Stabilization of Apoglobin by Low Temperature Increases Yield of Soluble Recombinant Hemoglobin in *Escherichia coli*

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Accumulation of soluble recombinant hemoglobin (rHb1.1) in *Escherichia coli* requires proper protein folding, prosthetic group (heme) addition, and subunit assembly. This served as a new model system for the study of the effects of temperature, protein synthesis rates, and protein accumulation rates on protein solubility in *E. coli*. Fermentation expression of rHb1.1 at 30°C from cultures containing a medium or high globin gene dosage (pBR-based or pUC-based plasmids with rHb1.1 genes under the control of the tac promoter) was compared. A medium gene dosage resulted in rHb1.1 accumulating to ~7% of the soluble cell protein, of which 78% was soluble. A high globin gene dosage resulted in a ≥3-fold increase in total globin to 23 to 24% of the soluble cell protein, but 70% was insoluble. Accumulation of insoluble rHb1.1 began immediately upon induction. The proportion of rHb1.1 from the high globin gene dosage that accumulated as insoluble globin was affected by reducing (i) the inducer concentration and (ii) the temperature. Reducing the inducer concentration reduced globin synthesis up to eightfold but increased the proportion of soluble rHb1.1 to 93%. In contrast, total globin protein synthesis was barely affected by reducing the temperature from 30 to 26°C, while soluble globin accumulation increased >2-fold to ~15% of the soluble cell protein. The contrast between the effects of reducing rates of protein synthesis and accumulation and those of reducing temperature suggests that low temperature stabilizes one or more folding intermediates. We propose a simplified physical model which integrates protein synthesis, folding, and heme association. This model shows that temperature-dependent apoglobin stability is the most critical factor in soluble rHb1.1 accumulation.

The need for an oxygen-carrying solution that can substitute for erythrocytes, can be administered without cross-matching, and carries no risk of transmission of infectious agents has led to the development of recombinant human hemoglobin that can be expressed in bacteria (16, 26). Production of high levels of functional recombinant hemoglobin tetramer (αβ) in *Escherichia coli* was achieved with the concomitant expression from a plasmid-borne operon of both α and β subunits and provision of exogenous heme (16). Recombinant human hemoglobin has also been expressed as a heterotetramer (rHb1.1) containing in each trimer one diapha globin plus two beta globins (26). Production of recombinant human hemoglobin in *E. coli* can result in formation of insoluble aggregates (14, 16, 38), a fate in common with that of many other recombinant proteins (reviewed in references 28 and 31). We have previously reported that insoluble globin is more protease sensitive (38). This has also been shown for insoluble aggregates of P22 tailspike endorhamnosidase (13) and murine Mx protein (32). For the P22 tailspike protein, sensitivity to proteases indicates a nonnative aggregate (13).

It is expected that rHb1.1 expressed to high levels within *E. coli* may form insoluble aggregates. Diapha or beta globin may misfold for a variety of reasons, including a limitation in heme availability and/or improper associations between diapha subunit domains. Weickert and Curry (38) observed that the absence of adequate heme production during globin protein biosynthesis results in insoluble aggregates. Consistent with this is the prediction by Mitraki and King (28) that aggregates are composed of partially folded apoprotein. The removal of heme from human hemoglobin in vitro results in partial unfolding and severely reduced solubility, which is reversed by heme addition (24). Inclusion bodies formed from unstable mutant hemoglobins have been seen in erythrocytes (5, 32). Inclusion bodies of recombinant human hemoglobin have been observed under conditions of high expression in *E. coli* (38). In the absence of heme, apoglobin can be considered a trapped folding intermediate.

Recombinant human hemoglobin may therefore be a useful molecule for in vivo protein folding and assembly studies because (i) the protein is well characterized, (ii) many mutants are available (23), (iii) it complexes with a prosthetic group, heme, and (iv) it associates as a heterotetramer. High-resolution X-ray structures of the hemoglobin tetramer in several different ligand states (3, 9, 27, 34) have assisted interpretation of many other studies which used hemoglobin as a model protein. These advantages are likely to assist interpretation of in vivo hemoglobin folding and aggregation studies, particularly future studies using mutants that influence these activities.

As a first step to investigate rHb1.1 as a model for in vivo protein folding and aggregation, we studied the effects of the rates of protein synthesis and accumulation of temperature on soluble and insoluble globin distribution during fermentation. Reducing the recombinant protein accumulation rate by reducing the inducer concentration improves the soluble expression of proteins in *E. coli* (2, 21, 35, 37). Lower temperature is well known to improve solubility of heterologous proteins in *E. coli* (1, 2, 6–8, 15, 17, 19, 30, 36, 37, 41; reviewed in references 28 and 31). Temperature is believed to work either by reducing protein synthesis rates through lower growth and reaction rates, so that they more closely match protein folding rates (21, 35), or as a critical parameter in protein folding and in partitioning of partially folded interme-
diate either native or aggregated states (28, 39). This study presents the effects of inducer concentration and temperature on rates of accumulation soluble and insoluble rHb1.1 in vivo in E. coli. In addition, a model to understand the effect of these rates on the hemoglobin assembly pathway is presented.

**MATERIALS AND METHODS**

**Bacterial strains.** E. coli SGE1662 (38) contained pSGE705, a medium-copy-number plasmid (pBR-based) containing the alpha and beta globin genes in an operon whose transcription is dependent upon the tac promoter (26), and was used to express rHb1.1, a recombinant human hemoglobin. A similar high-copy-number plasmid containing the pUC origin of replication was used to express rHb1.1 in SGE1464 (38).

**Fermentations.** Fermentations were performed in a defined medium in 15-liter BioLaffite fermentors generally as described by Looker et al. (25). Induction of expression was achieved by addition of IPTG (isopropyl-beta-D-thiogalactosidase) to between 5.5 and 1,000 nM. IPTG addition occurred at an optical density at 600 nm (OD600) of approximately 30. We examined the rates at which insoluble and soluble rHb1.1 accumulated under different temperature conditions (n = two or three independent fermentations per temperature; temperatures of 24, 26, 28, and 30°C were examined). One-milliliter samples were withdrawn into 1.7-mL Eppendorf tubes at 0, 3, 10, 30, and 60 min postinduction and at 2, 4, 6, 8, 10, 12, 14, and 16 h postinduction. These 1-mL samples were centrifuged in an Eppendorf centrifuge for 3 min, and the supernatants were removed. Pellets were stored at −80°C until processed.

**Dependence of percent soluble and insoluble rHb.** Cell mass in fermentation samples was determined directly or calculated from the measured cell density. Cells were lysed by lysozyme addition and incubation on ice, and the DNA was digested with DNase. The soluble and insoluble fractions were separated by centrifuging the lysate for 15 min in a microcentrifuge at top speed. The supernatant (soluble fraction) was transferred to another microcentrifuge tube. Hemoglobin was determined from the supernatant fraction of the lysate by analysis on a Biocad Perfusion Chromatography Workstation equipped with a Poros metal chelate fraction column charged with zinc or was quantified by difference spectroscopy as described by Looker et al. (25). The soluble fractions were assayed for total protein by using the bicinchoninic acid assay (Pierce no. 23225X) to establish a relationship between density (OD600) and total protein.

**RESULTS**

**Fermentation expression of rHb1.1 from a medium-copy-number plasmid.** In this study, production of rHb1.1 was examined primarily during 15-liter fermentations. Typical rHb1.1 production was accomplished in E. coli with a medium-copy-number plasmid (SGE1662) containing the diapha and beta globin genes on an operon under the control of the tac promoter (16, 26, 38). Twelve large-scale fermentations of SGE1662 were examined for the proportions of rHb1.1 present as soluble and insoluble protein. We measured globin protein solubility by using an enhanced chemiluminescence Western blot assay of the insoluble and soluble fractions from cell lysates. Nearly all of the globin protein detected was in the soluble fraction (~90% ± 8%). The percentage of each subunit that was soluble was relatively consistent (for diapha, ~89% ± 8%; for beta, ~90% ± 8%). This indicated that they were also stoichiometrically present in the insoluble fraction, suggesting that neither subunit accumulated preferentially as insoluble globin. Since the insoluble pellet represents approximately 7 to 8% of the total volume of the solution, some of the soluble hemoglobin may have been trapped in the insoluble pellet. Insoluble globin protein therefore represented ≤10%, and possibly as little as 2%, of the globin protein in these fermentations. Higher globin production was not correlated with a higher proportion of insoluble globin protein (r2 < 0.03 [data not shown]).

**Fermentation expression of rHb1.1 from a high-copy-number plasmid.** We next investigated expression of rHb1.1 by using a high-copy-number plasmid in strain SGE1464 (11, 38). We expected the increased gene dosage to result in higher globin protein accumulation rates. Fifteen-liter fermentations of SGE1464 performed at 30°C with 55 μM IPTG for induction yielded a greater-than-threefold increase in total globin protein over that in SGE1662 fermentations under identical 15-liter fermentor conditions (Table 1), consistent with the three- to fourfold-increased gene dosage (11). The increase

**TABLE 1. Effect of gene dosage, temperature, and induction on solubility of rHb1.1**

<table>
<thead>
<tr>
<th>Strain</th>
<th>IPTG (μM)</th>
<th>Temp (°C)</th>
<th>% Soluble rHb1.1 (mean ± SD)*</th>
<th>% Total globin (mean ± SD)*</th>
<th>% Soluble globin (mean ± SD)*</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>SGE1662</td>
<td>55</td>
<td>30</td>
<td>5.0 ± 1.2</td>
<td>6.8 ± 1.8</td>
<td>78.0 ± 20.5</td>
<td>8</td>
</tr>
<tr>
<td>SGE1464</td>
<td>55</td>
<td>30</td>
<td>6.7 ± 0.1</td>
<td>23.5 ± 4.3</td>
<td>29.5 ± 6.1</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>22.5</td>
<td>30</td>
<td>6.1 ± 1.0</td>
<td>17.7 ± 4.4</td>
<td>36.9 ± 10.5</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>30</td>
<td>5.9 ± 1.9</td>
<td>17.2 ± 7.5</td>
<td>40.3 ± 17.1</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>7.5</td>
<td>30</td>
<td>4.3 ± 0.4</td>
<td>5.2 ± 0.9</td>
<td>82.6 ± 7.5</td>
<td>2</td>
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<tr>
<td></td>
<td>5.5</td>
<td>30</td>
<td>2.8 ± 0.3</td>
<td>3.0 ± 0.2</td>
<td>93.3 ± 5.5</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>55</td>
<td>28</td>
<td>11.0 ± 1.3</td>
<td>22.4 ± 1.9</td>
<td>50.0 ± 10.1</td>
<td>2</td>
</tr>
</tbody>
</table>

* Percentages were performed in 15-liter BioLaffite fermentors as described in the text.

**a** The percentage of the total globin present in soluble form was determined for each fermentation sample. The averages of these independent determinations are shown. This average is not necessarily equivalent to dividing the percent soluble rHb1.1 by the percent total globin, since fermentations with higher globin accumulation would have greater weight in an average value.

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Each were performed. Blots were developed for 1 min with LumIGLO substrate (Kirkgaard & Perry Laboratories no. 54-61-00), covered with plastic wrap, and exposed to X-ray film (Amersham Hyperfilm-ECL no. RPN.203).

Developed films were scanned on a PDI (Huntington Station, N.Y.) scanning densitometer with the white-light setting recommended by the manufacturer. The densitometer is PDI’s model DNA 35 connected to a Sun Microsystems SPARC Station 2 computer running PDI’s Quantity One version 2.4 software. The software generates an image of the blot which is then analyzed. Image background was subtracted along a stripe down the center of each lane by a rolling-circle method. Each band was quantified as average density in the band times the band area. Quantitation values are in units of OD times square millimeters. Percent soluble rHb1.1 was calculated for each time point by the following formula: % soluble = (soluble value/soluble value + insoluble value) × 100.
was much less for the soluble rHb1.1; the percent cellular protein present as soluble rHb1.1 increased by only one-third. A large increase in the percentage of the globin protein persisting in the insoluble form was observed. In SGE1662 fermentations, 22% was insoluble, but in SGE1464 fermentations, ~70% was insoluble (Table 1).

**Inducer concentration.** The higher soluble rHb1.1 proportion from SGE1662 could result from a lower rate of globin accumulation due to the lower gene dosage relative to that for SGE1464. To obtain a lower rate of globin accumulation with the higher-gene-dosage strain, we reduced the level of IPTG used to induce rHb1.1 synthesis in SGE1464 in several steps from 55 to 5.5 μM. This resulted in an increase in the proportion of soluble globin from 30% at 55 μM IPTG to 93% at 5.5 μM IPTG and reduced the total rHb1.1 that accumulated by more than eightfold (Table 1). In spite of this, the 10-fold reduction in inducer reduced the soluble rHb1.1 by only a little more than 2-fold. Total globin production increased linearly in proportion to a logarithmic increase in IPTG over a 10-fold range of inducer concentrations (Fig. 1). Soluble rHb1.1 increased with increasing inducer concentration but at a much lower rate, especially at above 15 μM IPTG (Fig. 1). The percentage of total globin accounted for as soluble rHb1.1 was inversely proportional to the inducer concentration (Fig. 1), decreasing as total globin accumulation increased.

**Effect of temperature on soluble and insoluble rHb1.1 accumulation.** Another approach to reducing the rate of protein synthesis and/or accumulation is reducing the growth temperature. A 2°C reduction in the fermentation temperature, from 30 to 28°C, resulted in a >40% increase in the soluble rHb1.1 accumulation, to 9.4% of the soluble cell protein, without a significant reduction in the total globin accumulation (Table 1). This is in contrast to the IPTG reduction experiment described above, in which the increase in the soluble globin proportion was accompanied by a large reduction in total globin synthesis.

To build systematically on the effect of temperature on soluble rHb1.1 described above (Table 1), accumulation in both the soluble and insoluble fractions of the cell lysates was determined at different temperatures in otherwise identical fermentations. The initial rate of accumulation from induction through the first hour of accumulation and the overall rate of accumulation from 2 through 16 h postinduction were examined at four different temperatures covering a range from 24 to 30°C. No hemoglobin was detectable in initial samples taken immediately before induction, indicating that expression was strongly repressed (Fig. 2A and B). Hemoglobin accumulation in both the soluble and insoluble fractions was detected within 3 min after induction. The accumulation of insoluble and soluble globins occurred at a relatively constant rate for the first 60 min postinduction (Fig. 2).

The ratio of soluble to insoluble rHb1.1 early in the fermentation was roughly maintained throughout the fermentation (Fig. 3). The temperature dependence was observed early, and the proportional relationship between temperature and insolvability continued throughout the fermentations. Lower-temperature fermentations produced a higher proportion of soluble rHb1.1 than higher-temperature fermentations (Fig. 3), and at 26°C, the highest overall soluble rHb1.1 concentration, up to ~15% of the soluble cell protein, was observed (Fig. 3). This relationship is summarized in Fig. 4, which shows that although the proportion of insoluble protein dramatically changed, the total globin protein present in the cells remained relatively consistent throughout the temperature range studied, except for a modest decline at 24°C, the lowest temperature studied.

**A simplified model of hemoglobin synthesis.** To help understand these results, a model which integrates protein synthesis, folding, and heme association was developed. A numerical representation of the physical model enabled us to objectively understand the model’s properties and ability to account for the observed results. The rate constants represent complex multistep processes. The model (Fig. 5) relies on the following simplifying assumptions. (i) The concentration of heme (H) does not change significantly, since all fermentations were identically supplemented with heme. (ii) All reactions are irreversible. (iii) A single apo-hemoglobin (B) can either gain heme and become soluble (D) or interact with another apo-hemoglobin and precipitate as insoluble globin (E). (iv) Only those cells capable of growth can synthesize Hb. This fraction of the cell population is described by using Williams’ two-compartment description of cell growth (40) with the synthetic component (S) set to zero at induction. Numerical integration of the differential equations derived from this model, over the induction period, was carried out by using the predictor-corrector system of Gear (10). $k_s$ was estimated from cell growth kinetics, as were the starting concentrations for M and N. $k_s$ was taken from reference 12. $k_s$ is an empirical scaling factor to convert cell mass units (S) into apoglobin units (B), and along with $k_s$, $k_v$, and [H], was estimated by comparison of the simulated and observed soluble and insoluble hemoglobin kinetics at 30°C. Insoluble and soluble kinetics at 26°C were then simulated by decreasing only $k_s$ and $k_v$ until close agreement between the actual and simulated data was observed. The actual rate constants and starting concentrations used in the two simulations are shown in Tables 2 and 3, respectively.

The simulated and actual data correlated very well, with a clear conversion from a system producing mostly soluble product to a system producing mostly insoluble product as a result of increasing the fermentation temperature from 26 to 30°C (Fig. 6). The model is able to account for the increase in soluble globin at the expense of insoluble globin at lower temperatures primarily through an increase in the stability of the apoglobin species (B) (by a decrease in $k_s$) and, to a lesser extent, through a decrease in the growth rate ($k_v$), both of
FIG. 2. Accumulation of soluble and insoluble rHb1.1 immediately after induction at different fermentation temperatures. (A and B) Western blots of dialpha and beta globins in soluble (lanes S) and insoluble (lanes I) samples from the first 60 min after induction (times [in minutes] are given at the top) from fermentations at 26°C (A) and 28°C (B). A purified rHb1.1 sample standard (lane M) shows dialpha and beta globin bands on the Western blot (arrows). (C to F) Relative globin protein accumulation for soluble and insoluble rHb1.1. The highest measurement from the Western blot of each sample set was arbitrarily set at 1.0, and all other values were calculated relative to that value. Fermentations were at 24°C (C), 26°C (D), 28°C (E), and 30°C (F). Each graph represents the average values from three fermentations, except for panel C (n = 2).
which are expected at lower temperature. However, it is not possible to obtain the observed change in the soluble/insoluble globin ratio by changing the rate of cell growth ($k_4$) alone; it is the decrease in $k_2$ which most influences this ratio.

**DISCUSSION**

At a moderate globin gene dosage (SGE1662), globin synthesis resulted in rHb1.1 accumulation of up to 7% of the
soluble cell protein, and the globin itself was primarily soluble. The globin protein synthetic capacity was most likely limiting under the conditions of these fermentations; therefore, little excess globin protein was insoluble. At these expression levels, experimental variation in the level of soluble hemoglobin produced in fermentations represented primarily variation in the globin accumulation rather than variation in globin solubility. Under identical conditions, a high gene dosage (SGE1464) resulted in an increase in total globin accumulation, but most of the increased globin protein was insoluble. Insoluble globin accumulates in inclusion bodies, which are visible by electron microscopy (38), and contains approximately equal proportions of dia phila and beta globins as determined by Western blotting, suggesting that neither subunit accumulates preferentially in the insoluble fraction. The increase in globin accumulation was proportional to the gene dosage (11), and the increase in insoluble aggregates indicated that we had exceeded a putative limit for solubility of globin with these conditions.

Reducing the globin accumulation rate by lowering the inducer concentration increased the proportion of globin that accumulated in the soluble form, presumably by reducing the apoglobin concentration and therefore the products of the bimolecular insoluble globin formation reaction ($k_2$). This technique has been used to improve the soluble yield of other proteins in E. coli (21, 35). The protein accumulation rate is a key component of a kinetic model for protein aggregation in vivo (18), in which the proportion of aggregation is also determined by rates of folding and aggregation. This model predicts that at high levels of expression, the second-order aggregation reaction exceeds the first-order folding reaction, resulting in aggregation (18). Our inducer titration and simulation results seem to be consistent with this model.

Insoluble globin accumulated within a few minutes of induction, a time at which the globin concentration within the cell is extremely low. Several investigators have proposed that a high local concentration leads to aggregation (18, 31), perhaps through limited diffusion of partially folded intermediates or through interactions between nascent chains tethered to the polypeptide during translation. The observation of formation of insoluble globin at very low intracellular concentrations contradicts the assertion that the overall protein concentration alone drives aggregation, although Klein and Dharjati have reported evidence consistent with a solubility limit for a mutant CheY protein when the protein is overexpressed in E. coli (19). If a high local concentration was driving aggregation, fermentations performed at the same IPTG concentration and temperature with different gene dosages should result in the same local concentrations of folding intermediates, yet our results yielded very different solubility outcomes. Our results indicate that induction rapidly (in seconds to minutes) establishes a steady state of nascent globin chains yielding soluble or aggregated products. This reconciles our observations of modification of soluble globin proportions by different inducer concentrations or with different gene dosages. Since accumulation of soluble rHb1.1 began within 3 min of induction, the following mechanisms are unlikely to account for insoluble globin accumulation: (i) a critical concentration of hemoglobin (rather than nascent globin chains) must be reached to promote insolubility; (ii) a critical factor is exhausted, promoting insolubility; and (iii) protein synthesis in response to rHb1.1 synthesis is required to promote insolubility.

Reducing the temperature did not affect the total amount of globin synthesized, except at 24°C, the lowest temperature investigated, but it dramatically increased the proportion accumulating as soluble hemoglobin. The rate of accumulation of soluble and insoluble material was temperature dependent and relatively constant from induction through 16 h postinduction.

![FIG. 4. Total globin synthesis versus percent soluble rHb1.1. The range of temperature studied was 24 to 30°C. Temperature is plotted against percent rHb1.1 present as soluble protein (left vertical axis) and against total globin as a percentage of soluble E. coli protein (right vertical axis). rHb1.1 as percent soluble protein was the maximum average for the 14- or 16-h samples, whichever was higher, and percent soluble rHb1.1 was the average of the last four samples (10 to 16 h postinduction).](image)

![FIG. 5. Simplified model of hemoglobin synthesis. Comp., component; Div., division; Insol., insoluble. See text for details.](image)

**TABLE 2. Rate constants used for simulations**

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<thead>
<tr>
<th>Rate constant</th>
<th>Value used at:</th>
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<tr>
<td></td>
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<tr>
<td>$k_1$</td>
<td>$5 \times 10^{-4}$ h$^{-1}$</td>
</tr>
<tr>
<td>$k_2$</td>
<td>$6 \times 10^{10}$ M$^{-1}$ h$^{-1}$</td>
</tr>
<tr>
<td>$k_3$</td>
<td>$1.1 \times 10^{11}$ M$^{-1}$ h$^{-1}$</td>
</tr>
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<td>$k_a$</td>
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</tr>
<tr>
<td>$k_5$</td>
<td>$0.004$ h$^{-1}$</td>
</tr>
</tbody>
</table>

*a Units of $k_a$ are percentages of total biomass.

**TABLE 3. Starting concentrations used for simulations**

<table>
<thead>
<tr>
<th>Component</th>
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</tr>
</thead>
<tbody>
<tr>
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<tr>
<td>M</td>
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<tr>
<td>N</td>
<td>30</td>
</tr>
<tr>
<td>B</td>
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</tr>
<tr>
<td>H</td>
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</tr>
<tr>
<td>D</td>
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<tr>
<td>E</td>
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</table>

*a See Fig. 5.

*b The concentration for heme is given in molar units; all other units are percentages of total biomass.
At the higher temperatures, i.e., 28 and especially 30°C, a higher initial globin accumulation rate and then a lower rate of accumulation after 8 h postinduction was observed, indicating that later synthesis of globin was reduced or proteolysis was increased. The higher initial and lower later rates at 28 and 30°C account for the similar total globin accumulation achieved over a 6°C range of temperature. As the growth temperature increases from 25 to 30°C, the growth rate for *E. coli* increases by 40%, due primarily to increased elongation rates for rRNA and peptide chains (4). This is expected to have a proportional effect on the globin synthesis rate. This suggests that the mechanism by which IPTG reduction results in a higher proportion of soluble globin is fundamentally different from the mechanism by which temperature increases the proportion of soluble globin.

To help understand how temperature affects soluble globin accumulation in fermentations, the rate of accumulation of soluble and insoluble material was mathematically simulated to match very closely the observed temperature dependence of soluble and insoluble rHb1.1 accumulation. In our simplified model, the substantial increase in soluble yield at lower temperature can be accounted for very simply by the combination of an increase in the stability of the apoglobin intermediate and slower protein synthesis, both of which would be expected at lower temperatures. We also found that we could not account for the observed change in insolubility of globin due to temperature by any realistic adjustment in the growth rate (k₆) alone. The finding of similar total globin accumulations at various temperatures suggests that concentration-dependent association of subunits is unlikely to account for the differences observed, and this is therefore not included in the simplified model. Modeling suggests that stabilization of the apoglobin intermediate is the dominant effect of lower temperature. Although globin can accept heme before it is released from the ribosome in *E. coli* (29), it is unclear whether this occurs in bacteria. Unlike in eukaryotes, it is unlikely that globin can complete folding to its native configuration until its release from the ribosome in *E. coli* (29). Therefore, without heme, apoglobin can be likened to a trapped folding intermediate whose fate is influenced by temperature.

Although our simplified model helped us predict the effect of temperature on fermentation outcomes, understanding the mechanism of insoluble globin formation by in vitro kinetic studies would allow the development of a more complete and predictive model of soluble hemoglobin expression in *E. coli*. In addition, expression of recombinant human hemoglobin variants in *E. coli* may serve as a convenient in vivo model for studying aggregation of hemoglobin mutants observed in nature (5, 33).

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