The Activation of Glycerol Dehydrogenase from
Aerobacter aerogenes by Monovalent Cations*

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It has previously been reported that a capsulated strain of Aerobacter aerogenes, 1033, can utilize glycerol as sole source of
energy and of carbon (1), and that glycerol induces this organism
to form a diphosphopyridine nucleotide-linked glycerol dehydro-
genase (2). This enzyme may be identical with the glycerol dehydro-
genase discovered by Burton and Kaplan (3) in another
strain of A. aerogenes, whose partial purification was described
by Burton (4). The enzyme resembles also a glycerol dehydro-
genase of Escherichia coli described by Asnis and Brodie (5).

EXPERIMENTAL PROCEDURE

Bacteria—Aerobacter aerogenes strain P14 is a guanine-requiring
mutant of strain 1033 which has been described in an earlier
communication (6). The mutant was maintained by monthly
transfers on tryptone nutrient agar slants.

Base Medium—The basal culture medium contained 40 μg
of guanine per ml, 1.26% KH₂PO₄, 0.54% K₂HPO₄, 0.20%
(NH₄)₂SO₄, 0.02% MgSO₄·7H₂O, and 0.001% CaCl₂. The final
pH was adjusted to 6.8 by addition of NaOH. The solution
was sterilized by autoclaving. Glycerol and glucose, 20% solu-
tions, were autoclaved separately and added aseptically to the
basal medium. Media containing 0.2% glycerol and 0.01%
glucose were usually used.

Growth of Cells and Preparation of Enzyme Extracts—Cells
from agar slants were suspended and transferred aseptically into
the growth medium, 2-liter Erlenmeyer flasks each containing
1 liter of medium. Fully grown cultures were usually obtained
by incubating the inoculated flasks overnight on a New Bruns-
dwick rotary action flask shaker operated at about 150 cycles per
minute in a room maintained at 37°. The cells were collected
by centrifugation for 7 minutes at 4° in an International re-
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minute in a room maintained at 37°. The cells were collected
by centrifugation for 7 minutes at 4° in an International re-
suspended in 0.01 M sodium phosphate buffer at pH 7.4 (15 to
20 ml of buffer for every liter of culture). The suspension was
then sonically disrupted by treatment in a Raytheon 10 kc.-250
watt sonic oscillator at 0° for 4 minutes. This treatment did
not seem to inactivate the glycerol dehydrogenase because the
same specific activities were observed in extracts which had been
subjected to sonic disruption for 4, 6, or 8 minutes; 2 minutes of
sonic treatment released only 80% of the enzyme activity from
the cells. The disrupted preparation was centrifuged for 20
minutes at approximately 12,000 × g in a Servall angle centrifuge
in a cold room maintained at 5°. The supernatant fraction
contained the enzyme activity.

Chemicals—Metal-free NH₄Cl was generated by distilling NH₄
Cl from concentrated NH₄OH into constant boiling HCl solution.
The crystals were collected and recrystallized from glass distilled
water. (NH₄)₂SO₄ used in enzyme assays was first recrystallized
from a solution containing 2 g of disodium ethylenediaminetetra-
acetate per liter. The crystals were collected and recrystallized
from glass-distilled water. KCl was recrystallized twice in the
same manner. Other chemicals were obtained from commercial
sources without further purification; glycerol and other related
compounds were from Eastman Organic Chemicals, dihydroxy-
acetone from Mann Research Laboratories, crystalline pyridine
nucleotides from Pabst Laboratories, and pyrurate from Nutritional Biochemicals Corporation. Calcium phosphate gel
was prepared according to the procedure of Keilin and Hartree
(7). The Polia-Cloacalce phenol reagent was obtained from the
Hartman-Leddon Company.

Enzyme Assay—The activity of glycerol dehydrogenase was
measured by following the reduction of DPN at 340 μm in a
Beckman model DU spectrophotometer with a circulating water
constant temperature jacket maintained at 25 ± 1°. The reaction
was carried out in 1-cm light-path cuvettes. The enzyme
was first incubated for 1 to 2 minutes with 0.1 ml of neutralized
0.01 M DPN and 0.1 ml of 1 M (NH₄)₂SO₄. This was followed
by the addition of 0.6 ml of 0.5 M NaHCO₃-Na₂CO₃ buffer and
sufficient water to give a final volume of 3.0 ml. The reaction
was initiated by the addition of 0.3 ml of 1 M glycerol. The
reaction blank contained all components except the glycerol.
The initial slope during the first minute of reaction was used to
calculate the enzyme activity which was expressed as μmoles of
DPN reduced per minute. Glutathione had no appreciable
effect on the activity of the enzyme either in crude or in partially
purified preparations. Assays of crude extracts were carried out
at pH 10.0 in order to minimize the possible enzymatic destruc-
tion of DPNH. Purified enzyme preparations were usually
measured at pH 9.0, the optimum. The protein was determined

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by use of the Folin-Ciocalteu phenol reagent according to the procedure described by Lowry et al. (8)

RESULTS

Purification of Glycerol Dehydrogenase—Unless otherwise specified all the purification procedures were carried out at 0°C. Extracts of glycerol-grown cells from 4 liters of culture were used as the starting material for the enzyme purification. This extract, usually containing about 500 mg of protein, was gently swirled by magnetic stirring in an ice-cooled beaker, while 11.6 ml of 1% protamine sulfate (pH 7.0) were added dropwise from a pipette, and the resulting precipitate was then removed by centrifugation. The supernatant fluid contained almost all of the enzyme activity. To this fraction an equal volume of (NH₄)₂SO₄ saturated at 0°C, was added gradually with constant stirring. The precipitate was collected by centrifugation, redissolved in 20 ml of distilled water, and neutralized to pH 7.0. The solution was heated and kept at 60°C for 4 minutes, and was then immediately chilled in an ice bath. The coagulated material was removed by centrifugation. The supernatant fraction, usually containing about 40 mg of protein, was treated with 9 ml of calcium phosphate gel (16.6 mg dry weight per ml). The enzyme was adsorbed to the gel and was eluted by mixing the gel with 20 ml of 0.5 M KCl in 0.03 M potassium phosphate at pH 7.6. The results of a purification procedure are summarized in Table I. To study the effect of cations on the enzyme activity, the purified enzyme was dialyzed for 24 hours at 5°C against three changes of glass-distilled water with neutralized 0.001 M disodium ethylenediaminetetraacetate.

Effect of Monovalent Cations—This effect was accidentally discovered during the purification of the enzyme. It was noticed that after (NH₄)₂SO₄ fractionation an apparent 200 to 300% recovery of the enzyme activity was frequently obtained. Subsequently it was found that addition of (NH₄)₂SO₄ directly to the crude cell-free extract resulted in a pronounced increase of activity. Furthermore, dialysis of the purified enzyme resulted in almost complete loss of the activity unless NH₄⁺, K⁺, or Rb⁺ was added to the assay mixture. With the realization of this cation requirement all routine assays of glycerol dehydrogenase were carried out with (NH₄)₂SO₄, unless ionic effects were studied. Fig. 1 shows the activation of a purified enzyme preparation by NH₄Cl, (NH₄)₂SO₄, and KCl. The fact that two different NH₄⁺ salts gave almost the same activation at the same cation concentration and the fact that KCl at the same concentration was much less effective than NH₄Cl seems to rule out the action of anions.

A comparison of the effects of several monovalent cations on the glycerol dehydrogenase activity is given in Table II. The enzyme is activated by NH₄⁺, K⁺, and Rb⁺, but not by Li⁺. Na⁺ is presumably inactive since the preparation shown in Fig. 1 was almost completely inactive when measured in sodium carbonate buffer without the addition of other cations. The residual activity of the particular preparation in Table II is believed to be due to the presence of minute amounts of NH₄⁺ liberated gradually from the proteins, since this activity increased spontaneously during storage of the enzyme preparation. Indeed, this slow spontaneous activation of the enzyme made it difficult to study the ion effect, unless the studies were done quickly and immediately after the dialysis. Prolonged or repeated dialysis led to gradual and apparently irreversible loss of the enzyme activity.

Table III shows that the affinity of the enzyme for NH₄⁺ is greater than that for K⁺. Since ammonia is more effective at pH 8.5 (almost full ionization) than at pH 10 (about 20% ionization), and moreover, the enzyme can be activated by K⁺ and Rb⁺, it is likely that NH₄⁺ rather than NH₃ is the activating species for the dehydrogenase.

It was also observed that increasing the NH₄⁺ concentration
increased the apparent affinity of the enzyme for glycerol much more than the maximal velocity. It might be noted that the $K_m$ reported by Burton (4) ($3.9 \times 10^{-2}$ m) was higher than either of the values obtained in the present study. It would appear that the lower apparent affinity of the enzyme for the substrate in the former case was due to the lack of activating ions.

**pH Optimum**—The enzyme exhibited maximal glycerol dehydrogenating activity at pH 9 and was almost completely inactive at pH 11. Potassium instead of ammonium ions were used as the activator in these measurements since the concentration of hydrogen ions at pH 9 and was almost, completely inactive at pH 11. Potassium instead of ammonium ions were used as the activator in these measurements since the concentration of the latter is pH dependent.

**Specificity of Glycerol Dehydrogenase**—Table IV gives the initial rates of DPN reduction when the purified glycerol dehydrogenase was tested with various compounds at two different concentrations. The reactivity toward 1,2-propanediol and 1-chloro-2,3-propanediol confirms the observations made by Burton (4). It is noteworthy that at the lower substrate concentration, 1,2-propanediol is more rapidly dehydrogenated than glycerol (Table IV). The reverse reaction was studied by following DPNH oxidation. The enzyme reduced dihydroxyacetone but not D-glyceraldehyde.

**Inhibition of Glycerol Dehydrogenase**—In order to determine whether polyvalent cations also affect the enzyme activity, the assay was carried out in the presence of metal-complexing agents. The enzyme activity was found to be powerfully inhibited by 8-quinolinol and $\alpha,\alpha$-dipyridyl, but was relatively insensitive to diethyldithiocarbamate and ethylenediaminetetraacetate (Table V). On the other hand the activity of this enzyme was also markedly reduced by $\text{Na}^{+}$ ions, which caused a 50% inhibition at $2.1 \times 10^{-5}$ m. Direct evidence concerning the polyvalent cation requirement of glycerol dehydrogenase must await further purification and characterization of this protein.

**Discussion**

The glycerol dehydrogenase purified from *A. aerogenes* strain P14 resembles the enzymes previously isolated from *E. coli* (9) and *A. aerogenes* (ATCC 8724 (4)) in several of their properties. These include the relative stability to heat (4, 5), pH optimum in the alkaline region (4, 5), sensitivity to divalent cation inhibition (5), and relatively low substrate specificity (4).

We have in addition noted that the enzyme requires monovalent cations ($\text{NH}_4^+$, $\text{K}^+$, and $\text{Rb}^+$). This effect has not been reported by the previous workers, although Burton (4) has stated that “ammonium ions show a slight stimulatory effect from pH 6.8 to 8.8, but the reaction is inhibited at pH 8.9.”

The requirement of monovalent cations for activities of a number of different enzymes has been reported in the literature. In general it seems that the effects of $\text{NH}_4^+$, $\text{K}^+$, and $\text{Rb}^+$ are similar to each other and differ from those of $\text{Na}^+$ and $\text{Li}^+$. Although at present the properties of these two groups of ions responsible for their different biological activity are poorly understood, it is worthy of note that these two groups of ions differ from each other in radii of the hydration shell and relative mobility (9).

Among the enzymes reported to require the $\text{NH}_4^+$ group for maximal activity are several pyridine nucleotide-linked dehydrogenases: aldehyde dehydrogenase from bakers’ yeast (10, 11), inosine 5'-phosphate dehydrogenase (12) and inositol dehydrogenase from *A. aerogenes*. Several enzymes catalyzing reactions involving ATP were also reported to be activated by these ions. These include pyruvic kinase from rat (13) and rabbit muscle (9, 14), phosphotransacetylase from Clostridium kluyveri (15), acetate-activating enzyme from pig and rabbit heart (16), $\gamma$-glutamylcysteine synthetase (17) and glutathione synthetase from wheat germ (18), fructokinase from beef liver (19), and tyrosine-activating enzyme from pig pancreas (20). The hydrolytic enzymes, $\beta$ galactosidase from Saccharomyces fragilis (21), *E. coli* (22-25), and Bacillus megatherium (26), phenolsulfatase from *A. aerogenes* (27), and lipoprotein lipase from chicken adipose tissue (28) were also found to require these ions for maximal activity.

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1 A. P. Levin and B. Magasanik, unpublished results.
activity as was the pyridoxal phosphate-requiring tryptophanase from *E. coli* (29).

With β-galactosidase not only the activity but also the substrate specificity was dependent on the type of monovalent cation predominating in the reaction media (25). In the present studies we have noted an increase in the apparent affinity of the enzyme glycerol dehydrogenase for glycerol with an increase in NH₄⁺ concentration, which suggests that the monovalent cation is involved in the formation of the enzyme-substrate complex.

**SUMMARY**

An inducible diphosphopyridine nucleotide-linked glycerol dehydrogenase which converts glycerol to dihydroxyacetone was purified from a strain of *Aerobacter aerogenes*. The enzyme was fairly heat stable and its glycerol dehydrogenating activity was maximal at pH 9. The enzyme was found to require NH₄⁺, K⁺, or Rb⁺ for activity and was not activated by Na⁺ and Li⁺. The apparent affinity of the enzyme for glycerol was greatly increased by NH₄⁺. The enzyme activity was found to be strongly inhibited by Zn²⁺ and also by chelating agents such as α,α-dipyridyl and 8-quinolinol. In addition to glycerol, the enzyme was observed to attack 1,2-propanediol, 1-chloro-2,3-propanediol, and glycerol α-monomethyl ether at significant rates.

**REFERENCES**

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