A Phospholipid-requiring Enzyme, Malate-Vitamin K Reductase

PURIFICATION AND CHARACTERIZATION*

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SUMMARY

Malate-vitamin K reductase has been purified to near homogeneity from Mycobacterium phlei. The purified enzyme is dependent upon an added phospholipid for activity and requires FAD as a cofactor. The enzymatic activity was found to be affected by the degree of enzyme aggregation. At high salt concentrations the enzyme existed in a monomeric form which was more active than the aggregated form. The enzyme was reversibly aggregated into a less active form by either dilution or dialysis against a buffer of low salt concentration. An enzyme-phospholipid complex was isolated by glycerol gradient centrifugation. It is suggested that a phospholipid binding site (or sites) seems to be involved in the aggregation-disaggregation process. The molecular weight of the monomeric form was determined to be 53,000 by Sephadex G-200 chromatography and 51,000 by sodium dodecyl sulfate gel electrophoresis, whereas the aggregated form had a molecular weight of approximately 164,000, as estimated by Sephadex G-200.

The sonic extracts from Mycobacterium phlei contain an unique enzyme, malate-vitamin K reductase, which requires added phospholipid for activity (21-23). The partial purification and general properties of this enzyme were reported (24). In the present paper malate-vitamin K reductase was further purified and other characteristics of the enzyme, especially with respect to phospholipid binding, are described.

EXPERIMENTAL PROCEDURE

Materials—DEAE-cellulose was obtained from Sigma Chemical Co. Hydroxylapatite was the product of Bio-Rad Laboratories. Sephadex G-200 and QAE-Sephadex A-50 were purchased from Pharmacia Fine Chemicals. Asolectin (soy bean phospholipid) was obtained from Associated Concentrates and purified by the method of Kagawa and Racker (6). All other chemicals were of reagent grade.

Growth of Bacteria—M. phlei (ATCC 354) was grown as previously described (25). Cells were sonically disrupted and fractionated into particulate and supernatant fractions (26). The supernatant fraction was used as the starting material for purification of the enzyme.

Assay of Enzyme Activity—Malate-vitamin K reductase activity was measured spectrophotometrically in a cuvette of 1-cm light path with a Cary model 14 recording spectrophotometer (24). The reaction system contained 100 pmoles of Tris-HCl (pH 7.4), 40 pmoles of KCl, 12.5 nmoles of FAD, 0.24 pmoles of MTT; 1.1 pmoles of vitamin K1 (sonically dispersed with 2.5 mg of Asolectin), and an enzyme sample in a final volume of 1.5 ml. The reaction was started with m-malate (50 pmoles) and MTT reduction was followed spectrophotometrically at 565 nm.

A millimolar extinction coefficient of 15.0 was used for MTT (24). PMS (0.66 pmoles) and DCIP (0.15 pmoles) were also used as final electron acceptor. With PMS and DCIP as the electron acceptors, 2.5 mg of (sonically dispersed) Asolectin were added. A unit of activity is defined as 1.0 pmoles of final acceptor reduced per min at room temperature. Specific activity is in units per mg of protein.

Phospholipid Preparations—Phospholipid (Asolectin) was dispersed by a 30-min sonication as described previously (24). This preparation was used without further centrifugation. Phospholipid or phospholipid and vitamin K1 were also prepared by the method of Fleischer and Klouwen (27).

1 The abbreviations used are: MTT, thiazolyl blue tetrazolium; DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; SDS, sodium dodecyl sulfate.
Chemical Determinations—Protein concentration was determined by the method of Lowry et al. (28), with bovine serum albumin as the standard. Phospholipid was estimated according to the method of King (29).

Electrophoresis—Polyacrylamide gel electrophoresis was carried out according to the method of Weber and Osborn (30) in the presence of 0.1% SDS. Samples were usually treated with 1% SDS, 1% mercaptoethanol, and 4 mM urea for 45 min at 45°. The gels were stained overnight with 0.05% Coomassie blue in 50% methanol-10% acetic acid solution and then destained, first with the same concentration of the methanol-acetic acid solution and then with 7% acetic acid. Analytical gel electrophoresis in 5% gel was carried out by the method of Davis (31).

Glycerol Density Gradient Centrifugation—Glycerol gradient centrifugation was performed by the method of Martin and Ames (32). A 0.1-ml sample was layered on top of a 4.5-ml linear gradient of glycerol (5 to 25% v/v) in a 20 mM Tris-HCl (pH 8.2)-0.5 mM EDTA solution. At the bottom of the gradient, was placed 0.3 ml of 50% glycerol. The tubes were centrifuged at 38,000 rpm for 18 hours at 4° in a Beckman SW 50.1 rotor.

Molecular Weight Estimation by Gel Filtration—A Sephadex G-200 column (2.6 x 90 cm) was equilibrated with 10% glycerol-20 mM Tris-HCl (pH 8.2) with or without 0.3 M KCl. Gel filtration was performed by descending chromatography with fractions of 3 ml collected. The distribution coefficient (A_n) equals v_n - v_s/v_t - v_s and was experimentally determined for each protein standard (33).

Amino Acid Analysis—Samples were dialyzed overnight against water and then were hydrolyzed in 6 N HCl at 110° for 18 hours in vacuo. The hydrolyzed material was analyzed with a Joel-5AH automatic amino acid analyzer. Tryptophan was determined spectrophotometrically (34).

RESULTS

Purification—Nucleic acids and inactive proteins were removed from the starting material by precipitation with streptomycin. A 1/90 volume of 10% streptomycin sulfate solution was added dropwise with continuous stirring to crude supernatant fraction (300 to 350 ml). After additional stirring for 20 min the extract was centrifuged at 10,000 x g for 20 min. The supernatant was diluted with an equal volume of cold water and EDTA was added to a final concentration of 0.5 mM. The precipitate formed between 30 and 50% ammonium sulfate saturation was collected by centrifugation, dissolved in 100 ml of cold 10% glycerol, and dialyzed against 4 liters of 10% glycerol overnight.

The dialyzed material was applied to a DEAE-cellulose column (4 x 55 cm) equilibrated with 10% glycerol and 20 mM Tris-HCl (pH 8.2) solution. The column was washed with the buffer until the eluate was colorless. The gradient elution was accomplished by the use of a linear gradient of KC1 from 0 to 0.37 M in the same buffer. The total volume of the gradient was approximately 85% pure.

Effects of Glycerol and KCl—In an attempt to isolate the enzyme, it was found that the composition of the buffer solution used to dissolve the enzyme had a pronounced effect on the enzymatic activity. The enzyme was dissolved in different compositions of buffer solution at 4° and assayed for activity at the times indicated (Fig. 2). As shown in Fig. 2, 20% glycerol and 0.35 M KCl have a stabilizing effect on the enzyme.

After hydroxylapatite column chromatography, the enzyme preparation isolated in the absence of glycerol was chromatographed on a Sephadex G-200 column (2.6 x 50 cm). The column was equilibrated with three different buffer solutions:

| Step | Volume | Protein | Total Protein | Specific Activity | Recovery |
|------|--------|---------|---------------|------------------|----------|
| Sonic extract | 380 | 17.7 | 0.725 | 980 | 0.15 | 100 |
| Ammonium sulfate precipitate | 217 | 15.9 | 3.450 | 911 | 0.25 | 93 |
| DEAE-cellulose | 55 | 12.5 | 688 | 297 | 0.43 | 31 |
| Hydroxylapatite | 9.2 | 3.7 | 34.3 | 546 | 15.9 | 25 |
| Sephadex G-200 | 5.9 | 1.7 | 10.2 | 273 | 20.7 | 13 |
| QAE-Sephadex A-30 | 0.07 | 3.2 | 2.2 | 116 | 52.0 | 5.3 |
Solution 1, 5 mM Tris-HCl (pH 8.2) in 0.02 mM KCl; Solution 2, 10% glycerol in 20 mM Tris-HCl (pH 8.2); Solution 3, 0.3 mM KCl and 10% glycerol in 20 mM Tris-HCl (pH 8.2). With 5 mM Tris-HCl buffer in 0.02 mM KCl the enzyme activity pattern had a broad distribution, appearing in almost all of the fractions (Fig. 3A). Such a broad activity pattern suggests either that the enzyme is polydisperse or that the enzyme has a high affinity for other protein.

A single activity peak was observed upon the addition of 10% glycerol to the buffer (Fig. 3B). The addition of 0.3 mM KCl to the glycerol-Tris buffer produced a dramatic change in the gel filtration pattern (Fig. 3C). The specific activity was increased from that observed with 10% glycerol and buffer (Fig. 3B). At the same time, the peak activity migrated with the more slowly eluting fractions, indicating conversion of the enzyme to a smaller molecular weight species. Although 0.25 mM sucrose gave essentially the same result as 10% glycerol, the specific activity was only slightly improved. It should be noted that when the enzyme was isolated in the presence of 10% glycerol, gel filtration on a column equilibrated with 20 mM Tris-HCl (pH 8.2) gave exactly the same pattern as a column equilibrated with 10% glycerol in 20 mM Tris-HCl (pH 8.2).

The gel filtration studies indicate: (a) the enzyme is associated with other proteins and can be dissociated in the presence of glycerol or sucrose, (b) the enzyme exists in an aggregated form which is disaggregated in the presence of a high salt concentration, (c) the disaggregated form is much more active than the aggregated form.

Effect of Dilution—Dilution experiments were performed to provide further information on the effect of the medium or microenvironment on the enzyme activity. The enzyme was diluted...
in different media and then assayed immediately for activity. As shown in Table II, dilution of the enzyme in Tris-HCl buffer or water produced a decrease in specific activity. Whereas, when the enzyme was diluted with a mixture containing KCl, glycerol, and Tris-HCl buffer, the enzyme specific activity was higher. Moreover, when the enzyme was diluted in a phospholipid dispersion, the specific activity was higher than that observed in the presence of the KCl, glycerol, and Tris-HCl buffer.

The enzyme activity could be partially inactivated by the removal of KCl and reversibly reactivated in the presence of KCl-glycerol as shown in Fig. 4. A maximum reactivation was attained after 2 hours of incubation at 4°C. An analysis following glycerol density gradient centrifugation determined that the reactivation was dependent upon the KCl concentration and was accompanied with conversion of the enzyme to a more slowly sedimenting fraction (Fig. 5). These results suggest that the enzyme is spontaneously aggregated upon dilution in a salt-free buffer, and that the aggregated enzyme is reversibly disaggregated into a more active form at a slower rate. Moreover, after dilution in phospholipids, the enzyme seems to rapidly form an active enzyme-phospholipid complex having much higher activity.

**Phospholipid Binding**—The requirement for phospholipid for enzymatic activity suggests that a specific binding of phospholipids to enzyme may well be necessary for formation of an active complex. Evidence for complex formation was shown by the use of glycerol density gradient centrifugation (Fig. 6). When the enzyme alone was placed on a gradient, the enzyme sedimented as a single symmetrical peak. A more rapidly sedimenting peak resulted in the sedimentation of almost all of the enzyme to the bottom fractions (Fig. 7). The activity without the added phospholipids in the rapidly sedimenting fractions paralleled the sedimentation pattern as shown in Fig. 7. At low phospholipid-to-enzyme ratios two activity peaks were observed (Fig. 7, B and E), while only slight changes in the activity pattern were observed with lower amounts of phospholipids (Fig. 7, D and C). Addition of excessively large amounts of phospholipids resulted in the sedimentation of almost all of the enzyme to the bottom fractions (Fig. 7F). The activity without the added phospholipids in the rapidly sedimenting fractions paralleled the activity with phospholipids. Thus, no definite ratio of phospholipid to protein could be obtained for the formation of the complex.

**Molecular Weight**—The molecular weight was determined by gel filtration with a calibrated column of Sephadex G-200 (with standard protein markers) to be 53,000 in the presence of KCl (Fig. 8). Calculation of molecular weight from density gradient centrifugation in Fig. 6A gave a value of 60,800 in the presence of 0.5 M KCl. With gel filtration in the absence of KCl, the

### Table II

**Effect of dilution on malate-vitamin K reductase activity**

The enzyme preparation used was the second hydroxylapatite fraction after Sephadex G-200 gel filtration. Five microliters of the enzyme (42.5 μg) were diluted in the 95-μl of the solution described in the table. Twenty microliters of the diluted enzyme were assayed immediately for enzymatic activity with vitamin K1-MTT (I) and with PMS-DCIP (II) as acceptor.

| Dilution medium | Specific activity (units/mg) | Activity ratio |
|-----------------|-----------------------------|---------------|
| I. 20 mM Tris-HCl (pH 8.2) | 12.0 | 1.0 |
| 0.3 M KCl-10% glycerol-20 mM Tris-HCl (pH 8.2) | 19.0 | 1.6 |
| 0.083% Asolectin-0.37 mM vitamin K1 | 35.2 | 2.6 |
| 0.25% Asolectin-1.1 mM vitamin K1 | 33.8 | 2.8 |
| 0.25% Asolectin-1.1 mM vitamin K1-glycerol | 35.3 | 2.9 |
| Undiluted | 30.9 | 2.6 |
| II 20 mM Tris-HCl (pH 8.2) | 5.3 | 1.0 |
| 0.001% Asolectin | 6.7 | 1.3 |
| 0.01% Asolectin | 9.6 | 1.3 |
| 0.25% Asolectin | 12.6 | 2.4 |
| 2.5% Asolectin | 10.9 | 2.1 |
| Undiluted | 11.1 | 2.1 |

**Fig. 4 (upper).** Reactivation of malate-vitamin K reductase by KCl. The enzyme used was isolated by Sephadex G-200 column chromatography without KCl in the buffer. The enzyme solution was diluted one-third with a solution of 0.5 mM KCl, 25% glycerol, and 20 mM Tris-HCl (pH 8.2) and then incubated at 4°C. An aliquot of the solution was taken at the time indicated and assayed for activity.

**Fig. 5 (lower).** Effect of KCl concentrations on reactivation. The enzyme preparations, after Sephadex G-200 chromatography, were dialyzed against 5% glycerol in 20 mM Tris-HCl (pH 8.2). The enzyme (100 μg) in each tube was analyzed as described under "Experimental Procedure" except that KCl was included at a final concentration of (a) 0 (O—O); (b) 30 mM KCl (O—O); (c) 100 mM KCl (△—△); and (d) 500 mM KCl (△—△).
molecular weight was calculated to be 164,000 (Fig. 9). As can be seen in Fig. 3B, the elution pattern was rather broad as compared to the one in the presence of KCl. These results are compatible if it is assumed that the enzyme may exist in different degrees of aggregation. To determine the presence of subunit structure, the enzyme was subjected to gel electrophoresis in the presence of SDS. The enzyme was incubated in a solution of 1% SDS-1% mercaptoethanol and 4 M urea for 45 min at 45°. The relative electrophoretic mobility of the treated enzyme as well as those of standard proteins were used for a plot of the log molecular weight versus mobility (Fig. 10). The molecular weight calculated from such a standard curve was 51,000. Treatment of the enzyme at 100° for 2 min in 1% SDS, 1% mercaptoethanol-10 mM phosphate (pH 7.0) or dialysis overnight at room temperature against the same solution did not change the mobility of the enzyme. Thus it is highly unlikely that the enzyme is composed of subunits.

The higher activity in the presence of KCl indicates that the monomeric form of the enzyme with a molecular weight of 53,000 has a free binding site (or sites) available for phospholipids. However, in the absence of KCl the enzyme aggregates via phospholipid binding site (or sites), resulting in a decrease in the effective binding sites. The aggregated form appears to exist predominantly as a trimer.

**Absorption Spectrum and Amino Acid Compositions**—The absorption spectrum of the enzyme was determined and showed a peak at 280 nm. The amino acid composition was as follows: 11% glycine, 15% proline, 12% arginine, 12% glutamic acid, 11% aspartic acid, 9% serine, 9% threonine, 9% alanine, 8% valine, 8% isoleucine, 7% leucine, 7% phenylalanine, 6% lysine, 6% histidine, 4% cysteine, 4% methionine, 3% tryptophan, and 3% tyrosine.

**Fig. 6**. Phospholipid binding to malate-vitamin K reductase. A, thirty-seven micrograms of the enzyme (QAE Sephadex A-50 fraction) or the enzyme plus phospholipids, 100 µg, were layered on top of the gradient and centrifuged as described under "Experimental Procedure." Curves III and IV contained 0.5 m KCl in the gradient. The enzyme alone without KCl (O---O, I); enzyme plus phospholipids without KCl (O---O, II); enzyme alone with KCl (A---A, III); and enzyme plus phospholipids with KCl (A---A, IV). The arrow indicates the peak activity of the marker enzyme, yeast alcohol dehydrogenase (ADH). B, the same fractions obtained in A were assayed with PMS plus DCIP as acceptor. The closed circle and triangle represent the activities without the addition of phospholipid.

**Fig. 7**. Effect of phospholipid concentration on binding to malate-vitamin K reductase. The procedure was the same as that in Fig. 6, except that the amount of the enzyme was 25.4 µg in each tube, and the amount of phospholipids in micrograms was varied: 0 (A), 5 (B), 10 (C), 25 (D), 75 (E), and 250 (F).

**Fig. 8** (left). Molecular weight determination of malate-vitamin K reductase by Sephadex G-200 in the presence of KCl. The column (2.6 X 90 cm) was equilibrated with 0.3 m KCl in 20 mM Tris-HCl (pH 8.2) and 10% glycerol. The standards used were rabbit muscle pyruvate kinase (mol wt 237,000), yeast alcohol dehydrogenase (mol wt 190,000), bovine serum albumin (mol wt 68,000), ovalbumin (mol wt 43,000), and soybean trypsin inhibitor (mol wt 21,500). The void volume was estimated with blue dextran 2000. KMR, malate-vitamin K reductase.

**Fig. 9** (center). Molecular weight determination of malate-vitamin K reductase by Sephadex G-200 in the absence of KCl. The procedures were the same as those in Fig. 7, except that 0.3 m KCl was omitted from the buffer. The molecular weight values used were 64,500 for hemoglobin and 12,400 for horse heart cytochrome c. Other standards were the same as in Fig. 7. KMR, malate-vitamin K reductase.

**Fig. 10** (right). Estimation of the molecular weight of malate-vitamin K reductase by SDS gel electrophoresis. All the proteins used were treated as described under "Experimental Procedure" for gel electrophoresis. Electrophoresis was performed for 3 hours at room temperature. The molecular weights of the polypeptide chains of the standard proteins were 57,200 for pyruvate kinase, 43,000 for ovalbumin, 37,000 for yeast alcohol dehydrogenase, and 36,000 for rabbit muscle lactic dehydrogenase. KMR, malate-vitamin K reductase.
The absorption spectrum of malate-vitamin K reductase exhibits a maximum at 280 nm with a shoulder at 290 nm (Fig. 11). Since the enzyme requires FAD and vitamin K for activity (24, 35), the presence of these cofactors was examined with the purified enzyme. No spectral evidence was found for the presence of a flavin or quinone with 1.08 mg of purified enzyme even with an expanded full scale of 0.01 optical density on an Aminco DW-2 spectrophotometer.

The results of amino acid analyses are shown in Table III. Because of the difficulty in obtaining large quantities of purified enzyme only one analysis was obtained. It is of interest to note that tyrosine, phenylalanine, and half-cystine were not detected or were present in very small amounts, and that there was a relatively high content of nonpolar amino acids. Calculation of the polarity of the enzyme (36) gave a value of 42.7%, which is a value intermediate between that for soluble proteins (47-62%) and that for membrane-bound proteins (below 40%).

### TABLE III

| Amino acid     | Residues/32,000 g of protein |
|----------------|-----------------------------|
| Lysine         | 26                          |
| Histidine      | 11                          |
| Arginine       | 20                          |
| Hydroxylysine  | 6                           |
| Aspartic acid  | 35                          |
| Threonine      | 15                          |
| Serine         | 34                          |
| Glutamic acid  | 35                          |
| Proline        | 31                          |
| Glycine        | 54                          |
| Alanine        | 50                          |
| Half-cystine   | 0a                          |
| Valine         | 37                          |
| Methionine     | 7                           |
| Isoleucine     | 17                          |
| Leucine        | 43                          |
| Tyrosine       | 0a                          |
| Phenylalanine  | 0a                          |
| Tryptophan     | 5a                          |

*Not detectable.*  
*The content of tryptophan was estimated from the ultraviolet absorbance (34).*

Malate-vitamin K reductase has been characterized as an enzyme requiring phospholipids for enzymatic activity. Enzyme activity was not observed with the purified enzyme in the absence of phospholipids, while with less purified preparations, 3 to 5% of full activity was obtained without the addition of phospholipids. The determination of phospholipid content in the fractions obtained from different steps of purification gave values of 166, 8.4, and 2.3 μg of phospholipid per mg of protein in ammonium sulfate, DEAE-cellulose, and Sephadex fractions, respectively. Asloctin, whose major components are lecithin and phosphatidylethanolamine (40), has been shown to be the most effective phospholipid; individual lipids alone or cardiolipin are less effective (24). Differential centrifugation of sonicated cell extract was used to localize malate-vitamin K reductase. The enzyme activity was predominantly found in the supernatant fraction with some residual activity (resistant to 0.15 M KCl washing) in the particulate fraction. But ghost preparations, obtained by the treatment of cells with lysozyme in the presence of glycine, contained more than 85% of the enzyme activity, which can then be released by sonic oscillation (41). These results suggest that the enzyme is loosely bound to the cytoplasmic membrane and is not a “soluble cytoplasmic” enzyme. According to the classification of a membrane protein by Singer and Nicolson (42), this enzyme may be classified as a “peripheral” protein. Relevant to the nature of this association with the cytoplasmic membrane is the fact that the residual activity in the particulate fraction was completely released upon washing with either 10% glycerol or 0.25 M sucrose (data not shown). A bacterial coupling factor containing latent adenosine triphosphatase activity is associated with the particulate fraction from M. phlei and was also solubilized by the use of sucrose washing (43). Such an effect of sucrose or glycerol on a membrane-associated enzyme was also shown with acetyl-CoA.
carboxylase (44) and squalene synthetase (45). It is interesting to note that acetyl-CoA carboxylase was found to be slightly stimulated by phospholipid and was postulated to be attached weakly to a microsomal membrane (44). Thus, another type of chemical interaction, different from electrostatic bonding, seems to be an important force in associating these enzymes with the membranes.

In the present studies, complex formation between malate-vitamin K reductase and phospholipids has been successfully demonstrated. The lipid-protein complex dissociates completely into the separate components in the presence of KCl. It appears that electrostatic interaction is an important factor in the association between the enzyme and phospholipid. However, the enzyme activity associated with the particulate fraction can be released only by glycerol or sucrose, and not by KCl. This could be explained if the enzyme is not directly bound to phospholipid in the natural system, for example, if another protein (or proteins) is involved in the binding.

The effect of high KCl concentration on enzyme activity showed another aspect of enzymes with a requirement for phospholipid binding. The enzyme may undergo a KC-dependent aggregation phenomenon: the monomeric form (mol wt, 51,000 to 53,000) exists in the presence of KCl and the polymeric form (mol wt, 164,000) in the absence of KCl. Another important phenomenon is that the enzyme activity is correlated with the aggregation state; that is, the monomeric form has more than twice as much activity as the polymeric form. As shown in Fig. 6, the enzyme binds phospholipids, giving an enzyme-phospholipid complex which was dissociated with KCl. This was further verified by dilution experiments which showed that a higher specific activity was obtained upon dilution either in KCl-glycerol or in phospholipid. This provides a strong basis that electrostatic interaction is an important factor in the association between the enzyme and phospholipid. However, the enzyme activity associated with the particulate fraction can be released only by glycerol or sucrose, and not by KCl. This could be explained if the enzyme is not directly bound to phospholipid in the natural system, for example, if another protein (or proteins) is involved in the binding.

conductance (55) and surface pressure (52) are observed. Conformational changes in the protein were also suggested as a mechanism for protein phospholipid interaction (50, 54).

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