Activation and Stabilization of Halophilic Enzymes by Magnesium and Calcium Ions

The enzymes from extremely halophilic bacteria require high salt concentrations for both activity and stability[1,2]. Most experiments related to the activation and stabilization of halophilic enzymes in vitro have been performed with K^+ and Na^+, which are the main intracellular cations[3]. It has been demonstrated, however, that divalent cations are able to afford at least some activation and stabilization of particulate enzyme systems, such as NADH dehydrogenase[4,5] and cytochrome oxidase[6], and a partially purified catalase[7]; in these cases $MgCl_2$ was less effective than NaCl as an activator. We show here that $MgCl_2$ and $CaCl_2$ are efficient activators of seven halophilic enzymes, partially purified from *Halobacterium cutirubrum*. In some cases, the divalent cations were even better than K^+ or Na^+ as stabilizers or activators.

The enzymes studied were glycerol dehydrogenase (EC 1.1.1.6, GDH); malate dehydrogenase (EC 1.1.1.37, MDH); NADP-linked malic enzyme (EC 1.1.1.40, ME); NADP-linked isocitrate dehydrogenase (EC 1.1.1.42, ICDH); glutamate dehydrogenase (EC 1.4.1.3, GluDH); aspartate aminotransferase (EC 2.6.1.1, AAT); and citrate synthase (EC 4.1.3.7, CS). ME and ICDH were chosen as examples of enzymes requiring a divalent cation for activity in non-halophiles.

Table I. Activation of halophilic enzymes by divalent and monovalent cation salts

	GDH	MDH	ME	ICDH	GluDH	AAT	CS
No additions	0	8	0	53	0	0	9
$CaCl_2$ (*M*)							
0.005	57	38	—	53	36	—	49
0.010	58	49	—	64	47	—	66
0.020	60	64	—	68	52	—	72
0.050	57	78	—	72	69	29	87
0.100	51	92	—	59	93	54	69
0.200	48	98	—	35	100	84	45
0.400	31	100	—	1	81	100	24
$MgCl_2$ (*M*)							
0.005	58	21	11	62	45	—	42
0.010	60	31	26	72	47	—	55
0.020	57	41	35	76	58	—	64
0.050	45	51	31	73	81	31	71
0.100	40	61	2	60	97	54	46
0.200	27	66	0	27	92	72	30
0.400	10	46	0	1	56	76	16
NaCl (*M*)							
0.1	3	18	—	100	36	2	34
0.4	43	33	—	100	81	48	64
1.0	38	60	0	91	77	80	63
2.0	34	51	0	49	41	85	64
3.0	35	36	0	24	32	75	64
KCl (*M*)							
0.1	45	21	—	79	15	5	38
0.4	62	42	—	77	77	44	66
1.0	69	66	26	62	76	72	73
2.0	83	55	93	40	62	89	78
3.0	100	41	100	32	47	88	100

With the exception of ICDH, the enzymes were partially purified by ammonium sulphate fractionation and assayed spectrophotometrically at 30 °C, as previously described[12]. The enzyme preparations were exhaustively dialyzed against 0.05 *M* *Tris*-HCl buffer (ph 7.6), containing 5 *M* NaCl and 1 *mM* EDTA, and used for the experiments described. ICDH was purified 3-fold in fraction P_6 (ref.[12]) and assayed as described by HUBBARD and MILLER[13]. The enzymes were assayed in the presence of the salt concentrations stated in the Table. Reaction velocities are expressed as percent of the maximal activity attained for each enzyme. The amounts of protein (in μg) and the 100% of activity (in nmoles/min) were, respectively: GDH, 79 and 33; MDH, 21 and 41; ME, 1450 and 9; ICDH, 93 and 14; GluDH, 6.2 and 14; AAT, 83 and 5; CS, 10 and 11. $CaCl_2$ could not be tested on ME, because of precipitation in the reaction mixtures.

Table I shows the activation of the enzymes studied by concentrations of $MgCl_2$ or $CaCl_2$, ranging from 0.005 to 0.4 *M*; for comparative purposes, the enzyme activities in the presence of NaCl or KCl concentrations ranging from 0.1 to 3.0 *M* are also included. All the enzymes studied were activated by the divalent cations: $CaCl_2$ was the best activator of GluDH, MDH and AAT; KCl was the best activator of GDH, ME and CS, and NaCl was the best activator of ICDH. The concentrations of divalent cations required for maximal activation were, in general, lower than those of monovalent cations.

Table II shows the stabilization of the enzymes by the same salts. All the enzymes studied decayed very rapidly in the presence of 0.1–0.2 *M* NaCl at 30 °C ,but were stabilized with different degrees of effectiveness by 0.1 *M* $CaCl_2$ or $MgCl_2$ (or 0.4 *M* in the case of AAT). These salts were efficient protectors of GDH, MDH and AAT, and to a lesser extent of ME. 0.1 *M* $MgCl_2$ was better than 1 *M* NaCl or KCl as a protector for ICDH. On the other hand, the divalent cation salts were very poor protectors of GluDH and CS, when compared with NaCl and KCl.

The efficiency of divalent cation salts as activators, and at least in some cases as stabilizers, of 7 halophilic enzymes, at comparatively low salt concentrations, suggests that charge shielding by the cations[8] was an important factor in maintaining the native enzyme structures. In fact, divalent cations are considerably more effective than monovalent cations for charge shielding[9].

The enzymes studied by LANYI et al.[5,6] were better activated by high concentrations of NaCl than by $MgCl_2$. These authors suggested a role of the monovalent cations at high concentrations in the stabilization of hydrophobic bonds in the protein[9].

This seems not to be a general property of halophilic enzymes, however, since the enzymes studied here fall into 2 different groups in this respect. MDH, GluDH, ICDH and AAT showed a relative inhibition by high concentrations of monovalent cations, with the only exception of the effect of KCl on AAT. This suggests that stabilization of hydrophobic bonds by high monovalent cation concentrations was not required for full activity of these enzymes, and that the main factor in the activation process was the shielding of electronegative charges on the proteins. Accordingly, MDH, GluDH and AAT were best activated by $CaCl_2$, and efficiently activated by

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$MgCl_2$; ICDH was best activated by NaCl (0.1–0.4 *M*), and it was best protected by $MgCl_2$. On the other hand, GDH, ME and CS were maximally activated by KCl; at low concentrations $CaCl_2$ and $MgCl_2$ were efficient activators, but they caused a relative inhibition over 0.05 *M*. On the basis of the experimental data described, we suggest that GDH, ME and CS, but not the other enzymes studied here, require for maximal activity the stabilization of hydrophobic bonds by monovalent cations at concentrations higher than 1*M*, the divalent cations being ineffective for this purpose.

It is difficult to ascertain at present the role of the divalent cations in the maintenance of the native structure of halophilic enzymes in vivo. The intracellular concentrations of K^+ and Na^+ are undoubtedly much higher than those of Mg^{++} and Ca^{++}, but the concentrations of the latter cations required for activation are considerably lower. Moreover, preliminary experiments, not shown here, demonstrate that $MgCl_2$ or $CaCl_2$, at 0.1 *M*, can interact with halophilic enzymes even in the presence of 2.3 *M* KCl, causing in general a relative inhibition. The recent report by LANYI and SILVERMAN[10] on the state of the intracellular cations in *H. cutirubrum* may be relevant to this problem. They showed that, whereas K^+ seems to be free inside the cell, Mg^{++} is bound, probably to both the membrane lipids and the acidic proteins[11] present in extreme halophiles. The possibility cannot be excluded, then, that divalent cations, mainly Mg^{++}, might participate in the activation and stabilization of halophilic enzymes in vivo.

Table II. Stabilization of halophilic enzymes by divalent and monovalent cation salts

	GDH	MDH	ME	ICDH	GluDH	AAT	CS
Basal	0.1	0.4	3.7	< 0.1	< 0.1	5	<0.1
$CaCl_2$	1140	360	27	5.6	8	50	0.5
$MgCl_2$	2460	220	40	66	9	125	0.2
NaCl	960	450	310	10	5400	350	145
KCl	3240	75	180	3.3	132	120	53

The experiments were performed at 30 °C, as described in ref.[14]. The basal NaCl concentration was 0.1*M*, except for CS (0.2*M*). The protein concentrations in the preincubation mixtures (μg/ml) were, respectively: GDH, 158; MDH, 54; ME, 336; ICDH, 186; GluDH, 52; AAT, 83; and CS, 20. The salt concentrations tested as protectors were 0.4*M* Me^+ or Me^{++} (AAT), or 1*M* Me^+ or 0.1*M* Me^{++} for the other enzymes. The half-life values (in min) obtained from semilogarithmic plots of remaining activity *vs* time of preincubation are given as a measure of the stabilizing effect of the salts.

Resumen. Siete enzimas parcialmente purificadas de la bacteria halófila extrema *Halobacterium cutirubrum* fueron activadas, y la mayoría de ellas parcialmente estabilizadas, por concentraciones de Ca^{++} o Mg^{++} hasta 0.4 *M*. En algunos casos las sales de catión divalente fueron más eficaces que el ClNa o el ClK. Se discuten los resultados en relación con la posible participación del escudado de cargas y del refuerzo de uniones hidrofóbicas en el mantenimiento de la estructura nativa, y con el posible papel del Mg^{++} in vivo en la activación y estabilización de enzimas halofílicas.

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Lipid Metabolism in Suckling Rats with Fatty Liver Induced by Hypoxia

Previous studies have shown a fatty liver degeneration in baby rats born and kept at 3990 m above sea level or submitted to a simulated altitude of about 4600 m since 1-day-old. A depression of oxygen consumption was observed in these chronic hypoxic suckling rats[1] and the suggestion made that it could be due to decreased tissue oxidation. This would impair lipid utilization, which could result in fatty liver degeneration. To further investigate this hypothesis, plasma and liver total lipids and blood β-hydroxybutyrate and acetoacetate levels were measured in normal and in chronically hypoxic rats which were either sacrificed immediately after removal from the hypobaric chamber or kept at sea level pressure for 8 to 48 h before being sacrificed.

Material and methods. Litters of Sprague-Dawley rats were reduced to 10 rats at 1 day of age. Some litters were kept at sea level pressure as controls. The others were placed with the mother in a hypobaric chamber maintained at a simulated altitude of about 4600 m. The chamber was opened every other day to clean, replace food and water, and weight the litter.

The hypoxic and control rats were decapitated and the blood was collected in a heparinized container. The plasma sample was obtained by centrifuging the blood in microhematocrit tubes. Since the amount of blood obtained from each rat was too small to permit measurement of both acetoacetate and β-hydroxybutyrate, a different animal was used for each measurement. Total lipids in plasma and in liver were measured in each rat. Acetoacetate and β-hydroxybutyrate levels in the blood were determined using the enzymatic micromethod described by WILDENHOFF[2]. The standards used in the method were sodium β-hydroxybutyrate, which was recrystallized twice, and lithium acetoacetate, which was prepared according to the method described by HALL[3]. Total plasma lipids were measured by using the E. Merck Total Lipid test kit. Total lipids in liver were determined by a

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