Biochemistry (Moscow), Vol. 67, No. 8, 2002, pp. 914-917. Translated from Biokhimiya, Vol. 67, No. 8, 2002, pp. 1101-1105.

Effects of Osmolytes on Unfolding of Chicken Liver Fatty Acid Synthase

Y.-D. Park[1], B.-N. Wu[2], W.-X. Tian[2], and H.-M. Zhou[1,3]*

[1]Department of Biological Science and Biotechnology, School of Life Science and Engineering, Tsinghua University, Beijing 100084, P. R. China; fax: +8610 62772245; E-mail: zhm-dbs@mail.tsinghua.edu.cn
[2]Graduate School, Chinese Academy of Sciences, Beijing 100039, P. R. China
[3]Protein Science Laboratory of the Ministry of Education, School of Life Science and Engineering, Tsinghua University, Beijing 100084, P. R. China

Received May 29, 2001
Revision received October 28, 2001

Abstract—Urea-induced aggregation of chicken liver fatty acid synthase [acyl-CoA:malonyl-CoA *C*-acyltransferase (decarboxylating, oxoacyl- and enoyl-reducing and thioester-hydrolyzing), EC 2.3.1.85] was studied. The aggregation was facilitated at increased ionic strength. Methyl-β-cyclodextrin and some osmolytes, such as glycerol, sucrose, proline, glycine, and heparin, could effectively prevent the aggregation, implying an artificial chaperone role of those substances during fatty acid synthase unfolding. The osmolytes also protected the enzyme from inactivation.

Key words: fatty acid synthase, urea denaturation, aggregation, osmolyte, artificial chaperone

Fatty acid synthase [FAS; acyl-CoA:malonyl-CoA *C*-acyltransferase (decarboxylating, oxoacyl- and enoyl-reducing and thioester-hydrolyzing), EC 2.3.1.85] is an important enzyme in energy metabolism. Animal FAS includes two multifunctional polypeptide chains, each containing seven discrete functional domains with enzymatic activity, juxtaposed head-to-tail so that two separate centers for the FAS assembly are formed at the subunit interface [1-5]. In animals, *de novo* synthesis of fatty acids from acetyl-CoA and malonyl-CoA is catalyzed by a single protein, a FAS that consists of two identical 272 kD polypeptides [3-5]. Each polypeptide contains seven catalytic domains and an acyl carrier protein (ACP) arranged in the order (from the N-terminus) β-ketoacyl synthase, acetyl transacylase, malonyl transacylase, β-hydroxyacyl dehydratase, enoyl reductase, β-ketoacyl reductase, ACP, and thioesterase, as determined by sequencing [6-8] and mutagenesis [9]. Chicken liver FAS is a dimeric molecule composed of identical, multifunctional subunits, each with a molecular weight of 274 kD [10]. It has been recently reported that denaturation of FAS by SDS occurs in three steps: loss of overall activity, loss of component enzyme activity and, finally, conformational change (unfolding) [11].

Abbreviation: FAS) fatty acid synthase.

* To whom correspondence should be addressed.

Protein aggregation is a frequently observed phenomenon during protein unfolding and refolding [12-18]. Hydrophobic interactions and temperature are regarded as main factors in inducing aggregation. Correct folding *in vitro* or *in vivo* competes with unproductive side reactions such as misfolding or aggregation [17, 18]. Prevention of aggregation is important during purification. Recently, new approaches basing on "artificial chaperones" have been introduced to prevent aggregation through aiding protein folding [19-27]. Some osmolytes known to stabilize proteins against aggregation were recognized as protein folding helpers [25, 26]. Schein [28] described their effects on proteins and on the solvent properties of water. Osmolytes include polyols, sugars, polysaccharides, neutral polymers, amino acids and their derivatives, etc., which mainly affect the solvent properties of water as related to protein polarity and protein diffusion. Stability of an enzyme is determined by its tendency to participate in hydrophobic interactions, susceptibility to proteases, and by its isoelectric point. Osmolytes as solvent additives affect these properties and, hence, aggregation. Solvent additives can also favorably affect protein stability and solubility.

This study analyzes the effect of methyl-β-cyclodextrin and the osmolytes glycerol, sucrose, proline, glycine and heparin on FAS aggregation during urea denaturation. The results suggest a strategy to enhance correct protein folding through addition of chemical folding-aids

0006-2979/02/6708-0914$27.00 ©2002 MAIK "Nauka/Interperiodica"

into the buffer solution and also suggest an unfolding pathway for FAS. This investigation shows the importance of buffer components for protein unfolding.

MATERIALS AND METHODS

The isolation, storage, and use of chicken liver FAS were as described previously [29]. The preparation was homogeneous on PAGE in the presence and absence of SDS. Acetyl-CoA, malonyl-CoA, NADPH, acetoacetyl-CoA, urea of ultra-pure grade, heparin sodium, and methyl-β-cyclodextrin were obtained from Sigma (USA). All other reagents were local products of analytical grade.

The concentrations of the components of the reaction mixtures were determined by absorption measurements with the following extinction coefficients: chicken liver FAS, $4.83 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$ at 279 nm; acetyl-CoA, $1.54 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ at 259 nm, pH 7.0; malonyl-CoA, $1.46 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ at 260 nm, pH 6.0; NADPH, $6.02 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ at 340 nm and $1.59 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ at 259 nm, pH 9.0; and acetoacetyl-CoA, $1.59 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ at 259 nm, pH 7.0 [29]. FAS activity (in the overall reaction) was determined with a Perkin-Elmer Lambda Biospectrophotometer at 37°C by following the decrease in NADPH concentration at 340 nm. The reaction mixture contained a 0.1 M sodium phosphate buffer, pH 7.0, 1 mM EDTA, 3 μM acetyl-CoA, 10 μM malonyl-CoA, 35 μM NADPH, and chicken liver FAS in a volume of 1.0 ml.

Aggregation was monitored by measuring the light absorption at 400 nm and was initiated by adding 1.3 μM enzyme to the phosphate buffer, pH 7.0 (containing 3 M urea and 1 mM EDTA and preincubated at 37°C). Where indicated, osmolytes (glycerol, proline, sucrose, glycine, and heparin) or methyl-β-cyclodextrin were added to the unfolding medium.

RESULTS AND DISCUSSION

Aggregation and its inhibition by added artificial chaperones. Aggregation may primarily originate from hydrophobic interactions of unfolded polypeptide chains as second or higher order processes [16, 17, 30]. Aggregation of FAS was temperature and ionic strength dependent, as also observed with other proteins, and did not occur at low concentration of the buffer solutions (Fig. 1). The concentration of urea used (3 M) was optimal to study the aggregation.

Recently, attempts were made to aid protein folding using chemical additives [19-26]. Figure 2 shows that FAS aggregation was increasingly inhibited by methyl-β-cyclodextrin. FAS preincubated with 3 M urea for 1 h in the absence or presence of 300 mM methyl-β-cyclodextrin showed no enzymatic activity. Karuppiah and

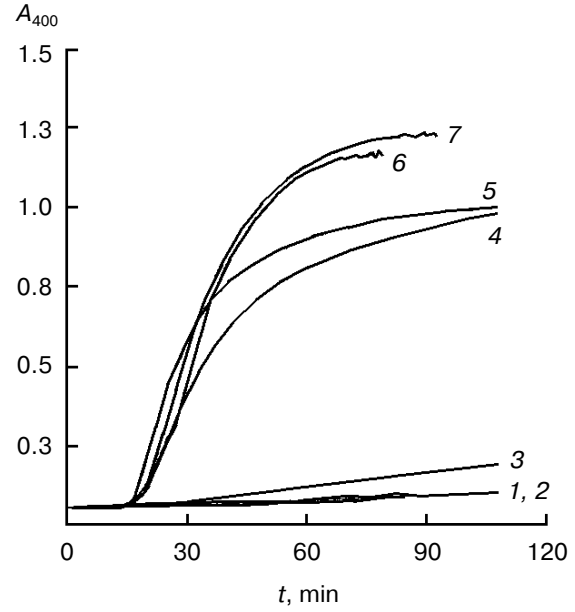

Fig. 1. Aggregation of FAS during denaturation with urea at different phosphate buffer concentrations. Enzyme solution was added to phosphate buffer, pH 7.0, containing 3 M urea and 1 mM EDTA and preincubated at 37°C. Curves *1-7* correspond to 0.1, 0.3, 0.5, 0.7, 0.8, 0.9, and 1 M phosphate buffer concentrations, respectively. The final enzyme concentration was 1.3 μM.

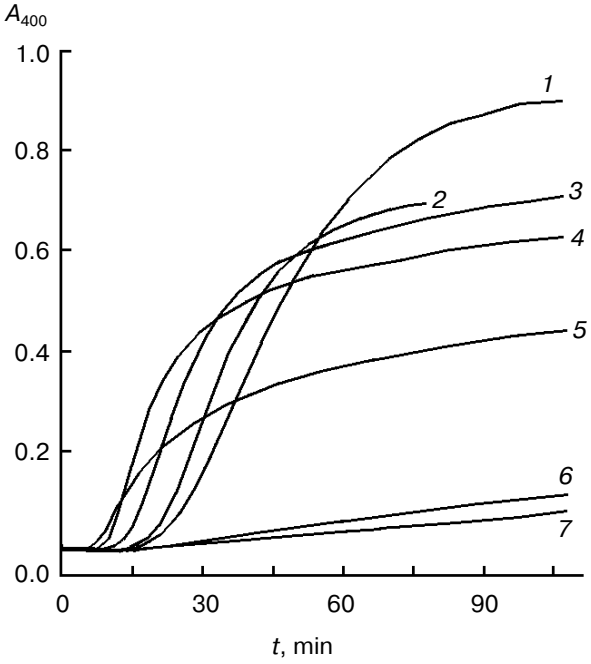

Fig. 2. Effect of methyl-β-cyclodextrin on FAS aggregation during 3 M urea denaturation. FAS was incubated with 3 M urea at 37°C in 1 M phosphate buffer including different concentrations of methyl-β-cyclodextrin (0, 10, 20, 80, 120, 200, and 300 mM for curves *1-7*, respectively). The final enzyme concentration was 1.3 μM.

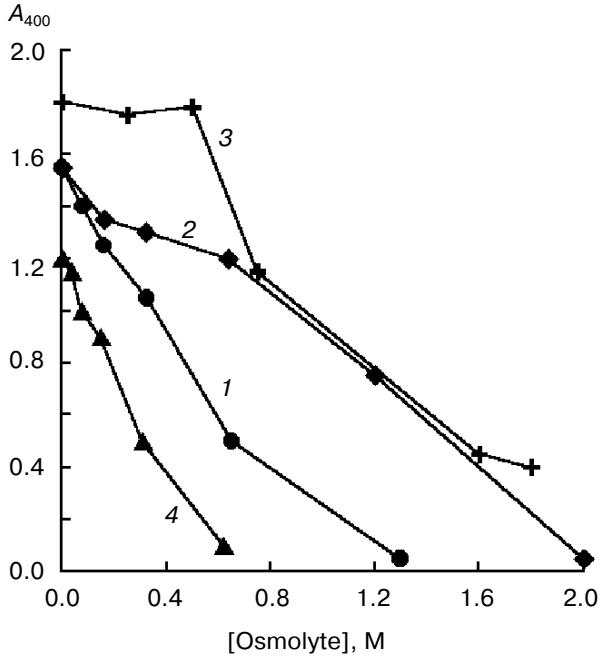

Fig. 3. Effect of different osmolytes on FAS aggregation. FAS was incubated with 3 M urea at 37°C in 1 M phosphate buffer containing different concentrations of osmolytes including glycerol (*1*), proline (*2*), glycine (*3*), or sucrose (*4*). The maximum turbidity of FAS at 400 nm was measured after 84 min. The final enzyme concentration was 1.3 μM.

Sharma [24] also reported the effect of cyclodextrins on the refolding of carbonic anhydrase under aggregating conditions. Cyclodextrins suppressed the aggregation of carbonic anhydrase even at high protein concentrations as well as enhanced protein reactivation.

Figure 3 shows the effect of glycerol, representing polyols, on FAS aggregation. Increasing glycerol concentrations effectively blocked FAS aggregation, with 1.3 M glycerol completely preventing aggregation. Glycerol not only caused large increases in solvent viscosity and dielectric constant with relatively small changes in pH, but usually protects the native structure of proteins even at large concentrations and is the most commonly used viscogenic cosolvent [31]. Glycerol stabilizes a folding intermediate in a maturation-competent state, either by inhibiting off-pathway reactions or by enhancing reactions that were on the folding pathway [32]. Glycerol may interact with a partially folded protein intermediate that otherwise would aggregate.

Proline, sucrose, and glycine also suppressed FAS aggregation (Fig. 3). Recently, proline has been used as a folding aid for bovine carbonic anhydrase and creatine kinase to effectively inhibit aggregation during refolding [25, 26], where high concentrations of proline enabled the protein to restore its native structure. Sucrose is a carbohydrate osmolyte commonly found in cyanobacteria, fungi, algae and vascular plants. Thermal stability of proteins increases in the presence of sugars [33, 34]. The much higher viscosity in the presence of high concentrations of sucrose likely blocks the interactions between protein molecules, thus preventing the formation of precipitates. The coating of proteins with a hydration shell around charged or polar groups to prevent self-binding was one possible strategy for suppressing aggregation and retaining protein stability [28].

Meng et al. recently reported that heparin prevents creatine kinase aggregation through forming a heparin–creatine kinase complex [26]. Although heparin partially blocked FAS aggregation when added at a low concentration (0.023 mg/ml), it stimulated aggregation in the concentration range of 0.047-0.18 mg/ml (data not shown).

Osmolytes as activity protectors during FAS inactivation. When FAS was incubated for 1 h with 1.5 M urea, the residual activity was only 3.8%. If, however, the incubation medium contained 0.62 M sucrose, 1.3 M glycerol, 2.4 M proline, 0.3 M methyl-β-cyclodextrin, or 1.8 M glycine, the residual activity was 19, 25, 30, 40, and 70% of native FAS activity, respectively.

Artificial chaperones such as methyl-β-cyclodextrin, glycerol, proline, glycine, and sucrose used in this study increased the viscosity of the refolding solution which affected protein unfolding dynamics so that the partially unfolded FAS intermediates successfully proceeded to their unfolding pathways, rather than being blocked by aggregation. Osmolytes may mainly affect a FAS intermediate that has a partially disrupted network of internal hydrophobic interactions, leading to a corresponding increase in the amount of solvent-exposed nonpolar surfaces. Solvent additives used in the present study appear to affect the hydrophobic interactions, which are regarded as the main factors inducing aggregation. The effect of osmotic stabilizers added to enhance the stability through the rehydration of proteins was related to the intermolecular hydrophobic forces. The coating of proteins with a hydration shell around charged and polar groups to prevent self-binding of the proteins is one strategy for preventing aggregation and for retaining protein stability [28].

The molecular chaperon GroEL assists folding by preventing unfavorable aggregation or misfolding both *in vivo* and *in vitro* [35]. However, proteins as large as FAS cannot pack into the cylinder cavity of GroEL. Therefore, one critical problem is to understand how large proteins like FAS unfold and refold *in vivo* or *in vitro*. We hypothesize that some osmolytes, including free amino acids, are not only energy substrates and organic components, but also play a chaperone role in assisting correct protein folding especially in large proteins during *in vivo* folding.

This investigation was supported in part by grant 39670174 of the China National Science Foundation and by grant G 1999075607 of National Key Basic Research Specific Funds, P. R. China.

REFERENCES

1. Stoops, J. K., and Wakil, S. J. (1981) *J. Biol. Chem.*, **256**, 5128-5133.
2. Stoops, J. K., and Wakil, S. J. (1982) *J. Biol. Chem.*, **257**, 3230-3235.
3. Wakil, S. J. (1989) *Biochemistry*, **28**, 4523-4530.
4. Chang, S. I., and Hammes, G. G. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8373-8376.
5. Smith, S. (1994) *FASEB J.*, **8**, 1248-1259.
6. Schweizer, M., Takabayashi, K., Laux, T., Beck, K. F., and Schreglmann, R. (1989) *Nucleic Acids Res.*, **17**, 567-587.
7. Amy, C., Witkowski, A., Naggert, J., Williams, B., Randhawa, Z., and Smith, S. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 3114-3118.
8. Holzer, K. P., Liu, W., and Hammes, G. G. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 4387-4391.
9. Joshi, A. K., and Smith, S. (1993) *J. Biol. Chem.*, **268**, 22508-22513.
10. Huang, W. Y., Chirala, S. S., and Wakil, S. J. (1994) *Arch. Biochem. Biophys.*, **314**, 45-49.
11. Shi, Y., Luo, W., Tian, W. X., Zhang, T., and Zhou, H. M. (1998) *Int. J. Biochem. Cell Biol.*, **30**, 1319-1330.
12. Speed, M. A., Wang, D. I. C., and King, J. (1996) *Nat. Biotechnol.*, **14**, 1283-1287.
13. Speed, M. A., King, J., and Wang, D. I. C. (1997) *Biotechnol. Bioeng.*, **54**, 333-343.
14. Cleland, J. L., and Wang, D. I. C. (1990) *Biochemistry*, **29**, 11072-11078.
15. Plomer, J. J., and Gafni, A. (1993) *Biochim. Biophys. Acta*, **1163**, 89-96.
16. Fink, A. L. (1998) *Fold. Des.*, **3**, 9-23.
17. Kiefhaber, T., Rudolph, R., Kohler, H. H., and Buchner, J. (1991) *Bio/Technology*, **9**, 825-829.
18. Jaenicke, R. (1998) *Biol. Chem.*, **379**, 237-243.
19. Rozema, D., and Gellman, S. H. (1995) *J. Am. Chem. Soc.*, **117**, 2373-2374.
20. Rozema, D., and Gellman, S. H. (1996) *Biochemistry*, **35**, 15760-15771.
21. Rozema, D., and Gellman, S. H. (1996) *J. Biol. Chem.*, **271**, 3478-3487.
22. Daugherty, D. L., Rozema, D., Hanson, P. E., and Gellman, S. H. (1998) *J. Biol. Chem.*, **273**, 33961-33971.
23. Couthon, F., Clottes, E., and Vial, C. (1996) *Biochem. Biophys. Res. Commun.*, **227**, 854-860.
24. Karuppiah, N., and Sharma, A. (1995) *Biochem. Biophys. Res. Commun.*, **211**, 60-66.
25. Kumar, T. K. S., Samuel, D., Jayaraman, G., Srimathi, T., and Yu, C. (1998) *Biochem. Mol. Biol. Int.*, **46**, 509-517.
26. Meng, F. G., Park, Y. D., and Zhou, H. M. (2001) *Int. J. Biochem. Cell Biol.*, **33**, 701-709.
27. Kurganov, B. I., and Topchieva, I. N. (1998) *Biochemistry (Moscow)*, **63**, 413-419.
28. Schein, C. H. (1990) *Bio/Technology*, **8**, 308-317.
29. Tian, W. X., Hsu, R. Y., and Wang, Y. S. (1985) *J. Biol. Chem.*, **260**, 11375-11387.
30. Rudolph, R., and Lilie, H. (1996) *FASEB J.*, **10**, 49-56.
31. Gonnelli, M., and Strambini, G. B. (1993) *Biophys. J.*, **65**, 131-137.
32. Sato, S., Ward, C. L., Krouse, M. E., Wine, J. J., and Kopito, R. R. (1996) *J. Biol. Chem.*, **271**, 635-638.
33. Back, J. F., Oakenfull, D., and Smith, M. B. (1979) *Biochemistry*, **18**, 5191-5199.
34. Gekko, K., and Koga, S. (1983) *J. Biochem.*, **94**, 199-208.
35. Hayer-Hartl, M. K., Ewbank, J. J., Creighton, T. E., and Hartl, F. U. (1994) *EMBO J.*, **13**, 3192-3202.