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Nature of the Inactivation of the Isocitrate Dehydrogenase from an Obligate Halophile

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The nicotinamide adenine dinucleotide phosphate-specific isocitrate dehydrogenase (ICDH) of *Halobacterium cutirubrum* is rapidly inactivated at low NaCl levels. From sucrose gradient analysis, it was estimated that the active ICDH has an $S_{20,w}$ of 5.3 and a molecular weight of 75,000. The inactivation by removal of NaCl causes an unfolding of the protein yielding a less-compact conformer with an $S_{20,w}$ of 2.0. This inactivation apparently causes internal sulfhydryl groups to be exposed. Over 90% of the initial activity can be restored by dialyzing the inactivated ICDH against 4 M NaCl, provided that the exposed sulfhydryl groups are protected with dithiothreitol. The ICDH is permanently inactivated when the sulfhydryl groups are oxidized or alkylated. The alkylation of the inactive ICDH was demonstrated by treatment with ^{14}C-*N*-ethyl maleimide. Sucrose gradient analysis showed that ^{14}C was bound to a protein with sedimentation properties identical to that of reversibly inactivated ICDH, i.e., an $S_{20,w}$ of 2.0. Much less ^{14}C was bound when active ICDH was treated with ^{14}C-*N*-ethyl maleimide. The *H. cutirubrum* ICDH resembles other bacterial isocitrate dehydrogenases in being susceptible to concerted feedback inhibition by oxalacetate and glyoxalate.

The nicotinamide adenine dinucleotide phosphate (NADP)-specific isocitrate dehydrogenase (ICDH; EC 1.1.1.42) of *Halobacterium cutirubrum* is typical of enzymes from extreme halophiles (3). This ICDH requires about 4 M NaCl for maximal stability and is rapidly inactivated when the NaCl concentration is reduced (2). Enzymatic activity can be restored by a slow readdition of 4 M NaCl. Mixtures of millimolar levels of isocitrate and $MnCl_2$, the substrate and metal activator, confer the same stability as the high salt levels. However, this mixture does not serve to reactivate the ICDH. The use of isocitrate and $MnCl_2$ to protect the ICDH at low ionic strength was advantageous in the purification and preliminary characterization of the halophilic ICDH (2). This paper presents further information on the conformational changes associated with the inactivation and reactivation of the protein.

MATERIALS AND METHODS

Enzyme preparation and assays. The conditions for growth of *H. cutirubrum*, preparation of 30-fold purified ICDH and measurement of ICDH activity have been described (2). The stock solutions of *H. cutirubrum* ICDH were dissolved in 0.02 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 7.5) containing 4.2 M NaCl. A unit of ICDH is the amount which catalyzes the reduction of 1 mmole of NADP per min at 30 C. A partially purified preparation of NADP-specific ICDH from pig heart was obtained from Calbiochem, Los Angeles, Calif. The pig ICDH was assayed under conditions described by Plaut (6).

Inactivation and reactivation of ICDH. Unless otherwise indicated, the halophilic ICDH was inactivated by dialyzing 0.4 ml of extract (0.002 units) for 2 hr at 4 C against 100 ml of 0.02 M Tris buffer (*p*H 7.5) containing 1 mM dithiothreitol (DTT). In some experiments the DTT was omitted or replaced by other reagents. The inactivated ICDH was maintained at 0 to 4 C. Reactivation was accomplished by dialyzing the inactive ICDH against 500 to 2,000 volumes of 0.02 M Tris buffer (*p*H 7.5) containing 4.2 M NaCl 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 by the procedure of Martin and Ames (4). 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 1 mM DTT. Samples were centrifuged for 16 hr at 124,000 × *g* in a Spinco model L centrifuge equipped with a swinging-bucket rotor (SW39).

Radioactive measurements. The *1*-^{14}*C*-*N*-ethyl

maleimide (^{14}C-NEM) with specific radioactivity of 10.3 mc/mmole was obtained from Schwarz BioResearch, Inc., Orangeburg, N.Y. Enzyme solutions (0.1-ml amounts) or 0.25 ml of sucrose gradient fractions were diluted to 2.0 ml with water and placed in 20-ml scintillation vials. A 10-ml amount of dioxane containing 10% (w/v) naphthalene and 0.5% (w/v) diphenyloxalone was added, and the radioactivity was measured in a liquid scintillation counter (Beckman LS-100). The counting efficiency was approximately 50%. All data are corrected for background radioactivity which was about 15 counts/min.

Other reagents. Trisodium isocitrate, NADP (sodium salt), glyoxalic acid, oxalacetic acid, and DTT were obtained from Calbiochem. Iodoacetamide (IAA) was obtained from J. T. Baker Chemical Co. Unlabeled *N*-ethyl maleimide (NEM) was obtained from Schwarz BioResearch, Inc. Glyoxalic acid and oxalacetic acid were neutralized with NaOH and were used within 1 hr after preparation. When glyoxalate and oxalacetate were used simultaneously, they were mixed for 4 min before the ICDH assay was initiated.

RESULTS

Sedimentation of ICDH. Figure 1 shows the differences in sedimentation of native and reversibly inactivated ICDH on sucrose density gradients. Pig heart ICDH was used as a control to determine whether the sucrose gradients prepared with the two different buffers gave different sedimentation values. This was not the case, since the pig enzyme was sedimented at the same rate on the gradient containing isocitrate and $MnCl_2$ or the one containing DTT.

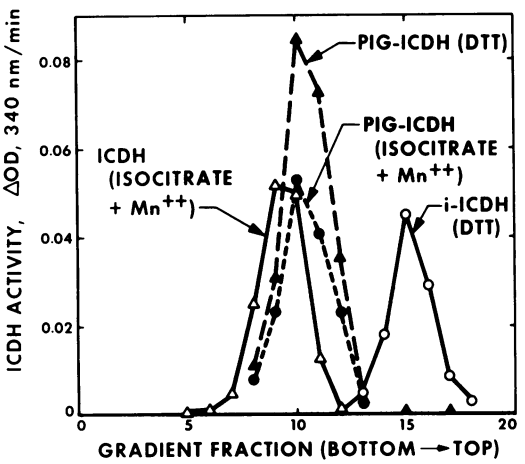

FIG. 1. *Sucrose gradient centrifugation of isocitrate dehydrogenase (ICDH). A 0.1-ml amount of a solution of active halophilic enzyme (ICDH), inactivated halophilic enzyme (i-ICDH), and pig heart enzyme (pig-ICDH) was layered on 4.6-ml sucrose gradients. As indicated, the sucrose solutions contained either dithiothreitol (DTT) or isocitrate and $MnCl_2$. Samples from the gradient run with i-ICDH were reactivated before the activity was measured.*

The pig ICDH is known to have an $S_{20,w}$ of 4.6 and a molecular weight of 61,000 (8). If it is assumed that the pig enzyme and native, halophilic ICDH have about the same shapes and partial specific volumes, then the $S_{20,w}$ and molecular weight of the latter can be calculated by the equations of Martin and Ames (4). Based on these assumptions, the native, halophilic ICDH has an $S_{20,w}$ of 5.3 and a molecular weight of 75,000. By similar calculations, the inactivated (expanded) ICDH has an $S_{20,w}$ of 2.0.

Exposure of sulfhydryl groups during inactivation. Dialysis of ICDH against dilute Tris buffer for 2 hr at 4 C caused complete inactivation (Table 1). A 44% recovery of activity was effected by dialyzing the inactive protein against 4.2 M NaCl. The extent of reactivation was less when the inactive protein was exposed to air, H_2O_2, IAA, or NEM. A greater degree of reactivation was achieved when the inactive enzyme was maintained in the presence of β-mercaptoethanol or DTT.

When the ICDH was stabilized with 4 M NaCl or isocitrate and $MnCl_2$, the loss in activity caused by IAA or NEM was only 8 to 22% (Table 2). Similarly, other experiments have shown that the

TABLE 1. *Reactivation of ICDH after various treatments*

Inactivation treatment[a]	Reactivation treatment	Activity recovered
		%
Tris	None	0
	NaCl	44
Tris + β-mercaptoethanol (1 mM)	None	0
	NaCl	73
Tris + DTT (1 mM)	None	0
	NaCl	99
Tris + aeration[b]	NaCl	11
Tris + H_2O_2[c]	NaCl	0
Tris + IAA (1 mM)	NaCl	11
Tris + NEM (1 μM)	NaCl	13

[a] Inactivated by dialysis against 0.02 M Tris buffer containing the indicated additions.

[b] After inactivation, the enzyme was removed from the dialysis bag and stirred for 10 min a 4 C.

[c] Inactivated enzyme was dialyzed against 0.02 M Tris containing 10% H_2O_2 for 1 hr at 4 C.

active conformer does not lose activity when aerated. Even when the enzyme is in the inactive conformation, DTT minimizes the permanent loss in activity caused by NEM. These observations suggest that the expanded, inactive protein has exposed sulfhydryl groups, and the enzyme can be permanently inactivated if these groups are oxidized or alkylated.

Binding of ^{14}C-NEM to ICDH. When partially purified ICDH was dialyzed against ^{14}C-NEM and then washed extensively, six times more radioactivity was bound to the inactivated enzyme than to the preparation protected with isocitrate and $MnCl_2$. To confirm that the label was bound to the ICDH, samples of the ^{14}C-NEM-treated proteins were analyzed on sucrose density gradients (Fig. 2). With the unprotected ICDH, the greatest concentration of radioactivity was sedimented to a distance identical to that previously observed with reversibly inactivated ICDH. This indicates that some ^{14}C-NEM is firmly bound to the inactive conformer. A smaller amount of radioactivity was detected in the corresponding fractions of the sucrose gradient run with the protected enzyme. It was shown (Table 2) that the NEM treatment caused some inactivation of the ICDH when isocitrate and $MnCl_2$ were present. Presumably, the low level of radioactivity corresponds to that partial loss in activity. The gradient fractions expected to contain the active ICDH did not contain appreciable levels of radioactivity. Apparently ^{14}C-NEM is not bound to the "compact" form of the enzyme.

The detection of radioactivity in gradient fractions 2 through 9 (Fig. 2) presumably reflects the binding of ^{14}C-NEM to contaminating proteins in this partially purified preparation. Since this binding was less pronounced with the "protected preparation," it appears that isocitrate or $MnCl_2$ (or both) also protects the contaminants from alkylation.

Calculation of the amount of binding of ^{14}C-NEM to inactivated ICDH is not particularly instructive. For example, if the assumptions that the ICDH has a molecular weight of 75,000 and a specific activity of 13.8 (2) are valid, the washed, inactivated preparation in Fig. 2 has retained only 0.19 moles of NEM per mole of enzyme. Moreover, the sucrose gradient analysis showed that all the label was not associated with the inactivated ICDH. Obviously, at least 1 mole of effector would be needed for permanent inactivation of 1 mole of enzyme. Perhaps some of the sulfhydryl groups may have been oxidized to disulfides and thus rendered nonreactive to the alkylation. Also, some NEM may have been displaced during the washing procedure.

TABLE 2. *Permanent inactivation of ICDH by alkylation reagents*

Inactivation treatment[a]	Activity recovered after reactivation
	%
DTT	90
NEM	9
NEM + DTT	75
NEM + NaCl	78
NEM + isocitrate + $MnCl_2$	81
IAA + NaCl	92
IAA + isocitrate + $MnCl_2$	89

[a] Enzyme was dialyzed against 0.02 M Tris containing the indicated additions. DTT, 1 mM; NEM, 10 μM; IAA, 1 mM; isocitrate, 4 mM; $MnCl_2$, 10 mM; NaCl, 4 M.

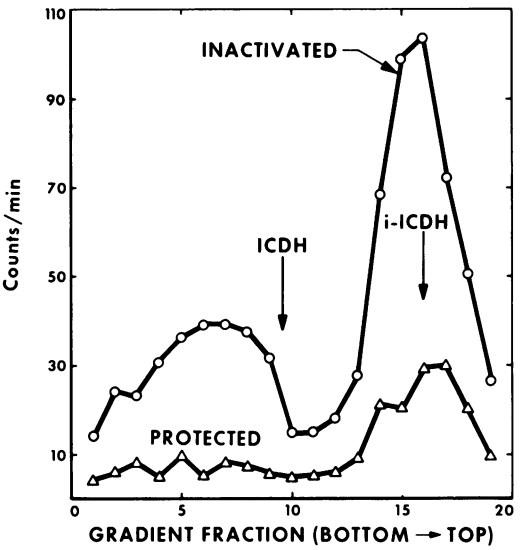

FIG. 2. *Distribution of radioactivity in sucrose gradient fractions after sedimentation of ICDH treated with ^{14}C-N-ethyl maleimide (^{14}C-NEM). A 0.4-ml amount of a solution (0.002 units) of enzyme was dialyzed against 50 ml of 0.02 M Tris containing either 0.97 μM ^{14}C-NEM (inactivated enzyme) or 0.97 μM ^{14}C-NEM, 4 mM isocitrate, and 10 mM $MnCl_2$ (protected enzyme). Treated enzymes were washed by dialysis against 1,000 ml of 0.02 M Tris containing 4.2 M NaCl for 3 hr at 31 C and then for 2 hr at 4 C against 100 ml of 0.02 M Tris containing 4 mM isocitrate and 10 mM $MnCl_2$. The protected enzyme retained 378 counts/min, and the inactivated preparation retained 2,228 counts/min. Samples (0.1 ml) were run on 4.6-ml sucrose gradients containing isocitrate and $MnCl_2$. The arrows indicate the distances that active (ICDH) and inactivated enzyme (i-ICDH) are normally sedimented.*

Feedback inhibition of ICDH. The *H. cutirubrum* enzyme is similar to the ICDH of other bacteria in being susceptible to a concerted feedback inhibition by glyoxalate and oxalacetate (Table 3). Neither of these effectors was inhibitory when tested individually. The degree of inhibition was greater when the lower level of isocitrate was used. This halophilic ICDH would fall in the category of the isocitrate dehydrogenases which are less sensitive to the inhibition than the *Brevibacterium flavum* enzyme (7).

DISCUSSION

In a previous study (2), gel filtration and disc electrophoresis experiments indicated that the inactivated ICDH (salt-free) has a larger molecular radius than the active enzyme. In the present study, the marked decrease in $S_{20,w}$ from 5.3 to 2.0 illustrates the extent to which the halophilic ICDH is expanded when salt is removed. The lower sedimentation rate is apparently caused by an increase in the frictional ratio (9).

Undoubtedly, the expansion of the enzyme from its active conformation would break hydrogen bonds. Recent studies (Hubbard and Miller, *unpublished data*) have shown that the halophilic ICDH can be inactivated by treatment with reagents which are known to break hydrogen bonds of proteins. Even when NaCl or isocitrate and $MnCl_2$ are present, exposure to guanidine hydrochloride (GuHCl) or urea causes a rapid loss in activity. The disc electrophoretic and sedimentation behaviors of this GuHCl-inactivated species are identical to that observed when the enzyme is inactivated by removal of NaCl. This same inactive conformer is obtained when ICDH is treated with GuHCl after the NaCl is removed. As expected, these GuHCl-inactivated species can be restored to the active conformation by dialysis against 4 M NaCl. Thus, exposure to GuHCl or to low ionic strength induces the same conformational change which apparently involves a major unfolding of the polypeptide chain(s).

TABLE 3. *Concerted feedback inhibition of H. cutirubrum ICDH*

Isocitrate	Oxalacetate	Glyoxalate	Activity[a]
mM	mM	mM	%
1.33	1.0	0	101
	0	1.0	107
	1.0	1.0	66
0.13	1.0	0	97
	0	1.0	103
	1.0	1.0	29

[a] 100%, Activity of assay containing neither inhibitor.

The extent of unfolding is further illustrated by the exposure of sulfhydryl groups which may be oxidized or alkylated. We refer to this as permanent inactivation, since the oxidized or alkylated protein cannot be refolded. Based on the sucrose gradient analyses, the permanently and reversibly inactivated ICDH appear to be indistinguishable. Obviously, the alkylation of a few sulfhydryl groups would not grossly affect the molecular weight or sedimentation rate. Differences in sedimentation would have undoubtedly been detected had the transition from reversible to permanent inactivation involved aggregation, disaggregation, or major conformational changes. Moreover, we previously showed that the disc electrophoretic mobilities of the reversibly and permanently inactivated ICDH are identical (2). This may not have been a valid comparison since the reversibly inactivated species could have been altered during electrophoresis. We have not been successful in attempts to elute the inactivated enzyme from disc gels and restore activity by dialysis against 4 M NaCl.

The exposure of sulfhydryl groups during inactivation of another halophilic enzyme was previously suggested from studies with the malate dehydrogenase of *H. salinarium* (1). For example, the malate dehydrogenase could not be reactivated when the salt-free protein was treated with NEM. The susceptibility of the inactive malate dehydrogenase to alkylation could also mean that its sulfhydryl groups were susceptible to oxidation. A partial oxidation could account for the relatively poor recovery of activity (50%) after normal reactivation with 4 M NaCl (1). Had the sulfhydryl groups of the inactive malate dehydrogenase been protected, this value might have been in line with the 90 to 99% recovery we routinely obtain with ICDH.

The susceptibility of the halophilic ICDH to concerted feedback inhibition by glyoxalate and oxalacetate would imply that the enzyme is an allosteric protein. Yet we have no indication that the ICDH conforms to the allosteric model formulated by Monod et al. (5). Although we have not excluded the possibility that the protein is composed of identical subunits, it is unlikely that any such protomers are held together by noncovalent bonds. For example, if the ICDH protomers were held together by hydrogen bonding, they would undoubtedly disaggregate when the protein is unfolded by exposure to low ionic strength or GuHCl. This was not observed. Moreover, in Monod's model (5), the disaggregation of protomers of allosteric proteins is favored when substrate is present. The halophilic ICDH is

stabilized by substrate (2). Conversely, the inhibitors of allosteric proteins would be expected to stabilize the polymeric state (5). In other experiments (Hubbard and Miller, *unpublished data*), the presence of glyoxalate and oxalacetate did not influence the rate of inactivation when salt was removed. These observations suggest that either the *H. cutirubrum* ICDH is an atypical allosteric protein or the allosteric transitions are masked by its halophilic properties.

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