JOURNAL OF BACTERIOLOGY, July 1969, p. 161-168
Vol. 99, No. 1
Copyright © 1969 American Society for Microbiology
Printed in U.S.A.

Purification and Reversible Inactivation of the Isocitrate Dehydrogenase from an Obligate Halophile

JERRY S. HUBBARD AND ALAN B. MILLER

Bioscience Section, Jet Propulsion Laboratory, California Institute of Technology, Pasadena, California 91103

Received for publication 20 February 1969

The nicotinamide adenine dinucleotide phosphate-specific isocitrate dehydrogenase of *Halobacterium cutirubrum* is rapidly inactivated at low NaCl levels. As much as 75% of the initial activity can be restored by dialyzing the inactive enzyme against 4 M NaCl. A mixture of 4 mM isocitrate and 10 mM $MnCl_2$ gives the same protection as 4 M NaCl but does not replace the NaCl requirement for reactivation. The reactivated and native enzymes have identical sedimentation rates on sucrose gradients, electrophoretic mobilities on polyacrylamide gels, and elution rates from Sephadex G-200. However, there are distinct differences between the active and inactive forms of the enzyme. Compared with the active enzyme, the inactive protein has a lower sedimentation rate, a lower electrophoretic mobility, and a faster elution rate from Sephadex. These differences indicate that inactivation causes a major conformational change in the protein. Presumably, the removal of NaCl permits the enzyme to expand into a less dense, inactive form. The isocitrate dehydrogenase was purified 69-fold by a procedure involving the following steps. When the enzyme is selectively protected with isocitrate and $MnCl_2$ at low ionic strength, most of the contaminating proteins are precipitated with $(NH_4)_2SO_4$ at 0.9 saturation. The enzyme in the supernatant fluid is then inactivated at low NaCl levels, precipitated with 0.5 saturated $(NH_4)_2SO_4$, and reactivated with 4 M NaCl. Minor impurities are removed by gel filtration on Sephadex G-200. The resulting preparation is more than 95% pure as judged by disc electrophoresis.

The enzymes of the extreme halophiles become rapidly inactivated if the salt concentration is reduced to low levels (3, 7, 8). Studies on the molecular basis of the salt dependency have been hampered by the fact that the halophilic enzymes are difficult to purify. This paper describes a method for the purification of the nicotinamide adenine dinucleotide phosphate (NADP)-specific isocitrate dehydrogenase (ICDH; EC 1.1.1.42) of *Halobacterium cutirubrum*. In addition, a comparison is made of certain physical properties of the active and inactive forms of the halophilic ICDH.

MATERIALS AND METHODS

Growth of cells. The strain of *H. cutirubrum* (No. 9) was obtained from N. E. Gibbons, National Research Council, Ottawa, Canada. Cultures were grown aerobically at 37 C for 24 hr in CSG medium (11) containing 4.2 M NaCl.

ICDH activity. The reduction of NADP was measured in a Calbiometer spectrophotometer by following the increase in absorbancy at 340 nm. The reaction was carried out in cuvettes of 1-cm light path at 30 C. Reaction mixtures contained 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 7.5), 1.33 mM $MnCl_2$, 0.167 mM NADP (sodium salt), 1.33 mM trisodium isocitrate, 0.5 to 0.65 M NaCl, and enzyme in a final volume of 3 ml. Determinations of absorbancy were made at 30-sec intervals after the addition of enzyme. The amount of enzyme was adjusted so that the initial rate of NADP reduction was linear with time and proportional to enzyme concentration. Specific activity is defined as micromoles of NADP reduced per minute per milligram of protein. Protein was determined by the method of Lowry et al. (9), with bovine serum albumin as the standard.

ICDH purification. All purification steps were performed at 4 C. In all cases, the Tris buffer refers to 0.02 M Tris-hydrochloride at *p*H 7.5. The harvest of cells from 4 liters of culture was suspended in 50 ml of Tris buffer containing 4.2 M NaCl. Cells were ruptured by treatment with a Sonifyer Cell Disruptor for 1 min, and the cell debris was removed by centrifugation at 18,000 × *g* for 30 min. The crude extract was dialyzed for 12 hr against 2 liters of Tris buffer containing 4.2 M NaCl and then was dialyzed

for an additional 12 hr against 9 volumes of Tris buffer containing 4.4 mM isocitrate and 11 mM $MnCl_2$. Solid $(NH_4)_2SO_4$ was added to make the extract 0.8 saturated. After 15-min equilibration, the extract was centrifuged for 30 min at 18,000 × *g*. The supernatant fluid (S_1) was saved, and to the pellet (P_1) was added 25 ml of Tris buffer containing 0.42 M NaCl, 4 mM isocitrate, and 10 mM $MnCl_2$ which had been made 0.8 saturated with $(NH_4)_2SO_4$. The P_1 fraction was stirred intermittently for 1 hr and then centrifuged at 18,000 × *g* for 30 min. The supernatant fluid obtained (S_2) was combined with S_1. The combined fraction (S_1S_2) was made 0.9 saturated with the addition of solid $(NH_4)_2SO_4$, equilibrated for 15 min, and then centrifuged at 130,000 × *g* for 1 hr. The resulting supernatant fluid (S_3) was dialyzed for 12 hr against 2 liters of Tris buffer containing 4.2 M NaCl, transferred to a fresh dialysis tubing, concentrated over a bed of Carbowax 6000 (polyethylene glycol) to a final volume of 95 ml, and dialyzed for 12 hr against 2 liters of Tris buffer containing 4.2 M NaCl. This concentrated S_3 fraction was used as the source of partially purified enzyme. With 4.2 M NaCl present, the ICDH activity was stable for several months when stored at 4 C. A rapid loss of activity occurred when the extract was stored at −18 C.

For further purification, 60 ml of the concentrated S_3 was inactivated by dialysis for 2 hr against nine volumes of Tris buffer containing 2.2 mM β-mercaptoethanol. The inactivated enzyme was precipitated by slowly adding 1.25 volumes of Tris buffer containing 2 mM β-mercaptoethanol and 0.42 M NaCl which had been made 0.9 saturated with $(NH_4)_2SO_4$. After 15-min equilibration, the extract was centrifuged at 18,000 × *g* for 15 min. The pellet (P_4) was carefully drained and then dissolved in 10 ml of Tris buffer containing 2 mM β-mercaptoethanol and 0.42 M NaCl. Residual $(NH_4)_2SO_4$ was removed by dialyzing the P_4 twice for 1 hr against 200 ml of the same buffer. The ICDH in the P_4 was reactivated by the procedure described below. After reactivation, the volume of P_4 was 7.7 ml.

A 5-ml amount of reactivated P_4 was placed in a dialysis bag and concentrated over Carbowax to 1.8 ml. The concentrated P_4 was added to a Sephadex G-200 column (330 × 23 mm) which was equilibrated with Tris buffer containing 4.2 M NaCl. The ICDH was eluted from the column with Tris buffer containing 4.2 M NaCl, and the enzyme activity was measured on 0.1-ml samples of the eluant. The highest specific activity of ICDH was in the fractions comprising the trailing half of the activity peak, i.e., an elution volume of 78 to 88 ml. These fractions were combined in a dialysis bag and concentrated over Carbowax to 1.4 ml.

Inactivation and reactivation of ICDH. Unless otherwise indicated, the ICDH was inactivated by dialyzing the extract for 2 hr at 4 C against 100 to 200 volumes of 0.02 M Tris buffer (*p*H 7.5) containing 2 mM β-mercaptoethanol. Reactivation was performed by dialyzing the inactive ICDH against 100 volumes of 0.02 M Tris buffer containing 4.2 M NaCl (*p*H 7.5) for 1.5 hr at 31 C, and then dialyzing against a second change of the same buffer for 2 hr at 31 C.

Sucrose gradient centrifugation. Sucrose density gradients (5 to 20%) were prepared and sampled according to the procedure of Martin and Ames (10). For experiments with active ICDH, a buffer containing 0.02 M Tris (*p*H 7.5), 4 mM isocitrate, and 10 mM $MnCl_2$ was mixed with the sucrose solutions. Inactivated ICDH was analyzed on gradients prepared with sucrose solutions containing 0.02 M Tris (*p*H 7.5) and 2 mM β-mercaptoethanol. Samples were centrifuged for 8 hr at 39,000 rev/min in a Spinco model L centrifuge equipped with a swinging-bucket rotor (SW 39).

Disc gel electrophoresis. The disc electrophoresis of proteins was performed by the procedure of Ornstein and Davis according to the instructions provided by Canalco Co., Silver Spring, Md. The procedure was modified in that the 7% gel and the Tris-glycine developing buffers were supplemented with 4 mM isocitrate and 10 mM $MgCl_2$. Before electrophoresis, samples of active ICDH were dialyzed for 2 hr against 200 volumes of 0.02 M Tris buffer (*p*H 7.5) containing 4 mM isocitrate and 10 mM $MnCl_2$. Samples of inactivated ICDH were in 0.02 M Tris-2 mM β-mercaptoethanol buffer (pH 7.5). The electrophoretograms were stained with water-soluble Nigrosin (0.0125%) in methanol-water-acetic acid (4:5:1) for 3 hr, destained with the same solvent, and stored in 7% aqueous acetic acid (1).

RESULTS

Stabilization of ICDH. Figure 1 shows activity measurements after partically purified ICDH was incubated with various levels of NaCl. Complete stabilization was achieved when the buffer contained more than 3 M NaCl. At lower concentrations, the degree of protection was roughly proportional to the NaCl level. For example, with 2.2 M NaCl about 50% of the activity was lost in 20 hr. With 1.2 M NaCl essentially all the activity was lost in 3 hr.

Certain other salts can protect the ICDH (Table 1). The effectiveness appears to be related to both the anion and cation employed. At 1.5 M levels, the order of effectiveness of the cations is $Na^+ > K^+ > Rb^+ > Cs^+ > Li^+$ and NH_4^+. Of the anions tested, acetate and SO_4^{2-} were more effective than Cl^-, and Br^- was ineffective.

From experiments in which assays were made with low NaCl levels, it was apparent that some component(s) of the reaction mixture replaces the high salt requirement for stabilizing the ICDH activity. Table 2 shows that complete stability was not conferred by isocitrate, NADP, or the metal activator when tested individually. However, the ICDH activity was protected when both isocitrate and $MnCl_2$ were added. A synergistic effect was also noted with isocitrate and $MgCl_2$,

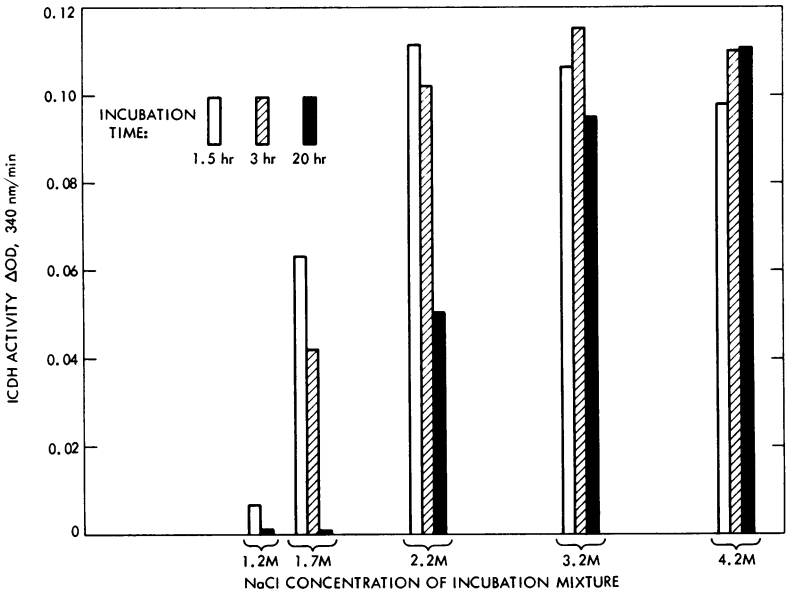

FIG. 1. *Stabilization of ICDH by various levels of NaCl. All incubations contained 0.02 M Tris (pH 7.5), 0.21 M NaCl, and enzyme (57 μg of protein) in a final volume of 0.4 ml. Incubations were performed at 0 C. Activity measurements were made on 0.1-ml samples of the incubation mixtures.*

TABLE 1. *Stabilization of ICDH by various salts*

Addition to incubation mixture[a]		Percentage of initial activity remaining after incubation for	
Salt	Concn (M)	1.5 hr	3 hr
None	—	0	0
NaCl	1.5	55	32
NaBr	1.5	0	0
Na_2SO_4	0.75	80	59
Na acetate	1.5	100	100
Na acetate	1.0	90	62
LiCl	1.5	0	0
LiCl	4.0	5	0
KCl	1.5	22	5
NH_4Cl	1.5	0	0
$(NH_4)_2SO_4$	0.75	25	8
RbCl	1.5	10	2
CsCl	1.5	4	2

[a] All incubation mixtures contained 0.02 M Tris (*p*H 7.5), 0.21 M NaCl, and enzyme (57 μg of protein) in a final volume of 0.4 ml. Incubations were at 0 C. Activity measurements were made at 30 C with the use of 0.1-ml samples of the incubation mixtures.

although a definite loss in activity occurred upon prolonged incubation. A slight protection was given by the mixture of NADP and isocitrate, but NADP did not act synergistically with isocitrate. The requirement for tricarboxylic acid appears specific for isocitrate. Other experiments showed that mixtures of $MnCl_2$ and sodium citrate or sodium *cis*-aconitate gave no more protection than $MnCl_2$ alone.

Reactivation of ICDH. The loss in activity caused by exposure to low ionic strength can be partially reversed by slowly increasing to high levels the concentration of NaCl. As much as 75% of the initial activity was recovered when the inactivated ICDH was dialyzed against 4 M NaCl (Table 3). Although a complete parametric study was not conducted, it appeared that the optimal conditions for reactivation involved dialysis at 31 C for 3 to 4 hr. Lower temperatures gave poorer recovery. Longer incubation at higher temperatures resulted in losses of activity which were presumably due to thermal denaturation. These conditions are quite similar to those reported for the reactivation of the halophilic malate dehydrogenase of *Halobacterium salinarium* (7). To insure good recovery, it was necessary to have β-mercaptoethanol present when the ICDH was inactivated. Presumably, this protects the sulfhydryl groups of the inactive protein from being oxidized and thereby causing permanent denaturation of the enzyme. Also, good recovery of activity was dependent upon the removal of the β-mercaptoethanol during the reactivation step. The mixture of isocitrate and $MnCl_2$ did

TABLE 2. *Stabilization of ICDH by substrates of the enzyme*

Addition to incubation mixture[a]	Percentage of initial activity remaining after incubation for	
	1.5 hr	20 hr
None	0	0
$MnCl_2$ (10 mM)	20	0
Isocitrate (4 mM)	0	0
$MnCl_2$ (10 mM) plus isocitrate (4 mM)	111	116
NADP (0.25 mM)	0	0
NADP (0.25 mM) plus $MnCl_2$ (10 mM)	64	6
NADP (0.25 mM) plus isocitrate (4 mM)	0	0
$MgCl_2$ (10 mM)	0	0
$MgCl_2$ (10 mM) plus isocitrate (4 mM)	105	77

[a] All incubation mixtures contained 0.02 M Tris (*p*H 7.5), 0.21 M NaCl, and enzyme (57 μg of protein) in a final volume of 0.4 ml. Incubations were at 0 C. Activity measurements were made at 30 C with the use of 0.1-ml samples of the incubation mixtures.

TABLE 3. *Reactivation of ICDH*

Reactivation buffer[a]	Percentage of initial activity recovered after dialysis at		
	4 C, 18 hr	31 C, 3.5 hr	31 C, 18 hr
None	0	0	0
NaCl (4 M)	22.9	74.9	53.4
Na acetate (4 M)	4.2	—	12.2
Isocitrate (4 mM) plus $MnCl_2$ (10 mM)	0	0	0

[a] Samples of inactivated ICDH were dialyzed against 160 volumes of 0.02 M Tris buffer (*p*H 7.5) containing the indicated additions.

not reactivate the ICDH (Table 3), even though this mixture effectively stabilizes the active enzyme (Table 2). Apparently, the latter effect is related to the ability of the substrates to hold the enzyme in its active conformation by virtue of their interaction at the active site. When the enzyme is in the inactive conformation, the substrate mixture cannot replace the high ionic strength needed to restore the active conformation. There is, however, some indication that $MnCl_2$ can interact with the inactive form of the enzyme. Other experiments showed that 10 mM $MnCl_2$ caused a 65% inhibition of the reactivation promoted by 4 M NaCl. Isocitrate did not affect the NaCl-promoted reactivation. Sodium acetate was less effective than NaCl in promoting reactivation (Table 3) even though the sodium acetate was effective in protecting the enzyme (Table 1).

The reversible inactivation could also be demonstrated by disc electrophoresis. In Fig. 2, gel F is a stained electrophoretogram of partially purified ICDH. The major protein bands were the ICDH and a nonhalophilic protein (NHP). Additional information on the NHP will be given in subsequent discussion. The identification of the other band as active ICDH was based on the detection of enzyme activity when the gels were sectioned and the enzyme was eluted with 4.2 M NaCl. To retain activity of the ICDH during electrophoresis, it was necessary to incorporate isocitrate and $MgCl_2$ in the gels and developing buffers. High levels of salt could not be used to stabilize the ICDH, because salts interfere with the polymerization of the polyacrylamide gels and with the development of electrophoretograms. When the ICDH was inactivated before electrophoresis, its electrophoretic mobility was altered. The inactive ICDH protein appeared concomitantly with the disappearance of the active ICDH (gel G). The lower mobility of the inactive protein suggests that the inactivation causes a major change in either the net charge or the shape of the protein molecule (12). The latter could be caused by aggregation of enzyme molecules to give a larger species or by a conformational change which increases the molecular radius of the protein. Gel H illustrates the partial reversal of the inactivation. After reactivation with 4.2 M NaCl, the band of inactive ICDH almost completely disappeared and the active ICDH band reappeared.

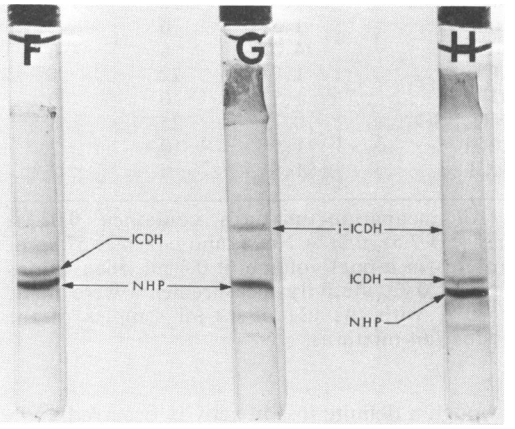

FIG. 2. *Disc electrophoresis of native (gel F), inactivated (gel G), and reactivated ICDH (gel H). NHP = nonhalophilic protein; i-ICDH = inactivated enzyme.*

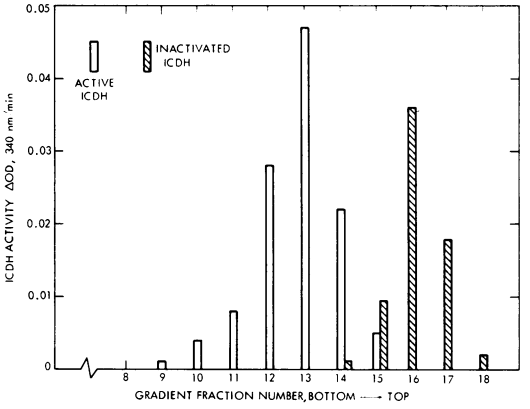

FIG. 3. *Sucrose gradient centrifugation of active and inactivated ICDH. Active and inactivated enzyme (0.1 ml, 0.22 mg of protein) were layered on 4.6-ml sucrose gradients. Samples from the gradient run with inactivated ICDH were reactivated before the activity was measured. In both experiments, 0.05 ml of the gradient fractions was assayed.*

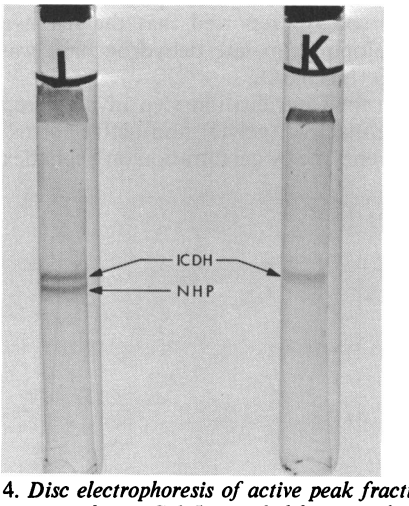

FIG. 4. *Disc electrophoresis of active peak fractions from sucrose gradients. Gel J is pooled fractions from a run with active ICDH. Gel K is ICDH which was inactivated, run on a sucrose gradient, reactivated, pooled, run on a second sucrose gradient, and then pooled for disc electrophoresis.*

Sedimentation of ICDH. Physical differences between the active and inactive forms of the enzyme could also be demonstrated by centrifuging the proteins on sucrose density gradients (Fig. 3). The inactivated ICDH was sedimented at a lower rate than the active enzyme. This suggests that the inactive protein is of lower molecular weight or is of lower density than active ICDH (15). In other experiments, the inactivated ICDH from sucrose gradients was reactivated and then centrifuged on a second sucrose gradient. The reactivated enzyme exhibited a sedimentation rate identical to that of the native enzyme. Figure 4 shows stained electrophoretograms of the ICDH recovered from sucrose gradients. When partially purified enzyme was sedimented in the active state (gel J), ICDH and NHP were detected in the same fractions. These two proteins must have similar sedimentation rates. When the inactivated ICDH was sedimented, reactivated, and run on a second sucrose gradient (gel K), the ICDH was the predominate protein. Apparently, the inactivated ICDH and the NHP had been separated because of the differences in sedimentation rates of the two proteins.

Gel filtration of ICDH. The two forms of the enzyme exhibit different rates of elution from Sephadex G-200 (Fig. 5). The more rapid elution of the inactive protein shows this species to be less restricted during its diffusion through the column of cross-linked gel particles. Apparently, the inactivated ICDH has a larger molecular radius than the active enzyme (15).

Purification of ICDH. The purification procedure is illustrated in Table 4 and Fig. 6. When the ICDH is selectively protected with isocitrate and $MnCl_2$ at low ionic strength, 0.9 saturated $(NH_4)_2SO_4$ precipitates large amounts of contaminating protein. Holmes and Halvorson (6) previously showed that halophilic proteins are easily precipitated with $(NH_4)_2SO_4$ once the proteins are denatured by the removal of NaCl. Thus, the components of the supernatant fluid should be either halophilic proteins which are protected by the isocitrate and $MnCl_2$ or nonhalophilic proteins which are not precipitated with 0.9 saturated $(NH_4)_2SO_4$. The effectiveness of this step is indicated by the 13-fold increase in specific activity with 68% recovery of activity.

After being inactivated, the ICDH can be precipitated with $(NH_4)_2SO_4$ at 0.5 saturation. The precipitated enzyme can then be partially reactivated by dialysis against 4.2 M NaCl (gel C). The NHP and other minor contaminants remained in the supernatant fluid. This poor recovery of activity at this step can be attributed to two causes. All of the inactivated ICDH was not precipitated and the efficiency of reactivation was low. The decrease in specific activity at step C (Table 4) is indicative of the incomplete reactivation. The relative intensities of the two forms of the enzyme (gel C) are not an accurate measure of the quantities of protein, since Nigrosin was found to stain the active ICDH more intensely than the inactive protein. Perhaps the $(NH_4)_2SO_4$ precipitation causes permanent inactivation of a large portion of the ICDH. Alternatively, small amounts of residual $(NH_4)_2SO_4$ may be inhibitory to the reactivation. Holmes and

Halvorson (6) reported that the reactivation of the halophilic malate dehydrogenase was inhibited by $(NH_4)_2SO_4$.

The final purification step involves separation of the inactive (gel D) and active forms (gel E) of the enzyme by gel filtration on Sephadex G-200.

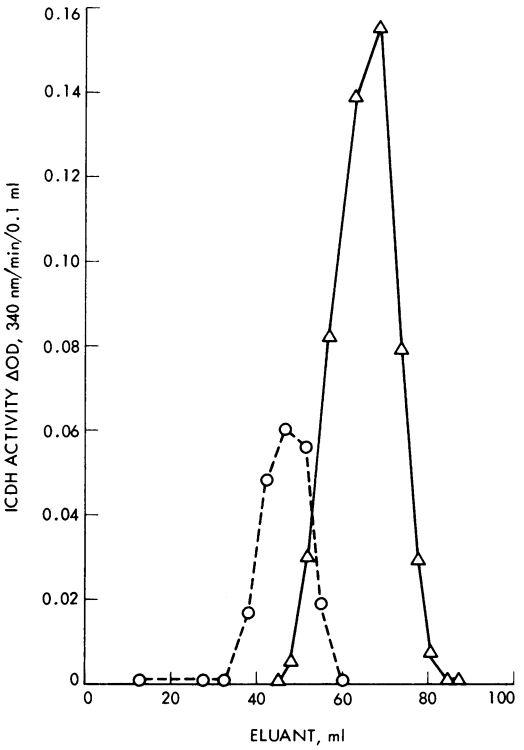

FIG. 5. *Gel filtration of active and inactivated ICDH on Sephadex G-200. The 23 × 225 mm column used for the active ICDH (△) was equilibrated and eluted with 0.02 M Tris (pH 7.5) containing 4 mM isocitrate and 10 mM $MnCl_2$. For the run with inactivated ICDH (○), the same column material was equilibrated and eluted with 0.02 M Tris (pH 7.5) containing 2 mM β-mercaptoethanol. The eluant fractions of the inactivated ICDH were reactivated before the enzyme activity was measured.*

Again, the inactivated ICDH was eluted from the column more rapidly than the active enzyme. The active ICDH (gel E) represents more than 95% of the material detected with the protein stain. The removal of the inactive ICDH and minor protein contaminants gave an eightfold increase in specific activity.

The entire procedure gave a 69-fold purification, and the resulting preparation had a specific activity of 13.8. This value is lower than the reported specific activities of 58 for purified ICDH from pig heart (14) and 21.5 for partially purified ICDH from *Brevibacterium flavum* (13).

DISCUSSION

On the basis of studies with the halophilic lactate dehydrogenase of *H. salinarium*, Baxter (2) proposed that lowering the salt concentration permitted the enzyme to expand to an inactive conformation. The salt was presumed to decrease the electrostatic repulsions between ionized groups within the enzyme molecule and thereby maintain the conformational structure which was catalytically active. Holmes and Halvorson (7) suggested a similar function for salt in the stabilization of the malate dehydrogenase of *H. salinarium*. Lowering the salt concentration caused a marked decrease in the sedimentation rate of malate dehydrogenase on sucrose gradients. The decreased sedimentation was interpreted as representing a decrease in the density of the enzyme. The alternate possibility that the inactivation caused a disaggregation of the malate dehydrogenase into inactive subunits was disregarded, since most of the enzyme activity could be regained by increasing the salt concentration. The latter assumption is unwarranted in view of findings that enzymes such as glutamine synthetase can be rapidly disaggregated and reaggregated with a near stoichiometric recovery of activity (16). The two interpretations, i.e., disaggregation or expansion, can be used to explain our demonstration that the inactivated ICDH has a slower sedimentation rate than the active

TABLE 4. *Purification of ICDH*

Fraction	Total activity	Total protein	Specific activity	Recovery corrected[a]
	units	*units*		%
(A) Dialyzed crude	627	3,120	0.2	100
(B) $(NH_4)_2SO_4$ supernatant fluid (0.9 saturated)	425	169	2.5	68
(C) $(NH_4)_2SO_4$ precipitate (0.5 saturated) of inactivated ICDH, reactivated	21.6	12.7	1.7	5.4
(E) Sephadex G-200 eluant (78–88 ml)	7.1	0.51	13.8	2.7

[a] Recovery values are corrected to the total volume of extract since only portions of fractions B and C were used in subsequent steps.

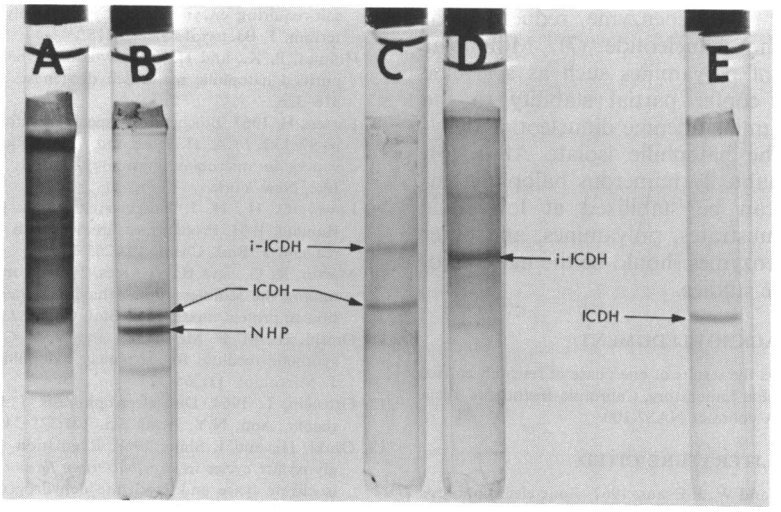

FIG. 6. *Disc electrophoresis of ICDH at different steps in the purification. Gels A, B, C, and E correspond to the purification steps in Table 4. Gel D shows the inactive form of ICDH (i-ICDH) eluted from the Sephadex G-200 column between 44 and 57 ml.*

enzyme. However, the additional data with gel filtration on Sephadex G-200 are more conclusive. The more rapid elution of the inactive enzyme indicates that the molecular radius of the protein has expanded either by aggregation of several molecules or by expansion of a single molecule. Thus, the interpretation that the removal of salt causes an expansion of the protein to a less dense state is consistent with the data on both sedimentation and gel filtration. Observation of the low electrophoretic mobility of the inactivated ICDH tends to support the molecular expansion theory, but the change in electrophoretic mobility could be explained in other ways.

It is probably an oversimplification to assume that salts stabilize the halophilic enzymes solely by the neutralization of repulsive, intramolecular charges. There is no apparent relationship between the protection afforded and the ionic radius or ionic mobility. Other factors must be involved. Moreover, different halophilic enzymes exhibit different specificities for salts. For example, the present study is the first report of NaCl being more effective than KCl in stabilizing a halophilic enzyme. Even more apparent are the differences in salt specificity in the activation of halophilic enzymes (4, 8). Other studies (Hubbard, *unpublished data*) have shown that the ICDH activity is stimulated by adding salts to the reaction mixture. For example, the activity with the optimal NaCl concentration of 0.5 M is 1.6 times that observed with 1 mM NaCl. An even greater degree of stimulation in activity is given with LiCl at its optimal concentration of 0.35 M. Again, the ICDH of *H. cutirubrum* differs from other halophilic enzymes in that KCl is a less effective activator than NaCl. Since LiCl was effective in activation and ineffective in stabilization, it would appear that salts occupy different roles in the two processes. A further indication that the functions are distinguishable comes from our observation and the observations of others (7) that different concentrations of a salt are needed for maximal activation and stabilization.

The use of isocitrate and $MnCl_2$ to selectively protect the ICDH was advantageous in our purification procedure. The number of purification steps that can be performed is quite limited when 4 M NaCl is present. However, with a buffer of low ionic strength the proteins can be fractionated by conventional methods. In the partial purification of halophilic malate dehydrogenase (6), the problem was circumvented by fractionating the enzyme in a salt-free state and then reactivating the enzyme. We were hesitant to use this technique for fear that the reactivated enzyme might be different from the native protein. Our attempts to purify the ICDH in its native state were unsuccessful, so one inactivation-reactivation step was used. However, the data from disc electrophoresis, sedimentation, and gel filtration experiments gave no indication that inactivation or reactivation altered the ICDH. Other studies (Hubbard, *unpublished data*) showed that native and once-reactivated enzyme had identical K_m values for isocitrate, $MnCl_2$, and NADP.

The ICDH of *H. cutirubrum* is not the only halophilic enzyme than can be stabilized with specific effectors as replacements for salt. The malate dehydrogenase of *H. salinarium* is par-

tially stabilized by its coenzyme, reduced nicotinamide adenine dinucleotide (7). Millimolar concentrations of polyamines such as spermine and polylysine confer partial stability to the reduced nicotinamide adenine dinucleotide dehydrogenase of the halophilic isolate AR-1 (5). There are undoubtedly numerous halophilic enzymes which can be stabilized at low ionic strength with substrates, polyamines, and other effectors. Such enzymes should be the material of choice for future studies.

ACKNOWLEDGMENT

This paper presents the results of one phase of research carried out at the Jet Propulsion Laboratory, California Institute of Technology, under NASA contract NAS7-100.

LITERATURE CITED

1. Altschul, A. M., and W. J. Evans. 1967. Zone electrophoresis with polyacrylamide gel, p. 179–186. *In* C. H. W. Hirs (ed.), Methods in enzymology, vol. 11. Academic Press Inc., New York.
2. Baxter, R. M. 1959. An interpretation of the effects of salts on the lactic dehydrogenase of *Halobacterium salinarium*. Can. J. Microbiol. 5:47–57.
3. Baxter, R. M., and N. E. Gibbons. 1956. Effects of sodium and potassium chloride on certain enzymes of *Micrococcus halodenitrificans* and *Pseudomonas salinaria*. Can. J. Microbiol. 2:599–606.
4. Hochstein, L. I., and B. P. Dalton. 1968. Salt specificity of a reduced nicotinamide adenine dinucleotide oxidase prepared from a halophilic bacterium. J. Bacteriol. 95:37–42.
5. Hochstein, L. I., and B. P. Dalton. 1968. Factors affecting the cation requirement of a halophilic NADH dehydrogenase. Biochim. Biophys. Acta 167:638–640.
6. Holmes, P. K., and H. O. Halvorson. 1965. Purification of a salt-requiring enzyme from an obligately halophilic bacterium. J. Bacteriol. 90:312–315.
7. Holmes, P. K., and H. O. Halvorson. 1965. Properties of a purified halophilic malic dehydrogenase. J. Bacteriol. 90: 316–326.
8. Larsen, H. 1967. Biochemical aspects of extreme halophilism, p. 97–132. *In* A. H. Rose and J. F. Wilkinson (ed.), Advances in microbial physiology, vol. 1. Academic Press Inc., New York.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
10. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behaviour of enzymes; application to protein mixtures. J. Biol. Chem. 236:1372–1379.
11. Onishi, H., M. E. McChance, and N. E. Gibbons. 1965. A synthetic medium for extremely halophilic bacteria. Can. J. Microbiol. 11:365–373.
12. Ornstein, L. 1964. Disc electrophoresis. I. Background and theory. Ann. N.Y. Acad. Sci. 121:321–349.
13. Ozaki, H., and I. Shiio. 1968. Regulation of the TCA and glyoxylate cycles in *Brevibacterium flavum*. I. Inhibition of isocitrate lyase and isocitrate dehydrogenase by organic acids related to the TCA and glyoxylate cycles. J. Biochem. 64:355–363.
14. Plaut, G. W. E. 1962. Isocitrate dehydrogenase (TPN-linked) from pig heart (revised procedure), p. 645–651. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 5. Academic Press Inc., New York.
15. Siegel, L. M., and K. J. Monty. 1966. Determination of molecular weights and frictional ratios of proteins in impure systems by use of gel filtration and density gradient centrifugation. Application to crude preparations of sulfite and hydroxylamine reductases. Biochim. Biophys. Acta 112: 346–362.
16. Woolfolk, C. A., and E. R. Stadtman. 1967. Regulation of glutamine synthetase. IV. Reversible dissociation and inactivation of glutamine synthetase from *Escherichia coli* by the concerted action of EDTA and urea. Arch. Biochem. Biophys. 122:174–189.