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Purification and Subunit Structure of Nicotinamide Adenine Dinucleotide Specific Isocitrate Dehydrogenase from *Neurospora crassa*†

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ABSTRACT: *Neurospora crassa* nicotinamide adenine dinucleotide specific isocitrate dehydrogenase (EC 1.1.1.41) has been purified to homogeneity by the criteria of disc gel electrophoresis and sedimentation equilibrium. Purification of the enzyme is facilitated by the presence of phenylmethanesulfonyl fluoride and by the use of a ribose-linked adenosine 5′-monophosphate affinity column. The enzyme appears to be composed of nonidentical subunits of molecular weights 42 800 and 38 300 as estimated by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate. From the intensity of each band and the native molecular weight, it is concluded that the enzyme is composed of either six or eight subunits, three or four of each type, respectively. The availability of pure enzyme will allow clarification of the structure of the enzyme by ligand binding studies.

The regulatory NAD^+ [1]-specific isocitrate dehydrogenase from *Neurospora crassa* has been suggested to bear at least three types of site: a substrate site specific for *threo*-D_s-isocitrate, NAD^+, and a divalent metal cation, and two regulatory sites, one specific for citrate, isocitrate, and structurally related molecules and a second specific for adenine nucleotides (Sanwal et al., 1963, 1965; Sanwal & Stachow, 1965; Sanwal & Cook, 1966). These conclusions have been based exclusively on results obtained from initial velocity kinetic studies. The regulatory characteristics of the enzyme,

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[1] Abbreviations used: IDH, isocitrate dehydrogenase; AMP, adenosine 5′-monophosphate; NAD^+, nicotinamide adenine dinucleotide; $NaDodSO_4$, sodium dodecyl sulfate; $PhCH_2SO_2F$, phenylmethanesulfonyl fluoride.

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i.e., the sigmoidal velocity vs. isocitrate plots and the activation by citrate and AMP, were originally explained in terms of a kinetic model involving no interaction between ligand-binding sites. The model, invoking no subunit interaction, to explain sigmoidal behavior was unique at the time (Sanwal et al., 1965; Sanwal & Cook, 1966). More recent, detailed, kinetic studies from this laboratory, however, have revealed a greater complexity in velocity–substrate curves than previously reported, suggesting a model for the enzyme involving interaction between ligand-binding sites (Barratt & Cook, 1978). In addition, the catalytic and regulatory properties of the enzyme have been shown to be significantly altered by the choice of metal cofactor, i.e., Mg^{2+} and Mn^{2+}. It has been suggested that the metal cofactors Mg^{2+} and Mn^{2+} stabilize two distinct forms of the enzyme which differ in response to varying substrate and activator concentrations (Barratt & Cook, 1978).

A definitive model for the enzyme could not be proposed in the absence of any data on the structure of the enzyme. Previous attempts to purify isocitrate dehydrogenase from *Neurospora crassa* to homogeneity have been hindered by the apparent instability of the enzyme during the isolation procedure (Cook & Sanwal, 1969; Barratt & Cook, 1978). The functionally identical NAD^+-specific isocitrate dehydrogenase from yeast (Kornberg & Pricer, 1951; Hathaway & Atkinson, 1963; Atkinson et al., 1965) has been purified to apparent homogeneity with an estimated molecular weight of 300 000 (Barnes et al., 1971). The enzyme appears to be composed of eight identical subunits of molecular weight 39 000. Ligand binding studies by Kuehn et al. (1971) on the yeast enzyme have indicated two binding sites for NAD^+, Mn^{2+}, and AMP and four binding sites for isocitrate. A model for the enzyme was not proposed, most likely due to the discrepancy between the number of binding sites and the subunit structure.

In the present paper, we report a purification procedure for obtaining homogeneous NAD^+-specific isocitrate dehydrogenase from *Neurospora crassa* as a prerequisite to a study of its ligand binding behavior. Sufficient homogeneous enzyme was available to determine the identity and number of constitutive subunits of the enzyme which is also reported in this study. The subunit structure differs markedly from the yeast enzyme but is similar to the structure recently reported for the pig heart enzyme (Ramachandran & Colman, 1978).

Experimental Section

Materials. Sepharose CL-6B was obtained from Pharmacia Fine Chemicals (Canada) Ltd. The protein assay kit, sodium dodecyl sulfate, and all reagents required for polyacrylamide gel electrophoresis were obtained from Bio-Rad Laboratories (Canada) Ltd. The ribose-linked AMP affinity matrix was purchased from Sigma Chemical Co. Guanidine hydrochloride was obtained from Schwarz/Mann.

Fumarase (pig heart), catalase (beef liver), alcohol dehydrogenase (liver), and glyceraldehyde 3-phosphate dehydrogenase (muscle) were purchased from Boehringer Mannheim and ovalbumin from Schwarz/Mann. Aspartate transcarbamylase was purified (Gerhart & Holoubek, 1967) from a diploid mutant of *E. coli* kindly provided by Dr. J. C. Gerhart.

Growth of the Organism. Neurospora crassa, wild-type strain STA-4, was obtained from the Fungal Genetic Stock Centre, Dartmouth College, NH. The organism was grown in large-scale fermentors (1000 L) through the courtesy of the Labatts Research Division, Labatts Brewery, London, Ontario, Canada. The organism was routinely grown in Vogel's Medium N (Vogel, 1956) at 28 °C with 1% sucrose as carbon source. After a 24-h fermentation, the organism was collected through cheesecloth, wrung dry, lyophilized, and stored at −20 °C until use.

Protein Determination. Protein was determined by the method of Bradford (1976) by using the Bio-Rad protein assay kit.

Enzyme Assay. Isocitrate dehydrogenase activity was determined by following NAD^+ reduction at 340 nm with a Gilford Model 2400 recording spectrophotometer equipped with dual thermoplates. A standard reaction mixture was used which contained 167 mM Tris–acetate buffer (pH 7.6), 0.33 mM AMP, 10 mM *dl*-isocitrate, 2 mM NAD^+, 3.5 mM $MgCl_2$, and enzyme preparation in a final volume of 1.5 mL. The reaction was initiated by the addition of *dl*-isocitrate. One unit of activity is the amount of enzyme which reduces 1 μmol of NAD^+/min under standard assay conditions. Specific activity is defined as the number of units of enzyme activity per milligram of protein.

Polyacrylamide Disc Gel Electrophoresis. Electrophoresis through 7.5% (w/v) and 3.5% (w/v) analytical polyacrylamide gels by using an anionic system (pH 9.5) was performed according to the procedure of Davis (1964). The inability to demonstrate any protein bands or bands of enzyme activity by using such a system resulted in the formulation of some modifications to the Davis (1964) procedure. In the modified procedure, the electrophoresis buffer contained 5 mM 2-mercaptoethanol to prevent the formation of any persulfate ion artefacts (Maurer, 1971). In addition, the samples applied to each gel were diluted to the appropriate range of protein concentration with 0.05 M monopotassium phosphate–NaOH buffer (pH 6.5) containing 0.1 mM EDTA, 0.1 M $PhCH_2SO_2F$, 5 mM 2-mercaptoethanol, 10 mM citrate, and 1% (v/v) Triton X-100 (Coutinho et al., 1966) and made dense with 40% (v/v) glycerol (50 μL). The total sample volume was kept constant at 0.15 mL. Suitable staining controls containing the diluent buffer (100 μL), 40% (v/v) glycerol (50 μL), and 0.05% (w/v) Bromophenol Blue dye (5 μL) were also run on separate gels. Electrophoresis was performed at 4 °C at 4 mA/gel. Gels were stained for protein by immersion in 1% (w/v) Amido-Schwarz in 7% (v/v) acetic acid for 2 h and destained electrophoretically in 7% (v/v) acetic acid. Isocitrate dehydrogenase activity in the gels was located by incubating the gels in the previously described assay mix containing 0.52 mM nitroblue tetrazolium and 0.24 mM phenazine methosulfate for 30 min in the dark at room temperature. All gels were stored in 7% (v/v) acetic acid. The lack of results with the normal Davis (1964) procedure led us to investigate the possibility that the enzyme was behaving as a cathodic rather than anionic species. Thus, electrophoresis of the enzyme in 3.5% (w/v) polyacrylamide gels was also performed with the cathodic gel system (pH 6.6) of Taber & Sherman (1964) as represented by Maurer's (1971) gel system no. 5.

Electrophoresis on 5% (w/v) polyacrylamide gels containing 0.1% (w/v) $NaDodSO_4$ was performed as described by Weber & Osborn (1969). Prior to electrophoresis, the standard proteins and the isocitrate dehydrogenase were incubated at 100 °C in 10 mM phosphate buffer (pH 7.0) containing 1% (w/v) $NaDodSO_4$ and 1% (v/v) 2-mercaptoethanol for 5 min to achieve dissociation. The standard proteins and the isocitrate dehydrogenase were run on separate gels. To ensure that the enzyme was completely dissociated and that no reaggregation had taken place during electrophoresis, the isocitrate dehydrogenase was alkylated with iodoacetic acid as described by Weber et al. (1972). The alkylated isocitrate dehydrogenase was subjected to electrophoresis on 5% (w/v) polyacrylamide gels containing 0.1% (w/v) $NaDodSO_4$ as

described by Weber & Osborn (1969).

Molecular Weight Determination. The molecular weight of the pure and impure NAD-specific isocitrate dehydrogenase was determined by their electrophoretic mobility on polyacrylamide gels at two gel concentrations (5% and 8%, weight by volume) of the acrylamide. The Bis/acrylamide monomer weight ratio of the small pore gel was kept constant at 1:30 (Hedrick & Smith, 1968). The procedure followed was as described by Davis (1964) with the buffer and sample diluent modifications previously described. The reference proteins (10 μg) were stained for protein and the isocitrate dehydrogenase position along the gel was demonstrated by using the specific stain previously described. For each protein, the relative mobility was measured as its migration distance divided by that of the Bromophenol Blue in the same gel. The retardation quotient, relative mobility in the 8% (w/v) gel/relative mobility in the 5% (w/v) gel, is completely independent of charge and depends only on molecular size (Thorun & Maurer, 1971). By plotting the retardation quotient against the log of the molecular weight for different reference proteins, a linear relationship is obtained (Matagne & Schlosser, 1977). All runs were done in duplicate, and the average values were fitted by the linear least-squares method.

The molecular weight of the impure and purified enzyme was also estimated by gel filtration on a Sepharose CL-6B column (2.5 × 92 cm) equilibrated in 0.05 M monopotassium phosphate–NaOH buffer (pH 6.5) containing 0.1 mM EDTA, 0.1 mM DTT, 0.1 mM $PhCH_2SO_2F$, and 10 mM citrate. The flow rate was 31 mL/h. The following reference proteins, their eluted positions established by appropriate assay, were used: lactate dehydrogenase (molecular weight 150000), pyruvate kinase (235000), xanthine oxidase (275000), aspartate transcarbamylase (310000), apoferritin (480000), and β-galactosidase (520000). The eluted positions of the proteins (v_e) relative to that of Dextran Blue (molecular weight 2000000) was plotted against the log of the molecular weight. The straight line was fitted by the linear least-squares method.

Further confirmation of the molecular weight of the purified NAD-specific isocitrate dehydrogenase was obtained by ultracentrifugation of the enzyme preparations in a Spinco Model E analytical ultracentrifuge. Prior to sedimentation studies, the enzyme was dialyzed for 48 h at 4 °C, with one buffer change, against a 500-fold excess of the appropriate buffer. The final buffer external to the dialysis tubing was employed as the solvent blank in each particular case.

The sedimentation behavior of the pure IDH, and, hence, the molecular weight of the enzyme was established by the meniscus depletion method of Yphantis (1964). The sedimentation was followed with Rayleigh interference optics. Conditions for the runs are described in the appropriate figure legends.

The refractive index patterns at equilibrium were used to determine four of the standard apparent molecular weight moments. These moments are the number (Mn), the weight (Mw), the $z(Mz)$, and the $z + 1$ ($Mz + 1$) average apparent reduced molecular weights (Yphantis, 1964). Thus, the apparent weight is defined as

$$M = \frac{RT}{(1 - \bar{V}\rho)w^2} \frac{\mathrm{d} \ln (Y - Y_0)}{\mathrm{d}(r^2/2)} = \frac{RT}{(1 - \bar{V}\rho)s^2}\sigma \quad (1)$$

In this equation, R is the gas constant, T the absolute temperature, $\bar{V}$ the partial specific volume of the solute which, in the specific case of IDH, is assumed to be 0.73 cm^3/g, ρ the solution density as determined from standard tables, w the angular velocity, and r the radius at which the Y and Y_0 values were taken corresponding, respectively, to the sample and blank solutions.

In terms of the average apparent reduced molecular weights, eq 1 can be rearranged:

$$\sigma n(r) = \frac{Mm, \mathrm{app}(r)(1 - \bar{V}\rho)w^2}{RT} \quad (2a)$$

$$\sigma w(r) = \frac{Mw, \mathrm{app}(r)(1 - \bar{V}\rho)w^2}{RT} \quad (2b)$$

$$\sigma z(r) = \frac{Mz, \mathrm{app}(r)(1 - \bar{V}\rho)w^2}{RT} \quad (2c)$$

$$\sigma z + 1(r) = \frac{Mz + 1, \mathrm{app}(r)(1 - \bar{V}\rho)w^2}{RT} \quad (2d)$$

Thus, by definition, the value of σ is an experimentally observable term which can be calculated for each apparent molecular weight moment by the derived relations defined by Roark & Yphantis (1969). Such relations along with the least-squares smoothing procedure and auxilliary procedures for estimating the integration constants and concentrations associated with these relations have been incorporated into a Fortran IV computer program (Dr. David Kells, personal communication) and applied to the data obtained with the sedimentation of isocitrate dehydrogenase from *Neurospora crassa*.

The computer-generated graphs are very comprehensive because of the number of data points involved, and, hence, for the sake of clarity and brevity these graphs have been redrawn in a consolidated and abbreviated format.

Results

Purification. All steps were performed at 4 °C except additions of absolute ethanol, which were carried out at the temperatures stated. All additions of ammonium sulfate were as the solid salt. Solvent additions and the required amount of ammonium sulfate were calculated by using formulae described by Noltmann et al. (1961). Unless otherwise indicated, all centrifugations were performed at 10000 rpm for 10 min in a Sorvall RC-2B preparative centrifuge using a GSA rotor. *Neurospora crassa* (50 g) was ruptured by suspension of the lyophilized powder in 750 mL of 0.1 M Tris–acetate buffer (pH 7.6) containing 0.1 mM DTT, 0.1 mM EDTA, and 0.1 mM $PhCH_2SO_2F$ (buffer A) and mechanical stirring for 90 min. The cell debris was removed by centrifugation, and the temperature of the supernatant solution was adjusted to +5 °C. The pH of this solution was carefully adjusted to 5.2 at +5 °C with 20% (v/v) acetic acid and the precipitate discarded after centrifugation. Cold absolute ethanol was added gradually with stirring to the supernatant solution to give a final concentration of 7% (v/v). Throughout this addition, the solution temperature was maintained at +5 ± 0.5 °C. After the final addition of ethanol, the precipitate was removed by centrifugation. The temperature of the supernatant solution was lowered to −3 °C and absolute ethanol (precooled to −20 °C) was added gradually to a final concentration of 15% (v/v) as the temperature of the solution is slowly lowered to −8 °C. After the last addition of absolute ethanol, the solution is allowed to stand in an ethanol:water bath (1:1 by volume) at −12 °C for 60 minutes. The precipitate, recovered by centrifugation, was redissolved in 125 mL of cold buffer A and the temperature of this solution was checked to be at +5 °C.

The pH of this solution was adjusted to pH 5.2 at +5 °C with cold 20% (v/v) acetic acid, and the precipitate was

Table I: Purification of NAD-Specific Isocitrate Dehydrogenase from *Neurospora crassa*

purification step	vol (mL)	protein (mg)	total act. (units)	sp act. (units/mg)	yield (%)
crude extract[a]	675	12717.4	418.83	0.033	100
7–15% ethanol fraction	125	1126.9	286.85	0.255	68.5
5–15% ethanol fraction	8.8	269.6	241.97	0.898	57.8
affinity pool	11.0	5.2	179.65	34.550	42.9
Sepharose CL-6B pool	27.9	1.8	90.25	50.140	21.5
concd Sepharose CL-6B pool	1.2	1.3	54.15	42.90	12.9

[a] From 50 g of lyophilized *Neurospora crassa*.

removed by centrifugation. Cold absolute ethanol was added gradually with stirring to the supernatant solution to give a final concentration of 5% (v/v). Throughout this addition the solution was maintained at +5 ± 0.5 °C. After the final addition of ethanol, the precipitate was removed by centrifugation. The ethanol concentration of the supernatant was increased to 15% (v/v) in a manner identical with that described for the previous ethanol addition to 15% (v/v). The resultant precipitate was collected by centrifugation and dissolved in a minimal volume (about 10 mL) of 0.02 M monopotassium phosphate–NaOH buffer (pH 6.5) containing 0.1 mM $PhCH_2SO_2F$, 0.1 mM DTT, 10 mM citrate, and 7.5 mM Mg^{2+} (as the chloride) (buffer B).

The preparation, whose temperature had been lowered to +1 °C, was applied to a 0.9 × 24 cm ribose-linked AMP affinity column equilibrated with buffer B at +4 °C. The flow rate, maintained with a Pharmacia peristaltic pump P-3 (Pharmacia Fine Chemicals), was kept constant at 3 mL/10 min.

After washing the nonbinding protein off the column with buffer B, the enzyme was eluted with 0.05 M monopotassium phosphate–NaOH buffer (pH 6.5) containing 0.1 mM EDTA, 0.1 mM $PhCH_2SO_2F$, 10 mM citrate (buffer C) containing 1 mM NAD^+ layered directly on top of the gel matrix (Figure 1). The enzyme is removed very sharply from the affinity column, and the resulting fractions with specific activities greater than 10 units/mg of protein were pooled and applied directly to a 2.5 × 92 cm Sepharose CL-6B column previously equilibrated with buffer C. The enzyme was eluted with the same buffer. The fractions demonstrating similar specific activities (Figure 2) were pooled and concentrated by adding solid $(NH_4)_2SO_4$ to 80% saturation. There is a constant discrepancy observed between the specific activity of the enzyme in individual fractions (sp act. 30) and the specific activity of the resultant pooled fractions (sp act. ~ 50). The simplest explanation of this discrepancy is that the activity of the enzyme is affected by protein concentration. Thus, the high protein dilution in several of the individual fractions may have resulted in partial inactivation of the enzyme which is overcome by pooling of the fractions. The final $(NH_4)_2SO_4$ precipitate was dissolved in a minimal volume of buffer C. The summary of a typical purification is presented in Table I.

Homogeneity. The homogeneity of the final isocitrate dehydrogenase preparation could not be directly established by using the standard Davis (1964) procedure due to the complete absence of protein-staining bands in the 3.5% (w/v) running gel. Such inability to penetrate the gel was evident at applied protein concentrations from 20 to 100 μg. Similar results were obtained with the cathodic system of Taber & Sherman (1964) by using 3.5% (w/v) polyacrylamide gels. The results with the standard Davis (1964) procedure indicate one sharp staining band at the interface of the sample mixture and the large pore (stacking) gel. Omitting the stacking gel does not result in any improvement in the mobility of the enzyme into the gel as it appears, under these conditions, at

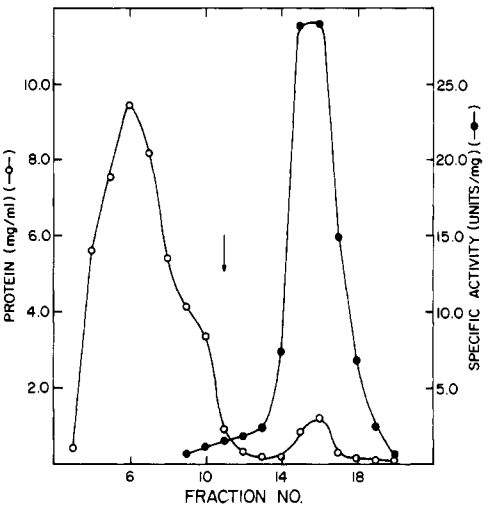

FIGURE 1: Elution profile of isocitrate dehydrogenase on a ribose-linked AMP affinity column (0.9 × 24 cm). The protein front was eluted with 20 mM monopotassium phosphate–NaOH buffer (pH 6.5) containing 0.1 mM $PhCH_2SO_2F$, 0.1 mM DTT, 10 mM citrate, and 7.5 mM $MgCl_2$ (buffer B). The enzyme was eluted with 50 mM monopotassium phosphate–NaOH buffer (pH 6.5) containing 0.1 mM EDTA, 0.1 mM $PhCH_2SO_2F$, 10 mM citrate (buffer C) as well as 1 mM NAD^+ layered directly on top of the column gel at fraction 11. The volume of each fraction was 2.0 mL.

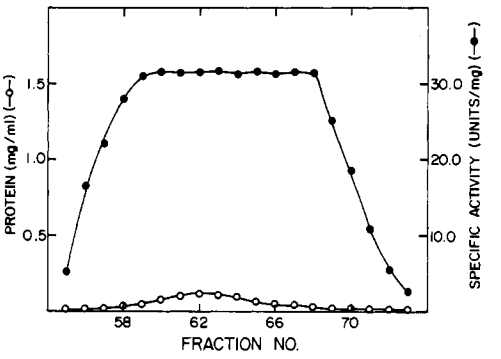

FIGURE 2: Elution profile of isocitrate dehydrogenase on a Sepharose CL-6B column (2.5 × 93 cm). The enzyme was eluted with buffer C (see legend to Figure 1). The void volume of the column, as determined with dextran blue, was 152 mL. The volume of each fraction was 4.5 mL.

the interface of the sample mixture and the running gels. In contrast, protein samples from earlier stages of the purification procedure (run in parallel gels) penetrate the various gels as expected. The addition of Triton X-100 to the samples and 2-mercaptoethanol to both the electrophoretic buffer and the samples results in penetration of the final enzyme preparation into the gels. With increasing times of incubation of the enzyme at 4 °C in the diluent buffer (buffer C) containing 1% (v/v) Triton X-100, 5 mM 2-mercaptoethanol, the resultant gel pattern changes from a "streak" (gel A, Figure 3) to one discrete band. However, some protein and IDH activity

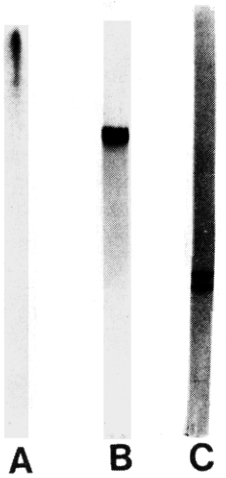

FIGURE 3: Polyacrylamide gel electrophoresis of isocitrate dehydrogenase. The current was discontinued when the marker, bromphenol blue, was almost at the anode end of each gel (bottom). (Gel A) A typical electrophoretic pattern obtained by using the standard Davis (1964) procedure. A sample (50 μg) of the final enzyme preparation was preincubated for 15 min in buffer C (see Figure 1) containing 1% (v/v) Triton X-100 and 5 mM mercaptoethanol prior to electrophoresis in a 7.5% (w/v) acrylamide gel. In the absence of Triton X-100 and 2-mercaptoethanol, no penetration of the sample into the gel occurred (not shown). (Gel B) The typical electrophoretic pattern obtained in a 5% (w/v) acrylamide gel by using the modified Davis (1964) procedure as outlined in the text. Sixty micrograms of the final enzyme preparation was applied. (Gel C) Sodium dodecyl sulfate gel electrophoresis of isocitrate dehydrogenase in 5% (w/v) polyacrylamide gels. The enzyme was treated with $NaDodSO_4$/2-mercaptoethanol at 100 °C as described in the Experimental Section, and 8 μg of protein was applied.

still remains at the original interface, immaterial of the incubation time. Polyacrylamide disc gel electrophoresis of the final enzyme preparation on 7.5% (w/v) and 5% (w/v) acrylamide gels using the modified Davis (1964) procedure described in the Experimental Section provides more direct evidence of the homogeneity of the final enzyme preparation (see gel B, Figure 3). A single protein band was demonstrable at both gel concentrations and at the two protein sample loads of 40 and 60 μg. Staining of the corresponding gels for isocitrate dehydrogenase activity establishes that the activity was located at the identical position on the gel as the single protein band. A control gel, incubated in the tetrazolium assay mixture without isocitrate, did not show the production of any formazan at the position of the protein band. Further evidence for the homogeneity of the enzyme preparation is provided by the lack of any minor bands on $NaDodSO_4$ polyacrylamide gel electrophoresis (Figure 3) and the constant specific activity across the Sepharose CL-6B peak indicative of purity with respect to molecular size (Figure 2). Similar evidence for molecular size homogeneity is provided by the interference pattern of the enzyme in the ultracentrifuge as represented in Figure 4.

Subunit Structure. The purified enzyme was treated with $NaDodSO_4$ and 2-mercaptoethanol, then subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis as described in the Experimental Section. The pattern obtained (gel C, Figure 3) reveals two closely spaced protein-staining bands which, by densitometric scan, stained with slightly differing intensity. However, the areas under each peak, as measured by triangulation, are similar relative to the total area.

The alkylated isocitrate dehydrogenase produced the same two-band pattern. By comparing the mobilities of these protein bands with the mobilities of several protein standards, the molecular weights of the subunits are established as 42 800

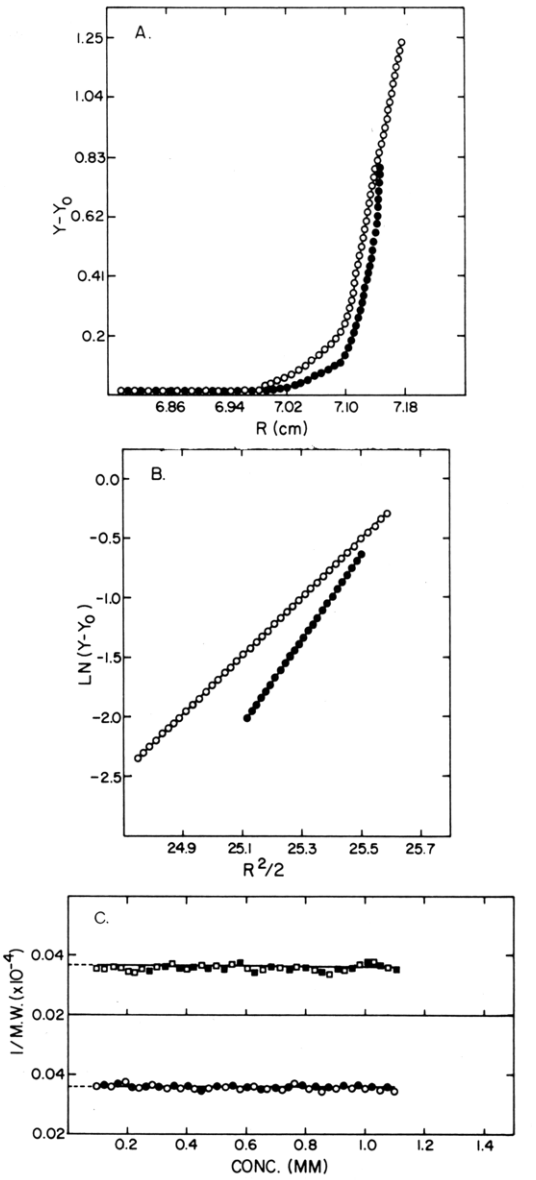

FIGURE 4: Sedimentation equilibrium studies of NAD-specific isocitrate dehydrogenase in 0.05 M monopotassium phosphate–NaOH buffer (pH 6.5) containing 0.1 mM EDTA, 0.1 mM $PhCH_2SO_2F$, and 0.1 mM DTT. The sample (0.45 mg of protein/mL) was centrifuged at 8547.01 rpm (○) and at 11 299.44 rpm (●) at 20 °C. (A) The experimental points ($Y - Y_0$) taken from the interference pattern plate are plotted vs. R, the radius at which the readings were taken. (B) Replot of the experimental points by using the Roark & Yphantis (1969) program base line. The line is fitted by the linear least-squares method. (C) Partial representation of the molecular weight data obtained from the Roark & Yphantis (1969) computer program analysis of the experimental data. Concentration is measured in terms of millimeters of fringe displacement above the base line. The points represent 1/mol wt (N) (○), 1/mol wt (W) (●), 1/mol wt (z) (□), and 1/mol wt ($z + 1$) (■). The line drawn is the least-squares line from the original computer graph containing greater than 100 points for each line.

± 324 and 38 300 ± 295. These values are taken as the average of six determinations from three individual electrophoresis runs on the same enzyme preparation. The corresponding percent deviations of the data from these averages are 1.6% for the larger molecular weight subunit and 1.2% for the smaller molecular weight subunit.

Molecular Weight. The molecular weight of the pure IDH as established by calculation of its retardation quotient on polyacrylamide gels of different porosity in comparison with that of known standards is 308 000 ± 15 000. The value

obtained using the Sepharose CL-6B column is 326 000 ± 70 000. This particular technique did not provide reproducible results as indicated by the large standard deviation obtained from five fractionations of the enzyme on the same column and the somewhat low coefficient of determination (0.921) of the regression line obtained with the protein standards fractionated through this column. By using both these techniques, no significant variation in the molecular weight of IDH was observed whether it was in a pure or relatively impure state. From sedimentation equilibrium experiments (Figure 4), the molecular weight of the pure enzyme was estimated to be 277 000 ± 1100 (assuming a partial specific volume of the enzyme to be 0.73 cm^3/g). In addition, the linearity of the data confirms the purity of the preparation and the lack of any aggregates being formed with increasing protein concentration. These sedimentation experiments were also performed in the presence of 10 mM citrate with identical results, thus confirming the molecular weight of the NAD-specific isocitrate dehydrogenase from *Neurospora crassa* to be 277 000 ± 1100.

Discussion

Previous efforts (Cook & Sanwal, 1969; Barratt & Cook, 1978) directed at purification of the NAD-specific isocitrate dehydrogenase from *N. crassa* to sufficient homogeneity to carry out physical studies on the enzyme had met with little success. Very large losses of enzyme activity invariably occurred during the column chromatographic fractionations. It was thus considered that the enzyme was very labile.

In designing the purification scheme described in this report, it was noted that recovery of enzyme from the lyophilized *N. crassa* and in subsequent steps was greatly improved by the inclusion of $PhCH_2SO_2F$ in the buffer systems. In addition, narrowing the first ethanol fractionation cut to 7–15% (v/v) from 7–20% (v/v) minimized the loss in enzyme activity and vastly improved the specific activity of the enzyme in the subsequent procedures, despite the loss of enzyme activity in the 7–15% (v/v) supernatant. The previously observed lability of the enzyme was due to the presence of proteases which are partially inhibited by $PhCH_2SO_2F$ and partially removed in the 7–15% (v/v) supernatant in this purification. Thus, the purification scheme described in this report has been devised to produce a rapid scheme (to minimize the contact time between protease and enzyme) which pays due attention to the well-documented array of proteases found in *N. crassa* (Yu et al., 1973; Siepen et al., 1975; Lumsden & Coggins, 1977).

The lack of penetration of the enzyme into a 3.5% (w/v) acrylamide gel in either an anionic or cationic gel system suggests the possible formation of a large molecular weight artifact during the electrophoretic process. We speculate that the addition of Triton X-100 to the sample partially prevented the formation of such aggregates by minimizing the extent of hydrophobic bonding between the molecules.

The molecular weight of the enzyme has been determined by several techniques, with resultant values of 277 000 (by sedimentation equilibrium), 307 000 (by acrylamide gel), and 326 000 (by Sepharose CL-6B fractiontion). However, the poor reproducibility and lack of good correlation in the standard line of the gel permeation technique would seem to indicate that the molecular weight value obtained by this technique is not very accurate. This is in complete contrast to the value obtained by sedimentation equilibrium, a technique inherent with a great degree of reproducibility, accuracy, and sensitivity (Yphantis, 1964; Roark & Yphantis, 1969; Chervenka, 1970).

The results of electrophoresis in the presence of $NaDodSO_4$ indicate that isocitrate dehydrogenase is composed of two different types of subunits of molecular weights of 42 800 and 38 300. Thus, from the standpoint of the types and size of the subunits, the NAD-specific isocitrate dehydrogenase from *N. crassa* is very similar to the enzyme from pig heart (Ramachandran & Colman, 1978). From the molecular weight determined by sedimentation equilibrium and the equal intensity of each band, it can be concluded that the enzyme is composed of six subunits, three of each type. However, the conclusion which can be arrived at using the molecular weight determined by the other two techniques is that the enzyme is composed of eight subunits, four of each type. It is well known that this enzyme is subject to regulation by AMP and citrate. Thus, it is possible that the enzyme is composed of a set of catalytic and a set of regulatory subunits. The structure of the enzyme could therefore be represented as either C_3R_3 or C_4R_4.

The yeast NAD-specific isocitrate dehydrogenase, suggested to be octameric, has been shown by ligand binding studies to have two catalytic sites and two binding sites for AMP (Kuehn et al., 1971). A molecular weight of 236 000 has, however, been proposed for the enzyme by Palm & Katzendobler (1972) which could indicate a hexameric structure.

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Kinetics of Association between Bisquaternary Ammonium Ligands and Acetylcholinesterase. Evidence for Two Conformational States of the Enzyme from Stopped-Flow Measurements of Fluorescence†

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ABSTRACT: Bisquaternary ammonium ligands in which the quaternary groups are separated by 14 Å are known to bind with high affinity to acetylcholinesterase. A series of bisquaternary ligands containing a benzoquinone moiety exhibit absorption spectra which overlap the fluorescence emission of the tryptophanyl residues on the protein. Upon complexation with acetylcholinesterase, quenching of protein fluorescence occurs along with a shift of emission maximum to shorter wavelength. The latter phenomenon suggests that a conformational change is associated with ligand binding. A study of the kinetics of complexation using stopped-flow instrumentation reveals that a rapid bimolecular step ($k = 9.7 \times 10^7$ M^{-1} s^{-1}) is followed by a relatively slow unimolecular ($k = 44$ s^{-1}) interconversion of enzyme species. The unimolecular step, which appears to involve an isomerization of two enzyme forms that exist prior to ligand binding, was found to occur for all the bisquaternary ligands examined. The kinetics of complexation, where a bimolecular rate below diffusion-controlled rates and a slow isomerization of the enzyme are observed, appear to be unique to the bisquaternary compounds since ligands which bind exclusively to the active center or peripheral anionic site exhibit association kinetics approximating that of a diffusion-controlled reaction. The change in conformation may well be associated with the necessity of obtaining the proper spatial arrangement between the two anionic sites on acetylcholinesterase at which the quaternary groups bind.

In recent years, it has become possible to measure the association of inhibitory ligands with acetylcholinesterase (AcChE)[1] by direct fluorescence (Mooser et al., 1972; Mooser & Sigman, 1974; Taylor & Jacobs, 1974; Taylor & Lappi, 1975) and magnetic resonance (Kato, 1972; Wee et al., 1976) techniques. These approaches have enabled investigators to confirm the existence of a peripheral site(s) for ligand binding which had previously been adduced from steady-state kinetic measurements of inhibition of substrate catalysis (Changeux, 1966). Hence, certain ligands such as edrophonium or the fluorescent compound *N*-methylacridinium will inhibit substrate hydrolysis by binding to the active center (Mooser et al., 1972) while other inhibitory compounds such as propidium at low ionic strength bind exclusively to a site peripheral to the active center (Taylor & Lappi, 1975). The stoichiometry of each ligand is 1:1 with the 80 000-dalton subunit on the tetrameric enzyme, and ternary complexes can be demonstrated where the respective ligands bind to the active and peripheral sites on each subunit (Mooser & Sigman, 1974; Taylor & Lappi, 1975). Bisquaternary ligands in which a 10 carbon methylene chain or approximately 14 Å separates the quaternary nitrogens are of particular interest since their binding is mutually exclusive with ligands that bind either to the peripheral site or to the active center (Taylor & Lappi, 1975). A complex where the bisquaternary ligand spans

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[1] Abbreviations used: AcChE, acetylcholinesterase; *o*ClB-BQ, 2,5-bis[[3-[diethyl(*o*-chlorobenzyl)ammonio]propyl]amino]benzoquinone; $p$$NO_2$B-BQ, 2,5-bis[[3-[diethyl(*p*-nitrobenzyl)ammonio]propyl]-amino]benzoquinone; B-BQ, 2,5-bis[[3-(diethylbenzylammonio)-propyl]amino]benzoquinone; DAP, 1,10-bis(3-aminopyridinio)decane; Et_3-BQ, 2,5-bis[[3-(triethylammonio)propyl]amino]benzoquinone; Me_3-BQ, 2,5-bis[[3-(trimethylammonio)propyl]amino]benzoquinone; *o*Br-ambenonium, *N*,*N*′-bis[[2-[diethyl(*o*-bromobenzyl)ammonio]ethyl]amino]-oxamide; ambenonium, *N*,*N*′-bis[[2-[diethyl(*o*-chlorobenzyl)ammonio]ethyl]amino]oxamide.

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