ACTIVATION OF DIHYDROFOLATE REDUCTASE BY 5, 5'-DITHIOBIS-(2-NITROBENZOIC ACID)*

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Most of the known dihydrofolate reductases (Huennekens, 1967) can be activated about 3- to 5-fold by treatment with chaotropic agents, such as salts (Bertino, 1962), urea (Kaufman, 1963) or guanidine (Kaufman, 1963; Perkins and Bertino, 1965) or with thiol-binding agents, such as mercurials (Perkins and Bertino, 1964, 1965; Kaufman, 1964) or iodine (Kaufman, 1966). Activation by the first class of compounds is a spontaneously reversible process (i.e. the enzyme reverts to its original activity when the concentration of the chaotropic agent is lowered), whereas the latter type of activation is not spontaneously reversible. Otherwise, both processes are similar inasmuch as they do not involve any significant change in molecular weight of the enzyme or the creation of additional binding sites for the substrates. Activation probably results, therefore, from conformational changes in the protein (Kaufman, 1963, 1964, 1966; Perkins and Bertino, 1964, 1965) which facilitate transfer of the hydride ion from TPNH to dihydrofolate.

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During our recent studies (Reyes and Huennekens, submitted to Biochemistry) on the salt-dependent activation of a purified dihydrofolate reductase from L1210 cells, we have observed that 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), ordinarily used for the quantitation of thiol groups (Ellman, 1959), is an effective irreversible activator of the enzyme, but only when it is added concomitantly with a chaotropic agent (Table I).

TABLE I

Activation of
Dihydrofolate Reductase by DTNB in the Presence
of Salts, Urea or Guanidine Chloride

Expt.	Additions to Preincubation Mixture	Activity
		nmoles dihydrofolate reduced/min
1	None	2.1
	NaCl	2.3
	DTNB	2.6
	DTNB + NaCl	10.6
	DNTB + NaBr	11.6
	DTNB + NaSCN	11.4
	DTNB + Na Acetate	3.4
	DTNB + NaCl + TPNH	3.9
2	Guanidine	2.3
	DTNB + Guanidine	8.6
	DTNB + Guanidine + TPNH	3.9
	DTNB + Guanidine + Dihydrofolate	3.6
	Urea	1.3
	DTNB + Urea	11.4

The preincubation mixture contained the following components, where indicated, in a total volume of 0.35 ml: enzyme solution (sp. act. = 2.1 units/mg protein; 0.6 mg/ml), 50 µl; 7×10^{-5} M DTNB; 2.8×10^{-4} M TPNH or dihydrofolate; salt (NaCl, 0.88 M; NaBr, 0.7 M; NaSCN, 0.26 M; NaAc, 0.44 M); guanidine chloride (0.3 M) or urea (3.4 M); and 0.047 M Tris buffer, pH 7.6. After preincubation at 37° for 10 min, a 50 µl aliquot was removed and assayed for dihydrofolate reductase activity (9). The assay mixture contained 0.05 M Tris buffer, pH 7.6, and 0.1 µmole each of TPNH and dihydrofolate in a total volume of 1.0 ml.

In these experiments, activation was carried out in a preincubation mixture and a small aliquot containing the enzyme was transferred to the assay mixture; this procedure separates the effects of reversible and irreversible activators. As seen in expt. 1, exposure of the enzyme to 0.88 M NaCl in the preincubation mixture produced no appreciable activation. In a separate experiment (not shown in the Table), the enzyme activity increased to 11.6 nmoles of dihydrofolate reduced per min when the NaCl concentration was 0.88 M in the assay mixture. DTNB alone was also unable to activate the enzyme but, when added in the presence of 0.88 M NaCl in the preincubation mixture, it was an efficient and irreversible activator. Similar results were obtained when DTNB was allowed to react in the presence of 0.7 M NaBr or 0.26 M NaSCN; these salts, at the concentrations used, have been shown previously (Reyes and Huennekens, submitted to Biochemistry) to be about as effective as NaCl for reversible activation. Alternatively, 0.44 M sodium acetate, which is a weak activator, was less able to potentiate the effect of DTNB.

Of interest was the observation (also shown in expt. 1) that TPNH markedly inhibited irreversible activation by DTNB although, as shown above, it clearly does not interfere with the salt-dependent, reversible activation. The second experiment in Table I demonstrates that the same results can be achieved when guanidine (0.3 M) or urea (3.4 M) is used as the reversible activator in place of salts. The noticeably lower activity of the enzyme following reversible activation by urea is due to the time-dependent decline in activity always observed when this agent is used. Confirming the results of the previous experiment, either of the substrates (TPNH or dihydrofolate) was able to block DTNB-activation in the presence of the chaotropic agent. Activation of the L1210 enzyme by DTNB causes the double pH optimum to be replaced by a

single, broad maximum at about pH 7; a similar effect has been noted previously in the activation of dihydrofolate reductases by other agents (Bertino, 1962; Kaufman, 1963, 1964, 1966; Perkins and Bertino, 1964, 1965).

The effect of DTNB appears to be rather specific, since it cannot be replaced by other disulfides, such as oxidized glutathione or cystine. Likewise, the reduced form of DTNB, 2-nitro-5-mercaptobenzoic acid, was unable to activate the enzyme. The assumption that DTNB, like mercurials or iodine, is reacting with one or more thiol groups on the enzyme is strengthened by the observation that the DTNB-treated enzyme can be deactivated by exposure to 2-mercaptoethanol or dithiothreitol (Table II). Deactivation by thiols, like activation by DTNB, is prevented by the presence of the substrates

TABLE II

Deactivation of the DTNB-Treated Enzyme by Thiols

Expt.	Additions to Assay Mixture	Activity
		nmoles dihydrofolate reduced/min
1	None	12.8
	Mercaptoethanol, prior to substrates	4.0
	Mercaptoethanol, after TPNH	11.4
	Mercaptoethanol, after dihydrofolate	11.8
2	None	16.7
	Dithiothreitol, prior to substrates	3.0
	Dithiothreitol, after TPNH	15.3
	Dithiothreitol, after dihydrofolate	14.5

The preincubation mixture contained the following components, where indicated, in a total volume of 0.35 ml: enzyme solution (sp. act. = 2.1 units/mg protein; 0.6 mg/ml), 50 µl; 7×10^{-5} M DTNB; 0.047 M Tris buffer, pH 7.6; and 0.88 M KCl. After preincubation

at 37° for 30 min, the mixture was cooled rapidly in an ice-bath and a 50 µl aliquot was removed and assayed for dihydrofolate reductase activity as described in the legend for Table I. Where indicated, 0.05 M 2-mercaptoethanol or 0.005 M dithiothreitol was added to the assay mixture 2 min prior to, or after, the addition of TPNH or dihydrofolate.

Reaction with DTNB may thus prove to be useful in providing an easily removed chromophoric marker on the enzyme.

The above results suggest that the enzyme can exist in different conformational states, as shown in Fig. 1. Salts, urea or guanidine disrupt the

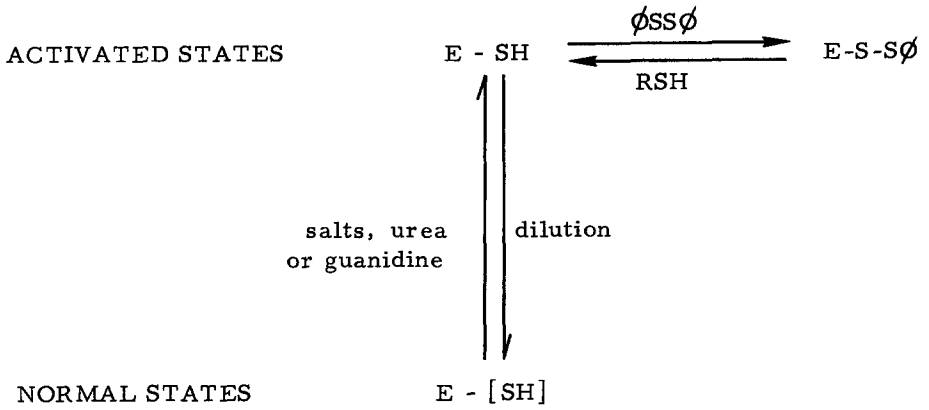

Fig. 1

Activation of L1210 Dihydrofolate Reductase

(ØSSØ and RSH represent DTNB and a thiol, respectively)

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tertiary structure of the enzyme and allow it to assume a more unfolded conformation. This effect is reversible, however, and when the concentration of the activator is decreased by dilution, the enzyme reverts to its normal state (E - [SH] in the Figure). In the activated conformation, one or more thiol groups becomes accessible to DTNB. Reaction of this group with DTNB then stabilizes the enzyme in an activated conformation that does not revert to the normal state when the chaotropic agent is removed. The assumption that the activated conformation involves a more unfolded structure is supported

by the observations that dihydrofolate reductases, after activation, become labile to heat and susceptible to proteolytic digestion (Mell et al., 1966).

Irreversible activation appears to depend upon two related factors: (a) the ability of salts, urea or guanidine to shift the equilibrium from the normal to a transient activated state; and (b) the reactivity of the activating agent toward an exposed thiol group. Preliminary experiments with the L1210 enzyme have shown that chloromercuriphenylsulfonate can activate in the absence of salts, although the effect is accelerated considerably when the latter are present. The inability of oxidized glutathione or cystine to replace DTNB probably reflects the relatively slow rate of reaction of these disulfides with the thiol group on the enzyme. The latter must be situated fairly close to the substrate binding sites since, even in the unfolded state, either substrate can shield the group from interaction with DTNB or from deactivation by thiols. Alternatively, though, it is possible that the binding of either substrate induces a conformational change that once again buries the thiol group.

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REFERENCES

Bertino, J. R., Biochim. Biophys. Acta, 58, 377 (1962).

Ellman, G. L., Arch. Biochem. Biophys., 82, 70 (1959).

Huennekens, F. M., in Biological Oxidations, ed. by Singer, T. P., John Wiley & Sons, Inc., New York, 1967 (in press).

Kaufman, B. T., Biochem. Biophys. Res. Comm., 10, 449 (1963).

Kaufman, B. T., J. Biol. Chem., 239, PC 669 (1964).

Kaufman, B. T., Proc. Nat. Acad. Sci. (U.S.), 56, 695 (1966).

Mathews, C. K., and Huennekens, F. M., J. Biol. Chem., 238, 3436 (1963).

Mell, G. P., Schroeder, G. M., and Huennekens, F. M., Federation Proc., 25, 277 (1966).

Perkins, J., and Bertino, J. R., Biochem. Biophys. Res. Comm., 15, 121 (1964).

Perkins, J. P., and Bertino, J. R., Biochem. 4, 847 (1965).

Reyes, P., and Huennekens, F. M., submitted to Biochemistry.