Bacterial Synthesis of Active Rat Stearyl-CoA Desaturase Lacking the 26-Residue Amino-terminal Amino Acid Sequence*

(Received for publication, June 22, 1987)

Philipp Strittmatter‡, Mark A. Thiede§, Craig S. Hackett, and Juris Ozols
From the Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032

Two clones containing inserts in pBR322 that together include the entire 1074-base open reading frame coding for the 358 amino acids of rat liver stearyl-CoA desaturase have been used to construct expression vectors for residues 3–358 and 27–358 fused to the first 6 residues of β-galactosidase and several amino acids of the multiple cloning site of pUC8. Growth of transformed Escherichia coli under conditions for suppression of the lac promoter, followed by subsequent induction of these cultures results in the synthesis of higher levels of desaturase proteins than those found in induced rat liver. The proteins are almost completely associated with the membrane fraction of cell homogenates. Posttranslational iron insertion into the apoproteins, either in vitro with membrane preparations or by iron addition during induction, results in the formation of active holoenzyme which can be reconstituted with NADH cytochrome b₅ reductase and cytochrome b₅ to form an active stearyl-CoA desaturase system. The deletion of the first 26 amino-terminal amino acid residues does not affect either enzyme activity or membrane binding. Therefore, the unusual sequence of 11 residues containing 10 amino acids with hydroxyl groups plays no apparent significant role in either protein insertion into membranes or iron chelation. Since the protein product for residues 3–358 is processed even further to delete the initial 33 amino-terminal residues, the limiting polypeptide primary structure required for an active membrane-bound catalyst is even smaller than this initial deletion mutation indicates.

The 26-Residue Amino-terminal Amino Acid Sequence

| Amino Acid | Sequence |
|------------|----------|
| 3          | FYTTKQ   |
| 4          | ELPL     |
| 5          | TPAL     |
| 6          | PTHL     |
| 7          | RAEE     |
| 8          | LKIF     |
| 9          | TLVI     |
| 10         | LLPR     |
| 11         | KQIV     |

The publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed.
§ Present address: Merck, Sharp and Dohme Research Laboratories, West Point, PA 19486.

* This work was supported by Public Health Service Research Grants GM-15924 (to P. S.) and GM-29595 (to J. O.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The Journal of Biological Chemistry
© 1988 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 263, No. 5, Issue of February 15, pp. 2532–2535, 1988
Printed in U.S.A.
Fig. 1. Structures of plasmids constructed to produce desaturase expression vectors. Closed and open bars indicate non-coding and coding segments of inserts, respectively. A, pDs3, insert in pBR322 at the Psrl restriction site. B, pDs27-358, insert into Psrl-HindIII restricted pUC8. C, pDs3-358, insert into BamHI-Sacl restricted pUC8.

to linear pUC8 produced by Psrl-HindIII digestion. Transformation of UT-481 cells produced clones on plates containing 0.5% glucose, to suppress expression, which contained the expected 1131 base pair insert. One of these, designated pDs27-358 (Fig. 1B), was shown to have the expected sequence from nucleotide 181-1131. Since ligation places this insert in frame with the initial 6 residues of β-galactosidase and 5 residues of the multiple cloning site, the expected protein product will be a fusion protein with these 11 residues amino-terminal to residue 27 of the desaturase (6) and continuing to residue 358 as determined by the termination codon for desaturase.

Construction of pDs3-358—Two restriction fragments were isolated from pDs1; a 384-base pair Aval-Acdl segment after digestion with Acdl, and a second by Nael digestion at 106 followed by addition of a 10 base pair BamHI linker to obtain the 453 base pair Aval-HindIII restriction fragment. Aval-HindIII digestion of pDs2, and BamHI-HindIII restriction of pUC8 were used to isolate the 367-base pair Aval-HindIII fragment and linear pUC8. Forced ligation, due to the sequence differences in the two Aval sites, produced a high frequency of clones containing the expected 1211-base pair insert. One of these, designated pDs3-358 (Fig. 1C) coding for a fusion protein with the 9 residues from nucleotide 181-193, was shown to have the expected sequence from nucleotide 181-1131. Since ligation places this insert in frame with the initial 9 residues of β-galactosidase and 5 residues of the multiple cloning site, the expected protein product will be a fusion protein with these 9 amino-terminal to residue 37 of the desaturase (6) and continuing to residue 358 as determined by the termination codon for desaturase.

Induction of Desaturase—UT-481 cells transformed with either pDs3-358 or pDs27-358 by dephosphorylation of the phosphatid acid promoters. In both clones grown in glucose with the elevated repressor levels of UT-481 cells there is no detectable desaturase antigen in the detergent extracts of the membrane fractions (lane 2, Mter-T of pDs3-358, and lane 4, Mter-T of pDs27-358). In contrast, antigen appears in both Mter-T preparations of the two clones (lanes 3 and 5). Clearly, the synthesized desaturase becomes associated with the membranes of these cells since the samples in Fig. 2 are detergent extracts of the membrane fractions. Moreover in preliminary experiments antigen was only found in membrane fractions and was not detectable in the supernatant fluid of homogenates following membrane breakage and sedimentation.

From the immunoblots shown in Fig. 2, as well as other similar experiments, the concentration of desaturase peptides was approximately 1.25-2.5 μg/mg bacterial protein. This constitutes approximately 2-4% of the total protein of the inner membrane and must be considered a minimum value since transfer of the desaturase from acrylamide gels to the nitrocellulose acetate sheets may be less efficient in the membrane preparations that contain a spectrum of other proteins compared to transfer of the purified desaturase standards (lanes 1 and 6). This value for bacterial expression of the desaturase is actually higher than the levels estimated for induced rat liver (approximately 0.75 μg/mg liver protein) (8). There is proteolytic processing of the desaturase synthesized from pDs3-358 (lane 3) since, as a fusion protein with the initial 9 residues derived from pUC8, the expected molecular weight is actually slightly larger than purified rat liver desaturase (lanes 1 and 6). There is a faint antigen band in the position of full length product in lane 5, but the major desaturase antigen has an apparent molecular mass corresponding to a decrease of approximately 3000-5000 daltons. Although both EDTA and a serine protease inhibitor were included in the preparations of cell-free extracts at a low temperature, it is not clear whether this proteolysis occurs in situ or during membrane isolation. The plasmid coding for
Bacterial Synthesis of Rat Stearyl-CoA Desaturase

FIG. 2. Immunoblot analysis of desaturase synthesis by induced cells transformed with either pDs3–358 or pDs27–358. Acrylamide gel electrophoresis, blotting, and preparative procedures are described under “Experimental Procedures.” Lanes 1 and 6, 0.06 nmol of purified rat stearyl-CoA desaturase; lanes 2 and 4, 10 μl of detergent extracts of membranes from repressed cells (M2–T) transformed with pDs3–358 and pDs27–358, respectively; lanes 3 and 5, 10 μl of detergent extracts of membranes from induced cells (M1–T) transformed with pDs3–358 and pDs27–358, respectively.

the product beginning at residue 27 of the desaturase, interestingly, is more resistant to proteolysis. The expected molecular mass for the pDs27–358 peptide, with 11 residues from pUC8 fused to Ala-27, should be approximately 2000 daltons lower than the rat liver standards and this is the major antigen detected in lane 5. A smaller amount of protein is processed further to a molecular weight similar to that of the major antigen from pDs3–358.

To determine the site of the processing of the desaturase from pDs3–358, the enzyme was purified from the membrane fraction as shown in Fig. 3. A major antigenic peptide (A, lane 2) representing the dominant protein species (B, lane 2) was isolated by preparative gel electrophoresis and subjected to sequence analysis (Table I). The sequence of the first 20 residues are identical to residues 34–53 of the primary structure of rat liver desaturase (6). The processed protein therefore represents a deletion of the amino-terminal 33 residues of the rat liver enzyme corresponding to 4200 daltons. The recent studies of Bachmair et al. (15) on the effect of the amino-terminal amino acid on the half-life of proteins in yeast have shown that amino-terminal Arg is particularly effective in destabilizing proteins to proteolysis. The coding sequence for the 4 amino acids preceding residue 3 of the desaturase in pDs3–358 yields an Arg-Gly-Ser-Arg sequence. Thus, any fortuitous initial processing that placed either arginyl residue amino-terminal might result in the rapid processing observed with this plasmid product.

Preliminary attempts to reconstitute the desaturase system by cytochrome b₅ and NADH cytochrome b₅ reductase addition to membrane fractions isolated from cells transformed with pDs27–358 and induced in the absence of iron yielded low levels of desaturase activity which were stimulated approximately 2–2.5-fold by preincubation in buffers containing 10 μM concentrations of ferric glycinate. Thus, cells grown in low iron medium produced membrane-bound apoprotein of the desaturase in significant quantities. For this reason subsequent induction media for ensuring posttranslational iron insertion to form the active holoenzyme included 50 μM FeCl₃.

The ability to sequester unladenated apodesaturase in the bacterial membranes, however, does offer a method for determining whether iron insertion in mammalian tissues is spontaneous or requires an iron carrier or catalysis to effect iron insertion at the ambient intracellular iron concentrations. Whereas repeated previous attempts to prepare unladenated apoenzyme from purified desaturase have failed, the bacterial membrane fraction containing apodesaturase represents the ideal substrate for monitoring the mechanism of such iron binding.

TABLE I

| Amino acid | pmol | Amino acid | pmol |
|------------|------|------------|------|
| 1 Met      | 585  | 11 Asp     | 323  |
| 2 Lys      | 464  | 12 Ile     | 237  |
| 3 Lys      | 364  | 13 Arg     | 189  |
| 4 Val      | 401  | 14 Pro     | 150  |
| 5 Pro      | 283  | 15 Glu     | 153  |
| 6 Leu      | 320  | 16 Met     | 150  |
| 7 Tyr      | 352  | 17 Arg     | 146  |
| 8 Leu      | 313  | 18 Glu     | 139  |
| 9 Glu      | 272  | 19 Asp     | 180  |
| 10 Glu     | 312  | 20 Ile     | 142  |

FIG. 3. Immunoblot analysis and gel electrophoresis of desaturase isolated from pDs3–358. A, immunoblots. Lane 1, 50 pmol of rat liver desaturase standard; lane 2, 1 μl of sodium dodecyl sulfate-purified desaturase from pDs3–358. B, sodium dodecyl sulfate gel electrophoresis and Coomassie Blue staining: lane 1, marker rat liver desaturase standard; and lane 2, 5 μl of purified desaturase from pDs3–358.
TABLE II

| Plasmid   | Cell fraction | Desaturase activity (nmol/min) | Desaturase content (μmol) per ml cells |
|-----------|--------------|-------------------------------|---------------------------------------|
| pDs3-358  | M<sub>R</sub> | <0.11                         | <0.4                                  |
|           | M<sub>R</sub> | 0.98-1.4                      | 3.5-5.0                               |
|           | S<sub>i</sub> | <0.05                         | <0.2                                  |
|           | M<sub>R</sub>-T | 1.12                         | 4.0                                  |
| pDs27-358 | M<sub>R</sub> | <0.10                         | <0.36                                 |
|           | M<sub>R</sub> | 1.0-1.33                      | 3.6-4.7                               |
|           | M<sub>R</sub>-T | 1.10                         | 3.9                                  |

Table II shows the levels of desaturase activity obtained with membrane fractions from cells induced in the presence of iron. The low levels of activity measured in preparations from repressed cells are near the limits of reliability of the assay method with crude enzyme preparations. Nevertheless, induced cells show at least an order of magnitude elevation of enzyme activities, concentrated almost completely in the membrane fractions. Virtually all of this activity is found in the Triton X-100 and sodium deoxycholate extract of membranes derived from induced cells as would be expected from the behavior of the apolar rat liver enzyme (8). Calculations of the amount of active desaturase in induced cells transformed with either plasmid using the turnover number of 28/min (14) yields values that are similar to the levels of antigen estimated from the immunoblots. Thus, virtually all of the desaturase is converted to active holoenzyme during protein synthesis in an iron-enriched medium.

The deletion of the 26-residue segment from the amino terminus of the desaturase represents the first mutational modification of the protein designed to identify the limiting sequences and particular residues required for membrane and iron binding to yield the substrate and cytochrome b<sub>i</sub> interactions involved in stearoyl-CoA desaturation. Clearly, the first 26 residues are not required for either membrane insertion or enzymatic activity. This peptide segment (6), which contains an unusual sequence of 11 residues (10-20) containing 10 residues with a hydroxyl group, therefore has no apparent functional significance. Moreover, the processing of the enzyme from pDs3-358 extends the limited catalytic significance of the amino-terminal segment of the desaturase through at least 33 residues.

The primary structure of the desaturase (6) is similar to that of one form of cytochrome P-450 (16). Both intrinsic membrane proteins contain several polar segments rich in cationic charges which are interrupted with several highly nonpolar sequences that may serve to orient the polar loops in the aqueous phase by insertion of the nonpolar segments in the hydrocarbon region of lipid bilayers. Extension of mutational deletions to selected “loop” or “anchoring” regions should identify domains that participate in specific binding or catalytic sequences. As the cationic residues involved in interactions with cytochrome b<sub>i</sub> (14), the 2 tyrosyl residues involved in iron binding (17), and the 2 arginyl residues required for stearoyl-CoA binding (17) are identified in the primary structure, selected site-directed mutagenesis will also address the questions of the functional roles of single amino acid residues.

In this respect, the recent success of Beck von Bodman et al. (18) in constructing an expression vector for cytochrome b<sub>i</sub> is of particular interest. The application of mutational methods to a detailed examination of structure-function relationships in this amphipathic heme protein will not only provide comparative data on a second oxidative membrane protein, but the combined information on both proteins should be complementary in understanding the precise protein-protein interactions that govern electron transfer between them.

Acknowledgment—We would like to thank George Korza for his excellent technical assistance in the peptide sequence analysis.

REFERENCES

1. Strittmatter, P., Spatz, L., Corcoran, D., Rogers, M. J., Setlow, B., and Redline, R. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 4565-4569
2. Rogers, M. J., and Strittmatter, P. (1973) J. Biol. Chem. 249, 895-900
3. Rachubinski, R. A., Verma, D. P. S., and Bergeron, J. M. (1980) J. Cell Biol. 84, 705-716
4. Thiede, M. A., and Strittmatter, P. (1985) J. Biol. Chem. 260, 14459-14463
5. Oshino, N., and Sato, R. (1972) Arch. Biochem. Biophys. 149, 369-377
6. Thiede, M. A., Ozols, J., and Strittmatter, P. (1986) J. Biol. Chem. 261, 13230-13235
7. Vierra, J., and Messing, J. (1982) Gene (Amst.) 19, 257-268
8. Strittmatter, P., Fleming, P., Connors, M., and Corcoran, D. (1978) Methods Enzymol. 51, 97-101
9. Kessil, C. R., Hediger, M., Ozols, J., and Strittmatter, P. (1983) J. Biol. Chem. 258, 14656-14663
10. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
11. Lasemoli, U. K. (1970) Nature 227, 680-685
12. Towbin, H., Stahelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350-4354
13. Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6-13
14. Dailey, H. A., and Strittmatter, P. (1979) J. Biol. Chem. 255, 5184-5189
15. Bachmair, A., Finley, D., and Varshavsky, A. (1986) Science 234, 179-186
16. Ozols, J., Heinemann, F. S., and Johnson, E. F. (1985) J. Biol. Chem. 260, 5427-5434
17. Enoch, H. G., and Strittmatter, P. (1978) Biochemistry 17, 4927-4932
18. Beck von Bodman, S., Sculler, M. A., Jollie, D. R., and Sligar, S. G. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 9443-9447