Published in final edited form as:

Arch Biochem Biophys. 2011 September 15; 513(2): 81–86. doi:10.1016/j.abb.2011.07.004.

The *Escherichia coli* alkylation response protein AidB is a redox partner of flavodoxin and binds RNA and acyl carrier protein

Scott B. Mulrooney^{a,*}, Michael J. Howard^a, and Robert P. Hausinger^{a,b}

^aDepartment of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824, USA

^bDepartment of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, USA

Abstract

Microorganisms are exposed to a wide variety of exogenous and endogenous chemical agents that alkylate DNA. *Escherichia coli* cells exhibit an adaptive response that recognizes and repairs alkylated DNA lesions using Ada, AlkA, and AlkB enzymes. Another alkylation response protein, the DNA-binding flavoprotein AidB, was proposed to repair DNA or protect it from chemical alkylating agents, but direct evidence for its role is lacking. Here, AidB was shown to form tight complexes with both flavodoxin and acyl carrier protein. In addition, electron transfer between 1-electron and 2-electron reduced flavodoxin to oxidized AidB was observed, although with very small rate constants. AidB was found to bind to RNA, raising the prospect that the protein may have a role in protection of RNA from chemical alkylation. Finally, the reagent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was eliminated as a direct substrate of the enzyme.

Keywords

AidB; adaptive response; alkylation damage; flavodoxin; protein complexes

Introduction

The chemical mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) alkylates DNA by reacting primarily with the *N*7 and *O*⁶ positions of guanine, *N*3 of adenine, *O*² or *O*⁴ of pyrimidines, and the non-phosphodiester oxygen atoms of the phosphate backbone [1, 2]. Microorganisms generate endogenous methylation compounds, such as S-adenosylmethionine [3], and nitrosated amino acids, peptides, or polyamines [4–6], and are exposed to exogenous methylating substances such as methyl halides [7] that can modify their DNA, RNA, and other cellular components. To overcome the mutagenic and toxic effects of DNA methylation, *Escherichia coli* possesses a variety of DNA repair enzymes, some of which are induced as part of the “adaptive response to alkylating agents” [8–11]. The Ada protein acts as a methyltransferase to irreversibly transfer a methyl group from the DNA backbone or from 4-methyl-T or 6-methyl-G to one of two critical Cys residues. Upon

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*Corresponding author. Address: Department of Microbiology and Molecular Genetics, 2215 Biomedical Physical Sciences, Michigan State University, East Lansing, MI 48824-4320, USA. Fax: +1517 353 8957. mulroon2@msu.edu (S.B. Mulrooney).

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accepting a methyl group on Cys38, Ada becomes a transcriptional activator of the adaptive response regulon [12, 13] which includes Ada [14–17], AlkA (a 3-methyl-A glycosylase) [18], AlkB (an Fe²⁺- and α -ketoglutarate-dependent oxidative demethylase) [19, 20], and AidB. In contrast to the situation for Ada, AlkA, and AlkB, the role of AidB in the adaptive response process remains obscure.

Overexpression of *aidB* is known to lead to reduced levels of mutagenesis caused by MNNG exposure [21], but the mechanism of protection against mutation is unknown. AidB has a close sequence relationship to acyl-coenzyme A (CoA) dehydrogenases, and extracts of overexpressing cells exhibit isovaleryl-CoA dehydrogenase activity [21]. Purified recombinant AidB possesses very low levels of this activity and contains FAD [22, 23]. The isolated protein tightly binds to double-stranded, but not single-stranded, DNA [22]. The AidB crystal structure shows high homology to acyl-CoA dehydrogenases; however, the FAD-containing active site possesses a Trp side chain that prevents binding of large acyl-CoA substrates [23]. Furthermore, the DNA-binding site, verified by the observed differences in fluorescence anisotropy of a synthetic oligonucleotide when using variant proteins, is located at a positively-charged surface located distant from the presumed active site [23]. Based on the structure of the putative substrate-binding pocket, AidB was hypothesized to play a protective role by detoxifying certain alkylating reagents. Finally, an analysis of the protein interaction network of *E. coli* [24] included results for AidB, as well as around 1,000 other proteins. Proteins found to interact with AidB include many components of ribosomes, several protein chaperones, various RNA- or DNA-binding proteins, and an assortment of other gene products.

Here we describe new properties of AidB and offer additional insights into its function. Two AidB-interacting proteins were chosen for further study. In particular, flavodoxin (FldA) could be a possible redox partner of AidB while acyl carrier protein (AcpP) is of interest because acyl-AcpP is used as substrate by some acyl-CoA dehydrogenases. In addition, since many proteins interacting with AidB are known to bind to or metabolize RNA we examined the ability of AidB to bind tRNA as a representative RNA molecule. Finally, we directly tested the hypothesis that AidB detoxifies MNNG.

Materials and Methods

Chemicals and media

Terrific broth (TB) medium was obtained from Fisher Scientific and used for large-scale growth of *E. coli* producing recombinant AidB. Ribonuclease, purchased from Roche Applied Science (Indianapolis, USA), was treated to inactivate deoxyribonuclease by dissolving it at 1 mg/ml in 10 mM sodium acetate buffer, pH 5.2, and placing in a boiling water bath for 15 min. Nitrilotriacetic acid (NTA) resin was obtained from Novagen All other enzymes and chemicals were obtained from Sigma (St. Louis, USA).

PCR cloning of *aidB* with a GTG start codon

Our previous study used AidB derived from a PCR fragment with an ATG codon as the translation start for the gene. Subsequent annotations in the NCBI Nucleotide database and the position of a presumptive Shine-Dalgarno sequence [21] showed that a GTG was the likely start codon. This codon resulted in a protein that was 5 amino acids shorter than what was used in our previous studies. All of the experiments described here were performed using recombinant AidB expressed from the authentic GTG start codon.

PCR primers were: 5'-AGG ATA TAC ATA TGG TGC ACT GGC AAA CTC-3' (forward), and 5'-TCT CGT AGA AGC TTT TAC ACA CAC ACT CCC CCC G-3' (which introduces a HindIII site at the 3' end of the gene). The PCR reaction, cloning of the product into

pET28a, and plasmid transformation into the host cells were performed as previously described [22].

Purification of AidB

E. coli C41(DE3) containing plasmid pET28aGTG-AidB was used for expression of recombinant AidB. Cultures were grown in TB medium and induced as described previously. AidB was purified as previously described by using phenyl Sepharose chromatography [22], or by heparin-agarose affinity chromatography [23]. Because AidB was known to bind DNA and could potentially be contaminated with small amounts of nucleic acids, some preparations were treated with benzonase to digest bound oligonucleotides. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli [25]. Although several purification protocols were used, the resulting protein preparations were equivalent as judged by SDS-PAGE, UV-Vis spectroscopy, and DNA binding ability.

Preparation of cell extracts containing FldA and AcpP

His-tagged FldA and AcpP were produced in *E. coli* strain JW0671 (*fldA* in plasmid pCA24N) or strain JW1080 (*acpP* in plasmid pCA24N), obtained from the Nara Institute of Science and Technology [26]. TB medium (20 ml) containing 50 µg/ml kanamycin was inoculated with an overnight colony and grown at 37°C while shaking until the absorbance at 600 nm was between 0.2 and 0.5. Isopropyl-β-D-thio-galactoside was added to a final concentration of 0.1 mM and growth was continued for 16–18 hours. Cultures were chilled on ice and the cells collected by centrifugation. Cells were resuspended in 20 ml of 20 mM Tris and 30 mM imidazole, pH 7.9, containing 300 mM NaCl plus 20% v/v glycerol, and disrupted by sonication. Insoluble material was removed by centrifugation at 14,000 g for 5 min at room temperature.

AidB interactions with FldA and AcpP

Ni-NTA was prepared by mixing the resin with 0.1 M NiSO₄ and washing it extensively with binding buffer (10 mM Tris and 30 mM imidazole, pH 7.2, containing 250 mM NaCl, 10% glycerol, and 0.02% (v/v) Triton X-100). Soluble cell-free extracts made from recombinant cultures producing His-tagged flavodoxin or acyl carrier protein were mixed with suspended Ni-NTA resin and allowed to incubate at room temperature for 15 min. Analogous studies were carried out with extracts from cells containing control plasmid with no cloned insert. The samples were centrifuged and the Ni-NTA resin pellets were washed two times with binding buffer. AidB (0.6 nmol) was added to the resuspended pellets in 1 ml binding buffer, and the samples were incubated at room temperature for 10 min. The samples were centrifuged and the pellets washed four times with binding buffer, followed by elution of proteins bound (specifically or in complex) to the Ni-NTA resin by using a buffer of 10 mM Tris and 500 mM imidazole, pH 7.9, containing 150 mM NaCl, 20% glycerol, and 0.02% Triton X-100. Portions of the eluted proteins were analyzed by SDS-PAGE using a 15% polyacrylamide gel.

Purification of His₆-flavodoxin

One liter cultures of *E. coli* strain JW0671 were grown and induced as described above. After overnight growth, the cultures were chilled on ice, the cells collected by centrifugation, and the pellets frozen. The cells were thawed and resuspended in binding buffer (20 mM Tris and 60 mM imidazole, pH 7.9, containing 500 mM NaCl) to give a final volume of ~20 ml. Flavin mononucleotide and the protease inhibitor phenylmethylsulfonyl fluoride were added to yield final concentrations of 50 µM and 0.1 mM, respectively. The cells were disrupted by sonication and centrifuged at 100,000 g for 45 min at 4°C. Cell-free

extracts were applied to a 3 ml column of Ni-NTA resin. After washing the column with binding buffer, His-tagged flavodoxin was eluted by using a buffer of 20 mM Tris and 500 mM imidazole, pH 7.9, containing 500 mM NaCl. Fractions containing the flavodoxin were dialyzed against 20 mM Tris buffer, pH 8, containing 200 mM NaCl and 20% glycerol then frozen at -80°C . An extinction coefficient of $8250\text{ M}^{-1}\text{cm}^{-1}$ at 466 nm was used to determine concentrations for the oxidized form [27].

Electron transfer between flavodoxin and AidB

Inter-protein electron transfer reactions were studied by using a cuvette equipped for anaerobic gas exchange with a side arm for addition of degassed samples into the main chamber. In some cases, His₆-FldA was placed into the 1 ml cuvette along with 1 nmole of 5-deazaflavin in a buffer containing 20 mM Tris, pH 8, plus 15 mM EDTA and 250 mM NaCl. AidB was added to the sidearm prior to sealing the cuvette. The system was made anaerobic by 10 vacuum/fill cycles using argon that had been scrubbed to remove trace oxygen, with at least 10 min of equilibration after each exchange. The flavodoxin was photoreduced to the 1-electron semiquinone state by exposing the main portion of the cuvette to a beam of white light from a 50 W halogen spotlight positioned 25 cm from the cuvette [28]. The sidearm containing AidB was shielded from the light source. At several times during the light exposure, absorbance spectra were recorded on a Shimadzu UV2401 spectrophotometer. Photoreduction was considered complete (after ~ 1.5 hours) when the characteristic absorbance of the blue semiquinone at 580 nm was fully developed (data not shown). Electron transfer from the flavodoxin semiquinone to oxidized AidB was initiated by tipping the cuvette to empty the contents of the sidearm into the flavodoxin, and spectra were taken at 5 min intervals. Data were input into Olis SpectralWorks software (Olis, Inc.) and analyzed both by single wavelength fitting at 580 nm and by singular value decomposition [29] of the changes in the 520–700 nm spectral region using $A \rightarrow ___$, B (A going to colorless with an absorbance background B) as the fit model. This spectral region reports only on changes involving the flavodoxin, with its blue semiquinone, whereas AidB forms a red semiquinone [22]. This approach allowed for straightforward quantification of changes involving one participant in a reaction that contained two flavoproteins.

In a variation of the above reaction, the flavodoxin was reduced to its fully reduced state by photoreduction and electron transfer to oxidized AidB was examined. Progress of the reaction was monitored by both development of the blue semiquinone of the flavodoxin and the red semiquinone of AidB.

Alternatively, AidB (33.4 nmoles) was placed directly into the cuvette and 613 nmoles of oxidized His₆-flavodoxin was added to the sidearm. After making the system anaerobic as described above, an anaerobic solution of sodium dithionite was titrated into the AidB sample until the FAD was fully reduced. The contents of the sidearm were added and spectra were monitored for 1 h.

RNA binding to AidB

Serial dilutions of purified AidB were prepared in a buffer consisting of 20 mM Tris, pH 7.9, containing 1 mM EDTA, 20% (v/v) glycerol, and 0.02 % (w/v) Triton X-100 in a volume of 10 μl . The tRNA (0.3 μg in a volume of 1 μl) was added and the mixture incubated at 37°C for 30 min. Samples were run on a 1% agarose gel for analysis.

Results and Discussion

AidB purifies with bound nucleic acids

The AidB protein investigated here was produced from a recombinant gene using an authentic GTG start codon, rather than from an ATG start codon as studied previously [22]. On the basis of the location of the amino terminus in the crystal structure [23], the additional five residues in the earlier version of the protein are unlikely to affect DNA binding or function of the active site. The protein was purified by using the published protocol and, as with the previous protein sample, exhibited instability and precipitation problems. For example, some new preparations of AidB that initially appeared soluble produced a yellow precipitate upon standing at 4°C for 1–2 days. Because AidB was known to bind DNA, we tested whether the purified enzyme possessed DNA and if its presence might affect stability. AidB was denatured by heating to 65°C for 55 min and the sample was centrifuged, yielding a white protein pellet and a yellow supernatant (presumably free FAD and any potentially bound nucleic acids). Portions of the supernatant were treated with deoxyribonuclease or ribonuclease. Agarose gel electrophoresis of the treated and control samples indicated that significant amounts of nucleic acids were released from the AidB by the heat treatment (Fig. 1). The supernatant treated with deoxyribonuclease showed substantial degradation of the nucleic acids, as indicated by the smaller apparent size of the nucleic acid smear on the gel, whereas treatment with ribonuclease had no effect. These results demonstrate that purified AidB contains bound DNA. A single DNA molecule could bind several AidB molecules and, because AidB is a multimer, large insoluble complexes could form. Indeed, Bowles *et al.* propose that AidB tetramers bind to and surround DNA in a protective role [23]. We observed that the addition of benzonase to samples of AidB confers enhanced stability to the protein.

AidB binds tightly to flavodoxin and acyl carrier protein

From a large-scale study of protein-protein interactions in *E. coli*, a list of proteins known to bind AidB was assembled [24]. Of the 32 proteins found to bind AidB, only flavodoxin had a definite redox function. AcpP, known to deliver acyl groups to some members of the acyl-CoA dehydrogenase family of enzymes, also was selected for further examination as an interacting partner of AidB. Both of these proteins are available as His-tagged fusions encoded by plasmid clones from the ASKA library [26]. Pull-down assays were carried out to test for co-elution of AidB with the His-tagged proteins using Ni-NTA resin, with the results shown in Fig. 2. His-tagged AcpP (presumably in the apo-ACP form) bound tightly to AidB (lane 3), even after multiple washes to remove unbound or weakly bound proteins. Interestingly, a second unidentified protein with a molecular weight of about 26 kDa, also bound to some preparations of the AidB:His₆-AcpP complex. Binding of AcpP may not have a functional role and may be an “evolutionary remnant” resulting from the structural similarity of AidB to acyl-CoA dehydrogenase [23]. Lane 4 shows that His₆-FldA also tightly bound AidB. The latter result led to speculation that FldA may be a redox partner with AidB.

Electron transfer between flavodoxin and AidB

Anaerobic FldA was reduced by exposure to white light in the presence of 15 mM EDTA and catalytic amounts of 5-deazaflavin. The reduction yielded a characteristic blue, 1-electron reduced flavin semiquinone spectrum of flavodoxin with a broad absorbance band in the 550–650 nm range [30]. Upon adding oxidized AidB, the long wavelength peak of the flavin semiquinone slowly decreased (Table 1), while absorbance increased in the 350–400 nm range (Fig. 3). This increase was accompanied by a minor peak at 400 nm, a feature observed in the anionic flavin semiquinone spectrum of 1-electron reduced AidB [22]. After recording spectra at 5 min intervals for 95 min, the cuvette was left in the dark overnight. A

spectrum recorded the next day showed no absorbance in the 550–650 range, while the 350–400 nm range had well defined features of the AidB flavin anionic semiquinone. After opening the cuvette to air and mixing thoroughly, the resulting spectrum resembled fully oxidized flavin as expected for a mixture of flavodoxin and AidB. These results show that electrons were transferred from the flavin semiquinone of flavodoxin to the oxidized AidB, yielding an AidB flavin semiquinone and oxidized flavodoxin.

Analogous studies were carried out by using fully reduced flavodoxin and oxidized AidB. Anaerobic mixing of these components led to the slow development (Table 1) of absorbance at 580 nm and 372 nm (Fig. 4). These results are interpreted in terms of the formation of the neutral flavin semiquinone in flavodoxin and the anionic flavin semiquinone in AidB.

By contrast to the above studies using reduced flavodoxin and oxidized AidB, no electron transfer was detected when using reduced AidB and oxidized flavodoxin. In particular, the combination of a 2.5 molar excess of chemically-reduced AidB and oxidized flavodoxin yielded no spectral changes over a 1 h period (data not shown).

Flavodoxins often are involved in electron transfer reactions with multiple protein partners [31]. *E. coli* flavodoxin, with redox potentials of -245 mV (oxidized/semiquinone) and -433 mV (semiquinone/fully reduced), similarly has several redox partner proteins. When electrons are supplied by flavodoxin NADP⁺ oxidoreductase, FldA functions mainly as a single-electron carrier by cycling its flavin between the oxidized and semiquinone states [30]. The redox potentials of AidB have not been reported, but our results are consistent with its oxidized/semiquinone potential lying significantly more positive than that of the flavodoxin oxidized/semiquinone or semiquinone/fully reduced flavin couples. In contrast, the semiquinone/fully reduced potential of flavin in AidB is unlikely to be significantly more negative than the oxidized/semiquinone potential of flavodoxin. It should be noted that the conditions used here included fairly high salt concentration (250 mM), the presence of a detergent in the stock solutions of AidB, and a temperature of 25°C. Changes in ionic strength and other conditions can alter the redox potentials of flavodoxin [32] and also may affect the redox behavior of AidB. The small rate constants observed in this study may be the result of purely thermodynamic events; however, they may also arise from suboptimal conditions, or the lack of yet unidentified factor(s) that facilitate the process. The stable binding of AidB to flavodoxin observed in the pull-down assays indicate that the binding event is not likely the rate limiting step in the electron transfer process. Furthermore, the binding interaction between these proteins may not be optimal for electron transfer. This conjecture is supported by the results in Table 1 which show little effect of flavodoxin concentration on the rate constants for electron transfer to oxidized AidB. Of related interest is the observation of similar rate constants for electron transfer whether using the flavin semiquinone or fully reduced states of the flavodoxin. While flavodoxin and AidB are redox partners, the small rate constants for electron transfer indicate this process might not have physiological significance. On the other hand, the presence of the (still unidentified) true substrate of AidB potentially could lead to dramatic enhancements of the electron transfer rate constants.

Binding of AidB to RNA

Electrophoretic mobility shift assay results showed that AidB bound to tRNA (Fig. 5). For this experiment, tRNA was selected because of its abundance in the cell, it is readily available in pure form, and is very stable. Earlier studies that used AidB as a bait protein showed interactions with many prey ribosomal proteins [24], possibly indicating that AidB binds to one or a few of these proteins with the other ribosomal proteins being indirect binding partners. Given the finding that AidB binds RNA, those results could alternatively be interpreted as AidB binding to ribosomal RNA, with the ribosomal proteins co-purifying

during the tandem affinity purification procedure. In a similar fashion, the reported interactions between AidB and RNA-binding proteins might be indirect and accounted for by simultaneous binding of these proteins to RNA. These results demonstrating RNA binding by AidB allow speculation that this protein may protect RNA, in addition to its previously proposed role in protecting DNA [23].

Test of MNNG as a direct substrate of AidB

The architecture of the binding pocket near FAD in the AidB crystal structure suggested that only small molecules would have access to the active site, leading to the proposal that this protein acts directly on DNA damaging chemicals [23]. This hypothesis was investigated by addition of MNNG to either anaerobic oxidized AidB or protein that had been photoreduced to the 1-electron semiquinone state. In both cases, MNNG addition led to no significant spectral changes over times extending to 70 min. Thus MNNG is not a direct substrate; however, it is possible that reactive intermediates generated from MNNG metabolic activation may be substrates for AidB. In addition, other alkylating agents not examined here may be substrates for AidB.

Conclusions

This report reveals several new insights about AidB, but its physiological function remains uncertain. We confirmed that both flavodoxin and acyl carrier protein bind to AidB. Furthermore, we extended the flavodoxin results to demonstrate that its reduced forms can transfer electrons to oxidized AidB; however, the rate constants for electron transfer are so small they are unlikely to be physiologically relevant unless accelerated by substrate binding. Although the AidB crystal structure indicated the active site is limited to small molecule substrates, raising the possibility of direct detoxification of alkylating agents, our testing of this hypothesis by addition of MNNG to oxidized and 1-electron reduced AidB demonstrated no effect; thus, it appears unlikely that MNNG is a direct substrate for AidB. Another discovery presented here is that AidB binds to RNA. This result suggests that future studies could test for a potential role of AidB in protecting RNA from chemical damaging agents.

Acknowledgments

This research was supported by the National Institutes of Health (GM063584 to R.P.H.). 5-deazaflavin was generously provided by Dave Arscott and Sumita Chakraborty (University of Michigan).

Abbreviations used

TB	terrific broth
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Highlights

- AidB binds flavodoxin acyl carrier protein, and RNA
- Reduced flavodoxin can donate electrons to oxidized AidB
- MNNG is not a substrate for AidB

Fig. 1. Purified AidB retains bound DNA. Isolated AidB was heat denatured and the resulting supernatant was directly analyzed (lane 2) or analyzed after treating with deoxyribonuclease (lane 3) or ribonuclease (lane 4). Standards (lane 1) and samples were subjected to electrophoresis in 1% agarose and stained with ethidium bromide.

Fig. 2.

Ni-NTA agarose pull-down assay of AidB binding to His-tagged flavodoxin and acyl carrier protein. Extracts of cells lacking His-tagged proteins (lane 2) or containing His₆-AcpP (lane 3) or His₆-FldA (lane 4) were mixed with purified AidB, Ni-NTA was added, the resin was washed, and the eluted fractions were analyzed by SDS-PAGE with staining by Coomassie blue. Lane 1, low range SDS-PAGE standards (Bio-Rad).

Fig. 3. Electron transfer between flavodoxin containing a flavin semiquinone and oxidized AidB. Spectra were recorded at 5 min intervals after mixing the proteins. Inset: The reaction was monitored by measuring the disappearance of the 580 nm absorbance associated with the flavin semiquinone form of flavodoxin (points) and fit as described in Materials and Methods.

Fig. 4. Electron transfer between fully-reduced flavodoxin and oxidized AidB. Spectra were recorded after mixing of the samples at 3 min intervals. Inset: The increase in absorption at 580 nm was monitored over time (points) and fit as described in Materials and Methods.

Fig. 5. Electrophoretic mobility shift assay of AidB binding to tRNA. Lane 1, 1 kb Plus DNA ladder (Invitrogen, USA); Lane 2, positive control with 1 µg 1 kb DNA ladder + 120 pmol AidB; Lanes 3–6, 0.3 µg tRNA mixed with 120, 40, and 13.3 pmol AidB.

Table 1

Electron transfer rate constants between flavodoxin and AidB

Flavodoxin form and concentration (μM)	Oxidized AidB concentration (μM)	Rate constant derived from 580 nm ($\Delta\text{A min}^{-1}$)	Rate constant derived from SVD (520–700 nm) ($\Delta\text{A min}^{-1}$)
Semiquinone, 16.1	9.3	$3.00 \pm 0.1 \times 10^{-2}$	$2.99 \pm 0.1 \times 10^{-2}$
Semiquinone, 32.4	27.4	$1.039 \pm 0.019 \times 10^{-2}$	$9.7 \pm 1 \times 10^{-3}$
Fully reduced, 11.9	9.3	$4.211 \pm 0.017 \times 10^{-2}$	$4.17 \pm 0.15 \times 10^{-2}$