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NUMBER AND REACTIVITY OF THE SULFHYDRYL GROUPS OF α-ISOPROPYLMALATE SYNTHASE OF *SALMONELLA TYPHIMURIUM**

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SUMMARY

With three different methods of titration, 16 ± 1 free sulfhydryl groups were found per molecule of α-isopropylmalate synthase from *Salmonella typhimurium*, assuming a molecular weight of 160 000. On the basis of their reaction rate with 5,5′-dithio-bis-2-nitrobenzoate and with 2,2′-dithiodipyridine, at least two types of sulfhydryl groups could be distinguished. The presence of the endproduct inhibitor leucine or, to a lesser extent, of the substrate α-ketoisovalerate caused a general decrease of the sulfhydryl reactivity. Enzymatic activity was lost after 4–5 sulfhydryl groups had been titrated with 5,5′-dithio-bis-2-nitrobenzoate. Incubation with thiol compounds partially restored activity. The restoration was complicated by the finding that the thiol compounds themselves caused inactivation of the unmodified enzyme.

INTRODUCTION

In *Salmonella typhimurium*, as in other organisms, the initial step in leucine biosynthesis consists of a condensation reaction between acetyl-CoA and α-ketoisovalerate. The enzyme catalyzing this reaction, α-isopropylmalate synthase, is subject to feedback inhibition by leucine[1]. It was previously observed that the enzyme was inactivated and partially desensitized against leucine inhibition by minute concentrations of mercuric salts[2]. This behavior indicated a direct or indirect participation of cysteinyl residues in the catalytic action and the regulatory function of the enzyme. It prompted a more detailed study of number and reactivity of the sulfhydryl groups, of which the present paper is a first account.

METHODS AND MATERIALS

Enzyme purification; activity assay. α-Isopropylmalate synthase from Strain CV-19 was purified to apparent homogeneity essentially as described previously[2]. The only major modification was made in the hydroxylapatite chromatography step

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where the 0.35 M phosphate solution used to elute the enzyme was replaced with a linear phosphate gradient between 0.16 and 0.40 M. This made it possible to eliminate the final DEAE-cellulose chromatography step. Enzyme activity was measured by determining the amount of CoASH liberated in a certain period of time[2].

Titrations with p-chloromercuribenzoate were performed according to the method of BOYER[3]. 250–450 μg of enzyme (1.56–2.81 nmoles) were incubated at 23° with 50 μmoles of sodium phosphate buffer (pH 7.0) in a total volume of 1 ml, and the $A_{250\text{ nm}}$ was recorded. Then, aliquots of 2–4 nmoles of *p*-chloromercuribenzoate were added and the increase in $A_{250\text{ nm}}$ was followed after each addition. The readings were corrected for the increase in volume, a blank without enzyme, and the $A_{250\text{ nm}}$ of the enzyme itself. Titrations in the presence of urea were begun after a pre-incubation period of 30 min at 23°.

Titrations with 5,5'-dithio-bis-2-nitrobenzoate were performed using between 150 and 250 μg of enzyme (0.94–1.56 nmoles). The enzyme was incubated at 23° in a total volume of 0.5 ml with 100 μmoles of Tris buffer (pH 8.5), 4 μmoles of KCl, and additions as indicated. The titration was usually started by adding 5,5'-dithio-bis-2-nitrobenzoate (final concentration 0.6 mM). The change in $A_{412\text{ nm}}$ was followed and the amount of 4-nitro-5-carboxythiophenolate formed was calculated using a molar extinction coefficient of 13 600 (ref. 4).

For titration with 2,2'-dithiodipyridine the conditions were as follows: 300–400 μg of enzyme (1.88–2.50 nmoles) were incubated at 23° in a final volume of 1 ml with 80 μmoles of Tris buffer (pH 8.0) and other compounds as indicated. The reaction was started by adding 2,2'-dithiodipyridine to give a final concentration of 0.5 mM. The increase in $A_{343\text{ nm}}$ due to the liberation of 2-mercaptopyridine was followed using a molar extinction coefficient of 7060 (ref. 5) for quantitation.

Protein concentration was measured by the biuret method[6] using as a standard bovine serum albumin which had been dried to constant weight and the nitrogen contents of which had been determined by the micro Kjeldahl method.

Special materials and their suppliers were: bovine serum albumin, crystallized, Pentex; sodium *p*-chloromercuribenzoate, and 2-mercaptopyridin, Aldrich; sodium dodecyl sulfate, and urea ultrapure, Mann; CoASH, lithium salt, chromatopure, PL Chemicals. 2,2'-Dithiodipyridine was a gift from E. H. Ulm of this laboratory. Acetyl-CoA was synthesized from CoASH and acetic anhydride, following the procedure of SIMON AND SHEMIN[7]. All other chemicals used were of the best available grade.

RESULTS

Titration of SH groups; influence of ligands on the reaction rates

The following estimations of the sulfhydryl contents of α-isopropylmalate synthase from *Salmonella typhimurium* are based on a molecular weight of 160 000 for the native enzyme. This molecular weight corresponds to an average value calculated from gel filtration experiments with calibrated Sephadex G-100 or G-200 columns using partially or highly purified enzyme (ref. 2, and unpublished results)*.

* Since recent analyses indicate the presence of an association–dissociation equilibrium which is strongly influenced by leucine and the protein concentration[10], an assessment of the "true" (*i.e.* fully associated) molecular weight of α-isopropylmalate synthase will have to await the completion of sedimentation equilibrium and related experiments.

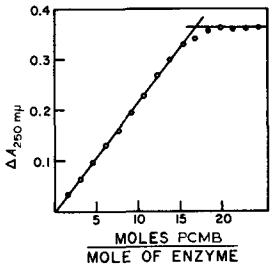
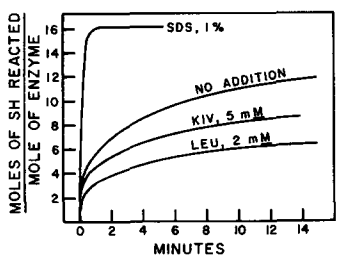


Fig. 1. Titration of α-isopropylmalate synthase with *p*-chloromercuribenzoate (PCMB). 427 μg of highly purified enzyme were used in the experiment presented above. The procedure outlined under METHODS AND MATERIALS was followed.

Three different methods were used to determine the number of free SH groups of the enzyme. They were reaction with *p*-chloromercuribenzoate, 5,5′-dithio-bis-2-nitrobenzoate, and 2,2′-dithiodipyridine, respectively. Fig. 1 shows the result of a titration of the native enzyme with *p*-chloromercuribenzoate. The values obtained with this method varied between 16 and 17 SH groups per enzyme molecule. During the titration of the first 6–7 SH groups it took less than 1 min for each increment of *p*-chloromercuribenzoate to react to completion, while in the later phases of the titration the reaction time increased to about 5 min. These differences in reactivity were not observed after the enzyme had been pre-incubated with 8 M urea; rather, reaction with each increment of *p*-chloromercuribenzoate was immediate. The presence of urea did not, however, change the end point of the titration; still, only 16–17 SH groups reacted per enzyme molecule.

Results obtained with 5,5′-dithio-bis-2-nitrobenzoate are shown in Figs. 2 and 3. The reaction of the native enzyme with this SH reagent was slow compared to the reaction with *p*-chloromercuribenzoate. The progress curve of Fig. 2 labelled "no addition" shows that after 14 min less than three quarters of the total number of free



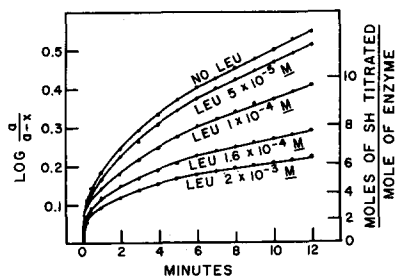


Fig. 2. Titration of α-isopropylmalate synthase with 5,5′-dithio-bis-2-nitrobenzoate. In each of the titrations presented above 219 μg of highly purified enzyme were used. The details of the procedure are given under METHODS AND MATERIALS. SDS, sodium dodecyl sulfate; KIV, α-keto-isovalerate; LEU, leucine.

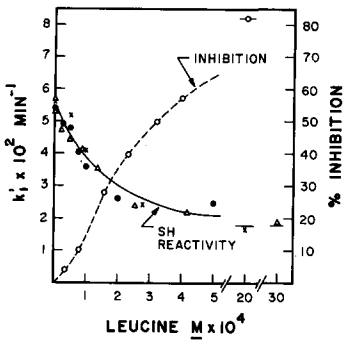
Fig. 3. The influence of leucine on the rate of reaction of 5,5′-dithio-bis-2-nitrobenzoate with α-isopropylmalate synthase. Data from experiments like those shown in Fig. 2 were plotted according to the equation for a (pseudo) first order process, $\log a/(a-x) = kt/2.303$, where a = the total concentration of SH and $a - x$ = the concentration of SH remaining after time t. LEU, leucine.

SH groups (taken as 16 per molecule) had been titrated. After 1 h, still only about 90% had reacted. Since the maximum decrease in the 5,5'-dithio-bis-2-nitrobenzoate concentration during any of the titrations was only 8%, it appeared feasible to plot the data according to the equation for a first order process (*cf.* Fig. 3). The slope of the resulting curve ("no leucine" curve of Fig. 3, shown for the first 12 min) was found to decrease continuously with time, until, after about 30 min, a constant slope was obtained. In order to quantitate the observed rate differences, pseudo first order rate constants were calculated from the slopes at different times. The rate of the very fast initial reaction between the SH reagent and the native enzyme could not be accurately determined. A crude estimation (obtained from an expanded-scale plot) yielded a constant of approx. 4 min^{-1}. After about 6 min of reaction, when between 9 and 10 SH groups had been titrated, the pseudo first order rate constant had dropped to 0.057 min^{-1}. The final value, reached after 30 min, was 0.014 min^{-1}. This supposedly reflects a different availability of individual sulfhydryl groups under the conditions employed. It would seem that at least two, but more likely several types of SH groups can be distinguished on this basis.

In the presence of sodium dodecyl sulfate the differences in SH reactivity were largely abolished, and 16–17 SH groups per molecule of mol. wt. 160 000 were titrated within a short period of time. With 1% sodium dodecyl sulfate and a pre-incubation time of 2 min, the titration was complete within 1 min (Fig. 2).

A considerable decrease of the SH reactivity took place when either leucine or α-ketoisovalerate were included in the reaction mixture (Fig. 2). α-Ketoisocaproate, a competitive inhibitor of the enzyme with respect to α-ketoisovalerate, slowed down the SH reactivity very much like the ketoacid substrate. Acetyl-CoA which was checked up to a concentration of 2 mM, had no effect. The apparent reaction rates seen with this substrate had to be corrected for a slow, constant reaction of the acetyl-CoA preparation itself with 5,5'-dithio-bis-2-nitrobenzoate. Valine, which is structurally related to leucine without being a feedback inhibitor, caused but a very slight decrease of the SH reaction rate, even at a concentration of 10 mM.

The effect of leucine on the SH reactivity, which was most striking, was analyzed in more detail. The reaction between the enzyme and 5,5'-dithio-bis-2-nitrobenzoate in the presence of excess leucine (2 mM) was followed for a total of 1 h under standard conditions. The slope of the (pseudo) first order rate curve decreased drastically during the first few minutes (Fig. 3, curve labelled LEU 2 mM) and, as was observed with no leucine present, reached a constant value after about 30 min, which in this case corresponded to a pseudo first order rate constant of 0.004 min^{-1}. After 1 h, 8–9 SH groups per molecule had reacted, and the reaction was still proceeding. As is shown in Fig. 3, decreasing the leucine concentration led to a gradual increase of the overall SH reactivity. When the pseudo first order rate constants were compared, it was found that the relative differences of these constants as a function of leucine concentration stayed essentially the same over the whole period of reaction (with the possible exception of the first 30 sec for which accurate values could not be obtained under the conditions employed). Therefore, the change of rate constants at any one moment could be chosen for an analysis of the leucine effect. The rate constants used in Fig. 4 (k_1') have for convenience been calculated from the slopes obtained between 6 and 10 min (Fig. 3). It is felt that these values adequately reflect the retarding effect of leucine on the reaction between 5,5'-dithio-bis-2-nitrobenzoate and α-isopro-



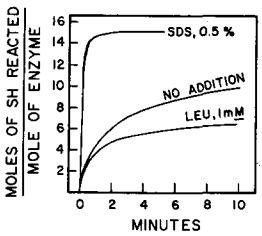


Fig. 4. Change of pseudo first order rate constant k_1' and inhibition of enzymatic activity as a function of leucine concentration. The rate constants were determined from the slopes of curves as in Fig. 3 after 6 min of reaction had passed (see text). The different symbols correspond to different series of experiments. Leucine inhibition was studied in 0.2 M Tris buffer (pH 8.5) with 80 mM KCl, 0.8 mM acetyl-CoA, and 4 mM α-ketoisovalerate. Temperature in inhibition studies 37°.

Fig 5. Titration of α-isopropylmalate synthase with 2,2'-dithiodipyridine. 380 μg of highly purified enzyme were used in the experiments shown. Details of the procedure are given under METHODS AND MATERIALS. SDS, sodium dodecyl sulfate; LEU, leucine.

pylmalate synthase. The range of leucine concentrations causing the decrease in the SH reactivity extended from about 0.05 to about 0.5 mM. As indicated in Fig. 4, this was exactly the range which, at the same pH, also encompassed the steep increase in inhibition of the enzymatic activity by leucine. This coincidence points to a relationship between the two leucine effects, *viz.*, inhibition of activity and protection of sulfhydryl groups, although it has to be realized that the conditions under which the two types of experiments were performed were not identical, especially with respect to the presence or absence of substrates and differences in enzyme concentration.

Titration of the sulfhydryl groups with 2,2'-dithiodipyridine according to the method of GRASSETTI AND MURRAY[5] yielded results which were qualitatively and quantitatively similar to, but not identical with, those obtained with 5,5'-dithio-bis-2-nitrobenzoate. In the experiment shown in Fig. 5, 15.2 moles of SH per 160 000 g of enzyme were titrated in the presence of 0.5% sodium dodecyl sulfate. Again, the reaction proceeded much more slowly in the absence of a denaturing agent and was slowed down even further when leucine was present.

Inactivation by 5,5'-dithio-bis-2-nitrobenzoate; partial reactivation by SH compounds

When the enzymatic activity of α-isopropylmalate synthase was measured while the enzyme reacted with 5,5'-dithio-bis-2-nitrobenzoate (under conditions identical to those employed for SH titration) it was found that within the limits of accuracy of the assay the activity had completely disappeared after about 40 sec (*cf.* Table I). At this time, 4–5 SH groups per molecule of mol. wt. 160 000 had reacted. If the inactivation was indeed due only to the reaction of 5,5'-dithio-bis-2-nitrobenzoate with 4–5 "essential" sulfhydryl groups, and if the effect of leucine was to generally decrease the SH reactivity, one would expect leucine to cause a delay of the inactivation corresponding to the additional time it takes for the "essential" SH

TABLE I

INACTIVATION OF α-ISOPROPYLMALATE SYNTHASE BY 5,5′-DITHIO-BIS-2-NITROBENZOATE (DTNB) AND EFFECT OF THIOL COMPOUNDS ON INACTIVATED AND NATIVE ENZYME

Treatment of enzyme before assay	*Duration of treatment*	*% Activity after treatment (appropriate control = 100%)*
DTNB (0.6 mM)*	30 sec	10
	40 sec or longer	< 5
DTNB (0.6 mM) *plus* leucine (2 mM)*	30 sec	32
	60 sec	13
	120 sec	9
	180 sec	< 5
DTNB (0.5 mM)	4 min	
followed by dithiothreitol (10 mM)**	30 min	35
DTNB (0.5 mM)	4 min	
followed by cysteine (20 mM)**	30 min	50
Dithiothreitol (10 mM)**	5 min	84
	30 min	33
Cysteine (20 mM)**	30 min	64

* Conditions were identical to those described for the titration of the enzyme with DTNB (*cf.* METHODS AND MATERIALS). At the indicated times, aliquots were diluted 80–100-fold into the assay mixture which contained 50 μmoles of Tris (pH 8.5) 20 μmoles of KCl, 0.2 μmole of acetyl-CoA, and 0.5 μmole of α-ketoisovalerate in a total volume of 0.25 ml. The final DTNB concentrations were not inhibitory.

** Approx. 200 μg of highly purified enzyme were incubated at 23° and pH 8.5 (0.1 M Tris buffer; final volume 0.6 ml) with the additions and for the periods indicated. Before any additions were made, an aliquot was withdrawn to determine activity. After the incubation the mixtures were layered on top of a 5-ml Sephadex G-25 column equilibrated with 50 mM potassium phosphate buffer (pH 6.8). The enzyme was eluted with the same buffer. The temperature during this operation was 4°. Total activity was then determined in the eluate. Control experiments were performed identically except that no DTNB, dithiothreitol, or cysteine was added. The procedure as outlined above was designed to remove the SH compounds which otherwise would interfere with the activity assay, itself based on the determination of CoASH (ref. 2).

groups to react in the presence of a given concentration of leucine. As shown in Table I, when leucine was present at a concentration of 2 mM, the inactivation of the enzyme was indeed delayed by the predicted 2–3 min.

Reactivation of the 5,5′-dithio-bis-2-nitrobenzoate-inactivated enzyme was attempted by incubating it with SH compounds. As demonstrated in Table I, at most 50% of the original activity could be restored after 30 min of incubation with either 10 mM dithiothreitol or 20 mM cysteine. Although it is possible that more time was needed for proper rearrangement of the enzyme, it was not feasible to extend the time of incubation with the SH compounds because of observations also listed in Table I; both dithiothreitol and cysteine were themselves inhibitory if left with the enzyme. As shown for dithiothreitol, the degree of inactivation increased with the length of time of incubation. The question of the existence of one or more essential disulfide bonds, put forth by these findings, is the subject of further investigations.

DISCUSSION

The different reactivity of the sulfhydryl groups of α-isopropylmalate synthase

seen especially with 5,5'-dithio-bis-2-nitrobenzoate and 2,2'-dithiodipyridine and reflected by the largely different pseudo first order rate constants at different times of reaction may be explained by assuming that the fast reacting SH groups are more exposed on the enzyme surface and/or less involved in side chain hydrogen bonding than the slowly reacting groups. The electronic environment of the individual SH groups does probably not play an important role in their reactivity since both disulfides used for titration produced similar effects in spite of carrying different charges.

As far as the influence of small molecules on the SH reactivity is concerned, the available evidence supports the notion that in the presence of leucine or, to a much smaller extent, of α-ketoisovalerate the enzyme undergoes conformational changes which result in a general decrease of the reactivity of the SH groups. A direct steric protection of all cysteinyl residues seems unlikely in view of the relatively large number of those residues: even if the enzyme were made up of four identical subunits, for which there is some evidence[10], there would still be 4–5 sulfhydryl groups per subunit. It is not clear at present whether or not the conformations generated by leucine and α-ketoisovalerate are similar. Since the two ligands are believed to bind to separate sites on the enzyme[2], one would expect the effects to be different. Obviously, additional criteria will have to be used to reach a more definite conclusion.

Other feedback inhibitors have been seen to exert a negative effect on the SH reactivity of their respective enzymes[8,9]. However, α-isopropylmalate synthase from *Salmonella typhimurium* is an especially intriguing example because of the observation that leucine also specifically favors a dissociation of the enzyme[2,10]. It will be interesting to find out in which way the two alterations are interrelated. Both occur at leucine concentrations which also cause inhibition.

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REFERENCES

1 C. Jungwirth, S. R. Gross, P. Margolin and H. E. Umbarger, *Biochemistry*, 2 (1963) 1.
2 G. Kohlhaw, T. R. Leary and H. E. Umbarger, *J. Biol. Chem.*, 244 (1969) 2218.
3 P. D. Boyer, *J. Am. Chem. Soc.*, 76 (1954) 4331.
4 G. Ellman, *Arch. Biochem. Biophys.*, 82 (1959) 70.
5 D. R. Grassetti and J. F. Murray, *Arch. Biochem. Biophys.*, 119 (1967) 41.
6 A. G. Gornall, C. J. Bardawill and M. M. David, *J. Biol. Chem.*, 177 (1949) 751.
7 E. J. Simon and D. Shemin, *J. Am. Chem. Soc.*, 75 (1953) 2520.
8 P. Truffa-Bachi, R. Van Rapenbusch, J. Janin, C. Gros and G. N. Cohen, *European J. Biochem.*, 5 (1968) 73.
9 J. C. Gerhart and H. K. Schachman, *Biochemistry*, 7 (1968) 538.
10 T. R. Leary and G. Kohlhaw, *Biochem. Biophys. Res. Commun.*, 39 (1970) 494.