# Alkylation of Adenosine Deaminase and Thioredoxin by Acrylamide in Human Cell Cultures

Thomas Schwend[a], Maren Möller[a], Jens Schabacker[a], Thomas Ruppert[b], and Michael Wink[a,*]

[a] Institut für Pharmazie und Molekulare Biotechnologie, Universität Heidelberg, Im Neuenheimer Feld 364, D-69120 Heidelberg, Germany. Fax: 0 62 21 54 48 84. E-mail: wink@uni-hd.de

[b] Zentrum für Molekulare Biologie Heidelberg, Im Neuenheimer Feld 260, D-69120 Heidelberg, Germany

* Author for correspondence and reprint requests

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Acrylamide is an $\alpha,\beta$-unsaturated vinyl monomer that causes cytotoxicity due to its alkylating properties. In recent years several proteins have been identified that are alkylated by acrylamide *in vivo*. This finding might explain the neurotoxic effects of acrylamide in humans. However, the list of potential acrylamide target proteins is far from being complete. In particular, the proteins that mediate the cytotoxicity of acrylamide in cell cultures remained unknown. Here we identify two novel acrylamide target proteins in human cell cultures (Jurkat, HepG2 and Caco-2), adenosine deaminase and thioredoxin.

*Key words:* Acrylamide, Alkylation, Cytotoxicity, Mode of Action

## Introduction

Acrylamide is a neurotoxic $\alpha,\beta$-unsaturated vinyl monomer. Laboratory animals treated with acrylamide develop ataxia and skeleton muscle weakness (LoPachin, 2005), a phenotype that is mainly caused by degradation of nerve terminal end (LoPachin *et al.*, 2002). Due to the electrophilic character of the terminal methylene group of acrylamide it was speculated that acrylamide induces neurodegeneration via alkylation of sulfhydryl groups in proteins (Lopachin and Decaprio, 2005). Recently it was shown that acrylamide indeed alkylates *N*-ethylmaleimide sensitive factor and SNAP-25 *in vivo* (Barber and LoPachin, 2004). Using ICAT as a competitive label for cysteines, Barber *et al*. (2007) showed that acrylamide treatment reduces the number of reactive cysteines in a variety of rat striatal synaptosome proteins indicating that a vast number of proteins are alkylated by acrylamide *in vivo*.

The alkylation of cysteine residues via Michael addition is in fact unspecific and occurs in all tissues which have a slight alkaline pH regime. For instance, haemoglobin is the major target for acrylamide alkylation *in vivo*. Unspecific alkylation by acrylamide may also be the reason for the observed cytotoxicity and reproductive dysfunctions in laboratory animals (Chapin *et al.,* 1995; Tyl and Friedman, 2003) and in cell cultures (Walmod *et al.,* 2004).

In the present study we tried to identify new proteins that undergo alkylation by acrylamide in three human cell cultures. We have treated three human cell lines (Jurkat, Caco-2 and HepG2 cells) with acrylamide and analyzed extracted proteins by MALDI-TOF for potentially alkylated candidates. With this approach we identified two novel acrylamide target proteins that may contribute to the toxicity of acrylamide in cell cultures.

*Abbreviations:* ADA, adenosine deaminase; MALDI-TOF, matrix-associated laser desorption/ionization-time of flight mass spectrometry; TRX, thioredoxin.

## Material and Methods

### *Cell cultures*

Jurkat (human leukaemia T cells) and HepG2 (human hepatocellular carcinoma cells) cells were maintained in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany), supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin (Invitrogen). Caco-2 cells (human colon carcinoma cells) were maintained in DMEM

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(Invitrogen), supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 1 mM sodium pyruvate (Biowittaker, Walkersville, MD, USA), 10 mM non-essential amino acids (Biowittaker), 100 units/mL penicillin, and 100 $\mu$g/mL streptomycin. Cells were grown at 37 °C in a humidified atmosphere of 5% $CO_2$ in 75-cm$^2$ culture flasks (Greiner bio-one, Frickenhausen, Germany).

*Analysis of cell viability*

Cytotoxicity was assessed with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; Sigma-Aldrich, Munich, Germany] assays. Exponentially growing cells were plated in 96-well plates and treated with various concentrations of acrylamide or propionamide (Sigma-Aldrich) dissolved in sterile water. Cells were incubated at 37 °C for 48 h. Then 0.05 mg/mL MTT were added to each well and incubation was continued for 2 and 4 h. MTT-formazan products were dissolved in water and absorbance was detected at 540 nm in an ELISA plate reader. Cell viability was calculated relative to untreated control cells. The assays were performed in 4 to 8 replicates and repeated at least 3 times.

*2D-Gel electrophoresis*

Cells were seeded in 75-cm$^2$ cell culture flasks. After 2 d of incubation 2 mM acrylamide was added and cells were incubated for 18 h. Cells were harvested after trypsin treatment, washed with PBS and resuspended in 50 $\mu$L lysis buffer (8 M urea, 4% CHAPS, 1% DTT, 1 tablet complete protease inhibitor cocktail/50 mL). Cell debris was removed by centrifugation at 16,000 × *g* for 1 h at 4 °C. 50 $\mu$L of the supernatant were added to 300 $\mu$L rehydration buffer (8 M urea, 2% CHAPS, 0.2% DTT, 0.5% IPG buffer) and the mixture was applied onto IPG strips, pH 4–7. Rehydration was performed at 50 V for 12 h. Isoelectric focusing was performed according to the following program: 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, linear gradient to 8000 V within 1 h and 8000 V for 6 h. After isoelectric focussing, IPG strips were equilibrated in SDS buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 2% DTT, 30% glycerol) for 30 min for the second dimension. Reduced cysteines were alkylated with iodoacetamide (2.5% in equilibration buffer) and IEF gels were placed on 12% SDS-PAGE gels. In order to exclude secondary alkylation with acrylamide monomers during the electrophoresis SDS-PAGE gels were polymerized overnight. Then SDS-PAGE (12%) was performed at 20 mA for 30 min and 40 mA for 3 h, and proteins were stained with colloidal Coomassie blue.

*MALDI-TOF analysis*

Excised gel plugs were washed with 100 $\mu$L water, 100 $\mu$L 50% acetonitrile and subsequently shrinked in 100 $\mu$L acetonitrile. Gel slices were rehydrated at 4 °C in 25 mM $NH_4HCO_3$, pH 8, containing 12 ng/$\mu$L trypsin (Promega, Madison, USA) for 1 h. Tryptic digestion was performed at 56 °C for 1 h. Peptides were extracted from the gel plugs with 20 $\mu$L of 5% formic acid and desalted using ZipTips (Millipore, Madison, USA). The eluted peptides were spotted onto the target plate, air-dried and covered with 0.5 $\mu$L alpha-cyano-4-hydroxycinnamic acid. MALDI-TOF analysis was performed on an Ultraflex mass spectrometer (Bruker Daltonics, Bremen, Germany). Mass spectra were obtained in the positive-ion mode at an acceleration voltage of 25 kV and a pulsed ion extraction time of 80 ns. Typically 300 shots were combined for one spectrum. MALDI-TOF and PSD spectra were searched against the NCBI non-redundant database using MASCOT (Matrix Science, London, UK).

*In vitro adenosine deaminase activity assay*

Increasing concentrations of acrylamide (0, 0.6, 1.3, 2.5, 5.0, 10.0 and 20.0 mM) were dissolved in 100 $\mu$L of PBS, pH 7.4, or 0.05 M Tris-HCl, pH 8.8, and applied into a 96-well plate. Adenosine deaminase (calf intestine mucosa, Sigma-Aldrich) was dissolved in PBS, pH 7.4 (final concentration 0.05 units/mL). 10 $\mu$L of enzyme solution were added to each well and incubated for 20 min at 37 °C. 10 $\mu$L of 0.45 mM adenosine solution (in PBS) were added to each well, and the enzyme kinetics was recorded at 265 nm against controls in one-minute steps at room temperature.

## Results

*Acrylamide exhibits dose-dependent toxicity on human cell cultures*

In order to investigate the toxicity of acrylamide on human cell cultures, different concentrations of acrylamide were added to Jurkat, Caco-2 and HepG2 cells. Cell viability was measured after

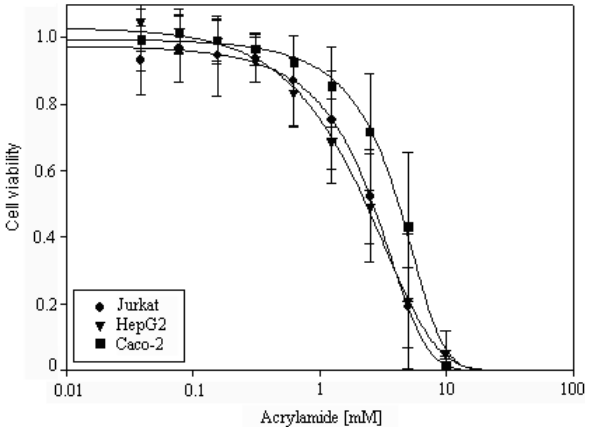


Fig. 1. Dose-dependent effect of acrylamide on Jurkat, Caco-2 and HepG2 cells. Cells were grown in 96-well plates and treated with acrylamide for 48 h. Cell viability was measured by the MTT assay (0.05 mg/mL MTT). Each bar represents the mean (plus standard deviation) of 24 wells. Experiments were performed in triplicate.

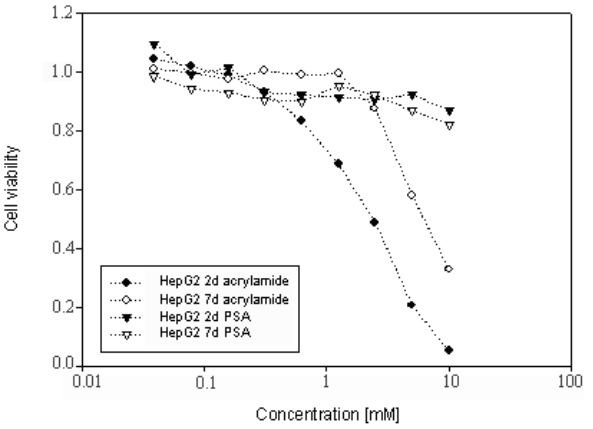


Fig. 2. Effect of propionamide (PSA) and acrylamide on HepG2 cells. Cells were grown in 96-well plates and treated with propionamide for 48 h. Cell viability was measured by the MTT assay (0.05 mg/mL MTT). Each bar represents the mean of 24 wells. Experiments were performed in triplicate.

acrylamide exposure with neutral red and the MTT assay. As shown in Fig. 1, acrylamide exhibited a significant dose-dependent toxicity on all three cell lines. Two days after seeding, HepG2 cells and Jurkat cells showed similar toxic effects, whereas Caco-2 cells exhibited a slightly, but significant ($p < 0.01$) higher cell viability (see calculated $IC_{50}$ values in Table I). Interestingly, the acrylamide-induced cell toxicity was significantly lower ($p < 0.01$) in 7-day-old HepG2 cells compared to 2-day-old cells. Hence our data are in agreement with previously reported results for acrylamide-treated RT4 cells (Tanii *et al.*, 1988). In order to test whether the cytotoxicity is based on the alkylating properties of acrylamide we treated the same cells with propionamide, an $\alpha,\beta$-saturated acrylamide analogue. In contrast to the effect of acrylamide, propionamide did not exhibit any toxic effects at the given concentrations (Fig. 2). This finding clearly indicates that the terminal methylene group of acrylamide is mediating the biochemical reactions in the cells.

Table I. $IC_{50}$ values of acrylamide toxicity. $IC_{50}$ values were calculated from dose-response curves 48 h after acrylamide treatment. Cell viability was monitored by MTT.

| Cell culture | $IC_{50}$ [mM] |
|---|---|
| Jurkat | 2 |
| HepG2 (2 d) | 2 |
| Caco-2 (2 d) | 4 |
| HepG2 (7 d) | 6 |
| Caco-2 (7 d) | not detectable |

### *Acrylamide alkylates adenosine deaminase (ADA) and thioredoxin (TRX)*

Modification of sulfhydryl groups in proteins appears to be the molecular mechanism of acrylamide toxicity. In order to identify proteins with a covalent bonding to acrylamide we separated total protein extracts of acrylamide-treated cells (10 $\mu$M acrylamide, cells treated for 20 h) by 2D-gel electrophoresis. From these 2D-gels, 32 high abundant spots were excised, digested with trypsin and analyzed by MALDI-TOF/TOF. The MALDI-TOF spectra were then searched for propionamide adducts (additional mass of 71 Da) using the MASCOT database. From the 32 analyzed proteins, two proteins, ADA (Cys-74, Jurkat cells) and TRX (Cys-73, Caco-2 cells) were found to be alkylated (Fig. 3; Table II). Both modifications were confirmed by PSD fragmentation of the alkylated peptide. An effect on other proteins is not excluded.

### *Acrylamide does not reduce ADA activity*

Cys-74 of ADA, which was found to be alkylated by acrylamide, is not located in the active pocket

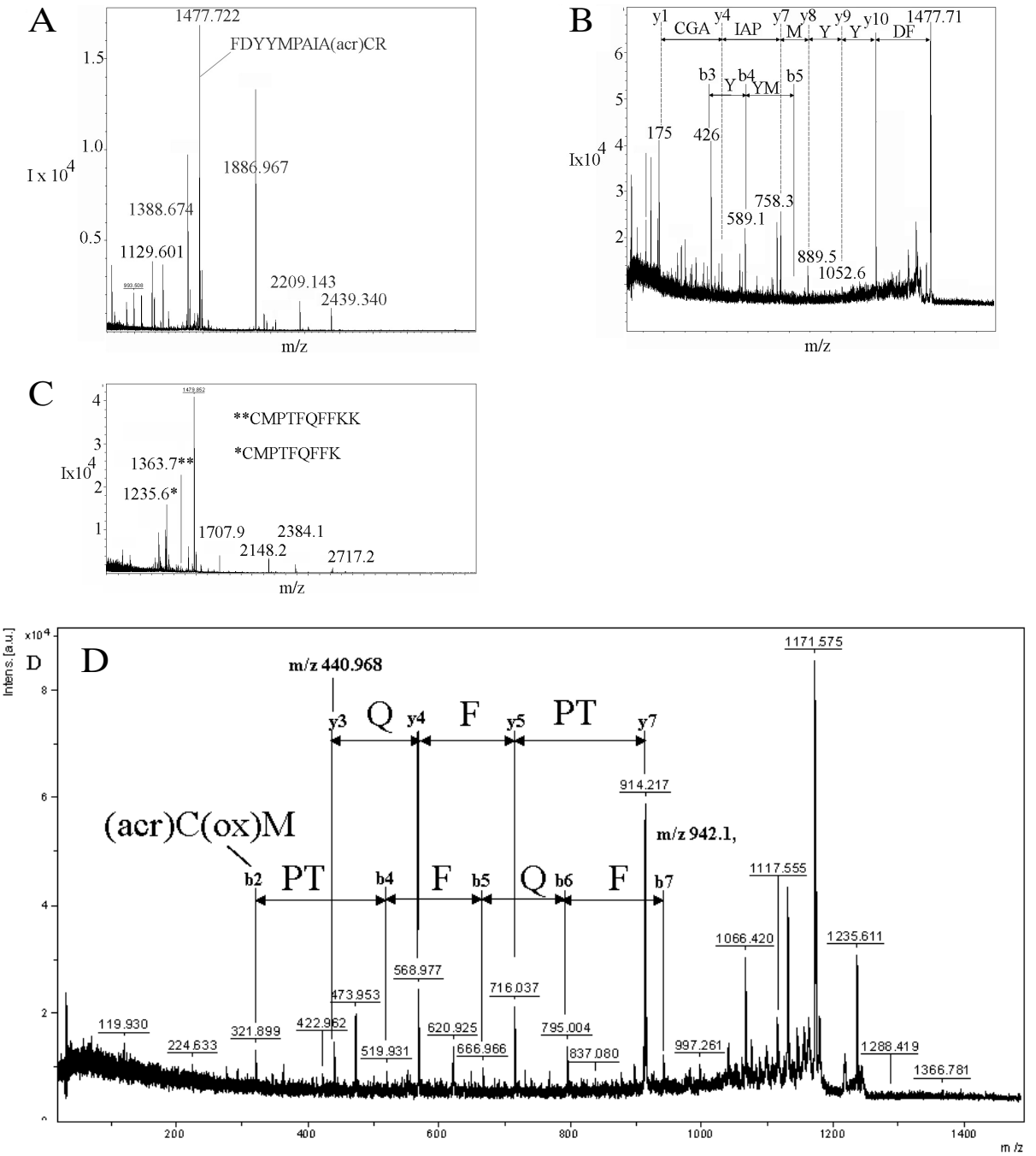


Fig. 3. (A) MALDI-TOF spectrum of adenosine deaminase and (B) PSD spectrum of the peptide *m/z* 1477.7. Adenosine deaminase (peptide tolerance 180 ppm, score 123) was identified by MALDI-TOF analysis. The spectrum contains a high abundant peptide (*m/z* 1477.7) which does not match to the sequence of the human adenosine deaminase. MASCOT database analysis shows that this peptide matches to the sequence FDYYMPAIA(acr)CR containing a propionamide modified cysteine. The peptide sequence was confirmed by PSD. (C) MALDI-TOF spectrum of TRX and (D) PSD spectrum of the peptide *m/z* 1235.6. The spectrum of TRX contains two potential alkylated peptides, *m/z* 1235.6 and *m/z* 1363.7 (labelled with asterisks). PSD fragmentation of the peptide *m/z* 1235.6 confirmed the predicted sequence of the acrylamide-modified peptide CMPTFQFFK.

(Wilson *et al*., 1991; Sharff *et al.,* 1992) and, therefore, we wondered whether alkylation of Cys-74 has an effect on ADA activity. In order to investigate if the enzymatic activity of ADA is reduced due to alkylation we treated commercial adenosine deaminase with different concentrations of acrylamide. Then we measured the activity of the enzyme with various concentrations of adenosine. However, no reduction of the ADA activity was detected.

## Discussion

Acrylamide is a well documented neurotoxin in humans and causes reproductive dysfunctions in animals. Acrylamide is also cytotoxic in cell-

Table II. Identified proteins and acrylamide-modified peptides. Proteins were identified after digestion with trypsin by MALDI-TOF/TOF. The MASCOT score refers to a peptide tolerance of 180 ppm and a significant score of 63 ($p < 0.05$) except * which were identified by a combination of TOF and PSD spectra (significant score 36). ** Sequences were confirmed by PSD; *** unconfirmed sequences; b, mixture of proteins; acr, acrylamide-modified cysteinyl.

| | Protein/peptide | MASCOT score | Accession number | Alkylated peptides |
|---|---|---|---|---|
| | *Jurkat cells* | | | |
| 1 | Ran-specific GTPase activating protein | 46* | 464544 | none |
| 2 | Stathmin 1 | 105 | 5031851 | none |
| 3 | B23 Nucleophosmin | 81 | 825671 | none |
| 4 | ATP synthase subunit d | 94 | 5453559 | none |
| 5 | PP protein | 77 | 33875891 | none |
| 6 | Rho GDP dissociation inhibitor | 146 | 10835002 | none |
| 7 | Adenosine deaminase | 144 | 113339 | FDYYMPAIAG(acr)CR ** FDYY(ox)MPAIAG(acr)CR *** |
| | *Caco-2 cells* | | | |
| 1 | Actin beta | 128 | | |
| 2 | Keratin 8 (cytokeratin) | 207 | 292059 | none |
| 2b | HSP75 | 207 | 4504919 | none |
| 3 | Chaperonin 60 | 179 | 31542947 | none |
| 4 | Tubulin beta 2 | 160 | 5174735 | none |
| 5 | Unidentified | – | – | – |
| 6 | Protein disulfide isomerase | 188 | 1085373 | none |
| 7 | Chaperonin 60 | 124 | 31542947 | none |
| 8 | Actin gamma chain | 140 | 71625 | none |
| 9 | Heat shock protein (HSPA8) | 83 | 13938297 | none |
| 10 | Tropomyosin 3 | 97 | 24119203 | none |
| 11 | Triosephosphate isomerase chain A | 254 | 999892 | none |
| 12 | Glutathione *S*-transferase | 65 | 2204207 | none |
| 13 | Thioredoxin peroxidase B | 85 | 9955007 | none |
| 14 | Unidentified | – | – | – |
| 15 | Tumour protein | 109 | 4507669 | none |
| 16 | Thioredoxin | 95 | 1827674 | (acr)CMPTFQFFk ** (acr)CMPTFQFFKK *** |
| | *HepG2 cells* | | | |
| 1 | Beta-5-tubulin | 106 | 18088719 | none |
| 2 | Prohibitin | 78 | 4505773 | none |
| 3 | Cathepsin D preproprotein | 165 | 4503143 | none |
| 4 | 6-Phosphogluconolactonase | 56* | 6912586 | none |
| 5 | Fatty acid binding protein 1 | 88 | 455757 | none |
| 6 | Superoxide dismutase (by similarity) | 57* | 2982080 | none |
| 7 | Stathmin 1 | 121 | 5031851 | none |
| 8 | Thioredoxin peroxidase | 236 | 9955007 | none |
| 8b | Cytidine monophosphate kinase | 236 | 12644008 | none |
| 9 | Chaperonin (HSP60) | 82 | 306890 | none |

cultured cells. Both, *in vivo* and *in vitro* toxicity is most likely the result of protein alkylation. In this paper we tried to identify proteins that are alkylated by acrylamide under cell culture conditions and might mediate the cytotoxicity of acrylamide. Therefore, we tested the toxicity of acrylamide in three well established human tumour cell cultures (HepG2, Jurkat and Caco-2 cells). Acrylamide showed dose-dependent cytotoxic effects in all three tested cultures. In contrast, the C1-C2 saturated analogue propionamide, a common negative control substance (Sickles *et al.*, 1996; Stone *et al.*, 1999), had no effect. The concentrations that caused serious cytotoxicity (2–4 mM) were comparable to those of other alkylating reagents tested in cultured human and rat hepatocytes (Boot, 1996). They were also similar to the $IC_{50}$ value of ifosfamide (a mustard alkylating agent) in rabbit proximal renal tubule cells (Springate *et al.*, 1999). Hence, our data confirms that acrylamide causes cytotoxicity effects in cell cultures and this cytotoxicity is most likely mediated by protein alkylation.

In order to identify alkylated proteins under cell culture conditions we extracted proteins from acrylamide-treated cells and analyzed them by 2D-gel electrophoresis and MALDI-TOF (Fig. 3). Using these techniques we identified two novel alkylated proteins. One alkylated protein was ADA in Jurkat cells (Table II). ADA degrades the nucleotide and messenger molecule adenosine to inactive inosine. In many cells ADA is associated with the receptor for adenosine, adenosine receptor (CD26), and there are strong evidences that ADA is involved in the regulation the CD26 via degradation of adenosine. Hence ADA is a negative regulator of ADA-induced release of acetylcholine in T-lymphocytes in motor neurons.

The mass spectrum of acrylamide-treated ADA showed a dominant peak that corresponds to the alkylated peptide FDYYMPAIACR (Fig. 3A). Fragmentation of this peptide confirmed the sequence and showed that Cys-74 is alkylated after acrylamide treatment (Fig. 3B). Cys-74, however, is not located in the active pocket of ADA (Wilson *et al.*, 1991; Sharff *et al.*, 1992) and it is unlikely that Cys-74 is necessary for complex formation with CD26 (Weihofen *et al.*, 2004). On the other hand, there are several evidences that modifications of cysteines affect the ADA activity. Lupidi *et al.* (1997) showed that the cysteine-modifying agent PCMBS reduces the ADA activity, and Arrendondo-Vega *et al.* (1998) isolated the ADA mutation G74C from cDNA of patients with severe and delayed onset combined immunodeficiency. The authors reported further that the mutation G74C shows reduced ADA activity *in vitro*. We did not observe any changes in the ADA activity after acrylamide treatment *in vitro*. The discrepancy between our data and those of Lupidi *et al.* (1997) might be due to the nature of the reagent: In contrast to propionamide, a small neutral irreversible modification, PCMBS is a relatively big, acidic compound that contains organic mercury. $Hg^{2+}$ is an inhibitor of ADA (Franco *et al.*, 1998), and traces of $Hg^{2+}$ in PCMBS might also contribute to the observed reduction in the ADA activity.

The other alkylation site is Cys-73 of the oxidoreductase TRX, found in Caco-2 cells (Table II). TRX Cys-73 facilitates the formation of enzymatically inactive homodimers (Gasdaska *et al.,* 1996; Weichsel *et al.*, 1996), and modification of Cys-73 affects the activity of TRX (Kirkpatrick *et al.*, 1998; Casagrande *et al.* 2002). Activated TRX promotes the proliferation of cancer cells, and recently it was shown that 2-imidazolyl disulfides, a class of anticancer agents, inhibit the growth of MCF-7 breast cancer cells by irreversible modification of Cys-73 (Kirkpatrick *et al.*, 1997, 1998). Alkylation of Cys-73 with acrylamide is also an irreversible modification and therefore most likely has similar effects. Therefore, TRX is a strong candidate for one of the proteins that mediate acrylamide-induced cytotoxicity.

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