NONHEME IRON: A FUNCTIONAL COMPONENT OF MALATE - VITAMIN K REDUCTASE*

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Two distinct respiratory pathways have been described for malate oxidation in Mycobacterium phlei (Asano and Brodie, 1963). One pathway utilizes malate dehydrogenase and makes use of the chain common to all NAD^+-linked substrates. The other pathway is mediated by a unique enzyme, malate-vitamin K reductase (MKR), which bypasses the bound NAD^+ and flavoprotein of the NAD^+-linked chain but converges with it at the naphthoquinone level (Asano and Brodie, 1964).

The bulk of the MKR activity is found in the soluble fraction following sedimentation of the electron transport particles from cell-free extracts. In the absence of the electron transport particles, the oxidation of malate by MKR has been shown to occur by a vitamin K-mediated reduction of thiazolyl blue tetrazolium (MTT) (Asano and Brodie, 1963). The enzyme is specific for L-malate, is activated by FAD and exhibits an absolute requirement for quinone and phospholipid for MTT reduction (Asano et al., 1965). During the course of studies on the nature and role of nonheme iron in the electron transport pathways of M. phlei it was found that the supernatant fraction contained malate-reducible nonheme iron. This observation prompted a study of the involvement of nonheme iron in MKR. The results presented in this communication indicate that nonheme iron functions as the terminal electron carrier of the enzyme complex.

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MATERIALS AND METHODS

M. phlei cells (ATCC 354) were grown and harvested by the procedures previously described (Brodie and Gray, 1956). The cells were disrupted by sonic oscillation and the particulate and supernatant fractions separated from the cell-free extract by differential centrifugation (Brodie, 1959). The supernatant fluid containing MKR activity was fractionated with ammonium sulfate according to the procedure of Asano, et al. (1965). Suspensions of vitamin K_1 in asolectin were prepared as described earlier (Asano et al., 1965). The enzyme activity was measured spectrophotometrically by following the rate of reduction of MTT (extinction coefficient at 565 mμ, 15.0 mM^{-1} cm^{-1}). The nonheme iron content of the preparation was determined by procedures previously described (Kurup and Brodie, 1967a). Sulfhydryl groups were determined by the method of Ellman (1959) and labile sulfide as described by Brumby et al. (1965).

The reduction of nonheme iron was measured in a Cary model 11 recording spectrophotometer. The reaction system consisted of 500 μmoles of Tris buffer, pH 7.4, 50 μmoles of $MgCl_2$, 30 μmoles of KCl, 50 mμmoles of FAD, 2.2 μmoles of vitamin K_1 suspended in asolectin (5 mg), enzyme (3 mg protein) and water to a total volume of 3 ml. The cuvettes, containing the same components were balanced and 100 μmoles of malate was added to the standard cuvette. A difference spectrum was taken after 100 seconds and 2 x 10^{-4} M o-phenanthroline (dissolved in 20% ethanol) was added to both cuvettes. The difference spectrum now exhibited a peak with a maximum at 510 mμ due to the formation of ferrous-o-phenanthrolinate. The extent of iron reduction was calculated from the extinction coefficient of the complex at 510-540 mμ, 7.8 mM^{-1} cm^{-1} (Kurup and Brodie, 1967b).

RESULTS

The particulate and soluble fractions of M. phlei have been shown to contain nonheme iron (Kurup and Brodie, 1967a). Reduction of the nonheme iron of the enzyme preparation obtained from the soluble fraction was found to occur with malate as electron donor (Fig. 1). The difference spectrum taken 100 seconds after the addition of malate (Fig. 1, A) exhibited a decrease of absorption in the 450 mμ region

due to the reduction of both nonheme iron and flavin. Following the addition of o-phenanthroline the difference spectrum exhibited a peak at 510 mμ (Fig. 1, B). This peak was not observed when succinate or β-hydroxybutyrate was used as electron donor. The enzymatically reducible nonheme iron was dependent on the protein concentration.

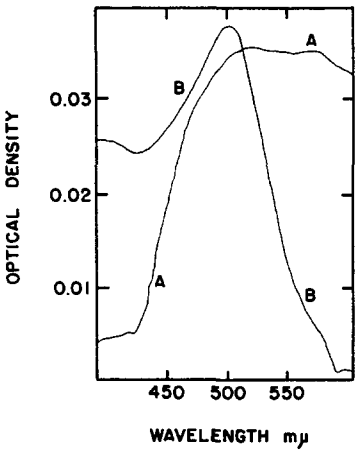

Fig. 1. Reduction of nonheme iron in malate-vitamin K reductase. The reaction system was the same as given in Materials and Methods. The protein concentration was 3 mg. A represents the difference spectrum 100 seconds after the addition of malate to the standard cuvette, and B 200 seconds after the addition of o-phenanthroline to both cuvettes.

Table 1. MKR Activity and Nonheme Iron Reduction

Reaction System	Enzyme activity (MTT reduced)	Nonheme iron reduced
	mμmoles/min	mμatoms
Complete	30.0	14.4
Complete minus FAD	8.0	2.7
Complete minus vitamin K_1 and phospholipid	0.0	0.0
Complete minus phospholipid	0.0	0.0
Complete minus malate	0.0	0.0

The reaction system for iron reduction was the same as given in Materials and Methods and contained 7.0 mg of enzyme protein. For the assay of enzyme 600 mμmoles of MTT and 1.5 mg of protein were used.

The conditions necessary for MKR activity and nonheme iron reduction were studied with the partially purified enzyme. The cofactor requirements for nonheme iron reduction were found to be similar to those necessary for the enzyme activity (Table 1). Both activities required the addition of FAD, vitamin K_1 and phospholipid.

An attempt was made to correlate the nonheme iron with the labile sulfide and sulfhydryl content. The content of labile sulfide was found to be much lower than the nonheme iron content of the preparation (Table 2). Only about one-fifth of the nonheme iron present in the preparation was found to undergo enzymatic reduction on the addition of malate. The malate-reducible nonheme iron, thus appeared to correlate with the labile sulfide content. The labile sulfide and nonheme iron in most proteins have been shown to exist in a 1:1 ratio (Brumby et al., 1965). The sulfhydryl content, on the other hand, was found to be of the same order as the content of nonheme iron. The sulfhydryl groups increased about 4-fold on denaturation of the protein with trichloracetic acid (Table 2).

Table 2. Nonheme Iron, Labile Sulfide and Sulfhydryl Content of MKR

Preparation	Nonheme iron		Labile	Sulfhydryl groups	
	Total	Reducible	Sulfide	Direct	TCA* treated
	mμatoms/mg protein		mμmoles/ mg protein	mμmoles/mg protein	
Malate-vitamin K reductase	10.7	2.2	2.8	16.7	60.0

TCA* - Protein sedimented following trichloracetic acid treatment.
The values were not corrected for contaminant proteins.

The involvement of a metal in MKR activity was further indicated by the effects of metal chelating agents (Table 3). Inhibition of MTT reduction was obtained with o-phenanthroline, 8-hydroxyquinoline and salicylaldoxime. Of the chelating agents tested o-phenanthroline appeared to be the best. This chelating agent has been shown to be an effective inhibitor of oxidative phosphorylation with the

cell-free systems from M. phlei. The inhibitory effect was specifically reversed by the addition of ferrous iron (Kurup and Brodie, 1967c).

Table 3. Effect of Metal Chelating Agents on MKR Activity

Chelating Agent	Concentration	Activity (MTT reduced)	Inhibition
	(mM)	mμmoles/min	%
None		31	
o-Phenanthroline	2.5	16	49
	5.0	5	84
	10.0	0	100
8-Hydroxyquinoline	5.0	10	66
Salicylaldoxime	10.0	11	65

The reaction system was the same as in Table 1 and contained 1.5 mg of enzyme. The system was incubated with the chelating agent for 5 min. before the addition of malate.

The site of nonheme iron in the sequence of electron carriers involved in the reduction of the dye was also determined by studying the "jump" in MTT reduction following preincubation of the system for various intervals before addition of the dye. The jump was previously ascribed to the reduced vitamin K_1 that accumulated in the reaction system during preincubation (Asano et al., 1965). The jump in MTT reduction was inhibited when o-phenanthroline was added before the dye (Table 4). Assay for the reduction of quinone with Emmerie-Engel reagent indicated that the enzymatic reduction of vitamin K_1 was not affected by the presence of the chelating agent in the incubation mixture.

The results indicated that the immediate electron donor for MTT reduction in the enzyme was nonheme iron. This was further confirmed by the observation that o-phenanthroline failed to inhibit the reduction of dichloroindophenol (DClP) in the presence of phenazinemethosulfate (PMS). This assay does not require vitamin K_1 for activity since PMS taps electrons at the flavoprotein level and reduces the DClP (Asano et al., 1965).

Table 4. Effect of o-phenanthroline on the Jump Reduction of MTT

Order of Addition	MTT Reduced
	mμmoles
Malate (300 sec) → MTT	40
Malate (300 sec) → MTT + OP	31
Malate + OP (300 sec) → MTT	5
Malate (300 sec) → OP (100 sec) → MTT	7

OP - o-phenanthroline.
The reaction system was the same as in Fig. 1 except that 50 μmoles of phosphate buffer pH 5.9 was added instead of Tris. The concentration of the chelating agent was 5×10^{-3}M. The figure in parenthesis denotes the time of incubation before the addition of the next component. The reduction of MTT was measured immediately after addition.

DISCUSSION

The requirements for the enzymatic reduction of nonheme iron in partially purified MKR are found to parallel those necessary for the reduction of MTT by the enzyme. Both require FAD, vitamin K_1, and phospholipid. Furthermore, the inhibition of dye reduction by chelating agents supports the finding that nonheme iron is a functional component of the enzyme. Studies of the site of interaction of nonheme iron in the electron transport sequence leading to dye reduction indicate that this component interacts between reduced vitamin K_1 and the dye (Fig. 2). Reduction of the quinone has been observed in the presence of o-phenanthroline whereas dye reduction is inhibited. Furthermore, the lack of inhibition of the PMS-DCIP assay system which does not require the addition of vitamin K_1, indicates that flavin reduction occurs before the quinone-nonheme iron level of oxidation-reduction. The site of interaction of nonheme iron in MKR appears to be similar to that ascribed for a number of nonheme iron containing enzymes where EPR studies have indicated that nonheme iron is the terminal electron carrier (Beinert and Palmer, 1965; Singer, 1966).

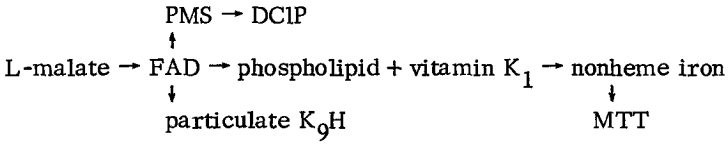

Fig. 2. Sequence of electron carriers for MKR activity

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