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Malate Dehydrogenases from Actinomycetes: Structural Comparison of *Thermoactinomyces* Enzyme with Other Actinomycete and *Bacillus* Enzymes

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Malate dehydrogenases from bacteria belonging to the genus *Thermoactinomyces* are tetrameric, like those from *Bacillus* spp., and exhibit a high degree of structural homology to *Bacillus* malate dehydrogenase as judged by immunological cross-reactivity. Malate dehydrogenases from other actinomycetes are dimers and do not cross-react with antibodies to *Bacillus* malate dehydrogenase.

Malate dehydrogenase (L-malate:NAD$^+$ oxidoreductase EC 1.1.1.37) occurs as a dimeric molecule (molecular weight, ca. 65,000) in most organisms and as a tetramer twice as large in some gram-positive bacteria, notably *Bacillus* spp. (8, 14). We have observed earlier that, as judged by its tetrameric structure and immunological cross-reactivity, malate dehydrogenase from *Thermoactinomyces sacchari* bears a strong structural resemblance to *Bacillus* malate dehydrogenase. Malate dehydrogenase from another actinomycete, *Thermomonospora fusca*, however, was found to be a dimer and does not cross-react with antibodies to the *Bacillus* enzymes (14). This structural similarity between the malate dehydrogenases from *Thermoactinomyces sacchari* and *Bacillus* spp. appeared to be another biochemical manifestation of a phylogenetic relationship between the two bacterial genera. We therefore undertook a more detailed study of malate dehydrogenases from actinomycetes and present here the results.

Malate dehydrogenases were isolated in pure state from the bacteria listed in Table 1 by the method described earlier (11). The course of purification from the six actinomycetes is summarized in Table 2. As with several bacteria studied previously, our method, in which the enzyme is selectively eluted as a ternary complex with NAD$^+$ and L-malate (11), produces pure malate dehydrogenase in a single step in high yield. The purity of the malate dehydrogenases was checked by polyacrylamide gel electrophoresis of the enzyme preparations in the native and denatured states. The specific activity of pure malate dehydrogenase from *Streptomyces lividans*, a mesophile, is appreciably higher than those of the other malate dehydrogenases, which are all from thermophiles (Table 2).

The sedimentation coefficients of the malate dehydrogenases, which were determined by sucrose density gradient centrifugation, are presented in Table 3. All these enzymes belong to one of two classes characterized by sedimentation coefficients of ca. 6.5S and 4.5S. A previous study (14) established that the malate dehydrogenases from *Bacillus caldotenax* and *Thermoactinomyces sacchari* are tetramers having a molecular weight of ca. 130,000 and that the malate dehydrogenase from *Thermomonospora fusca* is a dimer with a molecular weight of ca. 65,000. The subunit molecular weights of the malate dehydrogenases previously examined are ca. 33,000 (14). In the present investigation, the subunits of the malate dehydrogenases from the six actinomycetes had a similar size, with the molecular weights ranging between 32,800 and 34,100. It may therefore be assumed that a sedimentation coefficient approximating to 4.5S identifies the malate dehydrogenase as a dimer with a molecular weight of ca. 65,000 and that a sedimentation coefficient approximating to 6.5S shows the enzyme to be a tetramer with a molecular weight of ca. 130,000. By this criterion, the malate dehydrogenases from all the actinomycetes used in this study, except *Thermoactinomyces* spp., are dimers, and those from *Thermoactinomyces* spp., like the enzymes from *Bacillus* spp., are tetramers.

We purifed 12 bacterial malate dehydrogenases by adsorption to, and elution from, Procion Red HE3B immobilized on agarose. The tetrameric enzyme forms are eluted by 10 mM L-malate in conjunction with NAD$^+$ at a concentration of less than 0.2 mM, whereas the dimeric forms require a higher NAD$^+$ concentration for elution. It is therefore possible to predict the molecular size and subunit structure of a malate dehydrogenase reasonably well from its elution behavior.

Figures 1 and 2 summarize the results of immunotitration experiments. The titration profiles from enzyme neutralization tests (Fig. 1A and 2A) and those from experiments in which the antigen-antibody complex was sedimented in association with protein A (Fig. 1B and 2B) were similar except that the enzyme removal from the supernatant fraction in the latter experiments was more efficient than enzyme inactivation in the former experiments. This presumably is because complexes in which antibodies have combined with parts of the enzyme molecule not essential for enzyme activity may contribute to the residual enzyme activity determined in titration experiments in which they are not sedimented along with the protein A adsorbent. Antibodies to the malate dehydrogenase from *B. caldotenax* strongly cross-reacted with the enzymes from *Bacillus subtilis* and the two thermoactinomycetes (Fig. 1). These four malate dehydrogenases were all shown to be tetrameric by the results of sedimentation experiments (Table 3). No cross-reaction was observed (data not shown) between antibodies to the *B. caldotenax* enzyme and the malate dehydrogenases from *Thermomonospora fusca*, *Thermomonospora chromogena*, *Micropolyspora faeni*, and *S. lividans*, which were observed to be dimeric by the sedimentation criterion. Antibodies to *Thermomonospora chromogena* malate dehy-

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TABLE 1. Bacteial strains used

Bacterial strain	Source	Growth medium[a]
B. subtilis Marburg	Laboratory stock	(11)
B. caldotenax	(6); Centre for Applied Microbiology and Research	(10)
M. faeni ATCC 15347	A. McCarthy	Nutrient broth (1.25%) + glucose (0.4%)
Thermoactinomyces sacchari ATCC 27375	J. Lacey	Malt extract (0.3%) + yeast extract (0.3%) + Bacto-Peptone (0.5%) + glucose (1%), pH 7.2
T. candidus ATCC 27868	A. McCarthy	Nutrient broth (1.25%) + glucose (0.4%)
Thermomonospora chromogena NCIB 10212	A. McCarthy	Nutrient broth (1.25%) + glucose (0.4%)
T. fusca ATCC 27730	J. Lacey	Nutrient broth (1.25%) + glucose (0.4%)
S. lividans 66 (derivative)	(7); J. Cullum	Nutrient broth (2.5%) + yeast extract (0.5%)

[a] Growth temperatures were: *S. lividans*, 30°C; *B. subtilis*, 37°C; *B. caldotenax*, 65°C; all other bacteria, 55°C.

drogenase, a dimer, showed significant cross-reaction with the dimeric malate dehydrogenases from *Thermomonospora fusca* and *S. lividans* (Fig. 2) but not with tetrameric enzyme forms from *B. caldotenax*, *Thermoactinomyces sacchari*, and *Thermoactinomyces candidus* or with the dimeric enzyme from *M. faeni* (data not shown). The most important conclusion from these observations is that the malate dehydrogenases from the thermoactinomycetes bear strong homology to the *Bacillus* malate dehydrogenase on the basis of immunological cross-reactivity.

The present study of malate dehydrogenases from four genera considered to belong to the group of actinomycetes establishes that the dehydrogenase from *Thermoactinomyces* spp. is distinctive in its larger molecular size and tetrameric subunit structure from the smaller, dimeric malate dehydrogenase from the other three genera. The tetrameric malate dehydrogenase is the less common form of this enzyme, seen notably in *Bacillus* spp. (8, 14). It is therefore especially significant that antibodies to the tetrameric malate dehydrogenase from *B. caldotenax* cross-react strongly with the enzymes from the thermoactinomycetes but not with the malate dehydrogenases from the other actinomycetes included in this study (Fig. 1). The lack of cross-reaction between antibodies to the dimeric *Thermomonospora chromogena* malate dehydrogenase and the malate dehydrogenases from *Bacillus* spp. and the thermoactinomycetes further underlines the similarity between the genera *Bacillus* and *Thermoactinomyces*. It may be somewhat surprising that the dimeric malate dehydrogenase from *S. lividans* interacts with antibodies to the *Thermomonospora chromogena* enzyme but that the enzyme from *M. faeni*, also a dimer, does not, since there is no taxonomic indication at present that the genus *Streptomyces* is more closely related to the genus *Thermomonospora* than to the genus *Micropolyspora*. In the

TABLE 2. Purification of malate dehydrogenases from actinomycetes[a]

Bacterium	Step	Protein (mg)	Enzyme activity[b] (U)	Specific enzyme activity (U/mg of protein)	Yield (%)
Thermomonospora chromogena	Cell-free extract	2,030	975	0.48	100
	Procion Red chromatography	4	605	150	62
T. fusca	Cell-free extract	962	360	0.37	100
	Procion Red chromatography	1.65	241	146[c]	67
Thermoactinomyces candidus	Cell-free extract	830	1,528	1.84	100
	Procion Red chromatography	5.35	855	160	56
T. sacchari	Cell-free extract	1,897	3,182	1.67	100
	Procion Red chromatography	9.1	1,495	165[c]	47
M. faeni	Cell-free extract	1,190	2,705	2.27	100
	Procion Red chromatography	5.85	1,408	240	52
S. lividans	Cell-free extract	943	8,740	9.27	100
	Procion Red chromatography	3.6	3,038	845	35

[a] The purification was based on the method published earlier (11). Any variation from this method was in the concentration of NAD^+ in the buffers used to wash the Procion Red HE3B column and to elute the enzyme from the column. These concentrations were: *Thermomonospora fusca* enzyme, 0.2 mM; *Thermomonospora chromogena* enzyme, 0.25 mM; *Thermoactinomyces sacchari* enzyme, 0.17 mM; *Thermoactinomyces candidus* enzyme, 0.19 mM; *M. faeni* enzyme, 0.25 mM; and *S. lividans* enzyme, 0.25 mM.

[b] Enzyme activity unit (U) is the amount of enzyme causing the oxidation of 1 μmol of NADH per min at 30°C in the assay system (11).

[c] Previously (15), the specific activities of pure *Thermomonospora fusca* and *Thermoactinomyces sacchari* enzymes were reported to be 8.6 and 23.3, respectively. These low values were obtained because the concentration of oxaloacetate that was used in the enzyme assays is highly inhibitory. In this assay system, a strong activation of these enzyme forms by KCl was reported (14). We now find that the activation is weaker when an optimal concentration of oxaloacetate is used in the assays.

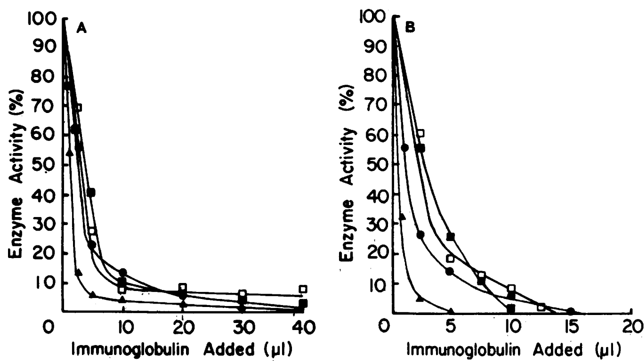

FIG. 1. Immunotitration of malate dehydrogenases against anti-*B. caldotenax* malate dehydrogenase immunoglobulin. (A) Each purified malate dehydrogenase (20 µl, 0.02 U) was incubated with the amount of immunoglobulin shown, 0.07 µmol of NADH, and sodium-potassium phosphate buffer (pH 7.5) in a volume of 0.45 ml for 30 min at 30°C. The residual enzyme activity was then determined after the addition of 0.05 ml of 6.6 mM oxaloacetate. The protein concentration of the immunoglobulin preparation was 7.5 mg/ml. Malate dehydrogenases titrated were: □, *B. caldotenax*; ▲, *B. subtilis*; ●, *Thermoactinomyces candidus*; and ■, *Thermoactinomyces sacchari*. (B) Each malate dehydrogenase (20 µl, 0.01 U) was incubated for 30 min at 30°C with the amount of immunoglobulin shown and NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-hydrochloride [pH 7.5], 0.02% sodium azide, and 0.05% Triton X-100) in a volume of 0.2 ml. Protein A adsorbent (0.2 ml) was added, and the incubation was continued at room temperature for 10 min. The protein A adsorbent and the immunoglobulin G complexed with it were spun down, and 0.2 ml of the supernatant fraction was assayed in a 1-ml system for malate dehydrogenase (11). The protein concentration of the immunoglobulin preparation was 3 mg/ml. Enzymes and symbols as in (A). The protein A adsorbent was prepared as follows: Formalin-fixed *Staphylococcus aureus* cells were washed with NET buffer containing Triton X-100 at a total concentration of 0.5% and then were suspended in NET buffer (containing 0.05% Triton X-100) to a concentration of 10% (vol/vol).

current state of flux of actinomycete classification (12), it must remain an open question whether this finding has any significance. In this study and previously (14), no appreciable immunological cross-reaction between the tetrameric *Bacillus* malate dehydrogenase and dimeric malate dehydrogenase from any organism has been observed. Thus, the

TABLE 3. Sedimentation coefficients of malate dehydrogenases[a]

Bacterial source of malate dehydrogenase	Sedimentation coefficient (S)
B. subtilis	6.7
B. caldotenax	6.5
Thermonomospora chromogena	4.6
T. fusca	4.5
Thermoactinomyces candidus	6.7
T. sacchari	6.7
M. faeni	4.5
S. lividans	4.4

[a] Sedimentation coefficients were determined from the positions of the malate dehydrogenases after centrifugation in a sucrose density gradient, relative to the positions of marker proteins. The enzymes (50 µg of protein) were centrifuged at 58,000 rpm (450,000 × g_{av}) through a continuous, linear, sucrose density gradient between 15 and 30% (wt/vol) in a volume of 3.6 ml, buffered with 10 mM Tris-hydrochloride (pH 7.2), at 4°C for 20 h.

dimeric and the tetrameric forms of this enzyme are distinct and stable structural entities. The significance of this divergence in evolutionary terms, however, is not clear. Immunological cross-reactivity is now widely regarded as a good indicator of structural resemblance. The high degree of structural homology that the *Thermoactinomyces* malate dehydrogenase bears to the *Bacillus* enzyme, as shown by this study and others, betokens at the molecular level a phylogenetic link between these two bacterial genera. This accords with recent thinking (4), based initially on the base composition of the DNA of thermoactinomycetes (3, 9) and on their ability to form heat-stable endospores (5), that these bacteria resemble *Bacillus* spp. more than they resemble the other actinomycetes. Further indications of this interesting relationship between the genera *Thermoactinomyces* and *Bacillus* have emerged from studies of other molecular parameters, e.g., rRNA oligonucleotide sequences (12), menaquinone composition (2), and partial sequences of proteinases (1, 13). Thus, the morphological property of hyphal growth, which thermoactinomycetes share with the other actinomycetes, may not have a fundamental significance from an evolutionary standpoint. It is reasonable to suppose that phylogenetically there is a basic kinship be-

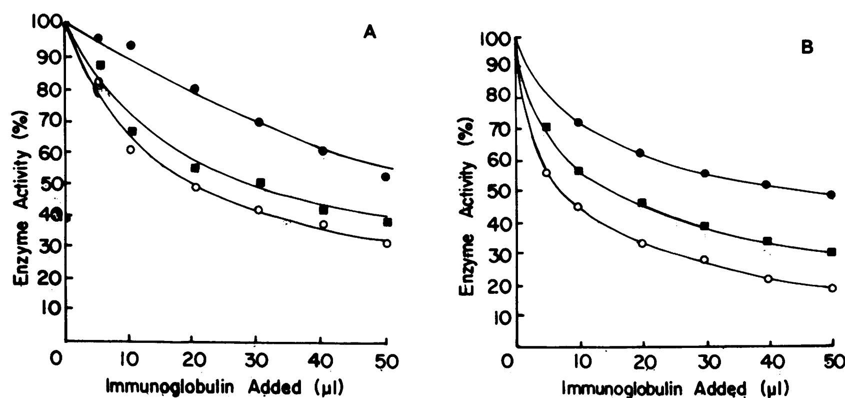

FIG. 2. Immunotitration of malate dehydrogenases against anti-*Thermomonospora chromogena* malate dehydrogenase immunoglobulin. (A) Experimental details as given in Fig. 1A. The protein concentration of the immunoglobulin preparation was 6.8 mg/ml. Malate dehydrogenases titrated were: ○, *Thermomonospora chromogena*; ■, *S. lividans*; and ●, *Thermomonospora fusca*. (B) Experimental details as outlined in Fig. 1B. The protein concentration of the immunoglobulin preparation was 2.7 mg/ml. Enzymes and symbols as in (A).

tween the genera *Thermoactinomyces* and *Bacillus* and that the organisms belonging to the former genus acquired the property of hyphal growth, perhaps during a process of convergent evolution with the other actinomycetes.

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