Proc. Nat. Acad. Sci. USA
Vol. 69, No. 9, pp. 2373–2376, September 1972

Glycerol as an Enzyme-Stabilizing Agent: Effects on Aldehyde Dehydrogenase

(thiol groups/polyhydric alcohols/arsenite)

SHELBY L. BRADBURY* AND WILLIAM B. JAKOBY

Section on Enzymes and Cellular Biochemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

Communicated by Earl R. Stadtman, June 21, 1972

ABSTRACT **The potassium-dependent aldehyde dehydrogenase (EC 1.2.1.3), from yeast is markedly altered by the addition of high concentrations of glycerol or other polyhydric alcohols to aqueous buffers. Several lines of evidence suggest that the three-dimensional structure near the active site is involved: (*i*) The stability of the enzyme when stored at 2°, or when subjected to repeated freezing and thawing, depends upon the presence of at least 30% (v/v) glycerol. (*ii*) In the same solvent, the K_m value for DPN and the binding constant for benzaldehyde decrease by 3- and 10-fold, respectively, compared with the values obtained for these substrates in fully aqueous media. (*iii*) Competitive inhibition by trivalent arsenicals with respect to DPN is no longer observed in glycerol; the inhibition becomes mixed and the K_i values increase by 5- and 50-fold, respectively, with arsenite and Mapharsen. (*iv*) Essential sulfhydryl groups, which are easily carboxymethylated in aqueous buffers, are not readily available in either glycerol or mannitol.**

The data are consistent with a change in topography induced by polyhydric alcohols in which sulfhydryl groups near the DPN-binding site are displaced to a more protected environment, where their reactivity is reduced. Since the stabilizing effects of such alcohols are frequently encountered, these results may have application to other enzymes.

The ability of glycerol and other polyhydric alcohols to confer stability on highly organized biological materials is a widely recognized phenomenon. Experimental evidence on the nature of these effects, however, is sparse, and the postulates of mechanism remain in the realm of speculation (1–4). Since potassium-dependent aldehyde dehydrogenase (EC 1.2:1.3) from yeast requires a polyhydric alcohol to prevent inactivation (5) and, since information is available on the binding of two substrates and one product to its two active sites (6, 7), the enzyme appeared suitable for a more detailed examination of the stabilizing influence of glycerol. Furthermore, the effect of glycerol on the dissociation and association of the four apparently identical subunits of aldehyde dehydrogenase has been evaluated (8). Consequently, it is possible to identify small perturbations affecting the integrity of the enzyme without interference from gross alterations in subunit structure.

MATERIALS AND METHODS

Aldehyde dehydrogenase *A* was prepared from baker's yeast in the presence of diisopropylfluorophosphate (9). Enzymatic activity was determined by measurement of the rate of formation of DPNH at 340 nm in either 0.1 M Tris–chloride buffer at pH 8.0, containing 0.6 mM benzaldehyde, 1.4 mM DPN, 0.2 M KCl, and 5 mM 2-mercaptoethanol (6), or in a solution containing 0.05 M potassium phosphate at pH 7.7, 0.1 M KCl, 5 mM 2-mercaptoethanol, 0.6 mM benzaldehyde, and 1.4 mM DPN, in a total volume of 1 ml. The Tris and phosphate buffers are referred to as A and B, respectively.

Treatment with Iodoacetate. Details of the alkylation reaction accompany the table or legend for the individual experiment; the procedure differed slightly depending upon the concentration of iodoacetate used. At a concentration greater than 2 mM, an aliquot from each reaction vessel was removed for assay of enzymatic activity before addition of the alkylating agent; at low iodoacetate concentrations the procedure was reversed. In all cases, recrystallized iodoacetic acid, obtained from Dr. Carol Letendre, was neutralized with KOH (final pH 5–7) immediately before use.

Carboxymethylation with Radioactive Iodoacetate. [2-^{14}C]-Iodoacetic acid, 32 Ci/mol (Amersham–Searle), was diluted and neutralized before use without further purification. The alkylation reaction was terminated by making the samples 0.2 M in 2-mercaptoethanol. Carboxymethylprotein was separated from carboxymethylmercaptoethanol by precipitation in a total volume of 150–500 μl of 5% Cl_3CCOOH, and recovered on Whatman GF/C glass-fiber paper (2.4 cm). The filters were prepared by treatment for 2 hr with 1 M iodoacetate at pH 8.5–9.0, soaked for 20 min in 0.1 M carboxymethylmercaptoethanol in Cl_3CCOOH, washed with 6% Cl_3CCOOH, and dried overnight. The protein precipitate on the filter was washed in the following sequence: four times with 5 ml of 6% Cl_3CCOOH, once with 0.1 M carboxymethylmercaptoethanol in 6% Cl_3CCOOH, once with 6% Cl_3CCOOH, once with 4 ml of acetone, and twice with 5 ml of ether.

Identification of [^{14}C]*Carboxymethyl Residues.* Highly labeled carboxymethyl aldehyde dehydrogenase for amino-acid analysis was prepared by treatment of 60 nM of enzyme for 15 min at room temperature with 2 mM iodoacetate in 0.1 M diethanolamine chloride, (pH 8.9)–0.2 M KCl–15 mM EDTA–1 mM 2-mercaptoethanol. The reaction was terminated by adjusting the mercaptoethanol concentration to 5 mM. Unlabeled carboxymethylribonuclease (1 mg) was mixed with the dehydrogenase before precipitation of both

* Present address: Laboratory of Biochemistry, National Institute of Dental Research, National Institutes of Health, Bethesda, Md.

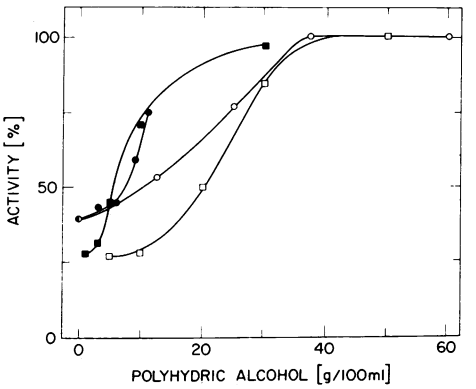

FIG. 1. Effect of polyhydric alcohols in stabilizing yeast aldehyde dehydrogenase. 130 nM dehydrogenase was incubated with polyhydric alcohols at 2° in standard buffer A made 50 mM in 2-mercaptoethanol. 10-μl aliquots were assayed for enzymatic activity in standard buffer A at zero time and at 45 hr. With glycerol (○), mannitol (●), sucrose (□), and ethylene glycol (■). The concentration of mannitol was limited by the relatively low solubility of the compound at low temperature.

enzymes with 10% Cl_3CCOOH. After the precipitate was washed twice with 10% Cl_3CCOOH and 20 μg of unlabeled carboxymethylglutathione was added, the mixture was hydrolyzed for 24 hr in 6 N HCl. The components of the hydrolyzate were separated on a Beckman model 120 C amino-acid analyzer with water substituted for the ninhydrin solution. The effluent was collected in a fraction collector and assayed for radioactivity (10); reaction with ninhydrin (11) was performed manually.

RESULTS

Stability studies

In the absence of a polyhydric alcohol, yeast aldehyde dehydrogenase undergoes irreversible denaturation, despite the presence of components otherwise conducive to stability (8, 12). Thus, incubation of the enzyme at 2° in the presence of 50 mM mercaptoethanol–0.1 M KCl–0.1 M Tris–chloride (pH 8.0) leads to a large loss in catalytic activity over 1 day. This loss can be entirely prevented by inclusion of one of several polyhydric alcohols in the storage solution. The addition of any one of several such alcohols at an appropriate concentration protects the enzyme from inactivation as shown in Fig. 1. Three of the compounds tested—glycerol, sucrose, and ethylene glycol—appear to be equally effective, each at a different, optimal concentration. The fourth compound, mannitol, is also active in stabilization, although it is limited in application by its relatively low solubility at low temperatures.

Equally dramatic stabilization was attained when the enzyme was subjected to repeated freezing in an acetone–dry ice bath, followed by rapid thawing. Only 9% of the initial enzyme activity was retained after five cycles of freezing and thawing during the course of 1 hr. However, in the presence of either 30% glycerol (v/v) or 50% sucrose (w/v), recovery of enzyme activity was 100%. 30% Glycerol was selected as the stabilizing agent for this enzyme because of its relative ease in handling.

TABLE 1. *Inhibition by trivalent arsenicals*

Time of incubation with inhibitor (min)	Glycerol % (v/v)	DPN K_m (μM)	Arsenite K_i (μM)	Mapharsen K_i (μM)
10	0	30	50	6
30	0	30	—	8
10	30	10	250	350*

2–4 nm Enzyme was incubated for the stated time at room temperature with 5 mM 2-mercaptoethanol–0.1 M Tris–chloride (pH 8.0)–0.2 M KCl. Enzyme activity was determined in the presence of 0.6 mM benzaldehyde and varied concentrations of inhibitor and DPN.

* K_i was calculated for inhibition of the mixed type, as described by Webb (14).

Dissociation and Michaelis constants for substrates

Under the usual conditions of assay, i.e., in the absence of glycerol and other polyhydric alcohols, the experimentally obtained Michaelis constant is, in fact, the dissociation constant for benzaldehyde; K_s for this substrate is 10 μM (6). In the presence of glycerol, the K_m obtained for benzaldehyde, 1 μM, is an order of magnitude lower than when K_s is measured under standard assay conditions. The K_m for DPN is not as sensitive to this change in medium; the value in glycerol, 10 μM, is one-third of that obtained in its absence. The K_m for DPN and benzaldehyde, as well as the K_i for an inhibitor, arsenite, are insensitive to pH in the range 6.5–9.0.

Comparison of inhibitor dissociation constants

The dissociation constant was determined for several inhibitors, including DPNH as a product of the catalytic reaction, and two trivalent arsenicals, sodium arsenite and Mapharsen.† These arsenicals inhibit aldehyde dehydrogenases by binding to two closely juxtaposed sulfhydryl groups at the active site (12, 13). DPNH acts as a competitive inhibitor with DPN for enzyme; the relevant K_i values are 50 μM in Tris–chloride at pH 8.0 (6) and 17 μM in the same buffer with glycerol as supplement.

A different pattern emerged when inhibition by trivalent arsenicals was studied. Arsenite is a competitive inhibitor of DPN, with a K_i of 50 μM, and is noncompetitive with benzaldehyde under conditions of limiting DPN. Similarly, Mapharsen, after 10 min of equilibration with enzyme, competes with DPN, binding more tightly than does arsenite (Table 1); a 10-min period of incubation is required for maximum inhibition by Mapharsen. Upon introduction of glycerol into the reaction mixture, inhibition by both Mapharsen (Table 1) and arsenite, with DPN as the variable substrate, is of the mixed type. Unlike other ligands that have been tested, the K_i for arsenite is 5-fold greater and, for Mapharsen, 50-fold greater in the presence of glycerol. A 10-min equilibration period with the enzyme was also sufficient to produce maximum inhibition by Mapharsen in glycerol; arsenite equilibrates immediately in both solvents.

The kinetics of inactivation by iodoacetate

A second approach involves modification of the sulfhydryl groups of the enzyme. Because inactivation of the dehy-

† Mapharsen, oxophenarsine hydrochloride, was a gift from Dr. J. R. Dice of Parke, Davis and Co., Ann Arbor, Mich.

drogenase by iodoacetate is strongly pH-dependent, i.e., a 20-fold difference in pseudo first-order rate constant is observed between pH 6.5 and 8.7, the effect of glycerol on the alkylation was studied at the more sensitive, higher pH.

Fig. 2 summarizes a series of experiments in which the effect of substrates and inhibitors on the kinetics of inactivation by iodoacetate was determined in both solvent systems. The first-order rate constants clearly fall into two classes, which depend entirely upon whether glycerol is present (Fig. 2*B*) or absent (Fig. 2*A*). Without glycerol, the enzyme is rapidly inactivated. Sodium arsenite protects the enzyme, DPNH protects to a lesser extent and, in the presence of both DPNH and benzaldehyde, the rate is identical with that in the presence of arsenite. Benzaldehyde alone increases the susceptibility of the enzyme to alkylation by iodoacetate. These data suggest that the inactivation reaction is sensitive to active site-related phenomena. In the presence of glycerol, inactivation by iodoacetate is slow. Moreover, the effects produced in glycerol by substrate, product, and inhibitor are qualitatively different. A mixture of DPNH and benzaldehyde protects the enzyme from inactivation by iodoacetate, much as it does in the absence of glycerol, and benzaldehyde increases the inactivation rate. However, arsenite and DPNH are ineffective in protecting against alkylation in the presence of glycerol.

Two assumptions are implicit: that alkylation of cysteine residues is responsible for loss in catalytic activity; and that the rate of reaction of iodoacetate is not *per se* affected by glycerol. Both assumptions were tested. Aminoacid analysis subsequent to alkylation of the dehydrogenase with highly labeled iodoacetate revealed that 82% of the radioactive residues cochromatographed with *S*-carboxymethylcysteine, and 12% with carboxymethylmercaptoethanol, the latter an artifact of the procedure resulting from carboxymethylation of 2-mercaptoethanol. The second control was designed to test the effect of glycerol on the reaction rate of iodoacetate with sulfhydryl groups in a simple model system. The compound chosen was 5-thio-2-nitrobenzoic acid, prepared by treatment of 5,5′-dithiobis (2-nitrobenzoate) with less than stoichiometric amounts of mercaptoethanol. The reaction rates with iodoacetate were the same regardless of whether or not glycerol was present.

Reaction of radioactive iodoacetate with dehydrogenase

Aldehyde dehydrogenase has twelve sulfhydryl groups, six to eight of which can be readily titrated with 5,5′-dithiobis (2-nitrobenzoate) (9). The multiplicity of these groups and the different environments that they occupy introduce ambiguities into the interpretation of alkylation experiments. These interpretative difficulties can be minimized by determination of the number of sulfhydryl groups that have reacted in a sample in which the loss of enzymatic activity can be measured. Toward this end, the enzyme was treated with radioactive iodoacetate.

The results indicate that the loss of enzymatic activity is, within the error of such experiments, a function of the number of alkylated sulfhydryl groups, both in the presence and the absence of glycerol (Table 2). Whereas these data, together with the specific activity of the iodoacetate, theoretically yield a measure of the number of carboxymethyl groups incorporated, no such estimate exists in actuality. In each experiment, the presence of a small amount of inactive enzyme

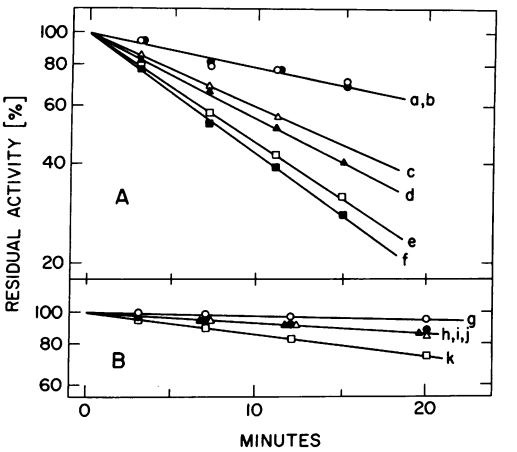

FIG. 2. (*A*) The kinetics of inactivation of 60 ± 8 nM dehydrogenase by 2 mM iodoacetate in solutions containing 0.1 M diethanolamine chloride (pH 8.9), 0.2 M KCl, 15 mM EDTA, 1 mM 2-mercaptoethanol, and the following additions: *a*, 1.0 mM sodium arsenite (○); *b*, 1 mM DPNH and 1.2 mM benzaldehyde (●); *c*, 1 mM DPNH (△); *d*, 10 mM glycoaldehyde (▲); *e*, none (□); and *f* 1.2 mM benzaldehyde (■). Aliquots were diluted 15-fold into standard buffer B for assay of residual enzyme activity. The data in each case have been corrected by as much as 30% for inactivation in the absence of iodoacetate. (*B*) The same experimental conditions as in part *A*, but with the addition of 30% glycerol (v/v). Additions are as follows: *g*, 1 mM DPNH and 1.2 mM benzaldehyde (○); *h*, none (●); *i*, 1 mM sodium arsenite (△); *j*, 1 mM DPNH (▲); and *k*, 1.2 mM benzaldehyde (□). Since the maximum correction for inactivation in this experiment was not greater than 3%, the data are uncorrected.

would complicate quantitative interpretation. Nevertheless, the data confirm the results of Fig. 2 by directly demonstrating that fewer sulfhydryl groups react with iodoacetate when glycerol is added to the reaction medium. Table 2 also shows that mannitol, like glycerol, protects the enzyme from carboxymethylation.

A separate experiment revealed that the amount of radioactivity incorporated into protein was directly proportional to enzyme concentration.

TABLE 2. [^{14}C]*Carboxymethylation of enzyme*

Additions		Reaction time	Incorporation	Residual activity
Ligands	Solvent	(min)	(cpm)	(%)
None	None	11	4640	17
None	None	22	4810	13
10 mM arsenite	None	11	1880	40
None	11% mannitol	11	1530	40
None	26% glycerol	11	1410	55

1.5 μM Enzyme was treated with 0.1 M iodoacetate (1.5 Ci/mol) in solutions containing 0.1 M potassium Bicine (pH 8.7), 0.1 M KCl, 15 mM EDTA, and the additions noted, in a volume of 100 μl. To stop the reaction, samples were made 0.2 M in 2-mercaptoethanol. Protein was precipitated in 6% Cl_3CCOOH, washed on Whatman GF/C glass-fiber filter paper (2.4 cm), and assayed for radioactivity (10). An appropriate control each for solvent was obtained by omission of enzyme and addition of 30 μg of bovine carbonic anhydrase after termination of the reaction with 2-mercaptoethanol. The data shown have been corrected by the value for the appropriate control, 150 ± 60 cpm.

DISCUSSION

We have presented indirect evidence from several approaches that is consistent with a glycerol-induced conformational change responsible, at least in part, for the stability of yeast aldehyde dehydrogenase. The finding that the kinetic parameters of the catalytic reaction and the effect of substrates and inhibitors on the kinetics of inactivation by an alkylating agent are both markedly altered by glycerol implicates groups in the vicinity of the active sites.

From a phenomenological point of view, these results emphasize the need for caution in interpreting measurements on proteins in the presence of high concentrations of polyhydric alcohols as stabilizing agents.

1. Jarabak, J., Seeds, A. E., Jr. & Talalay, P. (1966) *Biochemistry* **5**, 1269–1279.
2. Karow, A. M., Jr. (1969) *J. Pharm. Pharmacol.* **21**, 209–223.
3. Fridovich, I. (1963) *J. Biol. Chem.* **238**, 592–598.
4. Ruwart, M. J. & Suelter, C. H. (1971) *J. Biol. Chem.* **246**, 5990–5993.
5. Steinman, C. R. & Jakoby, W. B. (1967) *J. Biol. Chem.* **242**, 5019–5023.
6. Bradbury, S. L. & Jakoby, W. B. (1971) *J. Biol. Chem.* **246**, 1834–1840.
7. Bradbury, S. L. & Jakoby, W. B. (1971) *J. Biol. Chem.* **246**, 6929–6932.
8. Clark, J. F. & Jakoby, W. B. (1970) *J. Biol. Chem.* **245**, 6072–6077.
9. Clark, J. F. & Jakoby, W. B. (1970) *J. Biol. Chem.* **245**, 6065–6071.
10. Bray, G. A. (1960) *Anal. Biochem.* **1**, 279–285.
11. Moore, S. & Stein, W. H. (1954) *J. Biol. Chem.* **211**, 907–926.
12. Steinman, C. R. & Jakoby, W. B. (1968) *J. Biol. Chem.* **243**, 730–734.
13. Jakoby, W. B. (1963) in *The Enzymes*, eds. Boyer, P. D., Lardy, H. & Myrback, K. (Academic Press, New York), Vol. 7, pp. 203–221.
14. Webb, J. L. (1963) *Enzyme and Metabolic Inhibitors* (Academic Press, New York), Vol. 1, p. 162.