methyl-group peaks significantly until 40% methanol, whereupon they lost intensity rapidly. At this point haem methyl-group resonances due to the high-pH form were observed at very low intensity (less than 10%). The data indicate that ferriytochrome c unfolds in the presence of methanol via at least one intermediate form. This is the high-pH form of ferriytochrome c, in which the native haem ligand, methionine-80, is displaced, although the ferric ion is maintained in the low-spin state. At pH 5.3, the transition from native to intermediate form is co-operative.

Spectra of ferriytochrome c at 27 and 57°C were largely unaltered up to 50% methanol, after which a fairly sharp transition to a random-coil spectrum occurred. Before this transition small shifts to some resonances occurred on the addition of methanol. Most notable of the perturbed resonances are those of isoleucine-57 (at -0.43 p.p.m., 57°C) and tyrosine-74 (at 6.64 p.p.m., 57°C), which suffer 0.12 and 0.04 p.p.m. shifts from their native positions at 40% methanol and are the very residues that we have previously reported to be in a flexible part of the molecule (Moore & Williams, 1980c). There are smaller perturbations (less than 0.02 p.p.m.) to some other surface residues and to some resonances of the haem group. This group of resonances are also sensitive to temperature and pH variations (Moore & Williams, 1980c).

There are two important points from these observations. First, the observation of resonances of the high-pH form of ferriytochrome c at pH 5.3 and methanol/H2O (1:1, v/v) at 27°C strongly suggests that the sixth ligand in this form is not hydroxide. There has been considerable discussion of this point (Petittrew et al., 1976), but the nature of the ligand remains unclear. Secondly, the methanol unfolding of ferriytochrome c proceeds through the 'high-pH form' as an intermediate, which becomes less stable as the pH decreases, causing the transition to become more co-operative. The intermediate was not detected in the unfolding of ferriytochrome c, in which the iron–sulphur bond is broken a low-spin intermediate is formed, and no longer be stabilized, and consequently the protein becomes random-coil. In ferriytochrome c, however, when the iron–sulphur bond is broken a low-spin intermediate is formed, and this stabilizes the protein so that it does not become random-coil until the concentration of methanol is about 50%. In both oxidized and reduced cytochrome c the region around isoleucine-57 and tyrosine-74 is perturbed before the unfolding step, and we suggest that this region of the protein may be the single site of methanol binding involved in the denaturation step (Drew & Dickerson, 1978). We have found similar results in an n.m.r. study of the urea denaturation of cytochrome c and the temperature-induced denaturation of fully maleylated cytochrome c (A. P. Boswell, D. E. Harris, G. R. Moore and R. J. P. Williams, unpublished work).


Possible regulation of the methanol dehydrogenase from Methylophilus methylotrophus by its oxidized electron acceptor

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The ammonia-dependent dye-linked methanol dehydrogenase (EC 1.1.2.9) is found in all methylotrophs that are capable of growing on methanol as a source of carbon and energy. In vitro, this enzyme not only oxidizes methanol to formaldehyde, but also oxidizes this primary product to formate (see Sperl et al., 1974). However, in the obligate methylotroph Methylophilus methylotrophus most of the incoming formaldehyde is oxidized to CO₂ via an alternative cyclic mechanism involving other enzymes (Beardmore et al., 1978), so that in this organism the methanol dehydrogenase must function primarily as a methanol oxidase. We have purified the enzyme to homogeneity (Ghosh & Quayle, 1978), and have now examined, by the methods of steady-state kinetics, some of its possible regulatory properties.

Enzyme activity was measured in 0.125 M tetrasodium pyrophosphate buffer, pH 10.0, with phenazine ethosulfate as the primary electron acceptor, coupled to 2,6-dichlorophenol-indophenol (0.2 mM), and at near-saturating concentrations of the activator in vitro, ammonia (50 mM). Kinetic results were analysed by fitting the raw data to the exponential model for a regulatory enzyme (Ainsworth, 1977; Kinderlehrer & Ainsworth, 1978). This model enables any saturation function to be described by three parameters only: the association constant of the unbound enzyme for ligand A (αₐ); the co-operativity (kₛ), which bears a simple relation to the Hill coefficient, h[=4/(4-kₛ)]; and the apparent maximal velocity (Vₐ). The steady-state rate equation takes the form:

\[ v = \frac{Vₐ \cdot A \cdot \alphaₐ \cdot \exp(kₛ(v/Vₐ))}{1 + A \cdot \alphaₐ \cdot \exp(kₛ(v/Vₐ))} \]  

where \( kₛ \) may be zero (corresponding to the Michaelis–Menten case) or positive or negative (corresponding to positive or negative co-operativity respectively). The variation of \( \alphaₐ \) and \( kₛ \) with respect to the second substrate was followed by the re-plotting procedure of Ainsworth & Gregory (1978).

The results shown in Figs. 1(a) and 1(b) indicate that both methanol (M) and phenazine ethosulfate (P) exhibit negative and positive homotropic kinetics respectively, and also display mutual heterotropic interactions. These effects serve to justify the assumption that phenazine ethosulfate is binding specifically to the active site, possibly by mimicking the behaviour of the physiological acceptor (which may be cytochrome c; Duine et al., 1979).

The negatively co-operative kinetics of methanol binding is not due to the presence of independent non-identical sites, since the enzyme is a stable dimer of identical subunits (Ghosh &
Quayle, 1978), and $k_m$ varied considerably with the fractional saturation of the enzyme with phenazine ethosulphate. As the fractional saturation of the enzyme with phenazine ethosulphate was increased, $a_m$ experienced a minimum $[a_m(\text{min.}) = 22.2 \text{mm}^{-1}]$ but $k_m$ passed through a maximum, with the critical point at half-saturation with phenazine ethosulphate, corresponding to Michaelis–Menten kinetics. Thus, at the extremes of saturation with phenazine ethosulphate, the enzyme has the highest initial affinities for methanol, and also displays pronounced negative co-operativity for this substrate. This behaviour effectively lowers the ‘apparent $K_m$’ of the enzyme for the electron donor to typical values of $0.2-2.0 \mu M$,  so that even at low concentrations of methanol the enzyme is operating at the apparent maximal velocity.

The effect of increasing fractional saturations of methanol on the initial velocity was to decrease the initial affinity of the enzyme for phenazine ethosulphate and to make $k_m$ more positive. At high concentrations of methanol ($>0.05 \text{mm}$) the unbound enzyme possesses a low initial affinity for phenazine ethosulphate but responds rapidly to the presence of low concentrations of this substrate.

These results permit the construction of a simple model for the regulation of methanol oxidation in $M$. methylotrophus. The concentration of the oxidized electron acceptor in vivo is determined by the redox states of the other intermediates of the respiratory chain, and ultimately by the availability of molecular oxygen. At possible physiological concentrations of methanol ($0.05-5 \text{mm}$) the kinetic negative co-operativity of methanol binding will ensure that the methanol dehydrogenase is always operating at the apparent maximal velocity, with respect to methanol, determined by the concentration of the oxidized acceptor. In this way the methanol dehydrogenase in $M$. methylotrophus is probably acting as a ‘metabolic sensor’,  matching the production of formaldehyde for biosynthesis to the availability of oxygen for energy generation.

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