Valyl-tRNA synthetase from *Bacillus stearothermophilus*. Purification and Binding with the Substrates L-Valine and ATP†

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Valyl-tRNA synthetase (L-valine : tRNA\textsuperscript{val} ligase (AMP forming); EC 6.1.1.9) (VRS) was purified from *Bacillus stearothermophilus* NCA 1503 by streptomycin treatment, ammonium sulfate fractionation, and batch adsorption on DEAE-Sephadex A-50, then column chromatographies on hydroxylapatite, DEAE-Toyopearl 650S, BUTYL-Toyopearl 650S, and Sephacryl S-300 superfine. The enzyme preparation, purified approximately 1100-fold, was homogeneous on polyacrylamide gel disc electrophoresis and consisted of a single chain polypeptide. The molecular weight was about 100,000 and 110,000 by SDS-polyacrylamide gel electrophoresis and Sephacryl S-300 gel filtration, respectively. Enzyme activity in tRNA aminocacylation reaction increased as the temperature increased up to 52°C at pH 7.6 and pH 8.5. pH-optima of the aminocacylation activity were 8.5 at 30°C and 8.0 at 52°C under our conditions.

Ligand-induced decrease of the enzyme protein fluorescence was observed and used as a probe for studying the binding of substrates (L-valine and ATP) to VRS. Substrate bindings were also studied by equilibrium dialysis; two moles of L-valine or ATP were bound to a mole of enzyme when each substrate was examined separately. These two binding equilibria of L-valine or ATP seemed equivalent when each substrate is bound alone.

Aminoacyl-tRNA synthetases attach an amino acid to the 3' terminus of tRNA, generally according to the following reaction scheme\textsuperscript{11} (Eqs. (1) and (2)).

\[
\begin{align*}
\text{AA} + \text{ATP} + \text{E} & \rightleftharpoons \text{E.AA-AMP+PPi} \\
\text{E.AA-AMP+tRNA} & \rightleftharpoons \text{AA-tRNA} + \text{AMP+E}
\end{align*}
\]

where AA denotes an amino acid; E, the enzyme; and PPI, inorganic pyrophosphate. The aminoacylation reaction requires high specificity and plays a key role in the fidelity of gene expression. Accordingly our primary interest in the aminoacyl-tRNA synthetases resides in their high specificity in discriminating between many homologous compounds related to the substrates: amino acids, nucleotides, and tRNA's. Valyl-tRNA synthetase (L-valine : tRNA\textsuperscript{val} ligase (AMP forming); EC 6.1.1.9) (VRS) of *E. coli* has been known to show appreciable fluorescence change upon the binding of the substrates, either AA, ATP, or tRNA.\textsuperscript{2~4} The VRS of *B. stearothermophilus* also exhibits apparent change of protein fluorescence upon the binding of the substrates

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Abbreviations: VRS, valyl-tRNA synthetase; ORD, optical rotatory dispersion; BSA, bovine serum albumin; PPO, 2,5-diphenyloxazole; POPOP, 2,2'-p-phenylene-bis(5-phenyl-oxazole); EDTA, ethylenediamine tetraacetic acid; PMSF, phenylmethyl sulfonylfluoride; HEPES, N-2-hydroxyethylpiperazine-\textsuperscript{N}-2-ethanesulfonic acid; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.
(L-valine and ATP) as we report here. This property of the enzyme will be useful in the analysis of enzyme-substrate interaction.

Establishing the number of binding sites and the binding constants between enzyme and substrates is the first step for the analysis of enzyme-substrate interaction and has been done for several aminoacyl-tRNA synthetases using gel filtration column chromatography, nitrocellulose filter binding, equilibrium dialysis, equilibrium partition, sedimentation, fluorescence quenching, and ORD. There have been two inconsistent reports on the number of the L-valine binding sites and the $K_d$ values of VRS from B. stearothermophilus, which is a monomeric enzyme but is considered to have two repeated sequences in the primary structure: one has reported a single binding site with a single dissociation constant, $K_d = 19.6 \mu M$, and the other, two sites with two different dissociation constants, $K_{d1} = 17 \pm 11 \mu M$ and $K_{d2} = 184 \pm 52 \mu M$. The number of ATP binding sites has been reported to be two with an identical $K_d$ value of $67 \pm 2 \mu M$.

In this context, we have purified VRS from B. stearothermophilus to high homogeneity with a novel procedure giving a good yield, and examined the binding of the enzyme and each substrate in Eq. (1) (L-valine and ATP), in the absence of tRNA, by fluorescence titration and equilibrium dialysis.

MATERIALS AND METHODS

Bacillus stearothermophilus NCA 1503 cells were generously supplied by Unitika, Ltd., and stored at $-20^\circ C$ until use. Unfractionated tRNA (tRNA$_{am}$) from E. coli K-12 was prepared by the method of Zubay. L-[3,4,6$^3$H]valine was the product of Amersham International; [2,8-$^3$H]ATP and $^{32}$P-pyrophosphate, of NEN Research Products; ATP (disodium salt) and crystalline bovine serum albumin (BSA), of Sigma Chemical Company; and yeast RNA, of Kobjin Co., Ltd. Streptomycin sulfate was purchased from Meiji Seika Kaisha, Ltd., and hydroxylapatite, from Seikagaku Kogyo Co., Ltd. DEAE-Toyopearl 650S and BUTYL-Toyopearl 650S were the products of Toyo Soda MFG, Co., Ltd.; DEAE-Sephadex A-50 and Sephacryl S-300 superfine, of Pharmacia Fine Chemicals. N-Acetyl-L-tryptophan ethylster was the product of Aldrich Chemical Company, Inc. Multi-Cavity Microdialysis Cells were purchased from Shibata Scientific Technology, Ltd. Seamless cellophane tubing for dialysis was the product of Union Carbide Co., and glass microfiber filter (GF/C), of Whatman Ltd. All other chemicals were of reagent grade.

Protein concentrations were measured by the method of Lowry et al. at concentrations higher than 5 mg/ml and that of Bensadoun and Weinstein at lower concentrations, with crystalline BSA as the standard. It was measured also by UV absorption with $A_{275} = 12.8 \text{ cm}^{-1}$ (see RESULTS) at pH 7.5.

Enzyme activity assay. The acylation of tRNA with radioactive L-valine was used to measure the activity of VRS from B. stearothermophilus. The standard reaction mixture used for the measurement contained in 0.5 ml: 50 mm HEPES-KOH buffer of pH 7.6, 3 mm MgCl$_2$, 1 mm ATP, 1 mm diithioerythritol, 0.2 mm L-valine $(3.4 \times 10^3$ cpm/nmol), 10A$_{258}$ units of tRNA$_{am}$ obtained from E. coli, and 100 ng of crystalline BSA. After precubation at 52°C for 3 min, the reaction was started by the addition of 50 µl of the enzyme solution. The reaction was stopped, after incubation at 52°C for 5 min, by adding 3 ml of cold 5% TCA, and then immediately 50 µl of yeast RNA solution (10 mg/ml) was added as carrier. The mixture was kept at 0°C for 30 min and filtered on a Whatman GF/C glass fiber filter. The radioactivity remaining on the filter was measured in a Packard Liquid Scintillation Spectrometer Tri Carb 3320. One unit of the enzyme is defined as the amount which incorporates 1 µmol of $^3$H-L-valine into tRNA in 1 min under these conditions.

The enzyme activity was measured also by the ATP-PPI exchange reaction by the method of Seno et al. with some modifications. The standard reaction conditions were: 50 mm HEPES-KOH buffer of pH 7.6, 10 mm MgCl$_2$, 1 mm ATP, 1 mm diithioerythritol, 1 mm L-valine, and 1 mm $^{32}$P-pyrophosphate (300 cpm/nmol) at 30°C. ATP was adsorbed to charcoal, and the radioactivity was counted in the Packard Liquid Scintillation Spectrometer Tri Carb 3320 as described above.

Enzyme purification. All operations were done at 0~5°C. The standard buffer was potassium phosphate buffer containing 2 mm ethylenediaminetetraacetic acid (EDTA), 10 mm 2-mercaptoethanol, and 0.1 mm phenylmethylsulfonylfluoride (PMSF); the concentration and pH of the buffer were chosen as specified in each case.

Step 1. Cell extraction: B. stearothermophilus NCA 1503 cells (200 g), which had been kept frozen at $-20^\circ C$, were thawed and suspended in 400 ml of the standard buffer (10 mm, pH 7.5). The cell suspension was sonicated at 10 KC for 2 min in an ice bath with a Supersonicator Cell Destroyer T-A-4201. The sonication extract was centrifuged at 11,000 x g for 70 min and the supernatant fraction was collected (termed Crude extract).
Step 2. Streptomycin precipitation: Streptomycin sulfate (320 ml of 10% solution) was added with stirring to each liter of the crude extract. Stirring continued for 10 min in the cold and the precipitate that formed was removed by centrifugation at 11,000 × g for 30 min.

Step 3. Ammonium sulfate fractionation: solid ammonium sulfate was added slowly to the supernatant fraction obtained above and the protein fraction precipitated between 40~65% saturation of ammonium sulfate was collected. The precipitate was dissolved in 68 ml of the standard buffer (20 mM, pH 7.5), and the solution was desalted by gel filtration with a Sephadex G-25 column (5 × 45 cm) equilibrated with the same buffer.

Step 4. Batch elution from DEAE-Sephadex A-50: the desalted solution obtained in Step 3 was mixed with DEAE-Sephadex A-50 (40 g dry weight) equilibrated with the standard buffer (20 mM, pH 7.5). The slurry was mixed by stirring for 10 min and then left still. After the gel had settled down, the supernatant was discarded. The gel was washed with 1.1 liters of the standard buffer (20 mM, pH 7.5) and 2.2 liters of the standard buffer (100 mM, pH 7.2), consecutively. Then the enzyme activity was eluted from the gel with 4.5 liters of the standard buffer (350 mM, pH 6.5). Solid ammonium sulfate was added to 73% saturation slowly, and after 30 min of stirring, the suspension was centrifuged at 11,000 × g for 30 min. The precipitate was dissolved in 64 ml of the standard buffer (20 mM, pH 7.0) without EDTA. The suspension was desalted through a Sephadex G-25 column (5 × 45 cm) equilibrated with the same buffer.

Step 5. Hydroxyapatite chromatography: the product of Step 4 was chromatographed on a hydroxyapatite column (4 × 21.5 cm) equilibrated with the standard buffer (20 mM, pH 7.0) without EDTA. The column was washed after the enzyme was put on with a volume of the equilibration buffer 3 times the column volume. The enzyme was eluted with a linear concentration gradient of the standard buffer (pH 7.0) from 20 mM to 400 mM in a total volume of 2.6 liters with the flow rate of 100 ml/hr. The active fraction, which was eluted between 150 mM and 250 mM of the buffer, was pooled and concentrated by an Amicon ultrafiltration apparatus with a Diaflow Membrane YM 10.

Step 6. DEAE-Toyopearl 650S chromatography: the enzyme preparation obtained in Step 5 was dialyzed against the standard buffer (20 mM, pH 7.5). The dialyzed solution was put onto a column of DEAE-Toyopearl 650S (2 × 40 cm) equilibrated with the buffer used for dialysis. After washing with 500 ml of the same buffer, elution was done with a linear concentration gradient of KCl from 0 mM to 0.4 M in a total volume of 1 liter with the flow rate of 42 ml/hr. The active fraction, which was eluted at about 0.14 M KCl, was pooled and concentrated by ultrafiltration with a Diaflow Membrane YM 10.

Step 7. BUTYL-Toyopearl 650S chromatography: a column of BUTYL-Toyopearl 650S (2 × 40 cm) was equilibrated with the standard buffer (20 mM, pH 7.5) containing ammonium sulfate at 40% saturation. The product of Step 6 was dialyzed against the equilibration buffer and adsorbed onto a BUTYL-Toyopearl 650S column. After washing with 500 ml of the same buffer, elution was done with a linear concentration gradient of ammonium sulfate from 40% to 0% saturation in a total volume of 1 liter with the flow rate of 42 ml/hr. The active fraction was eluted at about 12% saturation of ammonium sulfate, and it was pooled and concentrated by ultrafiltration with a Diaflow Membrane YM 10.

Step 8. Gel filtration: the concentrated sample (3 ml) was subjected to gel filtration on a Sephacryl S-300 superfine column (2.5 × 84 cm) equilibrated with the standard buffer (0.1 M, pH 7.5) containing 0.5 M NaCl. The flow rate was 30 ml/hr. The active fractions were pooled and dialyzed against 50 mM HEPES-KOH buffer of pH 7.5 containing 50% glycerol, 10 mM MgCl₂, 10 mM 2-mercaptoethanol, and 0.1 mM PMSF, and this was stored at -20°C.

Molecular weight measurement: The molecular weight of the enzyme was measured both by gel filtration and by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Gel filtration was made on a Sephacryl S-300 superfine column (2.5 × 84 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 7.5, containing 10 mM 2-mercaptoethanol, 2 mM EDTA, 0.1 mM PMSF, and 0.5 M NaCl. The sample, in 3 ml of the same buffer, was put onto the column. The flow rate was 30 ml/hr. SDS-PAGE was done by the method of Laemmli using Coomassie Brilliant Blue.

Fluorescence titration. The purified enzyme was dialyzed, before the fluorescence measurement, against 50 mM HEPES-KOH buffer, pH 7.5, containing 10 mM MgCl₂, 10 mM 2-mercaptoethanol, and 0.1 mM PMSF. Fluorescence was measured with a recording spectrofluorometer, Union Giken FS-401. The absorbance of the enzyme solution was always less than 0.1 at the excitation wavelength, 295 nm. The fluorescence intensity at 340 nm was measured and corrected both for dilution of enzyme due to addition of the ligand solutions and for absorption of light at 295 nm by the nucleotide ligands. The enzyme solution (2 ml) was placed in an observation cell thermostated at 30°C or 15°C; then ligand solutions were added to the enzyme solution with a microsyringe and stirred. After allowing 3 min for equilibration, fluorescence was measured.

The values of the dissociation constant, Kd, for the enzyme-ligand complex, EL, and of the fluorescence intensity decrease at 340 nm (as a percentage) that would be observed when the enzyme is saturated with the ligand, ΔFmax, were determined by assuming a simple bimolecular binding equilibrium between the enzyme, E, and ligand, L, (Eqs. (3) ~ (5)) using the nonlinear least squares method.²¹

\[
E + L \rightleftharpoons EL
\]
\[ K_a = \frac{[E][L]}{[EL]} \]  
\[ \Delta F = \frac{\Delta F_{\text{max}}}{K_a + [L]} \]

where \( \Delta F \) is the fluorescence intensity decrease observed at 340 nm when a certain amount of the ligand is added, as expressed as a percentage of the fluorescence intensity of the enzyme; namely, \( \Delta F = 100 \left( \frac{F_{\text{EL}} - F_e}{F_{\text{EL}}} \right) \), where \( F_{\text{EL}} \) and \( F_e \) are the fluorescence intensity at 340 nm of the enzyme-ligand complex and the enzyme, respectively.

**Equilibrium dialysis.** Equilibrium dialysis experiments were done with microdialysis cells in a 4°C compartment. One chamber of each cell (100 \( \mu \)l) contained 18 \( \mu \)M VRS and the other contained 20 \( \mu \)M to 460 \( \mu \)M \(^3\)H-L-valine or 20 \( \mu \)M to 750 \( \mu \)M \(^3\)H-ATP, and the chambers were separated by cellophane dialysis membrane. The buffer used for the experiments was 150 mM Tris-HCl buffer, pH 7.5, containing 10 mM MgCl\(_2\), 10 mM 2-mercaptoethanol, and 0.1 mM PMSF. After the apparatus was slowly rotated for 7 days, samples of 10 \( \mu \)l were withdrawn from each chamber and the radioactivity of each sample was measured in a liquid scintillation spectrometer. In the preliminary tests we found that at least 3 days were necessary for these ligands to reach equilibrium under the conditions employed.

**RESULTS**

**Purification and molecular weight measurement**

Eight milligrams of purified VRS was obtained from 200 g of frozen cells of *B. stearothermophilus* (Table I). The yield of activity in this procedure was about 20% with a total purification of 1100-fold. A single band was obtained with the polyacrylamide disc-gel electrophoresis. SDS-PAGE showed that the enzyme was composed of a single chain polypeptide with molecular weight of about 100,000. The molecular weight of the enzyme estimated by gel filtration was about 110,000. In this report the molar concentration of the enzyme is calculated with the latter value, 110,000.

**pH--activity relationship**

The pH optima for the aminoacylation activity of VRS were determined with 3 mM MgCl\(_2\) and 1 mM ATP at 30°C and 52°C under the conditions described in METHODS (enzyme activity assay). The results (Fig. 1) demonstrate that the pH optima are 8.5 at 30°C and 8.0 at 52°C.

**Temperature--activity relationship**

The optimum temperature for the acylation reaction of VRS was determined with 3 mM MgCl\(_2\) and 1 mM ATP at pH 7.6 and pH 8.5 under the conditions described in METHODS (enzyme activity assay). The results (Fig. 2) demonstrate that the VRS activity increases up

| TABLE I. PURIFICATION OF VALYL-tRNA SYNTHETASE FROM B. stearothermophilus |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|
| Step                         | Total protein (mg) | Total activity (units\(^a\)) | Specific activity (units/mg) | Yield (%) |
| Crude extract                | 43000            | 180             | 0.0042           | 100          |
| Streptomycin precipitation   | 31000            | 150             | 0.0050           | 85           |
| Ammonium sulfate fractionation | 7900          | 160             | 0.021            | 91           |
| Batch elution from DEAE-Sephadex A-50 | 2600            | 140             | 0.053            | 76           |
| Hydroxyapatite chromatography | 870            | 94              | 0.11             | 51           |
| DEAE-Toyopearl 650S chromatography | 110           | 56              | 0.52             | 31           |
| BUTYL-Toyopearl 650S chromatography | 23            | 49              | 2.1              | 27           |
| Sephacryl S-300 chromatography | 8.3            | 37              | 4.5              | 20           |

\(^a\) 1 unit is defined as the amount of the enzyme that forms 1 \( \mu \)mol of \(^3\)H-val-tRNA in 1 min at 52°C.
Valyl-tRNA synthetase from B. stearothermophilus

Fig. 1. pH-Activity Profile of Valyl-tRNA Synthetase from B. stearothermophilus.

\[ [E]_0 = 3 \text{ mM}; [\text{MgCl}_2]_0 = 3 \text{ mM}; \rightarrow, 30^\circ C; \rightarrow \rightarrow, 52^\circ C; \bigcirc. \]

PIES buffer; ●, HEPES buffer; ○, glycine buffer. See the text (METHODS) for the other conditions of the activity measurement. The maximum value at each temperature is set at 100%.

Fig. 2. Temperature-Activity Profile of Valyl-tRNA Synthetase from B. stearothermophilus.

\[ [E]_0 = 3 \text{ mM}; [\text{MgCl}_2]_0 = 3 \text{ mM}; \bigcirc \bigcirc, \text{pH 8.5}; \bigcirc \cdot \bigcirc \bigcirc, \text{pH 7.6}. \]

See the text (METHODS) for the other conditions of the activity measurement. The maximum value at each pH is set at 100%.

to 52°C and then begins to decrease at both pH 7.6 and pH 8.5 under our conditions.

UV absorption spectrum

The UV absorption spectrum of VRS (Fig. 3) has a peak at 277nm at pH 7.5. The absorption coefficient, \( A_{277} \), calculated from the spectrum using the protein concentration by the Lowry method, is 12.8 cm\(^{-1}\) at pH 7.5.

Enzyme protein fluorescence

The emission spectrum of VRS excited at 295 nm shows a peak at about 340 nm (Fig. 4). The relative fluorescence quantum yield of the enzyme was obtained as 0.27 at pH 7.5 when the fluorescence quantum yield of N-acetyl-L-tryptophan ethylester chosen as the standard...
was assumed to be 0.2.

**Binding between VRS and substrates (L-valine and ATP)**

(a) **Fluorescence titration.** The addition of the substrates (L-valine and ATP) to VRS decreases the protein fluorescence. Figure 4 shows an example of the effects of L-valine on the fluorescence spectra of the enzyme. (1) Fluorescence titration of VRS with L-valine at 30°C is shown in Fig. 5(a). The $K_d$ and $\Delta F_{\max}$ values of L-valine, calculated by assuming a single bimolecular binding equilibrium between the enzyme and L-valine (Eqs. (3)~(5)), are $170 \pm 30 \mu M$ and $5.5 \pm 0.1%$ at 30°C, and $230 \pm 10 \mu M$ and $6.4 \pm 0.1%$ at 15°C, respectively, at pH 7.5 (Table II). (2) Fluorescence titration of VRS with ATP at 30°C is shown in Fig. 5(b). The values of $K_d$ and $\Delta F_{\max}$ of ATP are $51 \pm 5 \mu M$ and $7.1 \pm 0.1%$ at 30°C, and $91 \pm 9 \mu M$ and $7.6 \pm 0.2%$ at 15°C, respectively, at pH 7.5 (Table II).

(b) **Equilibrium dialysis.** (1) The Scatchard plot(22) for the binding of L-valine to the enzyme is presented in Fig. 6. The extrapolated values of $n$, the number of binding sites per mol of enzyme, is $2.3 \pm 0.8$. The $K_d$ value calculated from the plot is $210 \pm 60 \mu M$. The two binding equilibria seem equivalent (Table II). (2) The Scatchard plot for the binding of ATP to the enzyme is also presented in Fig. 6. The number of the binding sites per mol of enzyme is $2.1 \pm 0.5$ and the $K_d$ value of ATP is $130 \pm 40 \mu M$. The two binding equilibria are also equivalent in this case (Table II).

**DISCUSSION**

**Purification and properties**

There have been three reports which de-
scribe purification of VRS from *B. stearothermophilus*\(^{23-25}\); we present here, however, a novel procedure with about twice the yield as those previously reported. Hydrophobic chromatography with BUTYL-Toyopearl 650S (Step 7 in the purification method) was effective (Table I); the reproducibility of the chromatography was good and its high efficiency was comparable to that of the preparative isoelectric point electrophoresis which we had used previously.\(^{26}\) Two values have been reported so far on the molecular weight of VRS from *B. stearothermophilus* as estimated by gel filtration: 110,000\(^{3,15,24}\) and 89,000.\(^{23}\) Our results support the former. The effects of temperature and pH on the enzyme activity of our preparation (Figs. 1 and 2) agree with those observed by other investigators.\(^{15,23,24}\) The absorption coefficient, \(A^{\%}_{1}^{200 \text{ nm}}\), calculated from the UV absorption spectrum (Fig. 3) is 12.8 cm\(^{-1}\) at pH 7.5, while the value, \(A^{\%}_{175 \text{ nm}}\), calculated based on the amino acid composition reported by Koch *et al.*\(^{13}\) is 12.8 cm\(^{-1}\) at pH 7.1. Mulvey and Fersht\(^{14}\) gave the \(A^{\%}_{1}^{280 \text{ nm}}\) as 22 cm\(^{-1}\) without specifying the pH.

**Binding between enzyme and substrates, L-valine and ATP**

(a) Fluorescence titration. We found that the binding of the substrates (L-valine and ATP) decreased the protein fluorescence of VRS from *B. stearothermophilus*. The binding of a specific amino acid to an aminoacyl-tRNA synthetase does not always decrease the fluorescence of the enzyme: L-isoleucine did not influence the protein fluorescence of isoleucyl-tRNA synthetase from *E. coli*;\(^9\) but L-methionine induced a fluorescence increase of methionyl-tRNA synthetase from *E. coli*.\(^{10}\) Ligand-induced decrease in the protein fluorescence was observed with VRS of *E. coli*, and this property was used as a probe\(^2\) in the study of the substrate binding equilibrium; however, no investigation has been published on this aspect on VRS’s from other sources. Hêlène *et al.*\(^3\) reported for the VRS of *E. coli* that the \(K_d\) (L-valine) was 67 \(\mu\)M at pH 7.8, 50 \(\mu\)M at pH 6.5, and 333 \(\mu\)M at pH 5.5, and that the \(K_d\) (ATP) was 5 \(\mu\)M at pH 6.5. Our results on VRS from *B. stearothermophilus* show that the \(K_d\) (L-valine) is \(170 \pm 30 \mu\)M at 30°C and 230 \(\pm 10 \mu\)M at 15°C, and the \(K_d\) (ATP) is 51 \(\pm 5 \mu\)M at 30°C and 91 \(\pm 9 \mu\)M at 15°C, all at pH 7.5 (Table II).

(b) Equilibrium dialysis. Bindings between VRS from *B. stearothermophilus* and substrates (L-valine and ATP) had been examined by equilibrium dialysis,\(^{14,15}\) but those results were rather inconsistent. Our results here (Fig. 6) show that the number of binding sites for L-valine is 2.3 \(\pm 0.8\) with a \(K_d\) (L-valine) of 210 \(\pm 60 \mu\)M and the number of binding sites for ATP is 2.1 \(\pm 0.5\) with a \(K_d\) (ATP) of 130 \(\pm 40 \mu\)M at pH 7.5, 4°C. The number of substrate binding sites thus obtained (2 for each) is consistent with those reported by La Belle\(^{13}\); these values are reasonable in view that this enzyme, though a monomeric protein, has a repeated sequence in the primary structure.\(^{13}\) However, La Belle reported two \(K_d\) values for L-valine binding (17 \(\pm 11 \mu\)M and 184 \(\pm 52 \mu\)M) and one for ATP (67 \(\pm 2 \mu\)M), whereas we have found only one \(K_d\) value for each substrate. Our results indicate that the two binding equilibria of each L-valine and ATP are equivalent when each substrate is bound by itself to the enzyme. Mulvey and Fersht\(^{14}\) reported different results on the binding of L-valine: one binding site with \(K_d\) (L-valine) = 19.6 \(\mu\)M at pH 7.78, 25°C. These discrepancies may be related to the difference in experimental conditions for the equilibrium dialysis: Mulvey and Fersht, 25°C, 120 min\(^{14}\); La Belle, 4°C, 10 to 19 hr\(^{15}\); our investigation, 4°C, 7 days. We found in the preliminary test that at least three days were necessary for the ligands to reach the equilibrium under our conditions and that the enzyme activity did not change and the percentage of spontaneous degradation of ATP was less than 0.5% during 7 days.

The \(K_m\) value for L-valine and ATP in the ATP-Pi exchange reaction (Eq. (1)) are listed also in Table II. The value of Fersht and Kaether\(^{24}\) was \(K_m\) (L-valine) = 30 \(\mu\)M, at pH 7.78, 25°C. The \(K_m\) (L-valine) appears closer here to \(K_d\) (L-valine) obtained by Mulvey and
Fersht (19.6 μM) and to the smaller value obtained by La Belle (17 μM) rather than to our value. However, we have found (data not shown) that an apparent \( K_d \) (1-valine) obtained fluorometrically in the presence of saturated amount of ATP, the condition under which the ATP-Pi exchange reaction takes place, is much smaller than the value shown in Table II, which was obtained in the absence of ATP, thus becoming closer to the \( K_m \) value. We will report details of this aspect elsewhere. By comparing our results obtained by fluorescence titration and equilibrium dialysis, we can reasonably consider that the ligand-induced decrease of protein fluorescence observed here with VRS from \( B.\) steatorrhophilus is a genuine reflection of the specific binding of substrates and that this property can be used as a probe for the analysis of substrate binding. Since VRS from \( B.\) steatorrhophilus is much more stable than VRS from \( E.\) coli, this property of the former enzyme should facilitate further studies on the VRS reaction mechanism.

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