Structural Studies of *Escherichia coli* UDP-N-Acetylmuramate: L-Alanine Ligase

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**ABSTRACT:** Uridine diphosphate N-acetylmuramate: L-alanine ligase (EC 6.3.2.8, UNAM: L-Ala ligase or MurC gene product) adds the first amino acid to the sugar moiety of the peptidoglycan precursor, catalyzing one of the essential steps in cell wall biosynthesis for both gram-positive and gram-negative bacteria. Here, we report our studies on the secondary and quaternary structures of UNAM: L-Ala ligase from *Escherichia coli*. The molecular weight of the purified recombinant enzyme determined by electrospray ionization mass spectrometry agreed well with the molecular weight deduced from the DNA sequence. Through sedimentation equilibrium analysis, we show that the enzyme exists in equilibrium between monomeric and dimeric forms and that the dissociation constant of the dimer, $K_d$, was determined to be 1.1 $\pm$ 0.4 $\mu$M at 37 °C and 0.58 $\pm$ 0.30 $\mu$M at 4 °C. A very similar $K_d$ value was also obtained at 37 °C by gel filtration chromatography. The secondary structure of the enzyme was characterized by circular dichroism spectroscopy. No change in the secondary structure was observed between the monomeric and dimeric forms of the enzyme. The activity assays at enzyme concentrations both below and above the determined $K_d$ value lead to the conclusion that the enzyme is active both as dimers and as monomers and that the specific activity is independent of the oligomerization state.

In both gram-positive and gram-negative bacterial organisms, there exists a peptidoglycan layer that is part of the cell wall. The precursor for the peptidoglycan layer, UDP-N-acetylmuramate pentapeptide, is synthesized intracellularly by the Mur enzyme family (van Heijenoort, 1994). Uridine diphosphate N-acetylmuramate: L-alanine ligase (EC 6.3.2.8, UNAM: L-Ala ligase or MurC gene product) adds the first of five amino acids to UNAM (See Figure 1), catalyzing one of the essential steps in cell wall biosynthesis for all bacteria. Among many antibacterial approaches, interfering with the bacterial cell wall formation has been most effective. UNAM: L-Ala ligase, therefore, may be a novel target for antibiotic development.

Several Mur enzymes have been extensively studied. These include the enzymes involved in bacterial 3-nalamine metabolism (Wright & Walsh, 1992), the MurA gene product (Marquardt et al., 1994), and the MurB gene product (Benson et al., 1995; Dhalla et al., 1995). Relatively little has been known about the MurC gene product, UNAM: L-Ala ligase. The enzyme was partially purified first from *Bacillus subtilis* and *Bacillus cereus* (Hishinuma et al., 1971) and was purified close to homogeneity later from *Staphylococcus aureus* (Mizuno et al., 1973). The DNA sequences of the MurC gene from *Escherichia coli* (Ikeda et al., 1990) and from *Porphyromonas gingivalis* were reported (Ansai et al., 1995). Only recently was overexpression of the *E. coli* enzyme reported (Liger et al., 1995; Falk et al., 1996).

Though some basic characteristics of the enzyme have been reported (Mizuno et al., 1973; Liger et al., 1995; Falk et al., 1996), no structural information on the enzyme is available. In particular, the secondary and quaternary structures are not known. We report here secondary and quaternary structural studies on the purified recombinant enzyme. We present CD data on the enzyme and discuss the oligomerization state of the enzyme and its relationship to the catalytic activity. In particular, we address the following questions: (1) What is the predicted secondary structure? (2) What is the oligomerization state of the enzyme? (3) If there exist oligomers of the enzyme, what

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* To whom correspondence should be addressed.  ‡ Division of Macromolecular Structure, Princeton, NJ.  ‡ Analytical Research and Development, Princeton, NJ.  ‡ Department of Microbiology (104), Wallingford, CT.  © Abstract published in *Advances ACS Abstracts*, January 15, 1996.  1 Abbreviations: UNAM, uridine diphosphate N-acetylmuramate; UNAG, uridine diphosphate N-acetylglucosamine; UNAGEP, uridine diphosphate N-acetylglucosamine enolpyruvate; IPTG, isopropyl β-D-thiogalactopyranoside; ADP, adenosine diphosphate; ATP, adenosine 5′-triphosphate; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; NADH, β-nicotinamide adenine dinucleotide, reduced form; CD, circular dichroism; DTT, dithiothreitol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TEAB, triethylammonium bicarbonate; DEAE, diethylaminoethyl; NADP+, β-nicotinamide adenine dinucleotide phosphate; BSA, bovine serum albumin.
MATERIALS AND METHODS

Materials. Factor Xa and amylase affinity resin were from New England BioLabs. The Resource Q column was from Pharmacia. The Hi-Pore 318 reverse phase column and the protein dye binding assay were from Bio-Rad. The PacDiol 200 column was purchased from YMC (Wilmington, NC). Pefabloc, IPTG, pyruvate kinase, and lactate dehydrogenase were from Boehringer Mannheim. Mini-gels were from Novex. All other reagents, unless specified, were from Sigma and were of the highest reagent purity available.

Purification of the Recombinant UDP-N-Acetylmuramate: L-Alanine Ligase. The expression and overproduction of the enzyme will be reported elsewhere (Falk et al., 1996). The purification of the enzyme in this report is similar to the reported procedure, but with several modifications. Briefly, cell paste from 4 L of cell growth was thawed and diluted to a volume of 200 mL in lysis buffer containing 20 mM Tris, pH 8.0, and 200 mM KCl. The cells were lysed by sonication in a sonication horn using a Branson Sonifier 450 for 16 one-minute cycles at 50% power level with one-minute intervals between sonication cycles. The resulting lysates were clarified by centrifugation at 10 000 × g for 30 min at 4 °C. The supernatant was collected and passed over a 5 cm × 10 cm column containing 200 mL of amylase resin equilibrated in lysis buffer. After the column was washed with 200 mL of lysis buffer, the bound fusion protein was eluted with 100 mL of lysis buffer containing 10 mM maltose. Fractions containing fusion protein were pooled and were concentrated to 20 mL of 16 mg of protein/mL with an Amicon ultrafiltration cell using a YM10 filter under nitrogen pressure. Factor Xa protease was added to a final ratio of 250 µg/160 mg of fusion protein and was incubated with fusion protein for 72 h. Cleavage of maltose binding protein from UNAM:L-Ala ligase was assessed by SDS-PAGE on a 4%–20% gradient mini-gel. The protein solution was then dialyzed against 2 L of 20 mM Tris, pH 8.0. Maltose binding protein was separated from UNAM:L-Ala ligase by anion exchange chromatography on a 6 mL Pharmacia Resource Q column connected to a BioCAD liquid chromatography system (PerSeptive Biosystems, Cambridge, MA). The column was equilibrated in 20 mM Tris, pH 8.0, and injections of about 75 mg of fusion digest were made. Maltose binding protein was first eluted after injection while the column was re-equilibrated in 20 mM Tris, pH 8.0, and 100 mM KCl. UNAM:L-Ala ligase was then eluted with a gradient of KCl running from 100 mM to 300 mM over 18 min at a flow rate of 5 mL/min. In case of incomplete proteolysis of the fusion protein by Factor Xa, the uncleaved fusion protein, which tends to co-elute with UNAM:L-Ala ligase, was removed by passing the collected fractions through the amylase resin a second time. Enzyme from the anion exchange step was concentrated and dialyzed against storage buffer containing 20 mM Tris, 50 mM (NH₄)₂SO₄, 1 mM β-mercaptoethanol, and 20% glycerol and was then stored either at 4 or –20 °C. About 110 mg of homogeneous UNAM:L-Ala ligase was typically recovered. The highest specific activity of purified enzyme from several purifications was 1151 units/mg. One unit of activity corresponds to 1 µmol of ADP formed per hour.

Purification of UDP-N-Acetylglucosamine Enolpyruvyl Transferase and UDP-N-Acetylenolphosphorylglucosamine Reductase. The purification procedure for UNAM:L-Ala ligase up through the affinity step was used to purify the MurA gene product, UDP-N-acetylenolphosphorylglucosamine transferase fused with maltose binding protein or the MurB gene product, UDP-N-acetylenolphosphorylglucosamine reductase fused with maltose binding protein, as reported previously (Dhalla et al., 1995). Purified fusion protein for the MurA gene product was stored at –80 °C in 20% glycerol, while purified fusion protein for the MurB gene product was stored at –20 °C in 20% glycerol. They were used for synthetic reactions as an intact fusion protein, and no digestion to remove maltose binding protein was performed.

Liquid Chromatography/Mass Spectrometry. A Finnigan MAT TSQ 7000 (San Jose, CA) triple-stage quadrupole mass spectrometer equipped with a Finnigan MAT electrospray ionization (ESI) source was used for all mass spectrometric studies. All data was acquired in profile mode (10 data points per m/z unit) using positive ion detection. The mass spectrometer was scanned from m/z 500 to 2000 in 5 s in QIMS mode. Unit resolution was employed across the entire mass range. The instrument m/z calibration was performed daily using a mixture of polypropylene glycols (PPG 1000 and PPG 2000, 0.2 mg/mL each) in 80:20 methanol:water containing 10 mM ammonium acetate. Mass spectrometric data was processed with the Finnigan MAT ICIS software (version 8.1). Multiply charged ESI mass spectra were transformed using the BIOMASS deconvolution algorithm to determine the protein molecular weights. Several scans were averaged across the LC/MS peaks before applying the deconvolution.

Since samples analyzed contained high salt concentrations (e.g., typically 20 mM Tris, 250 mM KCl), on-line liquid chromatography was used as a means of effectively desalting the samples for compatibility with electrospray ionization mass spectrometry. A Michrom UMA microbore HPLC (Michrom BioResources, Auburn, CA) was used and was operated at a flow rate of 20 µL/min. The proteins were eluted using a linear gradient from 20% solvent B to 90% solvent B in 20 min, where solvent A contained 100 mM ammonium acetate:water containing 10 mM ammonium acetate. Mass spectrometric data was processed with the Finnigan MAT ICIS software (version 8.1). Multiply charged ESI mass spectra were transformed using the BIOMASS deconvolution algorithm to determine the protein molecular weights. Several scans were averaged across the LC/MS peaks before applying the deconvolution.

Synthesis and Purification of UDP-N-Acetylmuramate. The substrate for UNAM:L-Ala ligase was prepared by a two-step coupled enzymatic conversion of UNAG to UNAM. Reactions contained, in a total volume of 80 mL, 100 mM Tris, pH 8.0, 1 mM DTT, 20 mM UNAG, 30 mM NADPH, 30 mM phosphoenolpyruvate, 4 mg of MurA fusion protein, and 2.5 mg of MurB fusion protein. The reaction was continued at 25 °C and was more than 90% complete after 48 h, as determined by HPLC. A 5–25 µL amount of 1:100 diluted reaction product was injected onto a Bio-Rad HiPore reverse phase R318 column (250 mm × 4.6 mm) with an isocratic flow of 2.25 mL/min of 200 mM ammonium formate, pH 3.3. The retention times of UNAG, UNAGEP, UNAM, and NADP⁺ were 1.4, 1.8, 2.6, and 3.0 min, respectively. Enzymes were removed by ultrafiltration,
and products were separated from reactants by anion exchange chromatography on a 20 mm × 600 mm DEAE cellulose column. The column was equilibrated with 10 mM TEAB, pH 9, and then the filtered reaction products were loaded onto the column. The column was eluted with a TEAB gradient running at 5 ml/min from 10 to 110 mM for 700 mM, followed by an isocratic flow at 300 mM for 300 mL. Purity of the collected fractions was determined by HPLC before the fractions were pooled together. Purified UNAM was lyophilized three times to remove TEAB. The purified product was finally dissolved in water with a final concentration of 20–50 mM. The negative ion mass spectrometry gave a molecular weight of 679. Solutions of UNAM were quantitated by measuring the absorbance at 262 nm and using an extinction coefficient of $\varepsilon_{262} = 100$ cm$^{-1}$ M$^{-1}$ for UDP.

**Enzyme Assay.** Enzyme activity was measured by detecting formation of ADP in an enzyme reaction mixture. All assays were performed at 37 °C, except when the enzyme activity was measured as a function of the enzyme concentration at 4 °C. The standard conditions contained, in a final volume of 1 mL, 100 mM Tris, pH 8.0, 70 µg of pyruvate kinase, 70 µg of lactate dehydrogenase, 1 mM DTT, 0.38 mM NADH, 2 mM phosphoenolpyruvate, 20 mM MgCl$_2$, 25 mM (NH$_4$)$_2$SO$_4$, 2.5 mM β-mercaptoethanol, 5 mM ATP, 1 mM l-Ala, 1 mM UNAM, and 1.5 µg of UNAM:l-Ala ligase. Reaction mixtures without ATP and the ligase were incubated at 37 °C for 10 min before ATP was added and were incubated for another minute. Reactions were then started by addition of the ligase. The decrease in NADH absorbance at 340 nm was monitored on a Cary 3E spectrophotometer, and activities were determined from the linear portion of the progress curve within the first minute after addition of the ligase. The slope of the initial portion of the curve was calculated by linear least-squares analysis. An extinction coefficient of 6 220 cm$^{-1}$ was used to calculate the specific activity of the enzyme. One unit of activity corresponds to 1 µmol of ADP formed per hour. For the measurement at 4 °C, the assay mixture was the same except that 700 µg of pyruvate kinase and 700 µg of lactate dehydrogenase were used in a final volume of 1 mL.

**Determination of Extinction Coefficients.** The absorbance readings at 280 and 235 nm were measured as a function of concentration on a sample of the intact protein using 1 cm path length quartz cuvettes. Protein concentrations were determined by amino acid analysis. Routine measurements of the enzyme concentration were made with the dye binding assay from Bio-Rad using BSA as reference with the appropriate correction factor. A molecular weight of 54 486, based on the sum of the enzyme polypeptide and the eight amino acids (ISEFGSSR) N-terminal linker, was used for conversions of protein concentrations to molar units.

**Analytical Ultracentrifugation.** All sedimentation equilibrium experiments were performed in a Beckman XL-A using an AN 60 Ti rotor and three sample cells with six channel centripieces. The samples were prepared by dialyzing the enzyme either in 20 mM Tris, pH 8.0, and 100 mM KC1 or in 10 mM sodium phosphate, pH 8.0, and 100 mM NaF before dilution with the final dialysate to the desired concentrations. UNAM:l-Ala ligase was permitted to come to equilibrium at seven concentrations and three speeds. In the presence of individual substrates, enzyme samples at a loading concentration of 0.6 mg/mL were allowed to reach equilibrium at the same three speeds in separate runs. When samples contained individual substrates, at least one control sample that contained no substrate and reference samples with identical substrate concentration were also included in the same runs. Samples were equilibrated at either 4 or 37 °C for 24 h at 12 000 rpm, and duplicate scans 3 h apart were overlaid to ascertain that there were no further changes in the sample cell. The samples were then equilibrated at 16 000 rpm for the second data set and 20 000 rpm for the third data set. Concentration profiles were measured with optical absorbance at either 235, 280, or 295 nm, depending on the samples, with a step size of 0.001 cm. For experiments involving ATP or UNAM, the absorbance at 295 nm was used and 50 or 99 scans were averaged. For other samples, an average of 20 or 40 scans was taken and the absorbance at 280 nm was measured, except where the absorbance at 235 nm was taken for those samples with loading concentration less than 0.2 mg/mL.

Data analysis software running under Igor (WaveMatrics, Lake Oswego, OR) and incorporating a previously published nonlinear fitting algorithm (Johnson et al., 1981) was a generous gift from Dr. Preston Hensley at Smithkline Beecham, King of Prussia, PA. The equations and standard notation from the publication (Johnson et al., 1981) were followed. Unless specified otherwise, equations from Johnson et al. (1981) will be referred in the figures and tables listed below. The partial specific volume of UNAM:l-Ala ligase, 0.7362, was calculated by the method of Cohn and Edsall using partial specific volumes of amino acids as previously published (Laue et al., 1992). Extinction coefficients of $\varepsilon_{260} = 26 851$ cm$^{-1}$ M$^{-1}$ and $\varepsilon_{235} = 106 115$ cm$^{-1}$ M$^{-1}$ were used to convert dissociation constants into molar units.

**Gel Filtration Chromatography.** Measurements were made on a Hewlett-Packard 1090 HPLC system and a YMC PacDiol 200 HPLC column at 37 °C. The absorbance at 220 nm and the fluorescence signal at 350 nm with the excitation wavelength at 280 nm were used to monitor the elution of both UNAM: l-Ala ligase and molecular weight marker proteins. These molecular marker proteins included cytochrome C (12.4 kDa), myoglobin (17 kDa), carbonic anhydrase (29 kDa), ovalbumin (46 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa), and amylase (200 kDa). The elution of UNAM:l-Ala ligase was also monitored by the intrinsic fluorescence emission of the enzyme. Typically, 25 µL of protein was injected in each 20 min run with a flow rate of 0.5 mL/min. UNAM:l-Ala ligase samples were prepared the same way as those used for sedimentation equilibrium experiments. The dialysis buffer, 10 mM sodium phosphate, pH 8.0, and 100 mM NaF, was used both as the mobile phase and as dialution buffer. A best fit of the logarithm of the molecular weight of the marker proteins versus their retention times was used to estimate the molecular weight of UNAM:l-Ala ligase.

Analysis of the concentration dependence of the enzyme’s molecular weight was similar to that described previously (Fairman et al., 1995). Data were fitted by nonlinear least-squares methods to a monomer–dimer equilibrium model using a PC version of the program MLAB (Civilised Software, Bethesda, MD). The function used for fitting is

$$M_{\text{obs}} = M_{\text{mon}}[\text{Monomer}]/[P_{\text{tot}}] + 2M_{\text{dimer}}[\text{Monomer}]^2/K_d[P_{\text{tot}}]$$ (1)
where MW_{obs}, M_{mon}, M_{dim}, [Monomer], K, and [P_{tot}] are the observed molecular weight, monomeric molecular weight, dimeric molecular weight, monomer concentration, dissociation constant, and total concentration of the enzyme (an independent variable), respectively. M_{mon} was fixed at 54,486 during the fit.

Circular Dichroism Spectroscopy. All CD spectra were collected on an AVIV 62DS circular dichroism spectrometer (Lakewood, NJ). At 25 °C, spectra of the samples were collected in 25 mM potassium phosphate, pH 7.0. At 37 °C, the samples were prepared the same way as those used for both the sedimentation equilibrium and gel filtration experiments.

RESULTS

Mass Spectrometry. The purified recombinant UNAM: L-Ala ligase was electrophoretically homogenous as determined by SDS–PAGE and isoelectric focusing electrophoresis gel (pI = 6.0). The molecular weight determined from electrospray ionization mass spectrometry (Figure 2) was 54,486 Da. This is identical to the calculated molecular weight of the MurC gene product plus an amino acid linker (ISFGSSR) which is still attached to the gene product after proteolysis of the fusion protein. This indicates that the MurC gene was correctly expressed with the predicted sequence.

No Disulfide Bond. There are two cysteiny1 residues in each subunit, and they may or may not form disulfide bonds, either intra- or inter-subunits. It was found that both cysteines were present as free thiols as judged by the Ellman assay (Creighton, 1989) using the reagent DTNB. The average number of free thiols obtained from several experiments was 1.58 ± 0.14 per monomer of UNAM: L-Ala ligase.

This suggests that neither intra- nor inter-subunit disulfide bonds are likely. While the average number of free thiols is lower than the expected value of 2.0, it is possible that the cysteines undergo partial air oxidation during the assay since the assay was carried out in 0.1 M Tris, pH 8.3, and 6 M guanidine hydrochloride, in the absence of reducing agent. A similar number of thiols, 1.7 per monomer, was also obtained for the fully reduced enzyme.

In addition, electrospray ionization mass spectra of the enzyme gave no indication of a disulfide-linked dimer. No detectable dimer component was observed in electrospray ionization mass spectra of the native enzyme. Identical spectra were also obtained for the enzyme in the presence of 4 M guanidine hydrochloride.

Secondary Structure of the Enzyme. A CD spectrum for a 2.6 µM sample of UNAM: L-Ala ligase was collected at 25 °C (Figure 3). The CD spectrum was analyzed for secondary structure content using the singular value decomposition method (Hennessey et al., 1981) in conjunction with variable selection (Manavalan & Johnson, 1987) (see Table 1). This method takes advantage of a large basis set (33 proteins) containing proteins whose CD spectra penetrate down to 175 nm affording additional spectral information. To test the accuracy of this method, we compared the CD deconvolution to the method described by Sreerama and Woody (1993), using either a subset of the Johnson basis set (Manavalan & Johnson, 1987), the Kabsch and Sander basis set (1983), or the Kabsch and Sander basis set with the poly(Pro)II helix as an additional fitting parameter (1994). There is good agreement among these methods. The biggest difference in secondary structure prediction appears to be in the assignment of the β-turn and random coil components between the Johnson basis set and the Kabsch and Sander basis set. The inclusion of the poly(Pro)II helix can often account for a significant fraction of the random coil component in many CD deconvolutions. When the spectrum for this enzyme was evaluated, however, the random coil component did not change upon inclusion of this term.

No appreciable difference in CD spectra was observed when 0.16 mM L-Ala or 0.89 mM UNAM was added to the
enzyme. While the presence of excess amount of ATP masked the CD signal of the enzyme, the addition of 63 µM ATP to the enzyme in a tandem spectrophotometer cell did not alter the CD spectrum. Therefore, none of the individual substrates at the added concentrations induced any observable change in secondary structure.

CD spectra were measured at 37 °C to more easily look for difference in secondary structure between the monomeric and dimeric enzyme. No appreciable difference in CD spectra was observed when the enzyme concentration was varied from 10 to 0.36 µM.

Sedimentation Equilibrium Analysis. The native molecular weight and quaternary structure of the enzyme were studied by sedimentation equilibrium analysis. It was found that the enzyme exists in equilibrium between monomers and dimers. The dissociation constant of the dimer was determined at 4°C by sedimentation equilibrium analysis. It was found that the protein concentration and speed were obtained by fixing the monomeric molecular weight at 54 486. Unless specified otherwise, all equations referred in the figures and tables are from Johnson et al. (1981).

Table 1: Estimation of the Secondary Structure Content of UDP-N-Acetylmuramate: L-Alanine Ligase

<table>
<thead>
<tr>
<th>secondary structure</th>
<th>Johnson</th>
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<tr>
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<tr>
<td>poly(Pro)II helix</td>
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Figure 4: Typical sedimentation equilibrium data and fit for UDP-N-Acetylmuramate: L-Alanine ligase. Experimental conditions were as follows: sample concentration, 11 µM; rotation speed, 12 000 rpm; temperature, 4 °C; wavelength, 280 nm; scanning step, 0.001 cm; and 20 averages. The data were fitted using eq 2 and the monomeric molecular weight of 54 486. Unless specified otherwise, all equations referred in the figures and tables are from Johnson et al. (1981).

Table 2: Concentration and Speed Dependence of Apparent Molecular Weight of UDP-N-Acetylmuramate: L-Alanine Ligase

<table>
<thead>
<tr>
<th>speed (rpm)</th>
<th>wavelength (nm)</th>
<th>temp (°C)</th>
<th>[protein] (µM)</th>
<th>MW (kDa)</th>
<th>n-mer (%)</th>
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</thead>
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<td>12 000</td>
<td>280</td>
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<tr>
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<tr>
<td>20 000</td>
<td>280</td>
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<td>93 ± 1</td>
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<td>4</td>
<td>3.7</td>
<td>79 ± 4</td>
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</table>

* Concentration of protein loaded in centrifuge cells at the beginning of the experiment. * Molecular weights were obtained by fitting to eq 2, and only two significant digits were kept. (The error estimate represents the 65% confidence interval.) * Values for the n-mer at each protein concentration and speed were obtained by fixing the monomeric molecular weight at 54 486.

These variations are consistent with a mass action association, and are characteristic of a self-associating protein. The experimental data were fitted to self-associating models of monomer—dimer, monomer—trimer, and monomer—tetramer. Among the models examined, the monomer—dimer equilibrium provided the least χ² value, which was significantly smaller than any other oligomerization models (Table 3). On the basis of the observations it was concluded that there exists an equilibrium between dimeric and monomeric forms of the enzyme.

It is possible that the residuals can be further reduced by considering models with three or more species. However, the observation of a random distribution of residuals between the observed data and the fit based on monomer—dimer equilibrium suggests either that no additional oligomerization state exists or that the dissociation constant of any additional
oligomerization state is much bigger than 11 \mu M, the experimental concentration.

(B) Dissociation Constant. In addition to the apparent molecular weight, the dissociation constant of a self-associating system can be obtained from sedimentation equilibrium data. These data may be analyzed by eq 3 with the fixed monomeric molecular weight of 54,486. (The error estimate represents the 65% confidence interval.)

In the present case, the concentration traces was obtained at 12,000 rpm and at 4 °C. The \chi^2 values were summations of the squared difference between the observed and fitted values. The fitted values were generated by fitting eq 2 for single species or fitting eq 3 for monomer–n-mer equilibrium with the fixed monomeric molecular weight of 54,486. (The error estimate represents the 65% confidence interval.)

Concentration of protein loaded in centrifuge cells at the beginning of the experiment.

In this study, data at three different rotor speeds for each sample were merged and fitted to obtain either the apparent molecular weight or dissociation constant for the dimer, as illustrated in Figure 5. The monomer–dimer model correctly described this three-speed data set as judged by the distribution of residuals. Table 4 contains the fitted parameters determined from multiple data set analysis at seven concentra-

trations of the enzyme. As expected for a self-associating protein, the apparent molecular weight decreased as the loading concentration was decreased. When the loading concentration was varied from 11 to 0.27 \mu M—a 40-fold decrease—the value of \( K_d \) for the enzyme remained in the range 0.22–0.96 \mu M at 4 °C and remained in the range 0.70–1.7 \mu M at 37 °C. The average value of the dissociation constant is 0.58 ± 0.36 \mu M at 4 °C and 1.1 ± 0.4 \mu M at 37 °C. Samples in different buffers gave essentially identical dissociation constant.

(C) Effect of Substrates on the Oligomerization State of the Enzyme. To examine if the presence of substrates changes the oligomerization state of the enzyme, the effect of individual substrates was studied through sedimentation equilibrium experiments at both 4 and 37 °C. In the absence and presence of individual substrates, the molecular weight

| Table 3: Typical List of \( \chi^2 \) between the Observed and Fitted Concentration Trace for UDP-N-acetylmuramate: L-alanine Ligase
<table>
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<tr>
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</tr>
</tbody>
</table>

\* In the present case, the concentration traces was obtained at 12,000 rpm and at 4 °C. \* \chi^2 values were summations of the squared difference between the observed and fitted values.

Table 4: Apparent Molecular Weight, MW, and Dissociation Constant of the Dimer, \( K_d \), Obtained from the Multispeed Analysis of Sedimentation Equilibrium Data on UDP-N-Acetylmuramate: L-alanine Ligase

<table>
<thead>
<tr>
<th>[protein] (\mu M)</th>
<th>wavelength (nm)</th>
<th>MW (kDa)</th>
<th>( K_d ) (\mu M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>280</td>
<td>97 ± 1</td>
<td>94 ± 1</td>
</tr>
<tr>
<td>7.3</td>
<td>280</td>
<td>96 ± 1</td>
<td>92 ± 1</td>
</tr>
<tr>
<td>3.7</td>
<td>280</td>
<td>95 ± 2</td>
<td>90 ± 3</td>
</tr>
<tr>
<td>1.8</td>
<td>280</td>
<td>95 ± 3</td>
<td>80 ± 3</td>
</tr>
<tr>
<td>1.8</td>
<td>235</td>
<td>96 ± 1</td>
<td>89.4 ± 1.3</td>
</tr>
<tr>
<td>0.92</td>
<td>235</td>
<td>93 ± 1</td>
<td>80.7 ± 1.9</td>
</tr>
<tr>
<td>0.55</td>
<td>235</td>
<td>81 ± 1</td>
<td>0.96 ± 0.17</td>
</tr>
<tr>
<td>0.27</td>
<td>235</td>
<td>79 ± 3</td>
<td>0.55 ± 0.20</td>
</tr>
</tbody>
</table>

\* Concentration of protein loaded in centrifuge cells at the beginning of the experiment. \* Molecular weights were obtained by fitting to eq 2. (The error estimate represents the 65% confidence interval.) \* Values for the \( K_d \) were obtained by fitting to eq 3 with the fixed molecular weight of 54,486. \* Extinction coefficients used were 26,851 cm⁻¹ M⁻¹ at 280 nm and 106,000 cm⁻¹ M⁻¹ at 235 nm.
of the enzyme was determined and compared. The substrates were added to 11 μM enzyme to a concentration of 0.5 mM for L-Ala, 0.25 mM for UNAM, and 0.4 mM for ATP. These concentrations are significantly higher than their \( K_m \) values, which are 41, 57, and 140 μM at 37 °C for L-Ala, UNAM, and ATP, respectively (J. J. Emanuele et al., unpublished data). The absorbance was measured at 280 nm with L-Ala and at 295 nm with either UNAM or ATP. The detection at 295 nm was necessary because the substrates themselves had appreciable absorbance at 280 nm. Within the experimental errors, the presence of individual substrates did not change the apparent molecular weight of the enzyme at either temperature. Therefore, individual substrates imposed little or no effect on the enzyme’s oligomerization state.

**Gel Filtration Chromatography.** The monomer–dimer equilibrium was also studied by gel filtration experiments at 37 °C. The dissociation constant of the dimers determined from the experiments is consistent with the value obtained from sedimentation equilibrium analysis.

Experiments were performed at 37 °C with a sample concentration ranging from 0.20 μM to 0.20 mM. The chromatograms measured by optical absorption at 220 nm or by fluorescence were examined and compared in order to determine the peak position for the enzyme. As can be seen from the optical absorbance chromatograms (Figure 6), the retention time of the enzyme increased from 8.38 min to about 9.59 min as the sample concentration decreased from 0.20 mM to 0.20 μM.

The retention time was also obtained at 37 °C for the seven proteins with known molecular weight. These retention times were plotted as a function of the logarithmic of the molecular weight. The apparent molecular weights of UNAM:L-Ala ligase at different concentrations were determined by comparing the observed retention times to the linear fitted function. The distribution of molecular weight as a function of sample concentration was then fitted to eq 1 of this manuscript. The molecular weight values were obtained by comparing the retention times in Figure 6 to the retention times of molecular weight standard proteins (see Methods and Materials for further details). The fit was generated with the monomer molecular weight fixed to 54,486.

![Figure 6: Gel filtration spectra for UDP-N-acetylmutarate:L-alanine ligase at 37 °C. Experimental conditions were as follows: injected sample concentrations (from top), 0.2 mM, 50 μM, 20 μM, 10 μM, 2 μM, 1 μM, 400 nM, and 200 nM; detecting wavelength, 220 nm; mobile phase, 10 mM sodium phosphate, pH 8.0, and 100 mM NaF; flow rate, 0.5 mL/min.](image)

**DISCUSSION**

Sedimentation equilibrium analysis provides one of the most powerful and thermodynamically rigorous methods to determine the native molecular weight in dilute solution (Hansen et al., 1994; Laue & Rhodes, 1990; Yphantis, 1964). In contrast to gel filtration chromatography, it does not require the use of molecular weight standards. The parameters involved are either easily measured or easily estimated. This enables precise determination of the molecular weight for samples of interest. The molecular weights of UNAM:L-Ala ligase were determined at multiple speeds and at multiple concentrations. These data clearly demonstrated that UNAM:L-Ala ligase exists in equilibrium between monomeric and dimeric states.

Sedimentation equilibrium analysis also provides accurate determination of dissociation constant(s). In the present study, the accuracy is demonstrated by the consistency of the dissociation constants determined from global fits at
multiple sample concentrations. In principle, a dissociation constant, $K_d$, can be determined with a single loading concentration of the enzyme. Analysis at multiple loading concentrations enables verification of the determined $K_d$. Consistency of $K_d$ values over a wide range of loading concentrations ensures accuracy of the determined parameter. For UNAM:1-Ala ligase, the $K_d$ value of the dimer was determined at five to seven enzyme concentrations. These determined values fall into a narrow range around 1.1 $\mu$M at 4 °C and around 0.58 $\mu$M at 37 °C. This is a good indication that the average values of $K_d$ are reasonably correct. Concentration dependence of the observed molecular weight is also consistent with the determined $K_d$ values. For a monomer–dimer system, the apparent molecular weight decreases as the protein concentration is lowered. At protein concentrations comparable to $K_d$, the observed molecular weight is expected to be around 50%–80% of the expected value for the dimeric protein. Indeed, that is what was observed for this enzyme at both 4 and 37 °C.

A self-associating system can also be studied with gel filtration chromatography. It is one of the simplest and useful methods to determine the molecular weight of a protein, if the protein of interest possesses the same molecular shape as the proteins used as molecular weight markers. In the present case, the observed concentration dependence of the retention time of the enzyme enabled the determination of the $K_d$ value. As expected for a rapid monomer–dimer equilibrium, a single peak was observed. It is remarkable that the $K_d$ value determined from the gel filtration data, 1.4 ± 0.4 $\mu$M, is very close to the average $K_d$ value determined from sedimentation equilibrium analysis, 1.1 ± 0.4 $\mu$M. The close correspondence of the $K_d$ values further validates the conclusions drawn from sedimentation equilibrium analysis.

On the other hand, the association of monomers is not through disulfide bond(s). There are two cysteinyl residues in each subunit, and both cysteines are present as free thiols as judged by the Ellman assay. This suggests that neither intra- nor inter-subunit disulfide bonds are likely. In addition, electrospray ionization mass spectra of the enzyme gave no indication of a disulfide-linked dimer. Other experimental data are not consistent with the formation of inter-subunit disulfide bonds, either. If there exists an inter-subunit disulfide bond, the presence of reducing agent is expected to change observed molecular weight of the enzyme. In this study, the presence of reducing agent made no difference to the outcome of sedimentation equilibrium or electrophoresis experiments. In the presence or absence of DTT, sedimentation equilibrium data yielded the same molecular weight. Denaturing electrophoreses gels of the enzyme, with or without DTT in the running buffer, showed a single band at about 55 kDa, which is the expected molecular weight of a single subunit.

What is the relationship between the oligomerization state and catalytic activity of the enzyme? At 37 °C, the enzyme concentration used in the enzyme activity assay was typically around 1 mg/mL or about 20 nM. This is below the $K_d$ of the dimers at this temperature, which means that the enzyme exists predominantly as monomers. The enzyme, therefore, must be active as monomers. At concentrations higher than the $K_d$ value, the enzyme activity assay at 37 °C was not feasible because the product formation was too fast for the coupled enzyme assay. At 4 °C, the enzyme specific activity did not vary with the enzyme concentration, below or above the $K_d$ value. This means that both dimers and monomers are catalytically active and that the catalytic activity is independent of the quaternary structure of the enzyme. It can be argued that certain oligomerization states can only be found in the presence of all three substrates. Since no change, however, was observed in the presence of individual substrates, this seems unlikely.

The CD spectra did not change with the enzyme concentration, below or above the $K_d$ value, either. Therefore, there is no appreciable secondary structural change accompanying the changes in oligomerization state of the enzyme. This suggests that both dimer and monomer give rise to an identical CD spectrum.

Although both dimers and monomers of the enzyme are catalytically active, the physiological implication(s) of the dimerization is not understood. It is possible that the dimerization may stabilize the enzyme or that the dimerization may facilitate catalysis in the cell. Not enough information is available to address this issue yet.

In conclusion, through sedimentation equilibrium analysis, the quaternary structure of UNAM:1-Ala ligase has been characterized as an monomer–dimer equilibrium. The dissociation constant of the dimer was determined. It was found that the enzyme was active both as dimers and as monomers and that the specific activity is independent of the oligomerization state of the enzyme.

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REFERENCES


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