

Solubilization and Renaturation of Overexpressed Aggregates of Mutant Tryptophan Synthase α -Subunits

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Certain *Escherichia coli* tryptophan synthase mutant α -subunits encoded from mutagenized *trpA*-containing plasmids were overexpressed as insoluble aggregates which were seen as large, intracellular inclusion bodies. The insoluble aggregates were solubilized to various degrees by several neutral, chaotropic salts. The order of effectiveness of these salts (KSCN, NaI > NaNO₃, LiBr > CaCl₂) followed that for the Hofmeister series. Optimum conditions for the use of KSCN resulted in a maximum 70 to 75% solubilization of the aggregate forms for all mutant α -subunits examined. Removal of KSCN by dialysis resulted in the recovery of biological activity and of certain characteristic structural properties. Such salts may be a useful alternative for other recombinant protein aggregates which resist complete renaturation by commonly used treatments with guanidine or urea.

The cloning, amplification, and expression of genes in *Escherichia coli* have provided a potentially abundant source of proteins of commercial value and for experimental analysis of protein structure-function relationships. However, a recurring problem often encountered has been that the recombinant proteins are expressed as large, insoluble aggregates (3-6, 8, 9, 11-14, 16, 18, 21, 26, 28, 29, 32, 34-36). Frequently, these aggregates are large enough to be visualized as intracellular inclusion bodies (12, 26, 28, 29, 34). Although the exact nature of the aggregates remains unclear, it is generally thought that they consist of an array of misfolded polypeptide chains in which there are substantial intermolecular interactions, possibly including intermolecular disulfide bonding.

These aggregates are commonly solubilized after denaturation by 5 to 6 M guanidine or urea and renaturation, occasionally in the presence of glutathione redox buffers of suitable potential to promote disulfide bond reshuffling. A major concern with such treatments is whether the polypeptides can be renatured into a completely biologically active state. Although some apparent limited success with these protocols has been reported, it is clear that for some proteins, complete renaturation is not possible. More recently, a relatively gentle treatment with Q- or S-Sepharose followed by salt elution has been shown to be effective in solubilizing several recombinant proteins, but the resulting preparations appear to be only partially active (12).

During the course of in vitro mutagenesis studies (24) of the *E. coli trpA* gene and the subsequent overexpression in *E. coli* of the encoded tryptophan synthase (TSase) α -subunit, we have encountered a similar problem. Certain mutant α -subunits containing single amino acid differences from the wild-type protein appeared to be expressed very poorly when only soluble extracts were examined. We report here that for this class of mutant α -subunits, the host cells contain massive inclusion bodies consisting of insoluble, aggregated forms of these proteins. Such mutant α -subunits are of particular interest for structure-function studies because they may represent polypeptides with altered folded structures or decreased folding rates. Since the TSase α -subunit will not completely renature after guanidine

treatment and urea is not effective in solubilizing these aggregates, alternative methods were sought to obtain these mutant α -subunits in soluble form for further purification and subsequent analysis. We report here the successful use of several chaotropic neutral salts of the Hofmeister series. Substantial solubilization for all the mutant α -subunits of this class can be achieved. Such salts may be potentially useful for other proteins or enzymes that resist complete renaturation by previously reported protocols.

MATERIALS AND METHODS

Bacterial strain and plasmids. *E. coli* RB797 (F' *lacI^q proL8/arg Nal^r Rif^r recA sup lac pro*) served as the host strain for plasmids containing the mutant *trpA* genes. In all plasmid constructions (24), the *trpA* gene was under control of the *tac* promoter (2) and was inducible by isopropylthio- β -D-galactoside (IPTG) or lactose. Individual plasmids encode the wild-type α -subunit or mutant α -subunits designated by their alterations as follows: YC-4 (i.e., an α -subunit containing a Tyr \rightarrow Cys alteration at position 4 of the α -polypeptide), SP-6, PS-21, PL-28, SL-33, GS-44, DG-46, GD-51, PH-53, DN-60, TK-63, and RC-89. All except RC-89 contain the ampicillin resistance β -lactamase gene. The RC-89 *trpA* gene is present on a chloramphenicol acetyltransferase (CAT)-encoding plasmid (W.-K. Lim and J. K. Hardman, unpublished).

Culture conditions. Cells were grown at 37°C with shaking in modified L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing ampicillin (30 μ g/ml) or chloramphenicol (10 μ g/ml) to an OD₆₀₀ of 0.4 to 0.5 and induced with lactose (1%, final concentration). Following growth (usually in 40-ml cultures) for 20 to 22 h, at which time the OD₆₀₀ was 2.1 to 2.2, the cells were harvested and washed with 10 ml of a 0.1 M Tris hydrochloride buffer (pH 7.8) containing 0.5 mM disodium EDTA and 5 mM dithiothreitol (DTT). Except where noted, this buffer was used in all experiments. The cells were suspended in 3.0 ml of buffer, sonicated, and centrifuged at 28,000 $\times g$ for 15 min. The supernatant represents the soluble (S) extract. The pellet (P) was resuspended in 1.0 ml of buffer.

Solubilization conditions. A 50% pellet suspension was prepared with equal volumes, usually 200 μ l each, of the pellet preparations and salt (in buffer), gently agitated for 2 h

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(except where noted differently) at room temperature, and centrifuged at maximum speed in a microfuge. The supernatant was dialyzed overnight against 1,000 volumes of buffer; this represents the solubilized pellet (SP). The pellet was washed with 300 μ l of buffer and resuspended in 400 μ l of buffer; this represents the second pellet (2P).

SDS-PAGE. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 10 μ l of S and P preparations and 20 μ l of SP and 2P preparations was treated with SDS and β -mercaptoethanol and electrophoresed by the method of Laemmli (16). In order to quantitate the degree of solubilization, gel lanes were scanned with a model EC190 transmission densitometer (E-C Apparatus Corp.). When examined, the amount of stained protein estimated in this manner for the α -polypeptide bands in the SP plus 2P lanes was 85 to 100% of that estimated in the P lane.

Purification and assays of TSase α -subunits. α -Subunit purification was performed by the method of Milton et al. (24). The TSase β_2 -subunit was obtained from induced cultures containing a CAT-overexpressing plasmid carrying the *tac* promoter and *trpB* gene (M. McDaniel and J. K. Hardman, unpublished). Culture conditions and crude extract preparation were similar to those described above except that the buffer was 0.2 M potassium phosphate (pH 7.8) containing 5 mM disodium EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM β -mercaptoethanol, and 0.02 mM pyridoxal-5-phosphate. The crude extract was heat treated (22) and applied to a Sephadex A-50 column (2.5 by 40 cm) equilibrated with the above buffer. The β_2 -subunit was eluted with a linear gradient (500 ml of 0.2 M potassium phosphate buffer and 500 ml of 0.5 M potassium phosphate buffer, each at pH 7.8 and containing the above additions) at a flow rate of 15 ml/h. Fractions containing the β_2 -subunit were concentrated by ultrafiltration and stored at -70°C as a 60% saturated ammonium sulfate suspension.

The activity of the α -subunit was determined in two TSase reactions in which the fully constituted TSase $\alpha_2\beta_2$ complex activity was measured. In each assay, the α -subunit was limiting. The activity in the reaction in which indole plus glyceraldehyde-3-phosphate is converted to indoleglycerol phosphate was measured by modification of the Smith and Yanofsky method (30). The modification consisted of including DTT (0.2 mM), disodium EDTA (4 mM), pyridoxal-5-phosphate (0.02 mM), and NaCl (0.18 M) in the reaction mixtures. The activity in the reaction in which indole plus serine is converted to tryptophan was determined by the method of Kirschner et al. (15). One unit of activity represents the appearance of 0.1 μ mol of product or the disappearance of 0.1 μ mol of substrate in 20 min. Specific activities are given as units per milligram of protein.

Analytical assays. Preparations of mutant and wild-type α -subunits dialyzed against buffer containing no DTT were titrated with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) by the method of Malkinson and Hardman (20). Protein was estimated by the microbiret assay (19) or, for pure α -subunit preparations, by measuring the A_{278} (1).

Electron microscopy. Cells were grown, induced, and harvested as described above. After being washed with 0.1 M potassium phosphate buffer (pH 7), the cells were suspended in 4 ml of this buffer. A portion (3.0 ml) of the suspension was sonicated and centrifuged as described above. The pellet was suspended in 1.0 ml of the above buffer, incubated for 30 min on ice in the presence of 1% sodium cholate, centrifuged, and resuspended in 1 ml of the above buffer. Both the whole-cell suspension and this cholate-treated pellet preparation were fixed in 2.5% (vol/

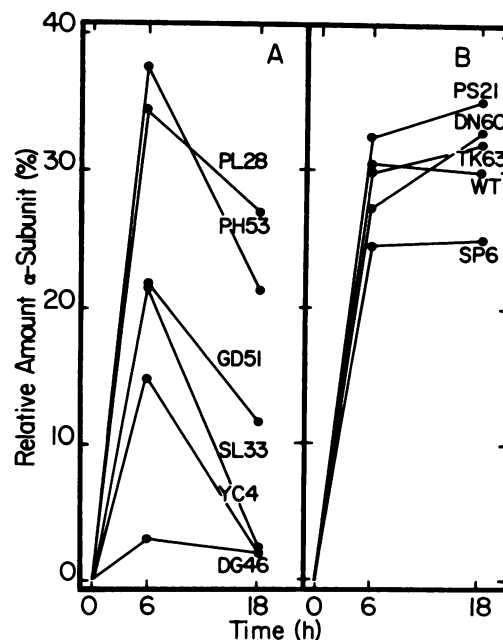


FIG. 1. Relative levels of α -subunits in soluble extracts. Cultures containing plasmids carrying wild-type and mutant genes were grown and induced with lactose. At 6 and 18 h after induction, cells were harvested and soluble extracts were prepared. Samples (50 μ g) of soluble extracts were electrophoresed by SDS-PAGE. The stained gels were scanned, and the relative amount of α -polypeptide was estimated for each preparation.

vol) glutaraldehyde in the above buffer, postfixed in 2% (wt/vol) osmium tetroxide, dehydrated in a graded ethanol series, and embedded in Spurr low-viscosity resin (31). Sections were stained in 0.5% uranyl acetate-lead citrate and examined with a Zeiss 10A transmission electron microscope operated at 60 kV.

RESULTS

Induction of wild-type and mutant α -subunits. All of the α -subunits studied here are encoded by plasmids containing *trpA* genes under control of the *tac* promoter. Thus, all are inducible by lactose or IPTG. Soluble extracts of cells induced for various lengths of time were examined by SDS-PAGE, and the relative amount of α -polypeptide was estimated after gel scanning. Two apparent stability types were noted. In one group (Fig. 1B), typical of the wild-type α -subunit, the mutant proteins were induced to steady levels (25 to 35% of the total soluble protein). Another group of mutant α -subunits (Fig. 1A) exhibited variable increases as soluble protein forms, but with longer induction times, marked decreases (25 to 90%) were observed. This decrease, originally attributed to proteolytic turnover (24), was due to the conversion of soluble forms of the proteins into insoluble particulate forms. These particulate forms were found among the cell debris in the pellet after sonication and centrifugation. Thus, all were induced to approximately the same level, but some, rather than being proteolysed, were sequestered into aggregates to a much greater, although variable, extent than others. It should be noted that even those in the wild-type group can be found in the pellet fraction but only at very low levels (1 to 2%). We selected mutant protein SL-33 as a representative of the unstable

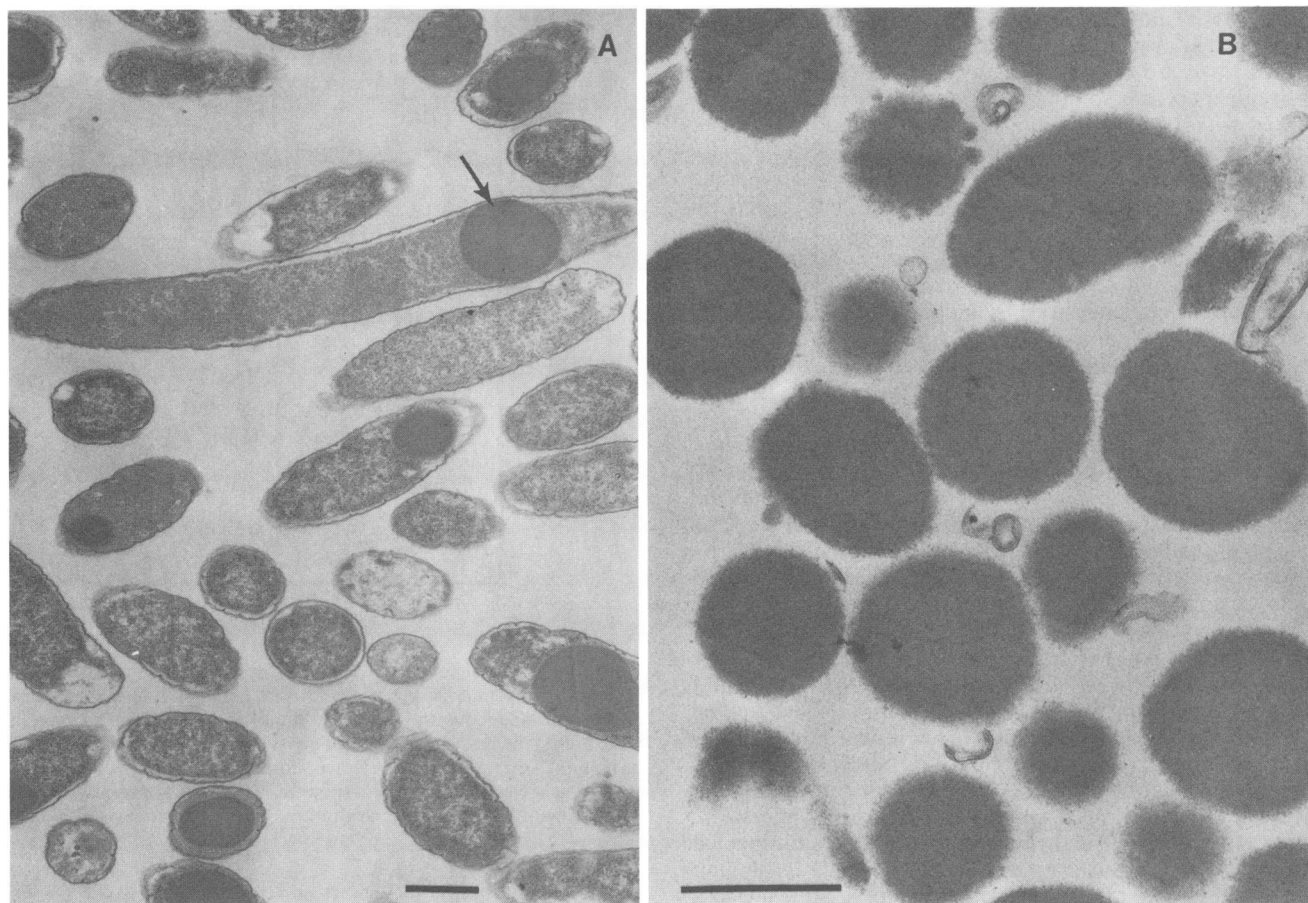


FIG. 2. Electron micrographs of mutant cells (A) and cholate-treated pellet fraction (B) containing SL-33 α -subunit. The arrow indicates an intracellular inclusion body. Bars, 0.5 μ m.

group for further analysis and for many of the solubilization experiments.

Concomitant with the loss of soluble SL-33 α -polypeptide, there appeared large, intracellular inclusion bodies (Fig. 2A, arrow). Figure 2A is an electron micrograph of intact cells containing fully induced (22 h) SL-33 α -subunit. No membrane surrounding these bodies is evident. Moreover, electron micrographs of pellet suspensions, treated with 1% sodium cholate to remove membranes, indicate (Fig. 2B) that the bulk of the particulate material remained as large, electron-dense particles. As will be shown below, 80 to 85% of the protein material in these pellet suspensions consisted of α -polypeptide.

Prior to attempts at solubilization, efforts were made to reduce the degree of aggregation by varying the culture conditions. Reasoning that excessive, rapid polypeptide synthesis together with potentially slow folding rates favored intermolecular interactions (i.e., aggregate formation), we attempted to reduce the rate and extent of α -polypeptide synthesis. The effects of lowering the growth temperature (from 37 to 22°C) and limiting the inducer IPTG concentration (from 1 mM through 1 μ M) was explored. A similar approach proved useful for the prosubtilisin-*ompA* fusion protein (32). Neither condition, however, was successful with the SL-33 α -subunit. At reduced temperature, no induction occurred. Although the reason for this remains obscure, it is clear that reduced plasmid content is not the explanation. When IPTG concentrations were lowered to a

range where induction levels became inducer dependent, no differences were evident in the distribution of SL-33 α -subunit between the soluble and pellet preparation. It should be noted here that the *tac* promoter is leaky, and under completely uninduced conditions, α -subunit production was still substantial (24).

Solubilization of SL-33 α -subunit aggregate by neutral salts.

The results of treating the SL-33 pellet fraction with a series of neutral salts are shown in Fig. 3. Five salts, each at a 1 M concentration, were used: KSCN, NaI, NaNO₃, LiBr, and CaCl₂. At these concentrations, KSCN and NaI appeared to solubilize 56 to 58% of the SL-33 polypeptide; NaNO₃ and LiBr were less effective (17 and 8% solubilization, respectively); and CaCl₂ was completely ineffective (data not shown).

Since KSCN had previously been employed to resolve the α - and β_2 -subunits of the fully constituted TSase (23) with no irreversible loss of activity, we focused most of our efforts on refining the conditions for the use of this salt. The optimum conditions come from the results shown in Fig. 4. Solubilization was optimal at 70 to 75% solubilization by treatment with 1 to 2 M KSCN for 1 to 2 h at room temperature. Similar results were obtained at 5°C. In each experiment, the percent solubilization value comes from the relative distribution of SL-33 polypeptide between the SP (solubilized pellet) and 2P (second pellet) fractions as determined by scanning of the gels. Variations in pellet suspension concentrations over a fivefold range had no effect, nor

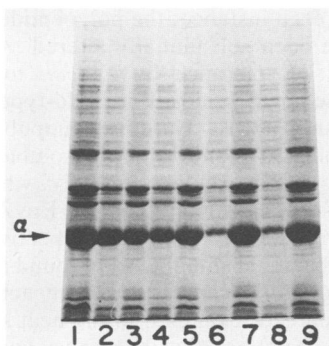


FIG. 3. Effect of neutral salts on solubilization of SL-33 α -subunit. Samples of the P fraction (10 μ l, lane 1) and the SP and 2P fractions (20 μ l) obtained after solubilization of P fraction by 1 M KSCN (lanes 2 and 3), NaI (lanes 4 and 5), NaNO₃ (lanes 6 and 7), and LiBr (lanes 8 and 9) were electrophoresed by SDS-PAGE. Each lane was scanned, and the relative distribution of the SL-33 polypeptide between the SP and 2P fractions for each salt represents the percent solubilization. The arrow indicates the mobility of the α -polypeptide.

did the presence or absence of DTT. Moreover, retreatment of 2P fractions did not further increase solubilization. Substitution of phosphate buffers reduced the solubilizing effectiveness by about 50% at all KSCN concentrations.

Solubilization of other mutant α -subunits. Table 1 presents the results of KSCN treatment of several additional mutant α -subunits of the unstable group. Each was treated with 1.5 M KSCN for 2 h. The S, P, SP, and 2P fractions were electrophoresed, the gel lanes were scanned, and the relative distribution of α -polypeptide between the S and P fractions (percent insoluble) and between the SP and 2P fractions (percent solubilized) was calculated. Figure 5 depicts a typical gel obtained in these analyses. For the majority of these mutants, 70 to 90% of the expressed α -polypeptide was found in the pellet, and for each, the degree of solubilization was approximately the same as the found with the SL-33 α -subunit.

Renaturation of solubilized α -subunits: recovery of activity.

It was of interest to determine whether the KSCN solubilization treatment resulted in complete renaturation. For the majority of the unstable mutant α -subunits, it is impossible

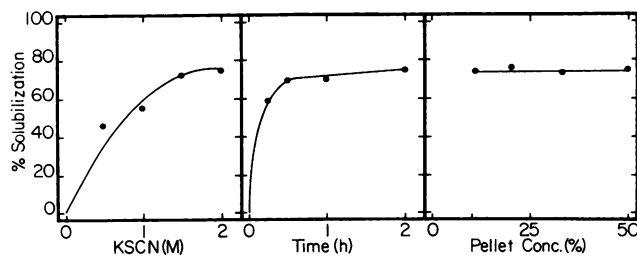


FIG. 4. Effects of KSCN, pellet concentrations, and time on solubilization of SL-33 α -subunit. (Left) The SP and 2P fractions were prepared with various concentrations of KSCN; (middle) the SP and 2P fractions were prepared with 2 M KSCN; (right) the amount of pellet material was decreased to various extents from the usual 50% pellet suspension used in all other experiments. In all three experiments, 20- μ l samples of the SP and 2P fractions were electrophoresed by SDS-PAGE, and the distribution of α -polypeptide between these fractions was estimated after scanning. The relative amount of α -polypeptide found in the SP fraction is shown ("percent solubilization").

TABLE 1. Solubilization of mutant α -subunits^a

Mutant subunit	% Insoluble	% Solubilized
YC-4	70	87
PL-28	68	69
SL-33	70	65
GS-44	69	74
DG-46	87	73
GD-51	23	73
RC-89	89	81

^a The data for mutant α -subunits PL-28, GS-44, and DG-46 were obtained by scanning the gel shown in Fig. 5. A similar analysis was made for the remaining α -subunits.

to predict the activities of the solubilized preparations, since "normal" preparations obtained from soluble extracts were not available. However, the PL-28 α -subunit has been purified from soluble extracts, and its specific activities in the different TSase assays can be determined. In addition, as noted above, a small fraction (1 to 2%) of the wild-type α -subunit appears in the pellet fraction; this material has been solubilized. Thus, a comparison of the activities of the PL-28 and wild-type α -subunit preparations obtained from soluble extracts and from solubilization treatments would provide a measure of renaturation. The PL-28 α -subunit solubilized from the pellet fraction was 80 to 85% pure and could be assayed directly. The KSCN-solubilized wild-type α -subunit was purified to homogeneity by our usual procedure (24). The specific activities in these assays were nearly identical for both soluble and KSCN-solubilized preparations of both wild-type and PL-28 α -subunits (Table 2).

Sulfhydryl titrations. One feature of α -subunit structure that has been employed to determine native structure is the availability of Cys residues for sulfhydryl reagents. There are three Cys residues in the native protein, and two of these appear to be topographically adjacent (7, 10). As a result of this conformation, one or the other of these two residues will react with sulfhydryl reagents such as *N*-ethylmaleimide and DTNB. Thus, for wild-type and many mutant α -subunits, there appeared to be a net of 1.0 to 1.2 mol of sulfhydryl group per mol of protein. There were no disulfide bonds,

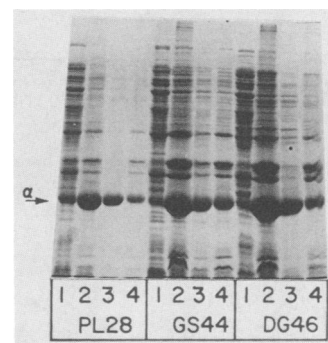


FIG. 5. Solubilization of other mutant α -subunits. S, P, SP, and 2P fractions were prepared for mutant α -subunits PL-28, GS-44, and DG-46. Samples (10 μ l) of the S (lanes 1) and P (lanes 2) fractions and 20- μ l samples of the SP (lanes 3) and 2P (lanes 4) fractions were electrophoresed by SDS-PAGE and the lanes were scanned. The arrow indicates the mobility of α -polypeptide. From the distribution of α -polypeptide between the S and P fractions, the percent insoluble value (Table 1) was estimated; from the distribution of α -polypeptide between the SP and 2P fractions, the percent solubilized value was obtained. The amount of α -polypeptide in the SP plus 2P lanes was 85 to 100% of that estimated in the P lane.

TABLE 2. Specific activities of α -subunits^a

Prepn	Sp act ^b (U/mg of protein)	
	In→Trp	In→InGP
Wild-type α -subunit		
Soluble	4,706	1,688
KSCN solubilized	4,778 (102%)	1,820 (108%)
PL-28 α -subunit		
Soluble	1,478	
KSCN solubilized	1,330 (90%)	449 (98%)

^a The specific activities of the KSCN-solubilized preparation of the PL-28 α -subunit have been corrected for its relative purity as determined by scanning of SDS-PAGE gels. The percent recovery of activity after KSCN treatment is shown in parentheses.

^b In→Trp, Indole plus serine to tryptophan; In→InGP, indole plus glyceraldehyde-3-phosphate to indoleglycerol phosphate.

since upon denaturation all three Cys residues could be titrated. Figure 6 presents the results of DTNB titrations of PL-28 α -subunit preparations obtained from soluble extracts and from KSCN solubilization in the presence and absence of DTT. All were identical and similar to wild-type preparations. In the presence of 6 M urea, the additional Cys residues were titratable. Thus, by these criteria, the KSCN solubilization resulted in virtually complete recovery of activity and structure.

DISCUSSION

The formation of inclusion bodies containing insoluble aggregates of certain TSase mutant α -subunits suggests that such structures are not exclusively characteristic of eucaryotic protein expression in *E. coli*. This phenomenon may be related to a number of factors. One of these may be the degree of abnormality of the structures as exemplified by both eucaryotic proteins and severely altered *E. coli* proteins, such as the mutant *E. coli* proteins reported here, β -galactosidase nonsense fragments (5), and abnormal *E. coli* proteins arising from the incorporation of amino acid

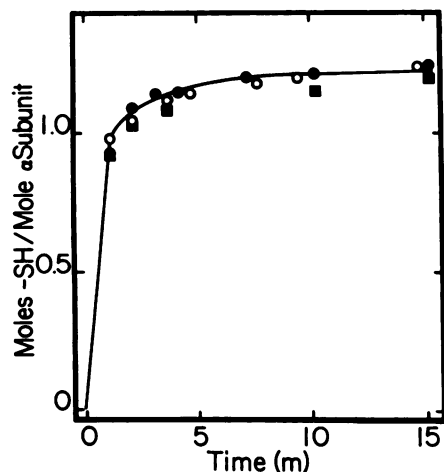


FIG. 6. Sulfhydryl content of soluble and solubilized PL-28 α -subunit. The sulfhydryl (SH) content of each preparation (250 to 350 μ g) was determined by DTNB titration. PL-28 α -subunit was obtained from purified, soluble preparations (●) and SP preparations treated with KSCN in the presence (■) and absence (○) of DTT.

analogous (27). In each instance, the polypeptide folding properties may have been substantially altered. Another factor may be a lack of tolerance by *E. coli* to the level of production of the polypeptides. Both wild-type *E. coli* TSase α - and β -subunits (J. K. Hardman, unpublished), when expressed beyond 30 to 40% of the soluble protein, are sequestered into such particles. Similarly, when the *E. coli* RNA polymerase sigma subunit and the EnvZ protein were expressed 100-fold and 10,000-fold, respectively, over the normal level, substantial amounts were found in the pellet (9, 21). These observations may suggest an active biological mechanism similar, for example, to the heat shock effect in *E. coli* (25). This notion, that excessive levels of certain abnormal proteins may trigger a heat shock response leading to an active sequestering event, was tested. We employed most of the known *E. coli* strains containing defects in heat shock protein as hosts for plasmids expressing the mutant α -subunits. Unfortunately, none showed any decrease in the aggregation of these proteins.

The mechanism(s) responsible for sequestering these proteins is still unresolved. Although aberrant disulfide bonding may be partially responsible in some instances, it clearly does not seem to be a major factor for the mutant α -subunits. The presence or absence of sulfhydryl compounds during the denaturation and renaturation steps neither increased the yield nor altered the sulfhydryl content of the proteins. It does appear, however, that there are multiple forms of the insoluble aggregates. The extent of solubilization reached a maximum at about 70 to 75% for all the mutant α -subunits examined. Neither decreasing the concentration of pellet material, increasing the concentration of denaturant, nor reextracting the pellet increased the yield. Thus, about 20 to 30% of the α -polypeptides remain in an insoluble, particulate form(s). This is consistent with our initial observations that 4 to 5 M guanidine treatment readily solubilizes virtually all (~90%) of the pellet fraction, but upon removal of guanidine by dialysis, about 30% or more of the protein becomes insoluble again. The remaining soluble fraction is only partially active. The residual particulate material may represent a separate type of complex or a core of differently misfolded polypeptides around which less severely misfolded polypeptides have aggregated.

The use of neutral salts as protein perturbants was described in some detail by von Hippel and Schleich in 1969 (33). These salts were shown to dissociate, denature, or dissolve a number of proteins. The strength of the perturbation generally followed the series originally described by Hofmeister for precipitation of euglobulins. The effectiveness of salting-in or denaturation of RNase, reported for several relevant anions, was $\text{PO}_4^{2-} < \text{Cl}^- < \text{Br}^- < \text{NO}_3^- < \text{I}^- < \text{SCN}^-$, for cations, it was: $\text{K}^+, \text{Na}^+ < \text{Li}^+ < \text{Ca}^{2+}$. The solubilizing effectiveness of the salts we employed was $\text{CaCl}_2 < \text{NaNO}_3, \text{LiBr} < \text{NaI}, \text{KSCN}$. Furthermore, the use of phosphate buffers antagonized the effectiveness of KSCN. Thus, the strength of these salts in dissolving the α -subunit aggregates appears to be consistent with the order in this series. Although the mechanism for solubilization of the α -subunit aggregates has not been examined, it has been suggested (33) that the salting-in characteristics of such salts involve their binding to and increased solubilization of the dipolar amide groups in proteins. In any event, they appear to work for α -subunit aggregates, and as noted earlier, they may serve as a worthwhile alternative to the use of guanidine or urea to solubilize similar recombinant protein aggregates.

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