KINETIC PROPERTIES OF THE TPNH-SPECIFIC SULFITE AND HYDROXYLAMINE REDUCTASE OF SALMONELLA TYPHIMURIUM*

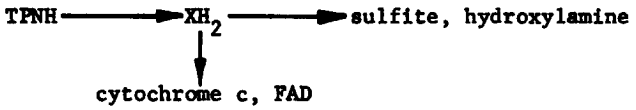
Lewis M. Siegel** and Kenneth J. Monty

Department of Biochemistry, University of Tennessee
Knoxville, Tennessee
and
McCollum-Pratt Institute, Johns Hopkins University, Baltimore, Maryland

Received July 14, 1964

In a preceding communication (Siegel, et al., 1964), evidence obtained from a study of mutants of Salmonella typhimurium was interpreted to indicate the existence of an intermediate electron carrier in the triphosphopyridine nucleotide (TPNH) specific sulfite reductase from that organism. This carrier was envisioned as being reduced by TPNH, and was in turn able to pass electrons either directly to flavine adenine dinucleotide (FAD) or cytochrome c, or indirectly to inorganic sulfite or hydroxylamine. The postulate is summarized by the flow diagram of Scheme I. In this communication we shall provide kinetic evidence which supports that postulate.

Scheme I



The enzyme preparations employed in these endeavors were obtained as follows: Cultures of wild-type cells (LT-2) and of certain mutants were harvested during the logarithmic phase of growth on a minimal

*Supported by Contract AT(40-1)-3082 from The U.S. Atomic Energy Commission and by Grant GM-17,008 from the National Institutes of Health, U.S. Public Health Service.

**Predoctoral Fellow of the National Institutes of Health.

medium which contained L-djenkolic acid as the sole source of sulfur (see Dreyfuss and Monty, 1963). The cells were washed once with sulfur-free medium, resuspended in 0.05 M sodium phosphate buffer, pH 7.7, and subjected to sonic disruption with a Branson Model S-75 Sonifier. The suspension thus obtained was subjected to a 30 minute centrifugation at 30,000 x g. The supernatant from this treatment served as the source of enzyme activity. Enzyme assays were performed essentially as described earlier (Siegel, et al., 1964).

The electron-flow diagram of Scheme I predicts that cytochrome c will compete with both hydroxylamine and sulfite for the electrons of the intermediate carrier "XH_2". This prediction is borne out by the following observations with enzyme prepared from wild-type cells. When the rate of cytochrome c reduction by TPNH is observed at 550 mμ, the addition of hydroxylamine results in a competitive inhibition. When these data were analyzed by the method of Lineweaver and Burk (1934), the following physical constants were obtained: K_M for cytochrome c, 7.7×10^{-6} M; K_I for hydroxylamine, 0.8×10^{-3} M. The same data, when examined through a plot of the reciprocal of the velocity of cytochrome c reduction versus the concentration of hydroxylamine (Figure 1), reveal

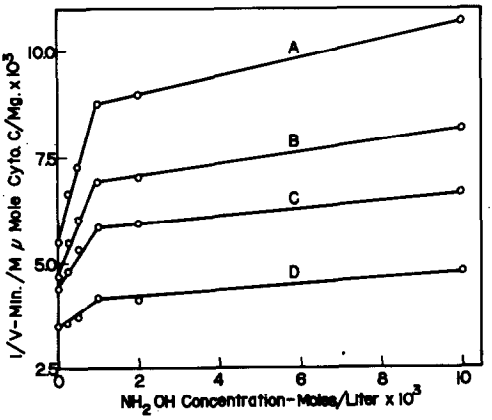


Figure 1. Inhibition of Cytochrome c Reduction by Hydroxylamine. Reaction mixtures contained 30 μ moles pyrophosphate, pH 8.0; 0.2 μ mole TPNH; 20 μ gm. wild-type extract; and 0.009 (A), 0.0135 (B), 0.018 (C), or 0.045 (D) μ moles oxidized cytochrome c, in a total volume of 1.0 ml.

---

that the maximum inhibition obtainable with hydroxylamine is always less than 100%, and is inversely related to the concentration of cytochrome c. This observation was to be expected in view of the earlier demonstration that the ratio of the maximal rates of reduction of cytochrome c and hydroxylamine is approximately 1.6 to 1. The K_I for hydroxylamine cal-

culated from the data of Figure 1 is 1.5 x 10^{-3}M, agreeing favorably with the K_I calculated from the Lineweaver-Burk transformations and with a K_M of 1.2 x 10^{-3}M calculated for hydroxylamine when the latter is employed as a sole terminal acceptor of electrons from TPNH.

As noted earlier (Siegel, et al., 1964), cytochrome c is reduced 11 times faster than is sulfite by the wild-type Salmonella enzyme preparations. In agreement with these data is the observation that sulfite produced only a negligible inhibition of cytochrome c reduction (Table I), even when the former is present at a concentration 250 times higher than the K_M concentration for sulfite (the K_M for sulfite serving as sole terminal electron acceptor is 2.0 x 10^{-5}M).

Two types of kinetic evidence suggest that sulfite and hydroxylamine are reduced at a common enzyme binding site. First, in studies of the hydroxylamine-dependent oxidation of TPNH, the addition of appropriate concentrations of sulfite yields data which conform to the classical picture of competitive inhibition. In spite of the fact that sulfite is

Table I

Effect of Arsenite, Hydroxylamine, and Sulfite on Cytochrome c Reduction

Additions	% Inhibition
None	—
10 mM NH_2OH	45
10 mM NH_2OH + 1 mM arsenite	8
1 mM arsenite	0
5 mM bisulfite	5

The basic incubation mixture contained in a 1.0 ml total volume: 30 μ moles pyrophosphate buffer, pH 8.0; 0.2 μ moles TPNH; 20 μg wild-type extract; and 0.023 μ moles oxidized cytochrome c. Absorbancy change followed at 550 mμ.

actually an alternative substrate to hydroxylamine, the former is reduced slowly enough in comparison to the latter (V_{max} for NH_2OH/V_{max} for Sulfite = 7) so that under the conditions employed in our experiments the total rate of TPNH oxidation is not significantly different from the rate due to the reduction of hydroxylamine. A Lineweaver-Burk plot demonstrating the competitive inhibition by sulfite of the TPNH-hydroxyl-

amine reductase activity is presented in Figure 2.

A second observation supporting the idea of a common binding site for sulfite and hydroxylamine is the fact that sodium arsenite is a competitive inhibitor of the reduction of either of the two acceptors. The K_I for arsenite in the TPNH-oxidation assay with either of the above acceptors is approximately 6-10 x 10^{-5}M. No detrimental effect of arsenite on the reduction of cytochrome c has been noted.

Extending these latter observations in evaluating the validity of the flow diagram of Scheme I, it is significant that arsenite alleviates the inhibition by hydroxylamine of the TPNH-cytochrome c reduction (Table I), apparently by virtue of the ability of arsenite to prevent

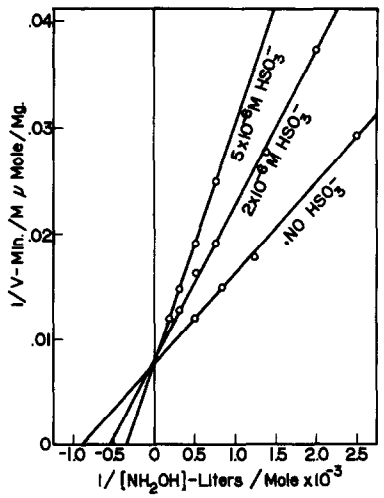


Figure 2. Inhibition of Hydroxylamine-Dependent TPNH Oxidation by Sulfite. Reaction mixtures contained 30 μ moles pyrophosphate, pH 8.0; 0.2 μ moles TPNH; 0.2 mg. wild-type extract; and hydroxylamine hydrochloride, in a total volume of 1.0 ml.

the binding of hydroxylamine to the enzymic site of reduction. Furthermore, extracts of the mutants cys G-439 and cys I-68, which have lost the ability to reduce sulfite and hydroxylamine, are insensitive to any negative influence by hydroxylamine on the reduction of cytochrome c.

From the data summarized above together with that presented earlier we conclude that the sulfite-reducing system of S. typhimurium contains at least two distinct enzymic sites. At one of these, cytochrome c, and probably FAD, can function as an electron acceptor. The reduction of sulfite occurs at an entirely separate site, as demonstrated by the selective effects of arsenite and by the differences in genetic depend-

ence (Siegel, et al., 1964). Three kinds of data pertain to the relationship between sulfite and hydroxylamine: (1) they are competitive as terminal electron acceptors; (2) in this role, they are mutually susceptible to arsenite as a competitive inhibitor; and (3) the ability of both to serve as terminal acceptors is apparently dependent upon the same group of cistrons. These observations are necessary, but not sufficient, criteria for the conclusion that sulfite and hydroxylamine are reduced at a common enzymic site.

Kemp, et al. (1963) noted the ability of the sulfite-reducing system from Escherichia coli to employ a number of compounds and ions as terminal electron acceptors, and presented a plausible argument that the reduction of all of these by TPNH occurred at a common site on a single enzyme. A major point in their argument was the copurification of the several activities to a specific activity about 100 fold above that of a crude bacterial extract. We have recently achieved an 35-fold purification of the TPNH-sulfite reductase from the 30,000 x g supernatant of wild-type S. typhimurium. These preparations retain the same relative abilities to employ sulfite, hydroxylamine and cytochrome c as terminal acceptors as found in the crude preparations. In spite of the tendency of the sulfite-reducing system to behave as a single entity during conventional purification procedures, the genetic and kinetic data present an overwhelming argument that the TPNH-sulfite reductase of S. typhimurium is a multi-site, multi-component system. It is probable that the system consists of an organized aggregate of component proteins which thus far have resisted separation.

REFERENCES

Dreyfuss, J., and K. J. Monty (1963) J. Biol. Chem. 238: 1019.
Kemp, J. D., D. E. Atkinson, A. Ehret and R. A. Lazzarini (1963) J. Biol. Chem. 238: 3466.
Lineweaver, H., and D. Burk (1934) J. Am. Chem. Soc. 56: 658.
Siegel, L. M., E. M. Click, and K. J. Monty (1964) Biochem. Biophys. Res. Commun. 17:125.