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Enhanced hydrogen peroxide sensitivity and altered stress protein expression in iron-starved *Mycobacterium smegmatis*

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***Mycobactericum smegmatis* ATCC 607 became iron starved and did not reach maximum population density when grown at an iron concentration of 0.1 μM, or less. Iron deficient cells were more susceptible than iron replete cells to H_2O_2 killing; 9 mM H_2O_2 killed about 80% of the population of cultures grown at 0.05 μM iron, while about 25 mM H_2O_2 was required for similar killing of cultures grown at 1 or 20 μM iron. In response to H_2O_2, iron sufficient cells produced major oxidative stress proteins of molecular masses of 90, 75, 65, 62, and 43 kDa (the 75 and 65 kDa proteins were identified as DnaK and GroEL homologs, respectively). Iron deficient *M. smegmatis* did not upregulate the DnaK and GroEL proteins when stressed with H_2O_2. Both iron deficient and iron sufficient *M. smegmatis* produced (at 48°C) major heat shock proteins of molecular masses of 90, 75 (DnaK), 65 (GroEL), 62, 43, and 16 kDa. The stress protein response induced by 2 M ethanol challenge was similar to the heat shock response except that ethanol induced a unique 55 kDa protein and the 16 kDa heat shock protein was not apparent. Induction of ethanol stress proteins was identical in high iron and low iron cells. All of the stress agents induced expression of a 62 kDa protein which may also be induced by iron insufficiency. The heat and ethanol shock responses of *M. smegmatis* were unchanged by iron deficiency; therefore, the absence of DnaK and GroEL from the response of iron starved *M. smegmatis* to H_2O_2 may be due to a specific defect (or alteration) of the oxidative stress response during iron starvation.**

Keywords: DnaK, GroEL, heat shock, iron, mycobacteria, oxy-radicals, peroxide, stress

Introduction

The oxy-radicals peroxide and superoxide (O_2^-) are by-products of aerobic metabolism (Keyer *et al.* 1995) and from these free radicals a powerful and highly toxic oxidant, the hydroxyl radical ($OH^\bullet$), can be formed (McCord & Day 1978). Certain metals have crucial catalytic roles in oxy-radical toxicity and the concentrations of these metals may determine the extent of oxidative damage as well as the ability of an organism to cope with the damage. Iron can have a pivotal dual role both as a protector and a destroyer. By transfer of an electron from Fe^{2+} to H_2O_2 (the Fenton reaction), iron mediates the formation of the destructive $OH^\bullet$ radical (McCord & Day 1978). The catalytic properties of iron are also employed protectively to form catalases, peroxidases and the iron-containing superoxide dismutases, all of which are enzymes that destroy oxy-radicals (Fridovich 1978).

Surplus iron has been implicated in various human diseases (Weinberg 1978, 1984, Byers 1987) and studies in bacteria have been useful in defining the cellular and molecular processes that are damaged by excess iron. In *Escherichia coli*, iron-generated $OH^\bullet$ radicals were implicated in DNA damage, which was suggested as one of the mechanisms of H_2O_2 induced lethal injury (Imlay *et al.* 1988, Keyer

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et al. 1995). Iron-laden bacteria are more likely to be injured by H_2O_2. Growth of *Staphylococcus aureus* at an elevated iron concentration yielded cells that were more susceptible to H_2O_2 killing (Repine *et al.* 1981, Hoepelman *et al.* 1990). An *E. coli* strain with deregulated iron assimilation (*fur* gene mutation) became iron overloaded and showed increased sensitivity to oxidative damage (Touati *et al.* 1995). Lowering of the intracellular iron level with a cell permeable iron chelating agent eliminated oxidative sensitivity of the *fur* mutant; $OH^{\bullet}$ radical scavengers also provided protection (Touati *et al.* 1995).

While excess iron may inflate injury from oxy-radicals, an insufficient iron level may also enhance oxidative damage by depressing the activities of the iron-containing protective enzymes. The need for iron to combat oxy-radicals might be apparent in some facultative microorganisms as an increased nutritional requirement for iron when switched from anaerobic to aerobic cultivation. *Streptococcus mutans* required an iron supplement for aerobic (but not for anaerobic) growth in a chemically defined medium that was treated to lower trace metal contamination (Martin *et al.* 1984). Manganese could substitute for iron in supporting aerobic growth of *S. mutans*; subsequently, it was shown that *S. mutans* has a cambialistic superoxide dismutase enzyme that is active with either iron or manganese as a cofactor, which may account for the functional exchange of iron and manganese (Martin *et al.* 1986).

Environmental pressures such as peroxides, other oxidative agents, heat shock, and ethanol trigger the syntheses of stress proteins. Some of these proteins (appropriately called molecular chaperons) process partially folded proteins and target them for secretion, proteolysis, or refolding as well as aid in assembly and disassembly of complex protein structures (Ellis & van der Vies 1991). In *E. coli*, exposure to H_2O_2 led to increased production of at least nine proteins, including catalase (KatG) and alkyl hydroperoxide reductase; the response to H_2O_2 was mediated by the OxyR protein, the product of the *oxyR* gene (Farr & Kogoma 1991). When exposed to H_2O_2, the saprophytic *M. smegmatis* displayed a response resembling the OxyR-initiated response of *E. coli* (Sherman *et al.* 1995). In the pathogenic species *Mycobacterium tuberculosis* and *Mycobacterium avium*, the response to H_2O_2 was limited to induction of a single protein, KatG (Sherman *et al.* 1995). On the other hand, in *M. tuberculosis* the O_2^- radical activated expression of some of the well-known heat shock proteins (Hsp) (Garbe *et al.* 1996). Heat shock of *M. tuberculosis* elevated synthesis of three major Hsp, identified as DnaK, GroEL, and GroES, as well as other Hsp of molecular masses of 90, 28, 20, and 15 kDa (Young & Garbe 1991). Although the heat shock response of *M. smegmatis* has not been fully described, synthesis of the 65 kDa Hsp (GroEL) has been noted in several mycobacterial species, including *M. smegmatis* (Shinnick *et al.* 1988).

Iron restricted growth of many bacteria prompts synthesis of the iron chelating siderophores and of several proteins, especially those involved in siderophore synthesis and in uptake of ferrisiderophores (Byers 1987). Iron starvation of *M. smegmatis* upregulated production of several envelope proteins and of two structurally different siderophore-like molecules called mycobactin and exochelin (Hall *et al.* 1987, Sharman *et al.* 1995). Iron regulated *M. smegmatis* exochelin synthesis and transport genes have been cloned and sequenced (Fiss *et al.* 1994). Sequences resembling the consensus binding site for the global iron dependent regulatory protein (DtxR) of *Corynebacterium diphtheriae* were identified upstream of the *M. smegmatis* exochelin genes (Fiss *et al.* 1994). A homolog of DtxR occurs in *M. tuberculosis* (Schmitt *et al.* 1995), suggesting that some of the effects of iron starvation noted in *M. smegmatis* may be mediated by an iron dependent global regulator related to the corynebacterial iron responsive repressor protein.

The present studies revealed that iron starved *M. smegmatis* were more susceptible than iron sufficient cells to H_2O_2 killing; such cells were also unable to synthesize the full complement of stress proteins triggered by oxidative challenge of iron replete cells. This difference (or defect) in iron deficient cells was specific for the oxidative stress response because the responses of iron limited cells to heat and ethanol shocks were essentially identical to those of iron sufficient cells.

Materials and methods

Microorganism

M. smegmatis ATCC 607, obtained from the American Type Culture Collection, was used for this research. It was routinely cultivated on tryptic soy agar (Difco Laboratories, Detroit, MI).

Growth curves

To determine the effect of various iron concentrations on growth of *M. smegmatis*, the microorganism was cultured in a glucose-mineral salts medium that contained (per liter): glucose, 10 g; K_2HPO_4, 3.5 g; KH_2PO_4, 1.5 g;

$(NH_4)_2SO_4$, 1 g. To lower trace metal contamination, the medium was treated with Chelex-100 (Bio-Rad Laboratories, Hercules, CA) by previously reported methods (Arceneaux *et al.* 1973). After Chelex treatment, the medium was supplemented with 0.1% Tween 80 (Sigma Chemical Co., St. Louis, MO) and with high purity sulfate salts (Johnson-Matthey, Inc., Seabrook, NH) of magnesium (830 μM), manganese (36 μM), zinc (0.3 μM) and iron (various concentrations). Cultures were grown with shaking at 37°C and growth was determined by turbidity (A_{600}) measurements and converting to milligrams dry weight per milliliter.

Hydrogen peroxide killing

To assess the effect of growth at different iron concentrations on killing of *M. smegmatis* by H_2O_2, the microorganism was grown in a Chelex-100 treated, glucose-mineral salts medium, prepared as described above, that contained (per liter): glucose, 2 g; K_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; $(NH_4)_2SO_4$, 1 g. The microorganism was grown (37°C, with shaking) at the desired iron concentration to a turbidity (A_{600}) of 0.35 (equivalent to 0.25 mg dry weight per ml). Aliquots (0.9 ml) of the culture were added to 0.1 ml of H_2O_2 (obtained as a 30% solution from Sigma Chemical Co., St. Louis, MO) at concentrations from 0 to 44 mM. After 15 min incubation at 37°C, the number of surviving cells was estimated by diluting the H_2O_2-treated cells in tryptic soy broth (Difco Laboratories, Detroit, MI) and then pipetting 10 μl of each dilution as a single drop on tryptic soy agar plates or by spreading 0.1 ml on the same agar medium. The plates (done in triplicate for each H_2O_2 dilution tested) were incubated at 37°C and the colonies were counted.

Radiolabeling of stress and iron regulated proteins

To visualize expression of stress proteins following their induction by application of a stress agent, the microorganism was first grown at 37°C (with shaking) in a glucose-mineral salts medium. This medium was made with high purity water (RO40/Milli Q system water treatment system, Millipore Corp., Bedford, MA). The medium contained (per liter): glucose, 2 g; K_2HPO_4, 10.5 g; KH_2PO_4, 4.5 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.2 mg; iron (as $FeCl_3$), either 20 μM (high iron medium) or no iron addition (low iron medium); and Tween 80, 0.1% . When cultures at either iron concentration reached turbidity (A_{600}) of 0.4 (equivalent to 0.29 mg dry weight per ml), 1 ml amounts were transferred to 1.5 ml microcentrifuge tubes. These samples were subjected to stress (either heat, H_2O_2, or ethanol) of the desired magnitude as indicated in Results. After 10 min incubation, 10 μCi of [^{35}S]methionine–cysteine (Dupont NEN, Boston, MA) were added to the sample. Incubation was continued for either 5 min (heat shock) or 10 min (H_2O_2 or ethanol shock). Nonradioactive methionine–cysteine was added to a final concentration of 100 mM and the samples were chilled in ice and centrifuged. The cell pellets were washed once with ice cold growth medium and the washed pellet was frozen at –20°C. Kinetics of heat shock protein induction were measured by radiolabeling proteins at 5 min intervals for 5 min (during 30 min of heat shock).

To examine regulation of protein expression by the level of iron added to the growth medium, cultures were grown to an A_{600} of 0.4 (equivalent to 0.29 mg dry weight per ml) at either high or low iron concentrations. Proteins were radiolabeled as described above for 20 min and the cells harvested, washed, and frozen at –20°C until preparation for electrophoresis.

Electrophoresis of labeled proteins

Radiolabeled cell pellets (prepared as described above) were suspended in 30 μl of high purity water and were then frozen in liquid N_2 and thawed in 60°C water for at least three freeze–thaw cycles. The proteins were then solubilized by heating the cell suspension in 0.0625 M Tris–HCl, pH 6.8, containing 4% SDS and 10% glycerol, at 100°C for 5 min as described by Lugtenberg *et al.* (1975). Amounts of radioactivity in the samples were determined by liquid scintillation counting using Ecolume (ICN, Costa Mesa, CA). Electrophoresis of samples was performed using 10% and 15% polyacrylamide gels containing 0.2% SDS and 0.375 M Tris–HCl, pH 8.8 (SDS–PAGE). Equivalent amounts of radioactivity were loaded in each lane. Radioactively labeled protein bands in the gels were visualized by autoradiography using Biomax MR film (Kodak, Rochester, NY). The iron regulated proteins were analyzed for their possible location in the cell envelope by the cellular fractionation and differential centrifugation protocol of Hall *et al.* (1987).

N-*terminal amino acid sequencing*

Protein bands in electrophoretic gels were transferred to polyvinylidene fluoride (PVDF) membranes (ProBlott, Perkin-Elmer Applied Biosystems, Inc., Foster City, CA) in a transfer buffer (composed of 25 mM Tris–HCl, pH 8.3, 190 mM glycine and 20% methanol) with a Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA) at 50 volts for 1 h. The blotted proteins on the PVDF membranes were then stained with Coomassie Brilliant Blue G-250 in 10% acetic acid and the membranes were destained with 10% acetic acid in 50% methanol. The desired bands were cut from the membranes. Amino acid sequencing of the excised protein bands was done by the sequencing laboratory in the Department of Biochemistry, University of Mississippi Medical Center, Jackson. Automated Edman degradation of the proteins was performed with an Applied Biosystems sequencer (model 470A) equipped with model 120A on-line Pth-amino acid analyzer.

Results

Growth of M. smegmatis *at various iron levels*

The nutritional requirement of *M. smegmatis* for iron was documented in a glucose-mineral salts medium that was treated with Chelex-100 to lower trace metal contamination and supplemented with high purity metals (Figure 1). Iron at 1 μM was adequate for full growth; higher concentrations did not increase the total growth density or the apparent division rate. Iron at 0.1 μM was restrictive, yielding a culture that failed to reach maximum growth density. Growth of *M. smegmatis* in a medium containing 0.1 μM (or less) iron should produce iron starved cells.

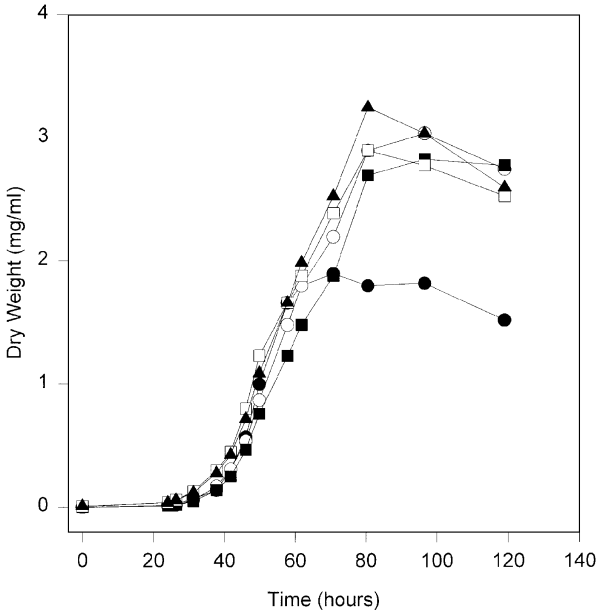

Figure 1. Iron requirement for growth of *M. smegmatis*. Cultures were grown in glucose-mineral salts medium (treated with Chelex-100 to lower trace metal contamination) that was supplemented with 0.1 (closed circle), 0.4 (open circle), 1 (closed square), 20 (open square) or 40 (closed triangle) μM iron.

Effects of growth medium iron levels on sensitivity of M. smegmatis *to* H_2O_2

When cultures of *M. smegmatis* that were grown at 1 or 20 μM iron were tested for H_2O_2 sensitivity, significant killing of the cultures occurred at H_2O_2 concentrations greater than 20 to 25 mM (Figure 2). Cultures grown at 0.2 μM iron (a concentration near that which restricted total growth) were less able to withstand insult from H_2O_2; the threshold level for significant killing was decreased to between 15 and 20 mM H_2O_2. Growth of cells at an even lower iron level (0.05 μM) resulted in marked sensitivity to oxidative damage with few survivors above 9 mM H_2O_2. These results suggest that too little cellular iron may preclude final synthesis of the iron-requiring protective enzymes that destroy oxy-radicals; however, iron starved cells may also be unable to respond in other ways to the challenge of H_2O_2.

Iron regulated proteins of M. smegmatis

Many bacterial species make (or increase production of) certain proteins during iron restricted growth. A strain of *M. smegmatis* produced five new envelope proteins of molecular masses of approximately 180, 84, 29, 25, and 14 kDa during iron limitation (Hall *et al.* 1987, Wheeler & Ratledge 1994). To confirm synthesis of iron regulated proteins in the strain and under conditions used here, radiolabeled proteins made during growth in high and low iron media were analyzed by SDS–PAGE (Figure 3). The iron limited cells expressed at least five proteins of molecular masses of 180, 84, 62, 48, and 38 kDa. With the differential centrifugation protocol of Hall *et al.* (1987), the 84 kDa iron regulated protein was found to be envelope associated (data not shown). This protein and the 180 kDa protein may be identical to the envelope proteins of similar size from *M. smegmatis* described previously (Wheeler & Ratledge 1994). At least one protein (of 140 kDa) was made only in high iron cells (Figure 3). The iron regulated 62 kDa protein which appeared in iron starved, nonstressed cells may also be made during stress of both high and low iron cells (described below).

Effect of growth medium iron level on expression of oxidative stress proteins

The enhanced susceptibility to H_2O_2 killing observed in iron deficient *M. smegmatis* may be due in part to inadequate levels of protective enzymes (iron-containing) as well as to the inability of the culture to initiate full expression of the oxidative stress response proteins. To determine the oxidative stress response of iron deficient cells, cultures were first grown in medium that was either supplemented with 20 μM iron (high iron) or which was given no iron

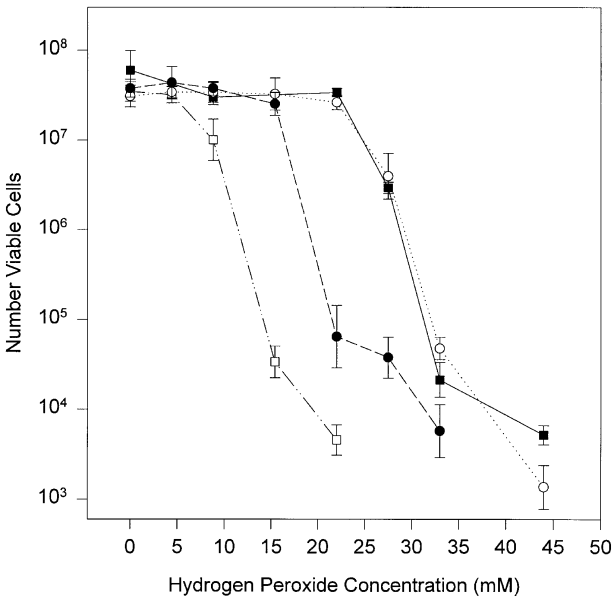

Figure 2. Hydrogen peroxide killing of *M. smegmatis* grown at various iron concentrations. Exponential growth phase cultures growing in a glucose-mineral salts medium (treated with Chelex-100) that was supplemented with 0.05 (open square), 0.2 (closed circle), 1 (open circle), or 20 (closed square) μM iron were exposed to the indicated concentrations of H_2O_2 and the surviving fraction of the population was determined by counting colony forming units. Each point represents the mean (with standard deviation) of at least six determinations.

supplement (low iron). They were then exposed to H_2O_2, and the radiolabeled proteins made in response to the stress were separated by SDS–PAGE (Figure 4). In iron deficient cultures, induction of oxidative stress protein synthesis was optimal at about 9 mM H_2O_2 (higher concentrations resulted in little radiolabeling of proteins). To achieve optimal induction of stress proteins in iron replete cells, an H_2O_2 concentration of 37 mM was required. High iron cells produced or upregulated major oxidative stress proteins of molecular masses of 90, 75, 65, 62, and 43 kDa. The iron deficient cultures were unable to mount a full oxidative stress response; the stress proteins of 65 and 75 kDa were not produced (or were produced in undetectable amounts). In iron replete H_2O_2 stressed cells, three protein bands at about 90 kDa were evident; in similarly stressed iron starved cells, only a single protein was evident at about 90 kDa. The iron regulated 62 kDa protein may be synthesized by oxidatively stressed cells regardless of iron level.

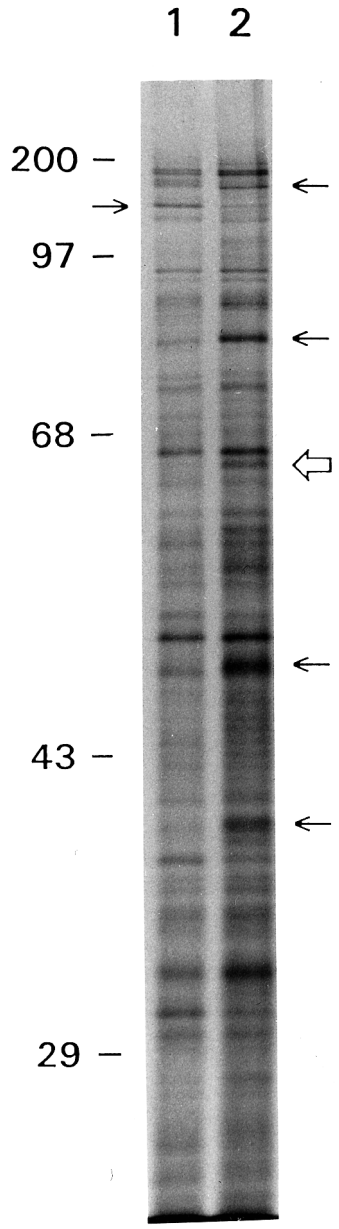

Figure 3. Iron regulated proteins of *M. smegmatis*. Proteins from high iron (lane 1) and low iron (lane 2) cultures were radiolabeled with [^{35}S]methionine–cysteine and separated by SDS–PAGE (10% polyacrylamide) and then visualized by autoradiography. Cultures were grown in medium that was not treated with Chelex-100 and which was supplemented with 20 μM iron (high iron) or no added iron (low iron). Arrows indicate the major iron regulated proteins and the open arrow indicates a possible 62 kDa iron regulated stress protein. Locations and sizes (kDa) of molecular mass markers are indicated to the left.

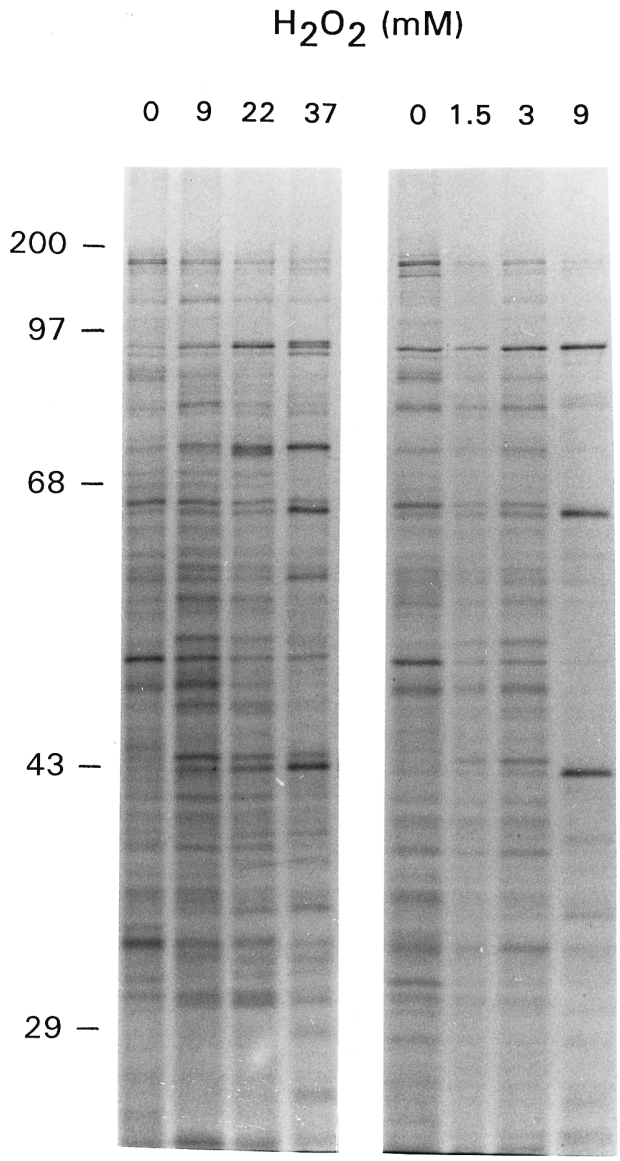

Figure 4. Oxidative stress proteins of *M. smegmatis* grown with high iron (left panel) or low iron (right panel). H_2O_2 concentrations are given above each lane. Cultures were grown in medium that was not treated with Chelex-100 and which was supplemented with 20 μM iron (high iron) or no added iron (low iron). Cells were radiolabeled with [^{35}S]methionine–cysteine and the proteins were separated by SDS–PAGE (10% polyacrylamide) and then visualized by autoradiography. Locations and sizes (kDa) of molecular mass markers are given to the left.

Effect of growth medium iron levels on induction of heat shock and ethanol shock proteins in M. smegmatis

To determine if the inability of iron deficient cells to produce at least two of the major oxidative stress proteins was due to a general failure of the stress response of such cells or to a specific defect in the response to oxidative stress, the response of low and high iron cultures to heat and ethanol shock was determined. The appropriate temperature for heat shock of the *M. smegmatis* strain used here was established by subjecting mid-log phase cultures (grown in high iron medium at 37°C) to heat shock at 42, 48, or 54°C for 10 min. Maximal induction of heat shock proteins (Hsp) of molecular masses of 90, 75, 65, 62, 43, and 16 kDa occurred at 48°C (Figure 5). To evaluate the effect of medium iron concentration on Hsp synthesis, mid-log phase cultures growing in high iron or low iron media were heat shocked at 48°C and protein synthesis was analyzed (Figure 6). Induction of Hsp was identical in iron starved and iron replete cells and was complete by 10 min. During the test period, synthesis of nonstress proteins declined. The pattern of stress protein synthesis induced by heat shock was nearly the same as that noted in iron replete cells that were oxidatively stressed (Figure 4). The rate of induction of Hsp in high and low iron cells also appeared similar, with the exception of that of the 62 kDa protein. The level of this iron regulated protein (Figure 3) reached maximum sooner in iron starved cells, probably due to expression of the protein prior to application of stress.

Similar experiments were performed with ethanol, an oxidant scavenger, as the stressing agent. Optimum expression of stress proteins occurred at 2 M ethanol and identical responses were noted in both iron starved and iron replete cells (data not shown). The pattern of stress proteins induced by ethanol was nearly the same as that induced by heat shock, except that the 16 kDa heat shock protein was not induced and a new stress protein of 55 kDa was made. Synthesis of this protein appeared to be provoked in cells treated with ethanol at concentrations as low as 0.2 mM (data not shown).

Identities of the M. smegmatis *65 kDa and 75 kDa stress proteins*

Proteins were blotted from electrophoretic gels onto PVDF membranes and the *N*-terminal amino acid sequences of the 65 and the 75 kDa stress proteins were determined. A sequence data base search, using the National Center for Biotechnology Information BLAST server, indicated that the 65 and 75 kDa proteins were GroEL and DnaK homologs, respectively (Figure 7). Sequence information on

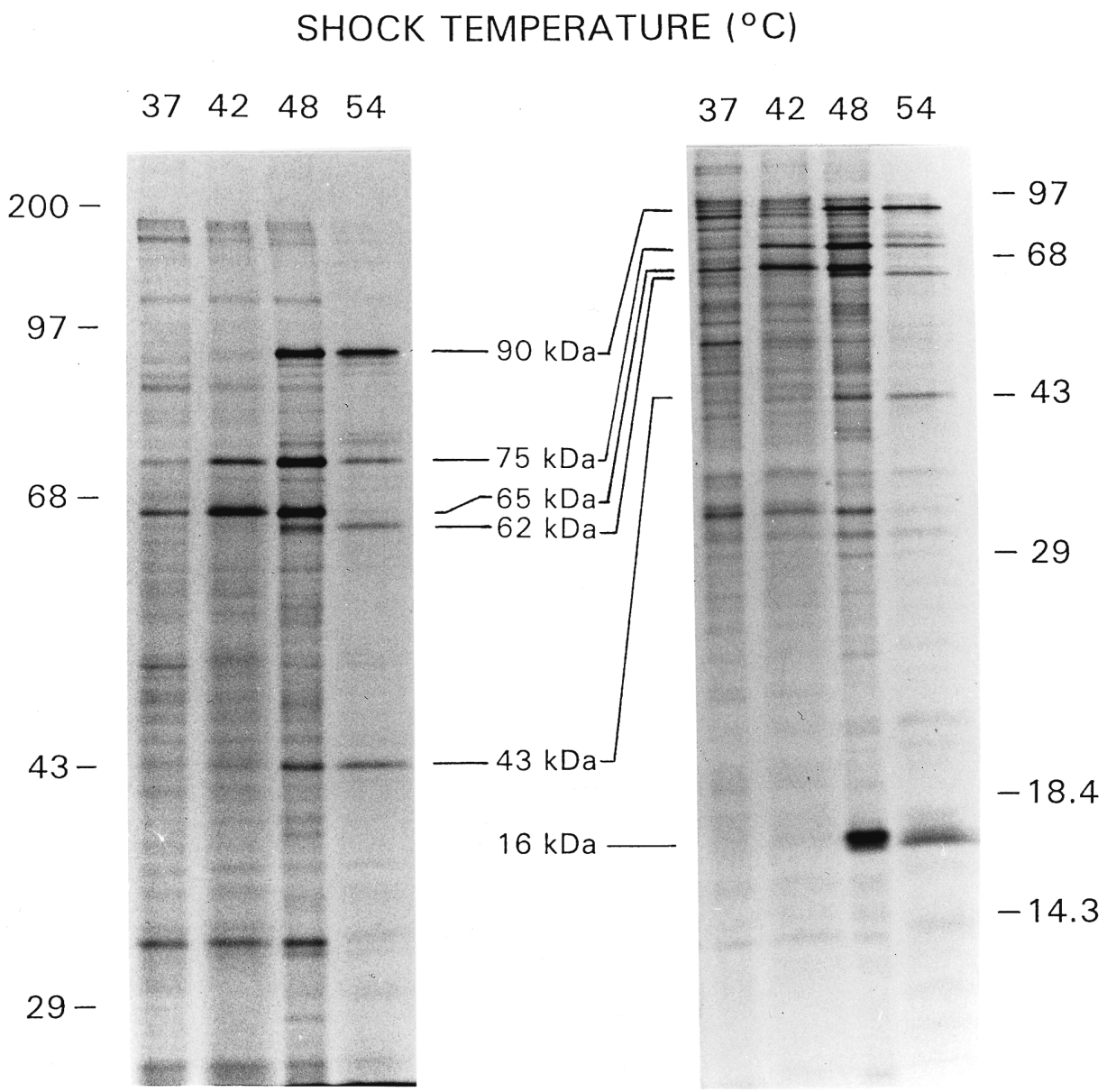

Figure 5. Heat shock proteins of *M. smegmatis*. Cultures were grown at 37°C in medium that was not treated with Chelex-100 and which was supplemented with 20 μM iron (high iron). Cells were then shocked at the indicated temperatures for 10 min and the proteins labeled with [^{35}S]methionine–cysteine. Radiolabeled proteins were separated by SDS–PAGE on 10% acrylamide (left panel) and 15% acrylamide (right panel) to resolve high and low molecular mass proteins, respectively; proteins were visualized by autoradiography. Major heat shock proteins are indicated and the locations of molecular mass markers are shown to the left and right of the panels.

the 62 kDa iron regulated, stress induced protein should be interesting; however, the concentration of this protein was too low for sequencing (it could be visualized only on autoradiography). Sequence data on additional *M. smegmatis* stress proteins were not obtained.

Discussion

Iron deficient *M. smegmatis* was more susceptible than iron replete *M. smegmatis* to the lethal effects of H_2O_2. The threshold levels of H_2O_2 necessary for killing of a significant portion of the population

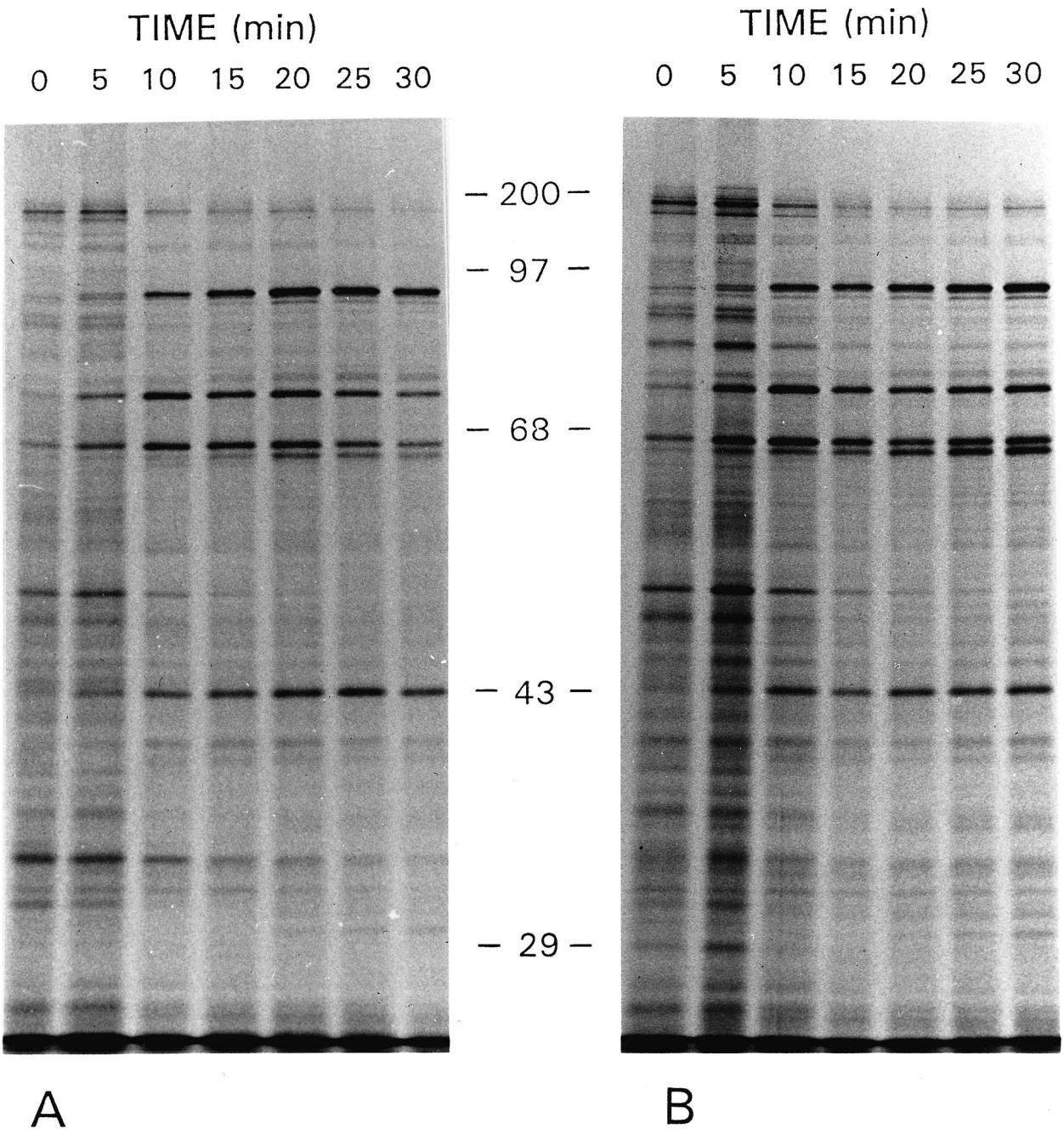

Figure 6. Kinetics of heat shock protein induction in *M. smegmatis* cultures grown in high iron (panel A) or low iron (panel B) medium. Cultures were grown in medium that was not treated with Chelex-100 and which was supplemented with 20 μM iron (high iron) or no added iron (low iron). Cells were placed at 48°C and at 5 min intervals (for 30 min), they were radiolabeled for 5 min, with [^{35}S]methionine–cysteine. The proteins were separated by SDS–PAGE (10% acrylamide) and then visualized by autoradiography. Locations and sizes (kDa) of molecular mass markers are given at the center.

were increased proportionally by growing the culture at higher iron concentrations. Greatest resistance to H_2O_2 killing was achieved at the iron level which supported full growth of the culture and increasing the iron concentration above this amount did not additionally increase resistance to H_2O_2. If it is true that both iron insufficiency and iron excess place cells at risk to damage from oxy-radicals, then present studies highlight the principle that the correct amount of cellular iron is 'just enough' to

GroEL (HSP60 Family)

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Ms 1 AKTIAYDEEARRGLERGLNSLADAVKVT 28
Ml 2 --------------------------- 29
Mt 2 -------------------A-------- 29
Sc 2 --ILKF--D---A----V-K---T---- 29
Ec 2 --DVKFGND--VKML--V-V-------- 29
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DnaK (HSP70 Family)

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Ms 1 ARAVGIDLGTTNXVXAV 17
Ml 2 ------------S-VS- 18
Mt 2 ------------S-LS- 18
Sc 2 ------------S-VS- 18
Ec 2 GKII--------SCV-I 18
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Figure 7. Alignment of the GroEL (65 kDa) and DnaK (75 kDa) *N*-terminal amino acid sequences of *M. smegmatis* (Ms) obtained in this study (X indicates no amino acid assignment was made), with sequences for *M. leprae* (Ml), *M. tuberculosis* (Mt), *Steptomyces coelicolor* (Sc), and *E. coli* (Ec). A dash indicates identity; nonidentical amino acids are indicated and the starting and final position numbers are shown. Sequence accession numbers (obtained from the SWISS-PROT Database, except as noted) for GroEL are: Ml; P09239; Mt, P06806; Sc, S37566 (from NBRF PIR Database); Ec, P06139; and for DnaK: Ml, P19993; Mt, P32723; Sc, Q05558; Ec, P04475. The Ms peptide sequence accession numbers (from EMBL-European Bioinformatics Institute) are: GroEL, P80673; DnaK, P80692.

supply the needs of the iron-requiring systems. While the vulnerability of iron starved cells to H_2O_2 may be due in part to a deficiency in iron-requiring protective enzymes, iron deficient *M. smegmatis* could also not produce the full complement of oxidative stress proteins seen in iron sufficient cells. The major stress proteins made by iron deficient and iron sufficient *M. smegmatis* during challenge with H_2O_2, heat, and ethanol are summarized in Figure 8. Syntheses of the *M. smegmatis* stress protein homologs of GroEL (65 kDa) and DnaK (75 kDa) were not upregulated in iron starved cells by H_2O_2 stress. On the other hand, GroEL and DnaK were made in iron deficient *M. smegmatis* during heat shock and ethanol stress, suggesting that the defective response of iron deficient cells was specific for oxidative stress. It is not clear why induction of some stress proteins was prevented by iron restriction. A possible explanation is disruption of signal transfer through the redox signalling system of *M. smegmatis*. In *E. coli*, at least one (SoxR) of the redox sensing regulatory proteins is an iron–sulfur protein (Hidalgo & Demple 1994). A similar iron–containing redox responsive regulatory homolog might be inoperative in iron deficient *M. smegmatis*. Another poorly understood relationship between stress protein synthesis and metal metabolism was previously reported in *M. bovis* BCG in which zinc restriction increased synthesis of the 65 kDa stress protein (De Bruyn *et al.* 1987).

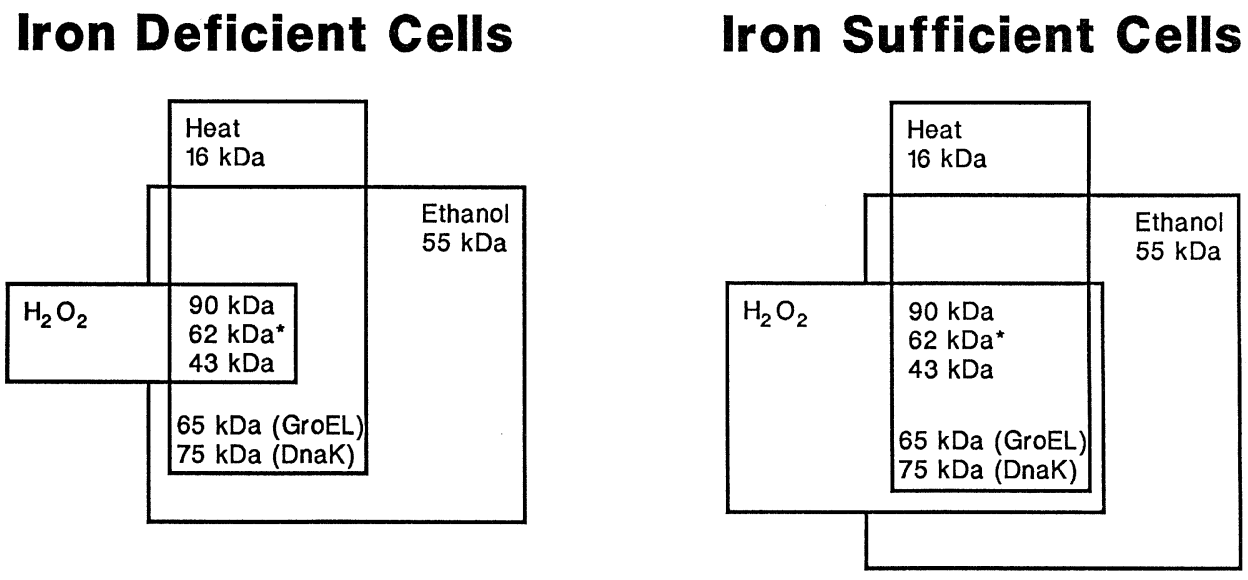

Figure 8. Proteins induced by heat, H_2O_2, and ethanol stress in iron deficient and iron sufficient *M. smegmatis*. The iron regulated, stress regulated protein is indicated (*).

Three additional stress response proteins (of 55, 62, and 16 kDa) of *M. smegmatis* deserve comment. Induction of these proteins in iron deficient and iron sufficient cells during challenge with H_2O_2, heat, and ethanol is summarized in Figure 8. The 55 kDa protein appeared during exposure of iron sufficient and iron deficient *M. smegmatis* to ethanol. The 16 kDa polypeptide apparently was induced only by heat shock. Synthesis of a 62 kDa protein was upregulated by all of the stressing agents and was also upregulated in iron deficiency. Expression of this protein was subject to multiple signals from iron and the various stressors.

The present studies illustrate the dramatic effects that can be exerted by iron on a biological system and reveal some of the complex and overlapping regulatory signals that control stress protein synthesis. If generally applicable to all microorganisms (including pathogens), the vulnerability of iron deficient *M. smegmatis* to oxidative damage may illustrate a defect that is exploited by vertebrates. Production of free radicals is assumed to be a major defense process of vertebrates and if iron starved pathogenic microorganisms are more susceptible than iron sufficient forms to oxy-radical damage, then this might explain some of the benefits of the strict iron limitation that vertebrate hosts attempt to impose on invading pathogens (Byers 1987).

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