Ethylene Glycol as a Very Slow Substrate for Xanthine Oxidase

STEPHEN J. TANNER and ROBERT C. BRAY

School of Molecular Sciences, University of Sussex, Falmer, Brighton BN1 9QJ, U.K.

The very low substrate specificity of xanthine oxidase has in the past caused much interest. The enzyme catalyses the oxidation of many aldehydes as well as that of various purines and other heterocyclic compounds (see Bray, 1975). More recently effects of certain alcohols on the enzyme (not obviously, however, involving turnover of the enzyme) have been studied. Under specific conditions methanol inhibits the enzyme (Rajagopalan & Handler, 1964) and also gives rise to a specific molybdenum(V) e.p.r. signal known as Inhibited (Pick et al., 1971). E.p.r. experiments, with methanol substituted in the methyl group with $^2$H, indicated that this group was not present, intact, in the signal-giving species and suggested that the group had probably been converted to an aldehyde group. [The $^{13}$C experiments of Tanner et al. (1978) confirm that the carbon atom is present.] We have also shown (Tanner & Bray, 1978; S. J. Tanner & R. C. Bray, unpublished work) that ethylene glycol behaves analogously to methanol, both in causing inhibition of the enzyme, and in giving rise to a specific e.p.r. signal. It is also pertinent that the inactive desulpho enzyme gives rise to a further molybdenum e.p.r. signal ['Resting(II)'] when it is treated with ethylene glycol (Lowe et al., 1976).

All these observations indicate that alcohols may make specific associations with the molybdenum centre of xanthine oxidase. This raises the possibility that they might be capable of serving as very slow substrates for the enzyme. The present work describes experiments in which $[^{14}$C]ethylene glycol was tested as a substrate.

$[^{14}$C]Ethylene glycol (from The Radiochemical Centre, Amersham, Bucks., U.K.) was diluted to a specific radioactivity of approx. 0.06 Ci/mol. Xanthine oxidase was prepared by the method of Hart et al. (1970). Desulpho enzyme was prepared from it by the method of Massey & Edmondson (1970). Reaction of the radioactive substrate with the enzyme was followed at an enzyme concentration of about 0.7 mm (total active centres) in a medium containing 50 mM-sodium pyrophosphate buffer, pH 8.2, in equilibrium with air, at 30°C, for a reaction period of 2 days. Examination of the products of the reaction was carried out by t.l.c., after denaturation of the enzyme with an excess of methanol. Radioactive spots were located on the plates by means of radioautographs. For quantitative evaluation, spots were cut out and radioactivity was determined by liquid-scintillation counting.

Fig. 1 shows typical experiments. E corresponds to the starting position. Spot A is the very large spot due to unreacted $[^{14}$C]ethylene glycol. The plates were distinctly overloaded with respect to glycol, and spot B is probably an artifact due to this, though it could be an impurity in the glycol. (According to the makers, the radiochemical purity was 94%.) Spot C ($R_f = 0.42$) was identified as glycollic acid, by comparison with the standard radioactive compound, run under identical conditions in the presence of the enzyme. Spot D was provisionally identified as oxalic acid, though the possibility that it might be due to glyoxylic acid was not rigorously excluded.

The experiments and controls illustrated in Fig. 1 (all of which were repeated many times) establish conclusively that xanthine oxidase catalyses the conversion of ethylene glycol into glycollic acid. The preliminary value for the catalytic centre activity under the conditions of the experiment is 0.23 mol of glycollic acid/mol of Mo per hour. This is some $10^5$-$10^6$ times slower than the corresponding catalytic rate for xanthine turnover by the enzyme (Bray, 1975).

Unexpectedly, desulpho enzyme also yielded glycollic acid, the turnover rate being apparently comparable with that for the functional enzyme. However, whereas the functional enzyme also appeared to produce oxalic acid (at a rate of about 0.08 mol/mol of Mo per hour) this product could not be detected when the desulpho enzyme was used.
Fig. 1. Radioautographs of thin layer plates showing conversion of [14C]ethylene glycol into glycollic acid by xanthine oxidase

Experiments (a), (b) and (c) are a control without enzyme after 2 days, native xanthine oxidase at zero time and native xanthine oxidase after 2 days respectively. The spots E, D, C, B and A correspond to the origin, oxalic acid, glycollic acid, an artifact of running and glycol respectively. Chromatography was carried out on cellulose plates in an ethanol/ammonia/water (20:1:4, by vol.) system, and the spots detected by radioautography. In the Figure, the density of the area within the spot corresponds roughly to the density of the spot on the original radioautograph.

The results thus show that xanthine oxidase has an even lower substrate specificity than was previously observed. The finding that the 'inactive' desulpho form of the enzyme has some catalytic potency, if a very low one, is consistent with the structural differences between the desulpho and functional forms being minimal (see Gutteridge et al., 1978).

Studies establishing that methanol is a substrate for the enzyme have yet to be completed, but preliminary experiments with the 14C-labelled compound indicate that its turnover must be even slower than that of ethylene glycol. Nevertheless, it does seem likely from the available e.p.r. data that it must be a substrate. Thus, we propose that the Inhibited signal (Pick et al., 1971) corresponds to a species obtained in the turnover of methanol by the enzyme, and appearing either on the direct reaction pathway or possibly on an inhibitory side path. Similarly the corresponding analogous species in the turnover of ethylene glycol gives the new signal recently observed by Tanner & Bray (1978). Then the analogue of this signal, observed in the turnover of ethylene glycol by the desulpho enzyme, is the Resting(II) signal of Lowe et al. (1976) (see also Tanner & Bray, 1978).
A New Molybdenum Electron-Paramagnetic-Resonance Signal from Treatment of Functional Xanthine Oxidase with Ethylene Glycol

STEPHEN J. TANNER and ROBERT C. BRAY
School of Molecular Sciences, University of Sussex, Falmer, Brighton BN1 9QJ, U.K.

It has been established that ethylene glycol is a slow substrate for functional as well as for desulpho xanthine oxidase, whereas methanol may be a substrate, though an even slower one, at least for the functional form of the enzyme (Tanner & Bray, 1978). Treatment of the functional enzyme with methanol gives rise to the molybdenum e.p.r. signal known as 'Inhibited' (Pick et al., 1971; Edmondson et al., 1972). It is believed (Tanner & Bray, 1978) that the signal-giving species represents an intermediate, or possibly an inhibitory side product, arising during enzymic turnover of methanol. This being so, we might have expected that an analogous signal would be obtainable during turnover by the functional enzyme of ethylene glycol. We now present evidence that such a signal is indeed detectable.

The new molybdenum(V) e.p.r. signal was generated by reductive titration of xanthine oxidase, with dithionite as reducing agent, in the presence of mediator dyes, with the addition of ethylene glycol. Techniques were generally those described by Cammack et al. (1976). The enzyme was reduced to a desired potential and held there for a suitable period. Samples were then withdrawn from the titration cell, under anaerobic conditions, and frozen for subsequent e.p.r. spectroscopy. The Inhibited signal was obtained with formaldehyde (which gives the same signal as methanol) or with its deuterium analogue (Pick et al., 1971). Fig. 1(a) shows the new signal. It has a striking resemblance in its g-values to the Inhibited signal (Fig. 1b). However, the new signal lacks proton hyperfine structure. Thus it is particularly similar to the Inhibited signal obtained with deuteromethanol or deuteroformaldehyde (Fig. 1c; see also Pick et al., 1971; Bray, 1971). Computer simulations of the signals are given in Figs. 1(a'), 1(b') and 1(c') and parameters are listed in Table 1. Though in due course the nomenclature of these signals may need some revision, we propose to refer to the new one as the 'glycol Inhibited' signal.

The discovery of the glycol Inhibited signal permits a number of new insights into the reactions of the molybdenum centre of xanthine oxidase. Since the Inhibited signal shows coupling of molybdenum to a single (non-exchangeable) proton derived from methanol, Pick et al. (1971) concluded that this signal corresponds to a species of the type enzyme–CHO. An analogous reaction of ethylene glycol with the enzyme would yield a species of the type enzyme–CO–CH₂OH. This would not be expected to show any strong coupling of molybdenum to a proton, since we no longer have a proton in the α-position. Though this formulation explains the general nature of the signal-giving species for glycol Inhibited, it must be added that the site, interacting with molybdenum, at which the substrate residue has become bound remains to be identified.