DISSOCIATION OF α-ISOPROPYLMALATE SYNTHASE FROM SALMONELLA TYPHIMURIUM BY ITS FEEDBACK INHIBITOR LEUCINE[1)]

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Summary. Results from sedimentation equilibrium experiments suggest that α-isopropylmalate synthase from Salmonella typhimurium exists in an association-dissociation equilibrium which is strongly influenced by its feedback inhibitor, leucine. It is shown that under appropriate conditions leucine can cause a dissociation of the enzyme into units having the size of the polypeptide chains, which appear to be similar and to have a molecular weight of 45,000 - 50,000. The size of the oligomer has not been unambiguously established, although the data available favor the existence of a tetramer under conditions where the association-dissociation equilibrium is shifted toward the aggregated form of the enzyme.

Research in recent years has brought to light a high degree of versatility for the way in which regulation of enzyme activity by modifiers operates. There are, however, two elements which appear to be common to most regulatory enzymes: they are composed of more than one polypeptide chain, and they possess distinct, separate sites for substrate(s) and modifier(s). As a consequence of the latter property, the effect of a modifier is likely to be indirect, involving conformational alterations of the protein molecule. The nature of these conformational changes has been found to vary greatly with different enzymes. Thus, it

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appears that the "classical" allosteric enzymes threonine deaminase and aspartate transcarbamylase of E. coli undergo relatively subtle changes in the presence of modifiers (1,2). On the other hand, there is an increasing number of regulatory enzymes which respond more drastically to their effectors, i.e. by association or dissociation of the protein (3 - 6).

The results presented in this paper suggest that α-isopropylmalate synthase from S. typhimurium is a multi-subunit enzyme and dissociates in the presence of its feedback inhibitor, L-leucine.

Methods

α-Isopropylmalate synthase was purified from S. typhimurium strain CV 19 as described previously (7).

Sedimentation equilibrium experiments were carried out in a Spinco Model E analytical ultracentrifuge equipped with Schlieren and Rayleigh optics and a temperature control unit. Detailed conditions for individual runs are given in the legend of Fig. 2.

The partial specific volume ($\bar{v}$) of the enzyme was determined with the density gradient method of Linderstrøm-Lang and Lanz (8), using the equation given by Schachman (9).

SDS[3]-polyacrylamide gel electrophoresis was performed essentially following published procedures (10,11). Specifically, the proteins were prepared for electrophoresis by incubation for 3 hours at 37° in the presence of 1% SDS and 1% 2-mercaptoethanol, followed by overnight dialysis against buffer containing 0.1% SDS and 0.5% 2-mercaptoethanol.

[3] The abbreviation used is: SDS, sodium dodecyl sulfate.

The gels contained 5% acrylamide. The ratio of acrylamide to methylene-bis-acrylamide was 28 : 1. After the run, a short piece of thin wire was used to mark the position of the tracking dye, thus eliminating the problem of changes in gel length during staining and destaining. Protein mobility (Fig. 3) is defined as distance of protein migration / distance of tracking dye migration.

Results and Discussion

The importance of the partial specific volume ($\bar{v}$) of a protein in determining an accurate molecular weight prompted us to determine the $\bar{v}$ of

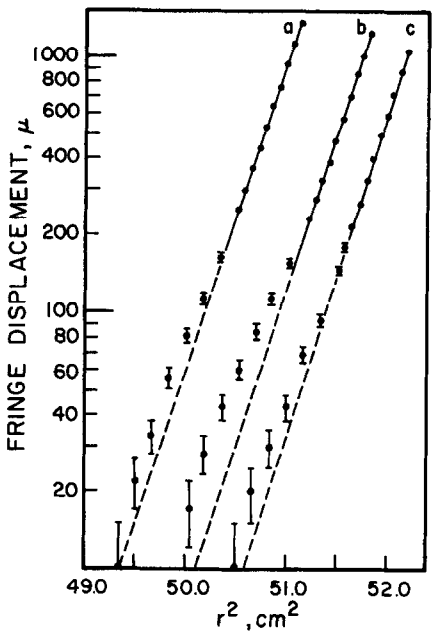

Fig. 1. Sedimentation equilibrium centrifugation of α-isopropylmalate synthase. The meniscus depletion method described by Yphantis (13) was used. The logarithm of fringe displacement is plotted vs. the squared distance from the center of rotation. The error bars represent an uncertainty of 5 μ in the fringe displacement measurements.
a: No leucine, 17,450 rpm; b: 0.06 mM leucine, 20,410 rpm; c: 10 mM leucine, 24,530 rpm. Initial protein concentration (biuret) 0.98 mg/ml. Other conditions as described in legend of Fig. 2.
Note: For the purpose of putting all curves on one graph, 0.5 abscissa units were added to the points of curve b, and 1 abscissa unit was added to the points of curve c.

α-isopropylmalate synthase experimentally (cf. "Methods"). Since the runs in the analytical ultracentrifuge were performed at 5° and in 0.01 M sodium phosphate buffer pH 7.0 containing 0.2 M NaCl, $\bar{v}$ was determined under these conditions and found to be 0.713 cc/g. This value did not vary appreciably over a range of enzyme concentration between 3 and 11 mg/ml. For conversion to higher temperatures an increase of 0.001 cc/g per degree may be used (12).

Fig. 1 shows the results of sedimentation equilibrium experiments performed according to the meniscus depletion method of Yphantis (13). The three experiments listed differ only with respect to leucine concen-

Table I

Weight-average and z-average molecule weights of α-isopropylmalate synthase as a function of leucine and protein concentration

Leucine conc. (mM)	Initial protein concentration mg/ml	$\bar{M}_w$ a)	$\bar{M}_z$ a)
0	6.25	166,000	192,000 b)
0	0.98	115,000	126,000
0.03	0.98	103,000	108,000
0.06	0.98	99,000	104,000
1	0.98	65,000	76,000
1	0.55	62,000	71,000
10	0.98	69,000	70,500
10	0.55	47,000	53,000

a) Weight-average ($\bar{M}_w$) and z-average ($\bar{M}_z$) molecular weights (over the entire cell) were calculated according to Yphantis (13).

b) This experiment was performed at 5° and at 7447 rpm according to the short column - low speed technique of Van Holde and Baldwin (15). Weight-average and z-average molecular weights (over the entire cell) were calculated according to methods I and II in this article.

tration and rotor speed. Point-weight-average molecular weights calculated from these and similar experiments, all with an initial protein concentration of 0.98 mg/ml, were plotted as a function of leucine concentration (Fig. 2). The values are seen to drop gradually from 132,000 in the absence of leucine to 71,000 at concentrations above 1 mM leucine[4]. As can be seen from the Yphantis plots (Fig. 1), a

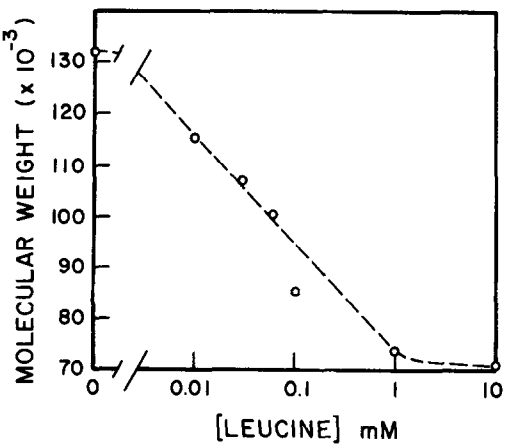

Fig. 2. Apparent molecular weights of α-isopropylmalate synthase as a function of leucine concentration. 10 μl of enzyme solution (50 mM Na-phosphate pH 6.8, 20% glycerol, 9.75 mg protein per ml) were diluted to 100 μl with 10 mM Na-phosphate pH 7.0 containing 200 mM NaCl and the appropriate concentration of leucine. Dialysis against 100 ml of the same solution for 5 to 8 hours followed. The sample was placed in a 2.5°, 12 mm double sector Kel-F centerpiece. The dialysate was placed in the reference channel, thus complying with the requirements of Casassa and Eisenberg (14). An An-D rotor, precooled to 5°, was used. All runs were performed at 5°. After overspeeding at 35,600 rpm for 1 hour, the speed was brought to 17,450 rpm for the no-leucine sample, 20,410 rpm for the 10 μM, 30 μM and 60 μM leucine samples, and to 24,530 rpm for the 0.1 mM, 1 mM and 10 mM leucine samples. Rayleigh interference photographs were taken on Kodak II-G spectroscopic plates. The samples were determined to be in equilibrium when a fringe displacement greater than 900 μ at a fixed distance from the center of rotation was within 10 μ in photographs taken 3 to 4 hours apart. Fringe displacements were corrected by a water blank. Point-weight-average molecular weights for the linear region were obtained from the slopes of lines as those in Fig. 1 using the equation

$M = \frac{RT}{(1-\bar{v}\rho)\omega^2} \frac{1}{r} \frac{d\ln c}{dr}$, where R is the gas constant, T is the absolute temperature, ρ is the density of the solution, ω is the angular velocity, c is the concentration of the solute, and r is the distance from the center of rotation.

[4] The values reported previously (T. R. Leary and G. Kohlhaw, Fed. Proc. 29, 541, 1970) had been calculated assuming $\bar{v}$ = 0.74 at 5°.

deviation from a straight line is observed below 150μ fringe displacement, which is indicative of a certain degree of polydispersity. This is also obvious from the differences in the cell-weight-average and the cell-z-average molecular weights (Table I). The same enzyme preparation, denatured with 6 M guanidine-HCl - 0.1 M 2-mercaptoethanol, showed no evidence of heterogeneity (T.R. Leary and G. Kohlhaw, unpublished experiments). The data suggest the existence of an association-dissociation equilibrium which is dependent on the leucine concentration as well as on the protein concentration (Table I). Because of the protein concentration dependence, the molecular weight extremes shown in Fig. 2 are neither representative of the fully associated enzyme nor of the degree of dissociation that can be caused by excess leucine. In fact, with decreasing initial protein concentration leucine appears to be able to cause complete dissociation of the enzyme, as suggested by the values in Table I which are in the region of the polypeptide chain weight (cf. Fig. 3).

The apparent molecular weight of 100,000 which was estimated previously from gel filtration of a crude extract of strain CV 19 in the presence of 5 mM leucine (7) may be explained by assuming that the relatively high protein concentration of the extract prevented further dissociation.

It is interesting to note that the leucine concentration required to cause 50% change in apparent molecular weight (60 μM) is of the same order of magnitude as the leucine concentration causing half-maximal inhibition at the same pH (7).

Evidence for the size of the polypeptide chain was obtained from SDS - gel electrophoresis (Fig. 3). A molecular weight of approximately 50,000 was seen repeatedly with or without carboxymethylation of the enzyme. A single protein band was observed in all experiments, suggesting that the subunits are similar. In addition, denaturation

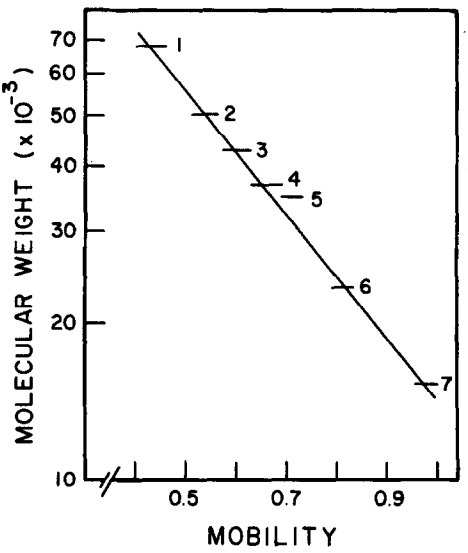

Fig. 3. SDS-Polyacrylamide gel electrophoresis. 1, bovine serum albumin (Pentex); 2, α-isopropylmalate synthase from strain CV 19 (7); 3, ovalbumin (Mann); 4, yeast alcohol dehydrogenase (Worthington); 5, pepsin (Sigma); 6, trypsin (Worthington); 7, bovine hemoglobin (Pentex). The molecular weights of the polypeptide chains used as standards were taken from (11). The horizontal bars indicate the spread of the protein bands.

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of the enzyme with 6 M guanidine-HCl - 0.1 M 2-mercaptoethanol led to a molecular weight of 46,000 $\pm$ 2000, assuming no change in the partial specific volume (T. R. Leary and G. Kohlhaw, unpublished experiments).

It is to be expected that the aggregated form of α-isopropylmalate synthase will predominate only at relatively high protein concentrations. The highest molecular weights observed with different methods have been in the region of 170,000 to 200,000. This is consistent with the existence of a tetramer. However, no definite conclusions can be drawn as to the number of subunits that make up the oligomer without a more elaborate analysis of the dependence of the state of aggregation on enzyme concentration. This analysis is now in progress.

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