PURIFICATION OF A LEUCINE-SPECIFIC BINDING PROTEIN FROM ESCHERICHIA COLI*

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Summary: A new binding protein for leucine was purified from E. coli and crystallized. It appears to be similar in size to a protein, previously isolated from E. coli, which binds leucine, isoleucine and valine. The new protein, however, binds only leucine. Furthermore, the amino acid analogue trifluoroleucine effectively inhibits the binding of leucine to the leucine-specific binding protein whereas, it is without effect on the protein that binds all three amino acids. The K_D for leucine binding is 0.7 μM. The nature of the inhibition of leucine transport by trifluoroleucine and isoleucine suggests that there is more than one transport system for leucine.

Two laboratories have reported the crystallization of a binding protein which binds leucine, isoleucine and valine (1,2); we shall refer to it as the LIV-binding protein. Anraku (1) observed a shoulder on the LIV-binding protein peak isolated by DEAE cellulose chromatography of shock fluid proteins. We have now resolved two leucine-binding proteins by DEAE cellulose chromatography, the second of which is highly specific for leucine (L-binding protein).

MATERIALS AND METHODS

Binding Assays - The binding assays were carried out in plexiglass cells containing two wells (0.2 ml each), separated by a disc of dialysis tubing. In the assay for column fractions a sub-saturating level of leucine was used (Assay 1). Side A contained protein, 0.05 M NaCl, 0.01 M phosphate buffer, pH 7.0, in a total volume of 0.10 or 0.05 ml. Side B contained 2×10^{-6} M

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^{14}C-leucine, 0.05 M NaCl and 0.01 M phosphate buffer in a volume equal to that in side A. When a saturating level of leucine was used for quantitative studies (Assay 2), side B contained 2 x 10^{-5}M leucine. After overnight equilibration at 2° on a rotating apparatus, aliquots were removed from each side and counted in a Triton-toluene counting solution (3). A filter assay similar to the one described by Riggs and Bourgeois (4) is also useful in locating peaks of binding activity in column fractions. In this assay, 0.05 ml of 2 x 10^{-5}M leucine was added to 0.1 ml of column fraction, followed by 0.05 ml of 1 M $MgCl_2$. The mixture was filtered on a 25 mm Schleicher & Schuell B-6 filter and washed with 0.5 ml of 0.2 M $MgCl_2$. The filters were dried and counted in a solution of 15 g PPO and 0.2 g POPOP (Packard Inst. Co.) per liter of toluene. (Transport filters were treated similarly).

Transport Assays - These were done at a saturating level of leucine at 23°. A suspension of mid-log phase cells twice washed with minimal medium was incubated for 5 minutes in minimal medium (5) supplemented with 10 mM glucose and 40 µg per ml of chloramphenicol. A sample of cells was then added to the reaction mixture which contained 10 mM glucose, 40 µg per ml chloramphenicol, 1 x 10^{-5}M ^{14}C-labeled amino acid and minimal medium to a final volume of 0.5 ml. To determine the initial rate of transport, aliquots were taken at 15 and 30 sec, filtered on a Millipore filter, and washed with 10 ml of 0.01 M Tris-HCl, pH 7.3, containing 0.15 M NaCl and 0.5 mM $MgCl_2$ (6) (temperature, 23°). For determination of K_m the number of cells used was such that less than 10% of the substrate was taken up at all concentrations of substrate used.

E. coli Strain 7 (derived from K10, from Dr. E. C. C. Lin) was grown to early stationary phase in minimal medium (5) supplemented with 1% succinic acid (Baker and Adamson). The cells were harvested and shocked as previously described (1) except that the concentration of EDTA was 2 mM and $MgCl_2$ was added to a final concentration of 1 mM within one minute after shocking with cold deionized water.

Radioactive isotopes obtained from New England Nuclear Corp. had specific activities from 20 to 275 mc per m mole. Trifluoroleucine was obtained from Dr. J. Calvo. The unlabeled amino acids were Mann "Assayed" grade.

Protein was determined by a modification of the method of Lowry et al. (7), with bovine serum albumin as a standard.

RESULTS

Purification of the leucine-specific binding protein. DEAE-cellulose chromatography - Concentrated shock fluids* from three preparations, representing 369 g wet cell paste were pooled and passed through a Biogel-P 10 column equilibrated with deionized water. The protein peak (1,344 mg of protein in 320 ml) was loaded on a 2.5 cm x 54 cm column of DEAE-cellulose (Brown and Co.) which had been prepared by the usual washing procedure (8), followed by equilibration with 5 mM Tris-HCl buffer, pH 7.6. The column was eluted with a 7 liter linear gradient from 0 to .15 M NaCl in 5 mM Tris-HCl buffer, pH 7.6. Two leucine-binding peaks were found to elute from the DEAE-cellulose column (Figure 1). Fractions from the first peak also bound isoleucine and valine, while fractions from the second peak bound only leucine. The same pattern was also observed when extracts from E. coli K12 were chromatographed in the same way.

Preparative polyacrylamide gel electrophoresis - The fractions from the peak which bound only leucine were pooled, concentrated by ultrafiltration, and dialyzed against a 1:4 dilution of stacking gel buffer (see below). Electrophoresis was carried out in a Canalco preparative electrophoresis apparatus (in two runs) using column number PD-2/320. A published procedure (9) was modified in that a buffer system developed by Jovin (10) was used. The 3 cm high resolving gel contained 0.083 M Tris-HCl, pH 7.4; the 4 ml

*Shock fluids were concentrated in an Amicon model 401 ultrafiltration cell fitted with a UM-10 filter.

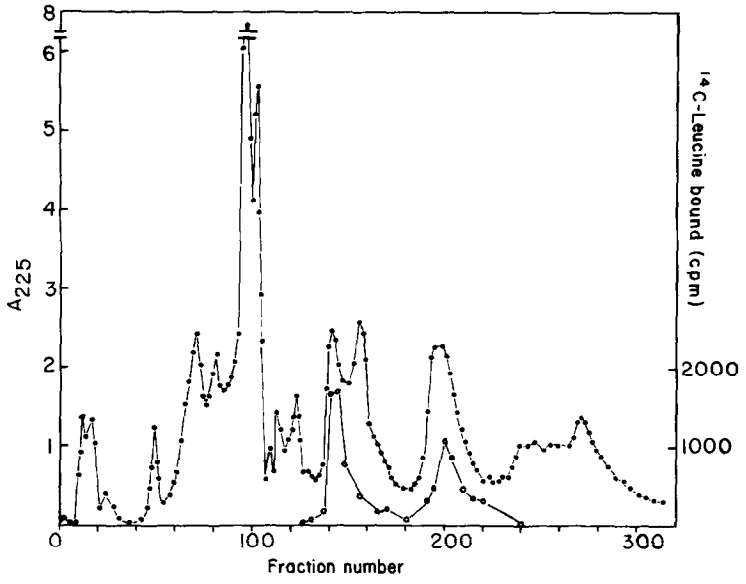

Fig. 1. DEAE-cellulose fractionation of the two leucine binding proteins. Closed circles, protein absorbance at 225 mμ; open circles, leucine binding activity (Assay 1).

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stacking gel contained 0.069 M Tris base-0.052 M H_3PO_4, pH 6.7; the upper buffer contained 0.05 M Tris base-0.06 M Tricine, pH 7.9; and the lower and elution buffers contained 0.06 M Tris-HCl, pH 7.3. A purification of 59 fold over the crude desalted shock fluid was obtained with an overall yield of 58%.

Crystallization of the leucine-specific binding protein - Crystallization (Fig. 2) by dialysis against a solution of ammonium sulfate did not alter the specific activity of material obtained from the preparative gel electrophoresis step. It is interesting that this value is essentially the same as that reported for the crystalline protein that binds leucine, isoleucine and valine (1,2). This material appeared to be homogeneous as judged by disc gel electrophoresis at two pH values and at different gel concentrations.

Estimation of size - The disc gel method of Hedrick and Smith (11) indicated that the L-binding protein was identical in size to the LIV-

Fig. 2. Crystals of the leucine-specific binding protein x 420.

binding protein which has a reported molecular weight of about 36,000 (2,12). The Sephadex gel filtration method of Andrews (13) also indicated that the molecular weight of the L-binding protein was identical to that of the LIV-binding protein. The molecular weight determined by this method with appropriate standards was 37,000.

Specificity of binding - Table 2 shows that leucine binding to the L-binding protein is not inhibited by isoleucine or valine. Further, the binding of leucine is not inhibited by threonine which we found to inhibit leucine binding to the LIV-binding protein, as well as to inhibit the transport of isoleucine, leucine and valine. The binding of leucine to the L-binding protein is, however, quite sensitive to inhibition by the analogue trifluoroleucine (TFL). In corroboration of the results of Penrose et al. (2), TFL did not inhibit the binding of leucine to the LIV-binding protein.

Table I

Purification of the leucine-specific binding protein (L-BP).

Data for the LIV-binding protein are also included since this makes it possible to estimate specific activities of both proteins in the shock fluid. One unit equals 1 mμmole of leucine bound under conditions of saturation (Methods).

Fraction	Volume ml	Units per ml	Total Units	Protein mg/ml	Specific activity	Yield %
Desalted Shock Fluid	320	3.6	1153	4.2	0.86 {.6(LIV-BP) .26(L-BP)}	100
DEAE LIV - BP	19.5	39.7	774	7.9	5.0	95
DEAE L - BP	9.2	35.8	330	13.2	2.7	
Prep Gel L - BP	8.8	21.9	192	1.4	15.6	58

Table II

Inhibition of Leucine transport and of Leucine binding to the LIV-binding protein and the L-binding protein.

Additions		Leucine Transport (Relative rate)	Relative Leucine Binding LIV-BP	Relative Leucine Binding L-BP
None		100	100	100
Isoleucine	100 μM	39		
	200 μM	29	10	97
Valine	100 μM	48		
	200 μM	42	13	91
Trifluoroleucine	100 μM	49		
	200 μM	39	100	6
Isoleucine + Valine	100 μM + 100 μM	15		
Isoleucine + Trifluoroleucine	100 μM + 100 μM	18		
Valine + Trifluoroleucine	100 μM + 100 μM	39		

The K_D for leucine binding to the L-binding protein is 0.7 μM compared to a K_D of 1 μM for leucine binding to the LIV-binding protein. None of the other 16 amino acids tested inhibited the binding of leucine to the L-binding protein.

Evidence for more than one transport system - The finding of a second leucine-binding protein prompted us to look for another transport system for leucine. Figure 3 shows that "cold" isoleucine does not completely inhibit the initial rate of leucine transport and further that the residual

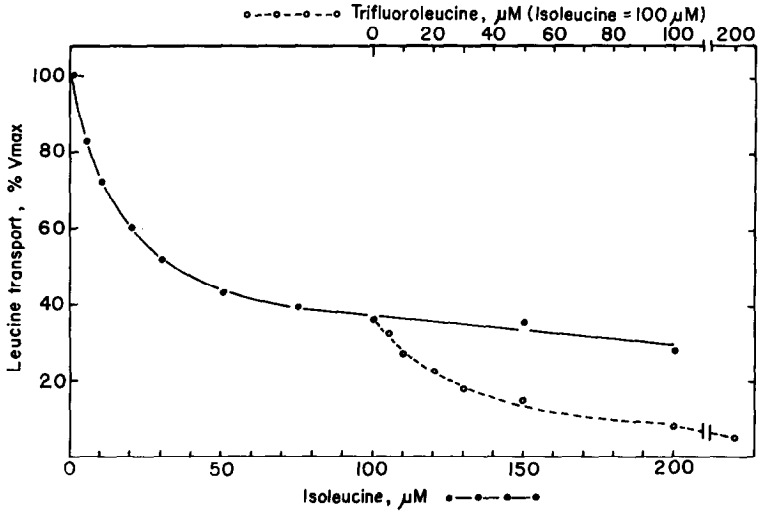

Fig. 3. Inhibition of leucine transport by isoleucine and isoleucine plus TFL. Solid circles solid line, leucine transport at the indicated level of isoleucine; open circles (broken line) leucine transport at 100 μM isoleucine plus the indicated level of trifluoroleucine.

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transport is very sensitive to inhibition by TFL. The reverse is also true, i.e., TFL does not completely inhibit leucine transport and the residual transport is very sensitive to inhibition by isoleucine. A second line of evidence for the existence of more than one transport system for leucine is that two K_m values were found for leucine transport, 0.2 and 2 μM. Further, in the presence of 20 μM unlabeled isoleucine, we found again two K_m's for leucine, 0.2 μM and 23 μM. Even in the presence of 200 μM unlabeled isoleucine the cells were still able to transport a considerable amount of leucine with a K_m of 2 μM.

Discussion These results are consistent with the existence of more than one transport system for leucine. The results of Anraku (1,6) and of Penrose et al. (2) strongly implicate the LIV-binding in leucine transport. In addition, unpublished data from this laboratory with a family of competitive inhibitors of the LIV-binding protein varying widely in inhibition constants have shown a good correlation between K_i for inhibition of binding and K_i for the inhibition of transport. It is tempting to speculate that

the leucine-specific binding protein may be involved in the second transport system for leucine. Most of the inhibition data are consistent with this concept. However, it is difficult to explain why valine and TFL appear to inhibit the same component of leucine transport (Table II). This matter is under investigation.

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