Biochemistry

 Volume 33, Number 26 July 5, 1994

Articles

Proline-Rich Domain and Glycosylation Are Not Essential for the Enzymic Activity of Bile Salt-Activated Lipase. Kinetic Studies of T-BAL, a Truncated Form of the Enzyme, Expressed in *Escherichia coli*†

Deborah Downs,‡ Yu-Yun Xu,‡ Jordan Tang,‡,§ and Chi-Sun Wang*,‡,§

Protein Studies Program, Oklahoma Medical Research Foundation, and Department of Biochemistry and Molecular Biology, Oklahoma University Health Sciences Center, Oklahoma City, Oklahoma

Received February 15, 1994; Revised Manuscript Received April 28, 1994®

ABSTRACT: We have expressed and purified a truncated recombinant human milk bile salt-activated lipase (T-BAL) from the T7 expression system in *Escherichia coli*. This T-BAL contains the N-terminal 538 residues of the 722-residue native enzyme. The purified T-BAL, when assayed with PANA (*p*-nitrophenyl acetate), had a specific activity of 64 ± 2 units/mg (n = 4), as compared to 52 units/mg for the native enzyme. Because the recombinant T-BAL expressed in *E. coli* is not glycosylated, these results indicated that the highly glycosylated C-terminal region of BAL is not essential for catalytic function. Heat inactivation patterns of native BAL and T-BAL were found to be similar, further suggesting that the folding of T-BAL is similar to that of the catalytic domain of the native enzyme. With the availability of a sufficient amount of recombinant T-BAL, the specificity and kinetics of T-BAL and native BAL were compared. Fluorescence studies of T-BAL indicated that it has a slightly higher affinity for the monomeric form of taurocholate with a dissociation constant (K_A) of 0.32 mM, compared with the reported 0.37 mM for the native enzyme. Further kinetic analysis indicated that there are enzyme specificity changes revealed with the use of PANA and PANB (*p*-nitrophenyl butyrate) as substrates. When assayed in the presence of taurocholate, T-BAL has a higher turnover rate constant with *p*-nitrophenyl acetate than with *p*-nitrophenyl butyrate, which was found to be in contrast to native BAL. However, similar to the native enzyme, T-BAL still has a higher substrate specificity constant with PANB than with PANA, because of the much lower Michaelis–Menten constant with PANB. The essential requirement of bile salt micelles as fatty acid acceptor in the BAL catalysis was also similar between T-BAL and native BAL. However, T-BAL was more resistant to the inactivation effect of a high concentration of taurocholate.

Human milk bile salt-activated lipase (BAL)[1] is the major lipolytic activity present in human milk (Wang & Hartsuck, 1993; Olivecrona & Bengtsson, 1984). The activity of this enzyme is not expressed in the milk but is activated by the bile salts present in the intestine. The physiological function of human milk BAL was suggested by Alemi et al. (1981) to be the digestion of milk fat in infants. This nutrition role of the

† This study was supported by research grant HD-23472 from the National Institutes of Health, Grant HR0-017/3924 from the Oklahoma Center for the Advancement of Science and Technology, and a grant from Astra Hässle.

* Please address correspondence to this author at Oklahoma Medical Research Foundation, 825 N. E. 13th Street, Oklahoma City, OK 73104. Tel. (405) 271-7284. FAX (405) 271-3980.

‡ Oklahoma Medical Research Foundation.

§ Department of Biochemistry and Molecular Biology, Oklahoma University Health Sciences Center.

® Abstract published in *Advance ACS Abstracts*, June 15, 1994.

[1] Abbreviations: PANA, *p*-nitrophenyl acetate; PANB, *p*-nitrophenyl butyrate; SDS, sodium dodecyl sulfate; BAL, human milk bile salt-activated lipase; T-BAL, truncated form of bile salt-activated lipase; PCR, polymerase chain reaction; TN, 50 mM Tris-HCl, pH 8.0, and 100 mM NaCl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FPLC, fast protein liquid chromatography; BSA, bovine serum albumin.

0006-2960/94/0433-7979$04.50/0

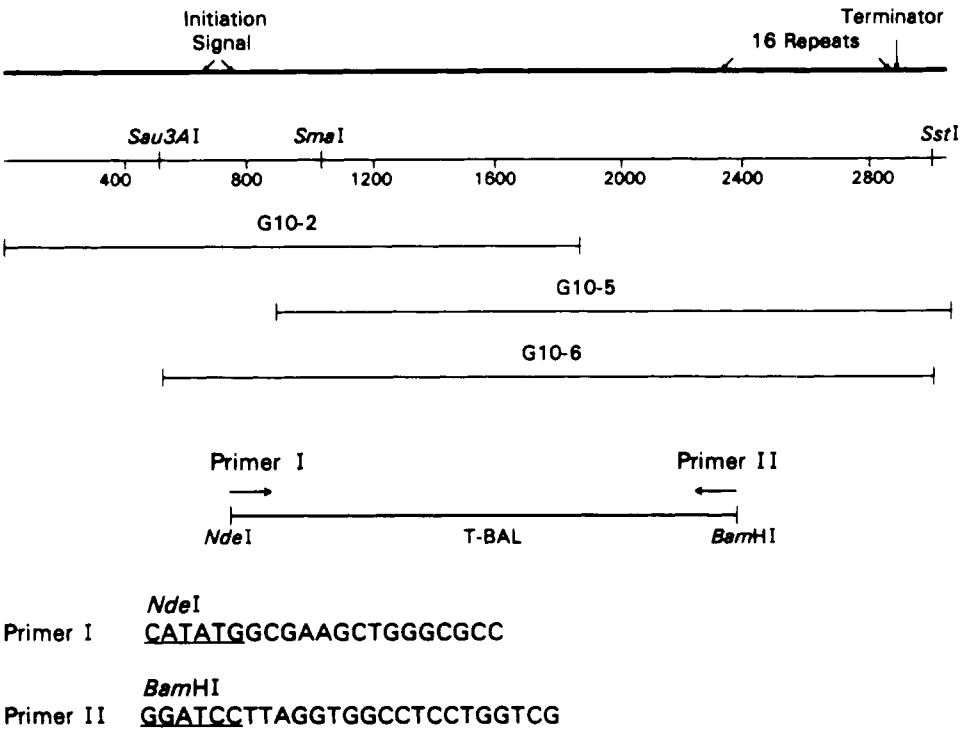


FIGURE 1: Map of BAL cDNA for the construction of the T-BAL expression vector. Partial clones G10–2 and G10–5 were isolated from a lactating human breast tissue cDNA library. G10–6 was derived from G10–2 and G10–5 using the unique *Sma*I site for the ligation. Oligonucleotide primers I and II provide the *Nde*I and *Bam*HI sites for the ligation of T-BAL to the pET11a expression vector.

enzyme has been clearly demonstrated in an animal model (Wang et al., 1989). Human pancreas supplies the intestine with a bile salt-activated lipase. However, the current understanding is that the pancreatic BAL is not well developed in human infants in general; thus the milk enzyme is nutritionally significant. The information on the structure and function relationship of this enzyme will therefore help in the understanding of the physiological roles of BAL.

Human milk BAL cDNA (Baba et al., 1991; Hui & Kissel, 1990; Nilsson et al., 1990) encodes a 722-residue mature enzyme. Interestingly, the cDNA structure is identical to that of the pancreatic BAL (Reue, 1991), suggesting that the enzyme from mammary gland and from pancreas may be expressed from the same gene. The enzyme is related to acetylcholinesterase and thyroglobulin (Baba et al., 1991) in amino acid sequence. The main difference in the structures of BAL and acetylcholinesterase is the presence in BAL of a mucin-like C-terminal region which consists of 16 similar repeating motifs composed of 11 amino acid residues with the consensus sequence of PVPPTGDSGAP (Baba et al., 1991). Evidence indicates that the threonine and serine residues in this region are *O*-glycosylated (Baba et al., 1991). Since neither acetylcholinesterase nor fungal lipase homologous to BAL possesses this mucin-like C-terminal region (Wang & Hartsuck, 1993), this region may serve some interesting physiological function besides catalyzing the lipolytic reaction. The crystal structures of acetylcholinesterase (Sussman et al., 1991) and fungal lipase (Ollis et al., 1992), however, suggest that BAL may be catalytically competent without the mucin-like region (Wang & Hartsuck, 1993). In order to test the functional requirements of the regions in BAL, we expressed the truncated BAL cDNA (without the mucin-like region, residues 539–722) in *Escherichia coli* and studied the properties of the truncated recombinant enzyme. The results are described in this paper.

EXPERIMENTAL PROCEDURES

Materials. The purification of BAL from human milk was performed as described previously (Baba et al., 1991). The cDNA library from human lactating breast tissue in λgt10 was purchased from Clontech. Glycerol tri[9,10-^{3}H]oleate was obtained from Amersham. The oligonucleotides primer I and primer II (Figure 1) were prepared by Dr. K. Jackson in the Molecular Biology Resource Facility, Oklahoma University Health Sciences Center. All other chemicals were purchased from Sigma Chemical Co.

Molecular Biological Methods. Techniques for sequencing analyses, restriction enzyme treatments, ligation, and subcloning are based on methods described in standard technique reference books (Sambrook et al., 1989; Ausbel et al., 1989). The cDNA clones G10-2 and G10-5 were isolated from human mammary gland cDNA as described previously (Baba et al., 1991). DNA sequencing analyses were performed using a DNA sequencing kit from United States Biochemical Corp. Polymerase chain reaction (PCR) was performed as described by Innis and Gelfand (1990). The PCR-generated DNA was subcloned into PCRII using the TA cloning kit from Invitrogen. Plasmid isolation was performed using the Magic Miniprep system supplied by Promega.

Construction of the Vector. The unique *Sma*I restriction site of the overlapping clones G10-2 and G10-5 was utilized for construction of the cDNA G10-6 to encompass the entire coding sequence region of BAL (Figure 1). PCR was utilized for preparing T-BAL cDNA with the use of primers I and II (Figure 1) for expression of the truncated form of BAL. The T-BAL cDNA was ligated to the pET11a cloning vector (Invitrogen) at the *Nde*I/*Bam*HI cloning site for the subsequent expression of T-BAL using the T7 expression system in *E. coli*.

Expression of T-BAL Using the pET11a Vector. The general approach and methodology for using T7 polymerase

to direct the expression of the cloned genes are described by Studier et al. (1990). The cDNA of T-BAL was ligated to the *Nde*I/*Bam*HI cloning sites of pET11a (Novagen). The vector was used to transform *E. coli* BL21(DE3) for the expression of T-BAL. The cells harboring the vector were cultured overnight in ZB medium (Studier et al., 1990) with ampicillin. The next morning 4 L of LB-ampicillin was incubated with 80 mL of the overnight culture, and the culture was shaken at 37 °C until absorbance at 600 nm reached 0.6. Isopropyl β-D-thiogalactopyranoside was then added to a concentration of 0.4 mM, and the culture was shaken for 3 h. These cells were then collected by centrifugation and resuspended in 800 mL of TN buffer (50 mM Tris-HCl, pH 8.0, and 100 mM NaCl) with 80 mg of lysozyme. The mixture was frozen at −70 °C and then thawed in a 37 °C water bath, for three cycles. The solution was then diluted to 2400 mL with TN and centrifuged at 16000*g* for 15 min. The pellet (inclusion bodies) was then washed twice with Triton X-100 (0.1%) in TN, and pellets were recovered by centrifugation and kept frozen at −20 °C for further processing.

Refolding and Purification of T-BAL. The frozen inclusion bodies were further solubilized by stirring with 60 mL of 8 M urea in 100 mM Tris-HCl, pH 12.5, containing 1 mM EGTA (ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid), 10 mM β-mercaptoethanol, and 5% glycerol (v/v). The mixture was further centrifuged at 105000*g* for 30 min, and the supernatant was then placed in dialysis tubing (exclusion MW = 10 000) and dialyzed against 1 L of a refolding buffer containing 1 M urea, 0.1 mM β-mercaptoethanol, and 5% glycerol in 10 mM Tris-HCl, pH 8.0. After dialysis for 20 h, the refolded T-BAL was assayed for esterase activity using 1 mM PANA as substrate and 2 mM taurocholate as activator to determine the amount of active enzyme in the dialyzed solution. The refolded T-BAL was then mixed with 60 mL of saturated ammonium sulfate (adjusted to pH 8.0 with NH_4OH), which caused the precipitation of T-BAL. The T-BAL was collected from the precipitate after centrifugation at 18 000 rpm for 1 h. The collected precipitate was solubilized with 40 mL of the refolding buffer and stirred at 4 °C for 30 min. The supernatant fraction was then concentrated to 3–5 mL in an Amicon Centriprep-10 concentrator. After further ultracentrifugation at 105000*g* for 30 min, the supernatant fraction (1 mL) was subjected to molecular sieving fractionation by fast protein liquid chromatography (FPLC). Two Superose 12 columns (Pharmacia) were linked in tandem for the FPLC fractionation. The columns were equilibrated and eluted with a buffer solution containing 1 M urea, 0.15 M NaCl, 0.1 mM β-mercaptoethanol, and 50 mM Tris-HCl, pH 8.0. The column was eluted with a flow rate of 0.5 mL/min. The eluate was collected in 1-mL fractions and monitored by measuring absorbance at 280 nm and by esterase activity with PANA as substrate.

N-Terminal Amino Acid Sequence Analysis. Automated Edman degradations were performed according to Hewick et al. (1981) in a Model 470A gas-phase protein sequencer equipped with a Model 120A on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems, Inc.).

Esterase Assay. The kinetic studies using *p*-nitrophenyl acetate (PANA) and *p*-nitrophenyl butyrate (PANB) were performed as described previously (Wang, 1991). For determination of the Michaelis–Menten kinetic parameters, the experiments were performed at 25 °C and the range of the substrate concentration in the final assay mixtures was 0.4–2 mM for PANA and 0.1–0.5 mM for PANB, with 2 mM taurocholate as activator. The rate of *p*-nitrophenol production was determined from the initial portion of the absorbance change (20 s) at 418 nm using a Hewlett-Packard diode array spectrophotometer equipped with a peltier temperature control. To monitor the refolding efficiency, as well as the esterase activity in column chromatography eluants and the specific activity of the purified enzyme, the enzyme assays were performed with 1 mM PANA as substrate and 2 mM taurocholate as activator. One unit of enzyme activity was defined as 1 μmol of the product released per minute.

Thermostability of T-BAL. For determination of the thermostability, 0.1 mg/mL each of T-BAL and native BAL in sodium phosphate (0.15 M, pH 7.4) was incubated for 10 min in a water bath with preset temperatures. After incubation, the solutions were cooled in ice–water. These treated samples were then assayed for remaining activity with PANA as substrate.

Lipase Assay. The lipase assay of BAL was performed according to a modification of the method of Nilsson and Schotz (1976) using glycerol tri[9,10-^{3}H]oleate as substrate. The 2-fold-concentrated stock substrate solution was prepared by emulsifying 28 μmol of trioleoylglycerol (specific activity, 1.4 μCi/μmol) and 2.8 μmol of dioleoylphosphatidylcholine in 10 mL of 50 mM NH_4OH–HCl buffer, pH 8.5. The mixture was emulsified using a W-380 sonicator (Heat Systems-Ultrasonics, Inc.) at a setting of 5 (50% maximum output) for 30 s in an ice bath. After cooling, the mixture was further sonicated for an additional 30 s. The assay mixture, with a final volume of 100 μL, contained 50 mM NH_4OH–HCl buffer, pH 8.5, 1.4 mM trioleoylglycerol, 0.14 mM dioleoylphosphatidylglycerol, taurocholate, and 10 μL of the enzyme solution. Following a 1-h incubation at 37 °C with agitation, the reaction was terminated by the addition of 3.2 mL of chloroform–heptane–methanol (5:4:5.6, v/v/v) and 1 mL of 0.2 M NaOH. Samples were centrifuged and mixed with 10 mL of Hydrocount (J.T. Baker, Inc., Phillipsburg, NJ), and the radioactivity was determined in a Beckman scintillation counter.

Sodium Dodecyl Sulfate (SDS)–Polyacrylamide Gel Electrophoresis. The SDS–polyacrylamide gel electrophoresis was performed using the LKB-Pharmacia Phast system with the use of an 8–25% polyacrylamide gel slab manufactured by Pharmacia. The samples were treated with 10 mM β-mercaptoethanol and 2% SDS at 100 °C for 6 min prior to electrophoresis.

Protein Analysis. The protein content of the enzyme preparation was determined by a previously reported modification (Wang & Smith, 1975) of Lowry's procedure using serum albumin as standard.

Fluorescence Study of the Interaction of T-BAL with Taurocholate. Fluorescence measurements were made at 25 °C with the Aminco-Bowman Series 2 fluorescence spectrometer. The T-BAL tryptophanyl fluorescence was utilized for studying the interaction of T-BAL with taurocholate. Fluorescence was recorded at 340 nm with the excitation wavelength at 280 nm. The bandwidths of excitation and emission were both set at 2 nm. The sensitivity of the instrument was set at 1000 V of the detector high voltage.

In the absence of taurocholate, the activator, the fluorescence intensity of T-BAL is F_0, and at the saturating concentration of taurocholate the fluorescence intensity is F_∞. On the basis of the fluorescence, F, at a specified taurocholate concentration, then, the molar fraction, x, of T-BAL which is associated with taurocholate can be determined in relation to F, as shown

```
AKLGAVYTEGGFVEGVNKKLGLLGDSVDIFKGIPFAAPTKALENPQPHPGWQGTLKAKNFKKRCLQATIT   70

QDSTYGDEDCLYLNIWVPQGRKQVSRDLPVMIWIYGGAFLMGSGHGANFLNNYLYDGEEIATRGNVIVVT  140
                                                    !       ∇
FNYRVGPLGFLSTGDANLPGNYGLRDQHMAIAWVKRNIAAFGGDPNNITLFGESAGGASVSLQTLSPYNK  210

GLIRRAISQSGVALSPWVIQKNPLFWAKKVAEKVGCPVGDAARMAQCLKVTDPRALTLAYKVPLAGLEYP  280
                                                  ∇
MLHYVGFVPVIDGDFIPADPINLYANAADIDYIAGTNNMDGHIFASIDMPAINKGNKKVTEEDFYKLVSE  350

FTITKGLRGAKTTFDVYTESWAQDPSQENKKKTVVDFETDVLFLVPTEIALAQHRANAKSAKTYAYLFSH  420
          ∇
PSRMPVYPKWVGADHADDIQYVFGKPFATPTGYRPQDRTVSKAMIAYWTNFAKTGDPNMGDSAVPTHWEP  490
                                                        ↓
YTTENSGYLEITKKMGSSSMKRSLRTNFLRYWTLTYLALPTVTDQEATPVPPTGDSEATPVPPTGDSETA  560

PVPPTGDSGAPPVPPTGDSGAPPVPPTGDSGAPPVPPTGDSGAPPVPPTGDSGAPPVPPTGDSGAPPVPP  630

TGDSGAPPVPPTGDAGPPPVPPTGDSGAPPVPPTGDSGAPPVTPTGDSETAPVPPTGDSGAPPVPPTGDS  700
                    ↓
EAAPVPPTDDSKEAQMPAVIRF    722
```

FIGURE 2: Primary amino acid sequence of mature human milk BAL (Baba et al., 1991). The amino acid sequence underlined is deleted from T-BAL. The arrows (↓) indicate the beginning and end of the proline-rich repeat sequence. The triangles (∇) indicate the active site triad residues. The exclamation point (!) indicates the *N*-glycosylation site.

in the following equation:

$$F = (1 - x)F_0 + xF_\infty \quad (1)$$

The deduced molar fraction, x, is then utilized for calculation of the taurocholate and T-BAL binary complex dissociation constant K_A. Conversely, we have used the computer least-squares curve-fitting procedure to treat K_A and F_∞ as the variable parameters for achieving the best agreement with the calculated and the experimentally determined values of F.

Data Analysis. Kinetic analysis was performed using a Lotus 1-2-3 spreadsheet program and an IBM-AT computer. The performance of least-squares nonlinear curve fitting was based on the approach described by Bevington (1969).

RESULTS

Refolding, Purification, and Characterization of Recombinant T-BAL. The amino acid sequences of T-BAL and native BAL are shown in Figure 2. As expected, the T-BAL synthesized in *E. coli* is inactive and is insoluble in aqueous buffer. Active T-BAL was obtained by refolding. This procedure includes the solubilization of the inclusion bodies with urea and β-mercaptoethanol at a high pH (12.5), followed by dialysis against a low concentration of urea at a lower pH (8.0). On the basis of the enzyme activity measurement, the yield of the active enzyme was about 10 mg per 4 L of the cultured LB medium. SDS–polyacrylamide gel electrophoresis (Figure 3) gave rise to a major protein (>90%) in the urea-soluble fraction corresponding to a molecular weight of 60 000, which approximated closely the expected molecular weight of T-BAL (59 270). N-terminal sequence analysis of this protein fraction gave the expected BAL N-terminal sequence of Ala-Lys-Leu-Gly-Ala-Val-Tyr-Thr-, indicating the successful synthesis of T-BAL and the removal of initiation methionine. The specific activity of T-BAL after the initial step of the refolding was about 5–10 units/mg, which is only 10–20% of that of the native BAL.

Purification of T-BAL is achieved by molecular sieving with FPLC (Figure 4) after prior partial purification of the refolded T-BAL with ammonium sulfate precipitation. As seen in the chromatography pattern, there are two major peaks

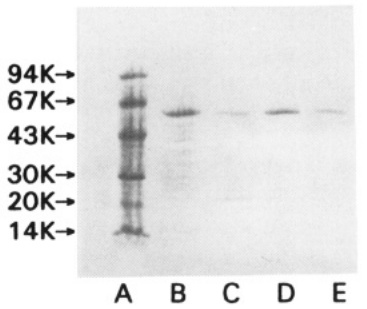


FIGURE 3: SDS–polyacrylamide gel patterns of T-BAL expressed from *E. coli*. Lane A, Molecular weight standards. Lane B, urea-soluble fraction of inclusion bodies. Lanes C, D, and E, fractions 27, 28, and 29, respectively, as shown in Figure 4.

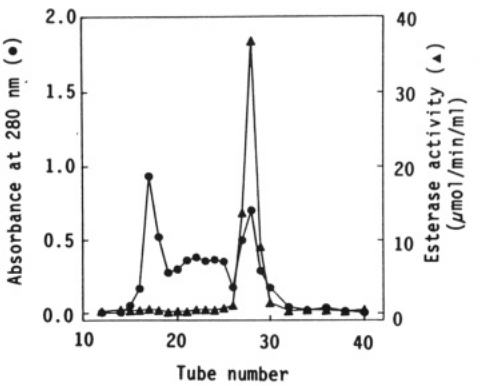


FIGURE 4: FPLC (fast protein liquid chromatography) fraction pattern of the refolded T-BAL after the step of ammonium sulfate precipitation. The fractionation was performed using two tandem linked Superose 12 molecular sieving columns. The columns were eluted and equilibrated with a buffer solution containing 1 M urea, 0.15 M NaCl, 0.1 mM β-mercaptoethanol, and 50 mM Tris-HCl, pH 8.0. The fractions contained 1 mL/tube. The peak tube (fraction 28) contained 0.56 mg/mL purified T-BAL.

found in the column fractions. The first peak, eluted at the void volume (17 mL), represents the major protein peak and contains mainly the aggregate form of the inactive T-BAL. The second peak (eluted at 28 mL) contains BAL activity. SDS–polyacrylamide gel patterns of fractions 27–29 (Figure 3) indicate that the T-BAL eluted in this peak was homogeneous. From four individual batches, we obtained an average

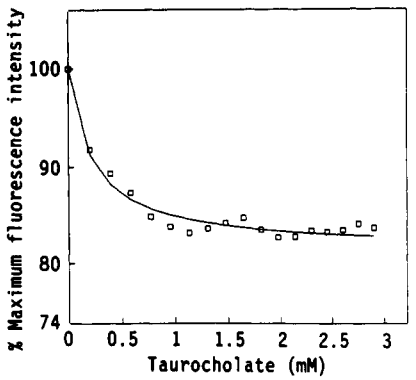


FIGURE 5: Fluorescence spectroscopy analysis of the interaction of T-BAL with taurocholate. The protein tryptophanyl fluorescence of T-BAL was measured at an initial concentration of 1 μM (60 μg/mL) in 0.15 M sodium phosphate (pH 7.4). Taurocholate (20 mM) was added in 25-μL increments to the enzyme solution (2.5 mL). The fluorescence intensity upon addition of taurocholate was corrected for the volume change. The wavelength of excitation was 280 nm, and the emission was recorded at 340 nm. The best-fitted curve was generated through the use of eq 1, treating K_A and F_∞ as the variable parameters.

specific activity of 64 ± 2 units/mg for the purified enzyme (fraction 28).

Thermostability of T-BAL. In order to compare the stability of T-BAL with the native enzyme, we treated these two enzyme forms at temperatures ranging from 30 to 50 °C. The inactivation patterns for T-BAL and native BAL were similar (results not shown), with both showing a loss of about 90% of activity with treatment at 50 °C for 10 min.

Taurocholate Binding Kinetics of T-BAL. We reported previously that upon binding with bile salt native BAL showed about 20% decrease of the protein tryptophanyl fluorescence at a saturating concentration of taurocholate (Wang & Kloer, 1983), which probably resulted from a conformational change of BAL upon ligand binding. In this study we have observed a similar decrease of tryptophanyl fluorescence of T-BAL (Figure 5). On the basis of a 1:1 stoichiometry, we have determined that the dissociation constant K_A is 0.32 ± 0.03 mM (n = 4) for the T-BAL and taurocholate interaction. There is an 18% decrease of fluorescence intensity at a saturating concentration of taurocholate.

Kinetic Properties of T-BAL with PANA and PANB. Similar to the native BAL, T-BAL was found to contain basal activity when assayed in the absence of bile salt with the esterase substrates. Therefore, taurocholate can also be considered as a nonessential activator of T-BAL. From the Lineweaver–Burk plots we have obtained the kinetic parameters K_S and k_{cat} for basal T-BAL and αK_S and βK_{cat} for taurocholate-activated T-BAL, with PANA and PANB as substrates (Table 1). Despite the fact that we obtained a specific activity of T-BAL (64 units/mg) slightly higher than that of native enzyme (52 units/mg) (Wang & Johnson, 1983), the derived k_{cat} and βk_{cat} of T-BAL are about 2–8-fold lower than those of the native enzyme (Table 1). However, the deduced K_S and αK_S of T-BAL are only slightly higher than those of the native enzyme (Table 1). Thus, we conclude that the presence of the proline-rich sequence plays a role mainly in enhancing the turnover rate of the enzyme, but has only a minor effect on the substrate binding affinity. In addition, we have observed that there is a change of the preferential reactivity of the enzyme. Previously we have reported that, among the short-chain acyl esters of p-nitrophenol, native BAL has the highest k_{cat} and βk_{cat} with PANB. In contrast, it was found in this study that T-BAL has a higher k_{cat} and

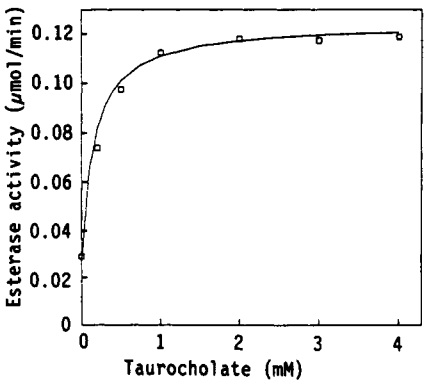


FIGURE 6: Effect of taurocholate on T-BAL activity with PANA as substrate. The parameters utilized for generating the calculated curve were based on eq 2: V_{max} = 16 μmol/min; α = 0.28; β = 3.06; K_A = 0.32 mM; K_S = 10.5 mM. The substrate concentration of PANA was 2 mM.

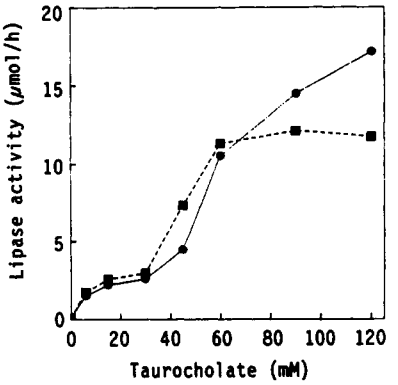


FIGURE 7: Effect of taurocholate on the native BAL (- -■- -) and the T-BAL activity (–●–) with 1 μg of the enzymes used per assay.

βk_{cat} with PANA than with PANB. Despite this, T-BAL also has higher substrate specificity constants (k_{cat}/K_S and $\beta k_{cat}/\alpha K_S$) with PANB than with PANA because of the much lower K_S and αK_S of T-BAL with PANB. This is similar to what is found for the native enzyme. Figure 6 shows the activation effect of taurocholate on BAL-catalyzed hydrolytic reaction with PANA as substrate. The results also clearly demonstrated that the proline-rich domain of BAL does not represent the bile salt binding site of the enzyme.

Lipase Activity of T-BAL. There is an essential requirement of bile salt for the BAL-catalyzed hydrolysis of long-chain triacylglycerol. In this respect, T-BAL was found to have a bile salt requirement for the hydrolysis of long-chain trioleoylglycerol similar to that of native BAL (Figure 7). There is apparent nonsaturable activation of T-BAL by taurocholate (up to 120 mM taurocholate tested). On the other hand, there is an apparent saturation of activation of the native BAL catalysis when the taurocholate concentration is above 60 mM. However, this can be attributed to the partial inactivation of the native BAL when taurocholate concentration is high (Wang et al., 1989).

DISCUSSION

Because of the lack of the carboxyl-terminal proline-rich domain in T-BAL, as well as the fact that *E. coli* expression does not cause glycosylation of the expressed foreign proteins, we concluded that the carboxyl-terminal domain, as well as *O*-glycosylation and *N*-glycosylation, is not absolutely essential for the enzymic function of BAL. On the basis of the crystal structure of acetylcholinesterase, it is likely that the proline-rich domain extends through the helix end of the catalytic

Table 1: Kinetic Parameters of T-BAL with *p*-Nitrophenyl Acetate (PANA) and *p*-Nitrophenyl Butyrate as Substrate

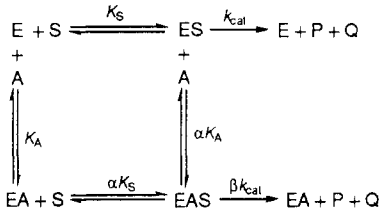
	k_{cat}[a] ($\times 10^{-3}$) (s^{-1})	K_S (mM)	k_{cat}/K_M (s^{-1} mM^{-1})	βk_{cat} ($\times 10^{-3}$) (s^{-1})	αK_S (mM)	$\beta k_{cat}/\alpha K_S$ (s^{-1} mM^{-1})
T-BAL						
PANA	0.16[b] ± 0.01	12 ± 2	13[c] ± 4	0.63 ± 0.03	4.0 ± 0.8	160 ± 20
PANB	0.49 ± 0.02	4.1 ± 0.4	120 ± 20	0.27 ± 0.02	0.42 ± 0.03	650 ± 30
Native[d] BAL						
PANA	0.630 ± 0.006	10.3 ± 0.9	61 ± 5	1.02 ± 0.04	2.4 ± 0.1	430 ± 20
PANB	3.1 ± 0.4	4.3 ± 0.8	700 ± 200	2.21 ± 0.02	0.38 ± 0.01	5800 ± 200

[a] The kinetic parameters are defined in eq 2. [b] Data are expressed as the mean ± SEM ($n = 3$). [c] The propagation of errors of ratios was performed as described by Bevington (1969). [d] From Wang (1991).

function unit (Wang & Hartsuck, 1993) and exists as an separate structural domain entity. In fact, Hansson et al. (1993) have recently shown that neither glycosylation nor the proline-rich repeats are essential for catalytic activity or bile salt activation of recombinant human milk BAL expressed in C127 cells. Since this specific activity of T-BAL is higher than that for the native BAL (52 units/mg) reported previously (Wang & Johnson, 1983), we concluded that the FPLC column chromatography effectively separated the active from the inactive forms of the enzyme.

With the availability of the purified T-BAL, we have also performed a detailed kinetic study of this enzyme. A direct binding study of the tryptophanyl fluorescence upon interaction with taurocholate indicated that there is about an 18% decrease of fluorescence intensity at a saturating concentration of taurocholate, which is similar to that found for the native enzyme (20%) (Wang & Kloer, 1983). Also, the determined dissociation constant, K_A, of 0.32 mM is very similar to that of native enzyme (0.37 mM) (Wang, 1981). Thus, the microenvironments of the taurocholate binding sites of T-BAL and native BAL must be very similar despite the deletion of the proline-rich sequence domain of T-BAL.

The kinetic scheme of T-BAL can be similarly described by the following reaction scheme:



(2)

In this equation, E is the enzyme, T-BAL; S is the substrate; A is the activator; P and Q are the alcoholic and carboxylic acid products, respectively; k_{cat} is the rate constant for the breakdown of ES to E + P + Q; and βk_{cat} is the rate constant for the breakdown of EAS to EA + P + Q. The K_A value, which is the dissociation constant for enzyme–activator interaction, was determined to be 0.32 mM (Figure 5). Here α and β are the two parameters utilized for expressing the activation effect of taurocholate by modifying the kinetic parameters K_S and k_{cat}, respectively (Segel, 1975).

The activation of T-BAL by taurocholate in the esterase reaction (Figure 6) was found to be well described by eq 2. Further kinetic analysis indicated that, despite the slightly higher specific activity of T-BAL than that of native enzyme assayed under a standardized assay condition, T-BAL showed a lower βk_{cat} than that of the native enzyme (628 vs 1020 s^{-1}). The lower βk_{cat} of T-BAL can be accounted for by the lower molecular weight of T-BAL vs native BAL (59 270 vs 100 000) utilized in the turnover rate calculation. Thus, the proline-rich sequence region must have contributed to the higher turnover rate of the enzyme, rather than simply contributing to a higher molecular mass for the native enzyme. Also, apparently the catalytic efficiency (k_{cat}/K_S and $\beta k_{cat}/\alpha K_S$) of T-BAL with PANB is much lower than that of native enzyme, which suggests that there must be some perturbation of the active-site pocket by the proline-rich sequence which contributes to the higher activity of native BAL. However, even with the k_{cat} and βk_{cat} being much lower than those of native enzyme, T-BAL still has a higher catalytic efficiency with PANB than with PANA (as seen in Table 1), which is similar to that of the native enzyme (Wang, 1991).

Because the major physiological substrate of BAL is long-chain triacylglycerols, we also examined the comparative aspect of the kinetics of the hydrolysis of trioleoylglycerol. We have shown previously that micellar bile salts can function as fatty acid acceptor (Wang et al., 1988). Since serum albumin is not a fatty acid acceptor for BAL catalysis, it is also a poor fatty acid acceptor in the BAL-catalyzed reaction; thus we included only taurocholate, and not BSA, in the lipase mixture as the fatty acid acceptor. There is an apparent nonsaturable effect of the micellar taurocholate on the activation of T-BAL. This would indicate that the transfer of fatty acid product from the enzyme active site is probably not through a receptor-mediated process but through second-order reaction kinetics involving the enzyme–fatty acid complex and the taurocholate micelles. The apparent saturation of the activation by taurocholate above 60 mM, on the other hand, is likely due to the partial inactivation of the enzyme when the taurocholate concentration is high.

Because functionally active BAL can be produced in the *E. coli* system, it will be possible to use this expression system for large-scale preparation of the recombinant enzyme at a low cost, compared to the mammary tissue culture expression system. Thus, it might be feasible to utilize the recombinant BAL from this bacterial expression system as a dietary supplement if such a recombinant enzyme can be proven to be beneficial to the infants fed with such supplements. For the structure-based functional interpretation of BAL catalysis, the availability of the X-ray crystal structure is essential. Probably due to the presence of microheterogeneity in the carbohydrate moiety, we have not been able to prepare the crystal of the purified native human milk BAL. Because the T-BAL as expressed in the *E. coli* expression system is not glycosylated, such microheterogeneity is not encountered in T-BAL. Thus, it is more suitable for crystallography studies. Currently, the attempt to prepare the T-BAL crystal is in progress in our laboratory.

ACKNOWLEDGMENT

We wish to thank Dr. E. C. Co for performing the refolding experiment in the early phase of this study, Dr. S. Foundling for the useful discussion in devising the strategy for the refolding of T-BAL, and the Molecular Biology Resource Center, University of Oklahoma Health Sciences Center, for performing the N-terminal sequencing analyses and preparing the oligonucleotide primers. We also wish to thank Ms. J. Pilcher for help in the preparation of this manuscript.

REFERENCES

Alemi, B., Hamosh, M., Scanion, J. W., Salzman-Mann, C., & Hamosh, P. (1981) *Pediatrics 68,* 484–489.

Ausubel, F. M., Porent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., & Struhl, K., Eds. (1989) *Current Protocols in Molecular Biology,* Green Publishing Associates and Wiley-Interscience, New York.

Baba, T., Downs, D., Jackson, K. W., Tang, J., & Wang, C.-S. (1991) *Biochemistry 30,* 500–510.

Bevington, P. R. (1969) in *Data Reduction and Error Analysis for the Physical Sciences,* pp 56–65 and 204–246, McGraw-Hill, New York.

Hansson, L., Bläckberg, L., Edlund, M., Lundberg, L., Strömqvist, M., & Hernell, O. (1993) *J. Biol. Chem. 268,* 26692–26698.

Hewick, R. M., Hunkapiller, M. W., Hood, L. E., & Dreyer, W. J. (1981) *J. Biol. Chem. 256,* 7990–7997.

Hui, D. Y., & Kissel, J. A. (1990) *FEBS Lett. 276,* 131–134.

Innis, M. A., & Gelfand, D. H. (1990) in *PCR Protocols. A Guide to Methods and Application* (Innis, M. A., Gelfand, D. H., Shinsky, J. J., & White, T. J., Eds) pp 3–12, Academic Press, New York.

Nilsson, J., Bläckberg, L., Carlsson, P., Enerbäck, S., Hernell, O., & Bjursell, G. (1990) *Eur. J. Biochem. 192,* 543–550.

Nilsson-Ehle, P., & Schotz, M. C. (1976) *J. Lipid Res. 17,* 536–541.

Olivecrona, T., & Bengtsson, G. (1984) in *Lipases* (Borgström, B., & Brockman, H. L., Eds.) pp 206–261, Elsevier, Amsterdam.

Ollis, D. L., Chea, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I., Schrag, J., Sussman, J. L., Verschueren, K. H. G., & Goldman, A. (1992) *Protein Eng. 5,* 197–211.

Reue, K., Zambaux, J., Wong, H., Lee, G., Leete, T. H., Ronk, M., Shively, J. E., Sternby, B., Borgström, B., Ameis, D., & Schotz, M. C. (1991) *J. Lipid Res. 32,* 267–276.

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning, A Laboratory Manual,* 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Segel, I. H. (1975) in *Enzyme Kinetics,* pp 227–231, John Wiley & Sons, New York.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorff, J. W. (1990) *Methods Enzymol. 185,* 60–89.

Sussman, J. L., Harel, M., Frolow, F., Oefner, C., Goldman, A., Toker, L., & Silman, I. (1991) *Science 253,* 872–879.

Wang, C.-S. (1981) *J. Biol. Chem. 256,* 10198–10202.

Wang, C.-S. (1991) *Biochem. J. 279,* 297–302.

Wang, C.-S., & Smith, R. L. (1975) *Anal. Biochem. 63,* 414–417.

Wang, C.-S., & Johnson, K. (1983) *Anal. Biochem. 133,* 457–461.

Wang, C.-S., & Kloer, H. U. (1983) *Biochim. Biophys. Acta 754,* 142–149.

Wang, C.-S., & Hartsuck, J. A. (1993) *Biochim. Biophys. Acta 1166,* 1–19.

Wang, C.-S., Martindale, M. E., King, M. M., & Tang, J. (1989) *Am. J. Clin. Nutr. 49,* 457–463.