Applied and Environmental Microbiology, Sept. 2004, p. 5159–5167 Vol. 70, No. 9
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.9.5159–5167.2004
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Assessment of Production Conditions for Efficient Use of *Escherichia coli* in High-Yield Heterologous Recombinant Selenoprotein Synthesis

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Received 7 April 2004/Accepted 13 May 2004

The production of heterologous selenoproteins in *Escherichia coli* necessitates the design of a secondary structure in the mRNA forming a selenocysteine insertion sequence (SECIS) element compatible with SelB, the elongation factor for selenocysteine insertion at a predefined UGA codon. SelB competes with release factor 2 (RF2) catalyzing translational termination at UGA. Stoichiometry between mRNA, the SelB elongation factor, and RF2 is thereby important, whereas other expression conditions affecting the yield of recombinant selenoproteins have been poorly assessed. Here we expressed the rat selenoprotein thioredoxin reductase, with titrated levels of the selenoprotein mRNA under diverse growth conditions, with or without cotransformation of the accessory bacterial *selA*, *selB*, and *selC* genes. Titration of the selenoprotein mRNA with a pBAD promoter was performed in both TOP10 and BW27783 cells, which unexpectedly could not improve yield or specific activity compared to that achieved in our prior studies. Guided by principal component analysis, we instead discovered that the most efficient bacterial selenoprotein production conditions were obtained with the high-transcription T7lac-driven pET vector system in presence of the *selA*, *selB*, and *selC* genes, with induction of production at late exponential phase. About 40 mg of rat thioredoxin reductase with 50% selenocysteine content could thereby be produced per liter bacterial culture. These findings clearly illustrate the ability of *E. coli* to upregulate the selenocysteine incorporation machinery on demand and that this is furthermore strongly augmented in late exponential phase. This study also demonstrates that *E. coli* can indeed be utilized as cell factories for highly efficient production of heterologous selenoproteins such as rat thioredoxin reductase.

Many organisms express selenoproteins, carrying a selenocysteine residue, the 21st naturally occurring amino acid (7, 16, 26, 34). Selenocysteine is cotranslationally inserted at the position of an opal (UGA) codon, which normally confers termination of translation. The UGA codon is recoded as selenocysteine by complex translation machineries that differ between gram-negative (7, 16) and gram-positive bacteria (13), archaea (31), and higher eukaryotes (9, 12, 30). The translation system in *Escherichia coli* is the most characterized (reviewed in references 7, 16, and 34). Briefly, the mRNA for an *E. coli* selenoprotein carries a specific sequence after the UGA codon, both encoding the amino acids following the selenocysteine residue and forming a stem-loop secondary structure, a so-called selenocysteine insertion sequence (SECIS) element.

The SECIS element binds the SelB elongation factor, the *selB* gene product. SelB is homologous to elongation factor Tu (EF-Tu) but, in addition, binds the loop of the SECIS element through an additional C-terminal domain. In terms of tRNA substrate, SelB is only functional with the selenocysteine-specific $tRNA^{Sec}$, the *selC* gene product, in its selenocysteinylated form. By analogy with EF-Tu, SelB catalyzes selenocysteine insertion at the specific position of the selenocysteine-encoding UGA codon. The $tRNA^{Sec}$ is, however, originally charged with a seryl residue, which by selenocysteine synthase, the *selA* gene product, is converted to selenocysteinyl utilizing selenophosphate as the selenium donor. Selenophosphate is provided by selenophosphate synthetase, the *selD* gene product.

In contrast to the bacterial situation, mammalian selenoprotein mRNA species have a SECIS element positioned in the 3′ untranslated region, with distinctly different structural features (22, 23, 24, 26, 39, 41). Hence, *E. coli* SelB does not recognize the selenocysteine-encoding UGA of a mammalian selenoprotein mRNA. The difference between SECIS elements therefore imposes a strict species barrier in direct heterologous synthesis of recombinant selenoproteins in *E. coli*, as further discussed elsewhere (1). The native UGA-directed selenocysteine insertion in *E. coli* is also inherently inefficient, with a significant translational pause at the UGA (35). It was therefore surprising that selenocysteine-containing rat thioredoxin reductase (TrxR) could nonetheless be expressed in *E. coli* at a level of 5 mg of selenoprotein produced per liter of culture, even if utilizing an engineered SECIS element compatible with the bacterial selenoprotein synthesis machinery and concomitant expression of accessory *selA*, *selB*, and *selC* genes under control of their endogenous promoters (3). The total amount of recombinant TrxR produced in that study was about 20 mg per liter of culture, i.e., a yield of 5 mg of selenoprotein produced was estimated from a specific activity of 25% compared to that of the native enzyme (3).

The exact stoichiometry between the separate factors involved in selenoprotein synthesis had previously been shown to

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be important for the bacterial selenoprotein translation efficiency; sole overexpression of either SelB, $tRNA^{Sec}$ or of an mRNA carrying a SECIS element can in fact reduce UGA readthrough, since SelB becomes tethered to nonfunctional complexes with either $tRNA^{Sec}$ or with SECIS elements of selenoprotein mRNA molecules (16, 37, 38). In this work, we therefore set out to titrate the levels of the selenoprotein mRNA with the aim to further increase the yield of the overproduced recombinant selenoprotein. Surprisingly, we found that the higher the mRNA was expressed, the higher both yield and specificity became. We therefore continued with a more detailed analysis of growth and culture conditions with the previously used pET system (3). The selenocysteine-containing mammalian thioredoxin reductase differs in size by only two amino acids from the prematurely truncated form at the UGA, since the carboxy-terminal motif of this protein is Gly-Cys-Sec-Gly-COOH (3, 41). Because the Sec residue is essential for enzymatic activity (3, 40, 41, 42), we have postulated that the specific activity of the recombinant protein becomes directly indicative of the extent of truncated versus full-length selenocysteine-containing protein present in a purified preparation of the enzyme. We have here utilized this property as a means to probe the ratio of selenocysteine-incorporation to UGA termination, and we furthermore confirm that the full-length Sec-containing protein has full enzymatic activity.

MATERIALS AND METHODS

Materials. Restriction enzymes were from New England Biolabs and Promega. Bovine serum albumin, insulin, 5,5′-dithiobis(2-nitrobenzoic acid), arabinose, L-cysteine, and guanidine hydrochloride were from Sigma Chemical Co. Sodium selenite was from Fluka Chemie AG. 1,4-Dithiothreitol was from Saveen Biotech AB. Plasmid purification kits were from Clontech (Nucleospin Plus Miniprep kit) and Qiagen (Maxi kit). The pBAD Myc/His A expression vector and the *E. coli* TOP10 strain (deficient in arabinose metabolism) used for the titration experiments were from Invitrogen. The BW27783 strain was kindly provided by J. D. Keasling, Department of Chemical Engineering, University of California, Berkeley. *E. coli* DH5 was used as the cloning strain. pSUABC, utilized previously (3), contains chromosomal fragments with the *selA*, *selB*, and *selC* genes under the control of their endogenous promoters. Phenylarsine oxide-Sepharose came from Invitrogen (ThioBond Sepharose), whereas dimercaptopropanol sulfonic acid was bought from Sigma Chemical Co.

Making pBAD-TRS$_{TER}$. The rat TrxR open reading frame fused with the engineered *E. coli*-type SECIS element was moved from the pET-TRS$_{TER}$ vector described previously (3) to the NcoI and HindIII sites of pBAD Myc/His A, which was then called pBAD-TRS$_{TER}$. Plasmids containing the insert were positively identified with colony PCR as described in the manual for the pET system (Novagen) with primers amplifying part of the rat TrxR open reading frame. All standard procedures were performed according to Sambrook et al. (32) Ampicillin (for pBAD-TRS$_{TER}$) was used at a final concentration of 50 or 100 μg/ml in all media and chloramphenicol (for pSUABC) at 34 μg/ml.

Expressing TrxR at different arabinose concentrations. Production of TrxR with the pET-TRS$_{TER}$ system was performed essentially as described previously (3) except for changes in culturing conditions as described herein. For the arabinose titration experiments, *E. coli* TOP10 or BW27783 was transformed with pBAD-TRS$_{TER}$ or with the combination of pBAD-TRS$_{TER}$ and pSUABC. Single colonies were inoculated in 2 to 5 ml of Luria broth (LB) medium and incubated at 37°C overnight with vigorous shaking; 50 μl or 1 ml of the overnight cultures was added to 5 ml or 100 ml of LB at 25 or 37°C as indicated. In all expression experiments 5 μM Na_2SeO_3 was added to ensure sufficient selenium supply and suppress Trp incorporation at the UGA (25) and 100 μg of L-Cys per ml was included to prevent unspecific incorporation of selenium into proteins through sulfur pathways (29). The cultures were grown with shaking until the optical density at 600 nm (OD$_{600}$) reached 0.50 to 0.55. Then arabinose was added, and incubation was continued at the indicated temperatures for 14 to 16 h. After this time, the optical density of the culture was measured, cells were harvested by centrifugation, taken up in 10 ml of 50 mM Tris–2 mM EDTA (TE), pH 8.0, and sonicated. The lysates were centrifuged for 90 min at 30,000 × *g* at 4°C. Soluble fractions (in a volume corresponding to soluble extract derived from 20 μl of culture at an OD$_{600}$ = 1) were directly assayed for mammalian TrxR activity with the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) reduction assay (2).

Purification and activity measurements of recombinant TrxR. Recombinant rat TrxR was purified from cleared bacterial cell lysates directly by affinity chromatography on 2′,5′-ADP-Sepharose as previously described (3), with identical procedures for all lysates. Enzyme concentrations were determined as calculated from the flavin adenine dinucleotide content by absorbance of the oxidized enzyme at 463 nm with an extinction coefficient of 11,300 M^{-1} cm^{-1}. Controls with either Bradford determination or by A_{280} to A_{310} gave essentially the same values, demonstrating that the purified enzyme had a correct flavin adenine dinucleotide content (one flavin adenine dinucleotide per subunit). Enzyme activity was determined by the standard DTNB reduction assay as described in detail elsewhere (2), with Ellman′s reagent (DTNB), concentrations for the k_{cat} and K_m determinations between 25 μM and 5 mM, and a recombinant TrxR concentration of 6 to 50 nM (depending on the specific activity of the preparations, in order to yield a gain in absorbance of approximately 0.1 A_{412}/min with the highest [5 mM] DTNB concentration).

Separation of full-length and truncated TrxR. First, 50 μg of purified recombinant mammalian TrxR preparations with the indicated specific activities was incubated with 0.5 mM dithiothreitol for 1 h at 4°C to reduce the selenenylsulfide bridge in the oxidized full-length enzyme. The reduced sample (600 μl) was then added to 100 μl of ThioBond (Invitrogen) resin, activated according to the manufacturer's instructions and packed as a minicolumn. The flowthrough was collected, whereupon the resin was washed with 1 ml of TE, followed by 1 ml of 5 mM β-mercaptoethanol in TE and subsequently 1 ml of 10 mM β-mercaptoethanol in TE. Bound protein was subsequently eluted by incubation of the column for 10 min at 4°C with 250 μl of 10 mM dimercaptopropanol sulfonic acid (2,3-dimercapto-1-propane-sulfonic acid) in TE, pH 6.0. The 500-μl elution fractions were desalted and concentrated into TE with a Nanosep 10,000-molecular-weight cutoff Omega (PALL) spin filter. Protein concentrations for the original sample, flowthrough, and concentrated eluate were determined with the Bio-Rad (Hercules, Calif.) protein assay with bovine serum albumin as the standard. Mammalian TrxR activity in each sample was measured with the standard DTNB assay (2) with 2 to 5 μl of each enzyme fraction in a reaction mixture of 500 μl with 2.5 mM DTNB and 300 μM NADPH in 50 mM phosphate buffer, pH 7.5, and 2 mM EDTA.

Multivariate principal component analysis. Principal component analysis is a statistical technique in which objects (in this case, the experiments) and variables are presented in a table. The experiments constitute the rows, and the variable data of the experiments are found in the columns. By considering the table to be a mathematical matrix, it is possible by standard linear algebra to interpret the data as vectors in a multidimensional space. Those vectors graphically describe the variation of the data and thus the information in the table, and the "cloud" of vector tips is generally prolonged in some direction(s) in the space. Principal component analysis is a method of finding orthogonal directions, principal components, in this space. The first principal component is the direction on which the sum of the projections of all variable vectors is at maximum. Graphically this corresponds to the direction in the space that best coincides with the maximum spreading of the cloud of vector tips. The second principal component is perpendicular to the first and still coincides with a direction of maximum spreading of data. Thus, a projection plane constituted by the first two principal components will always carry most of the information. A general description of the technique is found in reference 11 and interpretation of the results is found in reference 18. The computer program Sirius 3.0 (Pattern Recognition Systems) was used for the calculations. Before analysis, data were standardized by division with the standard deviation, resulting in equal weights for each variable in the analysis.

(i) Experimental design. To ensure an independent variation of the input variables, the variable values were designed by a variety of Placket-Burman design (8). The + values were chosen randomly between the mean value and the maximum value of the tested range, and − values were chosen accordingly between the mean and the minimum value of the range. See Table 3 for the resulting experimental set-up for multifactorial analysis of OD$_{600}$ at addition of IPTG (isopropylthiogalactopyranoside, 100 μM), temperature of culture at protein expression, and duration of IPTG-induced expression. In all cases, TrxR activity was measured with the DTNB assay with the same protocol as in the analysis of arabinose titration. The determined activities formed the basis for interpretation with principal component analysis.

(ii) Experimental setup. Small-scale bacterial culture (3 ml) of BL21(DE3) transformed with pET-TRS$_{TER}$ and pSUABC were grown as described (3). IPTG (100 μM) was added at different OD$_{600}$s, with different growth tempera-

TABLE 1. Total yield and kinetic parameters of purified TrxR induced at different expression levels in LB medium with the pBAD expression system

Arabinose (%)	pSUABC	Recovered[a] (mg/liter)	Recovered[a] (mg/liter/OD_{600})	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})	U/mg[b]	Recovered selenoprotein[c] (mg/liter/OD_{600})
0.0002	−	5.8	3.8	25	0.15	167	0.2	0.02
	+	9.3	5.7	159	0.13	1,223	1.4	0.23
0.005	−	13.4	9.5	123	0.18	683	1.1	0.30
	+	29.2	18.2	315	0.13	2,423	2.7	1.40
0.2	−	13.6	9.8	81	0.12	675	0.7	0.20
	+	24.2	15.2	646	0.17	3,800	5.6	2.43

[a] Total recombinant TrxR (mixture of truncated and full-length forms) recovered in purification, given as yield per liter of original medium or per liter and OD_{600} unit.
[b] One unit is defined as the reduction of 1 μmol of DTNB per min in the standard DTNB assay (2). The specific activity of native mammalian TrxR is approximately 35 U/mg (2).
[c] Selenoprotein (full-length TrxR) recovered in the purification, given as yield per original culture volume and OD_{600} unit, as calculated from units per milligram and total recovered yield, assuming 35 U/mg for the full- length selenoprotein (2).

tures and incubation time as indicated in Table 3. The amount of soluble active selenoprotein TrxR produced was subsequently determined as in Table 1 (values given are the means ± standard deviation of four separate cultures). The data presented in Table 3 were used as the basis for the multivariate analysis.

(iii) Interpretation with principal component analysis. In a projection plane, a loading plot constituted by two principal components, the independent and dependent variables, will be visualized as vectors pointing in several directions, and every vector represents the pattern of variation of a variable over all experiments. If two vectors point in the same direction or one of the vectors has a projection on the other that points in the same direction, then they have covariation and thus, e.g., high or low values simultaneously. If two vectors point in opposite directions or one vector has a projection on the other that points in the opposite direction, then one will have a high value and the other a low value. Perpendicular vectors show that variables have no correlation with each other. The significance of an interpretation is high if the original vectors are well described in the chosen principal component projection. For confirmation of a possible correlation, further experiments need to be performed in line with such suggested correlations (18). As explained, this was also done in the present study.

RESULTS

Titrating selenoprotein mRNA levels. To investigate the effect of the selenoprotein mRNA levels on total selenoprotein TrxR yield, an arabinose-titratable araBAD-driven expression vector was used. First we used TOP10 cells, regularly used with the pBAD vector with which it is supplied by the commercial vendor. However, the level of expression response under pBAD at a given arabinose concentration is dependent on the uptake of arabinose through the transporter protein AraE, which itself is under arabinose control (33). This may result in a protein expression response within each bacterial cell in an "all or none" fashion, although on the culture level expression may appear titratable. This effect is more pronounced at lower arabinose concentrations than at higher (20). We therefore also utilized BW27783 cells, which have constitutive AraE overexpression and thereby distinct arabinose titration response on the cellular level (19).

In a wild-type background for selenocysteine incorporation, we found that total TrxR activity was increased with increasing mRNA levels up to those induced by approximately 0.01% arabinose. There it reached a plateau with both TOP10 and BW27783 cells. At this and higher arabinose concentrations, the production of selenocysteine-containing TrxR corresponded to a yield of approximately 0.8 mg produced per liter of bacterial culture and OD_{600} (Fig. 1A and B, open circles). At this production level, the maximum capacity of the endogenous wild-type Sec incorporation machinery in both strains thereby seemed to have been reached. However, with concomitant overexpression of *selA*, *selB*, and *selC*, a higher maximum level of about 1.6 mg of TrxR/mg/OD_{600} was reached in both strains, with a faster response to arabinose in BW27783 than in TOP10 (Fig. 1A and 1B, solid diamonds). This total TrxR yield was lower than that obtained with a T7lac-driven system (3) but did not reveal the extent of full-length selenoprotein TrxR produced in relation to the UGA-truncated non-selenocysteine-containing species.

To examine whether the TrxR protein produced at lower arabinose concentrations had a higher extent of selenocysteine incorporation due to less truncation at UGA, we purified the recombinant protein produced under different concentrations of arabinose and determined its kinetic parameters. This approach was based on the rationale that truncated TrxR formed due to RF2-mediated termination at the UGA lacks enzymatic activity but is copurified with the full-length enzyme as it differs by only two amino acids, resulting in an overall decrease in the specific activity of the recombinant protein preparation (3).

Total yield and specific activity with arabinose induction. The recombinant protein could be purified essentially quantitatively upon expression induced by 0.0002, 0.005, and 0.2% arabinose (Fig. 1C). This purification showed, to our surprise, that the fraction of full-length selenoprotein increased in strict correlation with higher expression levels. This was clearly revealed by the highest specific activity in the enzyme produced in the presence of pSUABC with the highest arabinose concentration (Table 1). The specific activity of this enzyme was hence 5.6 U/mg (Table 1). This was less than the 7.7 U/mg previously achieved with the very high transcription levels derived from the pET vector-based expression system reported earlier (3).

Production in minimal medium. SelB-mediated incorporation of selenocysteine is less decreased than the efficiency of RF2-mediated termination at a Sec-encoding UGA codon in cells growing slowly in minimal medium (28). Therefore, we also examined production of enzymatically active TrxR under such conditions, i.e., aerobic growth in M9 minimal medium, with or without cotransformation with pSUABC. Either glucose or glycerol was used as the energy source, since glucose at lower degrees of expression suppresses the arabinose-driven promoter in the pBAD vector system (14). In M9 minimal medium containing glucose, pSUABC clearly increased sel-

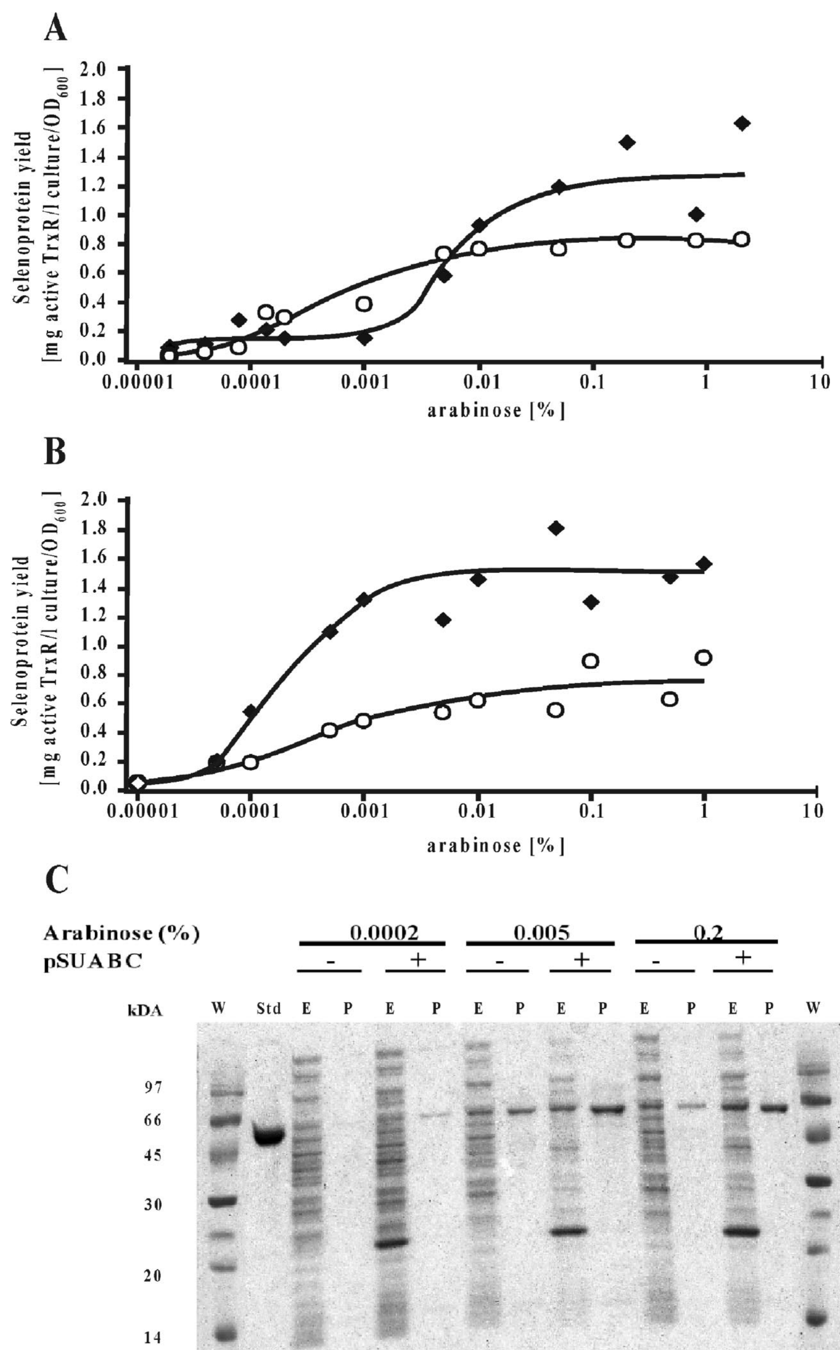

FIG. 1. Effect of expression levels on TrxR selenocysteine incorporation. (A) Effects of expression levels derived from a pBAD vector in TOP10 cells with different arabinose concentration in the absence or presence of pSUABC (as indicated) are shown. (B) Effects of expression levels derived from a pBAD vector in BW27783 cells with different arabinose concentrations in the absence or presence of pSUABC (as indicated). Cultures (5 ml) were grown aerobically at 25°C for 16 h. Full-length TrxR production was assessed with the DTNB assay (2). At 36°C, somewhat higher TrxR levels could be reached but with variable yield, probably due to inclusion body formation (not shown). (C) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of cell extract (E) and purified recombinant TrxR (P) derived from 100 ml of TOP10 cultures induced at 30°C is shown; each lane was loaded with a protein amount calculated to derive from 25 μl of culture at an OD_{600} of 1. Purified enzyme species from 0.0002% arabinose are hardly visible when the gel was loaded with this amount due to the low expression levels (see Table 1 for total yields). W, size markers. Std, standard recombinant TrxR.

enoprotein yield over that in the native background. However, the highest total TrxR activity with these production conditions corresponded to less than 140 μg of selenoprotein per liter of culture per OD_{600} (Table 2). With glycerol as an energy source, cotransformation with pSUABC had no effect. This could be explained by a lack of glucose-derived ribulose-5-phosphate needed for synthesis of the riboflavin precursor (5), essential for production of the flavin adenine dinucleotide cofactor of

TABLE 2. Yield of full-length TrxR in M9 minimal medium

% Arabinose	Energy source	pSUABC	Yield of active enzyme (mg/liter/OD_{600})[a]	Effect of pSUABC (fold increase)
0.005	Glycerol	−	0.024	
		+	0.051	2
	Glucose	−	0.019	
		+	0.128	7
0.1	Glycerol	−	0.059	
		+	0.063	No
	Glucose	−	0.014	
		+	0.130	9

[a] Determined as for Fig. 1A and B.

TrxR, or by a need for glucose in selenoprotein synthesis by an as yet unknown pathway.

Based on these experiments, we could conclude that incorporation of Sec neither under growth in minimal medium nor with use of arabinose-driven expression proved advantageous in terms of TrxR yield compared to growth in rich medium with the pET expression system (3). We therefore continued with closer analysis of the Sec incorporation in the latter expression system, aiming to identify conditions under which bacterial selenoprotein synthesis was further increased.

Optimizing yield with the pET vector system. We used a multivariate principal component analysis approach to optimize three parameters affecting selenoprotein yield with the pET vector-based production system together with cotransformaition of pSUABC: growth temperature, OD_{600} at time of IPTG addition, and duration of expression. The plot of principal component I against principal component II (8) showed that the yield of selenoprotein per liter of culture or per liter of culture and OD_{600} unit completely covaried, that a high OD_{600} at the time of IPTG addition would result in higher yield since the OD_{600} at IPTG vector had a positive projection on the yield vectors and covaried completely, and that temperature should be kept low within the tested interval (20 to 37°C) for highest yield, as the temperature vector projection pointed in the opposite direction of the yield vectors (Fig. 2); the data are presented in Table 3. Moreover, the duration of expression seemed to have no major impact, since there was a lack of covariation with the yield vectors (possibly because of the large interval of duration in the experimental set-up). In summary, the principal component analysis suggested that the highest yields of selenoprotein TrxR would be obtained with induction at a higher OD_{600} (i.e., in late exponential or stationary phase) with growth at lower temperatures.

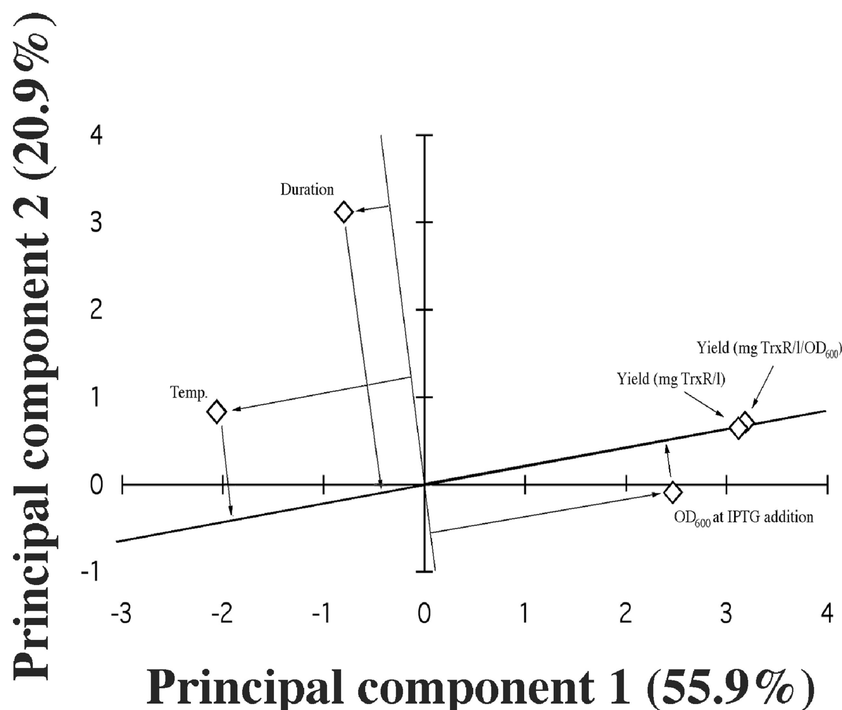

FIG. 2. Multivariate principal component analysis. The figure shows the loading plot of principal component 1 versus principal component 2, with the results presented in Table 3. The loading plot describes the relationship between the variables, with values in parentheses indicating how much of the variation is described by each component. The sum (76.8%) shows that most of the variation in the data is covered by this plot, suggesting that conclusions drawn from the plot have a high degree of reliability. Note that the yield of selenoprotein, given either as milligrams per liter or milligrams per liter per OD_{600} unit, covaried completely, illustrating that a higher total yield per liter of culture also illustrated a higher selenoprotein production per bacterial cell and was not only a reflection of a higher cell density. The projections of the variables duration of induction, OD_{600} at the time of IPTG addition, and temperature of the culture at time of induction are illustrated in the figure. Note that yield covaried inversely with temperature, indicating that a lower culture temperature would increase yield and that a higher OD_{600} seemed to have a positive effect on yield, while the duration of induction (within the 2- to 48-h time frame) seemed to lack a major effect on yield. For further interpretation of the principal component analysis plot, please see the text, and for subsequent experimental verification of the principal component analysis, see Fig. 3.

TABLE 3. Experimental set-up and results with the multivariate principal component analysis and the pET system

Expt no.	IPTG addition (OD_{600})	Temp (°C)	Duration (h)	Yield of active TrxR[a]	
				mg/liter	mg/liter/OD_{600}
1	2.3	37	10	13.7 ± 6.4	3.7 ± 1.6
2	0.4	30	48	1.5 ± 0.5	0.5 ± 0.1
3	1.8	20	40	13.6 ± 3.0	4.2 ± 1.2
4	1.8	30	5	4.6 ± 3.2	1.6 ± 1.3
5	0.7	37	24	3.3 ± 1.5	1.3 ± 0.4
6	3.0	37	48	3.0 ± 0.02	1.5 ± 0.2
7	1.7	20	24	7.8 ± 0.6	3.0 ± 0.2
8	1.8	25	2	2.9 ± 1.3	1.1 ± 0.4
9	0.6	30	16	5.3 ± 0.3	2.2 ± 0.8
10	0.0	37	48	6.0 ± 5.1	1.8 ± 1.2
11	1.8	20	24	13.6 ± 1.6	4.6 ± 0.3
12	1.5	25	16	8.6 ± 1.2	2.9 ± 0.5

[a] See Table 2, footnote *a*.

Large-scale overexpression of TrxR. Based on the principal component analysis data, we first attempted to induce overexpression of TrxR in stationary phase (when the OD_{600} had reached 4). This resulted in less than 3 mg of selenoprotein produced per liter of culture (not shown). Therefore, we examined the effect of induced expression of TrxR at different stages of the late exponential phase. Induction at later stages of the exponential phase led to a decrease of bacterial density, and within 8 to 36 h after the onset of induction selenoprotein TrxR levels reached a plateau. At all events within 24 h of expression, total yields per liter of culture and the yield per OD_{600} covaried completely. A large increase in yield was indeed obtained when production was induced at late exponential phase compared to the more standard induction in mid-log phase; this was in good agreement with the principal component analysis prediction. Figures 3A and B summarize these experiments. We conclude that IPTG addition at an OD_{600} of 2.4 with continued growth at 24°C for 24 h gave the highest total yield of selenoprotein TrxR, resulting in approximately 20 mg of selenoprotein being produced per liter of bacterial culture. This was a fourfold increase in total yield over the previously achieved production with regular induction in mid-log phase (3).

We next assessed the 2.4/24/24 protocol (i.e., induction of expression at an OD_{600} of 2.4 with production for 24 h at 24°C) for large-scale overexpression of TrxR with subsequent purification. This gave an enzyme preparation with a specific activity of 18 U/mg in the standard DTNB assay, which is approximately 50% of that of the native enzyme (2) and significantly higher than the activity previously obtained (22%) for the recombinant enzyme (3). A specific activity of about 50% was also confirmed with the insulin reduction assay coupled with human Trx (not shown). These results strongly suggested that not only total selenoprotein yield but also the specific incorporation of selenocysteine became highly increased upon initiation of production at late exponential phase. However, for that interpretation, we also needed to confirm our assumption that the specific activity of the Sec-containing recombinant rat TrxR indeed was similar to that of native TrxR and that the specific activity thereby can be used as a measure of its selenium content.

Separation of full-length and truncated recombinant TrxR. Proteins containing vicinal dithiols specifically bind to phenylarsine oxide-Sepharose (17). We reasoned that the selenothiol motif of reduced full-length TrxR would bind to phenylarsine oxide-Sepharose, whereas the truncated species would possibly bind with less affinity. This proved to be true, and in contrast to other vicinal dithiol-containing proteins (17), selenocysteine-containing TrxR could essentially not be eluted from the resin with either 1 M β-mercaptoethanol or 10 mM dithiothreitol. Thereby, washing the column with these reagents removed the truncated TrxR species that were probably species bound to the column via the N-terminal or other dithiol motifs present in the truncated enzyme (42). The full-length Sec-containing TrxR could subsequently be eluted with dimercaptopropanol sulfonic acid, a highly specific chelator for arsine. The specific activity of the enzyme in the eluted fractions was 37.8 ± 2.6 U/mg (Table 4), which is the same activity as obtained for native mammalian TrxR purified from tissues (4). This result proved that the full-length recombinant enzyme had full activity and hence that its specific activity could be used as a measure of Sec incorporation, as has been done herein. (The reversible binding of the selenothiol motif to phenylarsine oxide-Sepharose may also be developed for purification purposes [Johansson et al, unpublished data].)

DISCUSSION

Production systems for bacterial expression of recombinant selenoproteins are hampered by the species-restricted SECIS element and the competition between RF2 and SelB. Our prior TrxR overexpression in *E. coli* has remained the most productive system until the present study. Already in that prior study, we were surprised by the high reserve capacity of *E. coli* for selenoprotein synthesis (3). Expression of the *selA* and *selB* genes was later shown by Thanbichler and Böck to be regulated by a complex formed at the 5′ end of the *selAB* operon transcript consisting of a SECIS-like element and SelB, which inhibits translation of *selA* and *selB* when free SelB is available (36). That mechanism implies a downregulation of the selenocysteine insertion machinery when selenoprotein mRNA levels are low. Conversely, at high selenoprotein mRNA levels, the SelB-mediated block of the *selAB* operon becomes relieved, which gives room for significant upregulation as long as the *selAB* transcript is expressed. This mechanism should be a major factor explaining the very high yield of selenoprotein expression that we obtained here with the highest heterologous transcription level possible (the pET vector system) concomitant with coexpression of the *selAB* operon under its own promoter.

Nonetheless, we had reasoned that the stoichiometry of selenoprotein mRNA to the *selA*, *selB*, and *selC* gene products would still be of importance for yield, and we therefore attempted the titration experiments reported herein. The method of arabinose titration of a synthetic *araBAD* promoter is much used but has been debated. At lower arabinose levels, titration seems to occur not at the cellular but rather at the bacterial culture level, with the individual cell being on or off (19-21, 33). Therefore, we also performed experiments with *E. coli* strain BW27783 (21), which is believed to enable titration of arabinose-driven expression on the individual cellular level

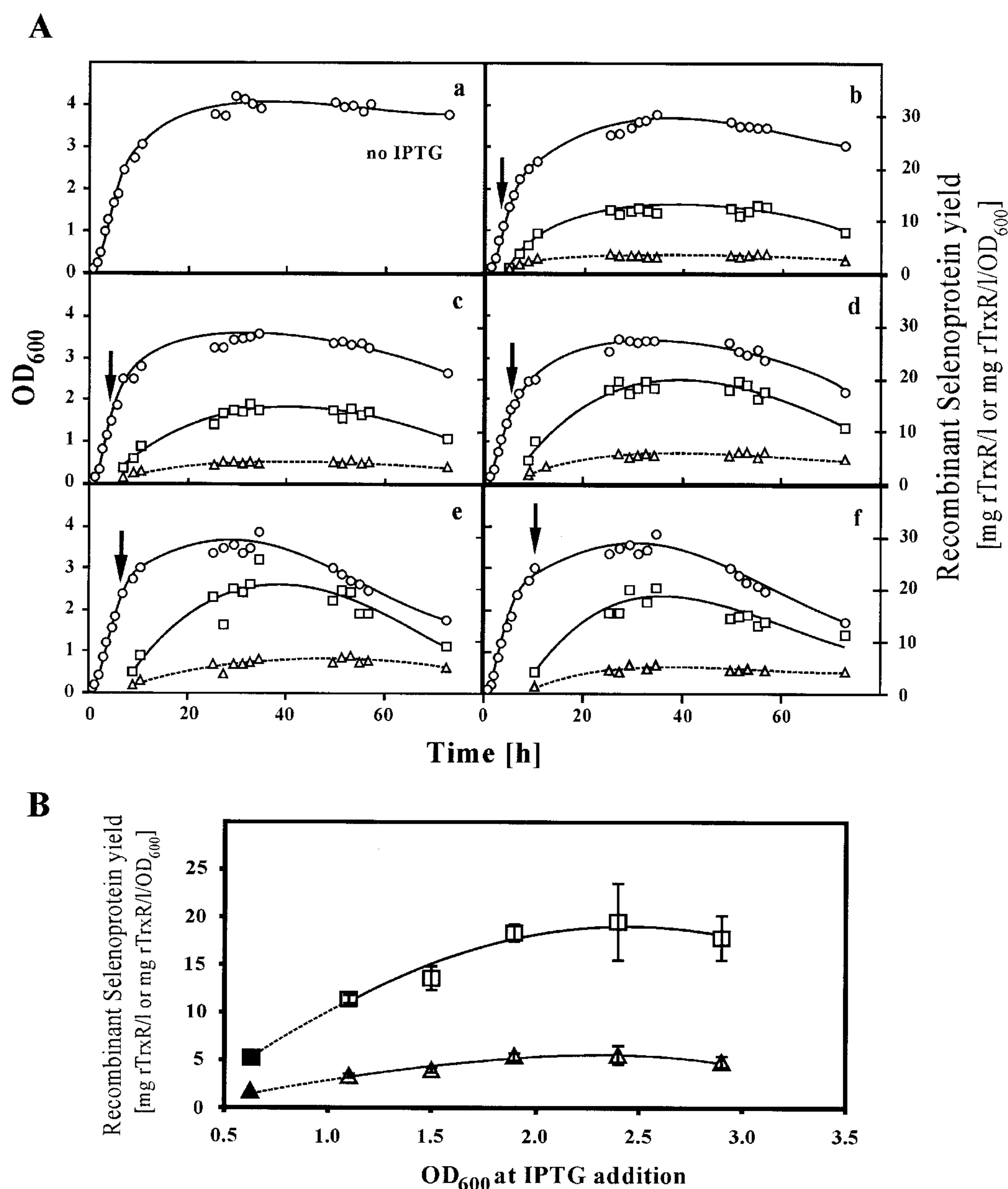

FIG. 3. Yield of recombinant selenoprotein is significantly increased when production is induced at late exponential phase. (A) Growth of 150-ml cultures of BL21(DE3) cells transformed with the pET-TRS_{TER} and pSUABC vectors is shown as OD_{600} (circles in panel a) in uninduced culture or cultures where selenoprotein production was induced with the addition of 100 μM IPTG at an OD_{600} of 1.1, 1.5, 1.9, 2.4, or 2.9 (b to f, resepctively), as indicated by arrows. Each culture was grown aerobically under vigorous shaking and moved from 37 to 24°C at the time of IPTG addition. To enable comparison of the effects on growth of IPTG addition, the uninduced culture shown in a was correspondingly moved from 37 to 24°C at an OD_{600} of 2.7. The production of recombinant selenoprotein was assessed subsequent to the addition of IPTG with determination of enzymatic activity, as described in Materials and Methods. The selenoprotein yields are given as milligrams of selenoprotein recombinant TrxR produced per liter culture (squares) as well as milligrams of selenoprotein recombinant TrxR produced per liter of culture per OD_{600} (triangles). Note that both the cellular and total yields increased upon induction at higher OD_{600} (i.e., panels d to f compared to panels b and c), while the cellular yield within each experiment reached nearly constant levels at 24 h of induction, resulting in the total yields' subsequently being solely dependent on the bacterial densities of the cultures. (B) Selenoprotein yields of the experiments shown in panel A as a function of OD_{600} at the time of IPTG addition and mean ± standard deviation of the 24- to 35-h time points, clearly illustrating that the highest yields were obtained when selenoprotein expression was induced at an OD_{600} of ca. 2.4, i.e., late exponential phase, just before transition into stationary phase, as revealed by the growth curves shown in panel A. The yields in panel B are given as milligrams of selenoprotein recombinant TrxR produced per liter of culture (squares) and milligrams of selenoprotein recombinant TrxR produced per liter of culture per OD_{600} (triangles). The solid symbols show, for comparison, the recombinant selenoprotein yield when expression was induced with IPTG at mid-exponential phase, i.e., OD_{600} of 0.5 (3), as in standard protocols.

TABLE 4. Isolation of full-length selenocysteine-containing recombinant rat TrxR and determination of its specific activity

Sp act (U/mg) of original TrxR sample	Expt no.	TrxR sample eluted with DMPS	
		Amount eluted (μg)	Sp act (U/mg)
16	1	3.7	37.0
	2	3.8	40.0
11	3	5.9	34.4
	4	6.4	39.8
Mean ± SD			37.8 ± 2.6

at low arabinose concentrations due to constitutive *araE* expression (19).

Interpretations from expression experiments with low transcription levels are further complicated by the general fact that under such conditions, identical promoters in the same bacterial host background and culture conditions display a wide range of transcriptional activity due to stochastic "noise" (10). Therefore, we simply conclude from our arabinose titration experiments that although they are difficult to interpret in terms of stoichiometries at the cellular level, no conditions could be found that yielded higher level or specific activities of the produced selenoprotein than the stronger T7-driven expression. Our results on the highest total yield possible of a recombinant selenoprotein somewhat complement the studies of Suppmann et al. (35), where the readthrough efficiency of the native *E. coli* Sec incorporation machinery was demonstrated to be highly inefficient compared to incorporation of the other 20 amino acids. However, in that study, the native bacterial readthrough SECIS element of formate dehydrogenase H was analyzed, whereas here a major difference is our use of an engineered SECIS variant not translated through the SelB binding loop (3). This seemed to result in a higher efficiency than seen by others engineering a variant readthrough SECIS element for Sec insertion into protein (15). In other, unpublished experiments of ours with variants of SECIS elements designed to insert Sec at internal positions of recombinant proteins, we also noted considerably lower yields than that which we reported here for rat TrxR.

It was surprising to us that the ratio of full-length TrxR selenoprotein over the UGA-truncated enzyme clearly increased with increasing transcription levels. This may indicate less efficient RF2-mediated termination at higher levels of selenoprotein mRNA, higher susceptibility of the truncated protein to protease digestion, or a combination of these and other factors. An increased stability of the full-length protein may be supported by the fact that the oxidized form of the full-length TrxR is highly resistant to carboxypeptidase digestion (41). However, it is not likely that the C-terminal motif of overproduced mammalian TrxR would stay in the oxidized state in the bacterial cytosol. As the endogenous bacterial thioredoxin is a good substrate for the mammalian enzyme (4), it is, however, conceivable that overexpression of this reductase may in fact contribute to the overall redox state of the bacterial cell and that it may to some extent also be present in the oxidized state.

The very high production level of approximately 20 mg of full-length TrxR selenoprotein per liter of culture achieved here is a valuable advancement forming the basis for further development of a useful bacterial system for the production of heterologous recombinant selenoproteins. The results are likely a reflection of decreased RF2 function when the bacterial cells are about to enter stationary phase, as the highest selenoprotein expression was obtained in cells induced in late exponential phase. This finding agrees with reports showing that RF2 has highest activity during the rapid initial phase of exponential growth (28). The very low yields of recombinant TrxR when IPTG was added to a culture that had already reached a steady-state stationary phase may be explained by a lack of efficient overall protein translation in such cells, illustrating the delicate balance between anabolic and catabolic pathways.

It should also be noted that the phases of a developing bacterial culture may be exceedingly more complex than previously believed. Up to 15 separate biochemically and physically distinct metabolic states of *E. coli* were recently demonstrated throughout the growth of a bacterial culture, each state with its own specific profile of expression of transcription factors and other phenotype determinants (27). The yield in expression of a recombinant selenoprotein may hence be highly dependent upon the specific growth phase of the bacterial culture, as also suggested by this work. Recent data indicate that aerobically grown *E. coli* may also induce formate dehydrogenase N in stationary phase (6). Hence, *E. coli* may have an augmented machinery for the synthesis of selenoprotein at the transition to stationary phase, at least in comparison to the efficiency of RF2-mediated truncation at a selenocysteine-encoding UGA. We have demonstrated here that this property may be utilized for efficient use of *E. coli* for production of mammalian TrxR and possibly also of other recombinant selenoproteins.

ACKNOWLEDGMENTS

We are grateful for helpful advice provided by Carlos Gitler and Edna Kalef, Weizmann Institute of Science, Rehovot, Israel, regarding the phenylarsine oxide-Sepharose separations and Jay Keasling, Department of Chemical Engineering, University of California, Berkeley, for kindly providing *E. coli* strain BW27783.

This study was supported by grants from the Karolinska Institute, the Swedish Society for Medicine, the Swedish Cancer Society (projects 3775 and 4056), and the Swedish Research Council for Medicine (projects 14527 and 14528).

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