A plasmid expression system for quantitative *in vivo* biotinylation of thioredoxin fusion proteins in *Escherichia coli*

Paul A. Smith[+], Brian C. Tripp[§], Elizabeth A. DiBlasio-Smith, Zhijian Lu, Edward R. LaVallie and John M. McCoy*

Genetics Institute Inc., 87 Cambridge Park Drive, Cambridge, MA 02140, USA

ABSTRACT

The high affinity binding interaction of biotin to avidin or streptavidin has been used widely in biochemistry and molecular biology, often in sensitive protein detection or protein capture applications. However, *in vitro* chemical techniques for protein biotinylation are not always successful, with some common problems being a lack of reaction specificity, inactivation of amino acid residues critical for protein function and low levels of biotin incorporation. This report describes an improved expression system for the highly specific and quantitative *in vivo* biotinylation of fusion proteins. A short 'biotinylation peptide', described previously by Schatz, is linked to the N-terminus of *Escherichia coli* thioredoxin (TrxA) to form a new protein, called BIOTRX. The 'biotinylation peptide' serves as an *in vivo* substrate mimic for *E.coli* biotin holoenzyme synthetase (BirA), an enzyme which usually performs highly selective biotinylation of *E.coli* biotin carboxyl carrier protein (BCCP). A plasmid expression vector carrying the *BIOTRX* and *birA* genes arranged as a bacterial operon can be used to obtain high level production of soluble BIOTRX and BirA proteins and, under appropriate culture conditions, BIOTRX protein produced by this system is completely biotinylated. Fusions of BIOTRX to other proteins or peptides, whether these polypeptides are linked to the C-terminus or inserted into the BIOTRX active site loop, are also quantitatively biotinylated. Both types of BIOTRX fusion can be captured efficiently on avidin/streptavidin media for purification purposes or to facilitate interaction assays. We illustrate the utility of the system by measurements of antibody and soluble receptor protein binding to BIOTRX fusions immobilized on streptavidin-conjugated BIAcore chips.

INTRODUCTION

Key features of the interaction between biotin and avidin/streptavidin are its extraordinarily high affinity (i.e. $K_d = 10^{-15}$ M) [1,2] and extremely slow dissociation rate. These properties have been exploited by researchers who have attached biotin 'tags' to proteins for purification purposes or to monitor specific protein–protein interactions [3,4]. Usually *in vitro* protein biotinylation is performed for these applications, whereby lysine residues of target proteins are modified by chemical agents such as biotinyl-*N*-hydroxysuccinimide ester [5]. A limitation of this approach, however, is that such chemical techniques may disrupt the protein conformation and/or its biological function, particularly if critical lysine residues are modified. An alternative site-specific *in vivo* biotinylation procedure would clearly be superior.

There are examples in nature of proteins which are specifically biotinylated *in vivo*. These proteins typically act as biotin transporters and are involved in metabolic carboxylate transfer processes within the cell [6]. *Escherichia coli* contains only one such biotinylated protein, the biotin carboxyl carrier protein (BCCP), a subunit of acetyl-CoA carboxylase [7]. BCCP is specifically biotinylated by biotin holoenzyme synthetase (BirA), the product of the chromosomal *birA* gene [8,9]. BirA functions by catalyzing activation of biotin by ATP to form biotin-5′-adenylate, with subsequent covalent linkage of the biotin moiety to the ε-NH₂ group of a specific BCCP lysine residue [10].

Specific biotinylation of fusion proteins *in vivo* has previously been reported by Cronan [11] and Yamano *et al.* [12]. These authors describe the construction of chimeric genes containing 'biotin acceptor domains' derived from portions of biotin acceptor-type proteins (e.g. BCCP) fused to genes encoding heterologous proteins (e.g. alkaline phosphatase, β-galactosidase). The approach has been successful in yielding biotinylated fusion proteins, but the sizes of the biotinylated domains used were large, e.g. 75–105 amino acids in length, intracellular proteolysis was problematical and the extent of biotinylation was <100%. In addition, 'biotin acceptor domains' do not function well as fusion partners when compared with other molecules typically used in this role, such as glutathione S-transferase (GST), maltose binding protein (MBP) and thioredoxin (TrxA).

A novel strategy for *in vivo* biotinylation was reported by Schatz [13], who identified a series of small 14–23 residue peptides that mimic the folded conformation of the BCCP biotin acceptor region and which are themselves substrates for biotinylation at specific lysine residues *in vivo* by *E.coli* BirA. However, this approach also has problems with low overall biotin incorporation.

We describe here a modification of the Schatz approach, whereby a biotinylation peptide is fused to the N-terminus of a

fusion partner protein with the intention of providing a universal affinity handle for the detection or capture of fusion proteins. Such an approach has recently been described for the maltose binding protein gene fusion expression system (14). Here we describe the addition of an N-terminal biotinylation peptide to *E.coli* thioredoxin to form a new fusion partner protein called BIOTRX. An important aspect of this work is inclusion of the *birA* gene as part of an operon fusion in the expression vector, resulting in quantitative *in vivo* biotinylation of the BIOTRX protein upon induction of protein expression. We show that, like thioredoxin, biotinylated BIOTRX retains the high thermal stability of native thioredoxin and its ability to be quantitatively released from bacterial cytoplasm upon osmotic shock. We also show production of two classes of protein fusions to demonstrate that, like native thioredoxin (15), BIOTRX can be used as an effective N-terminal and insertional fusion partner for soluble production of heterologous proteins in *E.coli*. We describe surface plasmon resonance experiments using BIOTRX fusions to illustrate the potential of the system to help in the study of protein–protein interactions.

MATERIALS AND METHODS

Chemicals and reagents

Chemicals and reagents were obtained from either Sigma or Fisher, unless otherwise noted. Monomeric avidin was a generous gift from Promega. Restriction enzymes, calf intestinal alkaline phosphatase, T4 DNA ligase and T4 polynucleotide kinase were from New England Biolabs. Reagents for PCR were obtained from Perkin-Elmer Cetus Instruments. DNA sequencing reagents (Sequenase 2.0 kit) were obtained from US Biochemical Corp. Avidin–alkaline phosphatase conjugate, alkaline phosphatase buffer, 5-bromo-4-chloro-3′-indolylphosphate *p*-toluidine (BCIP) and nitroblue tetrazolium chloride (NBT) were from Pierce.

Bacterial strains and plasmid construction

All expression work was performed in *E.coli* K-12 strain GI724 (15) (genotype W3110 F⁻, *lacI*q, *lacP* L8, *ampC*::λ *cI*⁺; ATCC no. 55151) or GI934 (16) (genotype GI724, *ilvG*⁺, *ompT*::*cat*, *ompP*::*kan*). Both strains contain the bacteriophage λ repressor (*cI*) gene stably integrated into the chromosomal *ampC* locus. The *cI* gene in *ampC* is under transcriptional control of an upstream synthetic *Salmonella typhimurium* tryptophan promoter. All plasmid vectors were constructed using standard techniques (17). The expression vector pBIOTRX (Fig. 1) was derived from pALtrxA-781 (18). Plasmid pALtrxA-781 is based on pUC-18 and contains the coding sequence of wild-type *E.coli* thioredoxin (*trxA*), as well as a colE1 origin of replication and the β-lactamase gene as a selectable drug resistance marker. The *trxA* gene is under transcriptional control of a bacteriophage λ pL promoter and is preceded by a λ cII ribosome binding site. The *E.coli aspA* terminator is positioned at the 3′-end of the pL transcription unit. In constructing pBIOTRX an oligonucleotide duplex encoding biotinylation peptide 81 (13), amino acid sequence MASSLR-QILDSQKMEWRSNAGGS, was inserted in-frame at the 5′-end of the *trxA* gene in pALtrxA-781 at the unique *Nde*I restriction site. pBIOTRX/IL-11, a *BIOTRX–IL-11* fusion expression vector (Fig. 1), was constructed by replacing the *trxA* gene of an existing thioredoxin–IL-11 fusion vector (15) with the *BIOTRX* gene.
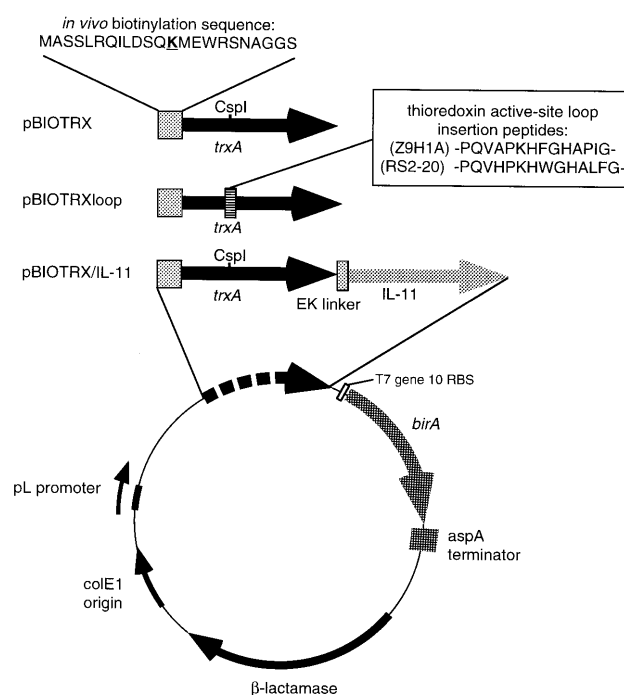
BIOTRX Expression Vectors

Figure 1. A diagram illustrating the main features and variants of BIOTRX expression vectors. pBIOTRX encodes a fusion of a biotinylation peptide sequence to the N-terminus of wild-type *E.coli* thioredoxin (TrxA). The N-terminal peptide is biotinylated *in vivo* at a lysine residue (underlined). pBIOTRXloop vectors contain internal in-frame DNA insertions into the unique *Csp*I site located within *trxA*. The inserted sequences encode two different peptides, Z9H1A and RS2-20, which interact with an anti-human IL-8 monoclonal antibody with differing affinities. pBIOTRX/IL-11 encodes a C-terminal fusion of human IL-11 to TrxA. The two domains of the fusion are connected by a linker peptide (GSGSGDDDDK), comprising a flexible glycine/serine spacer followed by an enterokinase cleavage site. The *BIOTRX*, *BIOTRXloop* and *BIOTRX–IL-11* genes are also each positioned upstream of *birA* in a series of operon fusion vectors. An intervening T7 gene10 ribosome binding site ensures efficient translation initiation of the downstream *birA* gene.

pBIOTRX-BirA (Fig. 1, GenBank accession no. AF044308), a *BIOTRX–birA* operon fusion vector, was made by insertion of the *E.coli birA* gene into pBIOTRX, downstream of the thioredoxin coding region. Oligonucleotides spanning the 5′- and 3′-ends of the coding sequence were used as primers to isolate the *birA* gene from *E.coli* genomic DNA using PCR. PCR primers each carried a *Xba*I restriction site and the 5′ primer contained in addition a bacteriophage T7 gene10 ribosome binding site, situated between the *Xba*I site and the 5′-end of the *birA* gene. Genomic DNA, prepared from *E.coli* strain W3110 (15) by a phenol extraction/CsCl gradient purification procedure, was used as the template for PCR. The resulting DNA product was then cleaved with *Xba*I and inserted downstream of the *BIOTRX* gene in pBIOTRX to create the operon fusion.

The operon fusion vector pBIOTRX/IL-11-BirA (Fig. 1) was constructed by insertion of the *birA* gene into pBIOTRX/IL-11 in a similar way to that used in construction of pBIOTRX-BirA.

For construction of BIOTRX active site loop insertion mutants (BIOTRXloop) pBIOTRX-BirA was cut at a unique site using *Csp*I (Stratagene, La Jolla, CA). The 5′-ends of the cut plasmid
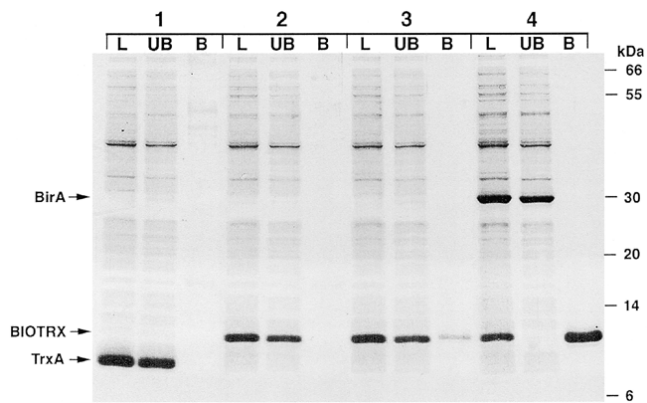
Figure 2. A 12% tricine SDS–PAGE gel demonstrating the avidin binding capacity of various thioredoxin and BIOTRX expression constructs under a variety of culture conditions. Lanes 1, induction of pALtrxA-781 (wild-type thioredoxin); lanes 2, induction of pBIOTRX; lanes 3, induction of pBIOTRX with 10 µg/ml biotin in the growth medium; lanes 4, induction of pBIOTRX-BirA with 10 µg/ml biotin supplement. Lanes L, clarified cell lysates; lanes B, avidin–agarose bound fractions; lanes UB, avidin–agarose unbound fractions. The gel was visualized by Coomassie blue staining. Bands corresponding to BirA, BIOTRX and TrxA proteins are indicated by arrows.

Figure 3. Osmotic shock release of TrxA and BIOTRX proteins from *E.coli* cells. Shown is a Coomassie blue stained 10% tricine SDS–PAGE gel. Lane A, total cellular proteins after 4 h induction of pALtrxA-781; lane B, total cellular proteins after 4 h induction of pBIOTRX-BirA with 10 µg/ml biotin in the growth medium; lane C, proteins released by osmotic shock from the cells of lane A; lane D, proteins released by osmotic shock from the cells of lane B; lane E, proteins retained within the cells of lane A following osmotic shock; lane F, proteins retained within the cells in lane B following osmotic shock. Bands corresponding to BirA, BIOTRX and TrxA proteins are indicated by arrows.

were dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN), which was later inactivated by phenol extraction. The following phosphorylated synthetic oligonucleotides, annealed to their complementary strands, were separately ligated with T4 ligase into the *Csp*I site of the cut pBIOTRX-BirA: Z9H1A, 5′-GTCCACAGGT AGCTCCAAAA CACTTCGGTC ACGCTCCAAT CG-3′; RS2-20, 5′-GTCCACAGGT ACATCCAAAA CACTGGGGTC ACGCTCTGTT CG-3′.

The two resultant BIOTRXloop proteins contain 14 amino acid insertions into the thioredoxin active site loop, with the inserted sequences being PQVAPKHFGHAPIG [BIOTRXloop(Z9H1A)] and PQVHPKHWGHALFG [BIOTRXloop(RS2-20)]. These peptide sequences are known to bind to the anti-human IL-8 monoclonal antibody hIL-8/NR7.

Expression of BIOTRX fusion proteins

Escherichia coli strain GI724 transformed with the expression vector of interest was inoculated into IMC broth [M9 medium containing 1 mM MgCl$_2$ and supplemented with 0.5% w/v glucose, 0.2% w/v casamino acids, 100 µg/ml ampicillin, 10 µg/ml biotin or biotin analog (if desired)] to give an initial optical density of 0.05 OD$_{550}$/ml. Cultures were grown at 30°C to an OD$_{550}$/ml of 0.5, at which time the growth temperature was raised to 37°C and fusion protein synthesis induced by addition of tryptophan to a concentration of 100 µg/ml. Four hours after induction, cells were harvested by centrifugation at 15 000 *g* for 2 min and were either used immediately or stored at –80°C.

Determination of the level of biotinylation of BIOTRX protein

The *E.coli* cell pellet was resuspended to 10 OD$_{550}$/ml in lysis buffer (20 mM Tris–HCl, pH 7.5, 1 mM *p*-aminobenzamidine, 0.5 mM phenylmethylsulfonyl fluoride). Resuspended cells were lysed in a French pressure cell at 20 000 p.s.i. and the lysate
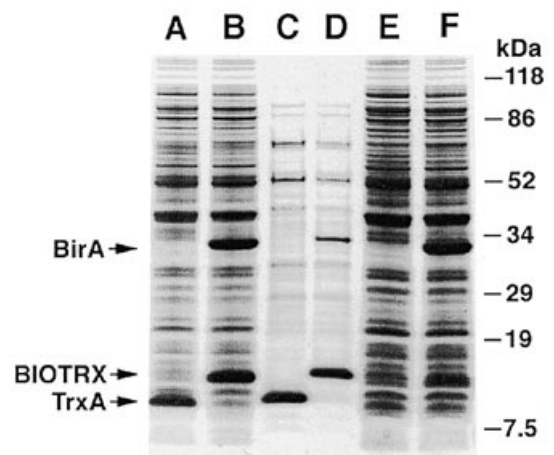
clarified by centrifugation at 15 000 *g* for 10 min at 4°C. Aliquots of 200 µl lysate supernatant were added to 100 µl 6% agarose bead slurry carrying immobilized tetrameric avidin (beads were pre-equilibrated with 1 ml lysis buffer containing 200 mM NaCl and 0.1% Triton X-100). After incubation of the mixture with gentle agitation at 4°C for 2 h the avidin–agarose beads were sedimented by a brief centrifugation at 15 000 *g* and washed twice with 1 ml lysis buffer (containing 200 mM NaCl and 0.1% Triton X-100). Aliquots of the lysate supernatant (total protein), the post avidin–agarose binding supernatant (unbound protein) and the avidin–agarose pellet after the final wash (bound protein) were heated in SDS gel loading buffer containing 1% β-mercaptoethanol at 90°C for 20 min and then analyzed on a SDS–PAGE gel (Fig. 2).

Investigation of the selective release of fusion proteins from cells by osmotic shock

Escherichia coli cells from induced cultures were resuspended to a density of 5 OD$_{550}$/ml in an ice-cold solution of 20% (w/v) sucrose, 20 mM Tris–HCl, pH 7.5, and 2.5 mM EDTA and incubated on ice for 10 min. The cells were then sedimented by centrifugation at 15 000 *g* for 1 min and resuspended in an equivalent volume of ice-cold 20 mM Tris–HCl, pH 7.5, and 2.5 mM EDTA. After another 10 min incubation on ice the cells were sedimented once more by brief centrifugation. Samples of the original cells, the final supernatant ('shockate') and the final cell pellet were analyzed by SDS–PAGE (Fig. 3).

Investigation of the heat stability of BIOTRX protein

Escherichia coli cells containing high levels of fusion protein were resuspended to a density of 100 OD$_{550}$/ml in lysis buffer and lysed by passage through a French pressure cell at 20 000 p.s.i. The lysate was then incubated at 80°C in a glass-walled tube and samples removed after 0.0, 0.5, 1, 2, 5 and 10 min. These samples were subjected to a brief centrifugation step at 15 000 *g* to

sediment insoluble material. Supernatants were then examined by SDS–PAGE analysis.

Detection of biotinylated compounds on nitrocellulose blots using an avidin–alkaline phosphatase conjugate

Protein samples to be analyzed by this procedure were first subjected to SDS–PAGE, followed by electrotransfer onto a nitrocellulose filter. The filter was then incubated at room temperature in a blocking buffer [3% w/v bovine serum albumin (BSA), 50 mM Tris–HCl, pH 7.5, 0.15 M NaCl] for 2 h to saturate all unoccupied protein binding sites, followed by an additional 2 h incubation at room temperature in blocking buffer containing a 1:1000 dilution of avidin–alkaline phosphatase conjugate. The filter was then subjected to three 10 min washes with blocking buffer followed by a rinse with alkaline phosphatase buffer. A substrate solution of 8 µg/ml BCIP and 33 µg/ml NBT was added and the filter incubated at room temperature until bands indicating the presence of bound alkaline phosphatase–avidin could be clearly seen.

Purification of BIOTRXloop proteins

After induction of protein synthesis in cells containing one of the BIOTRXloop expression plasmids (as above) bacteria were harvested by centrifugation, washed and then resuspended in 50 ml 25 mM Tris–HCl, pH 7.5, 2 mM EDTA containing one dissolved tablet of Complete Protease Inhibitor Cocktail (Boehringer Mannheim). All subsequent purification steps were performed at 4°C. Resuspended cells were lysed by passage through a French pressure cell at 20 000 p.s.i. and the lysate clarified by centrifugation at 100 000 g for 30 min followed by passage through a 0.22 µm filter. The clarified lysate was then loaded onto a 40 ml Q-Sepharose fast flow anion exchange column (Pharmacia) and BIOTRXloop protein eluted with a gradient of 0.0–0.5 M NaCl. Fractions were assayed by SDS–PAGE. Peak fractions were pooled and concentrated using an ultrafiltration cell (Amicon, Beverly, MA) fitted with a YM10 membrane. BIOTRXloop concentrates were then loaded onto a Pharmacia HiPrep Sephacryl S-200 size exclusion column pre-equilibrated with 300 mM NaCl, 50 mM Tris–HCl, pH 8.0, 2 mM EDTA buffer. Eluted fractions containing BIOTRXloop proteins were concentrated on a YM10 membrane and loaded onto a Toyopearl Phenyl 650-S column equilibrated in 2.0 M NaCl, 25 mM Tris–HCl, pH 8.0, and eluted with a decreasing gradient of 2.0–0.0 M NaCl. Eluted fractions containing pure BIOTRXloop protein were dialyzed against 10 mM HEPES, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween-20 buffer [HBS(T)], concentrated with Centriprep-10 concentrators (Amicon, Beverly, MA) and stored at 4°C.

Surface plasmon resonance measurement of BIOTRXloop binding to an anti-interleukin-8 antibody

Murine anti-human interleukin-8 (IL-8) monoclonal antibody (clone hIL-8/NR7) was obtained from Devaron (Dayton, NJ). A streptavidin-conjugated BIAcore chip (Pharmacia) carrying ~4000 response units (RU) of chemically coupled streptavidin (1000 RU = 1 ng/mm^2 coupled/adsorbed protein) was pre-conditioned with three 10 µl injections of 50 mM NaOH, 1.0 M NaCl and equilibrated with HBS(T). Purified BIOTRXloop proteins Z9H1A and RS2-20 were injected into separate lanes of the chip and the binding response measured as a function of time. Protein concentrations of the order of 1 µg/ml or lower and flow rates of 20 µl/min or higher were required to limit the amount of BIOTRXloop protein captured by the chemically immobilized streptavidin. Approximately 2000 RU of each BIOTRXloop protein were captured on separate lanes of the chip. A solution of anti-human interleukin-8 antibody hIL-8/NR7 at a concentration of 0.045 mg/ml was injected into the chip at a flow rate of 40 µl/min and the binding response again recorded as a function of time.

Surface plasmon resonance study of BIOTRX–IL-11 fusion protein binding to IL-11 receptor–Fc and IL-13 receptor–Fc fusion proteins

Escherichia coli cells expressing BIOTRX–IL-11 fusion protein were resuspended in 25 mM Tris–HCl, pH 7.5, 1% α-methyl-mannoside, 2 mM EDTA and Complete™ Protease Inhibitor Cocktail, followed by lysis in a French pressure cell at a pressure of 20 000 p.s.i. The lysate was cleared by ultracentrifugation for 35 min at 100 000 g and the upper 80% of clarified supernatant collected and filtered through a 0.2 µm filter. The clarified lysate was diluted 100-fold in HBS(T) buffer and 20 µl injected onto a pre-conditioned streptavidin-conjugated BIAcore chip at a flow rate of 20 µl/min. Aliquots of 20 µl 1 M NaCl, 50 mM HEPES, pH 7.4, 0.05% Tween 20 was then injected to wash off non-specifically bound *E.coli* proteins. Aliquots of 20 µl of 10 µg/ml solutions of purified soluble IL-13 receptor–Fc fusion protein (sIL13R-Fc, kindly provided by Debra Donaldson, Genetics Institute) and purified soluble IL-11 receptor–Fc fusion protein (sIL-11R-Fc, kindly provided by James Tobin, Genetics Institute) were then successively injected onto the chip at a flow-rate of 20 µl/min and the binding response monitored over time.

RESULTS

Expression and biotinylation properties of BIOTRX and BirA

A recombinant thioredoxin capable of being biotinylated *in vivo* by the host endogenous biotinylation machinery (BIOTRX) was constructed by inserting an oligonucleotide encoding a 23 amino acid biotinylation recognition peptide in-frame at the 5′-end of *trxA*. In initial experiments the production and solubility of BIOTRX protein derived from cells containing the pBIOTRX plasmid and grown in the absence of exogenous biotin were good, with estimated levels achieved of up to 10% of total cellular protein (Fig. 2, lane 2L). However, the amount of tritiated biotin incorporated into BIOTRX using this plasmid under these growth conditions was low (data not shown), as was BIOTRX binding to immobilized avidin (Fig. 2, lane 2B) or to avidin–alkaline phosphatase conjugates (not shown). Addition of 10 µg/ml biotin to the pre-induction growth medium of cells containing pBIOTRX resulted in an improvement in the overall extent of biotin incorporation (Fig. 2, lane 3B), although considerably less than 50% of the total BIOTRX protein (Fig. 2, lanes 3L and 3UB) was biotinylated even under these conditions. It was suspected that a limiting intracellular level of BirA might be the reason for the observed low extent of BIOTRX biotinylation. To test this hypothesis we inserted the *E.coli birA* gene into the pBIOTRX expression plasmid, creating the operon fusion vector pBIOTRX-BirA (Fig. 1). From this plasmid BIOTRX and BirA proteins are made separately at high levels under transcriptional control of the same λ pL promoter. A bacteriophage T7 gene10 ribosome

binding site ensured efficient translation of the downstream *birA* mRNA (Fig. 2, lane 4L, 30 kDa band indicated by arrow). The BirA protein produced by cells containing pBIOTRX-BirA was fully soluble under the conditions used and quantitative biotinylation of BIOTRX (estimated at >90%) was attained by combined use of this vector with supplemental biotin in the growth medium (Fig. 2, lane 4B). Under these circumstances very little BIOTRX protein remained in the unbound avidin–agarose supernatant (Fig. 2, lane 4UB). This finding was later confirmed in a Western blot, staining BIOTRX with an avidin–alkaline phosphatase conjugate (data not shown).

Thioredoxin-like properties of BIOTRX proteins

The osmotic shock release of BIOTRX protein was investigated using the procedure described above. SDS–PAGE analysis of a total lysate of cells producing wild-type thioredoxin or BIOTRX (Fig. 3, lanes A and B), of proteins released from these cells by osmotic shock treatments without lysis (Fig. 3, lanes C and D) and of the remaining intracellular proteins following osmotic shock treatment (Fig. 3, lanes E and F) indicated that BIOTRX retains the ability of native thioredoxin to be selectively and quantitatively released from *E.coli* cytoplasm by osmotic shock. A small amount of BirA also appeared to be released from cells by osmotic shock, possibly due to association with the BIOTRX (substrate) protein.

Wild-type *E.coli* thioredoxin is thermally stable; incubation at 80°C for 10 min does not adversely affect the structural conformation of the native protein (15). This property has been utilized in the purification of thioredoxin fusion proteins, since most other contaminating *E.coli* proteins are denatured and precipitate upon prolonged incubation at 80°C. The thermal stability of BIOTRX was investigated as described above, with the resulting heat-treated fractions analyzed by SDS–PAGE. As shown in Figure 4, most of the BIOTRX protein is still in solution after 10 min incubation at 80°C, while most other *E.coli* proteins denature and precipitate after a much shorter incubation period. Thus BIOTRX retains the high thermal stability of native thioredoxin.

Binding and release of biotinylated BIOTRX from immobilized avidin

The potential utility of BIOTRX for affinity purification using immobilized avidin was examined, with the goal of finding mild non-denaturing elution conditions for release of avidin-bound BIOTRX fusion proteins. In an attempt to overcome the high affinity of the biotin–avidin interaction two different schemes were explored. The first approach was to attempt *in vivo* incorporation of biotin analogs with lower affinity for avidin (1,19,20). The second approach was to capture BIOTRX proteins (biotinylated with normal biotin) using immobilized monomeric avidin, which has a much lower inherent affinity for biotin than native tetrameric avidin (21). Biotin analogs hexahydro-2-imino-1-thieno-3,4-imidazole-4-valeric acid (2-iminobiotin) and *cis*-3,4-diamino-2-tetrahydrothiophene valeric acid (diaminobiotin) were substituted for normal biotin in the usual cell culture and induction procedures. Both biotin analogs were non-toxic to *E.coli* and BIOTRX protein expression occurred at normal high levels in their presence. However, overall incorporation of the analogs was very low, leading to a complete absence of BIOTRX
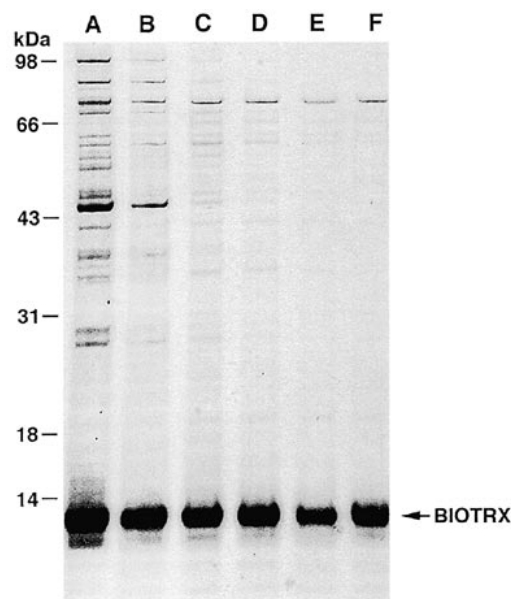

Figure 4. Heat stability of BIOTRX. Shown is a Coomassie blue stained 10% tricine SDS–PAGE gel. Whole cell lysates were incubated at 80°C for varying lengths of time, immediately chilled on ice and then fractionated by centrifugation. Lanes A–F represent the soluble fractions of the heated whole cell lysates removed after 0, 0.5, 1, 2, 5 and 10 min incubation time respectively.

binding to avidin–agarose in these experiments (data not shown). The second approach, normal biotin incorporation *in vivo* combined with the use of monomeric Soft-link™ avidin as capture ligand, was more successful. Approximately 50% of the total biotinylated BIOTRX protein could be captured by monomeric avidin and then specifically released upon addition of 4 mM free biotin (data not shown).

Expression, biotinylation and antibody binding properties of BIOTRXloop proteins

Both BIOTRXloop proteins described in this study were produced at high levels in *E.coli*, although significant amounts of both proteins were found in the insoluble fraction of lysed cells. Nevertheless, enough of each remained soluble to enable purification and characterization. Purified biotinylated BIOTRX-loop proteins could be rapidly captured by immobilized streptavidin, as indicated by a large increase in response signal (refractive index change) on a BIAcore surface plasmon resonance instrument equipped with a streptavidin-conjugated chip. Up to 4000 RU biotinylated BIOTRXloop protein could be captured, corresponding to an approximate ratio of four BIOTRXloop molecules bound per molecule of streptavidin, agreeing well with the theoretical number of available biotin binding sites of streptavidin (22). Desorption of bound BIOTRXloop proteins was minimal, being almost undetectable at lower values of captured BIOTRXloop protein, i.e. <1000 RU.

Previous work has demonstrated, using a nitrocellulose filter blot assay, that an anti-human IL-8 monoclonal antibody, hIL-8/NR7, could bind to IL-8-specific peptides inserted into the active site loop of native thioredoxin (23). In the present study the same antibody was tested for binding to two IL-8-specific peptides inserted into BIOTRX [i.e. BIOTRXloop(Z9H1A) and BIOTRXloop(RS2-20) proteins]. Nitrocellulose filter binding experiments performed on
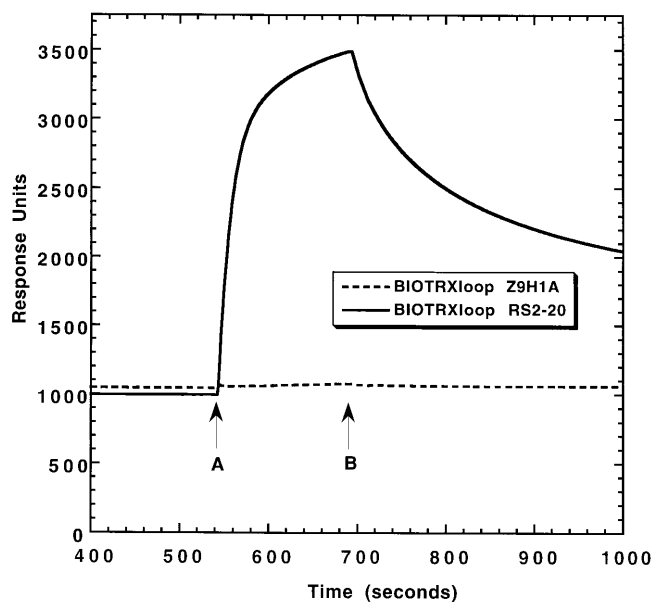
Figure 5. Surface plasmon resonance measurements of human anti-IL-8 monoclonal antibody binding to two BIOTRXloop proteins captured on a streptavidin-conjugated BIAcore chip. Approximately 1000 RU each BIOTRXloop protein were captured on separate lanes of the chip. Arrow A indicates injection of 0.045 mg/ml hIL-8/NR7 anti-human IL-8 antibody (hIL-8/NR7), arrow B initiation of a buffer wash.

Figure 6. Surface plasmon resonance measurement of streptavidin-captured BIOTRX–IL-11 fusion protein binding to soluble IL-11 receptor–Fc domain and IL-13 receptor–Fc domain fusion proteins. Approximately 1200 RU fusion protein were captured on a streptavidin-conjugated BIAcore chip during injection of a clarified *E.coli* cell lysate containing BIOTRX–IL-11. Arrow A indicates injection of IL-13 receptor–Fc protein, arrow B injection of IL-11 receptor–Fc protein.

BIOTRXloop(Z9H1A) and BIOTRXloop(RS2-20) proteins (not shown) showed that one of the inserted peptides (RS2-20) bound to hIL-8/NR7 antibody with much greater affinity than the other (Z9H1A). In this study binding of the antibody to the two immobilized BIOTRXloop proteins was monitored on streptavidin-conjugated BIAcore chips. A large increase in response signal was observed when hIL-8/NR7 was presented to the immobilized RS2-20 BIOTRXloop protein, while a minimal change in the response signal was observed for Z9H1A BIOTRXloop (Fig. 5), consistent with the nitrocellulose filter blot assay data.

Expression and biotinylation properties of a BIOTRX–IL-11 fusion protein

To investigate the expression efficiency, solubility and biotinylation efficiency of BIOTRX fusion proteins, the coding sequence of mature interleukin-11 (IL-11) was fused in-frame to the 3′-terminus of BIOTRX to produce a new vector, pBIOTRX/IL-11-BirA (Fig. 1). This plasmid, when induced for expression, directs production of both BIOTRX–IL-11 and BirA proteins. The BIOTRX–IL-11 fusion protein (molecular weight 34 kDa) was ~50% soluble under the conditions used.

The ability of the BIOTRX–IL-11 fusion protein to be biotinylated *in vivo* by BirA was investigated in an avidin–agarose binding experiment, identical to that previously used in determining the level of biotinylation of BIOTRX. It was found that the level of biotinylation of the N-terminal biotinylation peptide in the BIOTRX–IL-11 fusion was >90% and thus seemed to be unaffected by the presence of the large IL-11 domain fused to the BIOTRX C-terminus (data not shown).

Approximately 1200 RU BIOTRX–IL-11 fusion protein could be rapidly captured on a streptavidin-conjugated BIAcore chip simply by inje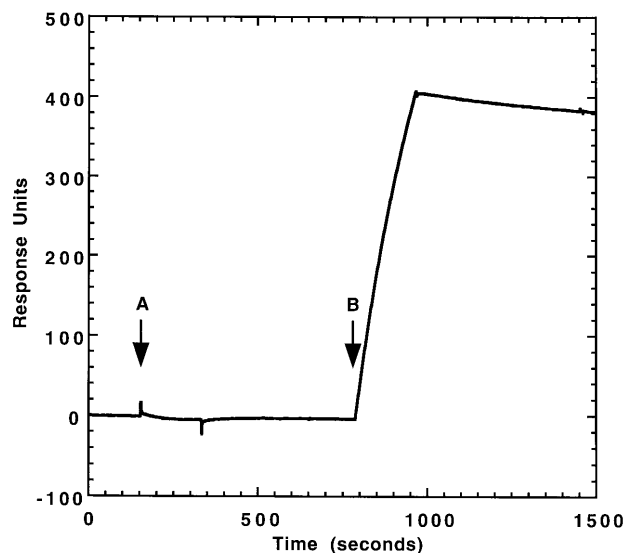cting the diluted soluble fraction of a crude cell lysate containing BIOTRX–IL-11. Endogenous biotinylated BCCP protein, present at low levels in *E.coli* lysates, was not thought to contribute significantly to the total captured protein in these binding experiments, since control cell lysates containing no BIOTRX yielded final bound protein only in the amount of 3 RU (data not shown).

Captured BIOTRX–IL-11 protein was functionally active for specific receptor binding, as assayed by surface plasmon resonance. As shown in Figure 6, ~400 RU soluble IL-11 receptor preparation (sIL-11R–Fc) was able to bind to captured BIOTRX–IL-11 protein, while a non-cognate soluble IL-13 receptor preparation (sIL-13R–Fc) did not bind. Thus the BIOTRX vector can function as a simultaneous capture and purification tool for use in measuring protein–protein interactions.

DISCUSSION

In this work we describe a system for quantitative *in vivo* biotinylation of thioredoxin protein fusions produced in *E.coli*. In our initial studies an expression vector was constructed, pBIOTRX, in which a short segment of DNA encoding a peptide substrate mimic for *E.coli* BirA (13) was fused in-frame to the 5′-end of the thioredoxin gene. Although this construct directed high level production of a soluble fusion protein (BIOTRX), under standard conditions only a small fraction was biotinylated. To explain this result we suspected that wild-type levels of BirA (11) in *E.coli* were insufficient to completely modify an over-produced biotinylation substrate such as BIOTRX. In addition, the host cell endogenous biotin biosynthetic capacity may also have been limiting on the overall extent of BIOTRX biotinylation. To address these two possibilities we constructed an operon fusion of *birA* with the *BIOTRX* gene, resulting in a new expression vector, pBIOTRX-BirA, which directed simultaneous

high level production of both BIOTRX and BirA under standard induction conditions. Combined with the addition of exogenous biotin to the culture medium, induction of pBIOTRX-BirA produces completely biotinylated BIOTRX protein in *E.coli*.

Fully biotinylated BIOTRX protein retains characteristics of native thioredoxin which are important for utility of the molecule as a fusion partner protein. For example, BIOTRX shares the ability of thioredoxin to be selectively and quantitatively released from *E.coli* cytoplasm by osmotic shock treatment and shares the inherent high thermal stability of thioredoxin. These properties suggest that the thioredoxin moiety within BIOTRX retains much of its native structure. The successful production of a BIOTRX–IL-11 fusion protein confirmed our expectation that BIOTRX would also work as an effective fusion partner, although the proportion of this particular fusion protein remaining in the soluble fraction (50%) was lower than that previously observed with a wild-type thioredoxin–IL-11 fusion (100%; 15). Nevertheless, we expect that many other proteins can be produced in *E.coli* as soluble fusions to BIOTRX. Further, we have shown that BIOTRX, like wild-type thioredoxin, can tolerate peptide insertions within its active site loop, although again the overall level of soluble expression of this class of fusion may also be lower than that achieved using wild-type thioredoxin.

The most important feature of the expression system we describe here is its ability to biotinylate soluble BIOTRX fusion proteins quantitatively *in vivo*. There are several advantages associated with this. First, *in vitro* chemical biotinylation procedures are unnecessary and reaction conditions potentially deleterious for protein activity are avoided (5). Secondly, the protein of interest itself is not modified by biotin, reducing the possibility of derivitizing critical 'active site' residues. Furthermore, in the BIOTRX system the biotin is linked to the N-terminus of thioredoxin, while the protein of interest is fused to the C-terminus (24). In this configuration it is unlikely that binding of avidin/streptavidin to BIOTRX will hinder interaction of the fused protein of interest with a cognate binding partner.

To demonstrate the utility of the BIOTRX system we made two types of fusion, C-terminal and internal, and tested both in protein–protein interaction studies monitored by surface plasmon resonance in a BIAcore instrument. In the IL-11 study biotinylated BIOTRX–IL-11 fusion protein was captured directly from bacterial lysates onto a streptavidin-conjugated BIAcore chip. This captured fusion interacted specifically with a soluble IL-11 receptor–Fc fusion, but not with an IL-13 receptor–Fc fusion. These data show how BIOTRX fusions may be used in detecting and characterizing specific protein–ligand interactions. To further illustrate this application of the system two forms of purified BIOTRX loop fusion bearing epitopes for antibody hIL-8/NR7 were loaded on the streptavidin BIAcore chip. Here they displayed relative binding affinities comparable with those seen in previous nitrocellulose filter binding experiments.

BIAcore experiments also revealed an extremely slow desorption rate for BIOTRX fusion proteins bound to the streptavidin chip, suggesting that immobilized BIOTRX fusion proteins will find broad utility in BIAcore, ELISA and many other molecular interaction assays which exploit the tightness of the biotin–avidin/ streptavidin interaction (3). In particular, the BIOTRX system might be useful in studying the interactions of thioredoxin active site loop peptide fusions with other molecules identified through FLITRX random peptide library pannings (21) or in yeast two-hybrid screens (25).

ACKNOWLEDGEMENTS

REFERENCES

1 Green,N.M. (1966) *Biochem. J.*, **101**, 774–780.
2 Green,N.M. (1975) *Adv. Protein Chem.*, **29**, 85–133.
3 Wilchek,M. and Bayer,E.A. (1990) *Methods Enzymol.*, **184**, 5–13.
4 Chilkoti,A. and Stayton,P.S. (1995) *J. Am. Chem Soc.*, **117**, 10622–10628.
5 Bayer,E.A and Wilchek,M. (1990) *Methods Enzymol.*, **184**, 138–160.
6 Samols,D., Thornton,C.G., Murtiff,V.L., Kumar,G.K., Haase,F.C. and Wood,H.G. (1988) *J. Biol. Chem.*, **263**, 6461–6464.
7 Fall,R.R. (1979) *Methods Enzymol.*, **62**, 390–398.
8 Barker,D.F. and Campbell,A.M. (1981) *J. Mol. Biol.*, **146**, 469–492.
9 Howard,P.K., Shaw,J. and Otsuka,A.J. (1985) *Gene*, **35**, 321–331.
10 Barker,D.F. and Campbell,A.M. (1981b) *J. Mol. Biol.*, **146**, 451–467.
11 Cronan,J.E.,Jr (1990) *J. Biol. Chem.*, **265**, 10327–10333.
12 Yamano,N., Kawata,Y., Kojima,H., Yoda,K. and Yamasaki,M. (1992) *Biosci. Biotechnol. Biochem.*, **56**, 1017–1026.
13 Schatz,P.J. (1993) *BioTechnology*, **11**, 1138–1143.
14 Tsao,K.-L., DeBarbieri,B., Michel,H. and Waugh,D.S. (1996) *Gene*, **169**, 59–64.
15 LaVallie,E.R., DiBlasio,E.A., Kovacic,S., Grant,K.L., Schendel,P.F. and McCoy,J.M. (1993) *BioTechnology*, **11**, 187–193.
16 Lu,Z., Diblasio-Smith,E.A., Grant,K.L., Warne,N.W., LaVallie,E.R., Collins-Racie,L.A., Follettie,M.T., Williamson,W.J. and McCoy,J.M. (1996) *J. Biol. Chem.*, **271**, 5059–5065.
17 Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
18 McCoy,J.M. and LaVallie,E.R. (1994) In *Current Protocols in Molecular Biology*, Unit 16.8. John Wiley and Sons, New York, NY, pp. 16.8.1–16.8.14.
19 Orr,G.A. (1981) *J. Biol. Chem.*, **256**, 761–766.
20 Heney,G. and Orr,G.A. (1981) *Anal. Biochem.*, **114**, 92–96.
21 Henrikson,K.P., Allen,S.H.G. and Maloy,W.L. (1979) *Anal. Biochem.*, **94**, 366–370.
22 Livnah,O., Bayer,E.A., Wilchek,M. and Sussman,J.L. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 5076–5080.
23 Lu,Z., Murray,K.S., Van Cleave,V., LaVallie,E.R., Stahl,M.L. and McCoy,J.M. (1995) *BioTechnology*, **13**, 366–372.
24 Katti,S.K., LeMaster,D.M. and Eklund,H. (1990) *J. Mol. Biol.*, **257**, 167–171.
25 Colas,P., Cohen,B., Jessen,T., Grishina,I., McCoy,J. and Brent,R. (1996) *Nature*, **380**, 548–550.