JOURNAL OF BACTERIOLOGY, Sept. 1971, p. 787-789

Vol. 107, No. 3
Printed in U.S.A.

Unusual Effects of Reducing Agents on *o*-Diphenoloxidase of *Mycobacterium leprae*

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Received for publication 12 April 1971

Reducing agents had no effect on the oxidation of 3,4-dihydroxyphenylalanine (DOPA) to quinone by *Mycobacterium leprae;* no quinone formation by *o*-diphenoloxidase of mammalian or plant origin was detected under similar experimental conditions. Ascorbic acid and reduced glutathione prevented further oxidation and polymerization of the quinone to melanin by *M. leprae;* cysteine was less effective. In the presence of reducing agents, the quinone (indole-5,6-quinone) formed from DOPA by *M. leprae* was not reduced back to diphenol. On the other hand, the quinone (dopachrome) produced from DOPA by mammalian or plant phenolase was rapidly decolorized by reducing agents. Oxidized glutathione and cystine had little effect on *o*-diphenoloxidase from all of the three sources. Cyanide, which completely inhibited mammalian and plant phenolases, had only a partial effect on the enzyme in the bacilli. Various lines of evidence suggest that the properties of *o*-diphenoloxidase in *M. leprae* are different from those of similar enzymes obtained from other sources.

Very little is known about the metabolic properties of *Mycobacterium leprae*, obviously because the only source of the organism for biochemical studies is the infected tissue of the human host. Our recent investigations (6, 7) with concentrates of acid-fast organisms separated from lepromatous material showed that the bacilli possess an active *o*-diphenoloxidase (EC 1.10.3.1), which converts various phenolic compounds to pigmented products in vitro. Several other mycobacteria tested did not have any phenoloxidase (2). The phenolase of *M. leprae* oxidized both D- and L-3,4-dihydroxyphenylalanine (DOPA) at the same rate and was different from similar enzymes of plant or mammalian origin in the range of substrates utilized, the products formed, and the effects of certain inhibitors (7, 8). Results on the unusual effect of reducing agents on the *o*-diphenoloxidase of the leprosy organisms are reported in the present paper.

MATERIALS AND METHODS

Materials. The method used for separation of *M. leprae* from infected tissues in a metabolically active state has been described before (5, 6). The tissues (biopsies of skin nodules or lepromatous spleen removed at autopsy) are collected aseptically and kept frozen at −20 C. The preparative procedures (homogenization of the tissue and differential centrifugation) are carried out at 0 C. As such, there is little chance of any contaminant organisms multiplying. The *M. leprae* preparation was used as intact organisms or was disrupted by ultrasonic oscillation. The sonified suspension was centrifuged at 125,000 × *g* for 60 min. The residue was washed once and finally suspended in water. Almost all of the phenolase activity was located in the particulate fraction.

The plant enzyme used was lyophilized mushroom tyrosinase (Nutritional Biochemicals Corporation, Cleveland, Ohio) or a crude preparation of potato phenolase. Water extracts of Harding-Passey or Cloudman S-91 mouse melanomas or a culture of melanocytes (from Cloudman S-91 melanoma) served as sources of mammalian *o*-diphenoloxidase. Commercially available chemicals of the highest purity were used without further purification.

Enzyme assay. The oxidation of DOPA or tyrosine to melanin pigment passes through a sequence of reactions involving several intermediates. The intermediates are highly unstable and have been identified and characterized by their absorption spectra (4). With mammalian or plant phenolase, the immediate reaction product that accumulates in the medium is dopachrome, which has an absorption maximum at 480 nm. With *M. leprae*, the product observed was indole-5,6-quinone, which absorbs maximally at 540 nm. The formation of this intermediate involves a decarboxylation step; the enzyme in *M. leprae* may be associated with an active decarboxylase (7). In the present study, the spectrophotometric method was adopted to assay the products formed, since it was sensitive enough for the small amounts of organisms available. In earlier experiments (5), oxidation of DOPA has been demonstrated by the Warburg manometric method. This technique could not be followed routinely because of

paucity of the human material needed. Details of the procedure used have been described before (8). The reaction system essentially contained the following constituents in the final concentrations indicated: Na_2HPO_4-KH_2PO_4 buffer (*p*H 6.8), 0.1 M; L-DOPA, 0.002 M; reducing agents or other inhibitors, 0.004 M; *M. leprae*, 1.5 to 2 mg of protein; melanoma extract, 15 mg of protein; and mushroom tyrosinase, 10 μg. The volume was 3 ml and the temperature was 37 C for *M. leprae* and melanoma extract and 25 C for mushroom tyrosinase. The incubation time was 30 min, unless otherwise stated. All experiments were carried out at least three times, and variability was in the range of 5 to 8%. Results reported are for representative experiments. All readings have been corrected for any absorbance due to enzyme, substrate, or inhibitor.

RESULTS AND DISCUSSION

In the presence of ascorbic acid, reduced glutathione (GSH), or L-cysteine, quinone formation from DOPA by the *o*-diphenoloxidase of melanoma extract and mushroom tyrosinase was not detected. The reducing agents, however, had no effect on the oxidation of DOPA to indole-5,6-quinone by *M. leprae*. Oxidized glutathione and L-cystine were without effect on the *o*-diphenoloxidase from all the three sources (Table 1).

TABLE 1. *Effect of reducing agents on oxidation of 3,4-dihydroxyphenylalanine (DOPA) to quinone by o-diphenoloxidase from different sources*

Reaction system[a]	Absorbancy 480 nm		Absorbancy 540 nm, *Mycobacterium leprae*	
	Melanoma extract	Mushroom tyrosinase	Intact	Disrupted
Enzyme + DOPA	0.237	0.220	0.135	0.100
Enzyme + DOPA + ascorbate	0	0	0.150	0.105
Enzyme + DOPA	0.208	0.220	0.100	0.090
Enzyme + DOPA + GSH	0	0	0.125	0.100
Enzyme + DOPA	0.200	0.160	0.100	0.085
Enzyme + DOPA + cysteine	0	0	0.120	0.090
Enzyme + DOPA	0.205	0.160		0.100
Enzyme + DOPA + GSSG	0.200	0.145		0.100
Enzyme + DOPA + cystine	0.210	0.150		0.105

[a] Abbreviations: GSH, reduced glutathione; GSSG, oxidized glutathione.

The lack of effect of reducing agents on the *o*-diphenoloxidase of *M. leprae* does not appear to be due to the permeability barrier of the bacterial cell wall. As the results show, both intact as well as disrupted organisms gave similar results. The purity of the enzyme preparation is also apparently not a critical factor in the effects observed. Crude potato phenolase behaved the same way as purified mushroom tyrosinase. Moreoever, the mammalian enzyme used was a crude preparation.

The effect of ascorbic acid and cysteine on the quinones formed from DOPA is shown in Table 2. Glutathione gave similar results as ascorbic acid. Melanoma extract and mushroom tyrosinase were preincubated with DOPA for 15 min; *M. leprae* was incubated with DOPA for 30 min. After the quinones were formed, the reducing agents were added and readings were taken at intervals. Reaction mixtures containing *M. leprae* or melanoma extract were centrifuged at 25,000 × *g* for 45 min before adding the reducing agents to the supernatant fluids. With melanoma extract and mushroom tyrosinase, the quinone produced (dopachrome) was rapidly decolorized in the presence of reducing agents. With *M. leprae*, indole-5,6-quinone formed was not reduced back to diphenol. However, the reducing agents prevented further oxidation and polymerization of the quinone to melanin; cysteine was less effective than GSH or ascorbic acid. When the reducing agents were preincubated with *M. leprae* before adding DOPA, formation of indole-5,6-quinone was not affected, but melanin formation was suppressed.

Phenolase is a copper protein, and sulfhydryl groups are known to bind the copper and inacti-

TABLE 2. *Effect of reducing agents on quinones formed from 3,4-dihydroxyphenylalanine by o-diphenoloxidase*

Time[a]	Absorbancy 480 nm				Absorbancy 540 nm, *Mycobacterium leprae*	
	Melanoma extract		Mushroom tyrosinase			
	Ascorbate	Cysteine	Ascorbate	Cysteine	Ascorbate	Cysteine
Zero time	0.125	0.165	0.080	0.070	0.100	0.090
15 min	0.100	0.120	0.050	0.050	0.100	0.095
30 min	0.080	0.090	0.040	0.040	0.100	0.100
60 min	0.070	0.070	0.040	0.035	0.100	0.100
2 hr	0.070	0.060	0.035	0.030	0.100	0.105
20 hr	0.040	0.030	0.025	0.015	0.110	0.260

[a] At 20 hr, absorbancy (480 nm) on melanoma extract was 0.8 and on mushroom tyrosinase was 0.76; absorbancy (540 nm) on *M. leprae* was 0.74 in the absence of reducing agents.

vate the enzyme; cysteine and reduced glutathione are reported to be effective inhibitors of phenolase (1, 3). Ascorbic acid and other reducing agents also interact with the quinones formed, reducing them back to diphenol and being oxidized in the process (3, 9, 10). In the conversion of a polyphenol to quinone, the cupric form of phenolase is reduced by the substrate to the cuprous form, which, in turn, is reoxidized by oxygen. Two reducing equivalents are removed from each substrate molecule. Sulfhydryl groups complex with the copper in the enzyme and inhibit the reversible oxidation-reduction process it undergoes.

The data reported here suggest that the *o*-diphenoloxidase of *M. leprae* probably has a higher affinity for the substrate, and the enzyme is not inactivated by the reducing agents. Probably its copper moiety is not readily accessible. In results reported earlier (8), penicillamine, a potent copper chelator, had no effect on phenolase of *M. leprae*, whereas the enzyme from mammalian or plant sources was inhibited. On the other hand, diethyldithiocarbamate, another copper chelator, completely suppressed *o*-diphenoloxidase of the bacilli. This compound possesses two ethyl groups (lipid-soluble, nonpolar masses) which shadow the two sulfurs (the polar region), enabling the compound to permeate lipid-predominant pores. This ability to permeate seems to be responsible for its inhibitory effect on the phenolase of *M. leprae*.

The suggestion that the copper moiety of the phenolase in the leprosy organisms may not be easily accessible gains added support from the effects of cyanide and azide on *o*-diphenoloxidase from different sources (Table 3). Cyanide, an inhibitor of metalloenzymes, completely suppressed the activity of mammalian and plant phenolase; however, it showed little effect on *M. leprae*. The organisms used had been disrupted by ultrasonic oscillation to obviate any permeability barrier of the bacterial cell wall. A partial inhibitory effect was observed when the *M. leprae* suspension was preincubated with cyanide before addition of DOPA. Sodium azide inhibited melanoma extract, but showed no effect on *o*-diphenoloxidase of the bacilli and mushroom tyrosinase.

The interpretations of the results given above are, of course, tentative. Purification of the enzyme in *M. leprae* has to await cultivation of the organisms in bacteriological media. However, the data presented here as well as the results already reported (7, 8) do suggest that in its properties the *o*-diphenoloxidase of *M. leprae* may be different from similar enzymes obtained so far from other sources.

TABLE 3. *Effect of azide and cyanide on oxidation of 3,4-dihydroxyphenylalanine (DOPA) to quinone by o-diphenoloxidase; increase in absorbancy*

Enzyme source	Wavelength (nm)	Enzyme + DOPA	Enzyme + DOPA + NaN_3	Enzyme + DOPA	Enzyme + DOPA + NaCN
Melanoma extract ..	480	0.205	0	0.225	0
Mushroom tyrosinase .	480	0.190	0.170	0.270	0
Mycobacterium leprae	540	0.095	0.100	0.115	0.105
M. leprae preincubated with NaN_3 and NaCN ...	540	0.090	0.095	0.115	0.055

ACKNOWLEDGMENTS

This investigation was supported by the United States-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases.

I thank W. F. Kirchheimer, Laboratory Research Department, USPHS Hospital, Carville, for his interest and encouragement.

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