Biochimica et Biophysica Acta, 410 (1975) 210—219

BBA 67637

BINDING OF α-KETOISOVALERATE TO α-ISOPROPYLMALATE SYNTHASE

HALF-OF-THE-SITES AND ALL-OF-THE-SITES AVAILABILITY*

ELIZABETH TENG-LEARY** and GUNTER B. KOHLHAW***

Department of Biochemistry, Purdue University, West Lafayette, Ind., 47907 (U.S.A.)

(Received May 15th, 1975)

Summary

Binding of α-ketoisovalerate to α-isopropylmalate synthase (3-hydroxy-4-methyl-3-carboxyvalerate 2-oxo-3-methylbutyrate-lyase (CoA-acetylating), EC 4.1.3.12) from *Salmonella thyphimurium* has been studied by equilibrium dialysis. When α-ketoisovalerate is the only ligand present, no more than two sites per enzyme tetramer can be saturated under the conditions chosen. The binding is non-cooperative with a dissociation constant of 6.6 ± 0.4 μM. Binding of α-ketoisovalerate has also been studied in the presence of propionyl-CoA. This compound was selected because of its close similarity to the natural substrate acetyl-CoA. It is a competitive inhibitor with respect to acetyl-CoA while reacting only extremely sluggishly as a substrate itself. The presence of propionyl-CoA has a profound effect on α-ketoisovalerate binding. The number of sites available to α-ketoisovalerate increases to about four per tetramer. At the same time, the dissociation constant for α-ketoisovalerate increases approx. 4-fold. These results suggest that the active conformation of α-isopropylmalate synthase is not obtained unless both substrates are present. They also support the notion, based on previous studies with the feedback inhibitor L-leucine, that α-isopropylmalate synthase has a tendency to form "functional dimers".

Introduction

α-Isopropylmalate synthase catalyzes the first committed step in leucine biosynthesis in microorganisms and plants. The product α-isopropylmalate is

* Journal Paper No. 5747 of the Agricultural Experiment Station, Purdue University.
** Present address: Department of Laboratory Medicine, University Hospital, University of Washington, Seattle, Wash., 98105, U.S.A.
*** To whom all correspondence should be addressed.

formed by a transfer of the acetyl group of acetyl-CoA to the α-carbon of α-ketoisovalerate. Enzyme from various sources has been shown to be subject to feedback-inhibition by L-leucine. We have recently shown that α-isopropylmalate synthase from *Salmonella typhimurium* appears to be saturated with leucine after only two molecules of the feedback inhibitor have bound per enzyme tetramer of molecular weight 200 000 [1]. The affinity of the enzyme for leucine depends on a number of factors such as presence or absence of substrates, pH, temperature, ionic strength, and enzyme concentration. Under all conditions, however, no more than two sites per tetramer are available to leucine. Since all available evidence is consistent with the polypeptide chains of the enzyme being identical, these results suggest that α-isopropylmalate synthase belongs to the growing class of enzymes with half-of-the-sites reactivity (see recent reviews by Seydoux et al. [2] and Lazdunski [3]). It seemed important to investigate whether half-of-the-sites reactivity would also be found with other ligands, particularly the substrates.

This account describes the interaction of α-isopropylmalate synthase with α-ketoisovalerate. It is shown that, when α-ketoisovalerate is the only ligand present, only two sites per tetramer are available to the substrate. However, in the presence of propionyl-CoA, the closest homologue of the natural substrate acetyl-CoA, the number of sites available to α-ketoisovalerate doubles. At the same time, the affinity for α-ketoisovalerate decreases. These results suggest that α-isopropylmalate synthase assumes a distinct conformation when both substrates are present, in agreement with earlier observations [4] which showed that the presence of acetyl-CoA and an analogue of α-ketoisovalerate shifted the association-dissociation equilibrium in which the enzyme exists in favor of the tetrameric state.

Experimental

Materials

^{14}C-labeled α-ketoisovalerate was prepared from uniformly labeled [^{14}C]valine of specific radioactivity 200 Ci/mol following in principle the procedure of Meister [5] established for α-ketoisocaproate. The starting material contained 0.053 mg [^{14}C]valine (in 0.9 ml), 0.2 mg carrier valine, 10 units L-amino acid oxidase, and 5 units bovine liver catalase in a total volume of 2 ml. The final pH was titrated to pH 7.2 with NaOH. The reaction mixture was incubated at 37°C with a continuous stream of air bubbling through the solution. The conversion of valine to α-ketoisovalerate was followed by (a) the disappearance of ninhydrin-positive material at the valine position on a thin-layer chromatography plate (Kodak silica gel sheet) developed in *n*-butanol/water/acetic acid (2 : 1 : 1, v/v) (Solvent A), and (b) the appearance of radioactivity at the α-ketoisovalerate position on a thin-layer plate also chromatographed in Solvent A. The reaction was more than 95% complete after 35 h. Fresh amino acid oxidase and catalase were added to the incubation mixture once during this period. The reaction mixture was diluted to 10 ml with water and concentrated to 2 ml using a 10 ml Amicon Diaflo concentrator with PM-30 ultrafiltration membrane. This procedure was repeated four times until only negligible radioactivity remained in the concentrator. The combined fil-

trates were then concentrated by a Buchi rotary flash evaporator to approx. 3 ml, keeping the temperature below 45°C. The concentrate was passed through a cation-exchange column (2 ml Dowex 50w-X2, 200—400 mesh). The column was eluted with distilled water until the eluate was basic on Congo red paper. The eluate was then titrated to pH 6.9 with KOH and concentrated to approx. 0.6 ml with the flash evaporator. The final yield of α-ketoisovalerate was 65%, based on the amount of radioactivity recovered. The purity was checked by thin-layer chromatography in both solvent A and solvent B (95% ethanol/chloroform/NH_4OH (28% assaying ammonia)/water in a ratio of 53 : 30.3 : 15.2 : 1.5, v/v) on Kodak silica gel or cellulose plates. The ^{14}C-labeled α-ketoisovalerate position was identified by (a) spraying with 2,4-dinitrophenylhydrazine solution (0.4% in 2 M HCl) followed by spraying with 40% KOH; (b) the iodine vapor procedure; (c) radioactivity. The chromatographed thin-layer plates did not react with ninhydrin. The R_F value of the synthesized ^{14}C-labeled α-ketoisovalerate was identical with that of authentic α-ketoisovalerate (R_F = 0.67 on silica gel plates in Solvent A and 0.76 on cellulose plates in Solvent B). The most likely decomposition product of α-ketoisovalerate, isobutyric acid, has R_F values of 0.15 and 0.16 in Solvent A and Solvent B, respectively. No radioactivity could be detected at any position other than the α-ketoisovalerate position. The concentration of the synthesized ^{14}C-labeled α-ketoisovalerate was determined by (a) complete turnover by purified α-isopropylmalate synthase in the presence of acetyl-CoA and subsequent determination of free CoA with 5,5′-dithio-bis-(2-nitrobenzoate); and (b) keto acid determination by 2,4-dinitrophenylhydrazine [6,7]. It was found to be 2.18 ± 0.03 mM. The specific radioactivity was $7.35 \cdot 10^{10}$ cpm/mmol or 42 Ci/mol, i.e. identical to that of the diluted [^{14}C] valine used as starting material.

Uniformly labeled L-[^{14}C]valine was purchased from ICN. Coenzyme A (lithium salt, "chromatopure") was a product of P-L Biochemicals. Acetyl- and propionyl-CoA were prepared from CoA and the appropriate acid anhydride following the general procedure of Simon and Shemin [8]. L-Leucine, L-valine, α-ketoisovalerate, α-ketoisocaproate and 5,5′-dithio-bis-(2-nitrobenzoic acid) were obtained from Sigma Chemical Co. α-Isopropylmalate was isolated from a *Neurospora crassa* mutant according to Burns et al. [9]. 2,5-Diphenyloxazole and 1,4-bis-2-(5-phenyloxazolyl)-benzene were from Arapahoe Chemicals, Inc. Scintillation grade toluene was obtained from Matheson, Coleman and Bell. The equilibrium dialysis membrane was size 20/32 from Union Carbide. All other chemicals were of the best available grade.

Methods

Enzyme isolation. α-Isopropylmalate synthase was purified from *S. typhimurium* strain CV-19 as previously reported [10,11]. The purity of the final preparations was determined by scanning gel patterns obtained after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and staining according to Fairbanks et al. [12]. Throughout this study, enzyme of purity 92—95% was employed.

Assay of α-isopropylmalate synthase activity. Activity was measured by determining the amount of CoA formed after a timed incubation of enzyme with 0.8 mM acetyl-CoA, 4 mM α-ketoisovalerate, 80 mM KCl, and 200 mM

Tris · HCl buffer, pH 8.5, at 37°C in a total volume of 0.125 ml. The CoA concentration was determined with 5,5'-dithio-bis-(2-nitrobenzoate) as described before [10]. Deviations from this standard assay are indicated where appropriate. Activity units are defined as μmol of CoA formed per h. Specific activity is defined as activity units/mg α-isopropylmalate synthase and is obtained by multiplying activity units/mg protein by 100/% purity to correct for the impurities present.

Protein determination. Protein concentration was determined by the biuret method with bovine serum albumin as standard [13]. Nitrogen determination of dried bovine serum albumin was performed to give the true protein content before a standard curve was made. Dry weight analysis of the purified enzyme corresponded closely to the biuret protein determination.

Equilibrium dialysis. The binding experiments were performed by equilibrium dialysis exactly as described previously for leucine [1]. The time required to reach equilibrium was between 2 and 3 h. The temperature was 4°C, the pH was 6.8 (50 mM potassium phosphate buffer with 10% (v/v) glycerol). Enzyme activity was determined at the start and conclusion of each experiment. No significant change in activity was observed.

Radioactivity measurements. A scintillation cocktail containing 9.8 g 2,5-diphenyloxazole, 0.244 g 1,4-bis-2-(5-phenyloxazolyl)-benzene, 1000 ml absolute ethanol and 1710 ml scintillation grade toluene was used. 92% counting efficiency for ^{14}C and 52% counting efficiency for ^{3}H were obtained with the above cocktail with a Beckman CPM-100 or Beckman LS-100 scintillation counter.

Results

Extrapolation of binding data to maximum specific activity of α-isopropylmalate synthase

Substrate binding data should ideally be obtained with enzyme of the highest possible specific activity and in the absence of inactive enzyme species. These conditions could not be achieved, however, in the present case. While the purity of the α-isopropylmalate synthase preparations used was between 92 and 95%, the specific activity was only 50—60% of the predicted maximum value since a significant portion of the activity was almost always lost during the last step in purification. This step consists of concentration by ultrafiltration and subsequent precipitation by 70% saturated $(NH_4)_2SO_4$ and is required in order to produce enzyme concentrated enough for use in equilibrium dialysis. Fortunately, as will be shown below (Fig. 1), the number of available α-ketoisovalerate binding sites exactly paralleled the specific activity of the enzyme. It was therefore possible to extrapolate the binding data to the maximum specific activity of the enzyme and thus obtain the number of available substrate sites per molecule of fully active enzyme. The likely maximum specific activity was arrived at from a large number of purifications without the final concentration step from which values were obtained which approached but never exceeded 1000 ± 10 units per mg of enzyme. Although this does not prove that enzyme with higher specific activity does not exist, the value of 1000 will be assumed to be the maximum specific activity attainable under assay conditions. That

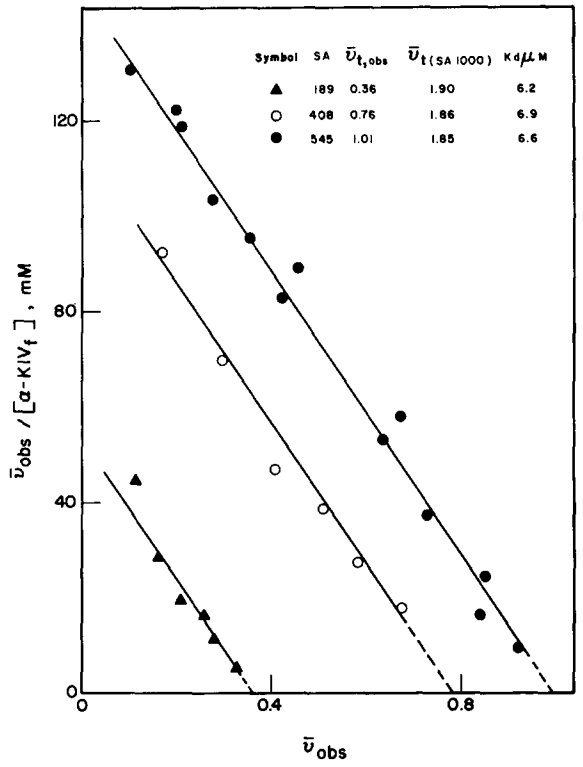

Fig. 1. Binding of α-ketoisovalerate to α-isopropylmalate synthase of different specific activities. $\bar{\nu}_{obs}$, mol of bound α-ketoisovalerate/mol of enzyme tetramer (experimental values); α-KIV$_f$, free α-ketoisovalerate; $\bar{\nu}_{t,obs}$, number of saturable α-ketoisovalerate binding sites per tetramer, as extrapolated from each straight line; $\bar{\nu}_{t(SA1000)}$ = calculated number of α-ketoisovalerate binding sites per tetramer of fully active enzyme (spec. act. = 1000), obtained by multiplying $\bar{\nu}_{t,obs}$ by the ratio $1000/SA_{actual}$ where SA is specific activity. K_d, dissociation constant. The enzyme preparation used had an original specific activity of 545. The lower specific activities were generated by incubating the enzyme at 37°C for 2 and 12 h, respectively. This was done to avoid contamination with extraneous protein. The enzyme concentration during equilibrium dialysis was 4 mg/ml.

this assumption is reasonable is suggested by the fact that a binding ratio of close to four molecules of α-ketoisovalerate per enzyme tetramer can be calculated (in the presence of propionyl-CoA, see below) if the data are corrected for a specific activity of 1000.

Binding of α-ketoisovalerate in the absence of other ligands

Correlation between number of available α-ketoisovalerate binding sites and specific activity of α-isopropylmalate synthase. The fact that concentrated enzyme with a specific activity near the predicted maximum could not be obtained raised questions as to the validity of the substrate binding data because of the possibility that catalytically inactive enzyme would still bind substrate or would have some effect on the binding behavior of the active enzyme. However, we proceeded with the binding studies when it was established that there existed a direct proportionality between specific activity and the number of available α-ketoisovalerate binding sites while at the same time

no significant variation of the dissociation constant for α-ketoisovalerate with enzyme of different specific activity was observed. Results with enzyme of three different specific activities are shown in Fig. 1. Linear Scatchard plots were obtained in all cases, indicating lack of cooperativity between binding sites, at least within the range of α-ketoisovalerate concentrations tested. While the dissociation constants remained practically unchanged at various specific activities, the number of binding sites saturable, $\bar{\nu}_{t,obs}$, was directly proportional to the specific activity (see inset in Fig. 1). Calculated on the basis of maximum specific activity (SA) of 1000, the number of α-ketoisovalerate binding sites per tetramer of fully active enzyme, $\bar{\nu}_{t(SA\,1000)}$, became approx. 1.9 in all three experiments. Increasing the protein concentration from 4 to 8 mg/ml did not change the binding parameters.

Binding in the presence of the substrate homologue, propionyl—CoA

As shown in the previous section, α-ketoisovalerate is apparently capable of saturating no more than two sites per tetramer in the absence of other ligands under the conditions employed. It was important to examine if the same held true in the presence of the second substrate, acetyl-CoA. Since binding in the presence of both natural substrates was not feasible due to rapid product formation, we synthesized the substrate homologue propionyl-CoA which was found, within experimental error, to behave as a competitive inhibitor of α-isopropylmalate synthase with respect to acetyl-CoA. The apparent K_i value for propionyl-CoA was 1.6 mM. Although there was no turnover of propionyl-CoA in the kinetic studies, a very sluggish formation of free CoA from propionyl-CoA was observed when the enzyme concentration was high. Thus, during the course of a binding experiment in the presence of 2 mM propionyl-CoA and 0.11 mM ^{14}C-labeled α-ketoisovalerate, and with 4 mg/ml of enzyme present, between 15 and 20% of the propionyl-CoA was hydrolyzed in 3 h, the duration of the experiment shown in Fig. 2. This probably meant that as much α-ketoisovalerate had been converted to the product analogue, α-isopropyl-β-methylmalate.

The (labeled) α-isopropyl-β-methylmalate might be expected to perturb the equilibrium in the dialysis cell. However, on the basis of additional experiments performed at an enzyme concentration (1 mg/ml) which resulted in only about 5% hydrolysis of propionyl-CoA, we concluded that the potential error caused by product formation was negligible: the binding parameters obtained with enzyme concentrations of 4 and 1 mg/ml were essentially the same (see below). Moreover, free CoA was shown by experiment not to interfere with α-ketoisovalerate binding. In two parallel trials, α-ketoisovalerate binding was studied at an enzyme concentration of 4 mg/ml in the absence of CoA and in the presence of 46 μM CoA, respectively. This CoA concentration was more than twice as much as could maximally be generated in a binding experiment. No effect was noted on either the dissociation constant or the number of α-ketoisovalerate binding sites available.

Fig. 2 shows that the presence of propionyl-CoA has a profound effect on the binding of α-ketoisovalerate. First, the number of α-ketoisovalerate binding sites available per tetramer of fully active enzyme ($\bar{\nu}_{t(SA\,1000)}$) went from 1.85 (no propionyl-CoA) to 3.50 (2 mM propionyl-CoA). Secondly, the disso-

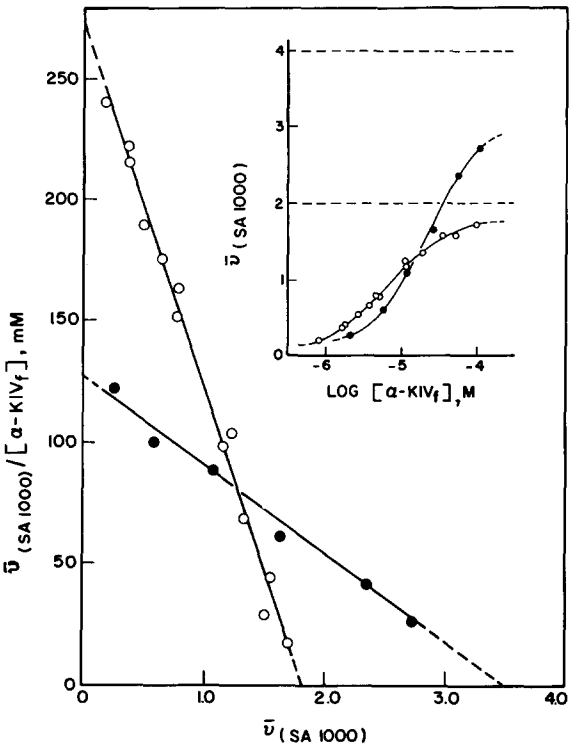

Fig. 2. Binding of α-ketoisovalerate(α-KIV) to α-isopropylmalate synthase in the absence of propionyl-CoA (○) and in the presence of 2 mM propionyl-CoA (●). $\bar{\nu}_{(SA1000)}$ is used as defined in the legend to Fig. 1. The straight lines were fitted to the points by the method of least squares. Enzyme concentration during equilibrium dialysis was 4 mg/ml.

ciation constant for α-ketoisovalerate increased from 6.6 μM in the absence of propionyl-CoA to 28 μM in the presence of 2 mM propionyl-CoA. Judging from the straight line obtained in the Scatchard plot, the α-ketoisovalerate sites remained non-cooperative. Shown as an inset in Fig. 2 is a semilogarithmic plot ($\log[\alpha\text{-KIV}_f]$ vs $\bar{\nu}_{(SA1000)}$ where α-KIV denotes α-ketoisovalerate) of the type recommended by Klotz [14,15] as a more reliable way to determine whether saturation with ligand is being approached or not. Saturation is clearly approached in the experiment without propionyl-CoA. In the presence of propionyl-CoA, a saturation plateau has not been reached, but a bending of the curve is evident. The inflection point in this case appears to be located at about −4.6 (x-axis), corresponding to a $\bar{\nu}_{(SA1000)}$ value of about 1.7. Since, as also pointed out by Klotz [15], the inflection point in this type of graph appears half-way toward saturation if the binding sites are non-interacting and equivalent, saturation would be expected to occur at a $\bar{\nu}_{(SA1000)}$ value of about 3.4.

Three additional binding experiments, performed at the same propionyl-CoA concentration of 2 mM but at an enzyme concentration of 1 mg/ml, yielded total α-ketoisovalerate binding site numbers per tetramer of fully active enzyme of 3.6, 4.2, and 3.8, with the corresponding dissociation constants

being 27, 29, and 30 μM, respectively. In all of these experiments, the binding of α-ketoisovalerate was non-cooperative, and the number of sites saturable was directly dependent on the specific activity of the enzyme.

Discussion

α-Ketoisovalerate is the second ligand of α-isopropylmalate synthase for which conditions are known under which only half as many binding sites are available as would be expected on the basis of the number of identical subunits. The feedback inhibitor L-leucine was previously shown to saturate no more than two sites per tetramer under a great variety of conditions, including the presence of α-ketoisovalerate, acetyl-CoA, or acetyl-CoA plus α-ketoisocaproate [1]. While it is possible that α-ketoisovalerate and leucine encounter (or generate) similar "functional dimers" when they bind to α-isopropylmalate synthase, we do not believe this to be the case, since there are important differences in the way the two ligands interact with the enzyme. First, unlike the feedback inhibitor, α-ketoisovalerate exhibits no positive cooperativity when studied in the absence of other ligands (although extreme negative cooperativity may exist and may indeed be the reason for the observed half-of-the-sites availability, see below). Also unlike what was seen with the feedback inhibitor, the number of available α-ketoisovalerate binding sites parallels the specific activity of the enzyme. This indicates that inactivation of the enzyme during purification or during incubation at 37° C (the method used to intentionally generate lower specific activities) involves destruction of the α-ketoisovalerate binding site. Thirdly, there is at least one condition, viz. the presence of propionyl-CoA, where four α-ketoisovalerate sites per tetramer become available. It should also be noted that the enzyme is dissociated by leucine while α-ketoisovalerate appears not to have any effect on the association-dissociation equilibrium [4]. Thus, while the enzyme exists primarily as a dimer in the presence of leucine, it behaves as a "dimer of dimers" in the presence of α-ketoisovalerate.

In attempting to explain half-of-the-sites availability, we face the same problem that other workers have faced before, i.e. to distinguish between several possible interpretations: (a) minor, so far undetected differences in the polypeptide chains that make up the enzyme; (b) generation of a binding site by association of two monomers; (c) electrostatic or steric exclusion of the second ligand after the first has bound; (d) subunit association-induced ("pre-existing") asymmetry; or (e) ligand-induced asymmetry. We have addressed ourselves to some of these questions before [1] and will not repeat all the arguments here. Genetic [16,17] and physical-chemical evidence [4,18] suggests only one type of polypeptide chain in α-isopropylmalate synthase, although crucial sequence and X-ray crystallographic data are not available for this enzyme. Therefore, no final judgement can be made on the absolute identity of the subunits. However, the fact that the presence of propionyl-CoA causes the appearance of two additional α-ketoisovalerate binding sites per tetramer and that the four sites per tetramer are apparently equivalent and independent, would tend to rule out possibilities (a) as well as (b) and (c). It is more likely that α-ketoisovalerate either encounters or generates an asymmetrical structure, according to possibilities (d) or (e), and that propionyl-CoA prevents or re-

verses such asymmetry. "Generating an asymmetrical structure" is of course equivalent to causing conformational changes of the enzyme. While no direct evidence for a conformational change caused by α-ketoisovalerate is available, it is known that this substrate decreases the reactivity of sulfhydryl groups [19] and gives rise to an ultraviolet difference spectrum which may reflect the net exposure of aromatic amino acid residues. α-Ketoisovalerate also increases the affinity of the enzyme for leucine [1].

The observation of a doubling of the sites available to α-ketoisovalerate when propionyl-CoA is present provides additional evidence for the notion that α-isopropylmalate synthase exists in a unique conformation in the presence of both substrates. It was noted before that the tetrameric state of the enzyme was strongly favored when enzyme which had been reacted with the potential active-site labeling reagent 3-bromopyruvate was incubated with acetyl-CoA [4]. Also, the pattern obtained after cross-linking with dimethyl suberimidate and subsequent sodium dodecyl sulfate gel electrophoresis was changed in favor of tetramers when enzyme was incubated with both (but not with either) acetyl-CoA and α-ketoisocaproate, a competitive analogue of α-ketoisovalerate. It is therefore clear that the exposure of new α-ketoisovalerate sites cannot be a consequence of enzyme dissociation, but rather involves changes in the tertiary (or secondary) structure of the enzyme.

It is of interest that no cooperativity between α-ketoisovalerate sites was observed, irrespective of the presence or absence of propionyl-CoA. This is in agreement with kinetic results which indicate that saturation with α-ketoisovalerate occurs in a seemingly straightforward Michaelis-Menten fashion [10,20].

Few other cases are known where a substrate analogue enhances the binding capacity for another ligand. One example is aspartate transcarbamylase where the number of available carbamyl phosphate sites was found to increase from approximately three to approximately six in the presence of the aspartate analogue succinate [21]. In a further striking analogy to α-isopropylmalate synthase, succinate also increased the dissociation constant for carbamyl phosphate. Another example is the binding of NAD^+ and $NADP^+$ to ox liver glutamate dehydrogenase [22]. In this case, glutarate (a glutamate analogue) increased the affinity for the coenzymes and also appeared to increase the maximum binding capacity for them. Unfortunately, the authors of both papers state that the experimental errors inherent in their experiments were too large to allow precise quantitation of their data.

In view of the complex binding pattern seen with two ligands of α-isopropylmalate synthase, i.e. leucine and α-ketoisovalerate, it will be of great interest to collect binding data also for the third major ligand of this enzyme, i.e. acetyl-CoA. Efforts to obtain such data are now underway.

Acknowledgement

This research was supported by Grant GM 15102 from the National Institutes of Health, U.S. Public Health Service.

References

1 Teng-Leary, E. and Kohlhaw, G.B. (1973) Biochemistry 12, 2980—2986
2 Seydoux, F., Malhotra, O.P. and Bernhard, S.A. (1974) CRC Crit. Rev. Biochem. 2, 227—257
3 Lazdunski, M. (1972) Current Topics in Cellular Regulation (Horecker, B.L. and Stadtman, E.R., eds), Vol. 6, pp. 267—310, Academic Press, New York
4 Leary, T.R. and Kohlhaw, G. (1972) J. Biol. Chem. 247, 1089—1095
5 Meister, A. (1953) Biochemical Preparations (Snell, E.E., ed.), Vol. 3, pp. 66—70, J. Wiley and Sons, Inc., New York
6 Friedemann, T. and Haugen, G. (1943) J. Biol. Chem. 147, 415—442
7 Parsons, S. and Burns, R.O. (1970) Methods in Enzymology (Tabor, H. and Tabor, C.W., eds), Vol. 17A, pp. 793—799, Academic Press, New York
8 Simon, E.J. and Shemin, D. (1953) J. Am. Chem. Soc. 75, 2520
9 Burns, R.O., Umbarger, H.E. and Gross, S.R. (1963) Biochemistry 2, 1053—1058
10 Kohlhaw, G., Leary, T.R. and Umbarger, H.E. (1969) J. Biol. Chem. 244, 2218—2225
11 Teng-Leary, E. (1973) Ph.D. Thesis, Purdue University, West Lafayette, Indiana
12 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606—2617
13 Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751—766
14 Thompson, C.J. and Klotz, I.M. (1971) Arch. Biochem. Biophys. 147, 178—185
15 Klotz, I.M. (1974) Acc. Chem. Res. 7, 162—168
16 Margolin, P. (1963) Genetics 48, 441—457
17 Calvo, J. and Worden, H.E. (1970) Genetics 64, 199—214
18 Bartholomew, J.C. and Calvo, J. (1971) Biochim. Biophys. Acta 250, 577—587
19 Kohlhaw, G. (1970) Biochim. Biophys. Acta 212, 58—64
20 Soper, T. (1974) Ph.D. Thesis, Purdue University, West Lafayette, Indiana
21 Rosenbusch, J.P. and Griffin, J.H. (1973) J. Biol. Chem. 248, 5063—5066
22 Dalziel, K. and Egan, R.R. (1972) Biochem. J. 126, 975—984