Expression, Purification, and Crystallization of the Adipocyte Lipid Binding Protein*

Zhaozhi Xu†, Melissa K. Buelts, Leonard J. Banaszak‡, and David A. Bernlohr§

From the Departments of Biochemistry, †Medical School, University of Minnesota, Minneapolis, Minnesota 55455 and the §College of Biological Sciences, University of Minnesota, St. Paul, Minnesota 55108

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The murine adipocyte lipid binding protein (ALBP/ aP2) has been cloned and expressed in Escherichia coli, purified to homogeneity, biochemically characterized, and crystallized for x-ray diffraction study. In the cloning, the ALBP coding region was placed under control of the recA promoter and downstream of the phase T7 g-10 translation enhancer sequence. Naldixic acid (50 μg/ml) induced the expression of ALBP 20-fold over that attained using the pT7 system previously reported (Chinander, L. L., and Bernlohr, D. A. (1989) J. Biol. Chem. 264, 19564–19572). Recombinant ALBP was purified to homogeneity using a combination of pH fractionation, gel filtration, and immobilized metal affinity chromatography. The fluorescent affinity ligand 12-(9-anthroyloxy)oleic acid bound to homologous ALBP with an apparent Kd of 0.5 μM. rALBP was devoid of endogenous fatty acid, and oleic acid inhibited cytosine 117 modification by 5,5′-dithiobis-(2-nitrobenzoic acid) indicating integrity of the ligand binding region. Recombinant ALBP was phosphorylated by the soluble kinase domain of the insulin receptor with a Vmax of 11 mmol·min⁻¹·mg of kinase and an apparent Kd of 270 μM. Purified protein was crystallized using the hanging drop method with seeding. Crystalline ALBP was orthorhombic with cell dimensions of a = 54.4 Å, b = 54.8 Å, and c = 76.3 Å. The space group was P2₁2₁2₁, and there was one molecule per asymmetric unit.

Intracellular hydrophobic ligand binding proteins are thought to be responsible for the uptake, transport, and metabolism of lipids. Different cell types appear to have different, but related, lipid binding proteins presumably fulfilling cell-specific functions. Fatty acid binding proteins are found in a variety of tissues including intestine, liver, heart, mammary, Schwann cell, and adipose (1–6). The adipocyte protein termed adipocyte lipid binding protein, abbreviated ALBP1 or aP2, is a fat cell specific protein that is considered to facilitate the bidirectional flux of fatty acids into and out of the adipocyte in response to insulin and epinephrine, respectively (6, 7).

It has been shown that in 3T3-L1 adipocytes ALBP is phosphorylated on Y19 in response to insulin (8, 9). Recently, insulin-stimulated tyrosyl phosphorylation of ALBP has been reconstituted in vitro using homogeneous ALBP and lectin-Sepharose-enriched insulin receptor (10). The characteristics of ALBP phosphorylation in vitro are very similar to those seen in situ leading to the consideration that ALBP is a primary substrate of the insulin receptor kinase. Because insulin regulates lipid metabolism in adipocytes, the flux of fatty acids into and out of the fat cell may be regulated by phosphorylation of the lipid carrier, ALBP. This hypothesis suggests some structural relationship between the site of phosphorylation, Y19, and the ligand binding domain.

Crystal structures of two related FABPs have been determined (11, 12). The intestinal fatty acid binding protein (IFABP) has 27% identity and myelin P2 protein 69% identity to ALBP. IFABP and P2 have very similar conformations. They have a single domain with secondary structures largely consisting of two 5-stranded orthogonal antiparallel β-sheets. The hydrophobic ligand binding site resides in the center of the molecule shielded from the aqueous environment (11, 12). The carboxylate of the bound fatty acid is coordinated with arginine residues in the internal cavity of the protein.

To address the structure-function relationship between tyrosine phosphorylation and lipid binding and to define the ligand binding region, the structure of ALBP must be determined. We report here the overexpression of recombinant ALBP in Escherichia coli, the characterization of its ligand binding and phosphorylation properties, the preparation of suitable crystalline specimens, and preliminary x-ray data from the resulting crystals.

EXPERIMENTAL PROCEDURES

Materials—[^1]Labeled protein A (200 Ci/mmol) was obtained from Du Pont-New England Nuclear. [γ-32P]ATP was obtained from Amersham. Oleic acid was purchased from Nu Check Prep, Inc. (Ely, MN). 12-(9-Anthroyloxy)oleic acid was purchased from Molecular Probes Inc. Bacto-tryptone, yeast extract, and casamino acids were purchased from Difco Laboratories. Sephadex G-75, IDA-Sepharose 6B, naldixic acid, and 5,5′-dithiobis-(2-nitrobenzoic acid) were obtained from Sigma. All components for the polymerase chain reaction were from Perkin-Elmer. Deoxynucleotides were synthesized by the University of Minnesota Microchemical facility. Plasmid pMON-5840 was generously provided by Dr. Jeffrey Gordon, Washington University (St. Louis, MO) and Dr. Peter Olins, Monsanto Co. (St. Louis, MO).

Molecular Cloning—Recombinant cDNA clone pT7-5 (10), encoding the full length insert corresponding to murine ALBP, was digested with PvuII, and the coding region was amplified via the polymerase chain reaction. To facilitate molecular cloning, two oligonucleotides that bracket the coding region were synthesized. A 15-base deoxyoli-
gonucleotide (5'-CCTTCTAGAAGAGGAGG-3') corresponding to part of the E. coli ribosome binding site and including an XbaI site was utilized as the 5' primer. A 23-base 3'-deoxynucleotigone (5'-TCTAGACATCCAGGCTCCTCC-3') was synthesized to anneal downstream of the translation stop codon and generated a second XbaI site. Amplification of the ALBP coding region by polymerase chain reaction was performed for 30 cycles. Each cycle utilized a 94°C denaturation for 1 min, 55°C annealing for 1 min, 72°C extension for 1 min, and a final 5-min 72°C elongation during cycle 30. The final product was extracted once with phenol/chloroform and precipitated with ethanol. The DNA was resolubilized with 10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0, and subjected to restriction endonuclease digestion with XbaI. A 450-base pair XbaI fragment was purified by electrophoresis from 1% agarose gel. The recovered DNA was ligated with dephosphorylated XbaI-linearized pMON 5840 and transformed into E. coli JM101. The transformants were selected at 37°C on the basis of ampicillin resistance, and putative transformants identified by self-matching XbaI were digested using different concentrations of digested colonies (13). All transformants harboring inserts were verified by restriction digest, and the insert amplified DNA was sequenced to ensure identity.

**Purification**—Concentrate harboring plasmid pMON containing the lipid binding protein insert were inoculated into 5 ml of LB medium containing 100 μg/ml ampicillin and grown overnight. The overnight culture was diluted 100-fold into 20 ml of M9 minimal salt medium containing 100 μg/ml ampicillin and grown at 37°C. The culture was then harvested by centrifugation at 4°C for 20 min at 8,000 rpm in a Beckman JA-10 rotor. Typically, 20 g of cell paste was recovered from 5 liters of 5.0 M EDTA. Homogeneous ALBP identified by its migration on SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to nitrocellulose membrane, and immunoblotting was as described by Chinander et al. (10). Murine ALBP was used as a positive control for ALBP expression. The colony identified by immunological analysis with the greatest ALBP expression was termed pMON-rALBP.

**Crystallization**—Large scale purification of ALBP was accomplished from E. coli JM101 harboring pMON-rALBP. The cells were grown as described above on minimal medium and harvested by centrifugation at 4°C for 5 min at 8,000 rpm in a Beckman JA-10 rotor. Typically, 20 g of cell paste was recovered from 5 liters of culture. 20 ml of buffer A, containing 25 mM imidazole HCl, pH 7.5, 50 mM NaCl, 1 mM 2-mercaptoethanol, 5 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride was added to the cell paste. The cell slurry was sonicated, and the cell debris was removed by centrifugation at 4°C for 30 min at 12,000 rpm. The soluble extract was recovered, and protamine sulfate was added over a 20-min period to a final concentration of 1% from a 5% stock prepared in buffer A. The supernatant was obtained by centrifugation, and the proteins were separated by 5–15% SDS-polyacrylamide gradient gel electrophoresis. The separated proteins were transferred to nitrocellulose membrane, and immunoblotting was as described. Data from different scans were integrated separately and frames were searched to find about 100 strong reflections which were then indexed, and the cell dimensions were refined by least square methods. Data from different scans were integrated separately and then merged together.

**Results and Discussion**—Murine ALBP cDNA was initially cloned and expressed in E. coli using the T7 system of Tabor and Richardson described in Ref. 10. However, the yield from this system was far below that necessary for crystallization trials. Thus, we turned our attention to the newly developed “g-10 leader” expression system. This expression system utilizes the phage T7 gene-10 product translation enhancer (20). The g-10 leader was placed upstream of the conventional E. coli ribosome binding site. Using this upstream translation enhancer under the control of the rea promoter, ALBP was overexpressed to a much larger extent than using the E. coli ribosome binding site alone. Induction of the rea promoter by naldixic acid resulted in the cytoplasmic expression of ALBP. This was verified by SDS-PAGE and immunoblotting (results not shown).

A purification protocol for recombinant ALBP had been established by Chinander and Bernlohr (10). An initial pH 5.0 fractionation was followed by Sephadex G-75 chromatography and CM-35W cation exchange HPLC column. The purification was monitored by SDS-polyacrylamide gel electrophoresis and immunobiochemical analysis. However, the procedure was time-consuming and difficult to scale up given the use of HPLC ion exchange chromatography. As an alternative to the existing protocol, we used immobilized metal affinity chromatography as the last step in the purification process.
In immobilized metal affinity chromatography, proteins are fractionated according to their affinity for transition metal ions, largely determined by their surface histidine content (21, 22). In the case of murine ALBP, the protein has no histidine residues suggesting that purification could be achieved by adsorption of contaminants onto a Cu²⁺-based affinity matrix.

Pooled protein fractions from the G-75 column were loaded onto the charged metal chelated column. ALBP flowed through the column, eluting during the washing of the resin, while most of the contaminant proteins did not elute until after the column was stripped with 0.5 mM EDTA. SDS-gel electrophoresis showed homogeneous ALBP in the flow-through fraction. Compared to the pT7-5 vector and coupled with the improved purification scheme, a 20-fold improved yield was achieved by using the new pMON-rALBP expression system. These results are summarized in Fig. 1.

To examine the functional properties of the ALBP, the fluorescent titration assay was utilized. Binding of the probe, 12-(9-anthroyloxy)oleic acid, to ALBP was a saturable process (Fig. 2). These results and previous studies based on radio-isotope ligand binding (6) demonstrated a stoichiometry of 1 mol of ligand per mol of ALBP. The apparent Kₐ for ALBP was calculated to be 0.5 μM.

Chemical modification of sulfhydryl groups of murine ALBP has shown that C117 resides within the ligand binding domain (23). In the presence of oleic acid, C117 of murine ALBP was protected from DTNB modification. Conversely, DTNB modification blocked fatty acid binding. To assess and compare the structural properties of rALBP to murine ALBP, sulfhydryl titration in the presence and absence of oleic acid was examined. Unlike murine ALBP, rALBP had only one free sulfhydryl group, C117; C1 is unreactive presumably due to its oxidation (10). The presence of oleic acid protected C117 in rALBP from modification. Upon denaturation of ALBP with SDS, the cysteine became reactive, indicating structural competence of the ligand binding domain (Fig. 3).

To further characterize rALBP, phosphorylation by the soluble insulin receptor kinase was studied, and the results are shown in Fig. 4. Lineweaver-Burk analysis of phosphorylation data revealed that the Vₘₐₓ was 11 nmol-min·mg of kinase, and the Kₘ was 270 μM. Given that the ligand binding and phosphorylation properties of rALBP faithfully mirrored that observed with the mammalian protein (24), we pursued crystallization trials.

Crystallization experiments using rALBP were immediately successful. With seeding, octahedral crystals of the apoprotein grew to a length of 0.4 mm and a height of 0.3 mm. These crystals give diffraction data to 2.4 Å. An entire data set was collected to 2.7-Å resolution using the area detector system. Statistical details of the combined x-ray data set are presented in Table I. The lattice type was orthorhombic with unit cell dimension of a = 34.4 Å, b = 54.8 Å, c = 76.3 Å.

The x-ray diffraction data were examined for systematic

**Fig. 1. Purification of ALBP from bacterial lysates.** A sample from each step in the purification was analyzed by SDS-polyacrylamide gel electrophoresis (protocols were described under "Experimental Procedures"). Lane 1, total E. coli extract; lane 2, protamine sulfate supernatant; lane 3, pH 5.0 soluble protein; lane 4, Sephadex G-75 pool; lane 5, Cu²⁺ IDA-Sepharose 6B purified protein. The gel was stained with Coomassie Blue. Numbers to the left of the gels show the apparent molecular mass in kilodaltons of the protein standards. The light smear migrating faster than ALBP corresponds to phospholipid which occasionally co-purifies but does not associate with ALBP.

**Fig. 2. 12-(9-Anthroyloxy)oleic acid binding of ALBP.** 12-AO (50 nM) was titrated with ALBP (0–5 μM), and 12-AO fluorescence was measured at an excitation wavelength of 383 nm and an emission wavelength of 460 nm. Inset, Scatchard analysis of ligand binding. Data shown are from a single experiment representative of at least 10 binding studies.

**Fig. 3. Sulfhydryl modification of ALBP.** ALBP (5 μM) was titrated with 40 μM DTNB in the presence (O) or absence (●) of saturating oleic acid. Arrow, addition of SDS to 1% final concentration to the oleic-containing sample. Data represent a single experiment performed four times with the same results.

C117 in rALBP from modification. Upon denaturation of ALBP with SDS, the cysteine became reactive, indicating structural competence of the ligand binding domain (Fig. 3). Additionally, in the absence of any fatty acid, 1 mol of sulfhydryl was titrated indicating that the purified protein is devoid of fatty acid.

To further characterize rALBP, phosphorylation by the soluble insulin receptor kinase was studied, and the results are shown in Fig. 4. Lineweaver-Burk analysis of phosphorylation data revealed that the Vₘₐₓ was 11 nmol-min·mg of kinase, and the Kₘ was 270 μM. Given that the ligand binding and phosphorylation properties of rALBP faithfully mirrored that observed with the mammalian protein (24), we pursued crystallization trials.

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the space group here, the volume of protein in 1 asymmetric unit would be 1.8

absences to determine the space group. Such absences were observed along the a*, b*, and c* axes. Only reflections with h, k, or l = 2n were observed along the reciprocal axes. This indicated that the space group is P2₁,2₁,2₁ (25). A unit cell with the dimensions described above has a volume of 1.44 × 10⁶ Å³. Assuming that half of the crystal volume is water, the volume of protein is approximately 7.2 × 10⁴ Å³. Considering the space group here, the volume of protein in 1 asymmetric unit would be 1.8 × 10⁶ Å³. By averaging the specific volume of constituent amino acids, the specific volume of ALBP is 0.715 ml/g. This led to the conclusion that the molecular mass of ALBP is approximately 15 kDa, there is only 1

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and translation functions have been obtained. Seeding techniques will allow us to obtain large crystals for further study of the holo- and phosphorylated protein. By comparing the crystal structures of these different forms, it should be possible to structurally determine the effects of protein phosphorylation on ligand binding and ligand binding on phosphorylation. These results will provide clues as to the effect of insulin on regulation of lipid trafficking in adipose cells.

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REFERENCES

**TABLE I**

X-ray data collection statistics for crystalline ALBP

| Merging R factor based on I | 0.0426 |
| Resolution limits | 2.2 Å |
| Number of observations | 20,478 |
| Number of unique x-ray reflections collected | 5,473 |
| Average number of observations for each reflection | 4.0 |
| % of possible reflections collected to 2.7 Å | 98 |
| % of possible reflections collected to 2.4 Å | 36 |

FIG. 4. Phosphorylation of ALBP with the soluble insulin receptor kinase. ALBP was phosphorylated in 25 mM HEPES, pH 7.5, containing 5 mM MnCl₂ and 100 μM [³²P]ATP by 0.25 μg of autophosphorylated soluble receptor for 10 and 22 min. The reactions were terminated, and the proteins were subjected to SDS-PAGE and autoradiography. The rate of [³²P] incorporation into ALBP was measured by excision of the radioactive protein and liquid scintillation counting. Results are presented from one analysis representative of three experiments.