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Heat shock protein 72 binds and protects dihydrofolate reductase against oxidative injury

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Abstract

Although heat shock protein Hsp72 confers resistance to oxidative injury, the mechanisms are unknown. These studies demonstrate that Hsp72 protects dihydrofolate reductase (DHFR) against injury caused by the thiol oxidant monochloramine (NH_2Cl). When exposed to NH_2Cl, DHFR catalytic activity is impaired and SDS–PAGE migration retarded. These may be blocked by prior addition of Hsp72 or the folate analog methotrexate. Methotrexate binding to DHFR is diminished by oxidant treatment, preventable by prior Hsp72 incubation. Hsp72 also protects DHFR in IEC-18 cells following oxidant exposure. Hsp72 co-immunoprecipitates with DHFR, especially after partial oxidation. The DHFR–Hsp72 interaction is modulated by cofactor/substrate binding for both Hsp72 (ATP) and DHFR (methotrexate). Thiol oxidation of DHFR increases susceptibility for tryptic proteolysis. Preincubation of DHFR with Hsp72 prevents the NH_2Cl-induced sensitivity to proteolysis. Thus, Hsp72 binds DHFR through enhanced protein–chaperone interactions upon oxidant exposure, a process that may protect against irreversible modification of DHFR catalytic and structural integrity.

Keywords: Heat shock proteins; Folic acid; Cimetidine; Methotrexate; Thiol oxidation; Proteolysis

The major inducible heat shock protein (Hsp) in many vertebrate cells is Hsp72, a protein that confers thermotolerance as well as resistance to a number of other forms of stress including oxidants [1–4]. With regard to the latter, the mechanisms underlying the protective effects of Hsp72 are not understood. In the inflammatory state, a variety of oxidants are physiologically produced in millimolar concentrations, including the cell permeant and long lived chloramine oxidants that are predominantly produced by activated neutrophils [5]. These cells produce large amounts of hypochlorous acid that react with amine groups of many molecules, including ammonia, resulting in the formation of the lipophilic monochloramine (NH_2Cl), a particularly damaging and physiologically important oxidant that rapidly denatures cellular proteins by thiol oxidation.

In the present study, we determined if the presence of Hsp72 mitigates the oxidative effects of monochloramine on the cytosolic protein, dihydrofolate reductase (DHFR). Dihydrofolate reductase (DHFR; EC 1.5.1.3) was selected for these studies because of its pivotal role as an essential cytosolic enzyme that catalyzes the formation of tetrahydrofolate (THF), a cofactor for many reactions requiring one carbon transfers such as amino acid and nucleotide biosynthesis and conversions (for review, see [6]). DHFR activity is particularly high in cells with a rapid turnover rate and its inactivation by the folate analog, methotrexate, can result in effective anti-cancer effects [6]. Methotrexate has a very high affinity for DHFR binding, competing for folate at its binding site [7].

DHFR has also been extensively studied as a model for how protein structure changes after exposure to denaturants such as acid, heat, and certain non-physiologic chemicals such as guanidine or urea [8–11]. Because a number of conformational states of DHFR have been characterized during its catalytic activity, a great deal is known about which regions of the protein form specific structural and functional domains. Recently, the interaction of native, as well as heat-denatured, DHFR

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with specific cellular protein chaperones, has been used to elucidate how the chaperone proteins operate. Although a number of protein chaperones may interact with DHFR, including heat shock protein 60 [12], Hsc (c for cognate, a non-heat inducible isoform) 70 [13], the greatest amount is known about the interaction with GroEL [14–17]. Vertebrate, but not prokaryotic, DHFR has a number of external loops which undergo conformational changes upon exposure to thermal stress. One loop, in particular, has been identified as a site of interaction between partially heat-denatured DHFR with GroEL [18], but other sites may exist as well.

Our studies show that Hsp72 and methotrexate (MTX) bind and protect DHFR under conditions of oxidant stress. Upon binding DHFR, Hsp72 prevents thiol oxidation of DHFR and retains its functions. Furthermore, it appears that MTX avidly displaces Hsp72 binding of DHFR, either through allosteric hindrance or direct competition at its binding site. We believe these actions provide important insights into the biochemical actions of Hsp72 in protecting cellular proteins against oxidative injury.

Materials and methods

Enzymes, antibodies, and other reagents. DHFR (chicken liver), dihydrofolic acid (DHF), tetrahydrofolic acid (THF), nicotinamide adenine dihydrophosphate (NADPH), cimetidine, *N*,*N*,*N*′,*N*′,-tetramethyl-*p*-phenyldiamine (TMPD), bovine serum albumin (BSA), *p*-hydroxymercuribenzoate (*p*-HMB), and *N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl)-ethylenediamine (IAENS) were purchased from Sigma Chemical (St. Louis, MO); chicken liver DHFR was also provided as a generous gift of Drs. J.M. Zhou and J. Li (Chinese Institute for Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China); IEC-18 were cells from American Type Culture Collection (Manasas, VA); Tet-Off Adenoviral System was from (Clontech, Palo Alto, CA); methotrexate (MTX) was from Lederle (Pearl River, NY); C92 monoclonal antibody was from Stressgen (Victoria, British Columbia, Canada), ADP and ATP were from Pharmacia (Piscataway, NJ), and rabbit anti-mouse IgG was from Zymed (South San Francisco, CA). Hsp72 was produced in BL21DE3 *Escherichia coli* (Novagen, Madison, WI) using the full length human Hsp72 cloned into pET-11 (a gift from R.I. Morimoto, Northwestern University, Evanston, IL). The purification was performed by the ATP-affinity method previously described [19]. Monochloramine was prepared freshly on the day of use by the NaOCl-induced oxidation of ammonium chloride and quantified by absorbance at 242 nm.

Measurement of DHFR activity. DHFR reduces DHF to THF in the presence of NADPH. Activity was assayed spectrophotometrically [20] by monitoring the decrease in absorbance (at 339 nm) caused by the DHFR-induced decrease in DHF and NADPH to form THF and $NADP^+$. All experiments were carried out using 0.05 U (5 μg) DHFR, 2 mM DHF, and 0.5 mM NADPH. Oxidant injury to the enzyme was achieved by DHFR exposure to NH_2Cl (0.25–3 mM) for 30 min at 37 °C, prior to the assay. To investigate the protective role of Hsp72, DHFR was incubated with 5 μg Hsp72 prior to oxidant exposure. Experiments involving NH_2Cl exposure were also conducted in the presence of cimetidine, an oxidant radical scavenger [21], to confirm that the activity changes observed were due to action of the oxidant on DHFR and eliminate the possibility of NH_2Cl acting as an interfering agent in the reduction assay.

To assess the extent of spontaneous changes in DHF and/or NADPH, control experiments included conducting the assay in the absence of DHFR, DHF or NADPH. There was no significant change in absorbance under these conditions. To exclude the possibility of NaOCl interfering with the assay, the mixture was exposed to a dilution of NaOCl equivalent to that observed with the highest concentration of NH_2Cl. The assay was also conducted in the presence of BSA replacing Hsp72 to assess the specificity of protection of DHFR activity.

Neutralization of oxidant activity using cimetidine. Autooxidation of the molecule TMPD in neutral solution (KH_2PO_4, 50 mM, pH 7.5, 1 mM TMPD) was monitored by measuring the change in absorbance at 610 nm over 5 min. NH_2Cl was added to increase the rate of oxidation and cimetidine was added to inhibit this oxidant-induced increase in TMPD oxidation. This control was performed to confirm that all NH_2Cl used to oxidize DHFR would be inactivated prior to addition to the assay. Stock solutions of TMPD and cimetidine were made up in ethanol on the day of use.

Determination of [^{3}H]MTX binding. The effects of NH_2Cl injury on DHFR were also investigated by a radioligand binding assay using [^{3}H]MTX [22]. The assay mixture contained NADPH (100 nM), [^{3}H]MTX (20 nM) with or without unlabeled MTX (20 μM) in potassium phosphate, 150 mM (pH 6.2). DHFR (20 μg) was added to the assay mixture and allowed to bind for 10 min at 37 °C prior to spinning through columns containing Sephadex G25. The Sephadex beads facilitate separation of protein bound to substrate (in eluant) from unbound substrate (remaining in column). Radioactivity in the eluant was counted by liquid scintillation and counts from samples containing unlabeled MTX and [^{3}H]MTX (non-specific) were then subtracted from those containing solely [^{3}H]MTX to indicate specific binding. In some experiments, DHFR was exposed to NH_2Cl (0.25 mM) before or after exposure to MTX to assess the effect of the oxidant on unbound or bound substrate states, respectively. Hsp72 protection was analyzed by preincubating DHFR (20 μg) with Hsp72 (20 μg at 37 °C for 10 min) prior to or after NH_2Cl treatment.

To determine the effect of Hsp72 and NH_2Cl on cellular [^{3}H]MTX binding, IEC-18 cells were grown in Dulbecco's modified Eagle's medium with 5% fetal bovine serum and 0.1 U/ml insulin. When 30–40% confluent, cells were infected with Hsp72 in the Tet-Off Adenoviral system along with another virus containing the tet operator protein. Tetracycline was included in the medium for two days and then removed while feeding from half the dishes. Tetracycline removal inactivates the tet-repressor protein, resulting in transcription of the gene due to a min-CMV promoter in this construct. Cells were allowed to grow for two more days with or without tetracycline and then challenged with 0.3 mM NH_2Cl as appropriate along with uninfected IEC-18 cells. Cells were treated with NH_2Cl for 30 min and then harvested by scraping in ice-cold PBS. Cells were pelleted (14,000*g* for 20 s at room temp) and cell pellets were resuspended in 50 μl lysis buffer (10 mM Tris, pH 7.4, 1 mM EDTA, with protease and phosphatase inhibitors as described previously). Cell pellets were homogenized gently in a 2 ml Teflon pestle homogenizer and snap-frozen and snap-thawed two cycles using a dry-ice alcohol bath and 37 °C water bath. Cytosolic extracts were isolated by centrifugation (100,000*g* for 20 min at 4 °C). Protein in the supernatants was measured and 100 μg was diluted to 20 μl for the [^{3}H]MTX binding assay. Binding reactions were performed in the cytosolic extracts as described for purified enzyme.

Immunoprecipitation of Hsp72 and DHFR. To determine potential binding between Hsp72 and DHFR, co-immunoprecipitation studies were performed with a specific monoclonal anti-Hsp72 (C92) antibody. DHFR (1 μg) was incubated with or without Hsp72 (1 μg) in the immunoprecipitation (IP) buffer (NaCl, 150 mM; Tris, pH 7.4, 10 mM; and PMSF, 1 mM) for 120 min at room temperature in a final volume of 50 μl. To examine the effect of oxidant, DHFR was exposed to NH_2Cl (0.25 mM) in the presence or absence of Hsp72, prior to immunoprecipitation. To analyze the effects of substrates on DFHR–Hsp72 interaction, in some experiments nucleotides or methotrexate was added to the incubation mixture before Hsp72.

To immunoprecipitate, C92 antibody was added (1 μg) and mixtures were incubated on a rotator wheel for 180 min at room temperature. Five micrograms of rabbit IgG was added and incubated for an additional 180 min. Immunoprecipitation was achieved by adding 50 μl of 10% (v/v) protein A-bearing *Staphylococcus aureus* cells and rotating for 180 min. Cells were pelleted by centrifugation (10,000*g* for 2 min at room temperature) and washed three times with wash buffer (NaCl, 150 mM; Tris, pH 7.4, 10 mM; and 0.1% v/v Triton X-100). Samples were eluted by boiling in Laemmli stop solution (10% v/v 2-mercaptoethanol, 0.01% w/v bromophenol blue, 25% w/v glycerol, 10% w/v SDS, and 50 mM Tris, pH 6.8). Insoluble material was pelleted (14,000*g* for 5 min) and sample supernates were analyzed by SDS–PAGE.

Tryptic proteolysis of DHFR. One microgram of DHFR was reacted with 250 μM *para*-hydroxymercuribenzoate (*p*-HMB), 1 mM *N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl) ethylenediamine (IAENS), or 1 mM NH_2Cl for 5 min at 37 °C. 100 U of trypsin (Worthington Enzymes) was added and the reactions were allowed to proceed for an additional 10 min. Reactions were stopped by addition of one half volume 3× Laemmli stop solution and heated to 65 °C for 10 min. In certain cases, 1 μg of purified Hsp72 was added to the DHFR prior to the reaction with NH_2Cl for 10 min at 37 °C. Samples were resolved on 12.5% Tris–Tricine PAGE gels, stained with Coomassie blue, and destained to visualize.

Results

Effect of NH_2Cl on DHFR activity and protection by Hsp72

DHFR activity, indicated by a decrease in absorbance, occurs rapidly upon addition of substrates and cofactors (Fig. 1A). Upon treatment with NH_2Cl, there was a dramatic and rapid loss of DHFR activity, occurring in a concentration-dependent manner. When the enzyme is allowed to interact with Hsp72 immediately prior to exposure to NH_2Cl (in a ratio of approximately 1:1, DHFR:Hsp72), the activity is partially restored (Fig. 1B). This is most evident at a NH_2Cl concentration of 0.3 mM where DHFR activity is completely abolished in the absence of Hsp72, but significantly preserved in its presence. However, high concentrations (greater than 1 mM NH_2Cl) abolish DHFR activity in the presence or the absence of Hsp72. Treatment with an approximately equal molar amount of BSA does not protect DHFR activity (Fig. 1B), suggesting that the protective effect is specific to Hsp72.

To ensure that the changes in DHFR activity are not a result of an effect of the oxidant on the assay substrates DHF and NADPH, cimetidine, a potent antioxidant [21], was used to quench oxidant radicals produced by NH_2Cl after oxidizing the protein but prior to the DHFR reaction. The oxidant indicator TMPD was used to assess the oxidative potential of the reaction solution. In the presence of 0.3 mM NH_2Cl, a large change in absorbance is observed (Fig. 2—*Y* axis indicates the change in absorbance). In the presence of the anti-oxidant, cimetidine, a dose-related decrease in oxidant-induced changes in TMPD absorbance was ob-

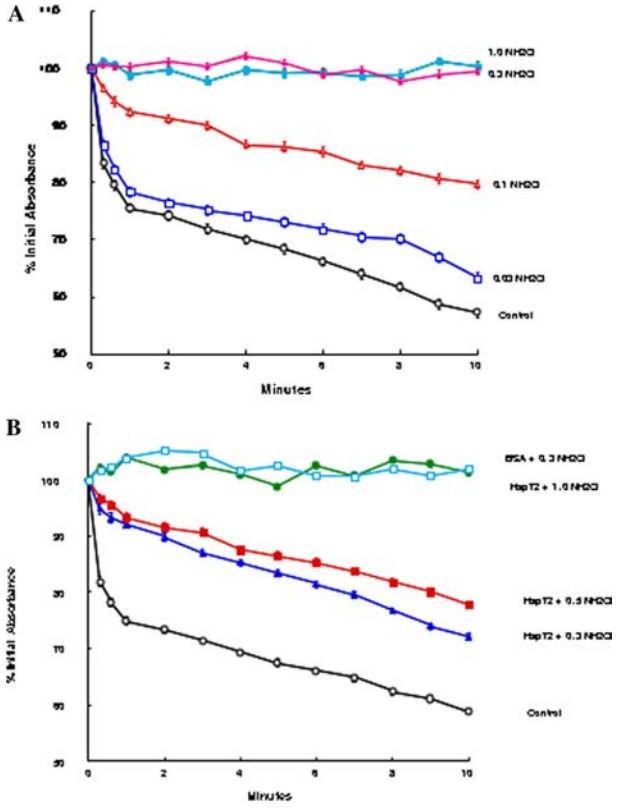

Fig. 1. Effect of NH_2Cl on DHFR activity and protective effect of Hsp72. DHFR activity was measured as described in Materials and methods by the decrease in $A_{339\,nm}$. (A) NH_2Cl at concentrations of 0.03–3 mM inhibits DHFR activity. (B) Prior incubation of DHFR with Hsp72 (5 μg), but not BSA (5 μg), prevents NH_2Cl inhibition of activity. Data shown are means ± SE for four experiments.

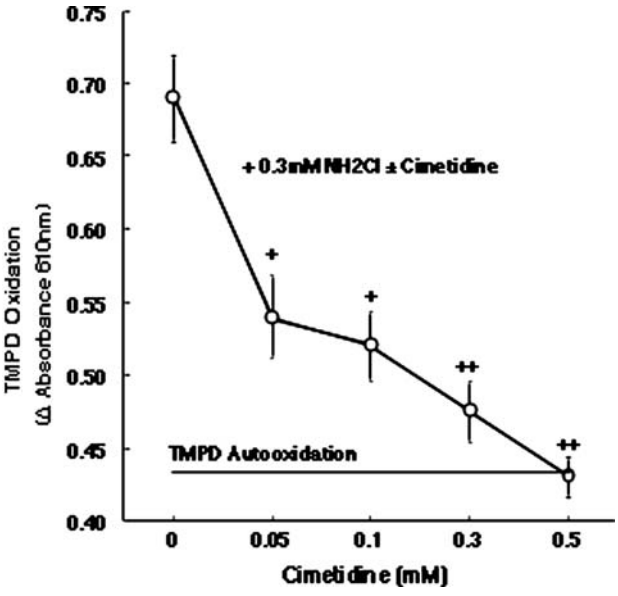

Fig. 2. Effect of cimetidine on TMPD oxidation. The oxidative indicator TMPD was used to determine the best concentration of cimetidine needed to quench the oxidative effects of NH_2Cl. Oxidation of TMPD was measured 5 min after exposure to NH_2Cl by absorbance at 610 nm. The dashed line indicates the rate of spontaneous autooxidation of TMPD. Data shown are means ± SE for three experiments.

served. It should be noted that this is not a quantitative assay, as TMPD undergoes rapid autooxidation (demarcated by the dashed line). Thus, the apparent neutralization of NH_2Cl at 0.5 mM cimetidine is likely an overestimation, as the TPMD is already irreversibly auto-oxidized within the time frame of the analysis (5 min). At this point, it is no longer indicative of the oxidative potential of the solution. Nevertheless, the data confirm the anti-oxidative properties of cimetidine which have been previously reported.

Cimetidine pretreatment was used to inhibit the ability of NH_2Cl to inhibit DHFR. Cimetidine addition (0.5 mM) to the assay did not inhibit the activity of DHFR (Fig. 3). Cimetidine addition (0.5 mM) to NH_2Cl (0.25 mM) prior to the assay (for 5 min) significantly reversed the NH_2Cl-induced inactivation of DHFR (Fig. 2). Therefore, the oxidizing potential of NH_2Cl appears to be central to its ability to inactivate DHFR.

Oxidants may cause modification of protein structure which can be observed as changes in electrophoretic mobility, even after denaturation by SDS. To determine if this occurred for DHFR, untreated and NH_2Cl-oxidized DHFR were run on 12.5% SDS–PAGE. As can be seen in Fig. 4, oxidation of DHFR resulted in a slightly slower migration of the protein (lane 2). When the NH_2Cl was inactivated with cimetidine prior to addition to DHFR this change in mobility did not occur (lane 3). Addition of Hsp72 prior to reaction of DHFR with NH_2Cl inhibited the conversion to the oxidized, more slowly migrating form (lane 5). When the Hsp72 was added after the reaction of the DHFR with NH_2Cl for 5 min, much less protection from oxidation was observed (lane 6).

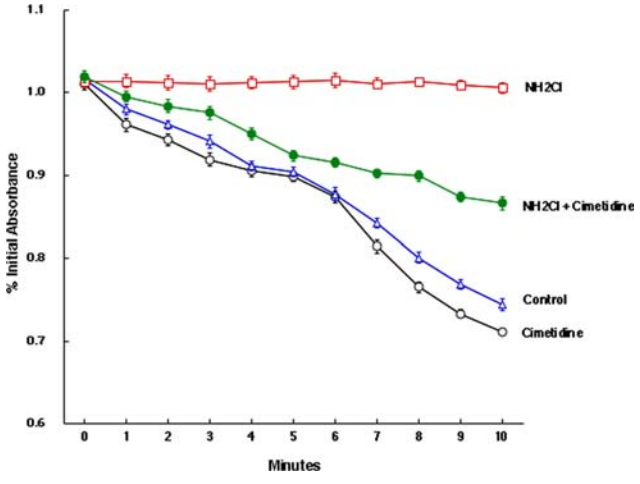

Fig. 3. NH_2Cl-mediated decrease in DHFR activity is blocked by addition of an anti-oxidant. DHFR activity was measured as described in Materials and methods with or without NH_2Cl pretreament (0.25 mM, 30 min). Cimetidine (0.5 mM) was added 10 min prior to NH_2Cl or by itself. Data shown are means ± SE for three experiments.

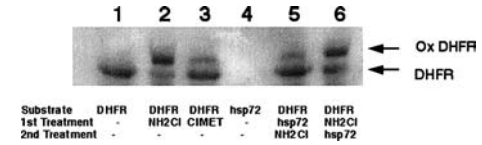

Fig. 4. Effect of NH_2Cl on electrophoretic mobility of DHFR and protection by Hsp72. Samples of DHFR (1 μg), either untreated (lane 1), oxidized with NH_2Cl (lane 2), incubated with NH_2Cl treated with cimetidine (lane 3), treated with Hsp72 prior to addition of NH_2Cl (lane 5), or treated with NH_2Cl for 5 min prior to addition of Hsp72 (lane 6), were resolved on 12.5% SDS–PAGE, stained with Coomassie, and destained. Lane 4 has Hsp72 alone. Image shown is representative of those of three separate determinations.

NH_2Cl causes increased access of DHFR sulfhydryl groups

Thiol modification of DHFR has been previously shown to increase the ability of trypsin to proteolytically digest the enzyme [23]. Because thiol groups are vulnerable to oxidation, we determined if NH_2Cl modified DHFR in a similar fashion. DHFR was treated with the thiol modifiers *p*-HMB, IAENS, or NH_2Cl prior to reaction with trypsin [23]. Untreated DHFR was resistant to tryptic proteolysis over 10 min. However, thiol modification of DHFR by *p*-HMB and to a lesser extent IAENS and NH_2Cl facilitated tryptic proteolysis (Fig. 5A). Proteolysis was characterized by variable formation of both smaller fragments (arrows) as well as large aggregates. The latter may have been caused by proteolytic fragments which were not sufficiently denatured prior to

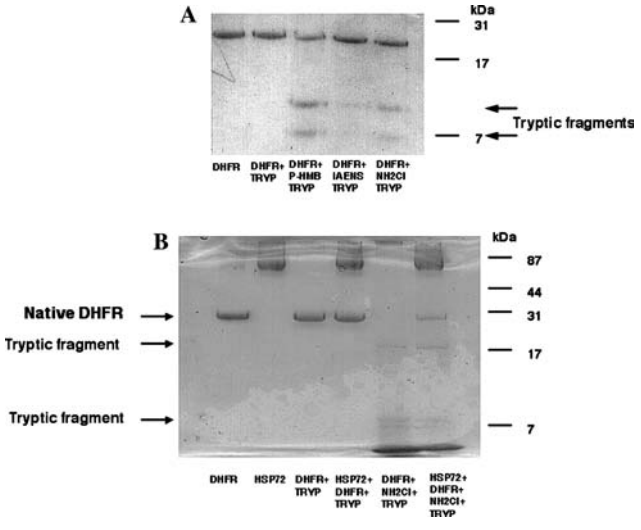

Fig. 5. Hsp72 prevents NH_2Cl-induced sensitivity to tryptic proteolysis. (A) Chicken liver DHFR was treated with trypsin either with or without thiol modification by *para*-hydroxy-mercuribenzoic acid (*p*-HMB), *N*-iodoacetyl-*N*′-(5-sulfo-1-naphthyl) ethylenediamine (IAENS), or NH_2Cl. Some of the DHFR degradative products are denoted by arrows. (B) For protection with Hsp72, Hsp72 was added to DHFR before addition of NH_2Cl. The order of addition of reagents occurred in the order shown. Image shown is representative of those of three separate determinations.

gel analysis (see Fig. 5B, lane 5 in particular). Preincubation of DHFR with Hsp72 prior to addition of NH_2Cl prevented the tryptic sensitization, similar to its effects on protection of activity (Fig. 5B, lane 6).

MTX and Hsp72 binding with DHFR protects it against NH_2Cl stress

Binding of MTX was tested as another characteristic of DHFR that might be altered by NH_2Cl-induced oxidation. MTX is a folate analog that binds the DHF binding site with high affinity. Because this affects the ability of proteases to degrade DHFR [24], a conformation change may occur. As shown in Fig. 6, NH_2Cl causes a significant decrease in DHFR–MTX binding when added prior to, but not after, addition of radiolabeled ligand (columns 2 and 3, respectively). Hsp72 addition, either after (column 4) or before (column 5) addition of radiolabel, does not affect DHFR–MTX binding. However, when DHFR is preincubated with Hsp72, the effect of subsequently added NH_2Cl is largely blocked, suggesting a protective effect of Hsp72 (column 6). One possible explanation for these findings is that oxidant injury may be directed at certain amino acids in the substrate pocket of DHFR which is protected by MTX and Hsp72. Alternatively, MTX or Hsp72 binding causes a conformational change which limits oxidation of functionally relevant amino acids by NH_2Cl.

To determine if oxidants affected cellular DHFR and whether Hsp72 could mitigate the damage, [^{3}H]MTX binding was used (a more sensitive measure than activity measurements). A normal diploid cell line from rat small intestine, IEC-18, was used. To specifically induce Hsp72, IEC-18 cells were infected with human Hsp72 cDNA cloned into the Tet-Off adenoviral system. Treatment with NH_2Cl decreased the amount of cytosolic [^{3}H]MTX binding (Fig. 7). Infection with Tet-Off Hsp72 adenovirus did not alter the amount of cytosolic [^{3}H]MTX binding either in the presence or absence of tetracycline. Omission of tetracycline two days before oxidant stress stimulated the induction of Hsp72 (Fig. 7, lower panel). This treatment prevented the NH_2Cl-induced decrease in [^{3}H]MTX binding. The adenoviral system used has the advantage that non-induced (tetracycline present) cells are appropriate controls as cells are treated identically.

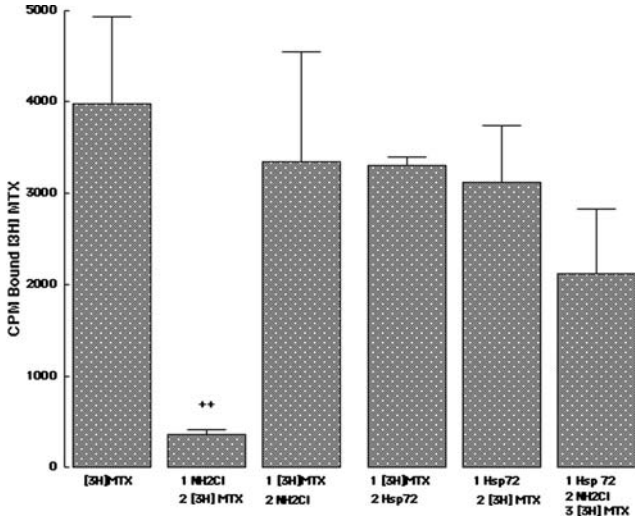

Fig. 6. Effect of NH_2Cl and Hsp72 on binding of [^{3}H]methotrexate (MTX) on DHFR. Binding assays were performed as described in Materials and methods. Binding presented is the specific binding of MTX, therefore it equals the difference in cpm [^{3}H]MTX bound per 20 μg DHFR in the presence and absence of 20 μM unlabeled methotrexate. The bottom of each column represents the order of addition of reagents to the assay. Values shown are means ± SE for three experiments.

Binding characteristics of Hsp72–DHFR binding

To determine if Hsp72 directly binds with DHFR, Hsp72 immunoprecipitations were performed from samples where DHFR was exposed to NH_2Cl, Hsp72, or a combination of Hsp72 and NH_2Cl. As shown in Fig. 8, no DHFR was immunoprecipitated by anti-Hsp72 antibody from samples containing only DHFR or Hsp72

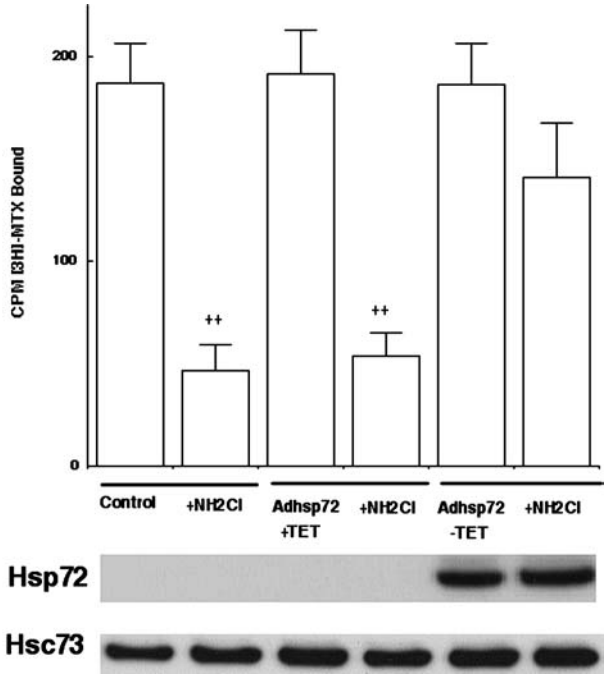

Fig. 7. Effect of adenoviral-mediated increased expression of Hsp72 on oxidant-induced changes in cellular [^{3}H]MTX binding. IEC-18 cells were infected with both Hsp72 in the Tet-Off adenovirus along with adenovirus containing the tet operator protein (last four lanes). After two days, tetracycline was removed to increase Hsp72 expression and, two days later, cells were challenged with 0.3 mM NH_2Cl for 30 min. Cellular cytosolic extracts and [^{3}H]MTX binding were performed as described in Materials and methods. Data shown are means ± SE for three experiments. $^{++}p < 0.05$ compared with control [^{3}H]MTX binding by analysis of variance.

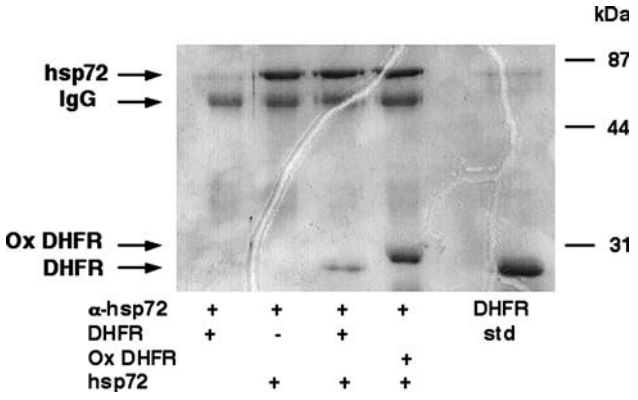

Fig. 8. Effect of oxidation on interaction of DHFR with Hsp72. DHFR, oxidized with NH_2Cl or not, was added to Hsp72 and co-immunoprecipitated using a specific anti-Hsp72 antibody. With only the antibodies and DHFR (lane 1) or Hsp72 (lane 2), no DHFR is detected. The co-addition of non-oxidized DHFR and Hsp72 results in some DHFR immunoprecipitating whereas a greater amount of DHFR co-immunoprecipitates with Hsp72 after the DHFR is oxidized (lane 4). The amount of DHFR used in each immunoprecipiation is shown as the purified DHFR standard in lane 5. The gel shown is representative of results of three separate immunoprecipitations.

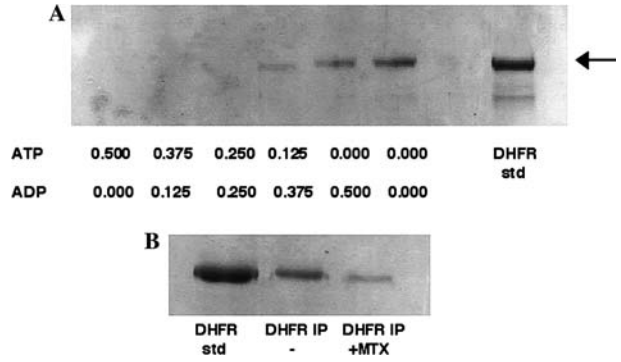

Fig. 9. (A) Effect of ATP on co-immunoprecipitation of DHFR and Hsp72. DHFR (arrow) was co-immunoprecipitated with Hsp72 as described in Materials and methods. Nucleotides (ATP or ADP) were added to the immunoprecipitation mixture prior to the addition of DHFR. The amount of DHFR used in each immunoprecipiation is shown as the purified DHFR standard in lane 5. The gel shown is representative of results of three separate immunoprecipitations. (B) Effect of methotrexate on co-immunoprecipitation of DHFR and Hsp72. DHFR and Hsp72 were co-immunoprecipitated with or without the addition of methotrexate (100 nM). Methotrextate was added to the DHFR prior to addition to the immunoprecipitation mixture. The amount of DHFR used in the reaction (DHFR std) is shown. The gel shown is representative of results of three separate immunoprecipitations.

(lanes 1 and 2, respectively). A small amount of DHFR was co-immunoprecipitated from a solution containing both DHFR and Hsp72 (lane 3). A greater amount of DHFR co-immunoprecipitated after the mixture was exposed to 0.25 mM NH_2Cl for 10 min (lane 4). As shown by the arrow, the slower gel mobility of the DHFR band in lane 4 is consistent with its oxidized state.

To test the nature of this association further, we examined the biochemical effect of substrates known to bind with Hsp72 or DHFR. Hsp72 has ATPase activity which regulates its interacting abilities with other proteins. In the absence of ADP or ATP, a small amount of DHFR co-immunoprecipitates with Hsp72, an interaction that is largely unaffected by the presence of 0.5 mM ADP alone. However, as the ATP/ADP ratio was increased, progressively less DHFR co-immunoprecipitated with Hsp72. Addition of ATP (0.5 mM) abolished the interaction between DHFR and Hsp72 (Fig. 9A).

Next, we examined the effects of MTX binding on Hsp72–DHFR interactions. The effects of methotrexate binding on Hsp72–DHFR binding MTX (at 100 nM which is 5× its K_d of 20 nM) also prevented the interaction of DHFR with Hsp72 (Fig. 9B). Thus, the substrates of both proteins seem to cause biochemical alterations that result in dissociation of the Hsp72–DHFR complex.

Discussion

In this study, we show that DHFR, a pivotal enzyme required for many metabolic pathways, is readily oxidized and denatured by the physiologically relevant reactive oxygen species, NH_2Cl, an effect that can be blocked by the presence of Hsp72. Hsp72 protection of DHFR is specific, as it is not seen with an equal molar amount of BSA. The nature of protection appears to be an interaction between DHFR and Hsp72, as these two protein co-immunoprecipitate. This interaction can also be modulated by the inclusion of factors which bind either the enzyme or chaperone.

Our studies show that MTX, by itself, may prevent NH_2Cl injury to DHFR, possibly by preventing exposure of oxidant-sensitive amino acid in the folate binding pocket of DHFR. A complex interaction of Hsp72, DHFR, and MTX may occur since MTX disrupts the DHFR/Hsp72 interaction. At least two hypotheses may explain this finding: (1) MTX causes an allosteric change in DHFR, disabling its association with Hsp72, or (2) MTX and Hsp72 share a common binding site on DHFR, but MTX by way of a higher affinity is able to preferentially bind to DHFR and displace Hsp72.

The dependence of the interaction on nucleotides brings further insights into the association of Hsp72 with DHFR in that it is consistent with previous data that show ATP affects substrate release by chaperones [25,26]. In the absence or low concentrations of ATP, Hsp72–DHFR binding is enhanced. This situation is analogous to the binding of DHFR to the Hsp72 prokaryote counterpart, GroEL, where the interactions of these two proteins are also modulated by ATP. This may be mediated through nucleotide-altered protein configuration of Hsp72, affecting its affinity to DHFR. This is an important finding because in cells under stress, changes

in cell ATP levels may trigger Hsp72 binding to partially denatured, but still functional, proteins, affording them protection against further denaturation or unfolding. Some insights into the nature of this interaction may be gained through data showing human and murine, but not *E. coli*, DHFR bind GroEL. Murine and human DHFR differ from prokaryotic DHFR by three distinct external loops that are not present in the prokaryotic form [15]. Changes in protein structure, either through substrate binding or oxidation of remote functionally unimportant and peripheral sites, could in theory affect the affinity of DHFR and Hsp72 at these sites.

Thermal injury has been established as a stimulus for chaperone-mediated binding and rescue of protein structure, including DHFR [10]. However, our study indicates that chemical reactions may also cause a similar response. We show that a physiologically relevant oxidant, monochloramine, might also stimulate chaperone-mediated rescue of protein structure and function. Monochloramine appears to react with DHFR in a similar fashion to other thiol oxidants. Maintenance of disulfides has been proposed to be important to maintain DHFR in the correct conformation [23]. Treatment of DHFR with either NH_2Cl or the well-characterized thiol oxidant *para*-hydroxy-mercuribenzoate acid partially unfolds the molecule, allowing trypsin access to previously inaccessible sites. The present results are novel in that they demonstrate the ability of heat shock proteins to prevent oxidant damage in addition to their well-characterized abilities to prevent thermal damage.

In conclusion, oxidants are a major class of effector molecules produced by inflammation that cause irreversible covalent alterations and denaturation of vital cellular proteins such as DHFR. We show here that Hsp72 binding of partially oxidized DHFR prevents further oxidant-mediated covalent modification of functional sites and denaturation of the protein, probably by blocking access and oxidation of critical amino acids located within more internal hydrophobic regions. We believe these are previously unrecognized mechanisms of action important in retaining cellular functions and enhancing cell survival in the context of oxidant-induced stress.

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