Site-Directed Mutagenesis of Lys[36] in Human Thioredoxin: The Highly Conserved Residue Affects Reduction Rates and Growth Stimulation but Is Not Essential for the Redox Protein's Biochemical or Biological Properties†

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Received July 26, 1994; Revised Manuscript Received December 20, 1994⊗

ABSTRACT: Previous studies have demonstrated that a recombinant form of the human redox protein thioredoxin can stimulate the growth rate of Swiss 3T3 murine fibroblasts and that this ability to promote cellular proliferation was dependent upon a redox-active form. A site-directed mutagenesis study of the highly conserved Lys[36] adjacent to the two active site cysteines of thioredoxin was performed to determine whether the basic residue was essential for the biochemical and mitogenic properties of human thioredoxin. Two mutants were generated in which the lysine residue was replaced with either glutamic acid (K36E) or leucine (K36L). While K36E and K36L were both redox-active in a thioredoxin-specific assay, the mutants exhibited decreased affinities for thioredoxin reductase relative to wild-type thioredoxin since their respective K_M values increased by a factor of 5 and 7. Examination of the secondary structure of the variants by circular dichroism spectroscopy revealed that both mutants had minor variations in the overall structural content when compared to thioredoxin, with K36L being most similar to the wild-type protein. Thermal equilibrium denaturation studies of the variants showed that K36E had a T_M of 69.5 °C. A T_M value for thioredoxin and K36L could not be established because the absence of a plateau above 83 °C rendered it difficult to establish an upper base line and, hence, the T_M. The two mutants were able to stimulate cellular proliferation, albeit with reduced efficiency when compared with wild-type thioredoxin. The results from this study indicate that Lys[36] is not essential for the biochemical or biological properties of human thioredoxin but removal of the positive charge does decrease the overall efficiency of thioredoxin-mediated events.

The intracellular redox environment is maintained by systems which utilize reducing equivalents (e.g., from NADPH) to reduce peptide thiols. One of these is the ubiquitous redox system comprised of thioredoxin and thioredoxin reductase (TR),[1] a flavoenzyme (EC 1.6.4.5). Human thioredoxin is an 11.5 kDa protein, and the active site is highly conserved among bacterial, plant, and vertebrate forms, with the consensus sequence Trp-Cys-Gly-Pro-Cys-Lys. The two vicinal half-cysteines in the active site of oxidized thioredoxin (thioredoxin-S_2) are reduced in an NADPH-dependent reaction catalyzed by TR, the final product of which [thioredoxin-$(SH)_2$] can reduce protein disulfides [for the most recent review, see Holmgren (1985)].

Studies have shown that thioredoxin can function *in vitro* as a cofactor in the reduction of such proteins as ribonucleotide reductase and methionine sulfoxide reductase (Laurent *et al.*, 1964; Gonzalez Porque *et al.*, 1970). This redox protein is capable of catalyzing the *in vitro* folding of proteins by a mechanism similar to that of protein disulfide isomerase (Lundström & Holmgren, 1990), the latter of which contains two thioredoxin-like active site domains [for a review, see Noiva and Lennarz (1992)]. Thioredoxin has also been found to reduce the nuclear redox factor, Ref-1, which in turn modulates the activity of AP-1 through reduction of cysteines present in the transcription factor (Abate *et al.*, 1990; Okuno *et al.*, 1993). Similar observations of redox regulation of transcription factor activity by thioredoxin have been made with TFIIIC (Cromlish *el al.*, 1989), BZLF1 (Bannister *et al.*, 1991), and NF-κB (Matthews *et al.*, 1992), as well as with steroid receptors (Grippo *et al.*, 1983; Peleg *et al.*, 1989).

While the above functions can be categorized as intracellular processes, two extracellular functions for thioredoxin have currently been established. Thioredoxin has been shown to be identical to the leukemic autocrine growth factor, ADF (Tagaya *et al.*, 1989, Deiss & Kimchi, 1991; Gasdaska *et al.*, 1994). Subsequent studies have found that thioredoxin can promote the growth of hepatoma cell lines (Nakamura *et al.*, 1992) and the transcript levels of thioredoxin were found to be elevated in some human solid lung tumors (Gasdaska *et al.*, 1994). We have found that recombinant human thioredoxin in mitogenic for Swiss 3T3 murine fibroblasts and that this property is dependent upon a redox-active form of the protein (Oblong *et al.*, 1994a). However, a 14-mer peptide spanning the active site of human thioredoxin failed to stimulate cellular proliferation, indicating that structural information in addition to that of an intact active site is more than likely required for stimulating proliferation (Oblong *et al.*, 1994b). Since mammalian cell lines transfected with thioredoxin cDNA secrete the protein through a nonclassical leaderless pathway (Rubartelli *et al.*, 1992), it

† This work was supported by NIH Grant CA 42286 (G.P.) and by NIH Training Grant in Cancer Biology CA 09213 (J.E.O.).

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⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1995.

[1] Abbreviations: ADF, adult T-cell leukemia-derived factor; CD, circular dichroism; DTT, dithiothreitol; FBS, fetal bovine serum; TR, thioredoxin reductase.

0006-2960/95/0434-3319$09.00/0

is possible that thioredoxin could be a general autocrine growth factor, even though some mammalian cell lines fail to respond in culture to thioredoxin.[2]

Extracellular thioredoxin has also been identified as a component in the "early pregnancy factor" system, a complex array of factors present in the sera of pregnant mammals which are capable of modifying lymphocytes (Clarke *et al.*, 1991). A recent mutagenesis study of human thioredoxin has determined that the active site Cys^{32} and Cys^{35} were not essential for this function but Cys^{74} was (Tonissen *et al.*, 1993). The latter is one of three non-active site cysteines (including Cys^{63} and Cys^{70}), all of which have been suggested to function in a structural capacity in eukaryotic homologues (Holmgren, 1985).

Human thioredoxin contains a highly conserved lysine residue at position 36, adjacent to the active site Cys^{35}. In this study, we wished to address several questions. First, what was the effect of mutagenesis at this position on human thioredoxin's reacivity with human TR? Additionally, was Lys^{36} essential for the growth factor properties of human thioredoxin? Since it has been previously shown from equilibrium denaturation studies that bacterial thioredoxin was a thermally stable protein, we also determined what effect the mutations would have on the overall structural stability of human thioredoxin as monitored by biochemical activity. Two mutant constructs of human thioredoxin were generated in which Lys^{36} was replaced by either Glu or Leu (K36E and K36L, respectively). Structural comparisons with wild-type thioredoxin, the ability of these mutants to be reduced by TR, and the mitogenic properties of the mutants were examined.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis. Single-stranded DNA of the sense strand of a human thioredoxin cDNA (Gasdaska *et al.*, 1994) ligated into the pBluescript KS(+) vector (Stratagene, La Jolla, CA) was isolated by PEG precipitation using R408 as helper phage (Sambrook *et al.*, 1989). The isolated single-stranded DNA was used in the Amersham Sculptor *in vitro* mutagenesis system (Amersham, U.K.) using the oligonucleotides 5′-GTTGATCATTTCGCAAGGCCC-3′ and 5′-GTTGATCATTAAGCAAGGCCC-3′ to generate K36E and K36L, respectively. The isolated constructs were confirmed by dideoxy sequencing of base-denatured double-stranded DNA using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH). To facilitate cloning into the pET-3a expression vector (Studier *et al.*, 1990), novel *Nde*I and *Bam*HI sites were introduced at the 5′ and 3′ flanking ends, respectively, of the mutated thioredoxin cDNA sequences by oligonucleotide-directed polymerase chain reaction mutagenesis. The 240 bp *NdeI*/*Bam*HI-digested fragments containing the mutated sites were isolated separately from agarose gels and ligated into *NdeI*/*Bam*HI-digested pET-3a vector. Mutant constructs were transformed into *E. coli* BL21 cells and confirmed by dideoxy sequencing of base-denatured double-stranded DNA as above.

Expression and Purification of Thioredoxin Mutants. BL21 cells containing the pET3a::thioredoxin variant constructs were grown to mid-log phase and then induced with 5 mM IPTG for an additional 3 h. Cells were sonicated in 50 mM Tris-HCl, 30 mM NaCl, and 5 mM DTT (pH 7.5) containing 10 μg/mL DNase I (Sigma Chemicals, St. Louis, MO), and cellular debris was removed by centrifugation at 15000*g*. The supernatant was applied to a DEAE ion-exchange column (3 × 11 cm) and bound protein eluted with a 200 mL 0−500 mM NaCl gradient in 50 mM Tris-HCl, 1 mM EDTA (pH 7.5) (solution A). Fractions containing recombinant protein were pooled, concentrated in a Centriprep-10 (Amicon Corp., Beverly, MA), reduced with excess DTT, and chromatographed on a Superose 12 HR 10/30 gel filtration column (Pharmacia LKB, Piscataway, NJ) in solution A containing 200 mM NaCl.

Circular Dichroism Spectroscopy. A stoppered 1-cm path length quartz cuvette was used in an Aviv Model 60DS spectropolarimeter containing a water jacket atttached to a circulating water bath. The actual temperature was read from the instrument's temperature probe. All protein samples were desalted into 5 mM potassium phosphate (pH 7.5) on G-25 spin columns and protein levels determined using a modified Lowry assay (Peterson, 1977). Initial scans were collected at 0.5 nm intervals with a bandwidth of 1.0 nm at a constant temperature of 26 °C. The dynode voltage remained below 600 V. A total of three scans were signal-averaged from which a buffer blank scan of 5 mM potassium phosphate (pH 7.5) was subtracted and smoothed to generate the final CD spectra. The temperature was increased from 2 to 3 °C at a rate of 1 °C/min, and the sample was allowed to equilibrate for a minimum of 10 min before the value at 223 nm was recorded. The T_M for K36E was calculated by establishing a lower and upper base line (Kahn *et al.*, 1992). The distance of each collected data point from the lower base line was divided by the distance to the upper base line to determine an equilibrium constant (K_{eq}). The derived K_{eq} values were used to calculate the van't Hoff enthalpy plot. The T_M was calculated by interpolating from the point where ln K_{eq} equals zero. The estimated percentages of secondary structure were calculated from the CD spectra with the program PROSEC (PROtein SECondary structure estimator v2.1; Aviv Associates, Lakewood, NJ) based upon the method of Chang *et al.* (1978).

Kinetic Analysis of Thioredoxin/TR Reduction. Human TR was purified from placental tissue as described previously (Oblong *et al.*, 1993). An insulin reduction assay which monitors the oxidation of NADPH at 340 nm by TR in the presence of insulin and thioredoxin (Luthman & Holmgren, 1982) was used to monitor the biochemical activity of the thioredoxin variants. An example reaction contained 250 μL of 100 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, and 0.2 mg/mL bovine serum albumin buffer mixed with 3 μL of NADPH (200 μM), 30 μL of bovine insulin (80 μM), 5 μL of TR (2.5 μM), and 15 μL of thioredoxin (2.5 μM) to a final volume of 300 μL. The concentration range of thioredoxin, K36E, and K36L used for calculation of kinetic constants was 2.5−100 μM. Initial enzyme rates were monitored over a 3 min period. Thioredoxin reductase kinetic constants were calculated as described in Luthman and Holmgren (1982).

Cell Growth Stimulation Studies. Cellular proliferation was measured by the increase in cell number relative to control cultures with time. Swiss murine 3T3 fibroblast cells [(1−2) × 10^5)] were allowed to attach overnight in 35-mm culture dishes with 2 mL of DMEM containing 10% FBS,

[2] Oblong, Berggren, and Powis, unpublished results.

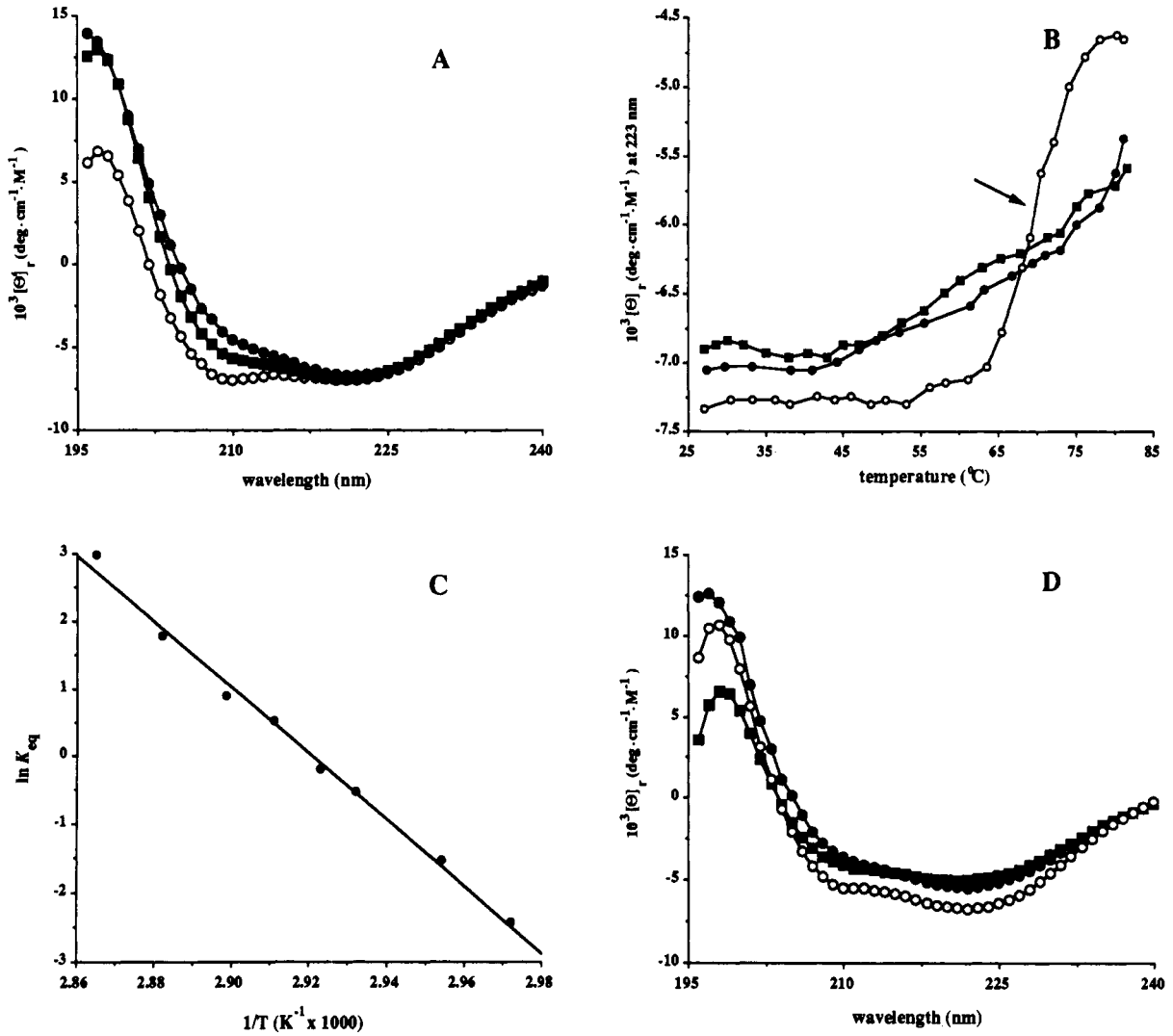

FIGURE 1: Far-UV circular dichroism spectra and thermal equilibrium denaturation of thioredoxin variants. (*Panel A*) Purified thioredoxin, K36E, and K36L (2 μM) samples collected from a final Superose 12 gel filtration column were desalted into 5 mM potassium phosphate (pH 7.5) and used to record the far-UV CD spectra at 26 °C (thioredoxin, *closed squares*; K36E, *closed circles*; and K36L, *open circles*). (*Panel B*) The effect of temperature on the ellipticity at 223 nm of 2 μM human recombinant thioredoxin, K36E, and K36L (*closed squares*, *closed circles*, and *open circles*, respectively). The arrow denotes the T_M value for K36E. (*Panel C*) The calculated van't Hoff enthalpy plot of K36E based on the change in ellipticity at 223 nm as a function of temperature. (*Panel D*) Far-UV CD spectra of the same samples used in *panels A* and *B* rescanned at 26 °C (thioredoxin, *closed squares*; K36E, *closed circles*; and K36L, *open circles*). The samples used in all panels were reduced with DTT (6 μM). The ellipticity in all panels is given as the molar ellipticity per residue.

washed with DMEM, and grown in DMEM containing 0.5% FBS for 24 h. Thioredoxin and mutant thioredoxin samples were reduced with a 3-fold excess of DTT prior to addition to the cultures for a maximal final concentration in the culture of 3 μM DTT. To each culture was added either 10% FBS, DTT (3 μM), or varying concentrations of thioredoxin variants and allowed to incubate for 48 h. Cell numbers were determined using a hemacytometer following detachment of the cells with 0.025% trypsin. All experiments were performed in triplicate for each data point.

RESULTS

Site-Directed Mutagenesis of Thioredoxin. Two specific mutants of human thioredoxin were generated at Lys[36] by site-directed mutagenesis and the recombinant forms purified from *E. coli* cellular extracts by chromatography. While the initiator Met was proteolytically removed from all recombinant forms of thioredoxin by the bacterial host system, the residue positions are numbered from the predicted amino acid sequence of the cDNA. Each of the purified samples gave a single 11.5 kDa band as seen by Coomassie blue staining on a 15% SDS−polyacrylamide gel (data not shown). The final yields of thioredoxin and K36E were 2−3 mg/L of culture whereas the yield of K36L was 0.8 mg/L of culture on average. The typical final yields of wild-type thioredoxin have been found to range from 3 to 5 mg/L of culture. At the present, we cannot explain the relatively low yields of recombinant K36L from this expression system. The p*I* of human thioredoxin, as determiend by "CHARGEPRO" (Intelligenetics, Inc., Mountain View, CA), is 4.42 whereas the p*I* values of K36E and K36L are decreased to 4.23 and 4.29, respectively. At pH 7.5, the net charges of the proteins are −6.61, −8.61, and −7.61 for thioredoxin, K36E, and K36L, respectively.

Circular Dichroism Spectroscopy. Examination of reduced thioredoxin, K36E, and K36L by far-UV CD spectroscopy showed relatively minor differences in the recorded spectra with the most distinct variation being in the spectrum of K36E (Figure 1A). The best estimates of the fractional components of the secondary structures of all three variants

Table 1: Structural Content of Human Thioredoxin Variants (%)[a]

sample	α-helix	β-sheet	turn	random coil
thioredoxin	28.91	28.42	30.08	12.59
	(27.73)	(26.85)	(30.17)	(15.25)
K36E	28.81	15.14	26.95	29.10
	(35.64)	(2.93)	(33.98)	(27.44)
K36L	32.52	18.85	29.78	18.85
	(25.39)	(15.63)	(33.49)	(25.49)

[a] Percentage values in parentheses were calculated from CD scans at 26 °C after equilibrium denaturation to 80 °C as described in the text.

Table 2: Kinetic Constants of Human Thioredoxin Reductase

substrate	K_M (μM)	V_{max}[b]
K36E	20.4	6.7
K36L	33.3	2.5
human thioredoxin	4.3[a]	10.8
E. coli thioredoxin	20.0[a]	3.1

[a] As previously reported (Oblong *et al.*, 1993). [b] Nanomoles of NADPH oxidized for minute (Luthman & Holmgren, 1982).

are presented in Table 1. As foreshadowed by the CD spectra, K36E had the greatest divergence in overall structural content from thioredoxin whereas K36L was found to be more similar to the wild-type protein. All scans were performed on samples prereduced with DTT since we have seen only minor changes in secondary structure between oxidized and reduced human thioredoxin, as previously reported (Dyson *et al.*, 1990; Forman-Kay *et al.*, 1991).

Insulin Reduction Assay with K36E and K36L. TR can reduce thioredoxin in an assay which monitors the oxidation of NADPH, contains insulin as the ultimate electron acceptor, and is NADPH-dependent (Luthman & Holmgren, 1982). Using this spectroscopic insulin reduction assay, the K_M values of human recombinant thioredoxin and *E. coli* thioredoxin for human TR have been previously reported to be 4.3 and 20.0 μM, respectively (Oblong *et al.*, 1993). When K36E and K36L were employed in the same assay, the K_M values with human TR increased to 20.4 and 33.3 μM, respectively (Table 2).

Equilibrium Denaturation of Thioredoxin Variants. The thermal denaturation profiles of K36E and K36L were compared with that of thioredoxin in order to see what effect Lys^{36} has on the structural integrity of the active site domain. A similar analysis of human recombinant thioredoxin has been performed (Oblong *et al.*, 1993) and was repeated in the present study so as to facilitate direct comparison of the denaturation profiles of all three proteins. It was not possible to establish a T_M value for thioredoxin and K36L since there was no observable transition in the denaturation curve (Figure 1B). In contrast, the thermal denaturation profile of K36E was more sigmoidal in shape, and a T_M value of 69.5 °C was determined from the van't Hoff enthalpy plot (Figure 1C). The same samples which were used to generate the thermal denaturation profiles were allowed to reequilibrate to room temperature and then rescanned at 26 °C to recored the differences in the secondary structures following the incubation up to 80 °C (Figure 1D). The rescanned CD spectra of all three thioredoxin variants were similar in shape to the original spectra, with observable differences occurring below 200 nm (compare Figure 1A with Figure 1D). The components of the secondary structures calculated from the CD spectra indicated that thioredoxin and K36L were able

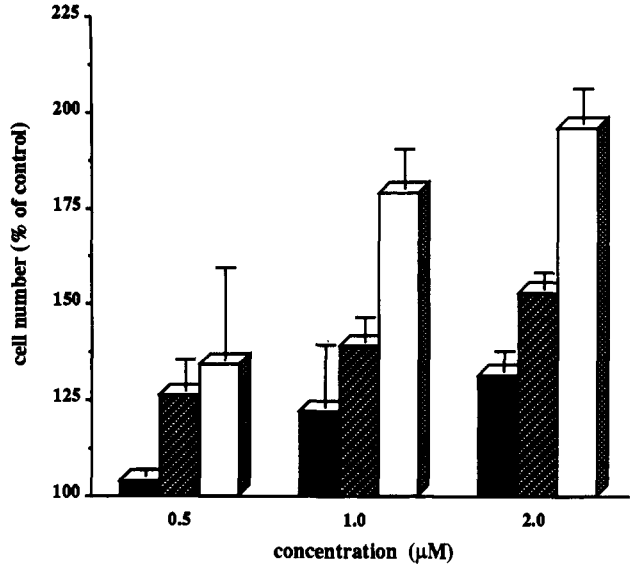

FIGURE 2: Effects of the variant forms of human recombinant thioredoxin on the cellular proliferation of Swiss 3T3 fibroblasts. The effect of the variant forms of thioredoxin on cellular proliferation was performed as described under Experimental Procedures. Cell number was determined 48 h after the addition of the indicated amounts of thioredoxin, K36E, and K36L prereduced with DTT (3.0 μM). The black, striped, and white bars represent thioredoxin, K36E, and K36L, respectively. The differences in cell numbers are expressed as a percentage relative to the number of cells in control cultures containing 3 μM DTT and 0.5% FBS alone. All data points were determined from triplicate samples.

to retain a majority of their native structures whereas K36E had a most pronounced change in the β-sheet content, which decreased from 15% to 3% (Table 1).

To determine if the structural changes induced by the high temperature altered the redox properties, thioredoxin, K36E, and K36L were incubated at 80 °C for 10 and 30 min, allowed to reequilibrate to room temperature, and assayed in the insulin reduction assay. All three functioned with the same catalytic efficiency in the assay as prior to incubation at 80 °C, indicating that the higher temperatures did not affect the biochemical properties. However, incubation at 100 °C resulted in complete inactivation of the proteins (data not shown).

Biological Activity of K36E and K36L. We examined the ability of K36E and K36L to stimulate cellualr proliferation. The dose response on the total cell number of serum-deprived Swiss 3T3 murine fibroblasts was determined with the thioredoxin variants (Figure 2). While reduced K36E and K36L were able to stimulate proliferation, it is evident that neither could stimulate to the level attained with reduced thioredoxin (Figure 2). At 1 μM concentrations, K36E and K36L stimulated to only 28% and 49% of the levels obtained with thioredoxin, respectively. The control cultures contained 0.5% FBS and DTT (3 μM), the latter of which has no effect on cellular proliferation when compared to control levels of cells incubated in the presence of 0.5% FBS alone (Oblong *et al.*, 1994a).

DISCUSSION

ADF, an autocrine growth factor which can stimulate the proliferation of leukemic cell lines (Tagaya *et al.*, 1989), has been shown to be identical to thioredoxin (Deiss & Kimchi, 1991; Gasdaska *et al.*, 1994). Using a recombinant form of human thioredoxin in growth stimulation studies, it

has been established that the protein has a mitogenic effect on murine fibroblasts and this property is dependent upon a redox-active form of the protein (Oblong *et al.*, 1994a). The thioredoxin/TR system may exert a key regulatory function for eukaryotic cell growth since it has been shown that TR is a target for inhibition by diazoquinone and doxorubicin, two quinoid anticancer drugs (Mau & Powis, 1992).

The positively charged amine group of Lys^{36} has been suggested to be critical in maintaining the thiolate anion of Cys^{32} (Kallis & Holmgren, 1980). However, pH titration curves of human thioredoxin analyzed by NMR spectroscopy have shown that the thiolate form of Cys^{32} is stabilized by interaction of the S^{γ} of Cys^{32} with the peptide backbone amide group of Cys^{35} (Forman-Kay *et al.*, 1992). Furthermore, mutagenesis of Lys^{36} to Glu in *E. coli* thioredoxin did not affect the ability of the mutant to be redox-active (Gleason *et al.*, 1990). Steady-state kinetic analysis of bacterial K36E reduction by *E. coli* TR indicated that the mutant analogue was a substrate for bacterial TR but had an increased K_M value from 3.0 to 5.6 μM (Navarro *et al.*, 1991). We have found similar biochemical results when utilizing identically mutated human thioredoxin analogues. The two mutants used in this study were redox-active, and the K_M values were increased from 4.3 for thioredoxin to 20.4 and 33.3 for K36E and K36L, respectively. Thus, Lys^{36} is not essential for the reduction of either prokaryotic or eukaryotic thioredoxn by TR, but is seemingly required for optimizing protein interactions with the flavoenzyme as evidenced by the increase in the K_M values for site-directed human mutants.

The solved crystal structure of *E. coli* K36E has shown that the largest deviation in structure from bacterial thioredoxin was apparent in the active site (Nikkola *et al.*, 1993). While the disulfide bond angles were found to be similar, the major change was due to the absence of a H-bond in K36E between Lys^{36} and the carbonyl oxygen of Thr^{30}. The CD spectral data from our mutagenesis study of Lys^{36} in human thioredoxin suggest that introduction of the hydrophobic Leu caused relatively minor changes in the structural content, presumably near the active site. In contrast, the introduction of the negatively charged group of Glu resulted in a larger deviation from the wild-type secondary structure. This suggests that the active site in human thioredoxin is more tolerant to the substitution of the positively charged residue with a neutral residue than substitution with a negatively charged one.

The CD rescan of K36E following incubation at 80 °C indicated that the observed structural changes were in large part reversible with no detectable decrease in catalytic efficiency. In general, proteins have been suggested to undergo transitions between three separate states during equilibrium denaturation. These include a reversible transition between native (N) and unfolded intermediates (U), and an irreversible transition from U to a denatured state (D) (Lepock *et al.*, 1992). Since incubation of the proteins at the elevated temperatures caused structural changes but not biochemical inactivation, the proteins presumably refolded to new native states (N′) which were biochemically very similar to N. Thus, the sigmoidal shape of the thermal denaturation curve of K36E can be interpreted as being the transition from N to U with a T_M value of 69.5 °C. Furthermore, the proteins did not irreversibly enter D at 80 °C since this would have led to biochemically inactive forms of the proteins, as occurs following incubation at 100 °C. The results from this study suggest that human thioredoxin is a thermally stable protein and mutagenesis of Lys^{36} does not seem to affect this property. It has been previously reported that *E. coli* thioredoxin is very resistant to thermal denaturation (Reutimann *et al.*, 1981) with T_M values of 86 and 75 °C for oxidized and reduced species, respectively (Sandberg *et al.*, 1991). We have found that human thioredoxin can be incubated at 80 °C for up to 17 h without any apparent decrease in catalytic efficiency in the insulin reduction assay.[2] It is intriguing that a human protein can withstand such elevated temperatures since most purified proteins resistant to denaturation at elevated temperatures without apparent loss of biochemical activity tend to be associated with organisms residing in habitats of extreme environmental conditions (i.e., *Thermophilus* bacterium). This high stability may be explained in part by the high degree of secondary structure present in the compact globular structure of thioredoxin (Kelley & Stellwagen, 1984). While bacterial thioredoxin has no structural disulfide bonds, human thioredoxin has three additional cysteine residues which have been suggested to have a structural role in the protein (Holmgren, 1985). The positively charged residue at position 36 does not have a critical function in maintaining the structural integrity of the active site at elevated temperatures. A more thorough thermal stability analysis would be beneficial for the characterization of this rather unique property of a human protein.

While Lys^{36} is not essential for the biological properties of thioredoxin, substitution with the negatively charged residue of Glu decreased the mitogenic potency of this redox protein. Presumably, one of the interactions between thioredoxin and its cellular binding site involves an electrostatic association. There is precedent for thioredoxin-like active site domains present in growth factors. It has been reported that the β-subunit of human follicle stimulating hormone contains a Trx-like active site in the receptor binding domain (Boniface & Reichert, 1990). Furthermore, using peptides of this domain, it was shown that cell signalling was dependent upon the "active-site" cysteines since redox-inactive peptides containing serines could not propagate a signal across the membrane, even though the mutant peptides could bind with the hormone's receptor (Grasso *et al.*, 1993). It is not yet clear if the target of extracellular thioredoxin is a thioredoxin-specific receptor, a surface-bound TR, or another proteinaceous factor requiring reduction of disulfides. While a membrane-bound form of TR has been reported to be present on the extracellular surface of some human cells (Schallreuter *et al.*, 1991), only a soluble form of human TR was employed in this study. Structure/function mutagenesis studies on thioredoxin utilizing biochemical and biological assays will allow for establishing whether thioredoxin-mediated stimualtion of cellular proliferation utilizes a similar reaction mechanism as that with TR.

ACKNOWLEDGMENT

We thank Edmundo Chantler for assistance in the purification of TR.

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BI941710I