Nitrite Reductase from *Achromobacter xylosoxidans* forms stable trimers in dilute solution

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Nitrite reductase is a key metabolic enzyme in denitrifying anaerobic bacteria. In the past, nitrite reductase purified from a number of microorganisms has shown variation in subunit composition. In this study we show definitively using sedimentation equilibrium in the analytical ultracentrifuge that nitrite reductase from the species *Achromobacter xylosoxidans* exists as stable trimers in dilute solution, confirming the observations of Godden et al [1]. The molar masses (whole distribution weight averages, M_W) obtained at a loading concentration of 0.5mg/ml via the "M*" procedure [2] were (110000±5000) g/mol and (105000±5000) g/mol in respectively phosphate and Tris HCl buffers, corresponding to trimers in both cases. From plots of point weight average molar masses [3] versus concentration it is clear that the association is very strong with apparent dissociation constants < 1μM.

There has been considerable discrepancy in the literature concerning the quaternary composition of the important enzyme nitrite reductase found in anaerobic denitrifying bacteria. Although the monomeric molar mass (molecular weight), M_1 is accepted to be ≈36500 on the basis of mass spectrometry and SDS-PAGE [4], results from the relative molar mass techniques of gel filtration and sedimentation velocity in the analytical ultracentrifuge on *Achromobacter xylosoxidans* nitrite reductase have suggested a dimer (M≈70000)[5] and tetramer (M≈149000)[6] respectively. Grossmann et al [7] reported trimers on the basis of x-ray solution scattering, apparently supported by sedimentation equilibrium measurements. Similar discrepancies have existed for the related enzyme from *Achromobacter cyclolastes* with gel filtration suggesting a dimer [8] apparently supported by the absolute (i.e. not requiring calibration standards) technique of sedimentation equilibrium in the analytical ultracentrifuge [9], although a later determination by Godden et al [1] indicated a trimer.

In an attempt to resolve this discrepancy we undertook a study on the *Achromobacter xylosoxidans* enzyme supplied by Z.H.L. Abraham of the University of Sussex, UK, on the basis of absolute molar mass measurement using low speed sedimentation equilibrium in the analytical ultracentrifuge, and using two different buffers: a phosphate-chloride buffer (pH7.2, I=0.10M) and a Tris-HCl (pH 7.5, 0.15M). A loading concentration of 0.5mg/ml was used in both cases. An Optima XL-A ultracentrifuge (Beckman Instruments, Palo Alto, USA) was employed at a rotor speed of 14000 rev/min, temperature of 20.0°C, and using 12mm optical path length cells. A partial specific volume of 0.7435 ml/g was calculated from the amino acid sequence [4].

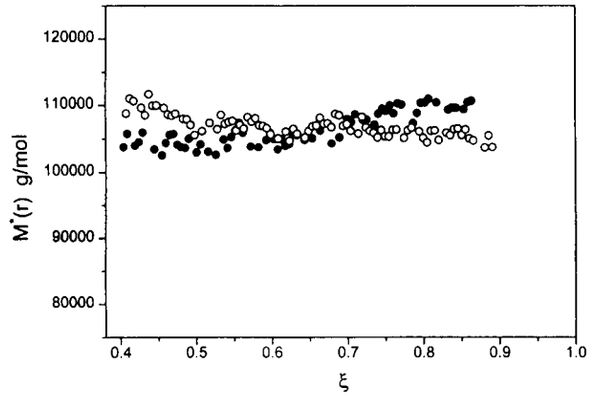

Fig.1. Extraction of weight average molar masses using the M* procedure. $\xi = (r^2-a^2)/(b^2-a^2)$ where r is the radial position in the ultracentrifuge cell and a,b the corresponding positions at the cell meniscus and base. $M_W = M^*$ (ξ=1). Filled circles, enzyme in phosphate buffer; open circles, Tris buffer

Equilibrium solute distributions were captured as an ASCII data set of concentration (expressed as ultra-violet absorbance at a wavelength of 278nm) versus radial displacement from the rotor centre, r (cm) and then analysed using the molar mass routine MSTARA recently adapted for PC [10]. Weight average molar masses, M_W were determined from extrapolation of the "M*" function to the cell base (Fig. 1). Using this procedure, M_W = (110000±5000) g/mol and (105000±5000) g/mol in phosphate and Tris HCl buffers respectively, *corresponding to trimers in both cases.* {Because of the low loading concentration in both cases (0.5mg/ml) non-ideality effects (which can suppress the measured apparent molar mass) can be assumed to be insignificant}.

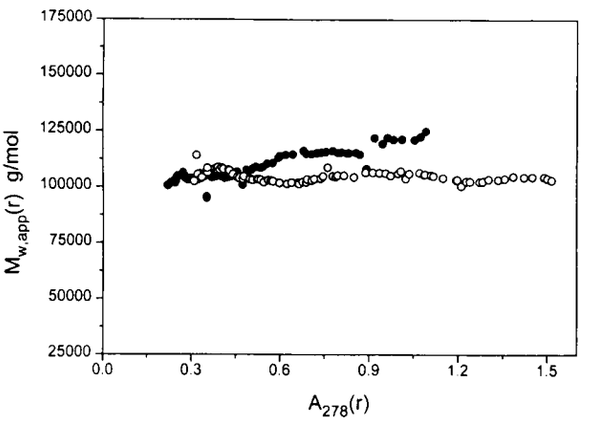

Fig.2. Plot of point weight average molar masses versus concentration (expressed in absorbance units) in the ultracentrifuge cell. Filled circles, phosphate buffer; open circles, Tris buffer

This observation is confirmed if we consider plots of "local" or point weight average molar masses, $M_{W,app}(r)$ {corresponding to radial positions r} versus local concentration $A_{278}(r)$ (expressed in absorbance units at 278nm). These are obtained from sliding strip fits of $\ln A_{278}(r)$ vs r^2 [3]. Fig. 2 clearly shows the existence of trimers throughout the ultracentrifuge cell, with no evidence for dissociation in the ultracentrifuge cell. This implies a very strong association, with dissociation constants less (and probably much less) than ≈ 1μM.

Nitrite reductase is thus another example of a stable trimeric protein system, an observation also seen, for example for chloramphenicol transacetylase [11] and for coiled-coil peptides.

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