ION-DEPENDENT ACTIVATION AND INHIBITION OF RIBONUCLEOTIDE REDUCTASE FROM LACTOBACILLUS LEICHMANNII*

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Summary: The activity of ribonucleotide reductase from Lactobacillus leichmannii is affected markedly by ions. These effects can be either stimulatory or inhibitory, depending upon the nature of the dithiol substrate. When dithioerythritol is the reductant, the enzyme is activated by a variety of anions, particularly fluoride, phosphate and acetate. The largest effect (approximately 17-fold) is obtained in the presence of 1.0 M fluoride. In contrast, anions are generally inhibitory when dihydrolipoic acid serves as the reductant. These results are considered in terms of the chaotropic (structure-disrupting) and anti-chaotropic (structure-forming) effects of anions on the conformation of the enzyme, particularly at the dithiol binding site.

During an investigation of the mechanism of the adenosyl-B_{12}-dependent ribonucleotide reductase from Lactobacillus leichmannii, we have observed that the activity of this enzyme is markedly affected by the ionic environment. These ion effects can be either stimulatory or inhibitory, depending upon the specific dithiol [$R(SH)_2$] that serves as the reducing substrate in the overall reaction:

$$GTP + R(SH)_2 \xrightarrow[\text{Ad-}B_{12}]{\text{ribonucleotide reductase}} dGTP + R\begin{matrix}S\\|\\S\end{matrix} + H_2O$$

* Paper IX in the series "B_{12} Coenzymes." This work was supported by a grant (AM 5458) from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health. Abbreviations used: Ad-B_{12}, adenosyl-B_{12} (5'-deoxyadenosylcobalamin); DTE, dithioerythritol; $Lip(SH)_2$, dihydrolipoic acid.

The present studies were carried out with a ribonucleotide reductase that was purified from cell-free extracts of L. leichmannii. The purification initially followed the procedure of Vitols, et al. (1967), viz. protamine sulfate treatment, ammonium sulfate fractionation and filtration through Sephadex G-100. The final step involved chromatography on DEAE-cellulose using a modification of the procedure of Goulian and Beck (1966). The purified enzyme showed a single symmetrical peak ($s_{20,w}$ = 5.3) upon ultracentrifugation (0.05 M phosphate buffer, pH 7.5, containing 0.15 M KF and 0.001 EDTA). It also migrated as a single band during electrophoresis on cellulose acetate using the above phosphate-KF-EDTA buffer system. Enzyme activity was determined either by: (A) a slight modification of the diphenylamine assay (Blakley, 1966); or (B) a radioactive assay developed in this laboratory (Schrecker, et al., 1968).[1] The standard incubation mixture, in which Mg^{++} and allosteric effectors were deliberately omitted, contained: GTP, 1.0 mM (assay A) or GTP-8-^{14}C (1.0 mM, specific activity = 4 mC/mM) (assay B); DTE, 30 mM (or $Lip(SH)_2$, 24 mM); Ad-B_{12}, 10 μM; Tris-Cl, 10 mM, pH 7.5; and EDTA, 1.0 mM. Assay (A) contained 48 μg of protein in a total volume of 0.25 ml, while assay (B) contained 4.8 μg of protein in a total volume of 0.025 ml. In the above system (which was essentially salt-free except for the 10 μM Tris-Cl), and with $Lip(SH)_2$ as the reductant, the purified enzyme had a specific activity of about 240 mμmoles of GTP reduced per min per mg protein at 37°.

The sensitivity of the L. leichmannii ribonucleotide reductase to the ionic environment is evident when enzyme activity is compared in two commonly used buffers (Tris-Cl and potassium phosphate). Pertinent results, using

1. Assay B was used in the presence of NaI, since this compound interferes with the diphenylamine assay.

DTE and $Lip(SH)_2$ as reductants, are shown in Table I. In agreement with the earlier studies of Vitols, et al. (1967), activities in the DTE-dependent system were found to be considerably higher when Tris buffer was replaced by phosphate buffer at pH 7.5 (optimum for the enzyme). In contrast, the $Lip(SH)_2$-dependent activity was increased to a lesser degree by substituting phosphate for Tris, and higher concentrations of either buffer were inhibitory.

Table I

Effects of buffer concentration
on ribonucleotide reductase with DTE or $Lip(SH)_2$

Addition*	Specific Activity**	
	DTE	$Lip(SH)_2$
None	14	238
0.05 M Tris-Cl	36	221
0.10 M "	53	176
0.20 M "	70	147
0.40 M "	48	46
0.05 M Phosphate	85	262
0.10 M "	141	280
0.20 M "	161	217
0.40 M "	159	200

* Assay A (see text) which includes in the system 0.01 M Tris-Cl, pH 7.5. All added buffers were also at pH 7.5.

** mμmoles/min/mg.

It was of interest to determine whether other cations and anions could also elicit these effects. Li^+, Na^+, K^+, Rb^+ and Cs^+ (present as the chlorides)

were all stimulatory in the DTE-depending system, the maximum effect being about 4-fold at a salt concentration of 0.2 M. However, these same salts were inhibitory when DTE was replaced by $Lip(SH)_2$. Divalent and trivalent cations were not tested because of their generally unfavorable interaction with other components of the assay system.

In the above experiment, the similarity in results obtained with each salt suggested that the effects were due to the common anion, Cl^-, rather than to the cations. Accordingly, chloride was compared with other anions (each present as the Na^+ salt). Representative results, shown in Fig. 1, are expressed in terms of the relative rate of ribonucleotide reduction as a function of anion concentration. On this basis, the relative activities of the

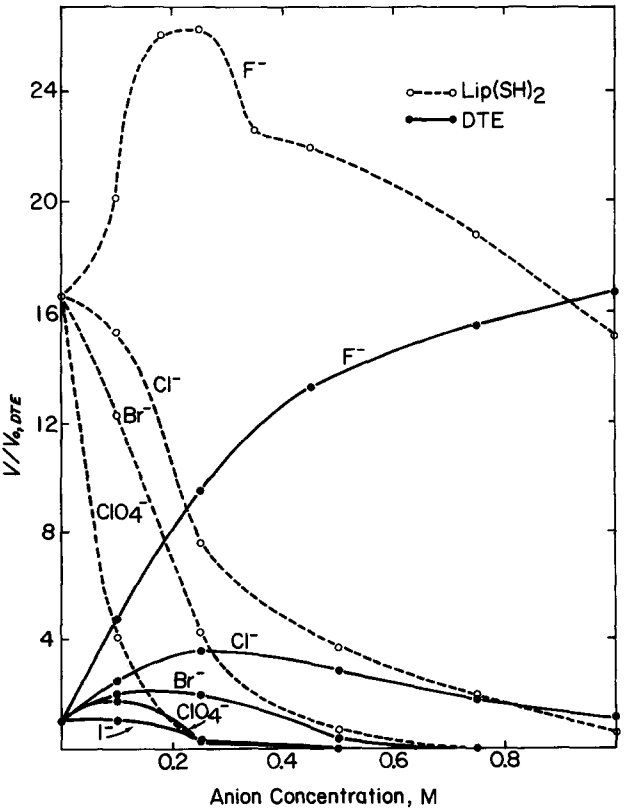

Fig. 1. Effect of anions on the rate of ribonucleotide reduction with $Lip(SH)_2$ and DTE as reductants. Assay conditions are described in the text with added anions at indicated concentrations. The rate with DTE as reductant and with no added anion ($V_{o, DTE}$) is taken as unity. All other rates (V) are expressed as the ratio ($V/V_{o, DTE}$).

$Lip(SH)_2$- and DTE-dependent systems were initially about 17:1 (i.e., in the absence of any added anions). When DTE was the reductant, the anions produced varying degrees of activation, the order of effectiveness being: $F^- \gg Cl^- > Br^- > ClO_4^- > I^-$. Higher concentrations of these anions caused some inhibition. When $Lip(SH)_2$ was the reductant, these anions were inhibitory with the exception of F^-, which was stimulatory at low concentrations.

Carboxylate ions were also found to be effective activators of the DTE-dependent system (Fig. 2). Na^+ and K^+ acetate were equally effective, while

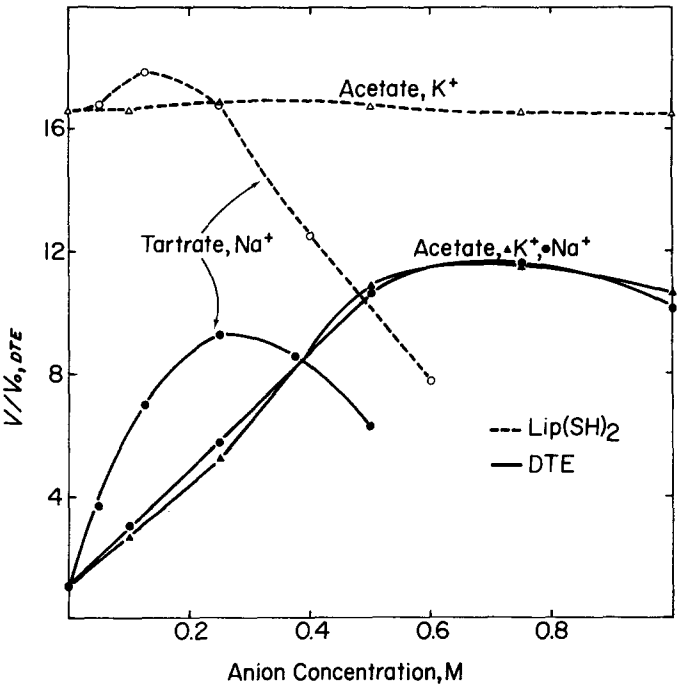

Fig. 2. Effect of carboxylate ions on activity of ribonucleotide reductase with $Lip(SH)_2$ and DTE as reductants. Assay conditions are described in the text with added carboxylate anions at indicated concentrations. Data are expressed as in Fig. 1.

a dicarboxylate ion tartrate achieved its maximum effect (about 80% of that observed with acetate) at about one-half the concentration required for full activation by acetate. Concentrations of tartrate higher than 0.2 M were inhibitory. Although not shown on the figure, formate and propionate activated

the DTE-dependent enzyme to a lesser extent (about 80% and 50%, respectively) of that observed with acetate. When tested with the $Lip(SH)_2$-dependent system, tartrate produced a slight activation, followed by inhibition. Acetate, on the other hand, was inert with this latter system.

Although $Lip(SH)_2$ or DTE are commonly used as the reductant for ribonucleotide reductase, the physiological reductant for this enzyme is probably a small molecular weight, dithiol-containing protein (Orr and Vitols, 1966), similar to thioredoxin from Escherichia coli (Laurent, et al., 1964). When tested in the presence of saturating levels of purified E. coli thioredoxin (and its TPNH-dependent reductase), the specific activity of the ribonucleotide reductase used in these experiments was 200 mμmoles/min/mg in the standard assay (unsupplemented by ions). This is about the same value as observed with $Lip(SH)_2$ (see Table I). NaF (0.15 M) and potassium acetate (0.20 M) each elevated the specific activity of the thioredoxin-dependent system to 300 (i.e., about the same effect as was observed with the $Lip(SH)_2$ system).

The above results may be considered in light of the chaotropic (structure-disrupting) effect of ions on proteins.[2] In terms of their effect on proteins, anions may be arranged in the following general sequence: SCN^- > ClO_4^- or I^- > NO_3^- or Br^- > Cl^- > $SO_4^=$, F^-, acetate or phosphate (Y. Hatefi, private communication). Anions at the beginning of the series are chaotropic; those at the end are anti-chaotropic (structure-forming). Although the interaction of anions with ribonucleotide reductase is undoubtedly a complex process, two principal modes of action are suggested by the

2. Hatefi and Hanstein (1969) have suggested that chaotropic agents actually disrupt the structure of water, and, as a consequence, hydrophobic groups on the protein are better able to penetrate into the solvent.

present data: (A) Effect on the overall conformation of the protein. The conformation of the enzyme (as isolated by the above procedure) appears to be suboptimal for catalysis since low concentrations of anti-chaotropic ions, such as F^-, acetate and phosphate activate the system, especially when DTE is the substrate. The inhibitory effects, noted at higher concentrations of these ions, are probably due to the enzyme assuming a structure that is too compact. (B) Effect on the dithiol binding site of the protein. This region may be somewhat hydrophobic as judged by the fact that $Lip(SH)_2$ is a much better substrate than the more hydrophilic DTE. Furthermore, the activity of the enzyme with $Lip(SH)_2$ is reduced by chaotropic agents which tend to weaken hydrophobic interactions. A further understanding of these processes must await more direct studies on the effect of chaotropic and anti-chaotropic anions on dithiol binding and on the conformation of the enzyme.

These results help to define optimal conditions for assaying ribonucleotide reductase from L. leichmannii and they may also be useful in enhancing the low levels of this activity that have been reported in mammalian cells. In addition, the striking effect of ions (relative to the reductant used) on the L. leichmannii ribonucleotide reductase may provide some clue to the complex mechanism of action of this enzyme.

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