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ARTICLE

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Low temperature solution behaviour of *Methylophilus methylotrophus* electron transferring flavoprotein: a study by analytical ultracentrifugation

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Abstract The solution behaviour of electron transferring flavoprotein (ETF) from *Methylophilus methylotrophus* was investigated at low temperature (4 °C) by analytical ultracentrifugation. The concentration dependence of the apparent weight average molecular weight, $M_{w,app}$, established the existence of the protein in heterodimeric state ($M = 63{,}700$ Da), but also signified the possible dissociation of the heterodimer at lower concentrations into its constituent subunits ($M = 28{,}900$ Da and 33,700 Da, together with FAD and AMP cofactors of collective $M = 1120$ Da). This similarity in subunit size allows approximate quantification of the dissociation in terms of expressions for a monomer-dimer equilibrium. The dissociative behaviour was confirmed by determination of the point average molecular weight, $M_{w,app}(r)$, as a function of the ETF concentration, c(r), throughout the sedimentation equilibrium distributions obtained with loading concentrations of 0.4 and 0.7 mg/ml. By means of the recently formulated "psi" procedure for direct analysis of solute self-association a value of (1.5 ± 0.1) μM has been obtained for the dissociation constant K_d. Sedimentation velocity experiments yielded an estimate of the heterodimer sedimentation coefficient, $s^0_{20,w}$, of (4.5 ± 0.2) S which for $M = 63{,}700$ Da suggests a globular structure.

Key words Electron transferring flavoprotein · Dissociation equilibrium · psi function · Gross conformation

Introduction

By virtue of its acceptance of electrons from the enzymic reduction of trimethylamine to dimethylamine and formaldehyde, electron transferring flavoprotein (ETF) is the physiological redox partner of trimethylamine dehydrogenase (TMADH) in bacterial metabolism. ETF is considered to be a heterodimer for which the molecular weight is 63,700 Da on the basis of the amino acid sequences of the two dissimilar subunits with molecular weights of 28,900 Da and 33,700 Da, together with a collective molecular weight (to two significant figures) of 1100 Da for the FAD and AMP cofactors (Chen and Swenson 1994). However the fact that sedimentation equilibrium distributions for relatively dilute solutions (<1 mg/ml) yielded apparent molecular weights below 63,700 Da has pointed to the possibility that the heterodimer may coexist in dissociation equilibrium with its separate subunits. This aspect of the solution behaviour of ETF clearly needs to be resolved before studies are attempted on the physicochemical characterization of the interaction between ETF and its redox partner, TMADH.

Experimental

ETF was purified from *Methylophilus methylotrophus* (bacterium W_3A_1) as described by Steenkamp and Gallup (1978), except that (i) the final gel chromatography step was performed on Sephacryl S-100-HR instead of Sephadex G-100 and (ii) PMSF (1 mM) was included throughout the preparation to suppress proteolysis. To determine the concentration of ETF, complete oxidation of the protein was effected by treatment with potassium

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ferricyanide, after which the sample was desalted by gel chromatography on Sephadex G-25. Concentrations of oxidised ETF solutions were determined spectrophotometrically on the basis of a molar absorption coefficient of 11,300 $M^{-1}\,cm^{-1}$ at 438 nm (Steenkamp and Gallup 1978). From the absorbance of solutions with concentrations so determined, the corresponding absorption coefficient at 280 nm is 66,100 $M^{-1}\,cm^{-1}$, which translates to a specific absorption coefficient of 1.04 L $g^{-1}\,cm^{-1}$ on the basis of the molecular weight of the heterodimer. For the ultracentrifuge studies ETF was dialyzed exhaustively against potassium phosphate-chloride buffer, pH 7.5, I 0.1. Concentrations in the range 0.05–2.7 mg/ml were used in sedimentation velocity studies designed to investigate the concentration dependence of the sedimentation coefficient, the corresponding range in sedimentation equilibrium studies being 0.1–1.0 mg/ml, with the upper limit being restricted by the usual Lambert-Beer restrictions.

Sedimentation studies were performed at 4 °C in a Beckman (Palo Alto, USA) Optima XL-A analytical ultracentrifuge equipped with the scanning optics set at a wavelength of 280 nm. Sedimentation velocity experiments were carried out at rotor speeds of 25,000 and 50,000 rpm in KEL-F double-sector centrepieces. All sedimentation coefficients were evaluated at least three times in order to minimize error in the evaluation of $s_{T,b}$ as $(1/\omega^2)\,(d\mathit{ln}\,r/dt)$, where r denotes the boundary position in a run conducted at angular velocity ω. These sedimentation coefficients were corrected to standard conditions of solvent density (ρ) and viscosity (η), viz., those of water at 20 °C, by means of the usual expression (Tanford 1961)

$$s_{20,w} = s_{T,b}(\eta_{T,b}/\eta_{20,w})[(1-\bar{v}\rho_{20,w})/(1-\bar{v}\rho_{T,b})] \qquad (1)$$

where $\bar{v}$, the partial specific volume, was taken as 0.738 ml/g on the basis of the amino acid composition (Edsall 1943; Laue et al. 1992).

Sedimentation equilibrium runs were performed at 4 °C and 17,000 rpm. Because of the length of time required to reach equilibrium (>24 h) six-channel centrepieces were used to allow concurrent examination of nine ETF solutions (nine solution/solvent pairs). Initially the resulting sedimentation equilibrium distributions were analyzed in terms of weight average and point weight average molecular weights by means of the MSTARA program (Harding et al. 1992; Cölfen and Harding 1997). However, the observation of a dissociation at low ETF concentration prompted quantitative analysis of the sedimentation equilibrium distributions in terms of heterodimer dissociation. Two procedures were used for direct analysis of the sedimentation distributions: (i) multi-exponential analysis of the concentration distribution (Kim et al. 1977) by means of the ASSOC4 software provided by the manufacturer of the ultracentrifuge, and (ii) analysis via the psi function (Wills et al. 1996).

Results and discussion

Sedimentation velocity studies

The fact that a single symmetrical boundary was invariably observed in sedimentation velocity experiments established that the ETF preparation was essentially homogeneous with respect to molecular size over the concentration range (0.05–2.7 mg/ml) examined. Linear regression analysis of the concentration dependence of the sedimentation coefficient (Fig. 1) yielded a limiting value, $s^0_{20,w}$, of (4.2 ± 0.1) S. We now consider if this value is likely to be influenced by any dissociative behaviour.

Sedimentation equilibrium studies

The dependence of the apparent cell-average molecular weight, $M_{w,app}$(cell), upon the loading concentration, c, in sedimentation equilibrium experiments is summarized in Fig. 2. Although the existence of heterodimer (M = 63,700 Da) is clearly demonstrated, there is also a clear indication of dissociative behaviour at concentrations below about 0.4 mg/ml. This evidence of ETF dissociation also showed up in plots of the point-average molecular weight, $M_{w,app}(r)$, as a function of local concentration, c(r), and radial distance r throughout the equilibrium distribution. A poor signal-to-noise ratio precluded analysis of the equilibrium distribution for the lowest loading concentration (0.1 mg/ml), but visual inspection of the results for loading concentrations of 0.4 and 0.7 mg/ml ETF (Fig. 3) suggests the operation of a dissociation constant (k_d) smaller than 0.1 mg/ml. In that regard, the overlap of results from the two experiments is consistent with the concept of self-association equilibrium (Roark and Yphantis 1969). Furthermore, these results are not in conflict with the infer-

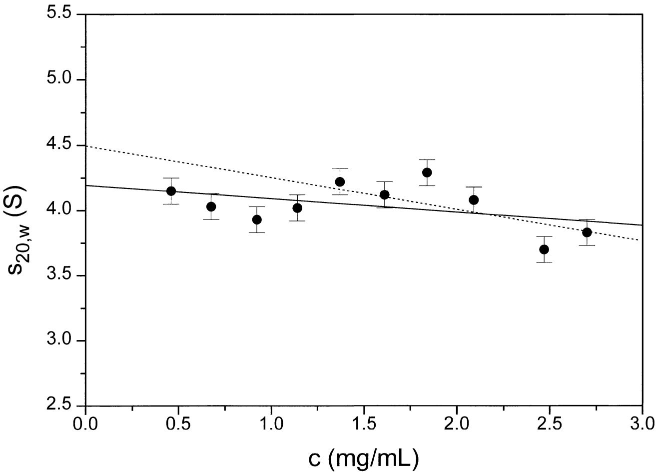

Fig. 1 Concentration dependence of the sedimentation coefficient, $s_{20,w}$, for ETF in phosphate/chloride buffer (pH 7.5, I 0.10) at 4 °C. *Full line*: linear regression line of all the points; *dashed line*: linear regression line disregarding those points of c < 1.0 mg/ml (and hence avoiding possible influence of dissociation phenomena)

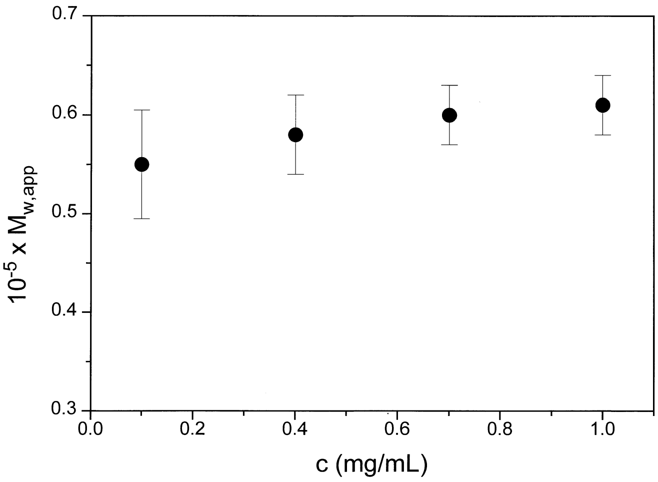

Fig. 2 Dependence of the apparent weight average molecular weight, $M_{w,app}$ (cell) upon loading concentration, c, of ETF in sedimentation equilibrium experiments. Conditions as in Fig. 1. The date given represent averages of several measurements

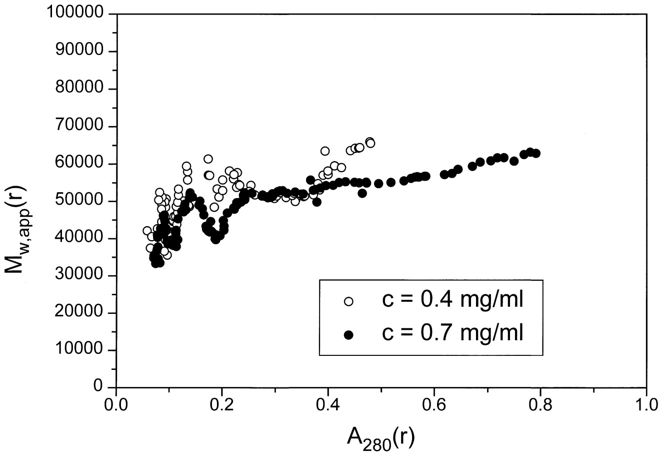

Fig. 3 Dependence of point-average molecular weight, $M_{w,app}(r)$ upon local concentration $A_{280}(r)$, expressed as absorbance units in a 12 mm optical path length, throughout sedimentation equilibrium distributions for loading concentrations, c_0; of 0.4 (○) and 0.7 (●) mg/ml ETF. Conditions as in Fig. 1

ence drawn from the sedimentation velocity experiments that ETF is essentially homogeneous with respect to molecular size: a system governed by such a small dissociation constant would be effectively dimeric throughout the concentration range examined by sedimentation velocity (Fig. 1).

From a quantitative viewpoint, the evidence of heterodimer dissociations at low ETF concentration is unequivocal, but treatment of the phenomenon in terms of a monomer-dimer equilibrium requires comment. This is an approximation made to allow advantage to be taken of quantitative treatments already developed for analysis of sedimentation equilibrium distributions. Furthermore, the similarity of subunit sizes ensures the essential identity of radial dependence of their relative concentrations, a factor which allows their presence to be considered in terms of a summed concentration distribution. Inasmuch as the latter is being assessed spectrophotometrically at 280 nm, the appropriate magnitude of the extinction coefficient is that for the heterodimer, because the dissociated subunits must also be present in 1:1 stoichiometry in the combined concentration distribution for dissociated subunits: the present analysis of the sedimentation distributions is thus independent of the relative absorption characteristics of the two subunits at 280 nm.

Detailed analyses of sedimentation equilibrium distributions

For more critical appraisal of the extent of ETF dissociation under the present conditions (pH 7.5, I 0.1, 4 °C), the sedimentation equilibrium distributions obtained with loading concentrations of 0.4 and 0.7 mg/ml were analyzed in terms of the expressions (Wills et al. 1996)

$$c(r) = c_1(r_F)\Psi_1(r) + k_2\,[c_1(r_F)\;\Psi_1(r)]^2 \tag{2a}$$

$$\Psi_1(r) = \exp[M_1(1-\bar{v}\rho)\;\omega^2(r^2-r_F^2)/2RT] \tag{2b}$$

in which the total concentration of solute, c(r), at radial distance r is written as the sum of the respective concentrations of “monomer” and dimer at that radial distance. We have taken the effective molecular weight of the monomer as 31,750 Da (half the dimer) and represents the mean of the 28,900 Da and 33,700 Da subunits together with 1100 Da for the collective molecular weight (to two significant figures) of the FAD and AMP cofactors. Although it is not certain whether the cofactors remain associated with the subunits following dissociation of the heterodimer, the effect on the calculated subunit association/dissociation constants will be small.

The monomer concentration is defined in terms of its concentration, $c_1(r_F)$, at fixed radial position r_F, and the psi function, Eq. (2b): that of dimer follows from the law of mass action for dimerization governed by the association constant k_2 (litre/g). The same partial specific volume ($\bar{v}$), of 0.738 ml/g is considered to apply to monomeric and dimeric states of ETF; and ρ, the solvent density (Wills and Winzor 1992), has been taken as 1.0032 g/ml on the basis of density measurements with the Anton-Paar instrument. Although the psi analysis (Wills et al. 1996) is designed to accommodate effects of thermodynamic nonideality, such effects are neglected in Eq. (2a) on the grounds that the assumed ideality should be a reasonable approximation for a globular protein over such a low concentration range (Ogston and Winzor 1975).

In Fig. 4, which summarizes the analysis of these two sedimentation equilibrium distributions in accordance with Eq. (2), the total ETF concentration, c(r), has been obtained from the measured absorbance at 280 nm, $A_{280}(r)$, on the basis of a pathlength of 1.2 cm and an absorption coefficient of 1.04 $l\,g^{-1}\,cm^{-1}$ (see Experimental). In that regard, the selection of the fixed radial positions (r_F) to achieve a common $c(r_F)$ of 0.30 mg/ml in the two experi-

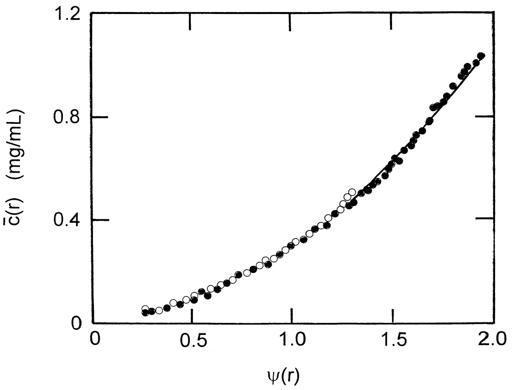

Fig. 4 Characterization of the dimerization of ETF by direct analysis [Eqn. (2)] of sedimentation equilibrium distributions (Wills et al. 1996) for which the loading concentrations were 0.4 mg/ml (○) and 0.7 mg/ml (●) protein, the monomeric molecular weight being taken as 31,750 Da. For evaluation of the abscissa parameter the values of the fixed radial position (r_F) in the two experiments were chosen on the basis of a common $c(r_F)$ of 0.30 mg/ml. The solid line is the best-fit description obtained by non-linear regression analysis of the results in terms of Eqn. (2). Conditions as in Fig. 1

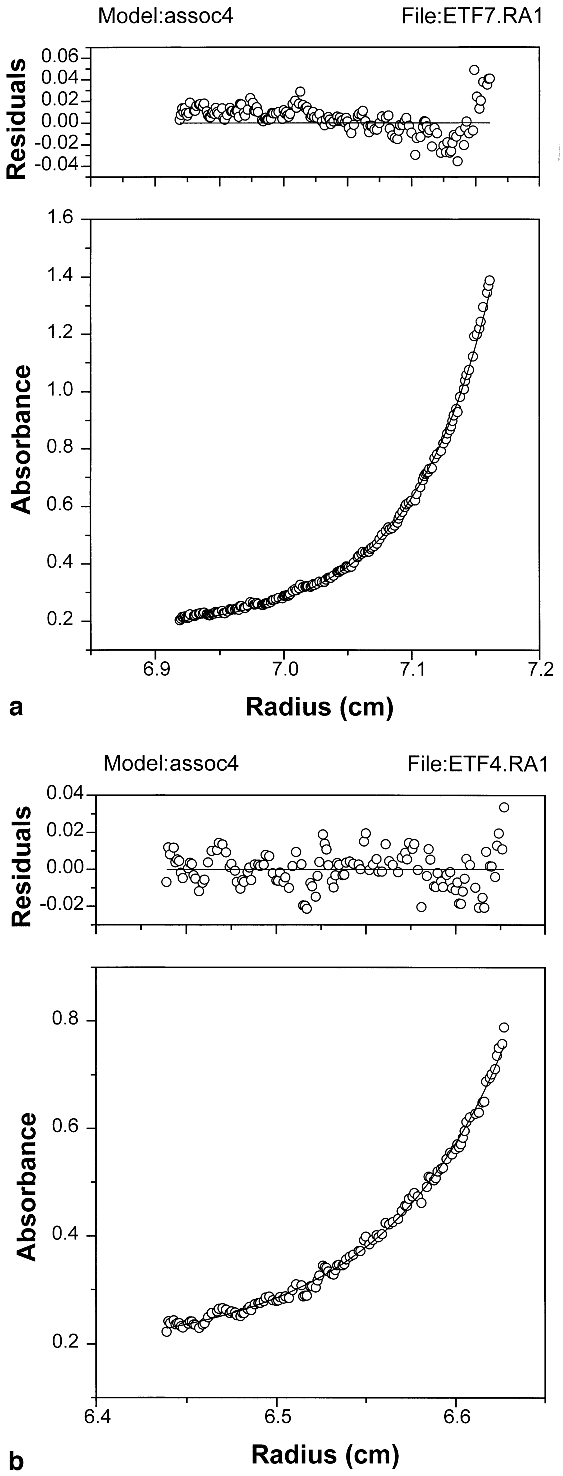

Fig. 5a, b Evaluation of the dimerization constant for ETF by means of the ASSOC4 routine to obtain the best-fit description of the sedimentation equilibrium distributions obtained with loading concentrations of (**a**) 0.4 and (**b**) 0.7 mg/ml protein. Conditions as in Fig. 1

ments ensures identical values of $c_1(r_F)$ for the two sets of data; and hence paves the way for their global curve-fitting. The solid line in Fig. 4 is the best-fit description obtained by nonlinear regression analysis of these results in terms of Eq. (2), which yields values of 0.073 (±0.002) mg/ml and 43 (±3) litre/g for $c_1(r_F)$ and k_2 respectively. Table 1 gives the results in associative (k_2) and dissociative (k_d) formats, as well as in molar terms.

As a check, the concentration distributions from the same two sedimentation equilibrium distributions were also analyzed with the aid of the ASSOC4 routine provided by the manufacturers of the ultracentrifuge: this software package, fits the data to Eqn. (III-27) of Kim et al. (1977). Such analysis of the distributions for ETF loading concentrations of 0.4 mg/ml (Fig. 5a) and 0.7 mg/ml (Fig. 5b) yielded respective dimerization constants of 13.8 (±1.6) and 9.3 (±1.6) litre/g, giving a mean of approximately 12 (±3) litre/g for k_2. Inasmuch as the quantitative expression used in the ASSOC4 program is essentially Eq. (2), the almost four-fold discrepancy between the estimates of the dimerization constant obtained by the two methods certainly requires comment. To that end it was noted that repetition of the psi analysis with a lower magnitude for $c(r_F)$ yielded a value of k_2 much closer to that obtained by means of the ASSOC4 program, for which the reference concentration was also low (0.04 mg/ml). Thus, whereas the psi analysis based on r_F values commensurate with a reference ETF concentration of 0.3 mg/ml yielded a dimerization constant of 43 litre/g (Fig. 4), the best-fit value decreased

Table 1 Equilibrium constants for the dissociation of ETF heterodimer

Analysis	k_2 (l/g)	10^{-5} K_2 (M^{-1})	k_d (g/l)	K_d (μM)
Psi[a]	43 (±3)	6.8 (±0.5)	0.023 (±0.002)	1.5 (±0.1)
Psi[b]	17 (±2)	2.7 (±0.3)	0.06 (±0.1)	3.8 (±0.4)
ASSOC4	12 (±3)	1.9 (±0.5)	0.08 (±0.02)	5.1 (±1.3)

[a] $c(r_F) = 0.30$ mg/ml
[b] $c(r_F) = 0.18$ mg/ml

to 16.7 (±1.5) litre/g on selection of reference radial positions such that $c(r_F) = 0.18$ mg/ml (Table 1). Our decision to favour the higher value of k_2 reflected its smaller relative uncertainty (7% cf 9%), and hence its slightly better description of the combined data sets.

In principle, the evaluated dimerization constant is independent of the magnitude of $c(r_F)$, which merely governs the value of the corresponding monomer concentration, $c_1(r_F)$, that is obtained as the second curve-fitting parameter in either analysis. Experimentally, the returned parameters are clearly reflecting different weightings of the errors associated with the measurements of c(r). Because of the sensitivity of analysis to any systematic error in c(r) [or its absorbance counterpart A(r)], the baseline was determined at the end of each experiment by high-speed centrifugation to pellet all protein. The search for potential sources of error distortion is not very rewarding in the sense that the ordinate parameter, c(r) or A(r), is untransformed in either analysis; and although $\psi_1(r)$ is a transformed variable, the essential absence of uncertainty in measurements of radial distance should preclude distortion of the analysis by such transformation.

Similar circumstances apply to the ASSOC4 analysis, which is formally identical with the psi procedure except that the reference radial position is anchored at the air-liquid meniscus – the restriction that precluded global analysis of the two sedimentation equilibrium distributions. In that regard the selection of a reference radial position at either end of a sedimentation equilibrium distribution certainly places heavy emphasis on the accuracy of the best-fit estimate of $c_1(r_F)$ because of its use to calculate the predicted c(r) at the other extreme of the distribution: the two concentrations are related by an exponential term involving the difference between the squares of the respective radial distances [Eq. (2)]. However, regardless of the consequent uncertainty inherent in the estimate of the dimerization constant for ETF, the important conclusion to emanate from this investigation is the conclusion (Figs. 4 and 5; Table 1) that quaternary protein structure formation in the ETF heterodimer is incomplete at low concentrations (<0.5 mg/ml) of the flavoprotein.

Gross conformation of ETF heterodimer

As done previously with ETF's redox partner TMADH, (Cölfen et al. 1996) based on just the sedimentation coefficient and the molecular weight, we can make some comments on the gross conformation of the ETF heterodimers, confirming its globular characteristics. There is a slight problem, in that, unlike TMADH, the ETF sedimentation coefficient data may be influenced by contributions from increasing proportions of dissociated subunits, as the concentration is reduced: this can lead to an underestimate of $s^0_{20,w}$ for the heterodimer. In Fig. 1 therefore we have included an extrapolation to zero concentration avoiding data points at concentrations <1.0 mg/ml: this leads to a revised estimate of $s^0_{20,w}$ for the heterodimer of ~(4.5 ± 0.2) S, with the larger uncertainty a feature of the much further extrapolation to the c = 0 axis. From $s^0_{20,w}$ and M_w we can estimate the shape or hydration of the ETF heterodimer via evaluation of the translational frictional ratio, f/f_o, and the corresponding Perrin function, P, for the hydrodynamically equivalent prolate ellipsoid of revolution:

$$\left(\frac{f}{f_0}\right) = \left[\frac{M(1-\bar{v}\rho_0)}{N_A(6\pi\eta_0 s^0_{20,w})}\right]\left(\frac{4\pi N_A}{3\bar{v}M}\right)^{1/3} \tag{3}$$

where N_A is Avogradro's number and ρ_0 and η_0 are the density and viscosity of water at 20 °C. Using values of $M = 63{,}700$ Da, $s^0_{20,w} = (4.5 \pm 0.2)$ S, $\bar{v} = 0.738$ ml/g, $\rho_0 = 0.9982$ g/ml and $\eta_0 = 0.01$ poise, we estimate $f/f_0 \sim (1.23 \pm 0.05)$.

P can then be calculated if the "hydration", δ (the amount of aqueous solvent chemically or physically associated to the protein per unit dry mass of protein) can be reasonably estimated or approximated:

$$P = \left(\frac{f}{f_0}\right)\left[\frac{\delta}{\bar{v}\rho_0} + 1\right]^{-1/3} \tag{4}$$

As before with TMADH (Cölfen et al. 1996), we consider two cases

1. Axial ratio a/b calculation of the ETF heterodimer, based on $f/f_0 = 1.23$ and assuming a value for the hydration δ of 0.35

Taking a so-called "typical" protein hydration value, an estimate for P of ~(1.08 ± 0.05) is predicted. This in turn yields, using simple ellipsoid of revolution inversion formulae (Harding and Cölfen 1995) (incorporated into the routine ELLIPS1 – see Harding et al. 1997) an estimate of the axial ratio for the hydrodynamically equivalent prolate ellipsoid of between 2 and 3, as with TMADH. The error quoted for P does not include any intrinsic error in the assumption we have made over δ.

2. Hydration δ of the heterodimer based on an $f/f_0 = 1.23$ and a spherical protein (P = 1.0)

This is the other extreme, and using Eqs. (3) and (4) we obtain an estimate for δ of ~0.6 g/g. This value is not completely implausible, since Squire and Himmel (1979) have estimated, by comparing predicted sedimentation coefficients for globular proteins from crystal structures, with hydrodynamically measured sedimentation coefficients, and assuming negligible difference between the shape of the protein in crystal and dilute solution states, estimated average protein hydrations of ~0.5.

In the absence of further hydrodynamic data, it is completely unjustified therefore to say anything more than the ETF heterodimer is a globular protein of axial ratio <3. *This illustrates the limitations of sedimentation analysis used in isolation* for conformation studies, particularly on globular structures, a limitation which is apparently not widely appreciated. More specific conclusions about solution structure await further analysis, and of course a crystal structure for ETF which can then be used as a starting estimate and "refined" accordingly.

Concluding remarks

This physicochemical study of *M. methylotrophus* ETF by analytical ultracentrifugation has served to confirm the existence of quaternary protein structure in the sense that ETF exhibits the solution characteristics of a globular protein with the molecular weight (63,700) of a heterodimer. However, on the basis that the extent of this quaternary structure formation is incomplete at concentrations below about 1 mg/ml, the macromolecular state of ETF is best described in terms of a monomer-dimer equilibrium governed by an association constant of ~40 litre/g (Table 1). Such considerations are likely to assume importance in studies of binding between ETF and its physiological redox partner, TMADH, this being an interaction (of reported K_d between 10–20 μM (Huang et al. 1995) on the basis of stopped-flow measurements) for which the appropriate ETF concentration range is unlikely to be sufficiently high for justifiable disregard of heterodimer dissociation.

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