overlaps. Enacyloxin and kirromycin bind in an area that is occluded in the GDP-bound conformation of EF-Tu, so that in the case of EF-Tu a protein surface that is meant to be buried is also a ligand-binding hot spot. However, there are no clusters in the interface between EF-Tu and the ribosome. This could be because this association is transient in nature, while the GDP-bound and GTP-bound conformations are inhabited for longer.

3.5. Compound collection considerations

Our compound collection is not ideal, but demonstrates the feasibility of identifying brominated fragments in low-resolution data.

A library better suited for the identification of ligand-binding hot spots could be assembled by searching the literature for fragments characterized as promiscuous and either buying their brominated counterparts or synthesizing them. The compounds should fulfil the general definitions of fragments, *i.e.* below a certain size and above a certain aqueous solubility. Maximal library diversity should be obtained using objective molecular descriptors such as chemical fingerprints (Blomberg *et al.*, 2009). Finally, the library should be screened for pan-assay interference compounds (PAINS; Baell & Holloway, 2010), since results from the library might be used as starting points for development of higher affinity ligands.

3.6. Applications

We identified one binding fragment from just 18 brominated fragments. Significantly, it bound at a site critically important for the recognition of tRNA. This is consistent with reported hit rates of 2–10% for fragment screening (Warr, 2009). The higher sensitivity of brominated-fragment X-ray crystallography allows the detection of fragments with lower affinity than non-brominated fragments and thus also of smaller fragments. Smaller fragments are presumed to have a higher hit rate owing to reduced complexity, which allows the use of a smaller fragment library to give the same number of hits. More importantly, it also allows the use of poorer resolution X-ray data for fragment screening and data sets can be screened for ligands

Figure 5
Overlap of BFA with the antibiotic-binding site. BFA (transparent spheres) superposes with the antibiotic GE2270 A from PDB entry 2c77.

quickly by performing an anomalous peak search after a molecular-replacement or rigid-body refinement step.

The bromide of BFA mediates a large proportion of the binding to our target protein, EF-Tu, and occupies a buried site, making it difficult to elaborate *via* a synthetic strategy of replacing the bromide with a C—C bond, but it would be possible to replace the bromide with an isostere. *FTMap* identifies many functional sites on EF-Tu, such as the switch hinge, the antibiotic sites and most of the adenine site, but fails to model the rotamer shifts of Ile231 and Leu289. We find that the two approaches complement each other well.

Using a small and very easily obtained bromo-fragment collection, we quickly pinpointed a documented binding pocket. This underscores the feasibility of using these methodologies in X-ray crystallographic research with high levels of automation or when only low-resolution diffraction data can be obtained.

We would like to thank the beamline scientists at MAX-lab for data-collection support. This work was supported by the Lundbeck Foundation and Novo Nordisk Foundation.

References

- Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.
- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). *Nucleic Acids Res.* **25**, 3389–3402.
- Antonyamy, S., Hirst, G., Park, F., Sprengeler, P., Stappenbeck, F., Steensma, R., Wilson, M. & Wong, M. (2009). *Bioorg. Med. Chem. Lett.* **19**, 279–282.
- Baell, J. B. & Holloway, G. A. (2010). *J. Med. Chem.* **53**, 2719–2740.
- Barelrier, S., Pons, J., Gehring, K., Lancelin, J.-M. & Krimm, I. (2010). *J. Med. Chem.* **53**, 5256–5266.
- Berchtold, H., Reshetnikova, L., Reiser, C. O., Schirmer, N. K., Sprinzl, M. & Hilgenfeld, R. (1993). *Nature (London)*, **365**, 126–132.
- Blank, J., Grillenbeck, N. W., Kreutzer, R. & Sprinzl, M. (1995). *Protein Expr. Purif.* **6**, 637–645.
- Blomberg, N., Cosgrove, D. A., Kenny, P. W. & Kolmodin, K. (2009). *J. Comput. Aided Mol. Des.* **23**, 513–525.
- Brenke, R., Kozakov, D., Chuang, G.-Y., Beglov, D., Hall, D., Landon, M. R., Mattos, C. & Vajda, S. (2009). *Bioinformatics*, **25**, 621–627.
- Brennfürher, A., Neumann, H. & Beller, M. (2009). *Angew. Chem. Int. Ed. Engl.* **48**, 4114–4133.
- Chessari, G. & Woodhead, A. J. (2009). *Drug Discov. Today*, **14**, 668–675.
- Chilingaryan, Z., Yin, Z. & Oakley, A. J. (2012). *Int. J. Mol. Sci.* **13**, 12857–12879.
- Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). *Acta Cryst.* **D66**, 486–501.
- Evans, P. R. & Murshudov, G. N. (2013). *Acta Cryst.* **D69**, 1204–1214.
- Gull, S. & Daniell, G. (1978). *Nature (London)*, **272**, 686–690.
- Hajduk, P. J. & Greer, J. (2007). *Nature Rev. Drug Discov.* **6**, 211–219.
- Heck, R. F. & Nolley, J. P. (1972). *J. Org. Chem.* **37**, 2320–2322.
- Hermange, P., Lindhardt, A. T., Taaning, R. H., Bjerglund, K., Lupp, D. & Skrydstrup, T. (2011). *J. Am. Chem. Soc.* **133**, 6061–6071.
- Jurnak, F., McPherson, A., Wang, A. H.-J. & Rich, A. (1980). *J. Biol. Chem.* **255**, 6751–6757.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 125–132.
- Kawashima, T., Berthet-Colominas, C., Wulff, M., Cusack, S. & Leberman, R. (1996). *Nature (London)*, **379**, 511–518.
- Kjeldgaard, M., Nissen, P., Thirup, S. & Nyborg, J. (1993). *Structure*, **1**, 35–50.
- Kjeldgaard, M. & Nyborg, J. (1992). *J. Mol. Biol.* **223**, 721–742.
- Landon, M. R., Lieberman, R. L., Hoang, Q. Q., Ju, S., Caaveiro, J. M. M., Orwig, S. D., Kozakov, D., Brenke, R., Chuang, G.-Y., Beglov, D., Vajda, S., Petsko, G. A. & Ringe, D. (2009). *J. Comput. Aided Mol. Des.* **23**, 491–500.
- Mattos, C., Bellamacina, C. R., Peisach, E., Pereira, A., Vitkup, D., Petsko, G. A. & Ringe, D. (2006). *J. Mol. Biol.* **357**, 1471–1482.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- Miyaura, N. & Suzuki, A. (1995). *Chem. Rev.* **95**, 2457–2483.
- Mizoroki, T., Mori, K. & Ozaki, A. (1971). *Bull. Chem. Soc. Jpn.*, **44**, 581.
- Morikawa, K., la Cour, T. F., Nyborg, J., Rasmussen, K. M., Miller, D. L. & Clark, B. F. (1978). *J. Mol. Biol.* **125**, 325–338.
- Murray, C. W. & Blundell, T. L. (2010). *Curr. Opin. Struct. Biol.* **20**, 497–507.
- Murray, C. W., Verdonk, M. L. & Rees, D. C. (2012). *Trends Pharmacol. Sci.* **33**, 224–232.
- Nilsson, J. & Nissen, P. (2005). *Curr. Opin. Struct. Biol.* **15**, 349–354.
- Nissen, P., Kjeldgaard, M., Thirup, S., Polekhina, G., Reshetnikova, L., Clark, B. F. & Nyborg, J. (1995). *Science*, **270**, 1464–1472.
- Parmeggiani, A., Krab, I. M., Okamura, S., Nielsen, R. C., Nyborg, J. & Nissen, P. (2006). *Biochemistry*, **45**, 6846–6857.
- Parmeggiani, A., Krab, I. M., Watanabe, T., Nielsen, R. C., Dahlberg, C., Nyborg, J. & Nissen, P. (2006). *J. Biol. Chem.* **281**, 2893–2900.
- Parmeggiani, A. & Nissen, P. (2006). *FEBS Lett.* **580**, 4576–4581.
- Rees, D. C., Congreve, M., Murray, C. W. & Carr, R. (2004). *Nature Rev. Drug Discov.* **3**, 660–672.
- Schmeing, T. M., Voorhees, R. M., Kelley, A. C., Gao, Y.-G., Murphy, F. V., Weir, J. R. & Ramakrishnan, V. (2009). *Science*, **326**, 688–694.
- Scott, D. E., Coyne, A. G., Hudson, S. A. & Abell, C. (2012). *Biochemistry*, **45**, 6846–6857.
- Sonogashira, K., Tohda, Y. & Hagihara, N. (1975). *Tetrahedron Lett.* **16**, 4467–4470.
- Vogele, L., Palm, G. J., Mesters, J. R. & Hilgenfeld, R. (2001). *J. Biol. Chem.* **276**, 17149–17155.
- Warr, W. A. (2009). *J. Comput. Aided Mol. Des.* **23**, 453–458.
- Wilcken, R., Zimmermann, M. O., Lange, A., Joerger, A. C. & Boeckler, F. M. (2013). *J. Med. Chem.* **56**, 1363–1388.
- Winter, G. (2010). *J. Appl. Cryst.* **43**, 186–190.