Redox-Dependent Regulation of the Conformation and Function of Human Heat Shock Factor 1†

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Received November 21, 2001; Revised Manuscript Received December 26, 2001

ABSTRACT: We present here evidence that redox-dependent thiol−disulfide exchange can provide a mechanism regulating the conformation and activity of the human heat shock transcription factor 1 (HSF1). Diamide and dithiothreitol were reagents used respectively to promote and reverse disulfide cross-link, and the resolution and detection of redox conformers of HSF1 were done according to recently published methods [Manalo, D. J., and Liu, A. Y.-C. (2001) *J. Biol. Chem. 276*, 23554−23561]. We showed that preincubation of the latent HSF1 monomer with diamide inhibited the in vitro heat-induced activation and trimerization of HSF1 and caused the formation of ox-hHSF1, a compact, disulfide cross-linked HSF1 conformer detectable in immuno-Western blot assay. These effects of diamide were dose-dependent and were rapidly and quantitatively reversed by dithiothreitol. Cysteine site-specific mutants of HSF1 were constructed and used to determine which of the five cysteine residues may be engaged in disulfide cross-link. Analysis of the in vitro transcribed and translated HSF1 proteins showed that while mutation of C1 (amino acid 36) and C2 (amino acid 103) had no effect on the redox sensitivity of HSF1, the mutation of C3 (amino acid 153) or double mutation of C4 and C5 (amino acids 373 and 378, respectively) to serine rendered HSF1 insensitive to diamide and prevented its conversion to ox-HSF1. HSF1 with a single cysteine to serine mutation at either the C4 or C5 position gave different ox-HSF1 conformers in the presence of diamide, suggesting that C3 could be disulfide cross-linked to either C4 or C5. The possibility that HSF1 may have integrated redox chemistry of cysteine sulfhydryl into its functional response in higher mammalian cells is discussed.

HSF1,[1] a member of the multigene heat shock factor family, is the principal heat shock transcription factor that mediates the cellular response to heat and other forms of stress. In higher eukaryotes, HSF1 is constitutively expressed as a latent monomer in the cytosol under unstressed conditions (*1*−*3*). The stress-induced activation of HSF1 is a multistep process that includes trimerization, gain of DNA-binding and transactivating activities, nuclear translocation, and phosphorylation of the protein; each of these steps may be regulated independently (*1*−*7*).

Studies on mutant HSF1 proteins have provided useful mechanistic information on the trimerization and activation of HSF1. For example, it has been shown that deleting or changing the carboxyl-terminal hydrophobic heptad repeat (LZ 4) of both the human and *Drosophila* HSF results in a constitutively active HSF trimer, suggesting that this domain may suppress trimerization by engaging in intramolecular coiled-coil interaction with the amino-terminal heptad repeat (LZ 1, 2/3) (*7*−*11*). Upon heat shock, forces that stabilize this intramolecular coiled-coil structure are presumably disrupted, and HSF1 is converted to a homotrimer stabilized by an intermolecular α-helical coiled-coil hydrophobic interaction (*7*, *8*). Other domains of the HSF1 protein and the cellular environment may affect the activation of HSF1 as well. Thus, activation of HSF1 can be modulated by a linker region that is C-terminal to the DNA-binding domain (*4*, *12*) and by the interaction of HSF1 with molecular chaperones (*13*, *14*) or a novel nuclear HSF-binding protein (*15*). Evidence that the activation of HSF1 may be modulated by cellular factors includes the following: (a) overexpression of HSF1 results in its constitutive activation (*6*, *7*); (b) expression of HSF1 in a heterologous cell system results in the reprogramming of the heat shock temperature to that of the host cells (*16*); (c) altering the temperature at which cells are grown prior to heat shock modulates the magnitude, temporal pattern, and temperature dependence of the activation of HSF1 (*17*); and (d) HSF1 can be activated in vitro by low pH and NP-40 (*18*). These results suggest that while the conversion from intra- to intermolecular hydrophobic interaction may drive the trimerization and activation of HSF1, other factors can modulate this activation. Clearly, the signaling mechanism(s) and molecular trigger(s) involved

† This work was supported in part by grants from the National Science Foundation (MCB 99-86189) and the Busch Research Foundation.

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[1] Abbreviations: HSF1, heat shock factor 1; ox-HSF1, oxidized HSF1; HSE, heat shock element; EMSA, electrophoretic mobility shift assay; DTT, dithiothreitol; SDS−PAGE, sodium docecyl sulfate−polyacrylamide gel electrophoresis.

10.1021/bi0159682 CCC: $22.00
Published on Web 01/31/2002

in the trimerization and activation of HSF1 are important but yet to be elucidated issues.

We have been interested in studying the role of redox in the regulation of HSF1 (*19*) and have recently established an experimental protocol to resolve and quantitate redox conformers of HSF1 (*20*). In this study, we report the creation and use of cysteine site-specific mutants of the human HSF1 protein to identify cysteine residues that participate in disulfide cross-links and to gauge the through-space distance of these cysteines in the native HSF1 protein.

EXPERIMENTAL PROCEDURES

Materials. Restriction enzymes were from New England Biolabs, Inc. The plasmid pJC20(HSF1) was from Dr. Carl Wu's laboratory of the National Institutes of Health (*21*). The mMessage-mMachine T7-Transcripton kit was from Ambion, Inc. Rabbit reticulocyte lysate for in vitro translation was from Promega, Inc. [^{35}S]Methionine (specific activity 1016 Ci/mmol; 10 mCi/mL) was obtained from Amersham Co. Diamide [diazenedicarboxylic acid bis(*N*,*N*-dimethylamide)], dithiothreitol, and β-mercaptoethanol were from Sigma or Pierce Chemical Co.

Cell Culture and Preparation and Handling of Cell Extracts. HeLa cells were grown as monolayer cultures at 37 °C in Dulbecco's-modified Eagle's medium supplemented with 10% fetal bovine serum and 50 units/mL penicillin plus 50 μg/mL streptomycin. The condition used for heat shock of cells was 42 °C for 60 min.

Whole cell and S100 cell extracts were prepared according to methods previously described (*20*). All buffers used for preparation and dialysis of cell extract contained 0.5 mM DTT. Extracts were aliquoted and stored at −70 °C until use. Protein concentration was determined using the BCA protein assay kit obtained from Pierce Chemical Co.

PCR-Mediated Mutagenesis and Plasmid Constructs. PCR-mediated mutagenesis of each of the five cysteine residues of HSF1 (C1−5, corresponding to cysteines at amino acid positions 36, 103, 153, 373, and 378 of the human HSF1, respectively) was done using a PE9600 Perkin-Elmer thermal cycler. Each cDNA mutant construct required four oligonucleotide primers: two primers containing restriction enzyme sites for subcloning purposes and two primers containing the sense and antisense mutation codon. For the first round of amplification, a restriction enzyme site and a mutant oligonucleotide primer were used to amplify each of the two pieces of DNA 5′ and 3′ of the intended mutation. The two amplified DNA fragments were gel purified, combined, and mixed with the two restriction enzyme site primers for PCR-mediated ligation and amplification. The C3 and C4 mutant each contained a new restriction site marker: the C3 mutant contained a new *Nco*I site (CCATGG) as a result of mutating cysteine to serine, and the C4 mutant had an engineered *Xho*I site (in bold italics in the primer sequence listed below) which simultaneously converted codon 369 from threonine (ACC) to serine (AGC). The following primers were used:

Restriction site oligonucleotide primers (the restriction enzyme site is underlined): C1T, C2Y, and C3S, sense primer (*Pfl*MI.5′) 5′-TTCCT GACCA AGCTG TGGA-3′ and antisense primer (*Nco*I.3′) 5′-GGGGG AGGCC ATGGG CT-3′; C4S and C5S, sense primer (*Nco*I.5′) 5′-TCCTG CCAGC CCCAT GGCCT-3′ and antisense primer (*Bgl*II.3′) 5′-GTTTA TAGAT CTCTG CCT-3′.

Mutant codon primers (mutant codons are underlined and in bold): C1 mutation (Cys36 to Thr36), sense 5′-ACGCG CTCAT C**ACC**T GGAGC CCGA-3′ and antisense 5′-TCGGG CTCCA **GGT**GA TGAGC GCGT-3′; C2 mutation (Cys103 to Tyr103), sense 5′-AGCAC CCA**TA** **C**TTCC TGCGT-3′ and antisense 5′-ACGCA GGAA**G** **TA**TGG GTGCT-3′; C3 mutation (Cys153 to Ser153), sense 5′-GGAAG CAGGA G**TCC**A TGGAC TCCA-3′ and antisense 5′-TGGAG TCCAT **GGA**CT CCTGC TT-3′; C4 mutation (Cys373 to Ser373), sense 5′-ACCTC ***GAG***CC CTGAA AAG**TC** **C**CTCA GCGTA-3′ and antisense 5′-TACGC TGAG**G** **GA**CTT TTCAG GG***CTC*** GAGGT-3′; C4,5 mutation (Cys373/Cys378 to Ser373/Ser378), sense 5′-TGAAA AGT**TC** **C**CTCA GCGTA GCC**TC** **C**CTGG ACAAG AAT-3′ and antisense 5′-TGTCC AG**GGA** GGCTA CGCTG AG**GGA** ACTTT TCA-3′; C5 mutation (Cys378 to Ser378), C5S is a subclone of the C4,5S construct.

The PCR-produced mutant HSF1 DNA fragments were restriction enzyme digested and inserted, casette style, into the wild-type pJC20(HSF1) plasmid DNA. For this, the PCR-amplified products of the C1T and C2Y mutations were digested with *Pfl*MI and *Nco*I restriction enzymes and subcloned into the wild-type pJC(HSF1)(*Pfl*MI/*Nco*I) plasmid vector. The C3S PCR fragment was digested with *Pfl*MI and *Sph*I restriction enzymes, subcloned into the pJC(HSF1)-(*Pfl*MI and *Sph*I) vector, and identified by *Nco*I restriction enzyme digest. The C4S and the C4,5S PCR fragment was each digested with *Nco*I and *Bgl*II restriction enzymes and subcloned into the pJC(HSF1)*(Nco*I/*Bgl*II*)* vector. To create the C5S mutant, the pJC(C4,5S) plasmid was digested with the *Bpu10*I and *Bgl*II restriction enzymes to isolate the C5S DNA fragment; this was subcloned into the *Bpu10*I/*Bgl*II-restricted pJC(HSF1) wild-type plasmid DNA. Transformation was done using the $CaCl_2$ precipitation method (*22*) with competent *Escherichia coli* BL-21(DE3) cells. All constructs were subjected to restriction enzyme mapping to ascertain the insert's orientation and size relative to the wild-type HSF1 DNA and were sequenced to confirm the intended mutations.

In vitro transcription, driven by the T7 promoter of the cloning vector, was done using the mMessage-mMachine T7-Transcription kit (Ambion Inc.) according to the manufacturer's instruction. All transcripts were analyzed on a 1% agarose gel to compare transcriptional efficiency of the constructs and to assess transcript size. We note that all constructs supported similarly robust transcription and yielded a transcript of ∼2.0 kb. In vitro translation of RNA was done in a 50% rabbit reticulocyte lysate mixture and, when indicated, in the presence of ∼1 μCi/μL [^{35}S]methionine (specific activity 1016 Ci/mmol; 10 mCi/mL) at 30 °C for 30 min. Translation efficiency was checked by trichloroacetic acid precipitation (generally 20−25K cpm/μL of translation mix), and translation fidelity was determined by SDS−PAGE analysis of the ^{35}S-labeled translated products; these parameters were similar for all HSF1 constructs. The translated HSF1 proteins were characterized with respect to their apparent molecular weight, activation of DNA-binding activity, and sensitivity to sulfhydryl-directed reagents, notably diamide and dithiothreitol. The concentration of

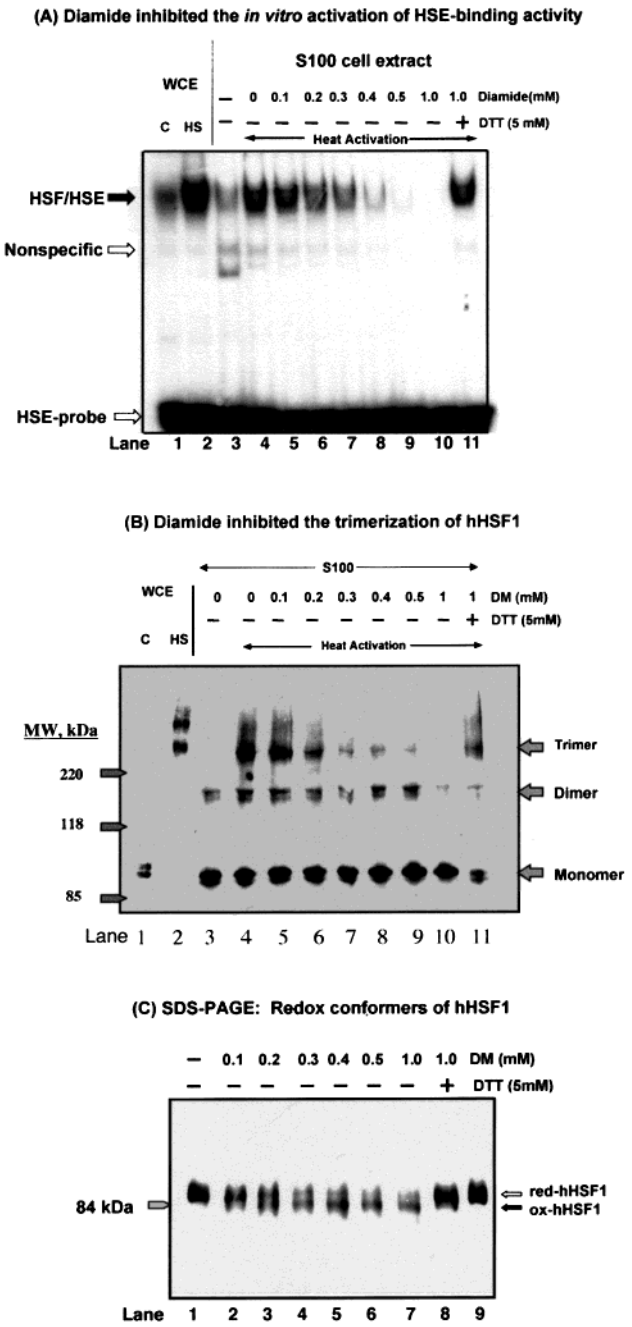

FIGURE 1: Diamide inhibited the in vitro activation and trimerization of HSF1 and promoted the formation of ox-HSF1, disulfide cross-linked, compact conformer(s) of HSF1. (A) Electrophoretic mobility shift assay of the effects of diamide treatment on the in vitro activation of HSF1 DNA-binding activity. S100 extract from control HeLa cells containing the latent HSF1 monomer was used. Aliquots of the extract containing 20 μg of protein were treated with increasing concentrations of diamide (0−1 mM; lanes 4−10) prior to subjecting the sample to in vitro heat activation (42 °C for 60 min). A sample incubated in parallel at 25 °C for 60 min served as the nonactivated control (lane 3). To test for reversibility of the effects of diamide, dithiothreitol was added to a sample (lane 11) to a final concentration of 5 mM and incubated at room temperature for 10 min prior to heat activation. For comparison, aliquots of the whole cell extract from control (lane 1) and heat-shocked (lane 2) HeLa cells were also used to assay for HSE-binding activity. The positions on the gel of the HSF−HSE complex, nonspecific protein-bound HSE, and the free HSE probe are as indicated. (B) Diamide inhibited the trimerization of HSF1. Aliquots of S100 extract were treated with concentrations of diamide as indicated. The extracts were then heat activated and oligomers of HSF1 cross-linked with glutaraldehyde according to methods described in the text. Samples were analyzed by gel electrophoresis and Western blot detection of HSF1. The positions on the gel of the HSF1 monomer, dimer, and trimer are as indicated. Lane 11 shows reversal of the effects of diamide by DTT (added to the sample prior to gel electrophoresis). For comparison, whole cell extracts from control (lane 1) and heat-shocked (lane 2) HeLa cells were included in this analyses. (C) Effects of diamide on the conformation/electrophoretic mobility of HSF1 in SDS−polyacrylamide gel. Aliquots of S100 extract treated with increasing concentrations of diamide were subjected to nonreducing SDS−PAGE and immuno-Western blot probing of HSF1. Reversal of the effects of diamide by DTT is shown in lane 8. The last sample (lane 9) represents extract mixed with a reducing (10 mM DTT), SDS sample buffer and heated at 100 °C for 5 min prior to gel electrophoresis. The positions of the reduced (red-HSF1) and oxidized (ox-HSF1) conformers of HSF1 are as indicated.

reagents used and time and temperature of incubation are as specified in the text and figure legends.

Polyacrylamide Gel Electrophoresis and Immuno-Western Blot Detection of Redox Conformers of HSF1. For determination of the effects of sulfhydryl-directed redox reagents on the conformation (as indicated by electrophoretic mobility) of HSF1, the treated samples were subjected to analysis by SDS−polyacrylamide gel electrophoresis or native polyacrylamide gel electrophoresis and immuno-Western blot detection of HSF1. Details of the experimental protocol used for the resolution, by gel electrophoresis, and detection, by immuno-Western blot probing, of redox conformers of HSF1 have been published (*20*). Briefly, aliquots of cell extracts containing 20 μg of protein were treated with a specified reagent for the concentration, time, and temperature indicated. The control and treated extracts were mixed with an equal volume of a 2× sample buffer of 125 mM Tris-HCl (pH 6.8), 20% glycerol, and 25 μg/mL bromophenol blue prior to being loaded onto the sample wells of a Bio-Rad mini-gel apparatus. For SDS−PAGE, the sample and separation gel buffer contained 2% and 0.1% SDS, respectively. For native gel electrophoresis, there was no SDS in the spacer or separation gel although it was necessary to include 0.02% SDS in the sample buffer; the omission of this SDS caused the HSF1 signal to appear as a smear in immuno-Western blots. Unless indicated otherwise, all electrophoresis buffers (sample and gel) contained no SH reducing reagents such as DTT or 2-mercaptoethanol. Gel electrophoresis was done as previously described (*22*) using either a 4% polyacrylamide spacer gel and a 5.5% separation gel or a 4% spacer gel and a 5−10% separation gel.

After electrophoresis, the polyacrylamide gel was equilibrated at 25 °C for 10 min in a buffer of 20 mM Tris (pH 8.0), 150 mM glycine, 20% methanol, and 5 mM DTT. Proteins were then transferred electrophoretically onto a nitrocellulose membrane in the same buffer that contained 1 mM DTT. Nonspecific protein binding sites on the nitrocellulose membrane were blocked by incubation of the membrane with 10% nonfat milk in a Tris−saline−Triton X-100 buffer [10 mM Tris (pH 8.0), 150 mM NaCl, and 0.1% Triton X-100]. Western blot detection of HSF1 was done using the enhanced chemiluminescence procedure of Amersham Co. with a rabbit polyclonal antibody (1:5000 dilution) that we have generated against a histidine-tagged HSF1 protein expressed in *E. coli* and affinity purified using nickel nitrilotriacetic acid (Ni-NTA; Qiagen) resin. In some experiments, the in vitro translated and [^{35}S]methionine-labeled HSF1 was directly visualized by fluorography after gel electrophoresis.

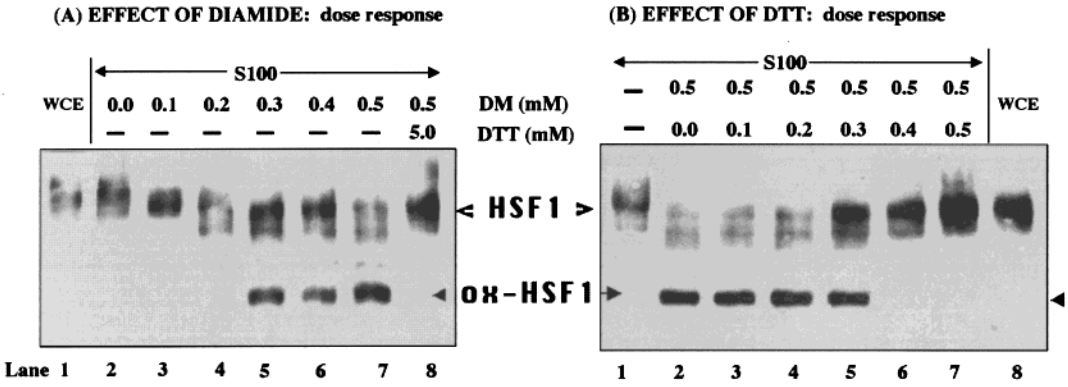

FIGURE 2: Resolution, detection, and quantitation of redox conformers of HSF1 in dose−response effects of diamide and dithiothreitol. (A) Effects of diamide. Aliquots of S100 cell extract from control HeLa cells were incubated with increasing concentrations of diamide (0−0.5 mM) as indicated at 25 °C for 10 min. To test for reversal of the effects of diamide in lane 8, DTT was added to a diamide-treated sample to a final concentration of 5 mM and incubated at 25 °C for 10 min. An aliquot of a whole cell extract (WCE) from HeLa cells was included in the experiment as a control. (B) Reversal of the effects of diamide by dithiothreitol. To aliquots of the 0.5 mM diamide-treated S100 extract, DTT was added to concentrations as specified and incubated at 25 °C for 10 min. All samples were subjected to electrophoresis in a 5.5% native polyacrylamide gel and immuno-Western blot probing for HSF1 according to methods described. The positions of the reduced and oxidized conformers of HSF1 are as indicated.

In Vitro Activation of HSF1. For assessment of the effects of mutation of cysteine residues or treatment with SH-directed reagents on the trimerization and activation of HSF1, the in vitro transcribed and translated HSF1 protein was heat activated by incubation at 42 °C for 60 min. The stoichiometry (monomer to trimer conversion) of HSF1 was determined by cross-linking of protein subunits with glutaraldehyde prior to SDS−polyacrylamide gel electrophoresis and immuno-Western blot probing for HSF1 as previously described (*19*). The DNA-binding activity was determined by electrophoretic mobility shift assay (EMSA) using a double-stranded synthetic oligonucleotide containing four inverted repeats of the NGAAN consensus heat shock element (HSE) sequence as previously described (*23*).

RESULTS

Effects of Diamide on Activation, Trimerization, and Conformation of HSF1. Figure 1 illustrates the in vitro effect of diamide, a reagent that promotes protein disulfide bond formation (*24*), on the activation, trimerization, and conformation of HSF1. We show in Figure 1A that diamide caused a dose-dependent inhibition of the in vitro activation of HSF1 and at 0.5 mM gave maximal and complete inhibition. The specificity of diamide's action was demonstrated by (1) the rapid and complete reversal of this inhibition by dithiothreitol (DTT), a sulfhydryl-reducing reagent (lane 11, Figure 1A), whereas DTT by itself was without effect, (2) the lack of effect of diamide when added after the in vitro heat activation procedure but prior to assaying for DNA-binding activity (data not shown), and (3) the absence of effect on protein binding to consensus Oct-1 and TATA sequences (*20*). Analysis of the stoichiometry of HSF1 before (Figure 1B, lane 3) and after (Figure 1B, lanes 4−11) in vitro heat activation demonstrated that diamide inhibited the trimerization of HSF1 in a dose-dependent manner. Further, the concentration of diamide needed to inhibit the activation of HSE-binding activity (Figure 1A) and trimerization (Figure 1B) was similar, a result consistent with the suggestion that trimerization of HSF1 is central to the acquisition of high-affinity binding to the HSE (*2*).

Immuno-Western blot detection of HSF1 in Figure 1C showed that diamide caused the HSF1 to assume a faster mobility in SDS−polyacrylamide gel electrophoresis. Since protein with intramolecular disulfide bonds usually migrates more rapidly during SDS−PAGE than when it is fully reduced perhaps because of a decrease in chain flexibility and hydrodynamic volume (*25*), one plausible interpretation of the result in Figure 1C is that diamide promoted intramolecular disulfide cross-link(s) of HSF1 and locked the protein in a more compact conformation. However, the resolution of the various redox conformers of HSF1 in SDS−PAGE as shown in Figure 1C was minimal, making it difficult to quantitate and relate this mobility shift to changes in the redox activity of the HSF1 protein.

In Figure 2 we showed, using native gel electrophoresis for protein separation, that diamide promoted the appearance of a distinctly high-mobility ox-HSF1. This effect of diamide was dose-dependent (Figure 2A) and was completely and readily reversed by adding dithiothreitol to the sample prior to gel electrophoresis (Figure 2B), confirming that the high-mobility ox-HSF1 is not a degraded product of HSF1. We further note that elution of the compact ox-HSF1 from the native gel followed by its reduction (10 mM DTT) and reelectrophoresis in SDS−PAGE resulted in the appearance of the normal 85−90 kDa HSF1 protein. The fact that the ox-HSF1 had a greater mobility than the reduced HSF1 in both SDS−polyacrylamide and native polyacrylamide gel electrophoresis strongly argues for intramolecular rather than intermolecular disulfide cross-linking. Together, the results in Figures 1 and 2 showed that diamide promoted formation of the disulfide cross-linked ox-HSF1 and inhibited the activation and trimerization of HSF1.

Mutation of C3 and of C4,5 Rendered HSF1 Insensitive to Redox-Dependent Regulation. To identify the cysteines engaged in this disulfide bond formation, we first evaluate the position and context of the five cysteine residues of HSF1. We note that C3 (amino acid 153) is in the midst of the amino-terminal hydrophobic domain (LZ 1,2/3) and that C4 and C5 (amino acids 373 and 378) are immediately upstream of the carboxyl-terminal hydrophobic region (LZ

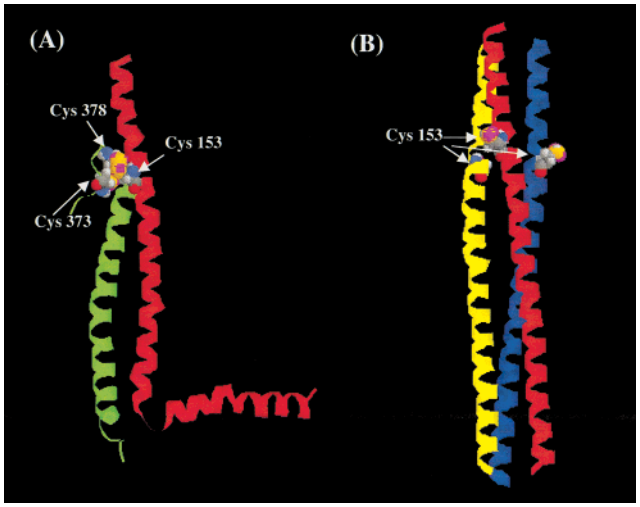

FIGURE 3: Computer-generated models of the coiled-coil domains of the HSF1 monomer and trimer. (A) Ribbon structure of amino acid residues V137− P210 (LZ 1, 2/3; red) and P370−V422 (LZ 4; green) of HSF1 as they may appear in an intramolecular coiled-coil domain of the HSF1 monomer. Cysteines 153, 373, and 378 are depicted in space-filled model and labeled. (B) Homotrimeric coiled-coil domain of residues V137−V197 (red, yellow, and blue) as they may appear in the HSF1 trimer. Preparation of the illustration: A blast search comparison of residues 137−197 of the human HSF1 against sequences in the PDB revealed that part of this sequence in HSF1 might be similar to the MHC-related Fc receptor (1exu) between residues 132 and 167. This portion of the Fc receptor contains two helices connected by a glutamine-rich turn. The highly conserved sequence Gln1181-Gln182-Gln183 of HSF1 was thus aligned with the three glutamines in the turn between the helices of the Fc receptor. The relative positions of residues illustrated in the turn and the adjacent helices of the HSF1 monomer were modeled on the coordinates of the backbone atoms in this region of the Fc receptor. The coiled-coil structure of the HSF1 monomer was constructed on the basis of coordinates of residues in the Fos-Jun heterodimer (1fos). During this process, backbone atoms of LZ 1, 2/3 (V137− P210) and LZ4 (P370−V422) of HSF1 were threaded through those of the Fos/Jun heterodimer in a fashion that maximized interactions between side chains of residues a and d. Construction of the homotrimeric coiled-coil domain of HSF1 was based on coordinates of influenza hemagglutinin (1qu1) in the region of its trimeric coiled coil. Again, backbone atoms of residues V137−V197 of HSF1 were threaded through those of the viral protein to maximize interactions between the hydrophobic side chains or residues a and d in the putative heptad repeat. As shown, the conformation of HSF1 residues 137−197 would differ considerably in the monomer and heterotrimer. All modeling was performed with the package Sybyl (Tripos Associates, St. Louis, MO).

4). Given that the amino- and carboxyl-terminal hydrophobic repeats most likely form an intramolecular coiled-coil structure in the HSF1 monomer (*8*), it would seem likely that C3 and C4,5 would be juxtaposed and capable of forming intramolecular disulfide cross-link(s) in the HSF1 monomer. Indeed, computer modeling of the α-helical coiled-coil domains of the HSF1 monomer and trimer presented in Figure 3 showed that the alignment of the N- and C-terminal hydrophobic repeats of the HSF1 monomer would bring C3 (amino acid 153) close to C4 and C5 (amino acids 373 and 378, respectively), in positions permissible for disulfide bond formation under the appropriate experimental condition. A likely consequence of this intramolecular disulfide cross-link is that HSF1 would be selectively stablilized in a compact, monomeric conformation, in a form incapable of undergoing the global conformation change necessary for the trimerization and activation of HSF1. These possibilities

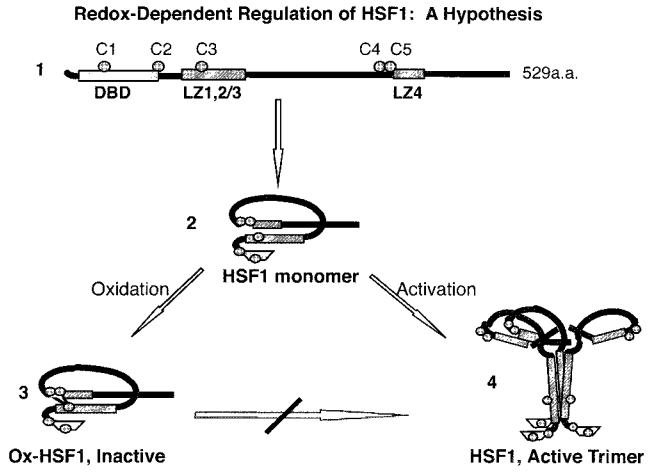

FIGURE 4: Schematic illustration of redox-dependent changes in the structure and function of HSF1. (1) Depiction of the 529 amino acid residue HSF1 and the location of the five cysteine residues relative to the functional domains of the protein (DBD, DNA-binding domain; LZ, leucine zipper). (2) The HSF1 monomer stabilized by intramolecular hydrophobic interaction. (3) The oxidized and disulfide cross-linked HSF1 that is resistant to heat activation. (4) The activated HSF1 trimer stabilized by intermolecular hydrophobic interaction. In the HSF1 monomer, LZ 1, 2/3 is expected to interact closely with LZ 4 to form a dimeric coiled-coil structure. In this model, C3 (amino acid 153) and C4/5 (amino acids 373 and 378, respectively) are likely to be adjacent, permitting them to be cross-linked in the presence of oxidizing agents. Activation of HSF1 is thought to be initiated by disruption of the intramolecular coiled-coil structure, thereby exposing the hydrophobic surfaces of the heptad repeats. Formation of a triple-helical coiled-coil structure involving V137−197 presumably drives the trimerization and activation of HSF1. Cross-linking Cys153 with Cys373 or Cys378 stabilizes the intramolecular coiled-coil structure and prevents its conversion to the intermolecular homotrimeric coiled coil.

are summarized in our working hypothesis presented in Figure 4.

To evaluate and validate our working model, we mutated each of the five cysteine residues (C1−5 at amino acid positions 36, 103, 153, 373, and 378, respectively) of HSF1 and tested the wild-type and mutant HSF1 proteins for their sensitivity to diamide. The design of the C1 and C2 mutations was to change the cysteine residue found in HSF1 to the corresponding amino acid residue found in HSF2 (*26*); thus C1 was mutated to threonine and C2 to tyrosine. In later experiments, we have also created the C1S and C2S mutants of HSF1; the behavior of these mutants toward diamide and DTT were similar to that of C1T and C2Y HSF1 mutants. C3, C4, and C5 were mutated to serine residues. Figure 5A illustrates the DNA-binding activity of the wild type and cysteine mutants of HSF1 under control (25 °C, lane 1 of each construct), in vitro heat-activated (42 °C, 60 min; lane 2), 2.0 mM diamide-treated (5 min, 25 °C) and then heat-activated (lane 3), and diamide- and DTT-treated (5 mM, 5 min at 25 °C) and then heat-activated (lane 4) conditions. While the latent HSF1 of HeLa S100 cell extract and the in vitro transcribed and translated HSF1 exhibited a slight difference in their electrophoretic mobility when bound to the HSE (perhaps because of differences in posttranslation modification), their behavior toward diamide and DTT was similar: DNA-binding activity was activated by heat; this activation was blocked by pretreatment with diamide, which in turn was reversed by dithiothreitol. The behavior of the

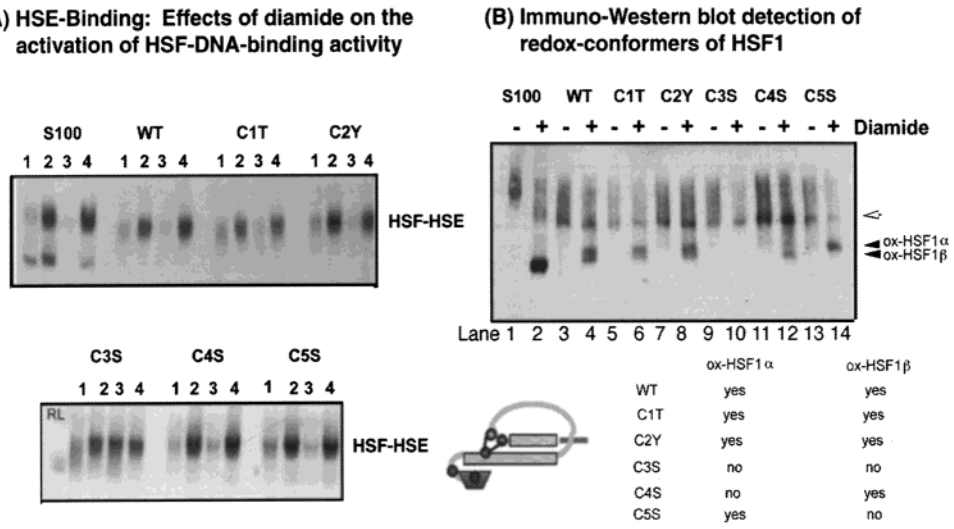

FIGURE 5: Effects of diamide on the in vitro activation and conformation of the wild type and cysteine mutants of HSF1. (A) EMSA of HSE-binding activity. The wild type and C1T, C2Y, C3S, C4S, and C5S mutants of HSF1 were transcribed and translated in vitro according to methods described in the text. HSE-binding activity of the wild type and cysteine mutants of HSF1 was determined under control (25 °C; lane 1 of each construct), in vitro heat-activated (42 °C, 60 min; lane 2), diamide-treated (2 mM, 5 min at 25 °C) and then heat-activated (lane 3), and diamide- and DTT-treated (5 mM, 5 min at 25 °C) and then heat activated (lane 4) conditions. For comparison, aliquots of HeLa S100 cell extract treated under identical conditions were included in this experiment. RL: reticulocyte lysate control. The position of the specific HSF−HSE complexes is as indicated. [Note: The difference in mobility of the HSF−HSE complex of the S100 extract versus the in vitro transcribed and translated HSF1 protein most likely is due to difference(s) in posttranslational modification.] (B) Resolution and detection of redox conformers of HSF1. The in vitro transcribed and translated HSF1 protein, without and with diamide treatment (2 mM, 5 min at 25 °C), was subjected to native gel electrophoresis and immuno-Western blot probing according to methods described. For comparison, S100 extract from HeLa cells was included in lanes 1 and 2. The positions of the disulfide cross-linked conformers of HSF1, ox-HSF1α and ox-HSF1*β*, are as indicated. Both the α and *β* ox-HSF1 were detected in the diamide-treated wild-type (lane 4), C1T (lane 6), and C2Y (lane 8) HSF1 protein, and both were absent in the diamide-treated C3S mutant HSF1 (lane 10). Of the two oxidized HSF1 conformers, the α form was absent in the C4S mutant (lane 12) and the *β* form was absent in the C5S mutant (lane 14). These results are summarized in a table format below the fluorogram.

C1T, C2Y, C4S, and C5S HSF1 mutants toward these reagents and treatment conditions was identical to that of the wild-type HSF1. Significantly, the behavior of the C3S mutant toward diamide was different; Figure 5A showed that activation of the DNA-binding activity of C3S HSF1 was unaffected by pretreatment with diamide.

The result of immuno-Western blot probing of the in vitro transcribed and translated wild-type and mutant HSF1 proteins without and with diamide treatment is shown in Figure 5B. For comparison, control and diamide-pretreated HeLa S100 extract were included in this experiment. We showed that diamide promoted the formation of the ox-HSF1 conformer in the wild-type HSF1 and the C1, C2, C4, and C5 cysteine site-specific mutants of HSF1. Upon closer examination, it was apparent that there were two ox-HSF1 in the diamide-treated wild-type HSF1, labeled as ox-HSF1α and ox-HSF1*β* (Figure 5B, lane 4). The ability of diamide to promote the formation of compact conformers of HSF1 was unaffected by the mutation of the C1 or C2 residues, suggesting that these cysteine residues were not targeted by diamide. On the other hand, formation of ox-HSF1 was critically dependent on C3 (amino acid 153), such that its mutation to serine rendered HSF1 insensitive to diamide. Interestingly, the ox-HSF1α conformer was absent in the diamide-treated C4S mutant of HSF1 (lane 12), whereas the ox-HSF1*β* conformer was absent in the diamide-treated C5S mutant (lane 14). These results, summarized in a tabular format in Figure 5B, suggested to us that C3 could form an intramolecular disulfide cross-link with either the C4 or C5 residue of HSF1. Disulfide cross-link between C3 and C4 yielded the ox-HSF1α, whereas cross-link between C3 and C5 gave the ox-HSF1*β* conformer.

To further confirm this interpretation, we constructed a mutant of HSF1 with both the fourth and fifth cysteine residues (amino acids 373 and 378) changed to serine. The effect of diamide treatment on the activation of HSF1 DNA-binding activity determined by EMSA is shown in Figure 6A, and the effects on electrophoretic mobility and conformation of HSF1 are shown in Figure 6B. As expected, diamide inhibited the activation of DNA-binding activity of the wild-type HSF1 and the C1T and C2Y mutants of the HSF1 protein and concomitantly promoted the appearance of a compact ^{35}S-labeled ox-HSF1 when analyzed in native gel electrophoresis (Figure 6B). Mutation of C3 (lane 8) or of C4,5 (lane 10) to serine rendered the protein completely insensitive to the effects of diamide. It may be noted that while the results in Figures 5B and 6B are qualitatively similar, Figure 5B is the result of immuno-Western blot detection of HSF1 whereas Figure 6B is a direct visualization of the in vitro translated [^{35}S]methionine HSF1. This comparison validates the reliability and quantitative nature of immuno-Western blot probing for HSF1 under our experimental conditions. Preliminary studies with two other mutants of HSF1 in which all cysteine residues were mutated with the exception of either the C3 and C4 pair or the C3 and C5 pair showed that diamide promoted intramolecular disulfide cross-link in both. Together, these results suggest that the C3 and C4/C5 residues of HSF1 are necessary and sufficient to form an intramolecular disulfide cross-link under an oxidizing condition.

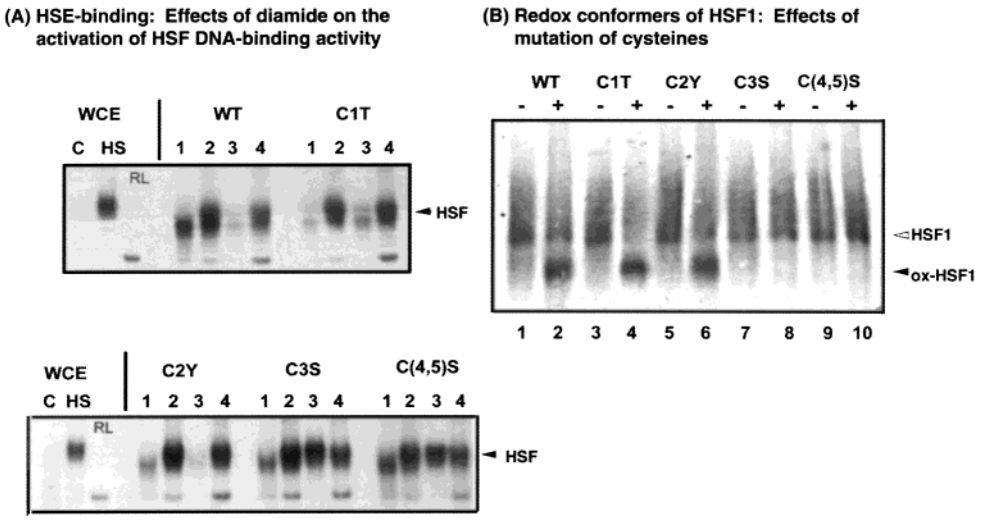

FIGURE 6: Double mutation of the cysteines at amino acid positions 373 (C4) and 378 (C5) of HSF1 to serine rendered HSF1 insensitive to the effects of diamide. (A) EMSA of HSE-binding activity. The wild type and C1T, C2Y, C3S, and C(4,5)S mutants of HSF1 were transcribed and translated in vitro according to methods described. For each of the HSF1 constructs, the following applies: lane 1, control (25 °C); lane 2, in vitro heat activated (42 °C, 60 min); lane 3, 2.0 mM diamide treated (5 min, 25 °C) and then heat activated; lane 4, diamide- and DTT-treated (5 mM, 5 min at 25 °C) and then heat activated. Aliquots of the whole cell extract from control (C) and heat-shocked (HS; 42 °C for 60 min) HeLa cells were included in this experiment for comparison. RL: reticulocyte lysate control (without RNA). (B) Redox conformers of HSF1. In vitro translation was carried out in the presence of [^{35}S]methionine. Diamide (2 mM) was then added to the translation mixture, and the mixture was incubated for 5 min at 25 °C. Samples were subjected to native gel electrophoresis, and the presence of ^{35}S-labeled protein was detected by fluorography. The positions of the reduced and oxidized HSF1 are indicated by an open and filled arrowhead, respectively.

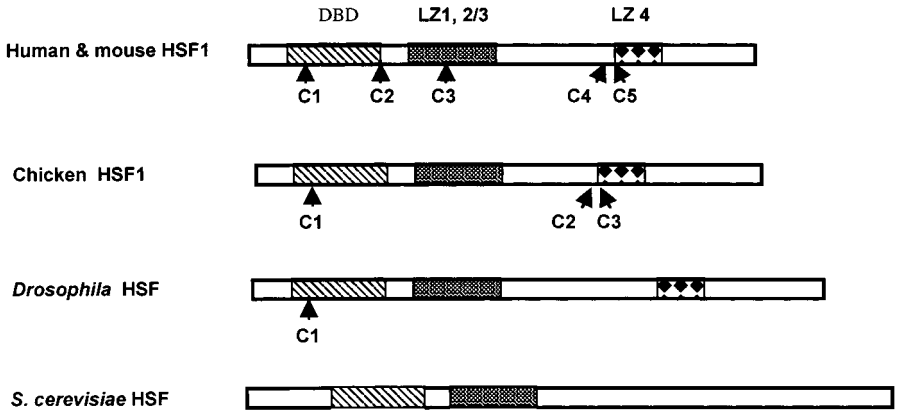

FIGURE 7: Diagrammatic illustration of the occurrence and placement of cysteines in HSFs from human, mouse, chicken, *Drosophila*, and yeast. The occurrence of cysteines (numbered from the N- to C-terminus of the protein) relative to the three functional domains of HSF, the DNA-binding domain (DBD; hatched box), the amino-terminal heptad repeat (LZ 1, 2/3; stippled box), and the carboxyl-terminal heptad repeat (LZ 4; diamond box), is as indicated.

DISCUSSION

It has long been suggested that thiol−disulfide exchange represents a redox-dependent, posttranslational modification mechanism that can regulate protein structure and function (*27*−*29*). The observation that reduction of the three cysteine bridges in bovine pancreatic trypsin inhibitor (BPTI) leads to its complete unfolding even in the absence of denaturants (*30*, *31*) and that a triple disulfide variant of the phage T4 lysozyme is significantly more stable and unfolds at a temperature 23.4 °C higher than that of the wild-type lysozyme (*32*) clearly demonstrates the importance of disulfide bond, perhaps by decreasing the entropy of the unfolded state, in the stability of protein structure. The notion that redox can provide a mechanism to regulate transcription factor activity has been clearly demonstrated in *OxyR*, a transcription factor that mediates the prokaryotic cell's response to peroxide/oxidative stress (*33*, *34*). Similarly, the structure and function of RsrA, an anti-sigma factor, changes as a function of its redox environment; an oxidizing environment promotes intramolecular disulfide bond formation in RsrA, causing its dissociation from the SigR factor, thus allowing for σ^R-dependent transcription to occur (*25*). These observations, together with the awareness that protein cysteine thiols confer responsiveness not just to O_2 and its reactive derivatives but to nitrogen radicals, nitric oxide, and its redox-related species as well (*35*), strongly argue for a fundamentally important role of cysteine redox centers in biological regulation.

In this study, we showed that cysteines of HSF1 can undergo redox-dependent disulfide cross-link; specifically, cysteine 153 (C3) of HSF1 can be reversibly disulfide cross-

linked to cysteine 373 or 378 (C4 and C5, respectively). This intramolecular disulfide cross-link selectively stabilizes HSF1 in a compact, monomeric form that is resistant to the heat-induced trimerization and activation. An examination of the contextual sequence of the cysteine residues of HSF1 shows that C3 (QECM) and C4 (EKCL) of HSF1 are bracketed by acidic and/or basic amino acids, residues that can enhance the reactivity of cysteines and act as acid−base catalysts for nitrosylation−denitrosylation of cysteine(s) (*35*−*37*). Nitrosylation of C3 (or perhaps of C4) would accelerate disulfide formation when in the immediate vicinity of an intramolecular thiol; i.e., the high effective concentration of vicinal thiols drives disulfide bond formation (*36*, *37*). This suggestion is consistent with our observation that HSF1 undergoes nitrosylation-dependent intramolecular disulfide cross-link (*20*).

Our results have other implications on the structure and regulation of the human HSF1 protein as well: (1) The experimental demonstration of disulfide cross-link between C3 and C4,5 helps in the structural alignment of the C-terminal hydrophobic repeat (LC4) with the much larger amino-terminal hydrophobic region (LZ 1, 2/3) and provides a framework to better understand the native structure of the HSF1 monomer. (2) We note that all cysteine mutants of HSF1 could be activated and bind DNA in vitro, suggesting that the cysteines are not required for the activation and DNA-binding function of HSF1. Rather, the cysteines residues, particularly C3, C4, and C5, most likely provide an off-switch, triggered by oxidation and disulfide cross-link, to prevent the activation of HSF1.

In considering redox as a mechanism regulating the function and activity of HSF1, a conundrum is that the five cysteines present in the human HSF1 protein are not evolutionarily conserved. A comparison of the occurrence and placement of cysteine residues in HSFs from human (*21*), mouse (*38*), chicken (*39*), *Drosophila* (*40*), and yeast (*41*), as schematically illustrated in Figure 7, shows that the number of cysteine residues in HSF correlates with the evolutionary hierarchy of the species: human and mouse HSF1 each have five, chicken has three, *Drosophila* has one, and yeast has none. Interestingly, we note that the yeast HSF, a protein devoid of cysteine residues, exhibits constitutive DNA-binding activity (*2*), whereas the *Drosophila* HSF, which has a single cysteine residue that maps to the DNA-binding domain and is normally regulated in a stress-dependent manner in *Drosophila* cells, becomes a constitutively active DNA-binding trimer when expressed in human cells (*16*). Perhaps the presence of cysteines in HSF1, notably C3,4,5 of the human (*21*) and mouse (*38*) HSF1, and their ability to undergo thiol−disulfide exchange in response to changes in the redox environment may offer a safeguard against the improper or inadvertent activation of HSF1. The flip side of this hypothesis is that thiol−disulfide exchange can limit or attenuate the activation of HSF1 under particular experimental or physiological conditions. In this context, we note that heat-induced activation of HSF1 is attenuated in a number of model systems for aging (*42* and references cited therein). Given the implicit importance of oxidative damage in the biology of aging (*43*), it is possible that oxidation and intramolecular disulfide cross-link of HSF1 may contribute to the attenuated heat shock transcriptional response in aging human cells. This is being investigated in our laboratory.

ACKNOWLEDGMENT

We thank Dr. Carl Wu of the National Cancer Institute, NIH, for providing us with the pJC20(HSF1) plasmid DNA. We are grateful for the help of Dr. William B. Moyle of the UMDNJ-Robert Wood Johnson Medical School in constructing the HSF1 model.

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BI0159682