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α-Isopropylmalate Synthase from *Alcaligenes eutrophus H 16*

I. Purification and General Properties*

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Abstract. α-Isopropylmalate (IPM) synthase, the first enzyme in the biosynthesis of L-leucine, was purified to a specific activity of 12 μmole/min × mg protein from the valine-isoleucine double auxotrophic mutant A-81 of the hydrogen bacterium *Alcaligenes eutrophus H 16*. The activity in crude extracts of derepressed cells was 0.106 μmoles of isopropylmalate formed per min and per mg protein. Gel electrophoresis and regel electrophoresis of the isolated main band resulted in several distinct bands, which were not altered by the additions of substrate α-ketoisovalerate, feedback inhibitor leucine or other effectors.

The isoelectric points of the enzyme protein was between 3.9 and 4.0. The molecular weight was 114500 daltons and 100000 respectively in the absence and presence of the feedback inhibitor leucine. The enzyme activity depended strongly on the pH, the optimum is at pH 8.2. The enzyme was could labile and exhibits temperature anomalies.

Key words: Hydrogen bacteria – *Alcaligenes eutrophus H 16* – Leucine biosynthesis – α-Isopropylmalate synthase – Temperature anomaly – Cold lability.

In *Alcaligenes eutrophus H 16* the branched chain amino acids are synthesized (Hill and Schlegel, 1969; Reh and Schlegel, 1969) via the pathway (Strassman et al., 1956) common to all organisms. Studies on trifluoroleucine resistant mutants of *A. eutrophus H 16* (Hill and Schlegel, 1969) indicated that the regulation of the branched chain amino acids biosynthetic pathway and the feedback regulation of the IPM synthase in this organism differs from those in other organisms studied so far (*Saccharomyces* spec.; Sai et al., 1969; Ulm et al., 1972, *Neurospora crassa*; Webster and Gross, 1965, *Salmonella typhimurium*; Kohlhaw et al., 1969). The feedback inhibition of IPM synthase by leucine and the growth inhibition of *A. eutrophus* by trifluoroleucine was overcome by L-valine (Hill and Schlegel, 1969). Experiments with crude extracts indicated that no leucine dependent dissociation-association equilibrium exists as in *S. typhimurium*. The IPM synthase from *S. typhimurium* was the only bacterial enzyme studied in detail. Therefore, the enzyme attracted further attention, and the present paper describes the purification and the properties of IPM synthase of the hydrogen bacterium *A. eutrophus H 16*.

Abbreviations Used. CoA = Coenzyme A; Tris = Tris(hydroxymethyl)aminomethane hydrochloride; DTNB = 5,5′-dithiobis-(2-nitrobenzoic acid); IPM = α-Isopropylmalate; KIV = α-Ketoisovalerate

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MATERIALS AND METHODS

Organisms and Culture Conditions. Alcaligenes eutrophus strain *H 16* (ATCC 17699; Wilde, 1962) was obtained from Deutsche Sammlung von Mikroorganismen, Göttingen. The isoleucine and valine double auxotrophic mutant *H 16*–A 81 was provided by Dr. Reh (Reh and Schlegel, 1969)

The wild type cells were grown heterotrophically or autotrophically in defined media described by Schlegel et al. (1961) in 9 l-batches in a Biostat-fermenter (Braun, Melsungen) at 32° C. The mutant cells were grown in the presence of 1 mM L-isoleucine and 1 mM L-valine. Batches of the mutant *H 16*–A 81 used for enzyme purification were grown under conditions which caused derepression of the enzymes of the branched-chain amino acids pathway; i.e. growth was limited by continually adding small amounts of L-valine or L-isoleucine. High enzyme activities were obtained, too, when limiting amounts of the amino acids (0.75 mM L-valine or L-isoleucine) were added to the medium before growth. The cells were harvested by centrifugation (Cepa-centrifuge, Padberg, Lahr; 32000 × g) in the late logarithmic growth phase and stored at −20° C.

Chemicals. α-Ketoisovalerate (KIV) and phenylmethane-sulfonyl fluoride (PMSF), were obtained from Sigma Chemical Company, St. Louis, U.S.A. Aquacide I was purchased from Calbiochem. Inc., Los Angeles, U.S.A. Alumina Alcoa 305 and 5,5′-dithiobis-

(2-nitrobenzoic acid) (DTNB) were purchased from Serva Entwicklungslabor, Heidelberg, Germany. The materials for column chromatography were obtained from W. & R. Balston Ltd., Maidstone, Kent, U.K. (Whatman DE 52), and from Pharmacia, Uppsala, Sweden (Sephadex). Coenzyme A (trilithium salt), acetyl phosphate (K, Li), phosphotransacetylase, combithek calibration proteins and citrate synthase (E.C. 4.1.3.7) were obtained from C. F. Boehringer, Mannheim, Germany. Ampholine Carrier Ampholyte was a product of LKB Produktor AB, Stockholm, Sweden. Acetyl-CoA was prepared by the method of Simon and Shemin (1953). D,L-α-Isopropylmalate (IPM) was a generous gift from Dr. W. R. Martin, Chicago, U.S.A.

Preparation of Crude Extracts. The packed frozen cells were resuspended in 1.5 parts (w/v) of extraction buffer containing 250 mM potassium phosphate, pH 7.5, 2 mM α-ketobutyric acid, 5 mM L-valine and 100 mM potassium fluoride. Disruption of cells was accomplished by two means: Small quantities were passed twice through a French pressure cell (Amicon) at 2000 kp/cm^2 and at 0–4° C. Larger quantities (50 g wet weight) of cells were continuously sonicated (15 s/ml, Schoeller & Co., Frankfurt/M.) in the presence of 10–20 mg alumina A 305/ml. The temperature was kept below 6° C throughout the treatment.

After sonication cell debris was removed by centrifugation at 20000 × g and 2° C for 30 min. The resulting supernatant liquid contained about 20 mg protein per ml. For experiments with crude extracts the disintegrated cells were centrifuged at 140000 × g and 2° C for 60 min and the supernatant was passed through a Sephadex G-25 column (13 ml gel/ml crude extract) previously equilibrated with 50 mM potassium phosphate (pH = 7.5) supplemented with 100 mM potassium fluoride and 100 mM potassium chloride. After gel filtration the enzyme was stabilized by addition of KIV (1 mM).

Protein Determination. Protein was determined by the method of Beisenherz et al. (1953) and Lowry et al. (1971) both after precipitation with trichloroacetic acid. Crystalline bovine serum albumin was used as a standard.

Enzyme Assay. In crude and partly purified extracts the IPM synthase activity was determined using a minor modification of the fluorimetric assay of Calvo et al. (1969). The standard incubation mixture contained in a volume of 1.00 ml: 250 μmoles Tris-HCl buffer pH 8.2, and 250 μmoles potassium chloride (or 250 μmoles potassium phosphate pH 8), 20 μmoles KIV, 20 μmoles acetyl phosphate (Li_3), 0.2 mg coenzyme A and 0.01 mg phosphotransacetylase. The incubation time was normally 20 min at 37° C. The following modified procedures were used for the fluorimetric measurements: Two aliquots of 2.0 ml ether extract were evaporated at 60° C, and two aliquots of each acidic isopropylumbelliferone solution were brought to a pH 10.2 with saturated sodium carbonate solution supplemented with 30 g boric acid/l. The fluorescence was measured in an Eppendorf photometer equipped with a fluorimetric attachment, which was calibrated with glasstandard No. 4617.

After the enzyme had been purified by chromatography on DEAE-Sephadex the activity was routinely measured by monitoring continuously the reaction of DTNB with liberated CoASH at 412 nm (Srere et al., 1963) (Zeiss PM 4 photometer with Servogor recorder, Goerz electro, Wien). The IPM synthase from *A. eutrophus H 16* was not inactivated by DTNB as is the enzyme from *Salmonella typhimurium* (Kohlhaw et al., 1969). The standard incubation mixture contained in a volume of 1.00 ml: 250 μmoles Tris-HCl buffer pH 8.2 or phosphate buffer pH 8.0, 20 μmoles KIV, 1 μmole acetyl-coenzyme A and 1 μmole DTNB. A molar extinction coefficient 11400 ± 600 given by Robyt et al. (1971) was used.

Alternatively, in some kinetic experiments and at pH values lower than 8.0, enzyme activity was measured by continuously monitoring the decrease of acetyl-CoA by following the absorption at 232 nm (Ochoa, 1955). For this test the substrate concentration had to be lowered to 1.5 mM KIV and 0.25 mM acetyl-CoA. The molar extinction coefficient was 5400. The rate of hydrolysis of acetyl-CoA was measured in parallel assays and considered when calculating enzyme activities.

All assays were started by addition of the enzyme, deviations resulted in lower activity. Close agreement was found between the fluorimetric end-point assay anf the continuous optical assay at 232 nm and at 412 nm with DTNB.

The photometer was equipped with a thermostat. When the temperature dependence of the enzyme activity was assayed, the temperature was measured directly in the cuvette using a Metratast P I with microdetector (Metrawatt, Nürnberg); the error was less than 0.1° C. The temperatures in the water baths of the fluorimetric assay were held constant by a Thermomix II (Braun, Melsungen) with an accuracy of better than 0.01° C. Specific activities are expressed as micromoles of products formed per gram of protein per min at 30° C.

The concentration of acetyl-CoA was determined using citrate synthase (E.C. 4.1.3.7) (Srere et al., 1963); Lithium ions and oxidized coenzyme A did not affect the activity and stability of IPM synthase and determination of coenzyme A.

Purification of IPM Synthase. The followed paragraphs describe the methods used in the purification of the enzyme.

Ammonium Sulfate Precipitation and Gel Filtration with Sephadex G-150. Procedures to remove the nucleic acids had to be omitted since treatment by cetyltrimethylammonium bromide, protamine sulfate, streptomycin sulfate as well as by DNase-RNase resulted in coprecipitation or inactivation of the enzyme.

Starting with the second ammonium sulfate precipitation all manipulations occurred at room temperature, since the enzyme was labile at low temperatures, especially at low ionic strength and at low protein concentrations during ion exchange chromatography. Only the initial purification steps had to occur at 0–4° C since at room temperature the proteolytic activity was too high even in the presence of inhibitors.

The soluble protein (20000 g supernatant) was fractionated by the addition of solid ammonium sulfate; the solution was stirred for 10 min before centrifugation at 20000 × g. The fraction between 35 and 70% saturation containing the IPM synthase was dissolved in a minimum volume of 100 mM potassium phosphate buffer (pH 7.5) supplemented with 250 mM potassium chloride, 5 mM L-valine and 4 mM α-ketobutyrate. Ammonium sulfate was removed by gel filtration with Sephadex G-25 (2.5 × 35 cm). Desalted extract (30 ml) was applied to a Sephadex G-150 column (5 × 140 cm) equilibrated with the above supplemented potassium phosphate buffer which was also used for elution. The fractions (20 ml) were collected at a flow rate of 35–40 ml/h. The IPM synthase was eluted from the column as a relatively small peak between the fraction numbers 60–85. These fractions were pooled and concentrated to 6–8 mg protein/ml using a Diaflochamber (filter UM 20 E). A second ammonium sulfate precipitation was carried out at 20–25° C; nearly all the activity appeared in the saturation fraction of 256–346 mg ammonium sulfate per ml extract. All following steps were carried out at 20–25° C.

Chromatography on DEAE-Sephadex A-50. The 45–60% ammonium sulfate fraction was desalted in a Sephadex G-25 column, which was equilibrated with 50 mM potassium phosphate (pH 7.2) containing 0.05% sodium azide and 2.5 mM L-leucine. The desalted protein was applied to a DEAE-Sephadex A-50 column (2.5 × 40 cm) equilibrated with the latter buffer. The enzyme was eluted by a linear gradient between 100 and 200 mM potassium chloride contained in the elution buffer. The total volume was 2 × 1000 ml and the flow rate was 5–6 ml/h. The enzyme was eluted at a concentration of approximately 150 mM potassium chloride. Fractions

containing enzyme activity were pooled and concentrated by Aquacide I. Thereafter the enzyme was passed through a Sephadex G-25 column which was equilibrated with 50 mM potassium phosphate (pH 7.8) supplemented with 4 mM L-valine, 0.05% sodium azide and 50 mM potassium chloride. This extract was applied to a second DEAE-Sephadex A-50 column (2.5 × 30 cm) equilibrated with the buffer of the latest Sephadex G-25 column. The potassium chloride gradient now used was between 100 and 250 mM potassium chloride (2 × 750 ml). The enzyme was eluted at a concentration of approximately 180 mM potassium chloride. Fractions containing IPM synthase activity were pooled and concentrated by Aquacide I and then passed through a Sephadex G-25 column equilibrated with 50 mM potassium phosphate (pH 7.5) supplemented with 20 mM potassium chloride, 0.05% sodium azide and 2.5 mM L-leucine. The enzyme solution was applied to a DEAE-cellulose column (2.5 × 60 cm). The elution of the protein was achieved by a linear potassium chloride gradient (2 × 500 ml) between 50 and 150 mM potassium chloride in the same buffer. The enzyme eluted at approximately 80 mM potassium chloride.

Gel Filtration on Sephadex G-200. The fractions from the DEAE-cellulose column containing enzyme activity were pooled and concentrated to a volume of 1–2 ml with Aquacide I. This concentrate was layered on top of a Sephadex G-200 fine column (2.5 × 100 cm) equilibrated with 50 mM potassium phosphate (pH 8.0), 100 mM potassium chloride, 0.05% sodium azide and 0.2 mM KIV. The enzyme was eluted in a symmetrical protein peak; the maxima for protein and IPM synthase activity corresponded exactly.

Gel Electrophoresis and Isoelectric Focussing. For disc gel electrophoresis the Shandon apparatus (Shandon Scientific Company Ltd., London, England) equipped with glass tubes 0.5 × 7.5 cm or plexiglass tubes 0.7 × 13 cm was used and run at 2–3 mM/tube. The systems of Davis (1964) and Williams and Reisfeld (1964) were used with 7% or 5% and 7.5% or 5% acrylamide, respectively. The amount of protein applied varied between 20 and 150 µg. The preparative plate-gel electrophoresis was done by the method of Stegemann (1972). For 0.6 cm gel a current of 20–30 mA was used and for 1.6 cm gel 60–70 mA. The IPM synthase was detected by incubating the gel in the DTNB-test mixture. The yellow bands were marked with thin wires before the protein was stained with Coomassie blue or Amido black. Electrofocussing was carried out according to the method of Wrigley (1968) in tubes 5 × 100 mm using Ampholine in the pH ranges 3–10 and 3–6 (LKB carrier ampholyte). A current of 1 mA/tube was applied for the first hour and 2 mA/tube for a further 2 h. The enzyme was detected by incubating the gel in the DTNB-assay mixture after shaking in Tris-HCl buffer (pH 8.2, 1.25 M) for 10 min; the protein was stained by the method of Hayes and Wellner (1969). For the measurement of pH and enzyme activity the gel was cut into 1 mm slices and leached in water (CO_2-free) for 1 h. For scanning the gels a densitometer (Chromosorb, Joyce Loebel and Co. Ltd., Gateshead, England) was used at a wavelength of 620 nm (after staining the protein with Coomasie Blue) and 459 nm (after the incubation in the DTNB assay mixture).

Ultrafiltration and Aquacide I Procedure. The 400 ml Diaflo chamber (Amicon corporation, Cambridge/Mass., U.S.A.) was used with UM 20 E filters and a working pressure less than 3 atm N_2. Filters of the type PM for phosphate buffer and organic acids caused precipitation of the protein. Since considerable losses of enzyme activity occurred with solutions containing less than 2 mg protein/ml the enzyme was usually concentrated in a shaken dialysis bag (Union Carbide Corporation, Chicago, U.S.A.) in Aquacide I.

Storage. The purified enzyme dissolved in the buffer of the Sephadex G-200 column was concentrated by Aquacide I, so that the final protein concentration was not less than 0.25 mg per ml. This solution was then frozen in thin layers and stored at −20° C. It was thawed quickly by shaking the tubes in a waterbath (30° C) and kept at room temperature for use.

RESULTS

Enzyme Level at Different Growth Stages

During growth in a batch-culture the highest specific activity of the α-isopropylmalate synthase of *Alcaligenes eutrophus H 16* was observed in cells harvested in the late exponential phase; during heterotrophic growth 10–12 units/g protein were reached. During the early stationary phase the enzyme activity decreased within a few hours to less than 40% of the maximum values. By growing an isoleucine, valine double auxotrophic mutant (*A. eutrophus H 16*-A 81; Reh and Schlegel, 1969) under L-valine or L-isoleucine limiting conditions for the purification of the IPM synthase, enzyme formation became derepressed and the specific activity increased up to 30-fold compared to that of the wild type.

Enzyme Stability

In crude extract enzyme activity was labile and lost about 50% of its activity in 5 h. However, it was stabilized partially (20–30% loss of activity per 5 h) in the presence of a combination of 5 mM L-leucine (or valine), 0.01 M KCl, and 0.5 mM α-ketoisovalerate (KIV) as well as in the presence of inhibitors of proteolytic enzymes such as phenylmethanesulfonyl fluoride (2 mM) or potassium fluoride (0.1 M). The purified enzyme was stable at 30° C and pH 7.5 for more than 10 days. During freezing (−20° C) and thawing, it lost about 10% of its activity, but the enzyme could be stored at −20° C for more than 5 months without further loss of activity (100 mM potassium phosphate, pH 7.5 or pH 8.0 and 0.2 mM KIV).

Purification of the Enzyme

The purification procedure is summarized in Table 1 and resulted in a specific activity of 12 µmoles/min per mg of protein. The overall purification was 113 and the yield 2.25%. The purification was complicated by the cold lability of the enzyme and its susceptibility to attack by proteolytic enzymes. Changes of the protein structure during purification were presumably avoided by the use of gel filtration and ion exchange chromatography (see "Effect of Temperature"). Disc gel electrophoresis of the enzyme purified by the indicated procedure and by preparative plate-gel electrophoresis suggested a homogeneous preparation (see "Gel Electrophoresis").

Table 1. Purification of the IPM synthase from 50 g of wet cells of *Alcaligenes eutrophus H 16*-A 81

Fraction	Total activity	Specific activity units/g protein	Overall purification	Yield (%)
Crude extract	1175	106	–	100
1. Ammonium sulfate (35–70%)	795	306	2.8	68
Sephadex G-150	632	584	5.5	54
2. Ammonium sulfate (45–60%)	480	917	8.6	41
1. DEAE-Sephadex	224	3790	35.4	19
2. DEAE-Sephadex	105	6830	63.9	9
DEAE-cellulose	31	11700	109	2.6
Sephadex G-200	26.5	12050	113	2.25
Preparative gel electrophoresis	–	12050	–	–

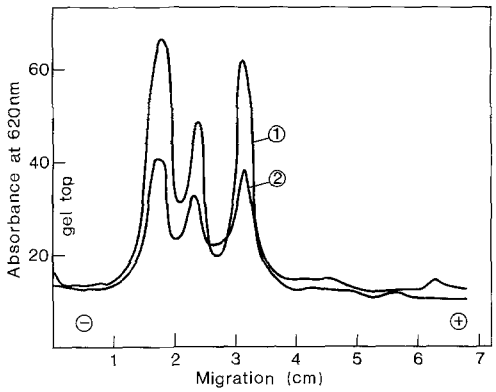

Fig. 1. Disc gel electrophoresis (densitogram) of purified IPM synthase after Sephadex G-200 step (*1*) and after preparative plate gel electrophoresis (*2*). For the re-gel electrophoresis (*2*) the main band of a preparative gel was cut out and eluted with 10 mM Tris-HCl buffer supplemented by 1 mM KIV. The system of Williams and Reisfeld (1964) was used in all cases. After staining with Coomasie Blue the gel was scanned for protein absorbance with a densitometer at a wavelength of 620 nm

During ion exchange chromatography the presence of L-leucine of L-valine in the elution buffer resulted in a slight alteration of the adsorptive properties of the enzyme. During gel filtration on Sephadex G-150 or G-200 the elution profile was altered by leucine, too; leucine was applied in varied concentrations (0.05–5.0 mM) and both pH 7.5 and 8.1. However, the effect was so small (see molecular weight determination) that it could not be applied for enzyme purification as reported for the enzyme from *Salmonella typhimurium* (Kohlhaw et al., 1969).

Gel Electrophoresis

The purified IPM synthase was subjected to analytical disc gel electrophoresis in 7% polyacrylamide gels. The gel exhibited one major (R_f 0.415) and three less intensive bands (R_f 0.46; 0.62; 0.65) in the system of Davis (1964) and one strong (R_f 0.28) and two less intensive bands (R_f 0.36; 0.46) in the system of Williams and Reisfeld (1964). The IPM synthase activity was localized in the main band in both systems. Less activity was found in the smaller band with the highest R_f-value. No further bands could be detected, when the gel (0.7 × 13 cm) was heavily overloaded. Thioesterases were proved to be absent.

The main bands were isolated by means of preparative "plate-gel electrophoresis" (Stegemann, 1972). Re-gel electrophoresis of the protein from the main bands gave again one major and three or two bands, respectively, as that of the original purified enzyme (Fig. 1). The addition of L-leucine, L-valine and/or KIV to the gel and to the gel electrophoresis buffer did not alter the number, the position or the intensity of the bands.

Molecular Weight, Isoelectric Point, SH-Groups and pH-Optimum

For IPM synthase an apparent molecular weight of 114500 daltons was found by gel filtration on Sephadex G-200 in the presence of reference proteins (Ackers, 1964; Bergmeyer, 1974). This molweight was in accordance with the sedimentation coefficient $S_{20,w} = 6.88 \times 10^{13}$ determined by using sucrose density gradient centrifugation (Martin and Ames, 1961). For both determinations 0.1 mM KIV had been added to the enzyme and to the buffer to stabilize the enzyme. A molecular weight of 100000 daltons was found by gel filtration on Sephadex G-200 (using the same columns as before) in the absence of KIV but in the presence of 2.5 mM leucine, using two different concentrations of protein. The purified enzyme (0.8 mg protein/ml) did not contain accessible free SH-groups as tested with DTNB in the absence and presence of 1% sodium dodecylsulfate. The presence

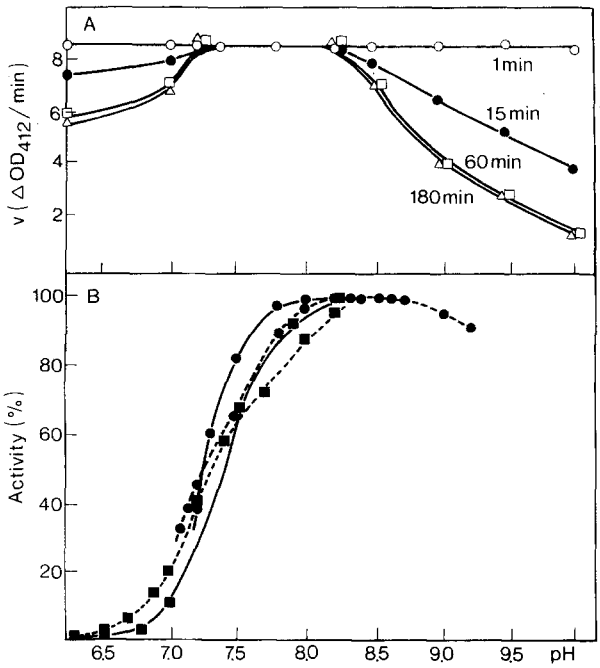

Fig. 2. (A) The effect of pH on the stability of the IPM synthase activity at 20° C. The incubation buffer contained 50 mM potassium phosphate (pH 6.0–8.0) or 50 mM Tris-HCl (pH 8.0–10.0). After 1 min (○), 15 min (●), 60 min (□) and 180 min (△) aliquots were withdrawn and assayed for residual IPM synthase activity. The DTNB assay with 250 mM Tris-HCl pH 8.1 at 30° C and with substrate concentrations of 1.5 mM KIV and 0.5 mM acetyl-CoA and the enzyme preparation after the DEAE-cellulose step was used. (B) Effect of pH on the activity of IPM synthase. The enzyme activity was determined using the fluorimetric assay (*solid lines*) started with crude extract after gel filtration on Sephadex G-25 (1.2 mg protein/ml volume of the assay). Using 110-fold purified enzyme (4.2 µg protein/ml volume of the assay) the activity was determined with the optical assay at 232 nm (*dashed lines*). The substrate concentration was 0.75 mM KIV and 0.188 mM acetyl-CoA. Activity at the pH optimum was taken as 100%, e.g. 150 rel. fluorimetric units/20 min or ΔOD_{232}/min = 0.25. Buffer used: 250 mM Tris-HCl containing 250 mM KCl (●) and 250 mM potassium phosphate (□)

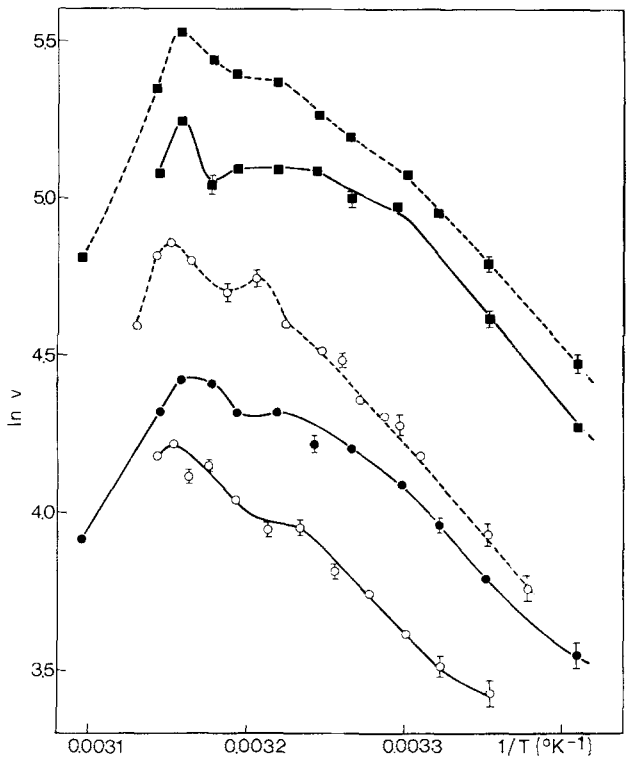

Fig. 3. The effect of temperature on IPM synthase activity (Arrhenius-plot). The fluorimetric assay was employed for determination of enzyme activity using crude extract after gel filtration on Sephadex G-25 (*dashed lines*) and partially purified enzyme (DEAE-cellulose step) (*solid lines*). At the indicated temperature the assay mixture was preincubated for 5 min without enzyme and for further 50 min after adding the enzyme. Buffer used: 250 mM Tris-HCl pH 7.5 ($\Delta pH/T = -0.01$) (Dawson et al., 1969) (●) and pH 8.0 ($\Delta pH/T = -0.0028$) (Weast, 1968/69) (■); 250 mM potassium phosphate pH 7.5 (○). To avoid crossovers the lower curves in the figure were shifted on the ordinate to other values. The values are the means of two parallel experiments with the range as indicated by vertical bars, otherwise the differences were in the range of the size of the symbols

of disulfide bonds (under further investigation) was detected by the methode of Robyt et al. (1971).

The isoelectric point was determined by isoelectric focussing with Ampholine and found to be between pH 3.9 and 4.0. The pH-optimum of enzyme activity measured in Tris-HCl buffer was between 8.0 and 8.5 (Fig. 2B). At pH 7.0 the enzyme activity was about 15% of maximal activity. In phosphate buffer, the highest activity was observed at pH 8.2. No significant differences in the pH dependence of the activity were encountered using crude extract (tested by means of the fluorometric enzyme assay) or purified enzyme (using the optical assay with DTNB). In the range from 7.2 to 8.2, the activity of the purified enzyme remained stable for several hours, whereas at higher and lower pH-values rapid inactivation occurred (Fig. 2A).

The Effect of Temperature

The temperature optimum of the purified enzyme was 44° C. The buffer (Tris-HCl or phosphate), the assay employed, the pH-value (7.5 and 8.1), and the concentration of the substrate (acetyl-CoA) had no marked influence on the temperature optimum. In the interval between 55–60° C, the activity of the enzyme decreased drastically and reached zero at 60° C. If, however, the enzyme was incubated for 5 min at 65° C in the presence of its substrates KIV and acetyl-CoA and was then quickly cooled to 40° C, the enzyme

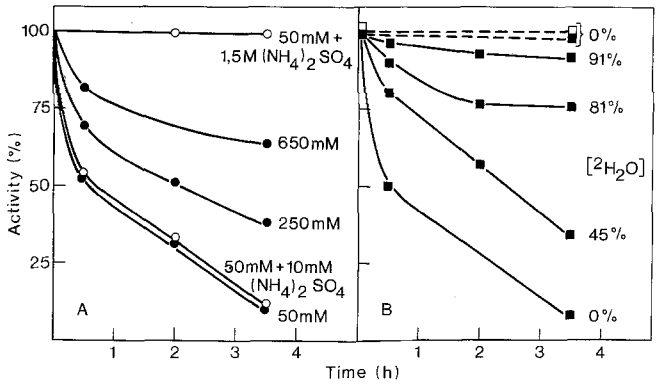

Fig. 4A and B. The effect of ionic strength (A) and of ^2H_2O concentration (B) on enzyme stability at 0° C. The 70-fold purified enzyme was stored on ice at pH 7.5 in solutions containing: (A) Potassium phosphate (50, 250 and 650 mM) and ammonium sulfate as indicated; (B) 25 mM (■) and 250 mM (□) potassium phosphate and ^2H_2O as indicated in percent. The dashed lines represent the control experiment at 23° C. The enzyme activity was determined by measuring initial velocities using the DTNB test (250 mM potassium phosphate, pH 8.1)

activity was not impaired. In the absence of the substrates incubation of the enzyme for 5 min at 60° C resulted in irreversible denaturation.

Anomalous, non-linear curves were obtained in the Arrhenius-plots (Fig. 3). This was observed especially in phosphate buffer at pH 7.5 and less in Tris-HCl buffer at pH 8.0. Identical results were obtained irrespective of whether the optical test at 232 and 412 nm or the fluorimetric test with crude extracts or purified enzyme were used. No alterations of the anomalous curves were observed when the enzyme was preincubated at 40° C for 20 min, and then cooled to 20° C before starting the assay. From the Arrhenius-plot (Fig. 3), an activation energy E_A of the enzyme reaction of 10.3 ± 0.8 kcal/mol was calculated for the temperature range from about 28 – 35° C.

At low temperature a rapid loss of enzyme activity was observed. The cold lability could be overcome by stabilizing the enzyme either by buffers of increased ionic strength or by replacing H_2O by ^2H_2O as is shown in Figure 4. The stabilizing effect of ^2H_2O on the enzyme at 0° C indicated that according to Lee and Berns (1968) hydrophobic effects between polypeptide chains are important for maintaining the native structure of this IPM synthase.

Cation Effects

In contrast to the stabilization of the IPM synthase at low temperature with KCl, no significant increase of activity was observed with the monovalent cations Li^+, K^+ or Na^+ added as chlorides or sulfates (Davis, 1964) using Sephadex G-25 treated enzyme (100 ml gel per ml of enzyme). High concentrations of KCl or K_2SO_4 inhibited the enzyme about 20% (pH 8.1, 400 mM; pH 7.5, 250 mM). No significant effect was detected with divalent cations, except with Mn^{2+}; the enzyme was slightly activated (25%) by 1 mM $MnCl_2$.

DISCUSSION

The specific activity of IPM synthase in extracts from *Alcaligenes eutrophus H 16* is similar to that of other organisms (12 – 15 units/g protein); *Neurospora crassa* (Webster and Gross, 1965); *Salmonella typhimurium* (Kohlhaw et al., 1969); *Saccharomyces* spec. (Sai et al., 1969; Ulm et al., 1972); *Paracoccus denitrificans*, (Fischer, 1973); maize seedlings (Oaks, 1965). Assuming that the purified enzyme was homogeneous at a specific activity of 12 units/mg protein, and that the crude extract represented the total soluble cellular protein, IPM synthase constituted 0.05% of the cell protein in autotrophically grown cells of *A. eutrophus H 16* and 0.1% in heterotrophically grown cells. For the mutant *H 16*-A 81 grown under conditions of derepression of IPM synthase a maximum value of 2.8% of the cellular protein was calculated. This value is comparable to 1.25% calculated for *S. typhimurium* CV-19 (Kohlhaw et al., 1969).

A significant change of the enzyme during the purification procedure seems improbable since neither the activation energy nor the temperature anomalies of the Arrhenius plot were altered. The temperature anomaly seems to be real. It is certainly not due to either a non-linear function of the enzyme test, or to a different influence of temperature on the forward and the reverse reaction or to the presence of isoenzymes, since different methods for measuring the enzyme activity have been applied since the IPM synthase reaction can be regarded to be an irreversible reaction (Webster and Gross, 1965) and since isoenzymes were not detected. An anomalous dependence of the reaction rate on the temperature is regarded as a sensitive indicator of reactions occurring at the enzyme, especially of minor changes of enzyme conformation, in the neighbourhood of the catalytic site (Han, 1972; Talsky, 1971). Therefore, both the described anomalous temperature dependence of enzyme activity and the reversible inactivation of the enzyme at a temperature higher than 44° C might be due to reversible changes of the enzyme conformation. The activity of all IPM synthases investigated depended strongly on the pH and decreased drastically within a narrow range of the pH below the pH-optimum which was in range of pH 8.0 – 8.5 for the enzyme from *A. eutrophus H 16*, *S. typhimurium* (Kohlhaw et al., 1969), and *Saccharomyces* spec. (Ulm et

al., 1972), and between pH 7.0 and 7.8 for the enzyme from *S. carlsbergensis* (Sai et al., 1969), *N. crassa* (Robyt et al., 1971), and *P. denitrificans* (Fischer, 1973).

The IPM synthase from *A. eutrophus* differs from those of other organisms in cold lability and anomalous temperature dependence. The apparent molecular weight determined by gelfiltration of about 114500 is the smallest so far known for IPM synthases; a rapid dissociation-association equilibrium of the subunits in the absence of leucine could not be excluded, therefore, the molecular weight might be higher. However, if this equilibrium of the subunits exists, it did not strongly depend on the concentration of leucine. This conclusion was drawn from several gelfiltrations with different leucine concentrations and analytical gel-electrophoresis in the presence and absence of leucine; no alteration of the bands was observed. In contrast, the association-dissociation equilibrium of the IPM synthase from *Salmonella typhimurium* was strongly dependent on protein and leucine concentrations, resulting in molecular weights between 50000 to 200000 daltons (Kohlhaw and Boatman, 1971) and in a single gel electrophoresis band only in the presence of leucine.

In analogy to the conclusions drawn by Webster and Gross (1965) for the IPM synthase from *N. crassa*, the relatively small differences of the apparent molecular weight of the enzyme from *A. eutrophus H 16* eventually resulted from a change of the Stokes' radius and the frictional rate during the binding of leucine.

In contrast to the enzymes from fungi (Ulm et al., 1972; Webster and Gross, 1965) the activity of the enzyme from *A. eutrophus* was not dependent on monovalent cations.

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