Complexes of the uracil-DNA glycosylase inhibitor protein, Ugi, with *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* uracil-DNA glycosylases

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Uracil, a promutagenic base, appears in DNA either by deamination of cytosine or by incorporation of dUMP by DNA polymerases. This unconventional base in DNA is removed by uracil-DNA glycosylase (UDG). Interestingly, a bacteriophage-encoded short polypeptide, UDG inhibitor (Ugi), specifically inhibits UDGs by forming a tight complex. Three-dimensional structures of the complexes of Ugi with UDGs from *Escherichia coli* (Eco), human and herpes simplex virus have shown that two of the structural elements in Ugi, the hydrophobic pocket and the $\beta_1$-edge, establish key interactions with UDGs. In this report the characterization of complexes of Ugi with UDGs from *Mycobacterium tuberculosis*, a pathogenic bacterium, and *Mycobacterium smegmatis*, a widely used model organism for the former, is described. Unlike the *E. coli* (Eco) UDG-Ugi complex, which is stable to treatment with 8 M urea, the mycobacterial UDG-Ugi complexes dissociate in 5–6 M urea. Furthermore, the Ugi from the complexes of mycobacterial UDGs can be exchanged by the DNA substrate. Interestingly, while *Eco* UDG sequestered Ugi into the *Eco* UDG-Ugi complex when incubated with mycobacterial UDG-Ugi complexes, even a large excess of mycobacterial UDGs failed to sequester Ugi from the *Eco* UDG-Ugi complex. However, the *M. tuberculosis* (Mtu) UDG-Ugi complex was seen when *Mtu* UDG was incubated with *M. smegmatis* (Msm) UDG-Ugi or *Eco* UDG(L191G)-Ugi complexes. The reversible nature of the complexes of Ugi with mycobacterial UDGs (which naturally lack some of the structural elements important for interaction with the $\beta_1$-edge of Ugi) and with mutants of *Eco* UDG (which are deficient in interaction with the hydrophobic pocket of Ugi) highlights the significance of both classes of interaction in formation of UDG-Ugi complexes. Furthermore, it is shown that even though mycobacterial UDG-Ugi complexes dissociate in 5–6 M urea, Ugi is still a potent inhibitor of UDG activity in mycobacteria.

**INTRODUCTION**

The specific recognition and removal of an RNA base, uracil, in DNA is the province of a highly specialized DNA repair enzyme, uracil-DNA glycosylase (UDG) (Lindahl, 1974; Krokan *et al.*, 1997). These enzymes belong to a highly conserved ubiquitous class of proteins (Aravind & Koonin, 2000). Interestingly, *Bacillus subtilis* phage PBS-1/2 which naturally contains uracil in its genome, encodes an early gene product, UDG inhibitor (Ugi) to protect its genome from host UDG by forming an extremely specific and exclusively stable complex. Ugi is a small (84 aa, 9.4 kDa), highly acidic (pI 4.2) and thermostable protein which interacts with UDGs in 1:1 molar stoichiometry (Cone *et al.*, 1980; Wang & Mosbaugh, 1989; Savva & Pearl, 1995; Mol *et al.*, 1995). Biochemical characterization of the *Escherichia coli* (Eco) UDG-Ugi complex has shown it to be irreversible under physiological conditions (Bennett & Mosbaugh, 1992). Furthermore, a stopped flow kinetic study suggested a two-step (‘docking’ and ‘locking’) mechanism of *Eco* UDG-Ugi complex formation (Bennett *et al.*, 1993). The co-crystal structures of various UDGs with Ugi have been resolved and show an extraordinary conservation in their overall architecture (Savva & Pearl, 1995; Mol *et al.*, 1995; Ravishankar *et al.*, 1998; Putnam *et al.*, 1999). Interestingly, these studies have identified Ugi as a transition-state substrate mimic, making it an attractive model system to understand the mechanistic aspects of protein–protein interaction. Ugi primarily uses two of its structural elements, the hydrophobic pocket between the $\alpha_2$ helix and the antiparallel $\beta$ sheet, and the unusually shaped...
β1-edge (beginning from the N-terminal side of Q19 to just past E28; Putnam et al., 1999) to form an exceptionally stable complex with UDGs. The hydrophobic pocket houses the L191 side chain of the DNA intercalation loop (187-HPSPLS-192) and the β1-edge interacts with the key active-site residues such as the water-activating loop, 63-QDPYH-67, the Pro-Ser loop; 84-AIPPS-88 and the DNA intercalation loop of EcoUDG (Putnam et al., 1999).

To understand the biochemical basis of UDG and Ugi interaction, extensive mutational analyses of Ugi have been carried out which have confirmed the predictions from crystal structures and provided new insights into the mechanism of their interaction (Lundquist et al., 1997; Acharya et al., 2002). In a complementary approach, we have carried out detailed mutational analysis of EcoUDG to understand the role of L191 in interaction with the hydrophobic pocket of Ugi (Handa et al., 2001). However, mutations in the side chains of UDGs that interface the β1-edge of Ugi have not yet been studied in detail (Handa et al., 2002). Primary structures of Mycobacterium tuberculosis (Mtu) and Mycobacterium smegmatis (Msm) UDGs (Cole et al., 1998; Acharya & Varshney, 2002) show that these enzymes possess substitutions at many of the conserved positions involved in interaction with the β1-edge of Ugi (Fig. 1, Table 1) and present an ideal genomics approach to investigate the consequences of substitutions in the DNA-binding groove of UDGs. Interestingly, this approach also avoids a commonly encountered problem of loss of enzymic activity upon mutating this critical region of UDGs. Furthermore, because of the high G+C richness of their genomes (Cole et al., 1998) and their habitat in host macrophages, mycobacteria (e.g. M. tuberculosis) are naturally at high risk of cytosine deamination, making UDG a crucial DNA repair enzyme. Thus, it was also of interest to carry out biochemical characterization of the inhibitory effects of Ugi on these UDGs, for its possible use as an inhibitor to control the growth of these bacteria.

**METHODS**

**Growth of E. coli, M. smegmatis and M. tuberculosis.** E. coli was grown in Luria–Bertani (LB) and M. smegmatis mc²155 (Snapper et al., 1990) was grown in LB supplemented with 0.2 % Tween 80. For growth on a solid surface, 1.5 % agar was included in the broth media. M. tuberculosis H37Ra was grown in Middlebrook 7H9-ADC (liquid) containing 0.05 % (v/v) Tween 80. Premixed media or the components were purchased from Difco. Liquid cultures were grown at 37 °C with shaking. When required, the media were supplemented with ampicillin at 100 µg ml⁻¹ for E. coli. Overproduction of various proteins was carried out in E. coli BL21 (DE3) with 0.5 mM IPTG.

**Preparation of cell-free extracts of M. smegmatis and M. tuberculosis for UDG assays.** Bacterial cells from 1 litre cultures grown to late exponential phase were harvested by centrifugation, washed with 50 mM Tris/HCl (pH 7.5) containing 25 % (w/v) sucrose, resuspended in 25 ml of the same buffer and frozen at −70 °C. The suspension was allowed to thaw on ice and lysed at 18 000 p.s.i. in a French press. The lyse was centrifuged in a super-speed centrifuge at 15 000 r.p.m. for 30 min in an SS34 rotor (Beckman) to prepare S20 supernatant, and then in an ultracentrifuge at 31 200 r.p.m. for 1 h using a Type 70Ti rotor (Beckman) to obtain S100 supernatant. The proteins in the S100 supernatant were precipitated with ammonium sulfate (0.35 g ml⁻¹), recovered by centrifugation, dissolved in R-buffer [50 mM Tris/HCl (pH 7.5), 1 mM Na₂EDTA, 2 mM β-mercaptoethanol and 10 % (v/v) glycerol], and dialysed against the R-buffer containing 50 mM Tris/HCl (pH 7.5), 0.70 M sucrose, resuspended in 25 ml of the same buffer and frozen at −70 °C. The suspension was allowed to thaw on ice and lysed at 18 000 p.s.i. in a French press. The lyse was centrifuged in a super-speed centrifuge at 15 000 r.p.m. for 30 min in an SS34 rotor (Beckman) to prepare S20 supernatant, and then in an ultracentrifuge at 31 200 r.p.m. for 1 h using a Type 70Ti rotor (Beckman) to obtain S100 supernatant. The proteins in the S100 supernatant were precipitated with ammonium sulfate (0.35 g ml⁻¹), recovered by centrifugation, dissolved in R-buffer [50 mM Tris/HCl (pH 7.5), 1 mM Na₂EDTA, 2 mM β-mercaptoethanol and 10 % (v/v) glycerol], and dialysed against the R-buffer containing 50 mM KCl. Total proteins were estimated by Bradford’s reagent using bovine serum albumin as standard (Sedmak & Grossberg, 1977).

**Generation of bicistronic constructs of UDG-Ugi.** A common forward primer (5'-AGGGATCCATGGCGCAAGGGC-3') was used in combination with reverse primers (5'-GGATTCCTTCTCAGGGCAACCG-3') to

**Fig. 1.** Sequence comparison of UDGs from M. smegmatis, M. tuberculosis and E. coli. The amino acid alignment was obtained by using the CLUSTAL W program. Identical residues are shown in light grey boxes. Amino acid position 1 in EcoUDG corresponds to the second position in the ung gene ORF. Asterisks below the EcoUDG sequence indicate positions involved in interaction with Ugi.
amplify *Msm*UDG or *Mtu*UDG ORFs from pTrc*Msm*UDG and pTrc*Mtu*UDG, respectively. Briefly, the samples were heated to 94 °C for 4 min and PCR was carried out for 29 cycles. Each cycle consisted of incubations at 94 °C for 1 min, 55 °C for 45 s, 68 °C for 1 min and a final extension of 68 °C for 10 min. The PCR products were digested with *Nol* and EcoRI, gel-purified and cloned into the same sites of pTrcEcoUDG-Ugi (Roy et al., 1998) to replace the EcoUDG fragment. The clones were confirmed as pTrcMsmUDG-Ugi and pTrcMtuUDG-Ugi. The *Msm*UDG-Ugi and *Mtu*UDG-Ugi constructs in pET11d were generated by subcloning *Nol*–*Hind*III fragments from the corresponding pTrc constructs into the same sites of pET11d (Sambrook et al., 1989). To generate an N-terminal tagged version of the *Mtu*UDG-Ugi overexpression construct, the *Nol*–*Hind*III fragment from pTrc*Mtu*UDG-Ugi was subcloned into the same sites of pRSETB*Mtu*UDG.

**Purification of proteins.** The purification of complexes of Ugi with *Eco*, *Mtu* and *Msm*UDGs was carried out by a protocol described by Acharya et al. (2002) using T7 RNA polymerase-based expression constructs in *E. coli* BL21 (DE3). Similarly, EcoUDG, MtuUDG and Ugi were also purified from T7 RNA polymerase-based hyperexpression constructs using standard protocols (Handa et al., 2001; Acharya et al., 2002). The purified proteins were estimated by Bradford’s reagent using bovine serum albumin as standard (Sedmak & Grossberg, 1977).

**PAGE.** The UDG-Ugi complexes, UDGs and Ugi were analysed by PAGE (15 %) with or without 0.1 % SDS. The protein was visualized by Coomassie brilliant blue R250 staining (Laemmli et al., 1970). The loading dye for native gels consisted of 50 mM Tris/HCl (pH 6.8), 10 % glycerol and 0.01 % bromophenol blue (Roy et al., 1998).

**Stability of UDG-Ugi complexes.** Total-cell lysates harbouring UDG-Ugi complexes (10 μg) were treated with different concentrations of urea (0–8 M) at 25 °C for 1 h in a 10 μl reaction. Native gel dye (5 μl) was added and the contents were loaded on to a 15 % non-denaturing polyacrylamide gel and stained with Coomassie brilliant blue R250.

**Unfolding of UDG-Ugi complexes in urea.** UDG-Ugi complexes (~1 OD280 unit) were incubated with different concentrations of urea (0–8 M) for 4 h at 25 °C. For fluorescence measurements, the samples were excited at 280 nm and the emission was recorded between 300 and 400 nm using a spectrofluorophotometer ( Shimadzu RF-5301PC).

**Exchange of UDG from UDG-Ugi complexes.** UDG-Ugi complexes (1–3 μg) were incubated with EcoUDG (0.05–2 μg) or *Mtu*UDG (1–5 μg) at 25 °C for 10 min and the reaction mixtures were analysed by native or 8 M urea PAGE (15 %).

**UDG assay.** UDG assays were carried out according to a protocol described previously (Acharya et al., 2002) using SSU9 (5'-CTC-AAGTGUAGGCATGCAAGAGCT-3') for reactions with the purified UDG-Ugi complexes or SSU12 (5'-A-TATACCGCGCGCGCGCGAAGCCTTAT-T-3') containing phosphorothioate bonds (~) between the first two and last two nucleotides, for reactions with total-cell lysates. The synthetic oligomers were 5'-end-labelled by T4 polynucleotide kinase and [γ-32P]ATP and purified by spin column chromatography on Sephadex G50 minicolumns (Kumar & Varshney, 1994; Sambrook et al., 1989). Purified substrate (20 000 c.p.m.) was used along with 2-5 pmol cold substrate for the assays per reaction with different amounts of UDG-Ugi complexes.

**RESULTS**

**Comparison of amino acid sequence of *E. coli* and mycobacterial UDGs**

Fig. 1 shows an alignment of UDGs from *M. smegmatis*, *M. tuberculosis* and *E. coli* obtained by CLUSTAL W analysis. The mycobacterial UDGs showed an overall identity and
similarity of 83.3 and 89%, respectively. However, when compared with EcoUDG, both the mycobacterial UDGs showed an overall identity and similarity of ~40 and ~53%, respectively. Such a high degree of homology and the observation that Ugi, a substrate mimic that binds in the active-site pocket of UDGs, is a common inhibitor of these enzymes (see below) strongly suggests that the overall architecture of the tertiary fold of the mycobacterial UDGs is similar to that of EcoUDG.

The co-crystal structures of the complexes of Ugi with UDGs from E. coli, human and herpes simplex virus (HSV) have revealed the details of interactions between the UDG and Ugi (summarized in Table 1). The UDG side chains of Q63, D64, Y66, H67, Q71, S88 and S189 (numbered according to EcoUDG), and the main chain atoms of A133, S (or T)166, H187 and P190 are among the highly conserved residues in E. coli and human UDGs that establish direct or water-mediated hydrogen bonds with various atoms in Ugi. In addition, another highly conserved interaction between UDG and Ugi is established through the insertion of side-chain residue L191 (or F191) into the hydrophobic pocket of Ugi, which contributes significantly to the stability of the UDG-Ugi complex (Handa et al., 2001). In Msm- and MtuUDGs, while most of these interacting residues are conserved, the equivalents of H67, Q71, A133 and S166 are represented by P, H, P and R, respectively. As shown in Fig. 2, all of these residues (except position 133) interact with the β1-edge of Ugi. The mycobacterial UDGs are thus naturally occurring fully active ‘mutants’ which allow us to investigate the significance of UDG interactions with the β1-edge of Ugi.

**Overexpression UDG-Ugi complexes and their stability in the presence of urea**

Fig. 3 shows various constructs used to overexpress UDGs or UDG-Ugi proteins. While abundant expression of the complexes of Ugi with Eco- and MsmUDGs was seen from pET11d constructs, appreciable levels were not seen for MtuUDG or its complex with Ugi from pTrc99C or the pET11d-derived expression constructs. Interestingly, expression of MtuUDG as a fusion protein containing an N-terminal tag from pRSETB resulted in abundant production, allowing direct analysis of the complexes formed in vivo. Also, as seen from the presence of free Ugi in the samples not treated with urea (Fig. 4a, lanes 1, 3 and 10), expression of Ugi from the bicistronic constructs occurred in slight molar excess to UDG (Handa et al., 2001).

We have previously described an assay system to monitor in situ dissociation of UDG-Ugi complexes by electrophoresis of cell lysates on polyacrylamide gels containing 0–8 M urea (Handa et al., 2001; Acharya et al., 2002). In this study, we treated cell lysates with different concentrations of urea (0–8 M) for 1 h, prior to resolution by native PAGE. An advantage of this system is that it is rapid and it allows direct comparison of the stability of the complexes on the same gel. In these native gels, UDG migrates much slower than Ugi and the complex of the two migrates between the two free proteins (Handa et al., 2001). Furthermore, among the three complexes analysed, the MsmUDG-Ugi migrates fastest and the N-terminal tag of MtuUDG confers slowest mobility to its complex with Ugi (Fig. 4a, compare lanes 1, 3 and 10).
As expected, the EcoUDG-Ugi complex was unaffected by treatment with 8 M urea, and no increase in the intensity of free Ugi was seen (Fig. 4a, compare lanes 1 and 2). The MsmUDG-Ugi complex was stable to treatment with 4 M urea (lanes 3–5). However, as deduced from the absence of the MsmUDG-Ugi band, it dissociated completely upon treatment with 5 M or higher concentrations of urea (lanes 6–9). As expected, the disappearance of the UDG-Ugi band resulted in a concomitant increase in the intensity of the Ugi band (compare lanes 3–5 with 6–9). Similarly, comparison of lanes 10–13 with 14–16 shows that the MtuUDG-Ugi complex is stable to treatment with 5 M urea, but dissociates at higher urea concentrations. However, because the MtuUDG was expressed as a fusion protein containing an N-terminal tag from pRSETB vector, it was important to confirm that the N-terminal tag did not influence the folding of MtuUDG and therefore the stability of its complex with Ugi. For this purpose, we analysed cell lysates wherein MtuUDG-Ugi was expressed from a pTrc99c construct by immunoblotting using anti-MtuUDG antibodies. As shown in Fig. 4(b), even the complex (C2) with an untagged UDG showed an identical profile of dissociation upon treatment with urea (compare lanes 1–7 with 8–14), suggesting that the stability of the MtuUDG-Ugi complex was unaffected by the presence of the N-terminal tag. Importantly, as discussed below, the presence of the N-terminal tag turned out to be quite useful in allowing a distinct separation of the MtuUDG-Ugi complex from the EcoUDG-Ugi complex in UDG/Ugi exchange studies. It may be noted that while both the Msm- and MtuUDGs share identical structural elements which interact with Ugi (Table 1), the MtuUDG-Ugi complex is marginally stronger than the MsmUDG-Ugi complex. The structural basis for this difference is unclear at present.

**Purification of UDG-Ugi complexes and spectrofluorometric analysis of urea-induced unfolding**

For further biochemical characterization, the UDG-Ugi complexes were purified and analysed by SDS-PAGE. As shown in Fig. 5, this preparation predominantly consisted of bands corresponding to UDG and Ugi, suggesting purification to near homogeneity. Previously we have shown that isothermal dissociation of EcoUDG-Ugi complexes in urea causes a red shift in the emission spectra that results from intrinsic fluorescence of tryptophan residues in the protein (Handa et al., 2001). We carried out similar studies with the mycobacterial UDG-Ugi complexes as well as EcoUDG-Ugi. As expected, when the EcoUDG-Ugi complex was subjected to treatment with up to 8 M urea, no significant changes in fluorescence profiles were observed (Fig. 6a). However, with increasing urea concentration, the mycobacterial complexes showed a notable quenching in fluorescence intensity and a red shift in the emission spectra. In agreement with the analysis of urea-treated total-cell lysates by native PAGE (Fig. 4a), the MsmUDG-Ugi complex was stable to 4 M urea and showed a red shift upon treatment with 5–8 M urea, while the MtuUDG-Ugi was stable to 5 M urea and showed a red shift upon treatment with 6–8 M urea (Fig. 6b and c, respectively).
Reversibility of mycobacterial UDG-Ugi complexes

Since the complexes of Ugi with mycobacterial UDGs dissociated in 5–6 M urea as opposed to the EcoUDG-Ugi complex which is stable at 8 M urea (Figs 4 and 6), it was of interest to investigate if the Ugi from these complexes could be sequestered to form a more stable complex with EcoUDG or exchanged with the substrate. Hence, the mycobacterial complexes were incubated with different amounts of EcoUDG and analysed on an 8 M urea gel. Although in the presence of urea EcoUDG and Ugi in their free state denature and do not form a complex, the preformed complex of the two is impervious to treatment with 8 M urea. In contrast, the complexes of Ugi with mycobacterial UDGs dissociate/denature at this concentration of urea. Thus, any EcoUDG-Ugi complex that formed from sequestration of Ugi from mycobacterial UDG-Ugi complexes (prior to electrophoresis) can be readily detected on 8 M urea gels (Fig. 7a). In these experiments, the intensity of the EcoUDG-Ugi complex increased upon incubation of the mycobacterial UDG-Ugi complexes with increasing concentrations of EcoUDG (Fig. 7a, lanes 3–8 and 9–14), suggesting that the mycobacterial UDG-Ugi complexes are reversible and the Ugi that dissociates from these complexes

Fig. 4. Stability of UDG-Ugi complexes. (a) Total-cell lysates (~10 μg) obtained from T7 promoter driven overexpression constructs were analysed by 15% native PAGE after incubation with different concentrations of urea and detected by Coomassie brilliant blue. Lanes: 1–2, EcoUDG-Ugi complex; 3–9, MsmUDG-Ugi complex; 10–16, MtuUDG-Ugi complex. The urea concentrations with which the lysates were treated are shown above each lane. (b) A Western blot analysis of a similar experiment, but the total-cell lysates were obtained from cells harbouring pRSETB_MtuUDG-Ugi (lanes 1–7, complex C1) and pTrcMtuUDG-Ugi (lanes 8–14, complex C2).
can be trapped into the EcoUDG-Ugi complex. On the other hand, as shown by the analysis on the native gel, incubation of the EcoUDG-Ugi complex with MtuUDG (with the N-terminal fusion), even when the latter was in 100-fold excess, did not result in any detectable formation of a MtuUDG-Ugi complex (Fig. 7b, compare lane 2 with lanes 3–6). However, when MtuUDG was incubated with complexes of Ugi with mutant EcoUDG (L191G, which dissociates in 6 M urea) a complex corresponding to MtuUDG-Ugi was seen on the native gel (lanes 7 to 10), increasing in amount with increasing concentrations of MtuUDG. As expected, the increase in the intensity of the MtuUDG-Ugi band was accompanied by a corresponding decrease in the intensity of the EcoUDG (L191G)-Ugi band and an increase in free EcoUDG (L191G) (lanes 7–10). Similar results were obtained when MtuUDG was incubated with the MsmUDG-Ugi complex (Fig. 7b, lanes 11–14). These observations suggest that the stability of complexes in different concentrations of urea can be taken as an index of their reversibility.

It may be noted that MtuUDG migrates slower than EcoUDG and as a smear (Fig. 7b). However, in the experiment shown in Fig. 7(a), the bands corresponding to MtuUDG and MsmUDG are prominent and migrate faster than EcoUDG. These apparent discrepancies in the relative mobility of the UDGs arise from the fact that in Fig. 7(a), the bulk of the mycobacterial UDGs enter the gel as UDG-Ugi complexes which dissociate to UDG and Ugi only upon migration into the 8 M urea-containing gel.

Fig. 5. Analysis of purified UDG-Ugi complexes. Proteins (~4 μg) were analysed by 15% SDS-PAGE. Lanes: 1, EcoUDG-Ugi; 2, MsmUDG-Ugi; 3, MtuUDG-Ugi.

Analysis of mycobacterial UDG-Ugi complexes

Fig. 6. Intrinsic fluorescence (tryptophan) changes in EcoUDG-Ugi (a), MsmUDG-Ugi (b) and MtuUDG-Ugi (c) complexes upon isothermal denaturation with urea. UDG-Ugi complex preparations (1 A280 unit ml⁻¹) were incubated with different concentrations of urea at room temperature for 4 h and the fluorescence spectra (300–400 nm) were recorded by using an excitation wavelength of 280 nm. Urea concentrations are indicated.

Analysis of substrate binding to UDG in mycobacterial UDG-Ugi complexes

The exchange of Ugi from mycobacterial UDG-Ugi to EcoUDG indicated that the mycobacterial UDGs form reversible complexes with Ugi. Since, Ugi is a substrate mimic and occupies the DNA-binding groove of UDGs, it was of interest to test if Ugi in these complexes could be exchanged with the substrate. Although substrate binding to UDG is short lived, it results in excision of uracil. Therefore, we assayed the amounts of complex needed to excise uracil from DNA as a measure of the dissociation of the two proteins in the complex. The greater the amount of complex needed for uracil excision, the less was the ability to
dissociate. As shown in Fig. 8, ~0.005 pmol of MsmUDG-Ugi and 1 pmol MtuUDG-Ugi were needed for ~50% uracil excision (a, lane 2; b, lane 6). However, even when a large excess of EcoUDG-Ugi (20 pmol) was used, uracil excision was seen to a lesser extent (c, lane 6). These observations are consistent with the results of dissociation of the complexes in urea and UDG exchange studies (Figs 4, 5 and 6).

Analysis of inhibition of UDG activity in *M. smegmatis* and *M. tuberculosis* cellular extracts by Ugi

It was important to analyse if Ugi, a potent inhibitor of *Eco* and human UDGs, could inhibit mycobacterial UDGs in the context of total-cell proteins. For this analysis we used both *M. smegmatis* and *M. tuberculosis* cell extracts. As shown in Fig. 9, use of as low as 20 ng Ugi with 50 μg total-cell proteins resulted in elimination of UDG activity (Fig. 9a and b, lanes 7). This observation suggests that even though the complexes of Ugi with mycobacterial UDGs are not as stable as the *Eco*UDG-Ugi complex, its (Ugi) expression in these bacteria, even to an extent as low as 0.04% of the total-cell protein, would be adequate to abolish UDG activity.

**DISCUSSION**

In these studies we have shown that complexes of Ugi with mycobacterial UDGs are reversible and dissociate in 5–6 M urea as opposed to *Eco*UDG-Ugi or human UDG-Ugi complexes that are stable to even 8 M urea (Bennett & Mosbaugh, 1992; Mol et al., 1995). Thus the mycobacterial UDGs belong to a distinct group of UDGs which form reversible complexes with Ugi. Furthermore, although the complexes of Ugi with *Mesoplasma* (formerly *Mycoplasma*) lactucae UDG have not been biochemically characterized, based on the observation that much higher levels of Ugi are needed to inhibit UDG activity in cellular extracts of *Mesoplasma lactucae* (Williams & Pollack, 1990), we suspect...
that even *Mesoplasma lactucae* UDG may belong to this group of naturally occurring UDGs that form a reversible complex with Ugi. This prediction is supported by the fact that at least the *Mycoplasma penetrans* UDG, whose sequence is known (Sasaki et al., 2002), like mycobacterial UDGs contains substitutions at many of the conserved positions which interact with Ugi (e.g. H67, Q71, A133 and S166 of *Eco* UDG are represented by Q, M, S and N, respectively).

Mechanistically, the formation of the *Eco* UDG-Ugi complex has been shown to involve two important kinetic steps: (i) a ‘docking’ step which defines a reversible pre-equilibrium step involving alignment of the two proteins, and (ii) a ‘locking’ step which leads to isomerization of the docked intermediate into an irreversible complex (Bennett et al., 1993). Several X-ray crystal structures of Ugi and its complexes with UDGs have elegantly demonstrated the structural features involved in their interaction (Savva & Pearl, 1995; Mol et al., 1995; Ravishankar et al., 1998; Putnam et al., 1999). The *Eco* UDG-Ugi interface is defined by two sets of predominant interactions: (i) an interaction between the hydrophobic cavity of Ugi (formed between the α2 helix and the β sheet) and L191 of the DNA intercalation loop of UDG, and (ii) the interactions of the β1-edge of Ugi in the DNA-binding groove of UDG via direct or the water-mediated hydrogen bonds (Ravishankar et al., 1998; Putnam et al., 1999). Other studies (Lundquist et al., 1997; Acharya et al., 2002) have shown that mutations in Ugi which compromise or abolish either the hydrophobic cavity interaction (M24K) or the β1-edge interaction (S21P, L23R, E20A) result in formation of complexes with *Eco* UDG that are reversible, suggesting that both classes of interaction are necessary to form a ‘locked’ complex. However, either class of interactions can foster the ‘docking’ step (Acharya et al., 2002). Thus, when mutations are made in Ugi that interfere with the hydrophobic interactions, the interactions between the Ugi β1-edge and the DNA-binding groove of UDG facilitate formation of the complex and vice versa (Acharya et al., 2002; Handa et al., 2001, 2002). Furthermore, in a complementary approach, we showed that the L191G mutation in the DNA intercalation loop in *Eco* UDG that compromises the hydrophobic cavity interaction, resulted in a complex that dissociated in 6 M urea and, as shown here, in a complex that is reversible (Fig. 7b). In this study, we show that a deficiency of conserved structural elements in mycobacterial UDGs that interact with the β1-edge of Ugi results in the formation of reversible UDG-Ugi complexes (Figs 7a and 8). Thus, based on mutations in both of the interacting partners (UDG and Ugi), it is clear

![Fig. 8. Exchange of Ugi in *Msm* UDG-Ugi (a), *Mtu* UDG-Ugi (b) and *Eco* UDG-Ugi (c) complexes with substrate. UDG assays were carried out for 10 min at 37 °C using SSU9 substrate in the presence of the indicated amounts of UDG-Ugi complexes. S and P represent the substrate and product bands, respectively.](http://mic.sgmjournals.org)
that either class of interactions facilitates the 'docking' step. However, it is the sum total of the extensive network of all the interactions between UDG and Ugi that leads to the formation of the 'locked' complex.

Importantly, UDGs play a crucial role in mutation prevention in various organisms (Olsen et al., 1991; Duncan & Weiss, 1982; Chen & Lacks, 1991; Impellizzeri et al., 1991). Furthermore, in poxvirus, attempts to inactivate the UDG gene have remained unsuccessful (Stuart et al., 1993). Also, a temperature-sensitive mutant of vaccinia virus, which did not replicate at the non-permissive temperature, was found to be defective in its UDG gene (Millins et al., 1994), demonstrating the essential nature of UDG in this virus. The essential role of UDG in efficient virus replication and latency of HSV-1 has also been documented (Mullaney et al., 1989; Pyles et al., 1994). Interestingly, in human immunodeficiency virus type-1 (HIV-1), the viral integrase (IN) enzyme, an essential enzyme for viral establishment, interacts with the host UDG (Willets et al., 1999), suggesting the need of this key DNA repair enzyme during packaging. These observations suggest that UDG plays an important role either in establishment or in survival of these viruses inside the hosts. In mycobacteria, which are G+C rich organisms that live under conditions that promote cytosine deamination, UDG is a crucial DNA repair enzyme.

In spite of the fact that Ugi forms complexes with mycobacterial UDGs that are not as stable as its complex with EcoUDG, the complex that is stable to treatment with 5–6 M urea is still a very stable complex. As shown in Fig. 9, the presence of Ugi in the total-cell extracts of M. smegmatis or M. tuberculosis, at a level as low as 0.04 % total-cell proteins, leads to abolition of UDG activity. Since in mammalian systems there are a number enzymes with UDG activity, and since a knockout mouse (ung/+ ung/) presented no distinct phenotypes or histopathological consequences (Nilsen et al., 2000), we believe that UDG could provide us with a new target to control the growth of mycobacteria. Finally, these studies have now set a stage for the crystal structure determination of mycobacterial UDG-Ugi complexes to further our understanding of the molecular mechanisms of their interaction and to allow the design of more potent and shorter peptide inhibitors.

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