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THE ABSENCE OF ZINC IN THE MITOCHONDRIAL AND SUPERNATANT FORMS OF MALATE DEHYDROGENASE

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SUMMARY

The malate dehydrogenase enzyme system from porcine heart (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) has previously been shown to be a multiple enzyme system[1]. This discussion is limited to a consideration of the two basic systems; *i.e.* enzyme isolated from the cytoplasm or supernatant form and enzyme of a mitochondrial origin. No consideration is made at this time of the microheterogeneity observed in each major type. An efficient method for the purification of the supernatant form of the enzyme in tandem with a previously reported procedure for the mitochondrial enzyme is described.

The possibility that either or both enzymes are zinc metalloenzymes has been investigated. Although instantaneous inhibition by known metal-chelating reagents was observed, atomic absorption analysis of samples of highly purified enzyme of both types failed to indicate the presence of a stoichiometric amount of zinc.

Inhibition of both enzymes has been observed in the presence of a non-chelating analogue of 1,10-phenanthroline, (1,7-phenanthroline). This information coupled with the analytical data indicating the absence of intrinsic metal ions in either enzyme system has suggested that the inhibition by metal-chelating agents observed by these authors and others was the result of hydrophobic interaction of the chelating agents at or near the enzymatic active center, and not due to the specific chelating properties of the reagents employed.

INTRODUCTION

Since the recognition of the presence of two types of malate dehydrogenase, several preparations for mitochondrial and supernatant enzyme have appeared. The first detailed isolation, reported by Straub[2], was for the mitochondrial enzyme. That preparation was considerably modified by Wolfe and Neilands[3], and more recently this laboratory has reported a method yielding enzyme exhibiting a specific activity of 420 units per mg with a 40% overall yield[4].

The supernatant enzyme has received less attention. Although it has been

isolated from many other sources[5–11], only two preparations have been reported for supernatant malate dehydrogenase from pig heart[12,13]. The supernatant enzyme used in this work was prepared basically by the Gerding and Wolfe[13] procedure but includes several important modifications. Due to the fact that the preparation of the mitochondrial enzyme was an ongoing procedure in this laboratory, it was considered advantageous to develop a method of preparation which could be incorporated into the procedure for mitochondrial enzyme.

An important consideration in any study of the characterization of an enzyme is its intrinsic metal content. The metal can serve a functional role, as in carboxypeptidase[14] or a structural role as well as a functional role as in alkaline phosphatase[15]. Among those enzymes determined as metalloenzymes are several which belong to the class of pyridine nucleotide dependent dehydrogenases. Indeed, zinc had been shown to be associated with enough of these dehydrogenases that a general scheme was once proposed suggesting a zinc requirement for enzymes of this group[16]. Though the scheme may be accurately applied to some of these enzymes, the data concerning the requirement of zinc in malate dehydrogenase is sparse and contradictory.

Malate dehydrogenase from porcine heart muscle has been shown to be significantly inhibited by exposure to the known chelating agents, 1,10-phenanthroline, 8-hydroxyquinoline and sodium diethyldithiocarbamate[16]. The results of analyses of this enzyme by emission spectroscopy showed significant quantities of zinc. The enzyme used was from a commercial source and in light of present knowledge of specific activities, its purity is certainly suspect.

Pfleiderer and Hohnholz[17] investigated the metal content of malate dehydrogenase by means of atomic absorption spectroscopy and, in contrast, found no zinc in their preparation. The enzyme was reinvestigated by Harrison[18] in 1964 and was reported to contain zinc in the amount of approximately 2 gatoms per mole of enzyme. The same author observed that his preparation of the enzyme was also inhibited significantly by 1,10-phenanthroline. Kosicki *et al.*[19] presented data which indicated the inability to observe inhibition of their preparation of the enzyme by the same chelating reagent.

In view of the above observations, the presence, and therefore the role, of zinc in malate dehydrogenase remains in doubt. The work reported here was begun with a two-fold purpose in mind; first to establish a convenient method to prepare highly purified samples of the two basic forms of malate dehydrogenase from porcine heart and secondly, as part of the characterization of this multiple enzyme system to reinvestigate the metal content of both enzymes.

MATERIALS

Porcine heart malate dehydrogenase (supernatant and mitochondrial) was purified from acetone powders of pig hearts as described below. NAD^+, NADH, L-malic acid, and oxaloacetic acid were purchased from Sigma. Chelex 100, Bio-Rex 70, and Bio-Gel HTP hydroxylapatite were purchased from Bio-Rad, Richmond, Calif. Electronic grade HCl, HNO_3 and NaOH were purchased from Baker and Adamson, Morristown, N.J. DEAE- and CM-cellulose were obtained from H. Reeve Angel and Company, Inc. Zinc standards for atomic absorption studies were purchased from Fisher Scientific and Instrumentation Laboratories.

METHODS

Enzymatic assay

Enzymatic assays were performed as previously described[4]. Specific activity is expressed as units of enzyme per mg. One unit of enzyme is defined as the quantity which catalyzes the reduction of 1 μmole of NAD^+ per min. Protein concentrations were determined spectrophotometrically at 280 nm, utilizing an extinction coefficient $E^{1\%}_{1\,cm}$ of 2.5 for the mitochondrial enzyme[4] and $E^{1\%}_{1\,cm}$ of 9.3 for the supernatant enzyme[13].

Amino acid analysis

Amino acid compositions were determined by the method of Spackman *et al.*[20] on a Beckman Model 116 amino acid analyzer. Determination of cysteine was performed by the method of Moore[21] as cysteic acid. Tryptophan was determined by the method of Edelhoch[22] and by the method of Barman and Koshland[23].

Electrophoresis

Protein samples were electrophoresed on Sepraphore III cellulose polyacetate strips as previously described[4].

Inhibition of malate dehydrogenase with chelating agents

Time-dependent inhibition studies were performed on both the supernatant and mitochondrial enzyme. Samples of enzyme (1.85 mg/ml) were either incubated with or dialyzed against either 15 mM 1,10-phenanthroline or 4.5 mM 8-hydroxyquinoline at 25 °C for periods up to 24 h. Aliquots were removed from the incubation mixtures at appropriate times and assayed for enzymatic activity and compared to a control sample incubated in the absence of chelating agent.

Instantaneous inhibitions were performed by assaying the enzymes under standard conditions with varying concentrations of chelating agents included in the assay media. The inhibition is represented as the log of the ratio $v_i:v_c$ (velocity of inhibited sample to velocity of a control sample) as a function of inhibitor concentration.

Preparation of glassware and buffers

All glassware used in metal analysis studies was soaked in concentrated HNO_3, followed by rinsing in deionized water and 0.03% diphenylthiocarbazone (dithiazone)[24] in carbon tetrachloride. All buffers used were passed through a Chelex-100 resin or extracted with 0.03% (w/v) dithiazone in carbon tetrachloride[25]. Buffers were adjusted to the desired pH with Electronic grade HCl or NaOH (metal free).

Metal analysis

Metal analyses were performed using the Perkin-Elmer atomic absorption spectrophotometer (Model 303). The hollow cathode voltage, burner flame and height were adjusted to maximize the signal. Standards were prepared from stock solutions and all dilutions were made with the buffer last used in dialysis of the enzyme sample.

As a reference, carboxypeptidase A, was assayed using the same zinc standards and a value of 1.03 gatoms of zinc per molecule of enzyme[14] was obtained.

Enzyme purification

Porcine hearts were obtained from large animals and converted into acetone dried powders[3]. Extraction of the acetone powders and the 50 and 75% $(NH_4)_2SO_4$ fractionations were performed as previously described[4]. All buffers utilized were made metal free and contained 1 mM 2-mercaptoethanol and 1 mM EDTA. All column fractionations were performed at 5 °C.

Bio-Rex 70 chromatography

The enzymatically active material from the $(NH_4)_2SO_4$ fractionation (pellet) was dialyzed for 24 h against four changes of 25 mM sodium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM 2-mercaptoethanol. A column (2.5 cm × 45 cm) packed with Bio-Rex 70 (200–400 mesh) was equilibrated with the above buffer and the enzyme preparation was applied to the column and washed until the eluant exhibited negligible absorbance at 280 nm. The mitochondrial enzyme is retained on the column under these conditions and may be removed selectively by gradient elution[4], while the supernatant enzyme does not bind and is present in the initial eluant. The purification outline given below concerns only the supernatant enzyme.

$(NH_4)_2SO_4$ fractionation

The supernatant malate dehydrogenase from the previous step was pooled and the protein concentration adjusted (diluted) to 15 mg/ml. The solution was brought to 50% saturation by the slow addition of 313 g of $(NH_4)_2SO_4$ per l, allowed to stand in the cold for an additional 2 h (ref. 26), centrifuged at 16 000 × *g* for 20 min and the pellet discarded.

The clear supernatant was then brought to 80% saturation by the addition of 214 g of $(NH_4)_2SO_4$ per l, allowed to stand in the cold for 2 h, centrifuged and the pellet retained. The pellet was dialyzed for 4 h against two changes of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 1 mM 2-mercaptoethanol.

DEAE-cellulose chromatography

The dialyzed preparation was applied to a column (2.5 cm × 45 cm) of DEAE-cellulose equilibrated with the Tris buffer described above. A linear gradient consisting of 550 ml of starting buffer and 550 ml of limit buffer, composed of starting buffer *plus* 200 mM NaCl, was utilized to develop the column.

The fractions exhibiting malate dehydrogenase activity were pooled and dialyzed against a solution of saturated ammonium sulfate containing no EDTA or 2-mercaptoethanol for purposes of concentrating the protein material.

$(NH_4)_2SO_4$ fractionation

The $(NH_4)_2SO_4$ suspension was then centrifuged and the pellet dissolved in 10 mM sodium phosphate buffer (pH 7.2) containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The protein concentration was adjusted to 12 mg/ml and brought to 35% saturation by the slow addition of 209 g of $(NH_4)_2SO_4$ per l, allowed to stand in the cold for 2 h, centrifuged and the pellet discarded.

The clear supernatant was then brought to 55% saturation by the addition of 129 g of $(NH_4)_2SO_4$ per l and allowed to stand in the cold for 2 h. The pellet from this step was discarded and the supernatant brought to 75% saturation by the

addition of 141 g of $(NH_4)_2SO_4$ per l. The pellet obtained from centrifugation of this suspension was dissolved and dialyzed for 24 h against four changes of 10 mM sodium phosphate buffer (pH 7.2) containing 1 mM EDTA and 1 mM 2-mercaptoethanol.

Bio-Gel HTP (hydroxylapatite) chromatography

A column (2.5 cm × 45 cm) packed with Bio-Gel HTP hydroxylapatite was equilibrated with 10 mM sodium phosphate buffer (pH 7.2) containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The protein sample from above was applied to the column. The column was developed with a linear gradient consisting of 550 ml of 40 mM sodium phosphate (pH 7.2) containing 1 mM EDTA and 1 mM 2-mercaptoethanol and 550 ml of a limit buffer consisting of 200 mM sodium phosphate (pH 7.2) containing 1 mM EDTA and 1 mM 2-mercaptoethanol. The fractions exhibiting malate dehydrogenase activity were pooled and precipitated by dialysis against a saturated solution of $(NH_4)_2SO_4$ and stored as a suspension.

RESULTS AND DISCUSSION

Purification and partial characterization of malate dehydrogenase

The isolation and purification of the mitochondrial and supernatant forms of malate dehydrogenase of porcine heart have been described[4,12,13]. However, in order to investigate the effects of metal-chelating agents and the metal content of both enzymes it was essential that enzyme of highest possible purity be available. Previous methods of purification of porcine heart mitochondrial malate dehydrogenase have utilized a zinc–ethanol fractionation in addition to ion-exchange chromatography. Due to the contradictory evidence as to the content of zinc in this enzyme it was deemed necessary to eliminate this step from the preparation of both enzymes, and hence to eliminate any possible addition of non-intrinsic metal. In the present work the purification scheme of Gerding and Wolfe[13] has been modified so that both forms of the enzyme can be isolated in parallel. The preparation has been made substantially shorter than that described by Gerding and Wolfe[13] whose enzyme exhibited a specific activity of approximately 100 units per mg. From the preparation reported here, it was not unusual to obtain enzyme with a specific activity of 300 units/mg. This may be due in part to the fact that all steps in this purification procedure were performed at 5 °C. In addition all buffers were made metal-free and contained EDTA and 2-mercaptoethanol. The addition of these two components is particularly relevant in light of a recent report by Cassman and King[27] in which a sulfhydryl-activated protease which was inhibited in the presence of EDTA was found to be present in their preparation of supernatant malate dehydrogenase. The difference in specific activity observed in this preparation as compared to that of Gerding and Wolfe[13] more than likely reflects a simple protection of the enzyme by our conditions rather than any higher degree of purity.

Since the majority of the malate dehydrogenase activity (85%) in the initial extract of acetone powders is mitochondrial in origin, the yields reported in Table I were calculated using the Bio-Rex 70 run through material as 100%. Utilizing the procedure previously reported by Gregory *et al.*[4], mitochondrial enzyme exhibiting a specific activity of 412 units per mg in an overall yield of 40% is prepared in less than 1 week. Table I indicates the yield and specific activity observed in each step

TABLE I

PURIFICATION OF PIG HEART SUPERNATANT MALATE DEHYDROGENASE

Step	*Total protein (mg)*	*Specific activity (units/mg)*	*Total units*	*Yield (% initial)*
Bio-Rex 70 chromatography	11 000	8	110 000	100
$(NH_4)_2SO_4$ fractionation	9 600	17	110 000	100
DEAE-cellulose chromatography	564	53	30 700	63.2
$(NH_4)_2SO_4$ fractionation	540	138	30 000	60
Hydroxylapatite chromatography	220	300	27 300	51.2

of the purification of the supernatant enzyme. As in the case of the mitochondrial enzyme, significant quantities of the supernatant enzyme (220 mg) can thus be easily prepared from the previously discarded portion of the mitochondrial enzyme preparation. The purified enzyme was observed to be homogeneous upon gradient elution from such chromatography systems as DEAE-cellulose and hydroxylapatite.

Electrophoresis on cellulose polyacetate strips indicates that this preparation of supernatant malate dehydrogenase contains two anodally migrating bands of protein, a finding in agreement with the work of Gerding and Wolfe[13]. An identical electrophoresis strip developed for enzymatic activity indicated two bands which were coincident with the protein bands. Electrophoresis of the mitochondrial enzyme under identical conditions yields three cathodally-migrating bands of protein[4].

The amino acid composition of this preparation of the supernatant enzyme was determined. The residue values in Table II represent the average of three independent

TABLE II

AMINO ACID COMPOSIITON OF MALATE DEHYDROGENASE

Amino acid	*Residues found per molecule*[a]		
	Mitochondrial[b]	*Supernatant*[c]	*Supernatant*[d]
Aspartic acid	46.6	78.2	80.0
Threonine	38.8	27.0	28.6
Serine	38.1	36.5	36.7
Glutamic acid	48.7	57.5	56.6
Proline	45.1	27.4	25.7
Glycine	56.8	46.0	45.8
Alanine	63.7	62.0	53.9
Valine	58.5	58.9	52.4
Isoleucine	44.0	48.1	36.9
Leucine	55.8	63.0	60.8
Tyrosine	9.7	14.2	14.3
Phenylalanine	21.1	22.9	22.3
Lysine	50.8	64.8	59.2
Histidine	14.7	8.4	11.4
Arginine	15.3	20.2	20.5
Cysteine	15.4	14.0	14.1
Tryptophan	0.0	9.4	—

[a] Calculations were based on a mol. wt of 70 000 for the mitochondrial[4] and 74 000 for the supernatant enzyme[13].
[b] Data are taken from Gregory *et al.*[4].
[c] Data are taken from Gerding and Wolfe[13].
[d] Data from this work.

samples. The composition agrees well with that reported by Gerding and Wolfe[13], with the exception of lower values for alanine, valine and isoleucine. For comparison Table II contains the amino acid composition previously reported for the mitochondrial enzyme for porcine heart[4].

Inhibition studies with metal-chelating reagents

The few reports concerning the presence of a metal atom in malate dehydrogenase have left the question unresolved. That data has been contradictory primarily because of varying degrees of enzyme purity, and because of differences in the analytical methods employed in determining the presence of the metal atom in biological material. In this study, there is access not only to highly purified enzyme but also to a highly sensitive method for determining the presence of a metal atom, *i.e.* atomic absorption spectroscopy. With these advantages in mind, an investigation was begun to determine whether or not either, both or neither malate dehydrogenase enzyme is indeed a metalloenzyme.

One method of approach to this problem would be the demonstration of a time-dependent inhibition by metal-chelating reagents, thus suggesting the functional or structural importance of an intrinsic metal atom. Time-dependent inhibition studies were performed on both enzymes using the chelating agents 1,10-phenanthroline and 8-hydroxyquinoline as described in Methods. Under the conditions reported here, pH 7.0, 6.0, and 5.0, no time-dependent inhibition was observed for either enzyme with either chelating agent.

Instantaneous inhibition studies were performed using both enzymes. The concentrations of 1,10-phenanthroline ranged from 0.66 mM to 18.5 mM (cuvette concentrations). The ranges for 8-hydroxyquinoline and diethyldithiocarbamate were 0.14 mM to 4.01 mM and 3.3 mM to 9.3 mM, respectively. The results of these inhibition studies are represented in Table III. For comparison with the results obtained

TABLE III

CONCENTRATION OF METAL CHELATOR REQUIRED TO ACHIEVE 50% INSTANTANEOUS INHIBITION

Enzyme	*Inhibitor (M)*		
	1,10-Phenanthroline	*8-Hydroxyquinoline*	*Diethyldithiocarbamate*
Mitochondrial	$1.5 \cdot 10^{-2}$	$7.5 \cdot 10^{-3}$	$5.0 \cdot 10^{-2}$
Supernatant	$1.2 \cdot 10^{-2}$	$8.0 \cdot 10^{-3}$	$4.0 \cdot 10^{-2}$

by others, the values represented in this table are the concentrations of chelators required to effect a 50% inhibition of the enzymatic activity as compared to a control sample which contained no chelator. Not included in this table are the results observed with EDTA. It was observed that concentrations as high as 100 mM EDTA affected no inhibition of the enzymatic activity of either enzyme.

In order to show that the chelating reagents were acting at or near the enzymatic active center of malate dehydrogenase, a competitive inhibition study utilizing 1,10-phenanthroline was carried out with the mitochondrial enzyme. Malate dehydrogenase was assayed in the presence and absence of 10 mM 1,10-phenanthroline utilizing concentrations of NADH from 0.14 to 0.56 mM. As is apparent from Fig. 1

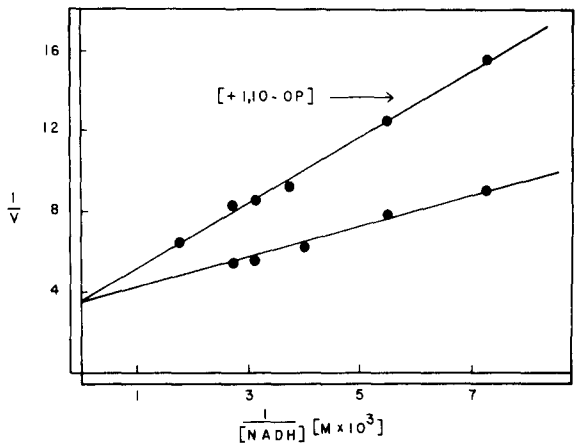

Fig. 1. Inhibition of mitochondrial malate dehydrogenase by 1,10-phenanthroline (10 mM) as a function of NADH concentration. Sodium phosphate buffer (pH 7.2, 46 mM); oxaloacetate, (5.0 mM), 25 °C.

the inhibitor 1,10-phenanthroline appears to be competitive with the coenzyme and thus is suggestive of an interaction at or near the enzymatic active center of this enzyme.

Although the presence of a metal atom is implied in the inhibition experiments, more conclusive evidence is needed to establish that the enzyme is indeed a metalloenzyme; that is that it contains an intrinsic stoichiometric amount of zinc. This can only be accomplished by measuring the metal content of the native enzyme.

To prepare the enzymes for atomic absorption studies samples were dialyzed against 10 mM Tris–HCl buffer (pH 7.0) containing 1 mM EDTA to remove any extrinsic metal. Immediately after dialysis, the enzymatic activity of the enzyme was determined and the zinc content determined based on a mol. wt of 70 000 and 74 000 for the mitochondrial and supernatant enzyme, respectively.

The results of these determinations indicated that these highly purified preparations of malate dehydrogenase were devoid of a stoichiometric amount of zinc. Three independent preparations of the mitochondrial enzyme were found to contain from 0.01 to 0.24 gatoms of zinc per mole of enzyme. The highest mole ratio of zinc to enzyme observed for the supernatant enzyme was 0.32. Similar analysis failed to indicate the presence of stoichiometric amounts of Mn^{2+}, Mg^{2+}, Co^{2+} or Fe^{2+}. Various attempts were made to affect the zinc content of both enzymes by dialysis *versus* zinc-containing buffers, followed by exhaustive dialysis *versus* metal-free buffer systems. In all studies of this nature no change in the specific activity or metal content was observed.

The data thus far lead to the paradoxical conclusion that an enzyme, devoid of zinc, is being inhibited by reagents which presumably inactivate enzymes by binding to metal atoms. 1,10-Phenanthroline and 8-hydroxyquinoline are aromatic, and highly hydrophobic; hence, it would not be unreasonable to propose that the chelators are mimicking the pyridine nucleotide and binding at or near the coenzyme binding site. Thus, the observed inhibition would not be due to metal chelation but rather to hydrophobic interactions, a finding consistent with similar observations by other investigators[28,29].

To test this hypothesis, 1,7-phenanthroline which retains the hydrophobic character, but is incapable of chelation was investigated. Instantaneous inhibition studies were limited to 3.1 mM to 4.7 mM due to solubility considerations. At a final concentration of 4.7 mM, the mitochondrial enzyme was 40% inactive while the supernatant enzyme had lost 25% of its enzymatic activity (Fig. 2). Thus, it appears that the inhibition caused by the chelators may be the result of hydrophobic inter-

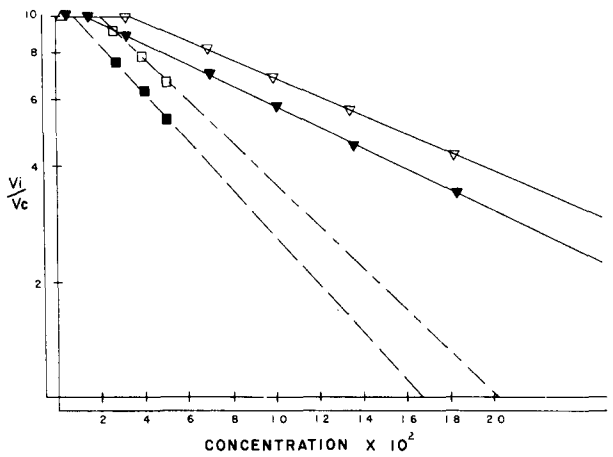

Fig. 2. Comparison of 1,10-phenanthroline with 1,7-phenanthroline as instantaneous inhibitors of malate dehydrogenase. Instantaneous inhibitions were performed as described in Methods. Concentrations ranged from 0.66 mM to 18.5 mM for 1,10-phenanthroline, and from 3.10 mM to 4.70 mM for 1,7-phenanthroline. Supernatant malate dehydrogenase (▲) and mitochondrial malate dehydrogenase (△) with 1,10-phenanthroline, supernatant malate dehydrogenase (■) and mitochondrial malate dehydrogenase (□) with 1,7-phenanthroline.

action rather than the binding to a metal required for enzymatic activity. Wedding *et al.*[30] in an investigation of the inhibition of mitochondrial malate dehydrogenase using aromatic phenolic compounds have suggested that a protein molecule has many hydrophobic areas capable of binding and that in the case of this enzyme one of these sites is the binding site for pyridine nucleotide.

Diethyldithiocarbamate is not aromatic and its inhibitory qualities therefore cannot be explained in this manner. It does, however, contain free sulfhydryl groups which are capable of forming disulfide linkages. Since a cysteine residue has recently been implicated at the active center of the mitochondrial enzyme[4,31], it is reasonable to suggest this type of interaction as the source of the observed inhibition. In conclusion this work has shown that neither forms of malate dehydrogenase isolated from porcine heart are zinc metalloenzymes, nor do they easily incorporate stoichiometric quantities of zinc. Furthermore inhibition data with chelating reagents can be reasonably explained in a manner consistent with this conclusion.

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