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CHARACTERIZATION OF THE HIGHLY ACTIVE ISOCITRATE ($NADP^+$) DEHYDROGENASE OF *AZOTOBACTER VINELANDII*

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SUMMARY

The highly active isocitrate dehydrogenase [*threo*-D$_s$-isocitrate:NADP oxidoreductase (decarboxylating), EC 1.1.1.42] was isolated and characterized from a clarified sonic cell-free extract of *Azotobacter vinelandii* Strain O. The purification was achieved by using a combination of $(NH_4)_2SO_4$ precipitation and preparatory disc electrophoresis. Analytical disc electrophoresis studies revealed that the purified enzyme was a single protein component having an isoelectric point of 6.1. The molecular weight of the *A. vinelandii* isocitrate dehydrogenase was 78 000 and amino acid analyses showed that 3 half-cystine residues were present per molecule of enzyme. The enzyme possessed an exceptionally high v_{max} value. It reduced 722 μmoles of $NADP^+$ per min per mg protein at 37°; the calculated turnover number was 56 000. The apparent K_m for the racemic DL-isocitrate was $3.6 \cdot 10^{-5}$ M and the apparent K_m for $NADP^+$ was $1.8 \cdot 10^{-5}$ M. The enzyme was specific for the *threo*-D-isomer and required Mn^{2+} ($5 \cdot 10^{-4}$ M) for maximum activity. Partial activation also was achieved by Mg^{2+}, Co^{2+} and Cd^{2+}. The active species appears to exist as a polymeric unit consisting of monomers with a molecular weight of 15 000–20 000. The enzyme was sensitive to *p*-chloromercuribenzoate and inhibition was also obtained with ATP, pyrophosphate and by the concerted action of glyoxylate and oxaloacetate.

INTRODUCTION

The resting cell preparations of *A. vinelandii* Strain O used for the isolation of the isocitrate dehydrogenase were grown under N_2-fixing conditions using acetate as the sole source of carbon. The isocitrate dehydrogenase present in this microorganism is unusual in that it possesses an exceptionally high specific activity. In the initial stages of purification, its specific activity is at least 10–100 times higher than any other NAD^+- or $NADP^+$-dependent dehydrogenase found in this organism. The purified enzyme exhibited specific activities at least 10–30 times higher than that of any other purified isocitrate dehydrogenase. The possibility exists that this

Abbreviation: PCMB, *p*-chloromercuribenzoic acid.

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unusually high activity is associated with the N_2-fixing capabilities of the Azotobacter species, the NADPH formed serving as the primary source of reducing equivalents. This possibility is further augmented by the fact that the Azotobacter also possesses relatively large amounts of an unusually active transhydrogenase[1], which would then allow for NADH generation by the reduction of NAD^+ *via* NADPH, should that form of reduced pyridine nucleotide be required as the prime energy source for the reduction of N_2. Thus it was of interest to isolate and characterize this enzyme in Azotobacter so that it could eventually be compared to other purified isocitrate dehydrogenases from bacterial and mammalian origin.

MATERIALS AND METHODS

Chemicals

The chemicals used, their respective abbreviations and sources, are listed below: $NADP^+$, DL-isocitrate, *threo*-D_5-isocitrate, *p*-chloromercuribenzoic acid (PCMB), Tris–HCl buffer and myokinase (Sigma Chemical Company, St. Louis, Mo.) enzyme grade $(NH_4)_2SO_4$, bovine serum albumin, chymotrypsinogen A (Mann Research Laboratories, New York, N.Y.); ATP (disodium salt), ADP (sodium salt), AMP (P-L Biochemicals, Incorporated, Milwaukee, Wisc.); ampholyte solutions (LKB Instruments, Incorporated, Rockville, Md.). Crystalline D-(−)-β-hydroxybutyric dehydrogenase was obtained as a gift from C. F. Boehringer Company, Tutzing, Germany. The acrylamide gel electrophoresis reagents were obtained from Canalco (Rockville, Md.).

Preparation of cell-free extracts

Cells of *A. vinelandii* Strain O were grown under N_2-fixing conditions in 15-l quantities using glass carboys and forced aeration at 33°. A modified Burk's nitrogen-free medium[2] was used with 1% sodium acetate (73 mM) as the sole source of carbon. The cells were grown to the late log phase and harvested. The cell pastes were washed, homogenized, and standardized turbidimetrically prior to disruption by sonic oscillation. The details of this procedure have been described previously[3,4]. The sonic extract was then subjected to differential centrifugation. The clarified supernatant fraction (designated S_3), which remained after the final centrifugation at 144 000 × *g* for 2 h exhibited the highest specific activity and contained essentially all of the activity units for the isocitrate dehydrogenase.

Acrylamide gel electrophoresis

Several types of acrylamide gel electrophoretic procedures were used in this study. Analytical disc electrophoresis was performed using 0.5 cm × 6.5 cm glass columns. The gel system consisted of a sample gel, spacer gel and a 7.5% acrylamide separating gel. The cathode was at the top of the gels and the anode at the bottom. This procedure has been described in detail by ORNSTEIN[5] and DAVIS[6]. Preparatory acrylamide gel electrophoresis was performed using the Canalco Model PDE-70 Prep Disc column. Other pertinent details are presented under RESULTS and in the legends of the figures as they apply to isolation and characterization of the isocitrate dehydrogenase.

Isocitrate dehydrogenase assay

The spectrophotometric assay used for measuring isocitrate dehydrogenase activity has been described previously[7]. These assay conditions were found to be optimal for the isocitrate dehydrogenase of *A. vinelandii*.

The assay system used to detect isocitrate dehydrogenase activity in acrylamide gels was a modification of that used for the lactate and malate dehydrogenases by GOLDBERG[8]. The final concentrations of the assay reagents were identical to those described previously[7] with the addition of 0.8 mg of *p*-nitrotetrazolium blue and 0.14 mg of phenazine methosulfate per ml of reaction mixture. The acrylamide gels were immersed in this mixture, and incubated in the dark at 37° for 15–45 min. A red band developed at the site of isocitrate dehydrogenase activity.

Amino acid analysis

Samples were first dialyzed against distilled water and evaporated to dryness. 0.1 ml of performic reagent (1 vol. of 30% H_2O_2 in 20 vol. of formic acid) was added to the dried sample and incubated for 1 h at 0°. After performic oxidation the protein sample was dried in a desiccator and hydrolyzed with 6 M HCl for 24 h at 110° (ref. 9). After acid hydrolysis, the protein sample was suspended in 0.7 ml of citrate buffer (pH 2.2) and 0.2 ml (approx. 50 μg of protein) was applied per column on a Beckman-Spinco Model 239) amino acid analyzer. Sulfhydryl content of the isocitrate dehydrogenase was also determined by the spectrophotometric procedure of ELLMAN[10]. Tryptophan content was not determined.

Electron microscopy

The samples used for electron microscopy were prepared by combining several peak fractions of the highly purified isocitrate dehydrogenase. These samples were concentrated to a small volume by $(NH_4)_2SO_4$ precipitation and dialyzed against 0.005 M phosphate buffer (pH 7.5) until all residual NH_4^+ were removed. The specimen was then prepared for negative staining[11]. The sample contained 52 μg protein and was stained with sodium methylphosphotungstic acid (0.25%) at pH 7.0. The final stain solution also contained 75 μl of 0.03% sucrose per ml.

RESULTS

A survey study on the dehydrogenase activities, present in the S_3 fraction of *A. vinelandii*, revealed that the isocitrate dehydrogenase possessed an unusually high activity. Its specific activity (10–12 μmoles $NADP^+$ reduced per min per mg protein at 37°) was at least one order of magnitude higher than that exhibited by any of the other dehydrogenases assayed. The *A. vinelandii* isocitrate dehydrogenase was approx. 40 times more active than the glutathione reductase, glyceraldehyde-3-phosphate and α-ketoglutarate dehydrogenases which were the second, third and fourth most active enzymes, respectively. The D-(−)-β-hydroxybutyrate dehydrogenases also exhibited a reasonable high specific activity, but was still 190-fold less active than the isocitrate dehydrogenase.

Purification of the isocitrate dehydrogenase

The isocitrate dehydrogenase of *A. vinelandii* was isolated in pure form by a

relatively simple procedure previously presented in a preliminary communication[12]. The details which were not previously described are as follows: the first stage of purification involved a stepwise $(NH_4)_2SO_4$ fractionation of the S_3 fraction. The fraction which contained the highest specific activity as well as 50% of the activity units, precipitated at the 70–80% $(NH_4)_2SO_4$ saturation levels, or the S_3 (70–80) fraction. The specific activity for the isocitrate dehydrogenase in this fraction usually ranged from 130 to 150 μmoles of $NADP^+$ reduced per min per mg protein at 37°. The S_3 (70–80) fraction was then subjected to preparatory disc electrophoresis for further purification. The gel system consisted of 8 ml of a sample gel which contained 38 mg of protein, 0.4 cm spacer gel, and a 1.5 cm separating gel consisting of 5% acrylamide. The recommended electrolyte, Tris–glycine (pH 8.3) buffer (Prep-Disc Instruction Manual, Canal Industrial Corporation, Rockville, Md.) was supplemented with $5 \cdot 10^{-4}$ M isocitrate. The fractions were eluted from the preparatory disc column with Tris–glycine buffer (pH 8.8) which contained $5 \cdot 10^{-4}$ M isocitrate and $2 \cdot 10^{-5}$ M

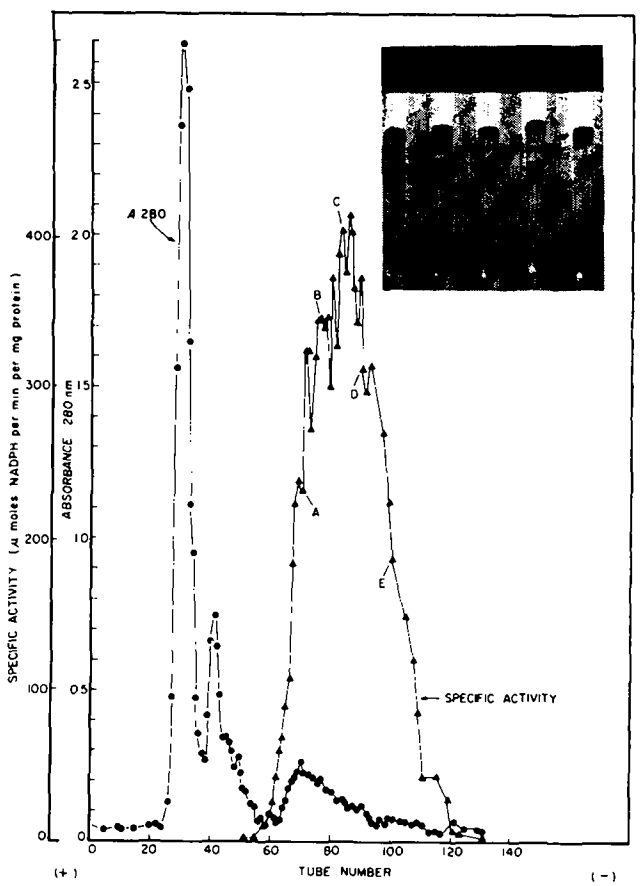

Fig. 1. Elution profile of the S_3 (70–80) fraction which contained the highly active isocitrate dehydrogenase of *A. vinelandii*. The purified enzyme is represented by the trailing peak in the Prep-Disc electrophoresis on 5% acrylamide gel. The inset shows the degree of protein homogeneity of the fractions designated A, B, C, D and E. These were determined by analytical disc electrophoresis on 7.5%. acrylamide gel and stained for protein.

$MnCl_2$. The flow rate for the elution buffer was 2.4 ml/min and a current of 5 mA was used. The total electrophoresis time was 8 h. The elution profile obtained by this type of electrophoresis is shown in Fig. 1. The protein concentration, measured as absorbance at 280 nm (solid line), and specific activity for the isocitrate dehydrogenase (dashed line), are shown on the left ordinate. Both were plotted as a function of the fraction number or volume of eluate.

Electrophoretic migration proceeded from the cathode (top) to the anode (bottom). Fig. 1 shows that the isocitrate dehydrogenase activity was exclusively associated with the trailing protein peak. The fractions indicated on the specific activity profile by the letters A, B, C, D, and E were selected for further analysis by disc electrophoresis using 7.5% acrylamide gels (inset of Fig. 1). Only the leading slope (Tubes No. 65–70) of the activity peak (A of Fig. 1) contained more than one protein band on analysis by disc electrophoresis. The amounts of contaminating proteins were relatively small as indicated by the arrows in Gel A (inset). As shown, the fractions beyond Tube No. 70 revealed only single protein species which represented the purified isocitrate dehydrogenase as shown by gel assays[12].

Molecular weight determination

The molecular weight of the isocitrate dehydrogenase was determined according to the procedure described by ANDREWS[13]. A Sephadex G-100 column (1.5 cm × 60 cm), was equilibrated with 0.02 M phosphate buffer (pH 7.5) and loaded with 1 ml of sample which contained 3–5 mg total protein. The marker proteins used to calibrate the column were the D-(−)-β-hydroxybutyrate dehydrogenase from *Rhodopseudomonas spheroides* (mol. wt. 85 000), bovine serum albumin (mol. wt. 67 000) and chymotrypsinogen A (mol. wt. 25 000). Each marker was passed through the column separately, and the absorbance measurements at 280 nm were used to determine the

TABLE I

AMINO ACID COMPOSITION OF THE ISOCITRATE DEHYDROGENASE OF *A. vinelandii*

Amino acid	*Residues/molecule**
Lysine	63
Histidine	16
Arginine	23
Aspartic acid	69
Threonine**	39
Serine**	87
Glutamic acid	90
Proline	28
Glycine	81
Alanine	80
Half-cystine***	3
Valine	35
Methionine	9
Isoleucine	30
Leucine	48
Phenylalanine	13

* Mol. wt. 78000.
** Corrected to zero time of hydrolysis.
*** Determined by the ELLMAN[10] procedure and as cysteic acid after performic oxidation.

elution profiles of bovine serum albumin and chymotrypsinogen A. Enzyme activity was used to establish the elution patterns for the D-(−)-β-hydroxybutyrate and isocitrate dehydrogenases. The volume of effluent obtained for the isocitrate dehydrogenase from *A. vinelandii* corresponded to a molecular weight of 78 000.

Amino acid analyses

Amino acid analyses were performed on the single protein fractions (Tubes No. 84 and 86) obtained from the Prep-Disc eluate in Fig. 1. The results of amino

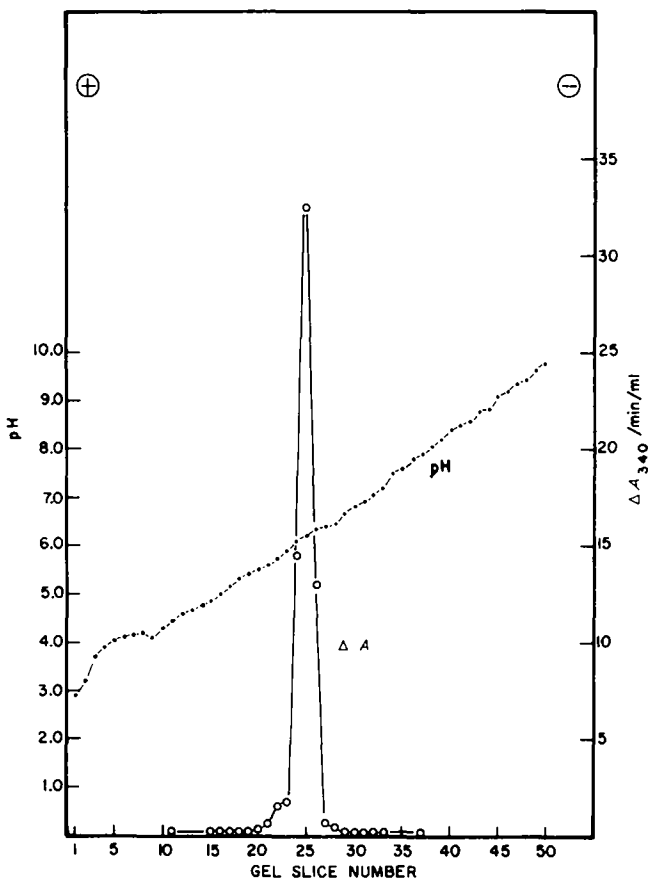

Fig. 2. Acrylamide gel (profile) showing the pH gradient (solid circles), and specific activity analyses (open circles) obtained after isoelectric focusing. Slice No. 25 had the highest specific activity and a pH of 6.1 which represents the isoelectric point of the isocitrate dehydrogenase of *A. vinelandii*.

acid analyses are shown in Table I. The values represent the average integral number of residues per molecule assuming a molecular weight of 78 000 for the isocitrate dehydrogenase. The value given for the half-cystine content is the average integral value obtained by both chromatographic (3.1 residues) and spectrophotometric (3.7 residues) determinations.

Isoelectric point determination

The S_3 (70–80) fraction was used for isoelectric focusing studies to determine the isoelectric point of the *A. vinelandii* isocitrate dehydrogenase[14,15]. Since this value can be obtained using enzyme activity as a marker, it was not necessary to use the highly purified dehydrogenase. The results of this study are shown in Fig. 2. The ampholyte mixture had a pH range of 3–10. The pH for the various gel slices (left ordinate) are plotted as a function of gel slice number. The pH gradient established by isoelectric focusing of the ampholyte mixture is shown by the broken line. The isocitrate dehydrogenase activity (right ordinate), obtained after eluting the enzyme from each slice, is shown by the solid line. The highest rate of activity was found in gel Slice No. 25, which corresponded to the fraction having a pH value of 6.1. Since the enzyme concentrated at this point, the p*I* of the isocitrate dehydrogenase from *A. vinelandii* is 6.1. This p*I* value was verified by use of another ampholyte mixture which exhibited a much narrower pH range closer to the isoelectric point. The electrofocusing profile pattern was almost identical to that shown in Fig. 2 except that the pH gradient ranged between pH 5 and 7.

Ultrastructure studies

The unstable nature of the highly purified *A. vinelandii* isocitrate dehydrogenase was evident upon examination by electron microscopy. Fig. 3 shows an electron micrograph of the isolated enzyme. The predominant protein structure is relatively small and corresponds to a molecular weight of 15 000 20 000. The larger species of protein, of which only one particle is clearly visible (see inset A), corresponds to a molecular weight of about 85 000. This latter value corresponds closely with the molecular weight value obtained for the isocitrate dehydrogenase by Sephadex chromatography. Inset B appears to be a side view of the larger molecular weight species shown in A. Assuming that the isocitrate dehydrogenase preparation examined

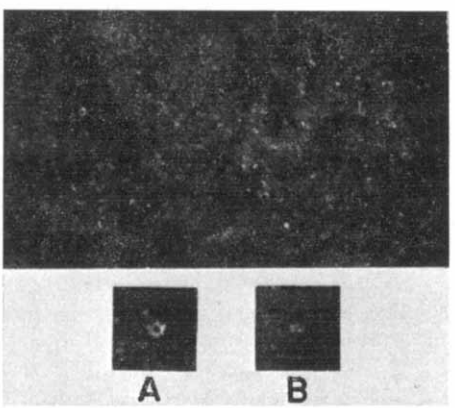

Fig. 3. Electron photomicrograph of the dissociated form of the highly purified isocitrate dehydrogenase of *A. vinelandii*. (magnification 200000×). The inset (A) shows the isolated view of what appears to be the intact polymeric enzyme molecule; while inset (B) is probably the side view of intact enzyme molecule. The magnifications for (A) and (B) are each 400000 —. Molecular weights were estimated from the above electron micrographs by use of the following formula: Outer diameter (sphere) $1.32\sqrt[3]{\text{mol. wt.}}$; an average value of 1.37 g/ml was assumed for the density of protein.

by electron microscopy was highly purified (a valid assumption based on disc electrophoresis analyses at pH 9.5 and 8.5), a high percentage of the species of mol. wt. 85 000 should have been found. Since this was not the case, one can conclude that the majority of the protein molecules observed in the electron micrographs examined did not represent the "native" or active state of the enzyme. This suggests that the enzyme was dissociated into subunits having a molecular weight of 15 000–20 000 which probably occurred during the preparatory phases during which the protein sample was treated for electron microscopic studies. Consequently the predominant protein species remaining after this treatment had a much lower molecular weight than the original enzyme protein. However, the electron micrograph does establish that if the structures seen in insets A and B of Fig. 3 are the natural forms of the *A. vinelandii* isocitrate dehydrogenase, the enzyme is a polymeric unit with a molecular weight of approx. 85 000, the monomers having a molecular weight of 15 000–20 000.

Kinetic characterization studies

The first kinetic parameter examined for the *A. vinelandii* isocitrate dehydrogenase was the effect of $NADP^+$ concentration. The apparent v_{max} was 714 μmoles of $NADP^+$ reduced per min per mg protein at 37°; the apparent K_m for $NADP^+$ was $1.8 \cdot 10^{-5}$ M. With varying substrate concentration, the apparent v_{max} was 729 μmoles of $NADP^+$ reduced per min per mg protein at 37°; the apparent K_m for DL-isocitrate was $3.6 \cdot 10^{-5}$ M. An average of the two maximum specific activities that were calculated at infinite substrate and acceptor concentration was 722 μmoles of $NADP^+$ reduced per min per mg protein at 37°. Therefore, the turnover number for the *A. vinelandii* isocitrate dehydrogenase, based on the v_{max} value of 722 μmoles of $NADP^+$ reduced per min per mg protein at 37° (mol. wt. 78 000), was calculated to be 56 000 at 37°. By comparison, the turnover number for the mammalian $NADP^+$-specific isocitrate dehydrogenase is 3500 at 25° (ref. 16).

Substrate stereospecificity of the *A. vinelandii* isocitrate dehydrogenase was demonstrated by use of the *threo*-D_S-isocitrate isomer as well as by the racemic mixture of DL-isocitrate. Total $NADP^+$ reduction was measured using known amounts of these substrates. The enzymatic reaction was allowed to proceed to completion in the presence of both excess $NADP^+$ and enzyme. In the presence of 0.1 μmole of the DL-isocitrate 0.05 μmole of NADPH was formed, indicating that only 50% of the isocitrate was enzymatically oxidized. With 0.1 μmole of *threo*-D_S-isocitrate, 0.09 μmole of NADPH was formed, indicating that 90% of this form of the substrate was oxidized in the presence of $NADP^+$ and the *A. vinelandii*-isocitrate dehydrogenase. Thus the active isomer is the *threo*-D_S-isocitrate, which is also the natural substrate for all other isocitrate dehydrogenases (mammalian and bacterial) studied to date.

The effect of metal ions

All isocitrate dehydrogenases (both NAD^+- and $NADP^+$-dependent) exhibit a requirement for added metal cations. Maximum activation of the isocitrate dehydrogenase from *A. vinelandii* was obtained when the assay reaction mixture contained 0.5 mM $MnCl_2$. This dependency for Mn^{2+} for activity and its concentration relationship for maximum turnover are shown in Fig. 4. Only 10% of the maximum activity was obtained when no Mn^{2+} were present in the assay mixture. This low degree of

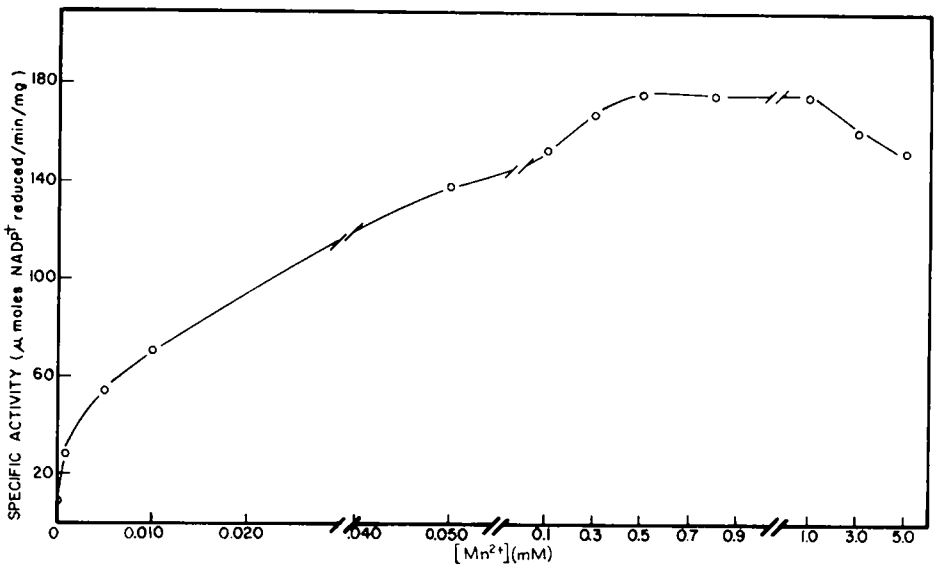

Fig. 4. The effect of Mn^{2+} concentration on the specific activity of the isocitrate dehydrogenase of *A. vinelandii*.

activity could have been due to trace amounts of cations present in the assay reagents. The increase in the specific activity of the isocitrate dehydrogenase was almost linear with respect to Mn^{2+} concentration in the range of 0.001–0.5 mM $MnCl_2$. Maximum stimulation was obtained when $MnCl_2$ reached a level of 0.5–1.0 mM. The enzyme reaction was distinctly inhibited when the amount of Mn^{2+} in the assay mixture was greater than 1.0 mM.

Stimulation of isocitrate dehydrogenase activity by other metal ions was also investigated. At a final concentration level of $5 \cdot 10^{-4}$ M, only Mg^{2+}, Co^{2+} and Cd^{2+} produced substantial activation, which represented 65, 47 and 40%, respectively, of that obtained with $MnCl_2$. Very slight stimulation (approx. 8%) was noted with Cu^{2+} and Zn^{2+}, while no activation was obtained with Fe^{3+}, Mo^{6+}, Ni^{2+}, Ca^{2+}, Al^{3+}, Ba^{2+}, Fe^{2+}, Hg^{2+} and Sr^{2+}. It is of interest to note that Cd^{2+} which has been reported to be a vicinal dithiol inhibitor[17] did not inhibit isocitrate dehydrogenase activity, but actually activated the enzyme when substituted for Mn^{2+}. This finding suggests that none of the 3 sulfhydryl groups contained per molecule of enzyme are functional vicinal dithiols.

Inhibition by ATP

A common inhibitor of isocitrate dehydrogenase activity is the nucleotide, ATP (refs. 18–23). ATKINSON[24] has shown that the NAD^+-specific isocitrate dehydrogenase from yeast is regulated by the ATP/AMP ratio in the presence of a myokinase, a phenomenon referred to as adenylate control. The myokinase added to known amounts of ATP and AMP equilibrated the ATP, ADP and AMP concentrations to their proper "natural" ratios. Regulation by adenylate was not exerted on the $NADP^+$-specific isocitrate dehydrogenase of *A. vinelandii*. Inhibition by an adenylate compound, however, was observed in this system which could be accounted for by

the action of ATP alone. The addition of AMP and/or myokinase did not contribute to this inhibition. At a 1 mM concentration, ATP caused no inhibition of the *A. vinelandii* isocitrate dehydrogenase. At 4 mM, ATP caused 49% inhibition and at a concentration of 10 mM it inhibited the reaction by 74%. Inhibition of the isocitrate dehydrogenase by ATP was competitive with regard to Mn^{2+}. Similar kinetic analyses revealed that ATP exerted a noncompetitive type of inhibition with respect to both $NADP^+$ and isocitrate. Since the pyrophosphate moiety of ATP enables it to act as a metal chelator, this mechanism was investigated to help explain the inhibition by ATP. At a concentration of 10 mM, pyrophosphate produced 89% inhibition while ATP, at this same concentration, caused only 74% loss of activity. The inhibition by pyrophosphate was noncompetitive with respect to Mn^{2+}, as well as with respect to both isocitrate and $NADP^+$. Thus it appears that the chelation of Mn^{2+} by the pyrophosphate moiety of ATP could account for inhibition exerted by ATP. However, ATP inhibition was competitive with respect to Mn^{2+} while pyrophosphate inhibition was not. This suggests that the adenosine moiety of ATP, which is similar to the adenosine moiety of $NADP^+$, allows it to act at or markedly influences the reaction site of Mn^{2+} on the enzyme.

Concerted inhibition studies

The bacterial isocitrate dehydrogenase may also be inhibited by the concerted action of glyoxylate and oxaloacetate[25]. This type of inhibition was noted also for the *A. vinelandii* isocitrate dehydrogenase. When 1 mM glyoxylate *plus* 1 mM oxaloacetate were added to the standard assay system, a 90% decrease in the rate of the reaction was observed. If a 10-fold excess of isocitrate was added after 4 min, the inhibition caused by the combined effect of glyoxylate and oxaloacetate was alleviated, and the rate of the reaction was restored to 87% of the control value. Neither glyoxylate nor oxaloacetate alone produced this marked inhibitory effect. At a concentration of 1 mM, glyoxylate alone produced 17% inhibition while oxaloacetate by itself caused 56% inhibition of the *A. vinelandii* isocitrate dehydrogenase.

Other metabolic intermediates also were examined as possible inhibitors of the *A. vinelandii* isocitrate dehydrogenase. Among these were pyruvate, glutamate,

TABLE II

THE EFFECT OF ISOCITRATE, $NADP^+$ AND Mn^{2+} ON INHIBITION OF THE *A. vinelandii* ISOCITRATE DEHYDROGENASE BY PCMB

Incubation system	*Component initiating reaction*	*Inhibition (%)*
Standard assay	Isocitrate	0
Standard assay	Enzyme	7
Standard assay + PCMB*	Enzyme	7
PCMB + enzyme	Isocitrate, $NADP^+$, Mn^{2+}	100
PCMB + enzyme + $NADP^+$	Isocitrate, Mn^{2+}	97
PCMB + enzyme + Mn^{2+}	Isocitrate, $NADP^+$	93
PCMB + enzyme + isocitrate	$NADP^+$, Mn^{2+}	89
PCMB + enzyme + $NADP^+$ + Mn^{2+}	Isocitrate	24
PCMB + enzyme + isocitrate + Mn^{2+}	$NADP^+$	0

* The final concentration of *p*-chloromercuribenzoic acid (PCMB) was $1 \cdot 10^{-6}$ M.

aspartate, citrate, α-ketoglutarate, succinate, malate, proline and arginine. Of these, only glutamate produced a significant effect, causing 43% inhibition at a 1 mM concentration level.

Inhibition studies with PCMB

The mammalian isocitrate dehydrogenase is known to be inhibited by PCMB (refs. 22, 26 28) and by 5,5′-dithiobis-(2-nitrobenzoic acid)[27], both of which react with sulfhydryl groups. In studying sulfhydryl inhibition with the isocitrate dehydrogenase of *A. vinelandii*, PCMB was used at a final concentration of $1 \cdot 10^{-6}$ M with the standard assay system.

The data in Table II show that if PCMB is added to the standard assay mixture, and the reaction is started by the addition of enzyme, there is essentially no loss of activity. However, if the enzyme is incubated for 8 min with PCMB and the reaction is then started by the addition of isocitrate, $NADP^+$ and Mn^{2+}, there is a complete or 100% loss of activity. This suggests that the reactivity of the enzyme sulfhydryl groups with PCMB was complete in the absence of substrate, $NADP^+$ and Mn^{2+}. Further studies revealed that incubation of the enzyme with Mn^{2+}, $NADP^+$ or isocitrate alone conferred little or no protection against PCMB inhibition. However, complete protection against PCMB inactivation was afforded when the enzyme was incubated in the presence of both isocitrate and Mn^{2+}, and the reaction was initiated by the addition of $NADP^+$. Thus it appears that substrate and Mn^{2+} react concurrently with the enzyme and prevent PCMB from reacting with the thiols associated with isocitrate dehydrogenase activity. Incubation of the enzyme with $NADP^+$ and Mn^{2+} together allowed only 24% inactivation suggesting that isocitrate Mn^{2+} binds more efficiently to protect the sulfhydryls associated with the active site than does the $NADP^+$–Mn^{2+} complex. These studies imply that thiol function, as reflected by PCMB inhibition, is more critical for the binding of substrate to the enzyme, in the presence of Mn^{2+}, than for nucleotide binding.

DISCUSSION

Recently, CHUNG AND FRANZEN[29] reported on the isolation of the isocitrate dehydrogenase from *A. vinelandii* Strain 9104 (ATCC). The purified enzyme had a specific activity of 135 μmoles of $NADP^+$ reduced per min per mg protein at 30°. In the studies presented here, on the isocitrate dehydrogenase from *A. vinelandii* Strain O, specific activities of 650 μmoles of $NADP^+$ reduced per min per mg protein (37°) were obtained for the highly purified enzyme under the standard assay conditions. Specific activities of 135, for *A. vinelandii* Strain O, were usually observed after the $(NH_4)_2SO_4$ precipitation step, or the S_3 (70–80) fraction. This difference between the specific activities of the two strains (Strain O *vs.* ATCC Strain 9104) may be due to: (a) the fact that different proteins may carry out the identical reaction; (b) may be reflections of the differences between the two isolation procedures or (c) differences in the assay conditions. CHUNG AND FRANZEN[29] used Mg^{2+} instead of Mn^{2+}, as the activating divalent cation, and their assays were performed at pH 7.0 (30°) instead of pH 8.0 (37°). The conditions used for assaying the isocitrate dehydrogenase from Strain 9014 would not have been optimal for the Strain O enzyme.

Amino acid composition

The isocitrate dehydrogenase from *A. vinelandii* Strain O was found to contain 3 half-cystine residues per molecule of enzyme (Table I), which confirms the value reported by CHUNG AND FRANZEN[29] for the enzyme isolated from *A. vinelandii* Strain 9104. In this respect, the two enzymes are identical. This low value is in sharp contrast to the 12 cysteine residues per molecule reported for the mammalian isocitrate dehydrogenase[28]. The mammalian isocitrate dehydrogenase is well known to be highly unstable and usually special precautions are taken to prevent its inactivation[16,30]. This instability could be attributed to the oxidation of some of these thiol groups. Conversely, the stability of the enzyme from *A. vinelandii* Strain O could be a reflection of its low thiol content.

The content of several amino acids reported for the *A. vinelandii* isocitrate dehydrogenase of Strain 9104 (ref. 29) differs markedly from the values obtained for the enzyme from Strain O (Table I). The values obtained for serine, glutamic acid and glycine content in the enzyme from *A. vinelandii* Strain O were 87, 90 and 81 residues per molecule, respectively, while the values reported for the isocitrate dehydrogenase of Strain 9104 were 42, 61 and 45 residues per molecule, respectively. The leucine content for Strain O was 48 residues per molecule while that reported for Strain 9104 was 60 residues per molecule. These major differences in amino acid content may be accounted for by the genetic differences between these two strains of *A. vinelandii*. The G C content of *A. vinelandii* Strain O is 66.8% (M. MANDEL, personal communication) while that reported for *A. vinelandii* Strain 9104 (ATCC) is 60.0% (ref. 38). Thus this difference in amino acid composition could reflect the genetic difference between these two strains of *A. vinelandii*. A more thorough study of the amino acid data reported by CHUNG AND FRANZEN[29] also revealed that their amino acid analysis accounted for only 88% of their reported molecular weight of the isocitrate dehydrogenase. The values reported for *A. vinelandii* Strain O (in Table I), which do not include a value for tryptophan, account for 98% of the molecular weight of the isocitrate dehydrogenase. Therefore the low recovery of amino acid residues given for the isocitrate dehydrogenase of *A. vinelandii* Strain 9104, could also be due to technical difficulties encountered in performing the amino acid analyses on the purified enzyme.

Of further interest in comparing the two isocitrate dehydrogenases is that maximal stimulation of the enzyme from Strain 9104 was obtained with Mg^{2+} at levels ranging from $6 \cdot 10^{-4}$ to $2 \cdot 10^{-2}$ M. It is unusual that Mg^{2+}, and not Mn^{2+}, gave optimum stimulation; and that Mn^{2+} stimulated maximally at $1 \cdot 10^{-2}$ M concentration. This finding differs markedly with those observed in this study (Fig. 4), as well as those reported for other bacterial isocitrate dehydrogenases[21,32,33]. Our results show that substantially higher activities were consistently obtained with Mn^{2+} rather than with Mg^{2+}. This is particularly true at the concentration range of $5 \cdot 10^{-4}$ $1 \cdot 10^{-3}$ M (ref. 34). In addition, the data in Fig. 4 shows that at levels above $1 \cdot 10^{-3}$ M, Mn^{2+} inhibited the isocitrate dehydrogenase from Strain O. SIEBERT *et al.*[30], reported that even the mammalian NADP$^+$-specific isocitrate dehydrogenase is also more effectively activated by Mn^{2+} than by Mg^{2+}. Thus the NADP$^+$-specific isocitrate dehydrogenases from *A. vinelandii* Strain O and beef heart are more similar in their activation response to Mn^{2+} and Mg^{2+} and this appears to differ markedly from the metal ion requirements reported for the enzyme isolated from *A. vinelandii* Strain 9104.

PCMB inhibition studies on the isocitrate dehydrogenase of *A. vinelandii* Strain O (Table II) undoubtedly reflect on the mechanism of action of this enzyme. The fact that $1 \cdot 10^{-6}$ M PCMB inhibits the reaction indicates that functional sulfhydryl groups are involved in enzyme activity. This has been shown to be the case for the mammalian isocitrate dehydrogenase[27,28,35]. There is also evidence which suggests that the active substrate for the dehydrogenating reaction is an isocitrate Mn^{2+} complex. LANGAN[36] was able to show that the addition of $1 \cdot 10^{-4}$ M Mn^{2+} ions increased the effectiveness of isocitrate binding to the dehydrogenase from porcine heart. His data indicated that isocitrate could displace NADPH in the presence of Mn^{2+}. This suggested that Mn^{2+} were needed for substrate binding and that the Mn^{2+} isocitrate complex had a higher affinity for the enzyme than the Mn^{2+} NADPH complex. COLMAN[27] reported that both isocitrate Mn^{2+} and NADPH Mn^{2+} could protect the mammalian enzyme from inactivation by 5,5′-dithiobis(2-nitrobenzoic acid) and that a methionyl residue was involved in the active site. The data in Table II show that only a combination of isocitrate and Mn^{2+}, when preincubated with the isocitrate dehydrogenase, was able to protect the enzyme completely against inactivation by PCMB. So it is possible that isocitrate Mn^{2+} may also be the "active substrate complex" for the isocitrate dehydrogenase of *A. vinelandii* Strain O. In this regard the isocitrate dehydrogenase from the Strain O *A. vinelandii* appears similar to the mammalian enzyme.

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REFERENCES

1 A. E. CHUNG, *J. Bacteriol.*, 102 (1970) 438.
2 J. T. DUFF AND O. WYSS, *J. Gen. Microbiol.*, 24 (1961) 273.
3 P. JURTSHUK, P. R. ASTON AND L. OLD, *J. Bacteriol.*, 93 (1967) 1069.
4 P. JURTSHUK, S. MANNING AND C. R. BARRERA, *Can. J. Microbiol.*, 14 (1968) 775.
5 L. ORNSTEIN, *Ann. N. Y. Acad. Sci.*, 121 (1964) 321.
6 B. J. DAVIS, *Ann. N. Y. Acad. Sci.*, 121 (1964) 404.
7 P. JURTSHUK, C. R. BARRERA AND S. MANNING, *Can. J. Microbiol.*, 15 (1969) 321.
8 E. GOLDBERG, *Science*, 139 (1963) 602.
9 S. MOORE AND W. H. STEIN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. VI, Academic Press, New York, 1963, p. 819.
10 G. L. ELLMAN, *Arch. Biochem. Biophys.*, 82 (1959) 70.
11 C. R. WILLMS, R. M. OLIVER, H. R. HENNEY, JR., B. B. MUKHERJEE AND L. J. REED, *J. Biol. Chem.*, 242 (1967) 889.
12 C. R. BARRERA AND P. JURTSHUK, *Biochim. Biophys. Acta*, 191 (1969) 193.
13 P. ANDREWS, *Biochem. J.*, 91 (1964) 222.
14 H. HAGLUND, *Sci. Tools*, 14 (1967) 17.

15 N. CATSIMPOOLAS, *Anal. Biochem.*, 26 (1968) 480.
16 G. SIEBERT, J. BUBUC, R. C. WARNER AND G. W. E. PLAUT, *J. Biol. Chem.*, 226 (1957) 965.
17 E. E. JACOBS, M. JACOB, D. R. SANADI AND L. B. BRADLEY, *J. Biol. Chem.*, 223 (1956) 147.
18 R. F. CHEN AND G. W. E. PLAUT, *Biochemistry*, 2 (1963) 1023.
19 H. GOEBELL AND M. KLINGENBERG, *Biochem. Z.*, 340 (1964) 441.
20 M. KLINGENBERG, H. GOEBELL AND G. WENSKE, *Biochem. Z.*, 341 (1965) 199.
21 J. J. MARR AND M. M. WEBER, *J. Biol. Chem.*, 243 (1968) 1973.
22 G. W. E. PLAUT, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 7, Academic Press, New York, 2nd. ed., 1963, p. 105.
23 A. M. STEIN, S. K. KIRKMAN AND J. H. STEIN, *Biochemistry*, 6 (1967) 319.
24 D. E. ATKINSON, in T. W. GOODWIN, *Metabolic Roles of Citrate*, Academic Press, New York, 1968, p. 23.
25 I. SHIIO AND H. OZAKI, *J. Biochem. Tokyo*, 64 (1968) 45.
26 W. D. LOTSPEICH AND R. A. PETERS, *Biochem. J.*, 49 (1951) 704.
27 R. F. COLMAN, *Biochemistry*, 8 (1969) 888.
28 R. F. COLMAN, *Biochem. Biophys. Res. Commun.*, 28 (1967) 222.
29 A. E. CHUNG AND J. S. FRANZEN, *Biochemistry*, 8 (1969) 3175.
30 G. SIEBERT, M. CARSIOTIS AND G. W. E. PLAUT, *J. Biol. Chem.*, 226 (1957) 977.
31 J. MARMUR AND P. DOTY, *J. Mol. Biol.*, 5 (1962) 109.
32 S. BARBAN AND S. AJL, *J. Bacteriol.*, 64 (1952) 443.
33 A. KORNBERG AND W. E. PRICER, JR., *J. Biol. Chem.*, 189 (1951) 123.
34 C. R. BARRERA, *Electrophoretic Resolution of the D (−)-β-hydroxybutyric and Isocitric Dehydrogenases from Azotobacter vinelandii strain O*, Master's, University of Texas at Austin, 1967.
35 R. F. COLMAN, *J. Biol. Chem.*, 243 (1968) 2454.
36 T. A. LANGAN, *Acta Chem. Scand.*, 14 (1960) 936.