SODIUM DITHIONITE REDUCTION OF FLAVIN

J.L. FOX
Department of Zoology, University of Texas at Austin, 78712, USA

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1. Introduction

The question of two- or one-electron reduction by sodium dithionite is still a hotly debated topic [1]. Sodium dithionite reduces many flavoenzymes to the free radical level, a one electron reduction [2]. On the other hand, reports of two-electron reduction by sodium dithionite have appeared recently [3–5] and are now generally accepted. The question of mechanism remains unclear, however, and several alternative explanations exist. Kolker and Waters [6] reasoned that dithionite ion disproportionated to two SO_2^- radical ions before it reduced *para*-nitrobenzene in their study. A recent study on cytochrome *c* reduction by Creutz and Sutin [7] surprisingly argues for reduction by the dithionite ion itself in a one-electron step.

Some years ago Burn and O'Brien [8] examined the aerobic reduction of riboflavin by sodium dithionite. These experiments were performed under conditions of great excess of sodium dithionite. In the range of neutral pH, at concentrations above 10^{-5} M the reaction rate was largely independent of flavin concentration and demonstrated complex behavior. In conclusion, the authors proposed the reaction:

$$2R \xrightarrow{2H} 2RH \xrightarrow{2H} 2RH_2$$
$$\downarrow\uparrow$$
$$(RH)_2$$

This reaction was recently reinvestigated using deoxygenated buffers [9] in the special deoxygenation chambers which we developed [10] for use in the Durrum–Gibson stopped-flow apparatus. The anaerobic reaction was approximately a thousand times faster than the reaction rate reported by Burn and O'Brien, so a more extensive analysis was performed.

2. Procedures

Potassium phosphate buffer, 0.1 M, pH 6.80 or pyrophosphate buffer, 0.01 M, pH 9.0 was prepared from high resistivity water obtained from the Continental Water Co. ion exchanger (greater than 10 M ohms from the tap). The buffer was deoxygenated by bubbling Matheson Research Grade argon (99.9998% pure) through it for 20 min. Identical results were obtained when sodium dithionite (British Drug House, purity assayed by lumiflavin anaerobic reduction) was dissolved in either 0.1 M phosphate buffer pH 6.8 or 0.01 M pyrophosphate buffer pH 9.0 and flavin mononucleotide (Sigma) was dissolved in 0.1 M phosphate buffer pH 6.8 under argon atmospheres. Reactions were monitored in a Durrum–Gibson stopped-flow instrument at 445 nm in a 1 mm pathlength cell and at 570 and 800 nm in a 10 mm pathlength cell. These reactions were conducted with concentrations in the general range of 10^{-3} M. The great dependence of reaction rate on concentration made it impossible to examine the reaction with this equipment over a range greater than one order of magnitude.

3. Results

Oxidized flavin reduction can be conveniently followed at 445 nm, flavin-free radical production at 570 nm and formation of the charge transfer complex between reduced and oxidized flavins at 800 nm [10, 11]. None of these reactions directly followed 1st, 2nd, or 1½ order reaction plots. At our lowest dithionite concentration early reaction times form a straight line to yield a pseudo first order rate constant of 0.17 sec^{-1}, but the reaction went to higher

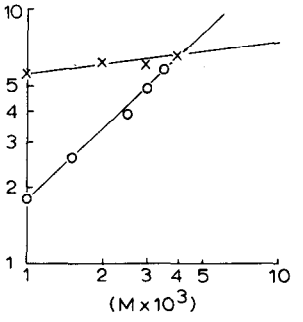

Fig. 1. Van 't Hoff log–log plots of initial reaction rates versus concentration. In both cases the excess reactant was held at 4×10^{-3} M and the other reactant was varied at lower concentrations. (o) are varied sodium dithionite concentrations yielding a slope of 0.94; (X) are varied flavin mononucleotide concentrations yielding a slope of 0.02.

Table 1
Initial reaction rate concentration order dependencies from Van 't Hoff plots.

	445 nm	570 nm	800 nm
FMN	0.02	0.81	0.88
$S_2O_4^{-2}$	0.94	0.54	1.09

order after that initial region. Reaction lags at 445, 570 or 800 nm are a control for the presence of oxygen and reaction data from experiments with lags were discarded.

Fig. 1 shows that the initial reaction rate of flavin with dithionite as followed by reduction of the 445 nm absorbance was linear with the dithionite concentration. Van 't Hoff plots at 445 nm for the flavin (table 1) conformed the zeroth order dependence of Burn and O'Brien [8] noted under aerobic conditions. It also yielded a first order dependence for dithionite. This excludes a direct reaction between dithionite and flavin and indicates that some other reducing species must be present.

Van 't Hoff plots of the initial rate kinetics at 570 and 800 nm produced non-integral values. This is in accord with the kinetic values known for the rapid disproportionation reactions which occur when flavin reduced species are mixed anaerobically [13]. Flavin-free radicals quickly convert to reduced flavin–oxidized flavin molecular complexes (characterized in part by charge transfer bonds) and finally fully oxidized and fully reduced flavins. The distribution of flavin species under these conditions are about 95% molecular complex (800 nm) 4% flavin free radical (570 nm) and 1% or so of fully oxidized and reduced flavins [12].

4. Discussion

The initial rate Van 't Hoff plots at 445 nm exclude dithionite ion from consideration as the reducing species. At least half a dozen decomposition products of dithionite are known to exist in equilibrium with dithionite in aqueous solution [14, 15]. The mechanism of oxygen in altering this distribution is also partially known [15] and is the rationale for reinvestigating the reaction under anaerobic conditions. The most likely candidates for the reducing species are sulfite and sulfur dioxide radical ion. The kinetics of sulfite reduction are published [16] and are too slow to be consistent with these findings by several order of magnitude.

The sulfur dioxide ion radical, SO_2^-, is well known in aqueous dithionite solutions. Its formation has recently been studied by electron spin resonance by Jensen [17]. The equilibrium $S_2O_4^= \rightleftharpoons 2SO_2^-$ easily can provide two electron equivalents per dithionite ion, but which may function by individual one electron reduction steps.

This conveniently explains the data. The disproportionation of dithionite ion to sulfur dioxide radical ion thus represents the rate limiting step. The flavin reduction is then proposed to proceed to the free radical state. At our pH 6.8, the flavin-free radical disproportionation is favored [11, 12] to yield very small concentrations of flavin-free radical:

$$S_2O_4^= \rightleftharpoons 2SO_2^- \text{ rate limiting step,} \quad (1)$$

$$H^+ + SO_2^- + FMN \rightleftharpoons SO_2 + FMNH\cdot\,, \quad (2)$$

$$2FMNH\cdot \rightleftharpoons (FMNH_2 \rightarrow FMN)_{ctc}\,, \quad (3)$$

$$(FMNH_2 \rightarrow FMN)_{ctc} \rightleftharpoons FMNH_2 + FMN\,. \quad (4)$$

This provides a cyclic path for flavin reduction. This mechanism is in accord with the interpretation of the data obtained from a study of the reduction of *para*-nitrobenzene derivatives by dithionite [6]. Those authors concluded that a sulfur dioxide radical ion must also be the active reducing agent.

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Note added in proof

S.G. Mayhew and V. Massey (Biochim. Biophys Acta 315, 181 (1973)) in studies with flavodoxin, and D.O. Lambeth and G. Palmer (J. Biol. Chem. 248, 6095 (1973)) in studies with several redox proteins conclude that a reduction mechanism analogous to that proposed here is operational through the sulfur dioxide radical anion intermediate ($SO_2^-\cdot$).

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