L23 protein functions as a chaperone docking site on the ribosome

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During translation, the first encounter of nascent polypeptides is with the ribosome-associated chaperones that assist the folding process—a principle that seems to be conserved in evolution[1–3]. In *Escherichia coli*, the ribosome-bound Trigger Factor chaperones the folding of cytosolic proteins by interacting with nascent polypeptides[4,5]. Here we identify a ribosome-binding motif in the amino-terminal domain of Trigger Factor. We also show the formation of crosslinked products between Trigger Factor and two adjacent ribosomal proteins, L23 and L29, which are located at the exit of the peptide tunnel in the ribosome. L23 is essential for the growth of *E. coli* and the association of Trigger Factor with the ribosome, whereas L29 is dispensable in both processes. Mutation of an exposed glutamate in L23 prevents Trigger Factor from interacting with ribosomes and nascent chains, and causes protein aggregation and conditional lethality in cells that lack the protein repair function of the DnaK chaperone. Purified L23 also interacts specifically with Trigger Factor *in vitro*. We conclude that essential L23 provides a chaperone docking site on ribosomes that directly links protein biosynthesis with chaperone-assisted protein folding.

The N-terminal domain of Trigger Factor (TF) mediates its binding to the 50S ribosomal subunit[6]. We aligned the N-terminal domains of TF homologues and found a conserved sequence of about 17 residues, including a completely conserved Gly-Phe-Arg-x-Gly-x-x-Pro motif, called the TF signature (Fig. 1a), which is predicted to be localized in an unstructured region. This prediction, and our observation that this stretch of sequence is susceptible to proteolysis, indicated that it could be exposed on the surface and thus might contribute to the interaction of TF with ribosomes.

To investigate whether this TF signature is involved in ribosome binding, we replaced residues Phe 44, Arg 45 and Lys 46 with alanine to form an FRK/AAA mutant of TF (Fig. 1a). Circular dichroism, partial proteinase K digestion and gel filtration verified the structural integrity of the FRK/AAA protein. We then tested whether it could associate with ribosomes purified from *Δtig* (which encodes TF) *E. coli* cells. After incubating the ribosomes with either TF or the FRK/AAA mutant, ribosome–TF complexes were separated from unbound TF by centrifugation. The FRK/AAA mutant protein was reduced in its association with ribosomes (Fig. 1b, lanes 3 and 5), indicating that the TF signature is involved in the interaction of TF with ribosomes.

To crosslink TF to its ribosomal binding site, we first replaced Asp 42, which is adjacent to the signature motif, with cysteine (TF D42C, Fig. 1a). The lack of additional cysteines in TF then allowed us to couple the thiol-specific ultraviolet-activatable crosslinker benzophenone-4-iodoacetamide (BPIA) to Cys 42. Because BPIA preferentially attacks C–H bonds, it can crosslink TF to ribosomal proteins and ribosomal RNA. The TF D42C mutant was not altered in structural integrity or in ribosome binding (data not shown).

After being coupled to BPIA, the TF D42C mutant was incubated with ribosomes from *Δtig* cells. Ultraviolet irradiation resulted in two additional bands with relative molecular masses (M_r) of 68,000 (68K) and 75K in the ribosomal pellet. These bands represented crosslinking products between TF and ribosomes because they depended strictly on the presence of ribosomes and were absent in the supernatant (Fig. 1b, lanes 6 and 7) and in non-irradiated samples (data not shown). RNase A treatment did not alter the mobility of these products, which indicated that ribosomal RNA was not present. The crosslinking bands were excised and digested with trypsin, and the resulting peptides were sequenced by nano-electrospray mass spectrometry (Supplementary Information Fig. 1a, b). We detected TF-derived peptides in both crosslinking bands. Unique peptides originating from ribosomal proteins L29 and L23 were identified in the 68K and 75K crosslinking products, respectively. BPIA is flexible and has a length of about 10 Å, which explains how it can form crosslinks to two adjacent proteins (see below).

The possibility that the TF D42C–BPIA mutant associated non-specifically with ribosomes was excluded by a competition experiment. We simultaneously added TF D42C–BPIA and a 2.5-fold molar excess of TF to ribosomes and analysed the crosslinking efficiency. Whereas wild-type TF competed with TF D42C–BPIA for ribosome binding and decreased the occurrence of both crosslinking products, TF FRK/AAA did not (data not shown). The

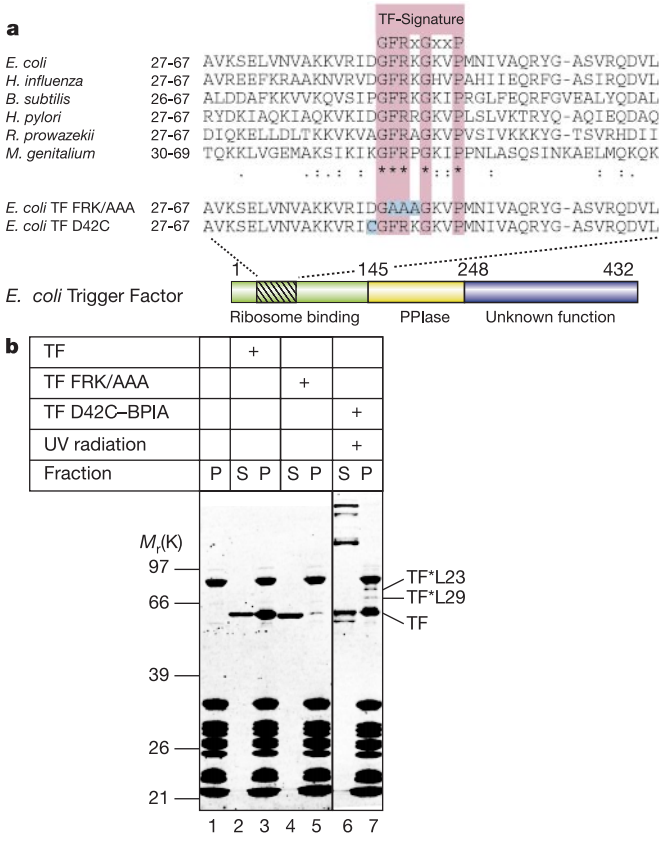

Figure 1 Trigger Factor is crosslinked to the ribosomal proteins L23 and L29. **a**, Alignment of the N-terminal regions of bacterial TF homologues using CLUSTALW[18], and the domain structure of *E. coli* TF. Identical amino acid residues are marked with an asterisk and shown in purple; conserved and semi-conserved residues are indicated by colons and dots, respectively. Amino acid residues that were replaced in TF mutant proteins are boxed in blue. **b**, Ribosomes from *Δtig* cells were incubated with TF variants followed by sucrose cushion centrifugation to separate the ribosomal pellets (P) from the soluble proteins (S). Fractions were separated by SDS–PAGE and stained with Coomassie blue. Where indicated, samples were irradiated with ultraviolet.

crosslinking products obtained with TF D42C–BPIA thus resulted from a specific interaction of TF with ribosomes.

There are homologues of L23 and L29 in all kingdoms. The crystal structure of the 50S subunit from *Haloarcula marismortui* shows that L23 and L29 are in direct contact with each other and are located next to the exit of the tunnel for nascent polypeptides[7,8]. Although *H. marismortui* lacks TF, the TF D42C–BPIA mutant crosslinked specifically to L23 of the *H. marismortui* 50S ribosomal subunits (refs 7, 8, and unpublished data), which ruled out the possibility that additional non-ribosomal or ribosome-associated factors are required for TF to bind to ribosomes.

To determine whether TF associates directly with L23 and/or L29, we generated *E. coli* strains with mutations in the genes *rplW* and *rpmC*, which encode L23 and L29, respectively. We reasoned that if there is a direct interaction between TF and L23 or L29, then we would observe reduced binding of TF to ribosomes originating from such strains. We first attempted to delete the *rplW* and *rpmC* genes in MC4100 by replacing the corresponding open reading frames with a nonpolar kanamycin resistance cassette[9]. MC4100 *ΔrpmC::kan* mutant cells were viable at all temperatures tested (23–37 °C), although they grew slightly slower than did wild-type strains on rich media plates (Fig. 2a). L29 is thus dispensable for the growth and protein biosynthesis of *E. coli*.

To test whether the absence of L29 influences TF binding, we purified ribosomes from *ΔrpmC::kan* mutant cells in the presence of a high salt concentration, which minimizes unspecific associations of proteins with ribosomes but allows the reduced but detectable binding of TF[10]. Similar amounts of TF were associated with ribosomes prepared from *ΔrpmC::kan* mutant and equivalent wild-type cells (Fig. 2b, lanes 4 and 12). We then analysed whether TF could rebind to mutant ribosomes under physiological salt conditions. Ribosomes purified in high salt were incubated with a twofold molar excess of TF in low-salt buffer followed by centrifugation. Ribosomes lacking L29 were not impaired in their association with TF (Fig. 2c, lanes 4 and 12), showing that L29 does not contribute significantly to TF binding to ribosomes.

Attempts to delete the chromosomal *rplW* gene failed unless the gene was expressed from a plasmid (pL23). Growth of MC4100 *ΔrplW::kan* cells containing pL23 was dependent on isopropylthiogalactoside (IPTG), which induced the expression of plasmid-encoded *rplW* (Fig. 2a). We designed L23 variants with alterations in residues whose side chains were judged to be exposed at the ribosomal surface (from the structure of the *H. marismortui* ribosome), and that are conserved among eubacterial L23 proteins. We identified two regions (1 and 2) in L23 that contain residues fulfilling both criteria (Supplementary Information Fig. 1c). The plasmid-encoded *rplW* gene was mutated to introduce alterations in region 1 (Glu 18 to alanine, yielding L23 E18A; Glu 18 to glutamine, yielding L23 E18Q; and Val 16, Ser 17 and Glu 18 to alanine, yielding L23 VSE/AAA) and region 2 (Glu 52 to lysine, yielding L23 E52K; Phe 51, Glu 52 and Val 53 to alanine, yielding L23 FEV/AAA). Each mutant *rplW* allele complemented the lethal phenotype of *ΔrplW::kan* cells at 30 °C and 37 °C (the results for L23 E18A are shown in Fig. 2a), which allowed us to isolate ribosomes containing mutant L23 proteins.

Ribosomes from MC4100 *ΔrplW::kan* cells that expressed L23 variants were tested for their co-purification with TF under physiological salt concentrations (Fig. 2d). Whereas alterations in region 2 did not influence TF association with ribosomes, all alterations in region 1 (E18A, E18Q and VSE/AAA) strongly reduced TF binding and resulted in a reduced co-purification of TF with ribosomes under high salt conditions (Fig. 2b). The rebinding of TF to these ribosomes under physiological salt conditions was also severely decreased (Fig. 2c, compare lanes 4, 6, 8 and 10). All ribosome preparations contained comparable amounts of L23 and S1 (Fig. 2c), which excluded the possibility that a smaller incorporation of mutant L23 proteins into ribosomes was responsible for the reduced binding.

To verify that TF interacted directly with L23, we purified an S-tagged L23 variant fused to thioredoxin to increase its solubility and coupled the protein (Trx–L23) to S-tag agarose columns. TF or the FRK/AAA mutant was applied to the columns, and bound proteins were eluted (Fig. 2e). Whereas TF bound to the L23 fusion protein, the FRK/AAA mutant showed a substantial decrease in its association with L23. Because TF and the FRK/AAA mutant have similar substrate-binding properties *in vitro* (unpublished data), we could

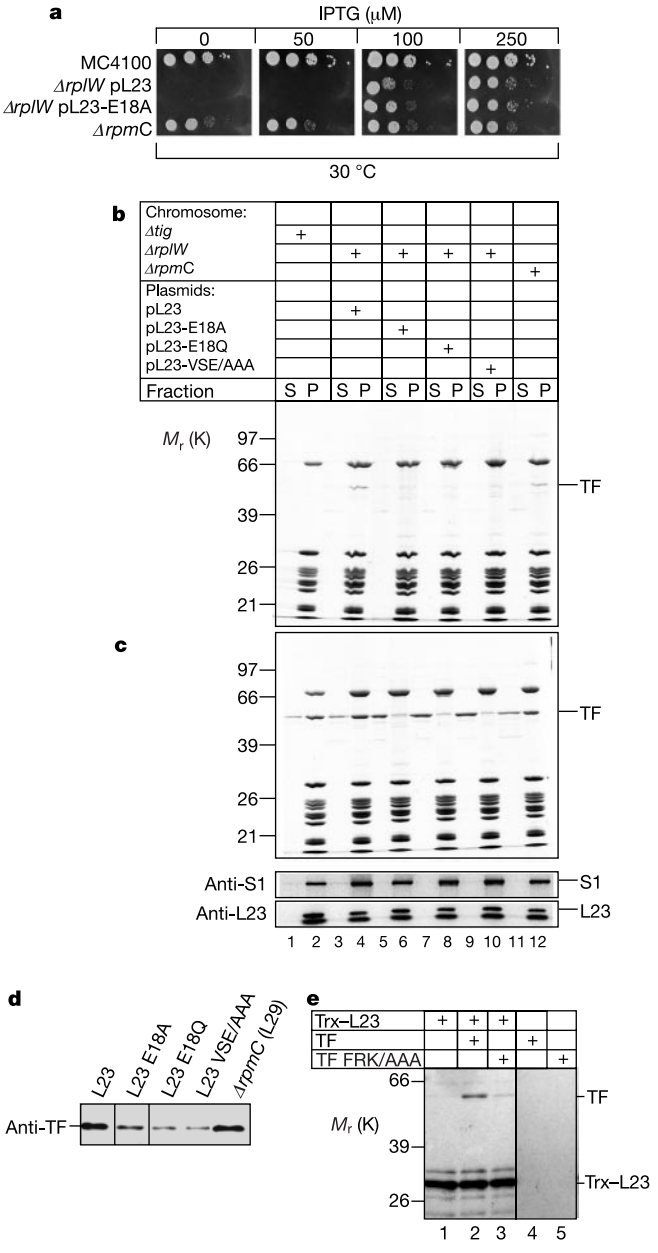

Figure 2 Ribosomes with mutations in L23 are deficient in TF binding. **a**, Growth analysis. **b**, Mutant ribosomes purified in high salt were analysed for TF association by SDS–PAGE and Coomassie blue staining. *Δtig* ribosomes are shown as control. **c**, Ribosomes (2 μM) were incubated with TF (4 μM) under physiological salt conditions. Ribosomal pellets (P) and supernatants (S) were analysed as described in **b**. L23 and S1 contents were analysed by western blotting. The lower signal in the anti-L23 blot is an unspecific crossreaction. **d**, Mutant ribosomes were purified under physiological salt conditions and TF content was analysed by immunoblotting. **e**, TF or the TF FRK/AAA mutant was incubated with Trx–L23 bound to S-tag agarose. Eluates were analysed by SDS–PAGE and Coomassie blue staining.

exclude the possibility of a chaperone–substrate interaction of TF and Trx–L23. Thus, L23 interacts directly with TF on ribosomes.

To investigate whether ribosome binding is a requirement for the interaction of TF with nascent polypeptides, we generated an *in vitro* transcription/translation system comprising a translation-competent (ribosome-free) fraction prepared from MC4100 *Δtig* cells[11], purified TF, and ribosomes containing either wild-type or mutant L23 proteins. Ribosome–nascent chain complexes (RNCs) carrying the ^{35}S-labelled N-terminal fragment (residues 1–173) of *E. coli* isocitrate dehydrogenase (ICDH), which is an *in vivo* substrate of TF[12], were produced. TF association with arrested nascent ICDH was analysed by crosslinking using the amino-reactive homobifunctional agent disuccinimidyl suberate (DSS). RNCs containing wild-type L23 produced two prominent crosslinking products (Fig. 3a). Both products were co-immunoprecipitated with antibodies specific for TF, which thus indicated that crosslinks had formed between TF and nascent polypeptides. Although observed previously with different RNCs[12], the reasons why there are two crosslinking products are unknown. The same products with comparable intensity were formed with L29-deficient RNCs (Fig. 3a, lanes 1–3 and 13–15). By contrast, ribosomes containing L23 mutant proteins with alterations at residue Glu 18 (E18A, E18Q and VSE/AAA) severely reduced TF interaction with nascent ICDH (Fig. 3a, lanes 4–12). The binding of TF to L23 through Glu 18 is therefore a prerequisite for the association of TF with nascent chains.

We next investigated whether the *in vivo* role of TF in protein folding requires its association with ribosomes. We took into account the fact that TF and the DnaK system cooperate in protein folding, which means that *Δtig ΔdnaK* mutants are synthetically lethal at 37 °C (refs 4, 5). We tested whether a *ΔrplW::kan* deletion could be introduced into *ΔdnaK52* cells that express plasmid-encoded wild-type or mutant L23 proteins. The rationale was that if TF association with ribosomes is a prerequisite for its function *in vivo*, then it should be possible to detect growth defects and/or protein aggregation in *ΔdnaK52* cells whose ribosomes contain L23 mutant proteins. The *ΔrplW::kan* allele could be introduced into *ΔdnaK52* mutants expressing wild-type or mutant L23 proteins without growth defects at 30 °C in Luria broth. At 34 °C, however, the *ΔrplW::kan ΔdnaK52* cells expressing the E18A, E18Q and VSE/AAA variants of L23 grew more slowly than did those expressing wild-type L23, and at 37 °C the expression of all three L23 mutant alleles was synthetically lethal (Fig. 3b).

To investigate the extent of protein misfolding in these cells, we grew *ΔrplW::kan ΔdnaK52* cells expressing L23 mutants from plasmids for four doubling times at 32 °C, 33 °C and 37 °C. These cells could grow for four doubling times at 37 °C in liquid media, perhaps because the aggregated protein did not reach a critical amount in this short period. In cells expressing L23 mutant proteins, the amount of insoluble protein aggregates increased markedly with rising temperature and was most severe in cells expressing the VSE/AAA mutant of L23 (Fig. 3c). In *ΔdnaK52 ΔrplW* cells complemented with L23 mutant proteins, the amount of ribosome-bound TF was thus too low to prevent protein aggregation at elevated temperatures. This is in agreement with our finding that strong overproduction of the FRK/AAA mutant complements lethality of *ΔdnaK Δtig* only up to 32 °C (data not shown).

We conclude that L23 is the docking site for TF and thereby

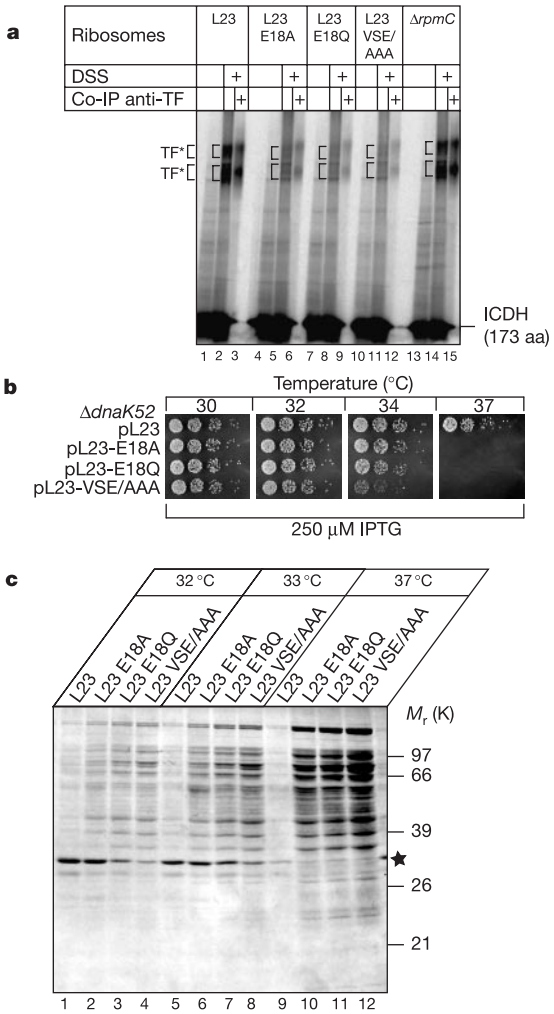

Figure 3 L23 docking is essential for the *in vivo* function of TF. **a**, L23 point mutations influence the association of TF with nascent polypeptides. Arrested ^{35}S-labelled nascent ICDH was synthesized *in vitro* using different ribosomes. After crosslinking and centrifugation, RNCs were co-immunoprecipitated to identify TF crosslinks. Brackets indicate TF crosslinks. aa, amino acids. **b**, Temperature sensitivity of *ΔdnaK52 ΔrplW* cells expressing different L23 alleles. **c**, *ΔdnaK52 ΔrplW* cells expressing different *rplW* alleles were collected in log phase. Aggregates were isolated[19] and analysed by SDS–PAGE and Coomassie blue staining. The band marked by an asterisk probably corresponds to an outer membrane protein. The reason for its disappearance at 37 °C is unknown.

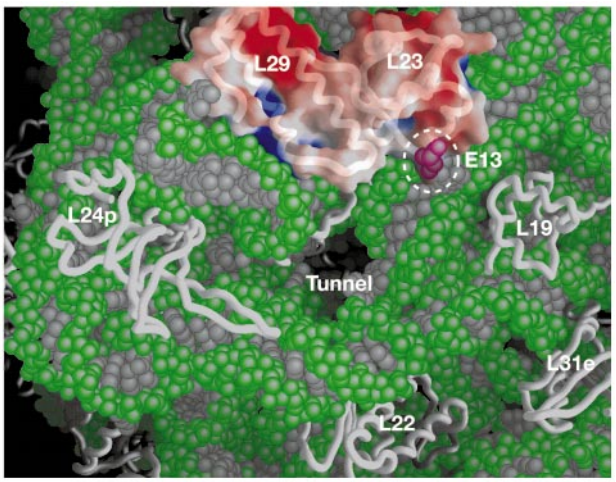

Figure 4 Positioning of the conserved glutamate in L23 at the polypeptide exit tunnel. Space-filling model of the ribosomal polypeptide exit region of the 50S subunit from *H. marismortui*. L23 and L29 are shown in a surface-charge distribution illustration[20] generated by GRASP. The surface-exposed Glu 13 (corresponding to *E. coli* Glu 18) residue that is involved in the interaction with TF is shown in purple. Other proteins in the exit region are visible by their Cα traces.

couples protein biosynthesis with chaperone-assisted protein folding. The involvement of Glu 18 in L23 (which corresponds to Glu 13 in *H. marismortui* L23)—a residue that is located at the rim of the peptide tunnel—in the binding process indicates that TF is positioned exactly where nascent polypeptides emerge from the tunnel (Fig. 4). This positioning is essential for the chaperone activity of TF. □

Methods

Strains and genetic manipulations

All *E. coli* strains were derivatives of MC4100. We grew cells in Luria broth medium supplemented with 100 μg ml^{-1} ampicillin, 40 μg ml^{-1} kanamycin and 250 μM IPTG when appropriate. The methods for P1 lysates, P1 transductions and disrupting chromosomal genes have been described[9,13]. Plasmids pL23, pHL23 and pL29 are derivatives of pTrc99B (ref. 14), and plasmid pFTR6 (encoding Trx–L23) is a derivative of pET32a (Novagen). We amplified *rplW* and *rpmC* by polymerase chain reaction using MC4100 chromosomal DNA as a template and cloned the products individually into pTrc99B to generate pL23 and pL29, respectively, and into pTrc-6His (lab collection) to generate pHL23 for expressing His_6–L23. Mutations in *rplW* were created using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) and pL23 as the template.

Protein purification and antibody production

We purified TF and TF fragments as described[6]. To raise antibodies against L23, His_6–L23 was expressed from plasmid pHL23 and purified under denaturing conditions using Ni^{2+}-NTA (Qiagen).

In vitro binding analysis

The Trx–L23 fusion protein was expressed from pFTR6 vector and purified using Ni^{2+}-NTA (Qiagen). S-tag agarose (Novagen) was saturated with purified Trx–L23 and unbound protein was removed by intense washing. We applied TF or the TF FRK/AAA mutant to the column and incubated it for 30 min at 23 °C. After washing with ten column volumes of buffer, bound proteins were eluted with 0.2 M sodium citrate, pH 2.

Crosslinking

We coupled BPIA (Molecular Probes) to the D42C mutant of TF protein as described[15]. Crosslinking mixtures were incubated for 30 min at 30 °C and subsequently irradiated on ice for 5 min with ultraviolet (365 nm, 100 W; Model B-100AP, Ultraviolet Products) at a distance of 5 cm. We separated ribosomal complexes by centrifugation through sucrose cushions as described[6].

Protein identification by mass spectrometry

Bands were excised from one-dimensional SDS–PAGE gels stained with Coomassie blue and digested in gel with trypsin as described[16]. We analysed the tryptic peptides by nanoelectrospray tandem mass spectrometry as described[17] using a QSTAR Pulsar (MDS Sciex) equipped with a nanoES ion source (MDS Proteomics). Sequence searches were done with the Protein and Peptide Software Suite (MDS Proteomics) in a non-redundant database.

In vitro transcription/translation

We carried out transcription and translation assays as described[11].

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Oxidative demethylation by *Escherichia coli* AlkB directly reverts DNA base damage

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Methylating agents generate cytotoxic and mutagenic DNA damage. Cells use 3-methyladenine-DNA glycosylases to excise some methylated bases from DNA, and suicidal O^6-methylguanine-DNA methyltransferases to transfer alkyl groups from other lesions onto a cysteine residue[1,2]. Here we report that the highly conserved AlkB protein repairs DNA alkylation damage by means of an unprecedented mechanism. AlkB has no detectable nuclease, DNA glycosylase or methyltransferase activity; however, *Escherichia coli alkB* mutants are defective in processing methylation damage generated in single-stranded DNA[3–5]. Theoretical protein fold recognition had suggested that AlkB resembles the Fe(II)- and α-ketoglutarate-dependent dioxygenases[6], which use iron-oxo intermediates to oxidize chemically inert compounds[7,8]. We show here that purified AlkB repairs the cytotoxic lesions 1-methyladenine and 3-methylcytosine in single- and double-stranded DNA in a reaction that is dependent on oxygen, α-ketoglutarate and Fe(II). The AlkB enzyme couples oxidative decarboxylation of α-ketoglutarate to the hydroxylation of these methylated bases in DNA, resulting in direct reversion to the unmodified base and the release of formaldehyde.

