DnaK dependence of mutant ethanol oxidoreductases evolved for aerobic function and protective role of the chaperone against protein oxidative damage in *Escherichia coli*

Pedro Echave*, M. Angel Esparza-Cerón†, Elisa Cabiscol*, Jordi Tamarit*, Joaquim Ros*, Jorge Membrillo-Hernández†‡, and E. C. C. Lin§

†Departamento de Biología Molecular y Biotecnología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, P.O. Box 70–228, 04510 Mexico City, Mexico; *Departament de Ciències Mèdiques Bàsiques, Facultat de Medicina, Universitat de Lleida, 25198 Lleida, Spain; and §Department of Microbiology and Molecular Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115

Edited by Susan Gottesman, National Institutes of Health, Bethesda, MD, and approved February 12, 2002 (received for review September 17, 2001)

The *adhE* gene of *Escherichia coli* encodes a multifunctional ethanol oxidoreductase (AdhE) that catalyzes successive reductions of acetyl-CoA to acetaldehyde and then to ethanol reversibly at the expense of NADH. Mutant JE52, serially selected for acquired and improved ability to grow aerobically on ethanol, synthesized an $AdhE^{A267T/E568K}$ with two amino acid substitutions that sequentially conferred improved catalytic properties and stability. Here we show that the aerobic growth ability on ethanol depends also on protection of the mutant AdhE against metal-catalyzed oxidation by the chaperone DnaK (a member of the Hsp70 family). No DnaK protection of the enzyme is evident during anaerobic growth on glucose. Synthesis of DnaK also protected *E. coli* from H_2O_2 killing under conditions when functional AdhE is not required. Our results therefore suggest that, in addition to the known role of protecting cells against heat stress, DnaK also protects numerous kinds of proteins from oxidative damage.

enzyme evolution | Hsp70 chaperone

Ethanol oxidoreductase (AdhE) of *Escherichia coli* is a multifunctional enzyme that catalyzes fermentative production of ethanol by two sequential NADH-dependent reductions of acetyl-CoA, as well as deactivation of pyruvate formate-lyase, which cleaves pyruvate to acetyl-CoA and formate. This large protein of 891 aa apparently emerged as a result of gene fusion. The NH_2-terminal region of this protein is highly homologous to the family of aldehyde:NAD^+ oxidoreductases, whereas the COOH-terminal region is homologous to the family of Fe^{2+}-dependent alcohol:NAD^+ oxidoreductases. The single Fe^{2+} bound to the alcohol oxidoreductase moiety of the AdhE protomer is also required for the deactivase activity (1–4). The estimated 3×10^4 AdhE molecules in an anaerobically grown cell are assembled into helical rod-like structures, or spirosomes, most of which contain 20–60 protomers. Residues 1–449 and 763–890 of AdhE are involved in its polymerization and may also be responsible for the propensity of the protein to aggregate amorphously. The biological significance of spirosomes, however, remains enigmatic (3, 5, 6).

Despite the reversibility of the two NADH-coupled reactions catalyzed by AdhE, wild-type *E. coli* is unable to grow on ethanol as a sole source of carbon and energy, because the *adhE* gene is transcribed aerobically at lowered levels (7–9) and the half-life of AdhE activity is shortened during aerobic metabolism by metal-catalyzed oxidation (MCO). During this process, the hydroxyl radicals locally generated by the Fe^{2+} bound to AdhE covalently attack the amino acid side chains near the active site (10, 11).

Mutants of *E. coli* capable of aerobic growth on ethanol as sole carbon and energy source have been isolated and characterized (7, 12, 13). All 16 independent first-stage mutants studied by us, typified by JE46, grew on ethanol with a doubling time of 240 min at 37°C and synthesized an AdhE with an Ala-267 → Thr substitution in the acetaldehyde:NAD^+ oxidoreductase domain ($AdhE^{A267T}$). Selection of each mutant for improved growth rate on ethanol resulted in a second-stage mutant. All of these second-stage mutants, typified by JE52, grow on ethanol with a doubling time of 90 min and synthesize a mutant protein with a second amino acid change, a Glu-568 → Lys substitution in the ethanol:NAD^+ oxidoreductase domain ($AdhE^{A267T/E568K}$). Neither the first- nor the second-stage mutants harbor mutations that affect transcriptional control of *adhE* (12).

$AdhE^{A267T}$ catalyzes the CoA-dependent dehydrogenation of acetaldehyde with an increased V_{max}. Apparently when catalyzing the two sequential reactions in a direction opposite to that of the physiological one, acetyl-CoA formation becomes rate-limiting for wild-type AdhE. The tradeoff for improving the V_{max} by $AdhE^{A267T}$ is decreased thermal enzyme stability and increased sensitivity to MCO damage. The second amino acid substitution in $AdhE^{A267T/E568K}$ partially restored protein stability and resistance to MCO damage, without further improvement of catalytic efficiency in substrate oxidation (12).

Clues that the altered properties of wild-type and mutant AdhE molecules observed *in vitro* may not in themselves be sufficient to account for the growth abilities of mutants JE46 and JE52 came from an interesting link between AdhE and the molecular chaperone DnaK (Hsp70). DnaK in conjunction with ClpB (Hsp104) has been shown to counteract the aggregation of thermolabile proteins under heat stress, and prominent among these is AdhE (14, 15). Upon inspection, we identified 16 DnaK-binding motifs in AdhE, based on the sequence proposed by Bukau and collaborators (14, 15). It thus seems possible that the functional integrity of the mutant AdhE proteins *in vivo* depends not only on their own properties but also on intervention by DnaK. In this study we address such a possibility.

Materials and Methods

Chemicals and Reagent Materials. Hydrogen peroxide, NAD, isopropyl β-D-thiogalactoside (IPTG), and *t*-butyl hydroperoxide were purchased from Sigma. Acrylamide/bisacrylamide solu-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: AdhE, ethanol oxidoreductase encoded by the *adhE* gene; MCO, metal-catalyzed oxidation; IPTG, isopropyl β-D-thiogalactoside.

‡To whom reprint requests should be addressed. E-mail: jmh@biomedicas.unam.mx.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

tion was supplied by Bio-Rad. Poly(vinylidene difluoride) membranes were from Millipore. The chemiluminescence detection kit (Western Light) was from Tropix (Bedford, MA).

Strains and Culture Conditions. The merodiploid strain *E. coli* ECL4000 [MC4100 *adhE*$^+$ Φ(*adhE-lacZ*)] was used as the wild-type parental strain (12, 16). A null *adhE*::*kan* strain (ECL4002) was used as a control for coimmunoprecipitation experiments to demonstrate physical interaction of the enzyme with DnaK. The selection of mutant strains JE46 and JE52 with acquired ability to grow on ethanol as sole carbon and energy source was described (12). An *E. coli* strain bearing the chromosomal locus P_{IPTG}*dnaK*$^+$*J*$^+$-*cat*-*lacI*^{q} (hereafter simply referred to as P_{IPTG}*dnaKJ*) was provided by B. Bukau (17). An *E. coli* strain bearing the Δ*dnaK52*::Cm^r locus (hereafter simply referred to as Δ*dnaK*), also provided by B. Bukau, has a deletion of 933 bp from the 5′ end of *dnaK* but a functional *dnaJ* (18, 19). The plasmid pBN15 (P_{tac}::*dnaK*$^+$*J*$^+$ *lacI*$^+$ *bla*$^+$) was provided by P. Blum (20). The chromosomal genetic loci and the plasmid were transferred to our experimental strains by following standard genetic procedures (21).

Minimal medium with 0.2% glucose and/or 2% ethanol as carbon and energy source was supplemented with 0.1% casein acid hydrolysate (21, 22). Culture optical density at 600 nm (OD_{600}) was determined in a DU640 Beckman spectrophotometer. Aerobic cultures of 10 ml were grown at 30°C with shaking (200 rpm) in 250-ml flasks. Anaerobic cultures were grown at 30°C in 10-ml test tubes filled to the brim. The BBL Gas-Pack system was used for anaerobic growth on solid media at 30°C. When used, IPTG was added at 1 mM, and ampicillin at 200 μg/ml.

Ethanol Oxidoreductase Assays. Cells were disrupted by sonication in an ice bath, and the centrifuged extracts were assayed for enzyme activity at 25°C as described (22). The assay mixture (1 ml) consisted of 1.6 M ethanol, 0.3 M potassium carbonate buffer (pH 10), and 0.66 mM NAD. The formation of NADH upon enzyme addition was monitored spectrophotometrically at 340 nm.

Coimmunoprecipitation Assays and Western Blot Analysis. Cells harvested at midexponential phase by centrifugation were resuspended in ice-cold immunoprecipitation buffer consisting of 10 mM Hepes–KOH at pH 7.9, 125 mM NaCl, 0.2% Nonidet P-40, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM *N*-tosyl-L-phenylalanine chloromethyl ketone, and 1 μM pepstatin A (all of the reagents were from Sigma). After disruption of the cells by sonication, the supernatant fraction was collected and the protein concentration was adjusted to 2 μg/μl with the same ice-cold buffer. One microgram of monoclonal anti-DnaK (StressGen Biotechnologies, Victoria, Canada) or polyclonal anti-AdhE antibodies (12) was added to 250 μl of cell extract and incubated for 60 min at 4°C. Protein A Sepharose CL-4B beads (40 μl; Amersham Pharmacia) were then added and the incubation was continued for 90 min at 4°C with gentle shaking. In control experiments, antibodies were omitted. Proteins bound to protein A Sepharose were eluted four times with the ice-cold immunoprecipitation buffer and loaded onto an SDS/9% polyacrylamide gel. The gel was subjected to Western blot analysis with anti-DnaK or anti-AdhE antibodies as described (11). Quantification of AdhE in extracts of cells grown aerobically or anaerobically was performed by Western blot analysis using anti-AdhE antibodies (12). The intensity of the bands was determined by using a Lumi Imager (Boehringer Mannheim).

Protein Derivatization and Immunodetection of Oxidatively Damaged Proteins. Oxidized proteins in cell extracts were revealed immunochemically by their carbonyl content (23) after derivatization with dinitrophenylhydrazine (11). Immunodetection was performed according to the instructions in the Tropix kit. The anti-2,4-dinitrophenyl antibodies were supplied by Dako and used at a 1:2000 dilution. The secondary antibody was a goat anti-rabbit antibody conjugated with alkaline phosphatase (Tropix) used at a 1:25,000 dilution.

H_2O_2 Treatment. JMH801 cells (P_{IPTG}*dnaKJ*/Δ*dnaKJ*) were grown aerobically at 30°C on glucose in the presence or absence of 1 mM IPTG. At midexponential phase of growth (OD_{600} 0.6, $\approx 1 \times 10^8$ cells ml^{-1}), H_2O_2 was added to give a concentration of 8.8 mM. Samples of 1 ml were then pipetted into each of nine Eppendorf tubes placed in a water bath at 30°C. At the desired time, a tube was withdrawn and the bacterial suspension was serially diluted with 10 mM $MgSO_4$ and then plated in triplicate on glucose agar for viable count.

Results

DnaK Dependence for Aerobic Growth on Ethanol by Strains JE46 and JE52. To determine whether the chaperone influences the *in vivo* stability of AdhE^{A267T} and AdhE$^{A267T/E568K}$, we first replaced the *dnaKJ* operon on the chromosome of the mutants JE46 and JE52 by $P1_{vir}$ transduction of an IPTG-inducible *dnaKJ* operon to yield JMH805 (P_{IPTG}*dnaKJ adhE*^{A267T}) and JMH803 (P_{IPTG}*dnaKJ adhE*$^{A267T/E568K}$), respectively. These strains were then tested for growth on glucose or ethanol agar in the presence or absence of IPTG. Like the wild-type parent ECL4000, the transductants JMH805 and JMH803 grew aerobically or anaerobically on glucose irrespective of the presence or absence of IPTG. By contrast, the transductants grew aerobically on ethanol only when their P_{IPTG}*dnaKJ* construct was induced by IPTG. A control experiment showed that ethanol, at the concentration used, did not inhibit aerobic or anaerobic growth on glucose (Fig. 1). It should be added that transformation of the wild-type strain ECL4000 with the plasmid pBN15 bearing P_{tac}::*dnaKJ* (20) did not confer aerobic growth ability on ethanol even when DnaK was induced by IPTG (data not shown). Thus, it seems that DnaK stabilizes AdhE^{A267T} and AdhE$^{A267T/E568K}$ and that, even if the wild-type enzyme is also stabilized, its specific activity remains insufficient to support growth on ethanol. Because the results from both mutant strains were essentially the same, further studies focused on mutant JE52 and its enzyme AdhE$^{A267T/E568K}$.

DnaK Protection of AdhE and AdhE$^{A267T/E568K}$ Specific Activity Occurs During Aerobic but Not Anaerobic Growth. We next assayed ethanol oxidoreductase activity level in extracts of cells with various combinations of *adhE* and *dnaK* alleles grown under different conditions. Data in Table 1 show that under anaerobic growth conditions, activity of neither the wild-type nor the mutant enzyme protein needed DnaK. Chromosomal deletion of *dnaK*, however, lowered the aerobic activity level of the wild-type AdhE by 65–70% (strain ECL4000 compared with strain JMH800). By contrast, the same deletion lowered the aerobic AdhE$^{A267T/E568K}$ activity level by 90% (strain JE52 compared with strain JMH802). As expected, after both Δ*dnaK* strains were transduced with the P_{IPTG}*dnaKJ* construct, induction by IPTG restored the aerobic AdhE and AdhE$^{A267T/E568K}$ activity levels to those observed in the *dnaK*$^+$ background. IPTG was without effect in the parental nontransduced Δ*dnaK* strains. The 5- to 6-fold increase of the ethanol oxidoreductase specific activity of AdhE$^{A267T/E568K}$ over that of AdhE in extracts of aerobically grown cells reflects the consequence of structural change of the protein and not the rate of gene transcription (12).

Protection of Preformed AdhE by DnaK after Shifting from Anaerobic to Aerobic Growth Conditions. The results from the aerobic growth experiments could reflect either the protection of AdhE from

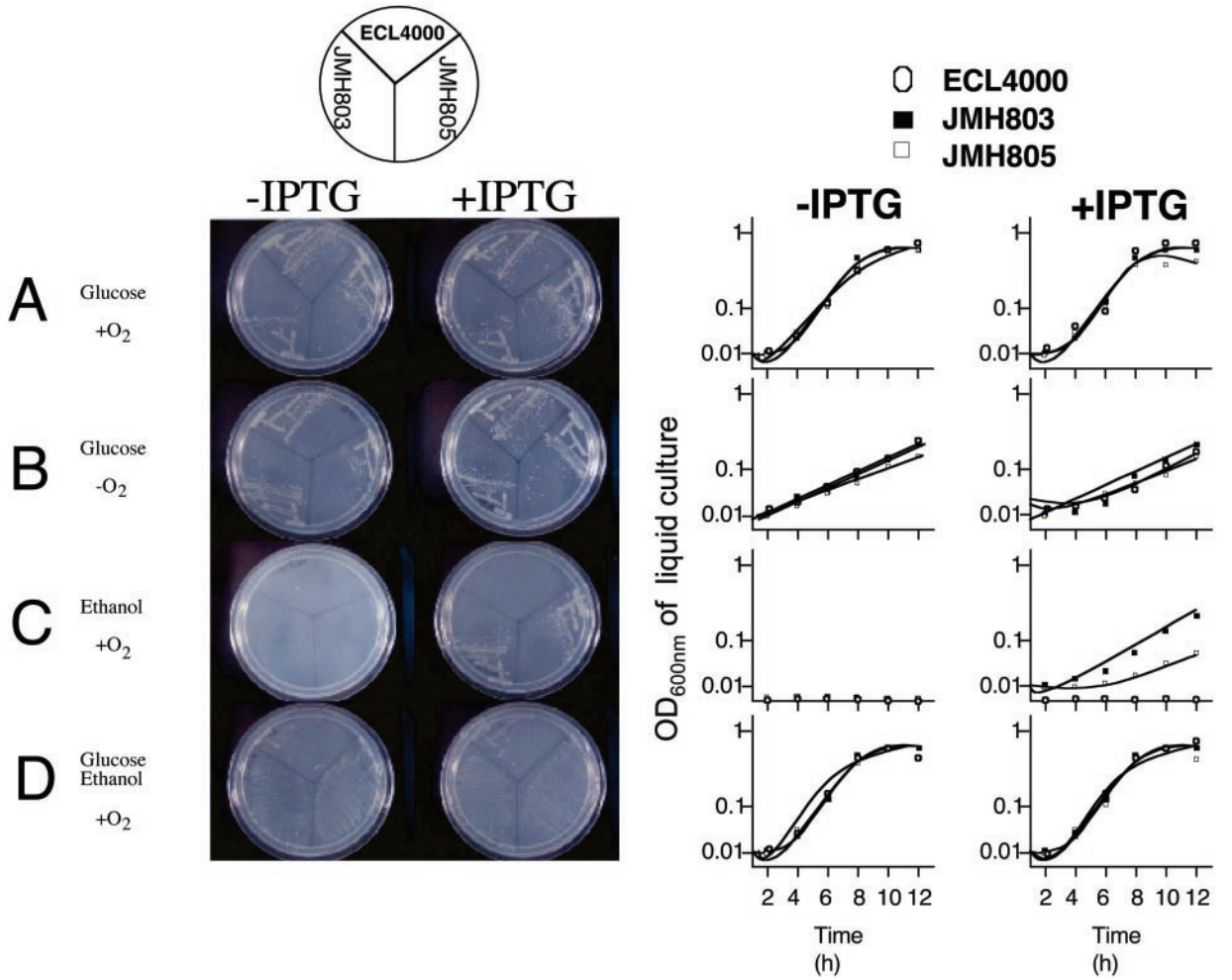

Fig. 1. Growth test of wild-type and various ethanol-mutant strains on different media and culture conditions. (*Left*) Cells of ECL4000 (*adhE*$^{+}$), JMH803 (P_{IPTG}*dnaJK adhE*$^{A267T/E568K}$), and JMH805 (P_{IPTG}*dnaJK adhE*^{A267T}) were seeded on agar containing different carbon sources in minimal medium, with or without IPTG. Plates containing glucose agar were incubated aerobically (*A*) or anaerobically (*B*). Plates containing ethanol agar (*C*) or ethanol plus glucose agar (*D*) were incubated only aerobically. The photography was taken after 48 h. (*Right*) Under conditions corresponding *A* to *D*, growth of ECL4000 (○), JMH803 (■), and JMH805 (□) cells in liquid medium was monitored.

inactivation by DnaK or the promotion of the synthesis and maturation of the enzyme by the chaperone. To distinguish between these two possibilities, cells synthesizing AdhE and AdhE$^{A267T/E568K}$ in the presence or absence of DnaK were first grown anaerobically on glucose. Exponentially growing cultures were then transferred to a large flask for aerobic incubation (further transcription of *adhE* repressed). After the shift, samples were withdrawn at different times for assay of the specific

Table 1. AdhE activity levels of different strains grown aerobically or anaerobically in glucose medium in the presence or absence of IPTG to induce DnaK

			Ethanol oxidoreductase specific activity, nmol/min per mg			
			Aerobic		Anaerobic	
Strain	Derived from	Relevant genotype*	−IPTG	+IPTG	−IPTG	+IPTG
ECL4000	MC4100	*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR attB*::Φ(*adhE-lacZ*)	86 ± 10	98 ± 12	660 ± 7	700 ± 20
JMH800	ECL4000	Δ*dnaK adhE*$^{+}$	31 ± 6	28 ± 08	640 ± 90	700 ± 10
JMH801	ECL4000	P_{IPTG}*dnaKJ adhE*$^{+}$	29 ± 10	97 ± 10	711 ± 70	900 ± 10
JE52	ECL4000	*dnaK*$^{+}$ *adhE*$^{A267T/E568K}$	580 ± 90	550 ± 70	1,600 ± 130	1,800 ± 200
JMH802	JE52	Δ*dnaK adhE*$^{A267T/E568K}$	49 ± 12	50 ± 16	1,600 ± 180	1,750 ± 300
JMH803	JE52	P_{IPTG}*dnaKJ adhE*$^{A267T/E568K}$	120 ± 11	670 ± 100	1,900 ± 260	1,900 ± 200

*For detailed description of *dnaK* alleles see *Materials and Methods.*

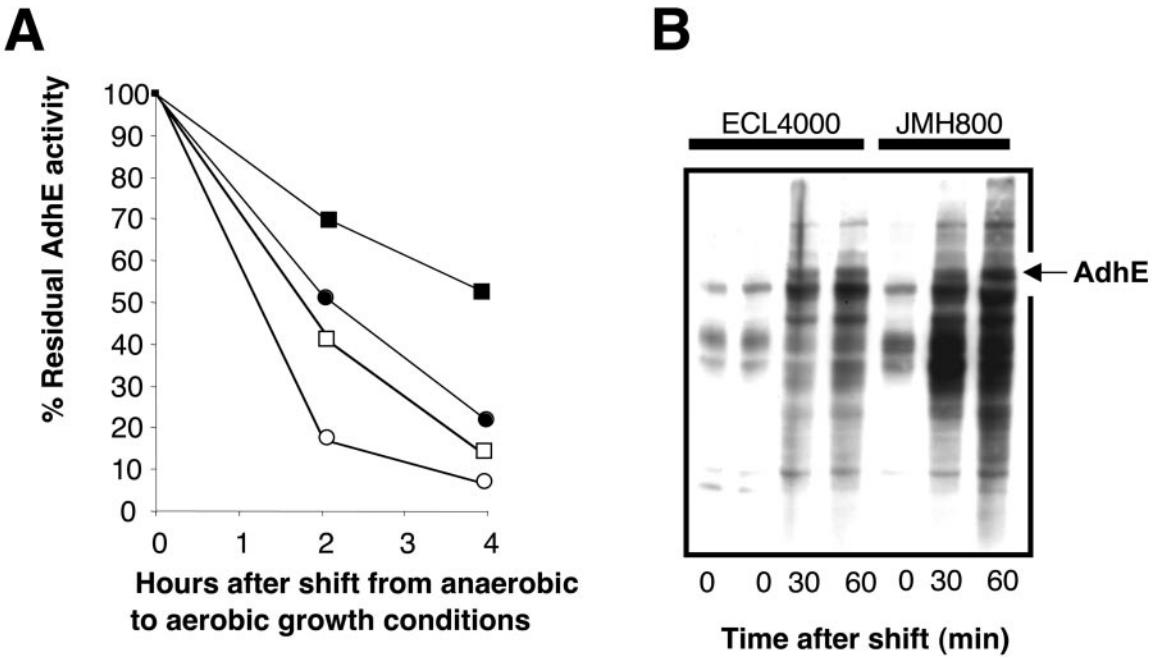

Fig. 2. Loss of specific ethanol oxidoreductase activity and carbonyl-content analysis in cells with or without DnaK, after a shift from anaerobic to aerobic growth conditions. Cultures of various strains, grown anaerobically until the OD_{600} reached 0.7 were transferred to flasks for aerobic incubation (see *Materials and Methods*). Samples were withdrawn from aerobic cultures at indicated times for analysis. (*A*) Specific ethanol oxidoreductase activity in cell extracts of cells: ECL4000 (*adhE*$^+$ *dnaK*$^+$; ■), JMH800 (*adhE*$^+$ Δ*dnaK*; ●), JE52 (*adhE*^{A267TE568K} *dnaK*$^+$; □), and JMH800 (*adhE*$^+$ Δ*dnaK*; ○). Data from each strain were obtained from at least three independent cultures, with a variation of less than 15%. Results from a typical experiment are shown. (*B*) Carbonyl-content analysis of strain ECL4000 (*adhE*$^+$ *dnaK*$^+$) and its isogenic JMH800 (*adhE*$^+$ Δ*dnaK*) mutant at 30 or 60 min after the shift to aerobic conditions. Location of AdhE protein is shown by an arrow.

activity of ethanol oxidoreductase. As shown in Fig. 2*A*, the specific activity of AdhE$^{A267T/E568K}$ dropped more rapidly than that of AdhE. Lack of DnaK accelerated the activity loss of both wild-type and mutant enzymes. We also monitored the change in carbonyl content of cellular proteins in *dnaK*$^+$ and Δ*dnaK* cells 30 and 60 min after the shift from anaerobic to aerobic growth conditions. As shown in Fig. 2*B*, carbonylation of proteins is consistently higher in Δ*dnaK* cells than in the isogenic *dnaK*$^+$ cells. Moreover, the percent residual enzyme activity after 60 min of aerobic exposure is consistently lower for AdhE$^{A267T/E568K}$ than for AdhE. It should be mentioned that the growth rate of *dnaK*$^+$ and Δ*dnaK* cells was not detectably different under the experimental conditions (data not shown). Thus, DnaK protects the preformed AdhE against MCO and the growth rate of the Δ*dnaK* mutant is not an issue.

Amount of the AdhE Protein and Enzyme Activity Units in *dnaK*$^+$ and Δ*dnaK* Strains. Because DnaK may protect AdhE from degradation, and from inactivation, the amounts of the AdhE protein in extracts of aerobically or anaerobically grown cells of ECL4000 (*adhE*$^+$ *dnaK*$^+$), JMH800 (*adhE*$^+$ Δ*dnaK*), JE52 (*adhE*$^{A267T/E568K}$ *dnaK*$^+$), and JMH802 (*adhE*$^{A267T/E568K}$ Δ*dnaK*) were also compared. Aerobic cultures growing on glucose were harvested during exponential phase (OD_{600} = 0.3), whereas the corresponding anaerobic cultures were harvested when fully grown. The AdhE protein content was then analyzed by Western blot with polyclonal anti-AdhE antibodies. In aerobically grown cells, the lack of DnaK lowered the AdhE or AdhE$^{A267T/E568K}$ content by about 40%. In anaerobically grown cells, no DnaK-associated difference in AdhE or AdhE$^{A267T/E568K}$ content was observed (data not shown).

When the aerobic levels of ethanol oxidoreductase activity were assayed, the lack of DnaK lowered the AdhE and the AdhE$^{A267T/E568K}$ specific activities by 3.3- and 10-fold, respectively (Table 1). The more rapid rate of MCO enzyme inactivation than the disappearance of the protein has also been reported for other Fe^{2+}-dependent enzymes such as glycerol oxidoreductase (24, 25).

Physical Interaction of AdhE and AdhE$^{A267T/E568K}$ with DnaK. If protection of AdhE by DnaK is stoichiometric or processive, a significant portion of AdhE should exist as a DnaK complex. We therefore assayed the amount of DnaK associated with AdhE by coimmunoprecipitation experiments with extracts of aerobically or anaerobically grown ECL4000 or JE52 strains. Fig. 3 shows the results of one such experiment in which anti-AdhE antibodies were first used for the precipitation, and subsequently, the amount DnaK associated with the precipitate was revealed by Western analysis using anti-DnaK antibodies. Wild-type AdhE showed negligible association with DnaK when extracted from cells grown anaerobically on glucose, whereas significant association was observed when the cells were grown aerobically on the same medium. The actual difference in the association should be even more striking, if one considers that anaerobically grown cells are expected to synthesize about 10 times more AdhE protein than aerobically grown cells (8). In AdhE$^{A267T/E568K}$, coimmunoprecipitation experiments showed a clear association with DnaK even when cells were grown anaerobically. This association increased almost 2-fold when the cells were grown aerobically, whether on glucose or ethanol. Consistent results were obtained when anti-DnaK antibodies were first used for the precipitation, and the amount of AdhE associated with the precipitate was determined subsequently (data not shown). As a control we used cell extracts from an *adhE*-null mutant strain. No significant coimmunoprecipitation of AdhE and DnaK was observed (data not shown). The results collectively indicate that (*i*) MCO damage of both wild-type and mutant AdhE molecules

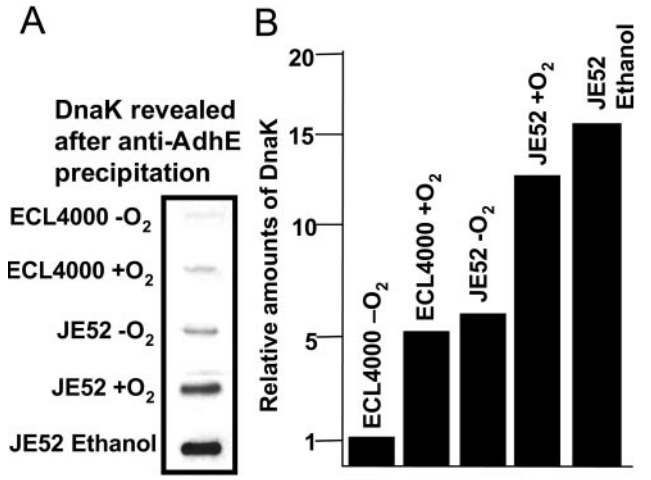

Fig. 3. Coimmunoprecipitation of AdhE with DnaK in crude extracts of cells grown under different culture conditions. Cell extracts were immunoprecipitated with anti-AdhE antibodies as described in *Materials and Methods*. The precipitated fraction was subjected to SDS/PAGE, and the amount of DnaK present in this fraction (i.e., coimmunoprecipiating with AdhE) was determined by Western blotting with anti-DnaK antibodies. (*A*) Western blot with anti-DnaK antibodies on cell extracts immunoprecipitated with anti-AdhE antibodies. In order from top to bottom, the samples were from strain ECL4000 (AdhE^{+}) grown on glucose anaerobically ($-O_2$) or aerobically ($+O_2$), strain JE52 (AdhE$^{A267T/E568K}$) grown on glucose anaerobically ($-O_2$) or aerobically ($+O_2$), and strain JE52 grown on ethanol aerobically. (*B*) Densitometry analysis of the Western blot. One arbitrary unit corresponds to the intensity of the signal given by the sample from wild-type cells grown under anaerobic conditions.

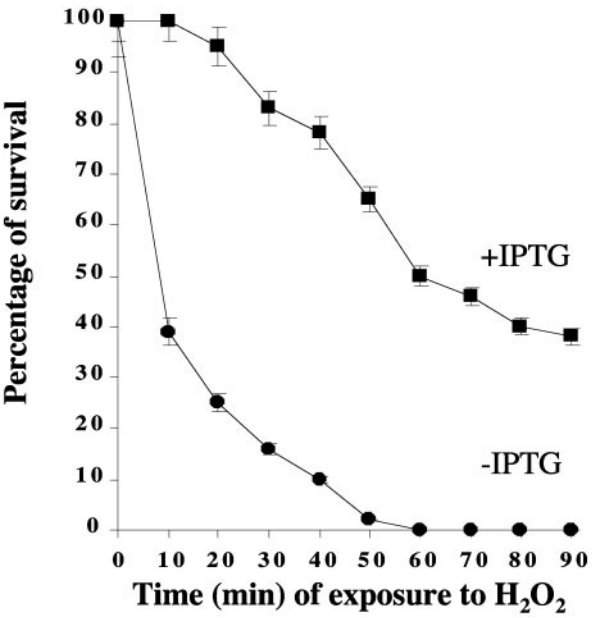

Fig. 4. H_2O_2 sensitivity of JMH801 (P_{IPTG}*dnaKJ*) cells induced or not induced in DnaK. Exponential cultures growing aerobically on glucose and casein amino acid hydrolysate were exposed to 8.8 mM H_2O_2 and cell survival was monitored at various times. Each point is a mean value based on determination of four independent culture suspensions. Error bars indicate SD.

is correlated with increased attraction for DnaK, and (*ii*) even undamaged AdhE$^{A267T/E568K}$ significantly attracts DnaK.

Cell Viability During Oxidative Stress in the Presence or Absence of DnaK. Because it seems unlikely that the protection by DnaK against MCO is limited to AdhE (see also Fig. 2*B*), we tested the importance of DnaK to cell survival when exposed to H_2O_2. Cultures of JMH801 cells (P_{IPTG}*dnaKJ*/Δ*dnaKJ*) were grown aerobically in the presence or absence of IPTG and then challenged with 8.8 mM H_2O_2. As shown in Fig. 4, cells induced in DnaK were more resistant than those that were not. Because aerobic growth on glucose does not require AdhE activity, the results indicate that DnaK plays a broad protective role against protein oxidation, although a role of shielding other macromolecular targets is not excluded.

Discussion

DnaK (Hsp70) has been regarded as a molecular chaperone that facilitates protein folding as well as assembly and disassembly of oligomeric protein complexes (26). The function of DnaK seems to be vital because its homologues have been found in all organisms. Biochemical data implicate the Hsp70 family of proteins in an even wider range of functions, including initiation of replication of bacteriophage λ DNA, protein secretion, clathrin uncoating followed by lysosomal protein degradation, and nascent peptide protection (17). Genetic evidence indicates that the Hsp70 proteins are important for cell growth of *Saccharomyces cerevisiae* and *E. coli*, especially under stressful conditions such as high temperature (26). The heat shock modulon significantly is also turned on by several kinds of oxidative stress (27–29). Chaperones preferentially bind stretches of hydrophobic amino acids that are usually buried in folded mature proteins. The inaccessibility of the buried regions in part accounts for the failure of DnaK to bind efficiently to most functional proteins. Some mature proteins, however, do bind DnaK, and a correlation exists between the weak affinity of these proteins for DnaK and their lack of affinity for hydrophobic-interaction chromatography adsorbents. This correlation is consistent with the idea that DnaK interacts with the exposed hydrophobic groups of the substrate proteins (15).

Five salient findings stand out from the present study. (*i*) DnaK has a protective effect on AdhE activity under aerobic but not anaerobic conditions. (*ii*) Under anaerobic conditions, AdhE$^{A267T/E568K}$ interacts more extensively with DnaK than does AdhE, even though a protective effect was not observed. (*iii*) AdhE$^{A267T/E568K}$ not only is more sensitive than AdhE to MCO inactivation [as reported for the purified enzymes (12)] but also is more dependent on DnaK stabilization than the wild-type enzyme. (*iv*) DnaK does not protect against degradation of the AdhE protein after its MCO inactivation. (*v*) Most importantly, DnaK protects a broad spectrum of proteins against irreversible oxidative damage.

A simple unifying working hypothesis for DnaK protection of AdhE against irreversible MCO damages can be described as follows: (*i*) A minor fraction of AdhE exists in a partially unraveled conformation that exposes its DnaK-binding domains, a conformation that also renders the protein vulnerable to MCO during aerobic metabolism. (*ii*) The fraction of the unraveled protein, magnified in AdhE$^{A267T/E568K}$, increases with temperature. (*iii*) DnaK counteracts the heat- or mutation-induced unraveling of AdhE and AdhE$^{A267T/E568K}$ by recognizing the exposed binding sites and driving their conformation back into the properly folded form at the expense of energy derived from ATP. Such a model would collaterally link the heat-chaperone role of DnaK with its protective role of proteins against MCO and may apply to many other cellular proteins as well. Unfortunately, because mutants lacking DnaK are temperature-sensitive for growth, protection of heat denaturation of AdhE by the chaperone cannot be readily tested. The proposed model may have to be revised or elaborated as more information becomes available. For instance, it is possible that the oxidation

of a single subunit of the multimer (oligomer or spirosome) attracts DnaK to protect the rest of the subunits.

Studies in the literature on protective mechanisms against oxidative damage have been heavily focused on induction of enzymes that catalytically destroy reactive oxygen species. Although such defense systems can be built up rapidly, the protection conferred can never be complete, and the covalent damages inflicted are irreversible (with the exception of oxidized methionine or disulfide bridge formation). Therefore, it should not be surprising that a supplemental chaperone defense mechanism has also evolved. We have reported that AdhE and DnaK in *E. coli* are two of the proteins most prone to be damaged by MCO, as indicated by their carbonyl content (10, 11). It is tempting to speculate that the physical interaction of DnaK with its protein substrate renders the chaperone itself vulnerable, particularly if metal ions are present locally (30). Because many proteins have acquired multiple functions during the course of evolution, it is conceivable that during the transitions from dark anaerobiosis to photosynthetic life and then to aerobic metabolism, DnaK has expanded its role from preventing thermal denaturation and aggregation of proteins to include their protection against oxidative damage. It is also possible that in each of these roles, DnaK is partnered with a different set of other proteins. In this regard, we have tested a *clpB* mutant strain and observed that ClpB is dispensable for the aerobic AdhE activity (data not shown).

Our studies have also revealed an unexpected aspect of biochemical evolution. DnaK enabled $AdhE^{A267T}$ and $AdhE^{A267T/E568K}$ to realize their acquired potential function. This is not a unique example. Recruitment of the mutated fermentative L-1,2-propanediol:NAD^+ 1-oxidoreductase of *E. coli* (encoded by *fucO*) for aerobic utilization of propanediol (ref. 31 and references therein) also required DnaK (J.M.-H., unpublished data). Similarly, Hsc66 (a DnaK homologue encoded by *hscA*) was reported to be necessary for protecting the dehydrogenase responsible for the acquired ability of a *Pseudomonas aeruginosa* mutant to grow aerobically on ethanol and other short-chain alcohols (32). When we checked whether the Hsc66 homologue in *E. coli* has a safeguarding role for wild-type and mutant AdhE proteins against oxidative inactivation, no effect was found (data not shown).

All three examples illustrate the importance of bracing the transitional structures during the selection before they can be stabilized by additional amino acid substitutions. Chaperone systems therefore can widen the evolutionary opportunity for proteins to acquire novel functions. The protection against MCO by chaperones may have implications in eukaryotic biology as well. For instance, indirect evidence relates the production of free radicals and aging with the presence of Hsp70 (the DnaK homologue). The induction of *hsp70* expression is reduced to approximately 50% with age in a variety of tissues from rats, as well as mononuclear cells from human subjects (33). Studies in *Drosophila* showed that the expression of *hsp70* decreases with aging and that the lack of Hsp70 is correlated with increased accumulation of damaged proteins (34).

We thank Dr. B. Bukau and P. Blum for the kind gifts of strains used in this study. This work was supported by Grants J33369-N from Consejo Nacional de Ciencia y Tecnología (Mexico) and IN205200 from the Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica Universidad Nacional Autónoma de México (to J.M.-H.); PB97–1456 from the Dirección General de Enseñanza Superior e Investigación Científica and the Comissionat per Universitats i Recerca de la Generalitat de Catalunya and Ajuntament of Lleida, Spain (to J.R.); and GM40993 from the National Institute of General Medical Sciences, National Institutes of Health, U.S. Public Health Service (to E.C.C.L.). P.E. is a recipient of a Ph.D. scholarship from the Ministerio de Educación y Cultura (Spain). J.T. is a recipient of a postdoctoral fellowship from the Generalitat de Catalunya (Spain). J.M.-H. acknowledges technical support from Marisela Aguirre-Ramírez and M. Paéz-Silva.

1. Cunningham, P. R. & Clark, D. P. (1986) *Mol. Gen. Genet.* **205,** 487–493.
2. Goodlove, P. E., Cunningham, P. R., Parker, J. & Clark, D. P. (1989) *Gene* **85,** 209–214.
3. Kessler, D., Herth, W. & Knappe, J. (1992) *J. Biol. Chem.* **267,** 18073–18079.
4. Kessler, D., Leibrecht, I. & Knappe, J. (1991) *FEBS Lett.* **281,** 59–63.
5. Matayoshi, S., Oda, H. & Sarwar, G. (1989) *J. Gen. Microbiol.* **135,** 525–529.
6. Knappe, J. & Sawers, G. (1990) *FEMS Microbiol. Rev.* **4,** 383–398.
7. Clark, D. P. & Cronan, J. E. (1980) *J. Bacteriol.* **144,** 179–184.
8. Chen, Y. M. & Lin, E. C. C. (1991) *J. Bacteriol.* **173,** 8009–8013.
9. Leonardo, M. R., Cunningham, P. R. & Clark, D. P. (1993) *J. Bacteriol.* **175,** 870–878.
10. Cabiscol, E., Aguilar, J. & Ros, J. (1994) *J. Biol. Chem.* **269,** 6592–6597.
11. Tamarit, J., Cabiscol, E. & Ros, J. (1998) *J. Biol. Chem.* **273,** 3027–3032.
12. Membrillo-Hernández, J., Echave, P., Cabiscol, E., Tamarit, J., Ros, J. & Lin, E. C. C. (2000) *J. Biol. Chem.* **275,** 33869–33875.
13. Holland-Staley, C. A., Lee, K., Clark, D. P. & Cunningham, P. R. (2000) *J. Bacteriol.* **182,** 6049–6054.
14. Rüdiger, S., Germeroth, L., Schneider-Mergener, J. & Bukau, B. (1997) *EMBO J.* **16,** 1501–1507.
15. Mogk, A., Tomoyasu T., Goloubinoff, P., Rüdiger, S., Röder, S., Langen, H. & Bukau, B. (1999) *EMBO J.* **18,** 6934–6949.
16. Casadaban, M. J. (1976) *J. Mol. Biol.* **104,** 541–555.
17. Deuerling, E., Schulze-Specking, A., Tomoyasu, T., Mogk, A. & Bukau, B. (1999) *Nature (London)* **400,** 693–697.
18. Bukau, B. & Walker, G. C. (1990) *EMBO J.* **9,** 4027–4036.
19. Paek, K.-H. & Walker, G. C. (1987) *J. Bacteriol.* **169,** 283–290.
20. Rockabrand, D., Livers, K., Austin, T., Kaiser, R., Debra, J., Burgess, R. & Blum, P. (1998) *J. Bacteriol.* **180,** 846–854.
21. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY).
22. Membrillo-Hernández, J. & Lin, E. C. C. (1999) *J. Bacteriol.* **181,** 7571–7579.
23. Levine, R. L., Williams, J. A., Stadtman, E. R. & Schacter, E. (1994) *Methods Enzymol.* **233,** 346–357.
24. Ruch, F. E., Lin, E. C. C., Kowit, J. D., Tang, C.-T. & Goldberg, A. L. (1980) *J. Bacteriol.* **141,** 1077–1085.
25. Chevalier, M., Lin, E. C. C. & Levine, R. L. (1990) *J. Biol. Chem.* **265,** 40–46.
26. Bukau, B. & Walker, G. C. (1989) *J. Bacteriol.* **171,** 2337–2346.
27. Estruch, F. (2000) *FEMS Microbiol. Rev.* **24,** 469–486.
28. Pappolla, M. A., Sos, M., Omar, R. A. & Sambamurti, K. (1996) *Mol. Chem. Neuropathol.* **28,** 21–34.
29. Dukan, S. & Nystrom, T. (1998) *Genes Dev.* **1,** 3431–3441.
30. Dean, R. T., Gieseg, S. & Davies, M. J. (1993) *Trends Biochem. Sci.* **18,** 437–441.
31. Lu, Z., Cabiscol, E., Obradors, N., Tamarit, J., Ros, J., Aguilar, J. & Lin, E. C. C. (1998) *J. Biol. Chem.* **273,** 8308–8316.
32. Campos-García, J., Ordoñez, L. G. & Soberón-Chávez, G. (2000) *Microbiology* **146,** 1429–1435.
33. Heydary, A. R., Wu, B., Takahashi, R., Strong, R. & Richardson A. (1993) *Mol. Cell. Biol.* **13,** 2909–2918.
34. Wheeler, J., Bieschke, E. T. & Tower, J. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 10408–10412.