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Inclusion body formation by interleukin-1β depends on the thermal sensitivity of a folding intermediate

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Abstract

We show that sequence and growth temperature effects on IB formation in the small, monomeric β-barrel protein interleukin-1β (IL-1β) can be quantitatively reproduced in an in vitro system in which IL-1β is refolded from denaturant at different temperatures. The results suggest that temperature and mutational effects on IB formation may be based on intrinsic properties of the protein sequence rather than interactions with chaperones or other cellular factors. We also report striking correlations of IB formation with mutation-dependent changes in residue hydrophobicity. The nature of these trends differs considerably with residue position, however, suggesting that they are mediated by particular local environments created by an ordered structure.

Key words: Inclusion body; Mutation; Interleukin-1β; Folding intermediate; Temperature-sensitive folding; Folding kinetics; Hydrophobicity

1. Introduction

High-level expression of foreign DNA in *E. coli* is often characterized by deposition of the protein product into electron-dense structures called inclusion bodies (IBs) [1–4]. Although of appreciable interest – as an aspect of protein folding in the cell [5], as a practical barrier to recombinant expression [1,2], and as a kindred process to amyloid formation in human disease [4,5] – inclusion body formation is little understood. Recently we described a series of point mutants in the small, monomeric β-barrel protein interleukin-1β (IL-1β) which exhibit wide variation in the extent to which they are deposited into IBs when expressed in *E. coli* [6].

Of the mutants previously characterized, the Lys → Val mutation at position 97 (K97V), on a long, surface-exposed loop [7–10], is of particular interest. Although exhibiting higher thermodynamic stability than wild-type IL-1β, a much larger percentage of total IL-1β (K97V) is deposited into IBs than is the wild-type molecule [6]. Like a number of other IB-forming proteins [4,11], the proportion of the K97V mutant deposited into IBs significantly decreases when growth temperature is decreased [6]. We report here excellent correspondence between these temperature effects on IB formation by sequence variants and the temperature effects on aggregation in vitro when the same proteins are refolded from denaturant. The results suggest that these IB effects derive from the influence of amino acid replacements on the kinetic partitioning of IL-1β, as it folds, into soluble and insoluble products, rather than on any impact of the replacements on thermodynamic stability or on interaction interfaces with other cellular components during biosynthesis.

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2. Experimental

2.1. Aggregation studies

Both WT and the K97V of IL-1β were purified from the supernatant of a native lysate of *E. coli* as described previously [6]. For refolding studies, proteins were exhaustively dialyzed against distilled water and lyophilized. The lyophilized powder was dissolved in a small volume of 3 M Gdn-HCl, 10 mM MES, 1 mM EDTA, 1 mM DTT, pH 6.5, to give a protein concentration of 10 mg/ml. Although no particulates were apparent, the solution was centrifuged in an Eppendorf microfuge to insure the absence of large aggregates. Aliquots of this solution were pipetted, with mixing, into 9 vols. of buffer (10 mM MES, 1 mM EDTA, 1 mM DTT, pH 6.5) equilibrated at different temperatures. After 5 min, the reaction was removed from the thermal bath and allowed to stand another 5 min at RT. Insoluble aggregate was assessed by the turbidity of the sample [11,12], by reading the apparent absorbence of the refolding reaction at 480 nm in a spectrophotometer. As a control, folded K97V IL-1β exposed to the endpoint buffer concentrations (including 0.3 M Gdn-HCl) at 42°C generates no turbidity.

The apparent plateau in aggregation at 48–54°C corresponds to aggregation of 91% of the available protein, as determined by a protein concentration assay (Biorad) on filtered samples after refolding at 48 and 24°C.

Concentration studies (Fig. 2) were performed by mixing the 3 M Gdn-HCl stock of the K97V mutant with either denaturation buffer alone or with the 3 M Gdn- HCl stock of WT IL-1β, before refolding at 48°C under conditions described above.

2.2. Folding and unfolding kinetics

Samples were folded/unfolded at 25°C in buffers containing 10 mM MES, 90 mM NaCl, 1 mM β-mercaptoethanol, pH 6.5. For refolding experiments, the stock protein solution was unfolded in 2 M Gdn-HCl overnight (16–24 h). Unfolding and refolding experiments were initiated by dilution of either folded or unfolded stock solutions into appropriate Gdn-HCl buffer mixes to give the desired final Gdn-HCl concentration after manual mixing. The final protein concentration was 0.1–0.3 mg/ml. The kinetics were monitored by fluorescence on an

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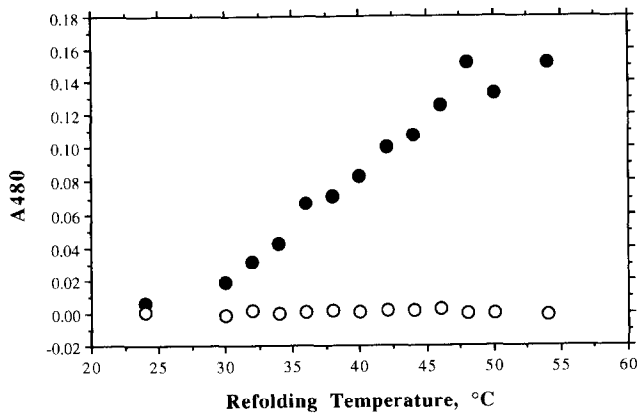

Fig. 1. Temperature dependence of aggregation during refolding of IL-1β [WT (○), K97V (●)]. An A_{480} of 0.165 corresponds to 100% aggregation of available IL-1β (see section 2). Based on this determination, A_{480} values of 0.100 (42°C) and 0.031 (32°C) correspond to aggregation of 61% and 19% of total available IL-1β, and these values are reported in Table 1.

SLM-8000 spectrofluorometer with excitation set at 295 nm (slit 1 mm) and emission at 343 nm (slit 4 mm). Data were fit to a sum of exponentials using the program NLIN (SAS, Cary, NC). The data fit best to a single exponential as determined by random deviation of the sum of the squares of the residuals. The aggregation and light scattering effects discussed in this paper did not interfere with these kinetic measurements since, as shown in Fig. 1, both wild-type and K97V IL-1β fold to soluble products at 25°C.

3. Results and discussion

Previously we reported that all of the IL-1β sequence variants tested are stable to thermally induced aggregation in vitro at temperatures greater than 50°C, yet at the same time support IB formation in vivo at temperatures as low as 32°C. We interpreted this data to suggest that differences in the thermodynamic stability of the native state cannot account for mutational effects on IB formation in this series of mutants [6]. Other ways in which mutational and thermal effects might be mediated are: (a) effects on the solubility or kinetic lifetime of a transient folding intermediate; or (b) effects on a chaperone interaction or some other component of the biosynthetic machinery. Here we compare the K97V mutant and the WT in an in vitro system designed to explore the behavior and solubility of the polypeptide *during* folding. Fig. 1 shows that the wild-type protein can be refolded to a soluble product throughout the temperature range 24– 54°C. Although the K97V mutant also folds efficiently and remains soluble at 24°C, it generates appreciable aggregate when refolded at 32°C, and the amount of aggregate increases linearly with temperature up to about 50°C. The aggregation seen at 50°C represents loss of about 90% of the total IL-1β from solution (see section 2). It should be emphasized that these temperature effects require that IL-1β be actively engaged in the folding process; as we have shown previously, thermally induced aggregation of folded IL-1β requires higher temperatures and also displays a much sharper temperature dependence [13]. The aggregation of the K97V mutant during folding in vitro is concentration dependent, as expected for a rate-limiting multi-molecular reaction step (data not shown).

Table 1
% aggregation of IL-1β in vivo and in vitro

	In vivo[a]		In vitro[b]	
IL-1β	32°C	42°C	32°C	42°C
WT	6	8–18	0	0
K97V	17	47–61	19	61

[a]Determined from SDS-PAGE on native lysis supernatants and pellets, developed by Coomassie blue staining and/or by immunoblots with an anti-IL-1β antibody (from [6]).
[b]Data from Fig. 1.

Table 1 compares the aggregation in this in vitro experiment with the temperature dependent inclusion body formation of these sequence variants [6]. The table shows a remarkably good agreement between the in vivo and in vitro aggregation results. This suggests that — at least for IL-1β sequence variants — thermal effects on IB formation do not depend on the temperature dependence of ribosomal synthesis rates, and mutational effects are not due to disruption of an interaction surface with a molecular chaperone.

Since aggregation of IL-1β during folding in vitro can account for several properties of IB formation, while the thermal aggregation of folded IL-1β cannot [6], we conclude that formation of IL-1β inclusion bodies depends on the properties of a folding intermediate. Independent evidence also supports the involvement of a folding intermediate in aggregation of IL-1β in vitro [13] and in vivo [13,14]. There are two broad mechanisms by which mu-

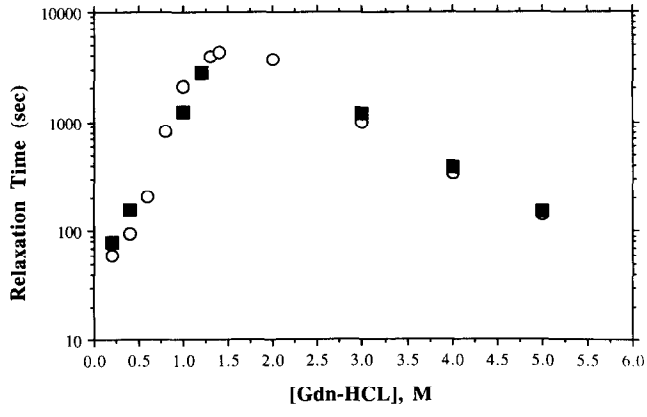

Fig. 2. Kinetics of folding/unfolding of WT (○) and K97V (■) IL-1β. See section 2. Data points between 0 and 1.5 M Gdn-HCl are from refolding experiments while data above 1.5 M Gdn-HCl were from unfolding experiments.

tations might impact aggregation of folding intermediates [4]: (a) by increasing the intrinsic tendency of a key folding intermediate to aggregate; or (b) by increasing the kinetic lifetime - and thus increasing the steady state concentration — of an aggregation-prone intermediate.

Although it is sometimes possible to determine the solubility properties of a folding intermediate populated at equilibrium [15,16], it is much more difficult to characterize solubilities or other physical properties of *transient* folding intermediates. We therefore explored the second possibility, that the K97V mutation affects the kinetic lifetime of an important intermediate. We determined the folding kinetics of both WT and K97V at 25°C and low protein concentration, conditions which allow folding of both proteins to proceed with good solubility. However, for the kinetic phase accessible using manual mixing techniques, we observed no difference in the refolding (as well as unfolding) rates of WT and K97V (Fig. 2). This does not rule out an effect on kinetic lifetime of an intermediate, however, since IL-1β is known to exhibit at least five steps in its folding, with kinetic half-lives for the reaction from 4 ms to 20 min at 4°C [17]. Comparison of the two proteins by rapid kinetics techniques will be required to further explore potential differences in folding rates[1].

Analysis of extents of IB formation with respect to sequence position suggests that the folding intermediate implicated in the aggregation process has significant structure. Fig. 3 shows that, for a collection of replacements at eight different sequence positions in IL-1β, there is no overall correlation of the extent of IB formation with the change in hydrophobic character brought about by the replacement, as quantified by changes in free energies of transfer [18,19]. This suggests that the aggregating species must possess enough structure to create different environments which exhibit unequal responses to similar hydrophobicity changes. In fact, quite different IB extents are associated with *identical* replacements at different residue positions in IL-1β[2]. These results are inconsistent with any model in which the aggregating species is a random coil in which sequence positions are equivalent.

Despite the absence of a universal trend, strong correlations are obtained between IB% and $\Delta\Delta G_{trans}$ at individual sequence positions in IL-1β, as shown in Fig. 3. At position 9 (located in the interior of folded IL-1β)

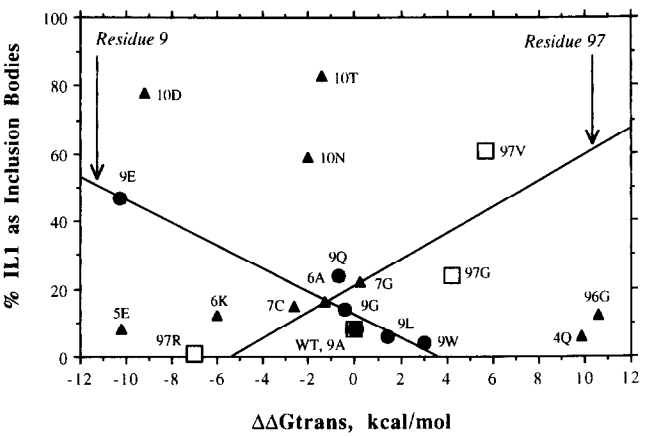

Fig. 3. Correlation of extent of inclusion body formation with the difference in free energies of transfer [18,19] between wild-type and single point mutant IL1β molecules for all mutants quantified. (The inclusion body data for replacements of Arg[4], Ser[5], Leu[6], Asn[7], Thr[9], Leu[10], Glu[96] and Lys[97] can be found in [6].) $\Delta\Delta G_{trans}$ was generated by subtracting the free energy of transfer [18,19] of the WT residue from that of the mutant residue. (□, position 97 mutants; ●, position 9 mutants; ▲, other mutants.

increasing IB formation correlates with decreasing hydrophobicity. In contrast, at position 97 (on the surface of the folded protein), increasing IB formation correlates with increasing hydrophobicity. The former effect might be compatible with the environment of position 9 in the native state, since placing a hydrophilic group in a hydrophobic pocket would be expected to be destabilizing. The latter effect, however, is incompatible with the environment of position 97 in the native structure: in general, replacement of surface residues is not normally found to greatly effect stability [20], and, in this case, the Lys → Val mutation at position 97 is known to be *stabilizing* to the native state [6]! Thus, the aggregating species in IB formation has both native-like and non-native environments by this analysis. Other data supports the role of a structured intermediate in IB formation by IL-1β. Recent FTIR studies indicate that the secondary structure of IL-1β in inclusion bodies — as well as in in vitro aggregates — is similar to, but clearly not identical with, that of the native IL-1β structure [14]. In addition, an aggregation-prone, native-like *unfolding* intermediate

[1]It is not clear whether all the intermediates detected at 4°C would be detectable at 25°C; however, the fact that much of the signal amplitude for folding of IL-1β is not detectable in our manual mixing experiments (B.A. Chrunyk and R. Wetzel, unpublished observations) suggests that one or more relatively rapid kinetic phases proceed the step we have been able to measure at 25°C. Since the generation of light scattering aggregate at 42°C is at least as rapid as the folding step shown in Fig. 2 (B.A. Chrunyk and R. Wetzel, unpublished observations), it is possible that the intermediate responsible for aggregation is populated earlier in the folding pathway.

[2]For example, three Lys→Gly replacements have been characterized in IL-1β. At position 94, wild-type levels of soluble protein expression are obtained; however, the same mutation at position 88 gives loss of stable expression (either in IBs or in the soluble fraction), and at position 97 gives an enhanced level of IB formation over that seen for the WT. At position 6, a Leu→Ala replacement leads to a slight enhancement of IB formation over WT; the same replacement at position 10, on the same face of the same β-strand, gives IB formation almost exclusively. The Thr→Ala mutation at position 9 gives no significant different in expression level or IB formation from WT, but the same replacement at position 79 gives little soluble expression and enhanced IB formation. See [6].

has been suggested based on the behavior of the K97V mutant in its unfolding-dependent aggregation in vitro [13].

Properties of folding intermediates have been invoked to account for the *temperature-sensitive folding* mutations, manifested by differences in extents of in vivo aggregation, described for the large, homotrimeric tailspike protein of phage P22 [3,21]. Recent in vitro folding studies support this interpretation [22,23]. The results presented here suggest that mutational effects on the kinetic lifetime or solubility of a folding intermediate can also account for effects on IB formation by the small, globular, monomeric protein IL-1β, and the availability of structural information has contributed to our understanding of these effects. The quantitative duplication of thermal and mutational effects on IB formation in the in vitro model suggests that measured extents of IB formation, at least in this case, reflect a property inherent to the structure of the polypeptide chain and the folding intermediates governed by that sequence, and are not the result of effects on chaperone binding sites, ribosomal synthesis rates, proteolysis rates, etc.

As an intrinsic aspect of polypeptide structure, the tendency to aggregate during folding can be viewed as a design flaw which has haunted organic evolution [5]. It is likely that aggregation during folding has provided a continuing, selective pressure on protein sequence evolution which operates *in addition to* a more generally recognized selection for net folding stability [5]. For at least some sequences which have proved incapable of avoiding aggregation, biosynthetic pathways appear to have been embellished to suppress aggregation during folding in vivo, for example through the use of molecular chaperones [24].

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References

[1] Marston, R.A.O. (1986) Biochem. J. 240, 1–12.
[2] Schein, C.H. (1989) Biotechnology 7, 1141–1149.
[3] Mitraki, A. and King, J. (1989) Biotechnology 7, 690–697.
[4] Wetzel, R. (1992) in: Stability of Protein Pharmaceuticals: In Vivo Pathways of Degradation and Strategies for Protein Stabilization (Ahern, T.J. and Manning, M.C. eds.) pp. 43–88, Plenum Press, New York.
[5] Wetzel, R. (1994) Trends Biotechnol. 12, 193–198.
[6] Chrunyk, B.A., Evans, J., Lillquist, J., Young, P. and Wetzel, R. (1993) J. Biol. Chem. 268, 18053–18061.
[7] Finzel, B.C., Clancy, L.L., Holland, D.R., Muchmore, S.W., Watenpaugh, K.D. and Einspahr, H.M. (1989) J. Mol. Biol. 209, 779–791.
[8] Priestle, J.P., Schar, H.-P. and Grutter, M.G. (1989) Proc. Natl. Acad. Sci. USA 86, 9667–9671.
[9] Clore, G.M., Wingfield, P.T. and Gronenborn, A.M. (1991) Biochemistry 30, 2315–23.
[10] Veerapandian, B., Gilliland, G.L., Raag, R., Svensson, L.A., Masui, Y., Hirai, Y. and Poulos, T.L. (1992) Proteins 12, 10–23.
[11] Wetzel, R. (1992) in: Protein Engineering — A Practical Approach (Rees, A.R., Sternberg, M.J.E. and Wetzel, R. eds.) pp. 191–219, IRL Press at Oxford University Press, Oxford.
[12] Mulkerrin, M.G. and Wetzel, R. (1989) Biochemistry 28, 6556–6561.
[13] Chrunyk, B.A. and Wetzel, R. (1993) Protein Eng. 6, 733–738.
[14] Oberg, K., Chrunyk, B.A., Wetzel, R. and Fink, A. (1994) Biochemistry 33, 2628–2634.
[15] Brems, D.N., Plaisted, S.M., Kauffman, E.W. and Havel, H.A. (1986) Biochemistry 25, 6539–6543.
[16] DeFelippis, M.R., Alter, L.A., Pekar, A.H., Havel, H.A. and Brems, D.N. (1993) Biochemistry 32, 1555–1562.
[17] Varley, P., Gronenborn, A.M., Christensen, H., Wingfield, P.T., Pain, R.H. and Clore, G.M. (1993) Science 260, 1110–1113.
[18] Nozaki, Y. and Tanford, C. (1971) J. Biol. Chem. 246, 2211–7.
[19] von Heijne, G. and Blomberg, C. (1979) Eur. J. Biochem. 97, 175–81.
[20] Matthews, B.W. (1993) Ann. Rev. Biochem. 62, 139–160.
[21] Haase-Pettingell, C.A. and King, J. (1988) J. Biol. Chem. 263, 4977–4983.
[22] Danner, M. and Seckler, R. (1993) Protein Sci. 2, 1869–1881.
[23] Mitraki, A., Danner, M., King, J. and Seckler, R. (1993) J. Biol. Chem. 268, 20071–5.
[24] Hendrick, J.P. and Hartl, F.-U. (1993) Annu. Rev. Biochem. 62, 349–84.